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UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

June 09, 2022

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APPLICATION NUMBER: *15/288,406*
FILING DATE: *October 07, 2016*
PATENT NUMBER: *10947555*
ISSUE DATE: *March 16, 2021*

Certified by

Performing the Functions and Duties of the
Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

DocCode – SEQ.TXT

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Application Number: 15288406 Document Date: 10/07/2016

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BARNES & THORNBURG LLP

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Indianapolis, Indiana 46204
(317) 236-1313

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No.	107540	
Group:	Unknown	
Confirmation No.:	Unknown	
Application No.:	Unknown	
Invention:	**NOVEL HERBICIDE RESISTANCE GENES**	**ELECTRONICALLY FILED ON:** **October 7, 2016**
Inventor:	Wright et al.	
Filed:	Herewith (October 7, 2016)	
Attorney Docket:	14764-258662	
Examiner:	Unknown	

PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Preliminary to the examination of the above-identified divisional patent application, Applicants respectfully request entry of the following amendments.

The Commissioner is hereby authorized to charge any fee necessary to affect this filing, to the account of Barnes & Thornburg, Deposit Account No. 10-0435, with reference to our matter 14764-258662.

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims begin on page 3.

Remarks begin on page 8 of this paper.

AMENDMENTS TO THE SPECIFICATION

On page 1, please replace the paragraph immediately following the section heading of CROSS REFERENCE TO RELATED APPLICATIONS, with the following amended paragraph:

This application is a continuation application of U.S. Serial No. 14/820,893, filed August 7, 2015, which is a continuation application of U.S. Serial No. 12/951,813, filed November 22, 2010, now patented as US 9,127,289, which is a ~~divisional~~ continuation of U.S. Serial No. 11/587,893, filed May 22, 2008, now patented as US 7,838,733, which is a national stage entry of PCT/US2005/014737, filed May 2, 2005, which claims the benefit of U.S. Provisional Application Serial No. 60/567,052, filed April 30, 2004, which are hereby incorporated by reference in their entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

COR-AAD1-00000004
Bayer EX1008 (Part 1 of 9)

AMENDMENTS TO THE CLAIMS:

Please cancel claims 1-14 without prejudice. This listing of claims will replace all prior versions, and listings of claims in the application:

15. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:
$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, wherein
X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;
$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;
$(X)_{139\text{-}243}$ represents a sequence of 131 amino acids; and
$(X)_{245\text{-}254}$ represents a sequence of 10 amino acids.

16. The plant cell of claim 15 wherein said AAD-1 motif has the general formula of:

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R(X)_{282\text{-}284}R$, wherein $(X)_{282\text{-}284}$ represents a sequence of 3 amino acids.

17. The plant cell of claim 15 wherein said AAD-1 motif has the general formula of:

$RVIGDDX_{110}HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, wherein $(X)_{110}$ represents a single amino acid at position 110, relative to the sequence of SEQ ID NO: 9.

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Bayer EX1008 (Part 1 of 9)

18. The plant cell of claim 15 wherein the AAD-1 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 9

19. The plant cell of claim 15 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an Arabidopsis cell.

20. The plant cell of claim 15 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

21. A transgenic plant comprising a plurality of the plant cell of claim 15, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

22. The plant of claim 21 wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

23. The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

24. The plant of claim 21 wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

25. The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, metamifop, cyhalofop, and clodinofop.

26. The plant of claim 21 wherein said plant further comprises a second herbicide resistance gene.

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Bayer EX1008 (Part 1 of 9)

27. The plant of claim 26 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

28. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 21, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide.

29. The method of claim 28 wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

30. The method of claim 28 wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

31. The method of claim 28 wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, metamifop, cyhalofop, and clodinofop.

32. The method of claim 28 wherein said method comprises applying a second herbicide.

33. The method of claim 32 wherein said first herbicide and said second herbicide are applied sequentially.

34. The method of claim 32 wherein said first herbicide and said second herbicide are applied concurrently.

35. The method of claim 32 wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

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Bayer EX1008 (Part 1 of 9)

36. The method of claim 32 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

37. The method of claim 32, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

38. The method of claim 32, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

39. The method of claim 32 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

40. The method of claim 39 wherein said second gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

41. The method of claim 40 wherein:

(a) said modified ALS gene is selected from the group consisting of a SurA, SurB, Csr1, Csr1-1, Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT; and,

(c) said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

42. The method of claim 32 wherein said method further comprises applying a third herbicide.

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Bayer EX1008 (Part 1 of 9)

43. The method of claim 42, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

44. The method of claim 43 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

45. A seed comprising a plurality of the plant cell of claim 15.

46. A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim 45 in said field within 14 days of applying said aryloxyalkanoate herbicide.

47. A plant grown from the seed of claim 45.

48. A part, progeny, or asexual propagate of the plant of claim 47, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

COR-AAD1-00000009
Bayer EX1008 (Part 1 of 9)

REMARKS

This Preliminary Amendment is being submitted to correctly set forth the relationship of the application filed herewith to previously filed applications as required under 37 C.F.R. §1.78.

Claims 1-14 have been cancelled without prejudice and replaced with claims 15-48. Support for the new claims is found throughout the specification, including for example in paragraphs [0050], [0078], [0079], [0082], [0085], [0089], [0093], [0099], [00107] and the original claims.

No amendment is believed to go beyond the disclosure in the international application as originally filed.

With the entry of the foregoing amendments, the application is believed to be in condition for examination and allowance. Consideration of the claims, leading to their allowance and passage of the application to issuance, is respectfully requested.

Respectfully submitted,

/John P. Breen/

John P. Breen
Attorney Reg. No. 38,833

JPB:glt
Indianapolis, Indiana 46204
317-261-7940

DMS 4389226v1

COR-AAD1-00000010
Bayer EX1008 (Part 1 of 9)

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Filer:	John P. Breen/Garla Twyman
Attorney Docket Number:	14764-258662

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	280	280
UTILITY SEARCH FEE	1111	1	600	600
UTILITY EXAMINATION FEE	1311	1	720	720
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		1600

Electronic Acknowledgement Receipt

EFS ID:	27149990
Application Number:	15288406
International Application Number:	
Confirmation Number:	5402
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Customer Number:	107540
Filer:	John P. Breen/Garla Twyman
Filer Authorized By:	John P. Breen
Attorney Docket Number:	14764-258662
Receipt Date:	07-OCT-2016
Filing Date:	
Time Stamp:	15:45:12
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1600
RAM confirmation Number	101116INTEFSW00002221100435
Deposit Account	100435
Authorized User	Garla Twyman
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	ADS.pdf	1823537 8c865c7f5df6ff0d8a862ded8a0fb3fbf54e82ab	no	10
Warnings:					
Information:					
2	Power of Attorney	Dow_Agrosciences_POA_3_73_c.pdf	270258 467ee24f10462b3468f4a38ba6e0a67253d4470b	no	2
Warnings:					
Information:					
3	Assignee showing of ownership per 37 CFR 3.73	373c.pdf	117797 bef3ca0565d1aed8a9b724274120770239bb389a	no	3
Warnings:					
Information:					
4	Oath or Declaration filed	Inventor_Declarations.pdf	425119 4cc17907be0ae3fc1e2df83ff0961e68e30a9a90	no	4
Warnings:					
Information:					
5		CON_Novel_Herbicide_Resistance_Genes.pdf	9384337 932998a63786ebe55856d26332db224ecb6b1cc6	yes	185

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	158
Claims	159	160
Abstract	161	161

	Drawings-only black and white line drawings		162	185	
Warnings:					
Information:					
6	Sequence Listing (Text File)	256882_ST25.txt	36696 29dcbcbf15406c2d40b398ac1a180f0d383a43c3	no	-
Warnings:					
Information:					
7		Preliminary_Amendment.pdf	25177 29dcbcbf15406c2d40b398ac1a180f0d383a43c3	yes	8
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Preliminary Amendment		1	1	
	Specification		2	2	
	Claims		3	7	
	Applicant Arguments/Remarks Made in an Amendment		8	8	
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	35161 a5b065c4dae77b6ae2a76a59cec181689f449867	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	12118082		

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/14 (11-15)
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Terry		WRIGHT	

Residence Information (Select One) ● US Residency Non US Residency Active US Military Service

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Address 2	

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Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
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Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

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Address 2	

City	State/Province	Postal Code	Country
Fishers	IN	46038	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Donald		MERLO	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

City	Carmel	State/Province	IN	Country of Residence	US

Mailing Address of Inventor:

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Address 2			
City	Carmel	State/Province	IN
Postal Code	46032	Country	US

Inventor 4 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Nicole		ARNOLD	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

City	Indianapolis	State/Province	IN	Country of Residence	US

Mailing Address of Inventor:

Address 1	2518 Chaseway Ct.		
Address 2			
City	Indianapolis	State/Province	IN
Postal Code	46268	Country	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	107540		
Email Address	INDocket@btlaw.com	Add Email	Remove Email

Application Information:

Title of the Invention	NOVEL HERBICIDE RESISTANCE GENES		
Attorney Docket Number	14764-258662	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	24	Suggested Figure for Publication (if any)	

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	● Customer Number	US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	107540		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	14/820893	2015-08-07

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14/820893	Continuation of	12/951813	2010-11-22	9127289	2015-09-08

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12/951813	Continuation of	11/587893	2008-05-22	7838733	2010-11-23

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
11/587893	a 371 of international	PCT/US2005/014737	2005-05-02

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2005/014737	Claims benefit of provisional	60/567052	2004-04-30

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 — Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

● Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.	Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Dow AgroSciences LLC		
Mailing Address Information For Applicant:			
Address 1	9330 Zionsville Road		
Address 2			
City	Indianapolis	**State/Province**	IN
Country	US	Postal Code	46268
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/John P. Breen/			Date (YYYY-MM-DD)	2016-10-07
First Name	John	Last Name	Breen	Registration Number	38833

Additional Signature may be generated within this form by selecting the Add button. Add

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

COR-AAD1-00000026
Bayer EX1008 (Part 1 of 9)

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POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

■ Practitioners associated with Customer Number: 107540

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

■ The address associated with Customer Number: 107540

OR

☐

Firm or Individual Name			
Address			
City		State	Zip
Country			
Telephone		Email	

Assignee Name and Address: Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268-1054

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be Filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of The practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature		Date	June 21, 2013
Name	Andrea T. Borucki	Telephone	(317) 337-4846
Title	General Patent Counsel		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Dow AgroSciences LLC

Application No./Patent No.: Unknown Filed/Issue Date: Herewith (October 7, 2016)

Titled: NOVEL HERBICIDE RESISTANCE GENES

Dow AgroSciences LLC, a Corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☑ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☑ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 021991, Frame 0527, or for which a copy thereof is attached.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☐ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/John P. Breen/	7 October 2016
Signature	Date
John P. Breen	38833
Printed or Typed Name	Title or Registration Number

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	Novel Herbicide Resistance Genes

As the below named inventor, I hereby declare that:

This declaration is directed to:
- [ ] The attached application, or
- [x] United States application or PCT international application number 14/820,893 filed on 08/07/2015 .

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Terry R. Wright Date (Optional): 10/6/15

Signature: [signature]

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

PTO/AIA/01 (06-12)
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	Novel Herbicide Resistance Genes

As the below named inventor, I hereby declare that:

This declaration is directed to:
- [ ] The attached application, or
- [x] United States application or PCT International application number 14/820,893 filed on 08/07/2015 .

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

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LEGAL NAME OF INVENTOR

Inventor: Justin M. Lira Date (Optional): 10/8/15

Signature:

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	Novel Herbicide Resistance Genes

As the below named inventor, I hereby declare that:

This declaration is directed to:
- [ ] The attached application, or
- [x] United States application or PCT international application number 14/820,893 filed on 08/07/2015 .

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

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LEGAL NAME OF INVENTOR

Inventor: Donald J. Merlo Date (Optional): Oct 21, 2015

Signature: Donald J. Merlo

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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LEGAL NAME OF INVENTOR

Inventor: Nicole L. Arnold Date (Optional): 10 OCT 2015

Signature: Nicole L Arnold

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

NOVEL HERBICIDE RESISTANCE GENES

Cross-Reference to Related Application

[0001] This application is a continuation application of US Serial No. 12/951,813, filed November 22, 2010, which is a divisional of U.S. Serial No. 11/587,893, filed May 22, 2008, now patented as US 7,838,733, which is a national stage entry of PCT/US2005/014737, filed May 2, 2005, which claims the benefit of U.S. Provisional Application Serial No. 60/567,052, filed April 30, 2004, which are hereby incorporated by reference in their entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

Background of the Invention

[0002] Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

[0003] Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant corn, for example, can be sprayed to control weeds without significantly damaging the corn plants.

[0004] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (*e.g.*, wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (*e.g.*, alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; *see* "isb.vt.edu/cfdocs/fieldtests1.cfm , 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few

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conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

[0005] Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

[0006] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Ng *et al.*, 2003; Simarmata *et al.*, 2003; Lorraine-Colwill *et al.*, 2003; Sfiligoj, 2004; Miller *et al.*, 2003; Heap, 2005; Murphy *et al.*, 2002; Martin *et al.*, 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past 3-5 years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis,* and *Plantago lanceolata.* Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

[0007] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-diclorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its

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selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

[0008] 2,4-D is in the phenoxy acid class of herbicides, as are MCPA, mecoprop, and dichlorprop. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known.

[0009] 2,4-D has different levels of selectivity on certain plants (*e.g.*, dicots are more sensitive than grasses). Differential metabolism of 2,4-D by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

[0010] Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D is currently formulated to have a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

[0011] One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-

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D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.* (1989), Lyon *et al.* (1989), Lyon (1993), and U.S. Patent No. 5,608,147).

[0012] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with α-ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

[0013] One unique example with low homology to *tfdA* (28% amino acid identity) is *rdpA* from *Sphingobium herbicidovorans* (Kohler *et al.*, 1999, Westendorf *et al.*, 2002). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Although the organisms that degrade phenoxypropionic acid were described some time ago, little progress had been made in characterizing this pathway until recently (Horvath *et al.*, 1990). An additional complication to dichlorprop degradation is the stereospecificity (R *vs.* S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.*, 2003). Heterologous expression of *rdpA* in other microbes, or transformation of this gene into plants, has not heretofore been reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids (*e.g.*, 2,4-D).

[0014] Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

[0015] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxy auxins (such as 2,4-D and dichlorprop), pyridyloxy auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among

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these chemical classes. Discovery of a multifunctional enzyme for the degradation of herbicides covering multiple modes would be both unique and valuable as an HTC trait.

Brief Summary of the Invention

[0016] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidazolinone-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0017] In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, AOPP, and one or more commercially available herbicides (*e.g.*, glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

[0018] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and AOPP herbicides, in an enantiospecific manner. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade

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herbicides of different chemical classes and modes of action. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant HTC trait and selectable marker opportunities.

[0019] There was no prior motivation to produce plants comprising an *AAD-1* gene (preferably an *AAD-1* polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-1 enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides (such as quizalofop, haloxyfop, *et al.*). Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0020] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoate auxin herbicides, and aryloxyphenoxypropionate herbicides by a recombinantly expressed AAD-1 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more AOPP, phenoxy auxin, or other aryloxyalkanoate herbicides to plants comprising an *AAD-1* gene. The subject invention also provides methods of using an *AAD-1* gene as a selectable marker for identifying plant cells and whole plants transformed with *AAD-1*, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

Brief Description of the Figures

[0021] **Figure 1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

[0022] **Figure 2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

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[0023] **Figure 3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

[0024] **Figure 4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

[0025] **Figure 5** shows 2,4-dichlorophenol production by recombinant *AAD-1*.

[0026] **Figures 6A and 6B** show phenol production by recombinant *AAD-1* from various herbicide substrates.

[0027] **Figure 7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

[0028] **Figures 8A and 8B** show that *AAD-1 (v3)* was expressed equally in *Arabidopsis* leaves of different ages but continued to accumulate throughout the 25 days of experiment. Plants that were not sprayed with the herbicide 2,4-D (panel A) expressed a little more AAD-1 (v3) than those had been sprayed (panel B). Bars represent the mean ± SEM of 5 leaves from 5 different plants, with percent expression of AAD-1 (v3) normalized to total soluble protein. Light bars represent the third young leaves (N-3) collected from the top, dark bars represent the 5th oldest leaves from the bottom.

[0029] **Figures 9A, 9B, and 9C** show injury of *Arabidopsis* plants after 2,4-D treatment. Four different lines were each treated with four different doses of 2,4-D and their injury was graded 4 (panel A) and 14 (panel B) days after treatment. Their expression of AAD-1 (v3) in leaves was also determined using ELISA (panel C). The results were mean ± SEM of five leaves from five different plants received the same treatment.

[0030] **Figure 10** illustrates that pDAB3230-transformed *Arabidopsis* (*AAD-1* + *EPSPS*) shows >14-fold level of glyphosate tolerance 7 DAT vs. wildtype and transformed control *Arabidopsis* lines.

[0031] **Figure 11** shows dose response of callused maize suspensions to R-haloxyfop.

[0032] **Figure 12** shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol.

[0033] **Figure 13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

[0034] **Figure 14** shows the responses of several *AAD-1 (v3)*-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied as a postemergence spray 1 week prior.

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[0035] **Figure 15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

[0036] **Figure 16** shows *AAD-1 (v3)*-transformed corn tolerant to 8X field rates of quizalofop (Assure II) under field conditions.

[0037] **Figure 17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

[0038] **Figure 18** shows Western Blotting analysis on soybean calli transformed with *AAD-1 (V3)* gene indicating that the callus cells are expressing AAD-1 (v3) protein.

[0039] **Figure 19** shows fitted curves for 2,4-D degradation rates by AAD-2 (v1) vs. AAD-1 (v1).

[0040] **Figure 20** shows the response of *AAD-1 v3* (plant optimized), or *AAD-1 (v2)* (native), *AAD-2 (v1)* (native), or *AAD-2 (v2)* (plant optimized)-transformed T_1 *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T_1 family.

[0041] **Figure 21** shows western blot analysis of individual native *AAD-2 (v1)*-transformed T_1 *Arabidopsis* plants. This shows that plants expressing the AAD-2 (v1) protein are suffering severe injury from 200 g ae (acid equivalent)/ha 2,4-D treatments, which normally causes little injury to native *AAD-1 (v2)* or plant optimized *AAD-1 (v3)*-transformed *Arabidopsis*. AAD-2 protein is identified on the gel. Several background bands were detected in *AAD-2*-transformed and *Pat/Cry1F*-transformed samples.

[0042] **Figure 22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D = dichlorprop > (R,S)-haloxyfop >> (R)-haloxyfop.

Brief Description of the Sequences

[0043] **SEQ ID NO:1** is the sequence of a forward primer used to amplify the *rdpA/AAD-1 (v1)* gene.

[0044] **SEQ ID NO:2** is the sequence of a reverse primer used to amplify the *rdpA/AAD-1 (v1)* gene.

[0045] **SEQ ID NO:3** is the nucleotide sequence of *AAD-1 (v1)* from *Sphingobium herbicidovorans*.

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[0046] **SEQ ID NO:4** is the nucleic acid sequence of the native *AAD-1* gene with internal NotI restriction site removed. This gene is designated *AAD-1 (v2)*. DNA sequencing confirmed that the correct PCR product was generated, but an inadvertent change was made at amino acid #212 from arginine to cysteine.

[0047] **SEQ ID NO:5** is a "plant-optimized" DNA sequence *AAD-1 (v3)*. This "gene" encodes SEQ ID NO:11, which is the same as SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations.

[0048] **SEQ ID NO:6** ("rdpA(ncoI)") and **SEQ ID NO:7** ("3'saci") were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[0049] **SEQ ID NO:8** is another PCR primer ("BstEII/Del NotI") that was used with the "3' SacI" primer.

[0050] **SEQ ID NO:9** is the native amino acid sequence encoded by the *AAD-1 (v1)* gene from *Sphingobium herbicidovorans*.

[0051] **SEQ ID NO:10** is the amino acid sequence encoded by the *AAD-1 (v2)* DNA sequence of SEQ ID NO:4.

[0052] **SEQ ID NO:11** is the amino acid sequence encoded by the *AAD-1 (v3)* plant-optimized DNA sequence of SEQ ID NO:5.

[0053] **SEQ ID NO:12** is the DNA sequence of the native *AAD-2 (v1)* gene.

[0054] **SEQ ID NO:13** is the amino acid sequence of the AAD-2 (v1) protein.

[0055] **SEQ ID NO:14** is a forward primer used to amplify *AAD-2 (v1)* DNA for cloning.

[0056] **SEQ ID NO:15** is a reverse primer used to amplify *AAD-2 (v1)* DNA for cloning.

[0057] **SEQ ID NO:16** is the M13 forward primer.

[0058] **SEQ ID NO:17** is the M13 reverse primer.

[0059] **SEQ ID NO:18** is a forward primer used to amplify *AAD-2 (v1)* DNA for cloning.

[0060] **SEQ ID NO:19** is a reverse primer used to amplify *AAD-2 (v1)* DNA for cloning.

[0061] **SEQ ID NO:20** is the native soybean EPSPS protein.

[0062] **SEQ ID NO:21** is a doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine).

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[0063] **SEQ ID NO:22** is the soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21.

[0064] **SEQ ID NO:23** is primer Pat 5-3.

[0065] **SEQ ID NO:24** is primer Pat 3-3.

[0066] **SEQ ID NO:25** is forward primer AAD-1 PTU.

[0067] **SEQ ID NO:26** is reverse primer AAD-1 PTU.

[0068] **SEQ ID NO:27** is the forward primer for the Coding Region PCR AAD-1.

[0069] **SEQ ID NO:28** is the reverse primer for the Coding Region PCR AAD-1.

[0070] **SEQ ID NO:29** is the *AAD-2 (v2)* nucleotide (plant optimized).

[0071] **SEQ ID NO:30** is the translated AAD-2 (v2) protein sequence.

[0072] **SEQ ID NO:31** is the Southern fragment PCR AAD-1 forward primer.

[0073] **SEQ ID NO:32** is the Southern fragment PCR AAD-1 reverse primer.

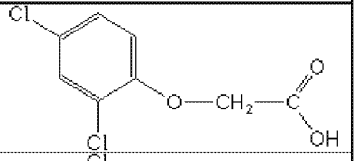
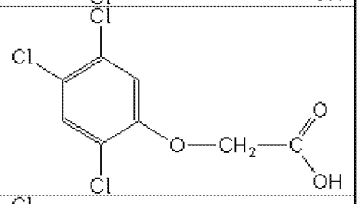
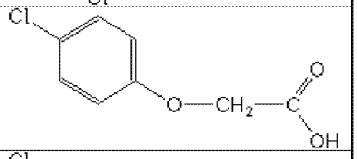
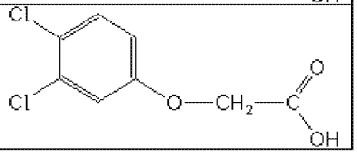
Detailed Description of the Invention

[0074] The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of an herbicide tolerance trait for 2,4-D would be its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism and the like. An additional benefit of the *AAD-1* gene is that unlike all *tfdA* homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (*e.g.*, dichlorprop and mecoprop) in addition to achiral phenoxy auxins (*e.g.*, 2,4-D, MCPA, 4-chlorophenoxyacetic acid). *See* **Table 1**. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the *AAD-1* gene in plants would afford protection to a much wider spectrum of phenoxy auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled, protecting from drift or other off-site phenoxy herbicide injury for the full breadth of commercially available phenoxy auxins.

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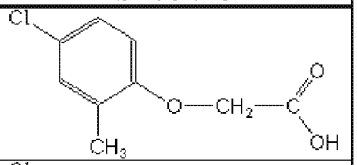
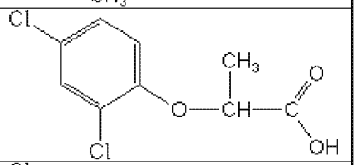
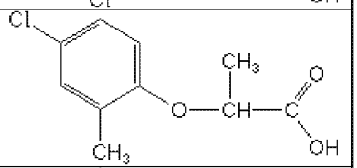
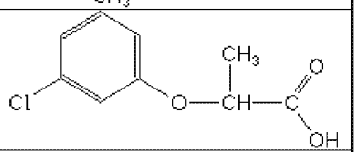
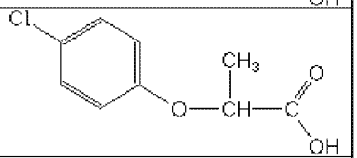
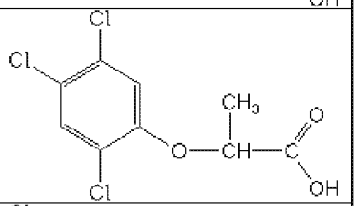
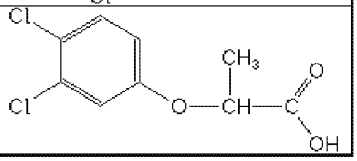
[0075] **Table 1.** Commercially available phenoxy auxins. Reference to phenoxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Table 1. Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	

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Table 1. Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
MCPA	94-74-6	25 – 4000	125 - 1550	
Dichlorprop	120-36-5	25 – 12000	100 - 2240	
Mecoprop	7085-19-0	25 – 4000	250 - 3360	
Cloprop	101-10-0	25 – 4000	25 – 4000	
4-CPP	3307-39-9	25 – 4000	25 – 4000	
Fenoprop	93-72-1	25 – 4000	25 – 4000	
3,4-DP	3307-41-3	25 – 4000	25 – 4000	

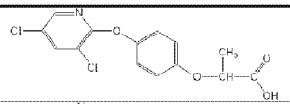
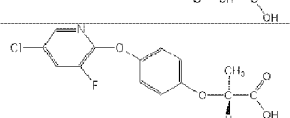
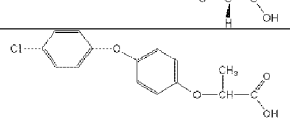
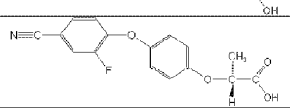
[0076] An additional benefit of the *AAD-1* gene is its unprecedented ability to concomitantly degrade a host of commercial and non-commercial graminicidal compounds of the general

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class aryloxyphenoxypropionates (AOPPs). *See* **Table 2**. This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing *AAD-1*, where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, oats, sorghum, warm and cool-season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

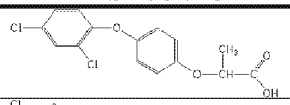
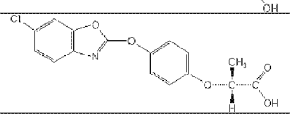
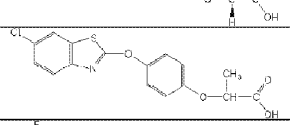
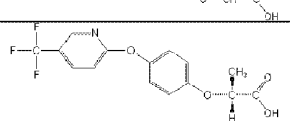
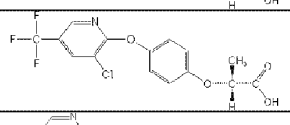
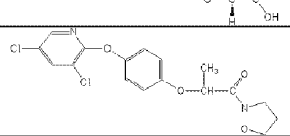
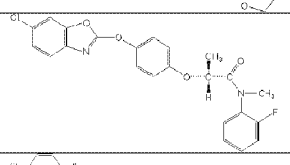
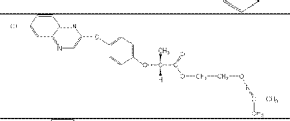
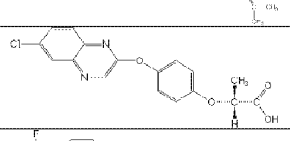
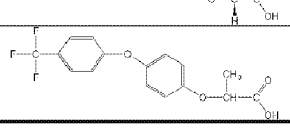
[0077] **Table 2.** AOPP graminicidal compounds listed by accepted common names. Reference to AOPP herbicides is generally made to the active acid but most are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Table 2. AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	

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Table 2. AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Diclofop	71283-65-3	10-2000	280-2000	
Fenoxaprop	66441-23-4	10-2000	20-200	
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	
Isoxapyrifop	87757-18-4	10-2000	30-240	
Metamifop	256412-89-2	10-2000	35-280	
Propaquizafop	111479-05-1	10-2000	30-240	
Quizalofop	76578-14-8	10-2000	20-240	
Trifop	58597-74-4	10-2000	10-2000	

[0078] A single gene (*AAD-1*) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, *AAD-1* can provide protection *in planta* to AOPP herbicides

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where natural tolerance also was not sufficient to allow selectivity. Plants containing *AAD-1* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several AOPP graminicidal compounds may be applied to plants expressing AAD-1 with reduced risk of injury from said herbicides. The rate for each AOPP may range from 10 to 2000 g ae/ha, and more typically from 20-500 g ae/ha for the control of a broad spectrum of monocot weeds. Combinations of these different chemistry classes and herbicides with different modes of action and spectra in the same field (either sequentially or in tank mix combination) shall provide control of most potential weeds for which herbicidal control is desired.

[0079] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking *AAD-1* with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant weed species (either grass weed species with one or more AOPP herbicides, or broadleaf weed species with one or more phenoxy auxins) in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (*e.g.*, 2,4-D) and AOPP herbicide(s) (*e.g.*, quizalofop) selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from 2 hours to 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0080] It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a

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glyphosate resistance gene/*AAD-1* stack could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0081] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0082] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant species or resistant weed species could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (*e.g.*, resistant plant or bacterial *EPSPS, GOX, GAT*), glufosinate resistance (*e.g.*, *Pat, bar*), acetolactate synthase (ALS)-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (*e.g.*, *PPO-1*), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), dicamba-degrading enzymes (*see, e.g.*, US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

[0083] Regarding additional herbicides, some additional preferred ALS inhibitors include the triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include flumiclorac,

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flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0084] Additionally, *AAD-1* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0085] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. Even though this enzyme has very low homology to *tfdA*, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes.

[0086] More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also AOPP herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0087] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed AAD-1 enzyme.

[0088] The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical and biological assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP (ranging from 50-100%

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conversion) *in vitro*. An additional advantage that *AAD-1*–transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0089] The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an *AAD-1* gene.

[0090] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0091] Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0092] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.*, 1989; Lyon *et al.*, 1989; Lyon *et al.*, 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α-ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0093] *RdpA*, from *Sphingobium herbicidovorans* (Westendorf *et al.*, 2002), is one unique example with low homology (28% amino acid identity). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as

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2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Although the organism responsible for phenoxypropionic acid degradation has been known for some time, little progress has been made in characterizing this pathway until recently (Horvath *et al.*, 1990). An additional complication to dichlorprop degradation is the stereospecificity (R *vs.* S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.*, 2003). Heterologous expression of *rdpA* in other microbes or transformation of this gene into plants, heretofore, was not reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids. There was no prior expectation that *rdpA* or *AAD-1* genes could be successfully expressed in plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

[0094] As described in more detail in the Examples below, *rdpA* was cloned from *Sphingobium herbicidovorans* and tested for substrate promiscuity among various herbicide chemical classes. This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and dichlorprop (Westendorf *et al.*, 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. *RdpA* has never been expressed in plants, nor was there any motivation to do so because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0095] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides *in vitro*. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance are the aryloxyphenoxypropionate (AOPP) grass herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities.

[0096] The broad spectrum grass AOPP herbicides are reported herein to be excellent substrates for AAD-1 as well as 2,4-D, dichlorprop, and other phenoxy auxins. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum

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broadleaf herbicides (phenoxy auxins) and a range of broad spectrum, highly active grass herbicides (AOPPs).

[0097] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxy-alkanoate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-1). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

[0098] The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf and grass weeds. *AAD-1* can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits (*e.g.*, glyphosate resistance, glufosinate resistance, imidazolinone resistance, bromoxynil resistance, *et al.*), and insect resistance traits (*Cry1F, Cry1Ab, Cry 34/45, et al.*) for example. Additionally, *AAD-1* can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0099] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with *AAD-1*-containing constructs and have demonstrated high levels of resistance to both the phenoxy auxin and AOPP herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention. As shown below in Example 6, the exemplified rebuilt gene was more efficacious in conveying herbicide resistance to the plant, as compared to the bacterial gene.

[00100] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-1. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[00101] HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give

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rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

[00102] Additionally, glyphosate tolerant crops grown worldwide are prevalent. Many times in rotation with other glyphosate tolerant crops, control of glyphosate-resistant volunteers may be difficult in rotational crops. Thus, the use of the subject transgenic traits, stacked or transformed individually into crops, provides a tool for controlling other HTC volunteer crops.

[00103] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[00104] The transgenic expression of the subject *AAD-1* genes is exemplified in, for example, *Arabidopsis*, corn (maize), tobacco, cotton, rice, soybean, and canola. However, the subject invention can be applied to any other desired types of plants. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be applied to multiple other grass and other broadleaf crops. Likewise, 2,4-D can be more positively utilized in grass crops where tolerance to 2,4-D is moderate, and increased tolerance via this trait would provide growers the opportunity to use 2,4-D at more efficacious rates and over a wider application timing without the risk of crop injury.

[00105] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed (auxins) and grass weeds (AOPPs). This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-1 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

[00106] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell

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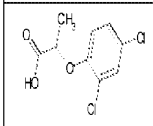
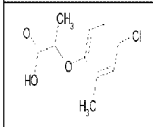
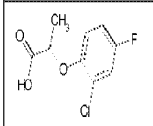
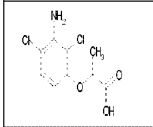
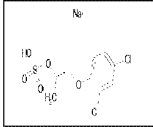
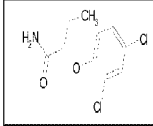
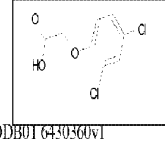
cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-1 for other phenoxyalkanoic auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

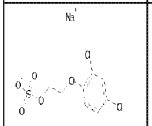
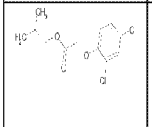
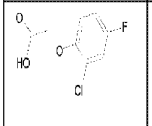
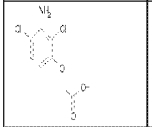
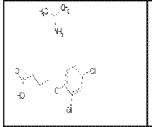
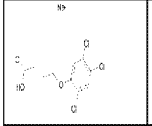
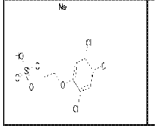
[00107] One gene of the subject invention, referred to herein as *AAD-1* (aryloxyalkanoate dioxygenase), was cloned from *Sphingobium herbicidovorans* (ATCC 700291) by PCR into pET 280-S/S (designated pDAB 3203) and expressed in BL-21 Star *E. coli*. When this gene is overexpressed (by induction of 1mM IPTG and culture lysate combined with the following reaction mix: 112.5 µg/ml 2,4-D, 1 mM Ascorbic acid, 1 mM α-ketoglutarate, 50 µM $Fe(NH_4)_2(SO_4)_2$, the recombinantly produced enzyme degrades 2,4-D into herbicidally inactive DCP (as determined by HPLC, mass spectrometry, colorimetric assay, and *Arabidopsis* plate assay). Additionally, AAD-1 has been demonstrated to convert the following herbicides into their corresponding inactive phenol: dichlorprop, mecoprop, haloxyfop, dichlofop, and others (See **Tables 3 and 4**).

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[00108] **Table 3:** Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2-}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Table 3: Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

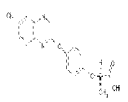
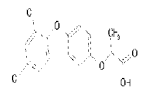
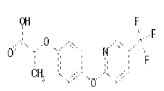
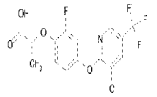
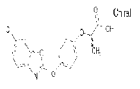
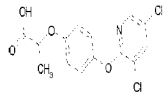
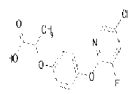
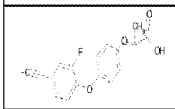
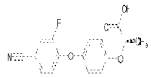
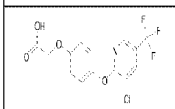
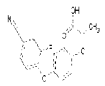
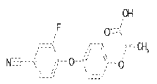
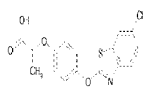
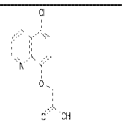
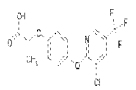
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058
	11263526		0.004	0.069
	178577		0.003	0.021
	178587		0.003	0.02
	188527		0.003	0.036

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[00109] **Table 4:** Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 µM Fe^{2+}, 200 µM Na ascorbate, 1 mM α-ketoglutarate using either 1 µg or 10 µg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Table 4. Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)	STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalofop	0.43	2.1		460511	(R,S)-diclofop	0.155	1.663
	67131	(R,S)-fluazifop	0.427	2.17		25646		0.144	1.69
	11044492	(R)-fenoxaprop	0.408	0.597		70222	(R,S)-chlorazifop	0.128	1.584
	34697	(R,S)-clodinofop	0.295	1.98		199608	cyhalofop	0.114	1.26
	14603	(R)-cyhalofop	0.222	1.989		43865	haloxyfop-oxyacetate	0.004	0.053
	14623	(R,S)-cyhalofop	0.215	1.815		7466	(S)-cyhalofop	0.003	0.017
	62942	(R,S)-fenthiaprop	0.199	1.055		204558	Cloquintocet	0	0.001
	66905	haloxyfop	0.172	1.63					

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[00110] Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

[00111] Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. Optimized polynucleotide can also be designed based on the protein sequence.

[00112] The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe

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or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

[00113] There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

[00114] One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria. Other members of the orders Firmicutes and Proteobacteria, and specific genera with known *rdpA*'s, such as *Sphingobium*, *Delftia*, *Rodoferax*, and *Comamonas* for example, can be used as source isolates.

[00115] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[00116] A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example),

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and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

[00117] Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook *et al.*, 1989, which is incorporated herein by reference.

[00118] Polynucleotides and probes. The subject invention further provides nucleotide sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

[00119] The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *AAD-1* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

[00120] As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense

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strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

[00121] In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

[00122] Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

[00123] Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, *etc.*).

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[00124] As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

[00125] Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[00126] Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

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[00127] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see*, *e.g.*, Maniatis *et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

Tm = 81.5° C + 16.6 Log[Na+] + 0.41(%G+C) - 0.61(%formamide) - 600/length of duplex in base pairs.

[00128] Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20° C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

[00129] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

Tm (°C) = 2(number T/A base pairs) + 4(number G/C base pairs)

(Suggs *et al.*, 1981).

[00130] Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1x SSPE, 0.1% SDS (moderate stringency wash).

[00131] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

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Low:	1 or 2x SSPE, room temperature
Low:	1 or 2x SSPE, 42° C
Moderate:	0.2x or 1x SSPE, 65° C
High:	0.1x SSPE, 65° C.

[00132] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

[00133] PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.*, 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

[00134] Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the

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scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

[00135] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target pests and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[00136] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[00137] "Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus,

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"focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[00138] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, or 297 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[00139] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained

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using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

[00140] It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, *B.t.* proteins can be used in a truncated (core protein) form (*see, e.g.*, Höfte *et al.* (1989), and Adang *et al.* (1985)). As used herein, the term "protein" can include functionally active truncations.

[00141] In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

[00142] Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93,

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94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

[00143] Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.,* 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.,* 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

[00144] Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 5** provides a listing of examples of amino acids belonging to each class.

Table 5

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[00145] In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

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[00146] As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

[00147] Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

[00148] Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

[00149] Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf and pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

[00150] In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this

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invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

[00151] Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

[00152] Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

[00153] A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

[00154] Vectors comprising an *AAD-1* polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc*. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids.

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Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley *et al.* (1986); and An *et al.* (1985).

[00155] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

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[00156] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[00157] In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

[00158] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate, (*PAT*), glyphosate (*EPSPS*), imazethyapyr (*AHAS*), and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[00159] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Patent Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Patent Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Patent Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S.

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Patent No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Patent No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Patent Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Patent Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. *See* U.S. Patent Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Electroporation technology has also been used to transform plants. *See* WO 87/06614 to Boyce Thompson Institute; U.S. Patent Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Patent No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Patent Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[00160] As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

[00161] In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta *et al.* (1980) and EPO 0 120 515.

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Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00162] For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

[00163] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[00164] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

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[00165] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00166] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin, CaMV 35S, and the like). Tissue specific

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promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[00167] Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[00168] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00169] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[00170] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Method for Identifying Genes That Impart Resistance to 2,4-D *In Planta*

[00171] As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence

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already identified that encodes a protein with the desired characteristics (*i.e.*, α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤ 50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[00172] PCR and cloning of gene into pET. A single gene (*rdpA*) was identified from the NCBI database (*see* the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 28% amino acid identity to *tfdA* from *Ralstonia eutropha*. Percent identity was determined by first translating both the *rdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00173] The strain of *Sphingobium herbicidovorans* containing the *rdpA* gene was obtained from ATCC (American Type Culture Collection strain #700291). The lyophilized strain was revived according to ATCC protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 536. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days.

[00174] A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' TCT AGA AGG AGA TAT ACC ATG CAT GCT GCA CTG TCC CCC CTC TCC CAG CG 3' [(SEQ ID NO:1) (added Xba I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' CTC GAG TTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2)(added extra stop codon and Xho I site)].

[00175] Twenty microliter reactions were set up as follows: MasterMix 8 µl, ea. primer 1 µl (50 pmoles/µl), gDNA 2.5 µl, H_2O 7.5 µl. PCR was then carried out under the following

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conditions: 94° C 45 sec, 52° C 1.5 minute, 72° C 1.5 minute, for 30 cycles, followed by a final cycle of 72° C 5 minute, using Eppendorf's Master Taq kit (Eppendorf cat. #0032 002.250). The resulting ~1kb PCR product was cloned into pCR 2.1 (Invitrogen cat. # K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00176] Ten of the resulting white colonies were picked into 4 ml Luria Broth + 50 μg/ml Kanamycin (LB K), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Promega Wizard Plus SV kit (Promega cat. #A1460) and following the included protocol. Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward (5' GTA AAA CGA CGG CCA GT 3') (SEQ ID NO:16) and Reverse (5' CAG GAA ACA GCT ATG AC 3') (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence (SEQ ID NO:3), and its corresponding protein (SEQ ID NO:9) was given a new general designation for internal consistency *AAD-1 (v1)* (AryloxyAlkanoate Dioxygenase).

[00177] Using the restriction enzymes corresponding to the sites added with the primer linkers (Xba 1, Xho 1) *AAD-1 (v1)* was cut out of the pCR2.1 vector and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates. To differentiate between *AAD-1 (v1)*:pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37° C for 4 hours with agitation.

[00178] Each culture was then spotted onto LB K plates, which were incubated at 37° C overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3203.

Example 2 – Expression and Testing

[00179] 2.1 – HPLC analysis.

[00180] Plasmid pDAB 3203 was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR 1878. For expression, plasmid DNA purified from TOP10F' culture using Promega's Wizard kit (Fisher cat. #PR-A1460) was transformed into BL-21

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Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 µl of the cells were plated onto LB S/S agar plates and incubated overnight at 37° C.

[00181] The next morning, all colonies from the entire plate were scraped into 100 mls LB in a 500 ml tri-baffled flask and incubated at 37° C/200 rpm for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C/200 rpm. All 100 ml of culture was centrifuged at 4000 rpm for 20 min. The supernatants were then discarded, and the pellets were resuspended in 10 ml of 50 mM MOPS. These were then subjected to three 45-sec rounds of sonication to lyse the cells. Following this, lysates were centrifuged at 15,000 rpm to remove cell debris. The supernatant was pipetted off and stored at 4° C. To check for recombinant expression, a 20 µl aliquot was run on a 4-20% Tris Glycine gel (Invitrogen cat. #EC60255).

[00182] After expression was confirmed, enzyme activity was tested as follows. First, an aliquot of the cell extract was desalted with a PD-10 cartridge (Amersham cat. #17-0435-01). This was then used for subsequent herbicide enzyme reactions.

[00183] For each reaction, the following were combined: 2,4-D (125 µg/ml), [Ascorbate (1 mM), Ferrous ion (50 µM), α-ketoglutarate (1 mM), in 100 mM MOPS], cell extract (100 µl). This reaction was then incubated at room temp for 30 min., after which the reaction was stopped with the addition of 0.1 N HCl until pH was between 2 and 3. Half of the reaction volume (~500 µl) was set aside for bioassay, the remaining volume was organically extracted using Solid Phase Extraction tubes (Fisher cat. #11-131-6), eluting with 400 µl of Acetonitrile + 0.05% TFA.

[00184] The extracts were then tested on HPLC for loss of the 2,4-D peak or presence of any additional peaks resulting from the degradation or modification of 2,4-D. Conditions for the HPLC were: Luna 10µ C18(2) 250 x 4.6mm (Phenomenex cat. #00G-4253-E0), run at 50% ACN + 0.05% TFA: 50% H_2O + 0.05% TFA to 100% ACN + 0.05% TFA over 5 min.

[00185] 2.2 – Plate test bioassays for herbicide degradation.

[00186] Plant bioassays were used to determine if *in vitro* enzymatic herbicide transformation resulted in a concomitant loss in herbicidal activity. Because of the selective nature of the herbicides being tested (*i.e.*, monocot plants controlled by AOPP herbicides and dicot plants controlled by auxinic herbicides), wildtype *Agrostis palustris* var. Pencross and *Arabidopsis*

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thaliana var. Columbia were used as monocot and dicot test species, respectively. Each species is amenable to germination and growth in small Petri dishes.

[00187] *Arabidopsis* seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 μL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @ 250 rpm). Bleach solution was decanted inside a sterile hood and rinsed three times with sterile water. Bentgrass seeds were surface sterilized for 20 minutes in a like manner.

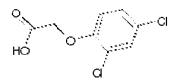
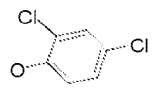
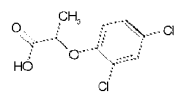
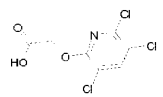
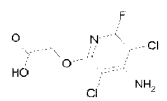
[00188] Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4 \cdot 2H_2O$, 10 μM NaCl, 10 nM $CoCl_2 \cdot H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60x15-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR_{50} rate (50% growth reduction) approximately in the center of the range.

[00189] For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed and maintained under sterile conditions in a low light growth chamber (24 h day^{-1}, 100 $\mu E/m^2s^1$, 23° C) for 7 days. Root length or root+shoot length were measured for five randomly chosen *Arabidopsis* and bentgrass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR_{50} determined.

[00190] This bioassay was used to confirm the loss of herbicidal activity as a result of AAD-1 (v1) degradation of the oxyalkanoate side chain from various agronomically relevant herbicides. In several instances, the anticipated phenol product co-eluted with the parent acid on HPLC and the bioassay served as the primary screen for herbicide degradation. **Tables 6 and 7** represent herbicidal substrates tested.

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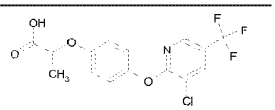
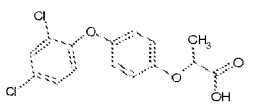
Table 6. *Arabidopsis* plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
2,4-D	22	17	267	16	
DCP	>1000	nd	nd	nd	
Dichlorprop	nd	30	1000	33	
Triclopyr	255	1000	1000	1	
Fluroxypyr	2200	2250	1825	<1	

* Blank vector represents of cell lysate treatment where E. coli pET vector had no gene insert.

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥ 2 is considered the threshold for detecting herbicide activity loss with this assay.

Table 7. Bentgrass plate test bioassay for commercial aryloxyphenoxyalkanoate ACCase-inhibiting substrates.

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
Haloxyfop-RS	28	21	520	25	
Diclofop-RS	nd	20	130	7	

* Blank vector represents of cell lysate treatment where E. coli pET vector had no gene insert.

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥ 2 is considered the threshold for detecting herbicide activity loss with this assay.

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[00191] 2.3 – HPLC results.

[00192] From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see **Figure 1**) and ferrous ion to bind in the active site. Other experiments in the literature have shown that the addition of ascorbate increased the enzymatic activity by maintaining the iron in the reduced state, thus preventing the enzyme for being degraded. Based on this previous work, initial assays were set up under the assumption that the subject enzyme would work in the same way as other members of this general class of enzyme.

[00193] Surprisingly, the initial HPLC results showed the presence of a new peak at 6.1 minute, in addition to a reduced 2,4-D peak at 5.5 min. This new peak was not present in the control assay. For an initial identification of the peak at 6.1 minutes, a DCP control was run under our assay conditions and predictably this also eluted at 6.1 minutes. The formation of this product was confirmed using a colorimetric assay to detect phenols (see example 3.1) as well as mass spectrometry. As expected, AAD-1 (v1) carries out a similar reaction as other members of this enzyme class. In the bioassay, these same samples were also shown to have an almost complete loss of 2,4-D herbicidal activity in the *Arabidopsis* plate assay (**Figure 2**). Regardless of the specific conditions of the assay (*i.e.*, longer incubations, more enzyme), only 50-75% of the 2,4-D could be degraded to DCP as measured by HPLC. In fact, longer induction of the BL-21 *E. coli* cells with IPTG only resulted in less active enzyme, even though more total recombinant protein was expressed.

[00194] After demonstrating degradation of 2,4-D, additional substrates were tested with similar ring substitutions (*i.e.*, oxyacetates and oxypropionates). The first compounds tested were the pyridine analogs fluroxypyr and triclopyr, which are pyridinyloxyacetates. No enzyme activity was detected on either of these as substrates. Additional tests on various analogs of these two pyridinyloxyacetates with either the fluorine or the amino groups removed also were not degraded. Interestingly however, adding a fluorine to the 5 position of 2,4-D resulted in an almost total loss of enzyme degradation (see next section for additional results).

[00195] ACCase inhibitors, haloxyfop and diclofop, were then tested using the same conditions as with 2,4-D. (The corresponding phenol metabolites co-eluted with the parent compound under the HPLC conditions used.) The bioassay results from these samples showed loss of herbicidal activity against both haloxyfop (**Figure 3**) and diclofop. These

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results were also confirmed by the colorimetric assay, which was also used to test a wider sampling of these compounds.

[00196] 2.4 – Plate test bioassays for herbicide degradation.

[00197] Bioassay tests results corroborated initial HPLC results that indicated loss of 2,4-D parent following incubation of 2,4-D solutions with unpurified recombinant AAD-1 (v1) extracts (**Figure 2**). Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR_{50} for herbicide+enzyme solution versus the herbicide solution alone served as measure of loss of parent herbicide activity resulting from enzyme activity. A ratio of 2-3 typically correlated with 50-75% loss of parent herbicide activity (**Table 6**). Often a GR_{50} could not be determined following enzyme treatment; *de facto*, no detectable herbicide activity remained.

[00198] The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (v1) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (**Figure 3** and **Table 7**). These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

Example 3 – *In vitro* assay of AAD-1 (v1) activity via colorimetric phenol detection

[00199] 3.1 – AAD-1 (v1) assay.

[00200] AAD-1 (v1) enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides such as haloxyfop and cyhalofop (see **Figure 4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so were readily

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detected. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μM phenol.

[00201] Enzyme assays were performed in a total volume of 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 15 minutes of incubation at 25° C, the reaction was terminated by addition of 10 μl 100 mM sodium EDTA. Color was developed by addition of 15 μl pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH), 1.5 μl 2% 4-aminoantipyrine and 1.5 μl 8% potassium ferricyanide. After 10 to 20 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols. Later assays were made more convenient by consolidating the additions as follows: the reaction was quenched by addition of 30 μl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer and 0.2 % 4-aminoantipyrine, then adding 10 μl 0.8 % potassium ferricyanide.

[00202] 3.2 – Extraction.

[00203] Activity of recombinant AAD-1 (v1) expressed in *Escherichia coli*. *E. coli* cell pellets were resuspended in 0.1 M Tris, pH 7.4 + 1mg/ml lysozyme (5 ml/cells from 250 ml culture; 20 ml/cells from 1 liter) at room temperature. After about 15 minutes with occasional shaking, the suspension was frozen in liquid nitrogen then thawed. DNase was added to 0.02 mg/ml final concentration and $MgCl_2$ to 1 mM. After the extract was no longer viscous, the extract was centrifuged for 15 min. The supernatant was passed over a BioRad 10DG column pre-equilibrated with 20 mM MOPS pH 6.75 and the eluant stored in aliquots at –70° C. Assays were either performed with these unpurified desalted extracts or with purified enzymes.

[00204] A cell pellet from a 250 ml culture of induced *E. coli* cells expressing pDAB3203 containing the gene encoding *AAD-1 (v1)* was extracted and assayed using the previously described protocols. The 2,4-D cleaving activity in the AAD-1 (v1) extract was compared to that from *E. coli* cells expressing a vector without *AAD-1 (v1)* using 1 mM 2,4-D and is

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shown in **Figure 5**. The amount of 2,4-dichlorophenol formed is clearly proportional to the amount of extract added to the assay whereas the control extract contains no 2,4-D cleaving activity.

[00205] The activity of this extract was tested on four additional herbicides, (R,S)-dichlorprop, (R,S)-mecoprop, (R,S)-haloxyfop and (R,S)-diclofop in comparison to 2,4-D (all at a final concentration of 0.5 mM) using 4 µl of the *E. coli* extract per assay with a 15 min assay period. **Figure 6A** shows that AAD-1 (v1) cleaved all five herbicides to yield a phenol with the relative activity on the substrates being dichlorprop = mecoprop > diclofop > haloxyfop > 2,4-D. Thus AAD-1 (v1) has activity on graminicidal aryloxyphenoxypropionate herbicides as well as phenoxy auxins.

[00206] The AAD-1 (v1) extract was then tested using racemic (R,S)-haloxyfop, the R enantiomer of haloxyfop and the S-enantiomer of cyhalofop (all at 0.5 mM) as potential substrates to ascertain the likely enantiomeric specificity of AAD-1 (v1). The results are shown in **Figure 6B**. The activity of the enzyme on (R)-haloxyfop was equivalent to that on (R,S)-haloxyfop whereas no activity could be seen on the S-enantiomer of cyhalofop indicating that AAD-1 (v1) has R specificity on AOPPs.

Example 4 – Substrate Specificity of AAD-1 (v1)

[00207] 4.1 – Additional substrates of AAD-1 (v1).

[00208] The substrate specificity of AAD-1 (v1) toward a variety of commercial and experimental herbicides was tested. Purified AAD-1 (v1) was used at either 1 or 10 µg per 160 µl assay and each substrate was tested at 1 mM with an assay time of 15 minutes. **Table 3** shows the A_{510} detected after the action of AAD-1 (v1) on a variety of aryloxyalkanoate auxinic herbicides and auxin analogs. The best substrate tested was dichlorprop, with mecoprop also being efficiently cleaved. Two other phenoxypropionates, the 4-fluoro and 3-amino analogs of dichlorprop, were also acted on effectively by AAD-1 (v1). AAD-1 (v1) produced small amounts of phenol from a variety of phenoxyacetates including 2,4-D. The relative rates on these substrates are better gauged from the assays using the higher amounts (10 µg) of AAD-1 (v1). From these data, 2,4-D is cleaved by AAD-1 (v1), as are two phenoxyalkylsulfonates, X188476 and X398166 (sesone).

[00209] **Table 4** shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly

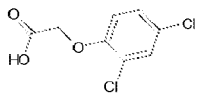
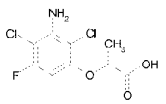
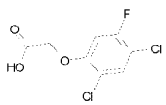
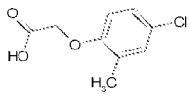
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increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 µg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

[00210] Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 µg crude recombinant AAD-1 (v1) per assay. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness (**Table 8**). X11115427 was slightly better as a substrate than 2,4-D (125% of the 2,4-D rate) in contrast to the close analog 3-amino-dichlorprop, which is ~7- fold better than 2,4-D as a substrate (**Table 3**). The 5-F substitution appears to decrease the effectiveness of X11115427 as a substrate for AAD-1 (v1). The rates of product formation from 5-F-phenoxyacetate and MCPA were 32% and 55% that of 2,4-D respectively.

[00211] **Table 8:** Effect of AAD-1 (v1) on three substrates relative to 2,4-D. Substrates were assayed as in Table 6 at 1 mM using a crude recombinant AAD-1 (v1) extract from *E. coli*.

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Table 8. Effect of AAD-1 (v1) on three substrates relative to 2,4-D.				
Registry ID	**MOLSTRUCTURE**	**Compound**	**A510**	**% 2,4-D**
195517		2,4-D	0.177	100
11115427		(R,S)-3-amino, 5-F-dichlorprop	0.221	125
124987		5-F, 2,4-D	0.056	32
192711		MCPA	0.097	55

[00212] 4.2—Kinetic characterization.

[00213] The K_m and k_{cat} values of purified AAD-1 (v1) (see Example 10) were determined for four herbicide substrates, (R)-dichlorprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 μM Na ascorbate; 200 μM Fe^{2+}; 1 mM α-ketoglutarate; 25° C). The dose response curves were fitted using Grafit (Erithacus Software, UK), and the graphs and derived constants are shown in **Figure 7** and **Table 9** respectively. The K_m values for the four substrates were fairly similar (75 – 125 μM), but the k_{cat} values varied significantly. (R)-dichlorprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichlorprop). These k_{cat} values were consistent with the range of values seen in the substrate specificity tests shown in **Table 3** and **Table 4** as these were performed at high (saturating) substrate concentrations (1 mM).

[00214] **Table 9:** Kinetic Constants for AAD-1 (v1) Substrates. Kinetic constants were derived from the data in Figure 7 using Grafit fitting to the Michaelis-Menten equation.

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Table 9. Kinetic Constants for AAD-1 (v1) Substrates.

Substrate	K_m (µM) ± SE	V_{max} (µmol min^{-1} mg^{-1} AAD1) ± SE	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
R-dichlorprop	75 ± 10	0.79 ± 0.03	26.1	348
R-quizalofop	125 ± 20	0.57 ± 0.03	18.9	151
R-haloxyfop	120 ± 54	0.34 ± 0.04	11.2	94
2,4-D	96 ± 8	0.57 ± 0.00	2.7	28

[00215] The relative K_m and k_{cat} values for dichlorprop and 2,4-D differ significantly from those published for the R-specific dioxygenase from *Delftia acidovorans* by Westendorf *et al.* (2003) (*Acta Biotechnol.* 23: 3-17). The published k_{cat}/K_m value for 2,4-D is 0.6% that of dichlorprop, whereas in our studies, the k_{cat}/K_m value for 2,4-D is 8% that of dichlorprop. Thus, in this study, AAD-1 (v1) is unexpectedly effective at catalyzing the cleavage of 2,4-D. This increases its potential utility for conferring diverse herbicide tolerance traits in transgenic applications.

[00216] 4.3 – Additional substrates for AAD-1 (v1).

[00217] Three additional substrates were tested at 1 mM using 27 µg crude recombinant AAD-1 (v1) per assay; X11115427, X124987 and MCPA. The results are shown in **Table 8**. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness. X11115427 was only slightly better (125%) as a substrate than 2,4-D. This is in contrast to 3-aminodichlorprop which is >7 fold better than 2,4-D as a substrate (**Table 3**). Thus, the 5-F substitution has significantly decreased the effectiveness of X11115427 as a substrate for AAD-1 (v1). A similar pattern is seen with 5-F-2,4-D which is only 32% as effective as a substrate relative to 2,4-D. In this assay, MCPA was also less effective as a substrate of AAD-1 (v1) (55% relative to 2,4-D).

Example 5 – Optimization of Sequence for Expression in Plants

[00218] 5.1 – Background.

[00219] To obtain high expression of heterologous genes in plants, it may be preferred to reengineer said genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an

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additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

[00220] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.*, TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.*, polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00221] **Table 10** illustrates how high the G+C content is in maize. For the data in **Table 10**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

Table 10: Compilation of G + C contents of protein coding regions of maize genes

Protein Class.sup.a	Range % G + C	Mean % G + C.sup.b
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+-.5.2)

.sup.a Number of genes in class given in parentheses.
.sup.b Standard deviations given in parentheses.
.sup.c Combined groups mean ignored in mean calculation

[00222] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant

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codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

[00223] In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 11**. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, *etc.* with the next preferred codon of choice.

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Table 11. Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

[00224] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00225] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled for the gene sequences for the

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particular plant. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

[00226] 5.2 – Rebuild analysis.

[00227] Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native *AAD-1 (v1)* coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID NO:5) was developed that encodes SEQ ID NO:11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and **Table 12** shows the differences in codon compositions of the native and plant-optimized sequences.

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Table 12. Codon composition comparison of native *AAD-1 (v1)* coding region and Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %	Amino Acid	Codon	Native Gene #		Plant Opt Gene #	Plant Opt Gene %
ALA (A)	GCA	2	9.1	5	22	LEU (L)	CTA	0	0.0	0	0
	GCC	10	45.5	8	35		CTC	5	22.7	7	32
22	GCG	9	40.9	0	0		CTG	16	72.7	0	0
	GCT	1	4.5	10	43	22	CTT	0	0.0	8	36
ARG (R)	AGA	0	0.0	7	30		TTA	0	0.0	0	0
	AGG	0	0.0	7	30		TTG	1	4.5	7	32
	CGA	0	0.0	0	0	LYS (K)	AAA	1	14.3	2	29
23	CGC	16	69.6	5	22	7	AAG	6	85.7	5	71
	CGG	4	17.4	0	0	MET (M)	ATG	8	**100**	8	**100**
	CGT	3	13.0	4	17	PHE (F)	TTC	10	76.9	9	69
ASN (N)	AAC	8	100.0	4	50	13	TTT	3	23.1	4	31
8	AAT	0	0.0	4	50	PRO (P)	CCA	1	5.9	8	47
ASP (D)	GAC	15	78.9	10	53		CCC	9	52.9	1	6
19	GAT	4	21.1	9	47	17	CCG	7	41.2	0	0
CYS (C)	TGC	3	100.0	2	67		CCT	0	0.0	8	47
3	TGT	0	0.0	1	33	SER (S)	AGC	11	73.3	4	27
END	TAA	0	0.0	0	0		AGT	0	0.0	0	0
1	TAG	1	100.0	0	0	15	TCA	0	0.0	4	27
	TGA	0	0.0	1	100		TCC	2	13.3	3	20
GLN (Q)	CAA	0	0.0	7	54		TCG	2	13.3	0	0
13	CAG	13	100.0	6	46		TCT	0	0.0	4	27
GLU (E)	GAA	8	50.0	5	31	THR (T)	ACA	1	4.3	6	26
16	GAG	8	50.0	11	69		ACC	16	69.6	9	39
GLY (G)	GGA	0	0.0	6	29	23	ACG	5	21.7	0	0
	GGC	15	71.4	7	33		ACT	1	4.3	8	35
21	GGG	3	14.3	2	10	TRP (W)	TGG	5	**100**	5	**100**
	GGT	3	14.3	6	29	TYR (Y)	TAC	7	70.0	6	60
HIS (H)	CAC	6	60.0	5	50	10	TAT	3	30.0	4	40
10	CAT	4	40.0	5	50	VAL (V)	GTA	2	7.1	0	0
ILE (I)	ATA	0	0.0	3	25		GTC	11	39.3	8	29
12	ATC	12	100.0	5	42	28	GTG	15	53.6	10	36
	ATT	0	0.0	4	33		GTT	0	0.0	10	36
	Totals	148		149			Totals	148		148	

[00228] 5.3 – Completion of binary vectors.

[00229] 5.3.1 – Rebuilt *AAD-1 (v3)*. The plant optimized gene *AAD-1 (v3)* was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:5) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-1 (v3)* DNA sequence. The *AAD-1 (v3)* gene was cloned into pDAB726 as an Nco I – Sac I fragment. The resulting construct was designated pDAB720,

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containing: [AtUbi10 promoter: Nt OSM 5'UTR: *AAD-1 (v3)*: Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB721, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: *AAD-1 (v3)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] was restriction digested (with Bam HI, EcoR I, EcoR V, HinD III, Pac I, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

[00230] 5.3.2 – Native *AAD1 (v1)* and modified *AAD-1 (v2)*. The *AAD-1 (v1)* gene (SEQ ID NO:3) was PCR amplified from pDAB3203. During the PCR reaction, alterations were made within the primers to introduce the NcoI and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "rdpA(ncoI)" [CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC] (SEQ ID NO:6) and "3'saci" [GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA] (SEQ ID NO:7) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[00231] The PCR amplicon was ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents.

[00232] Sequence data identified a clone with the correct sequence. During analysis a superfluous NotI restriction site was identified toward the 3' end of *AAD-1 (v1)*. This site was removed to facilitate cloning into pDAB3038. To remove the additional site a PCR reaction was performed with an internal 5' primer. The NotI site was altered by incorporating a new codon for an amino acid to remove the spurious NotI site. This change would alter the arginine at position 212 to a cysteine. The PCR primers "BstEII/Del NotI" [TGG TGG TGA CCC ATC CGG GCA GCG GCT GCA AGG GCC] (SEQ ID NO:8) and "3' saci" (SEQ ID NO:7) were used.

[00233] A PCR reaction was completed using the Fail Safe PCR System (Epicenter) and the resulting fragment was cloned into the pCR 2.1 TOPO TA cloning kit (Invitrogen). Confirmation of the correct PCR product was completed by DNA sequencing, and the "fixed" gene was given the designation *AAD-1 (v2)* (SEQ ID NO:4).

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[00234] A sequencing reaction using the M13 Reverse primer (SEQ ID NO:17) and the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents indicated that the correct PCR fragment had been isolated. This construct was digested with the BstEII and SacI enzymes. The resulting fragment was cloned into the pCR2.1 *AAD-1 (v2)* construct (pCR2.1 Delta NotI) and confirmed via restriction enzyme digestion.

[00235] The modified *AAD-1 (v2)* gene was then cloned into pDAB726 as a NcoI/SacI DNA fragment. The resulting construct (pDAB708) was verified with restriction digests. This construct was then cloned into the binary pDAB3038 as a NotI - NotI fragment. The final resulting construct was given the designation pDAB766, containing the [AtUbi10 promoter: Nt OSM5'UTR: *AAD-1 (v2)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] and was restriction digested for verification of the correct orientation. The completed construct was then used for transformation into *Agrobacterium*.

[00236] 5.3.3 – Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is presented herein as SEQ ID NO:20. A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is presented herein as SEQ ID NO:21.

[00237] A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in **Table 13**. Columns D and H of **Table 13** present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the

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protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in **Table 13.** Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

Weighted % of C1 =1/(%C1 + %C2 + %C3 + *etc.*) x %C1 x 100

where C1 is the codon in question, C2, C3, *etc.* represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of **Table 13** (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of **Table 13.** TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Indiana).

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Table 13. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A	B	C	D	E	F	G	H
Amino Acid	**Codon**	**Weighted %**	**Soybean %**	**Amino Acid**	**Codon**	**Weighted %**	**Soybean %**
ALA (A)	GCA	33.1	30.3	**LEU (L)**	CTA	**DNU**	**9.1**
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	**DNU***	**8.5**		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	**DNU**	**9.8**
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	**DNU**	**8.2**	**LYS (K)**	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	**DNU**	**6.0**	**MET (M)**	ATG	100.0	100
	CGT	16.9	14.5	**PHE (F)**	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	**PRO (P)**	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	**DNU**	**8.3**
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	**SER (S)**	AGC	16.0	15.1
END	TAA	**DNU**	40.7		AGT	18.2	17.1
	TAG	**DNU**	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	**DNU**	**6.1**
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	**THR (T)**	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	**DNU**	**8.3**
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	**TRP (W)**	TGG	100.0	100
	GGT	30.4	30.4	**TYR (Y)**	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	**VAL (V)**	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

***DNU** = Do Not Use

[00238] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall

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weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21.

[00239] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is given as bases 1-1575 of SEQ ID NO:22. Synthesis of a DNA fragment comprising SEQ ID NO:22 was performed by a commercial supplier (PicoScript, Houston TX).

[00240] 5.3.4 - Cloning of additional binary constructs. The completion of pDAB3295 and pDAB3757 incorporated the use of the GateWay Cloning Technology (Invitrogen, cat #11791-043 and cat #12535-019). The GateWay Technology uses lambda phage-based site-specific recombination to insert a gene cassette into a vector. For more information refer to *Gateway Technology: A universal technology to clone DNA sequence for functional analysis and expression in multiple systems*, © 1999-2003, Invitrogen Corp., 1600 Faraday Ave., Carlsbad, CA 92008 (printed - 2003). All other constructs created for transformation into appropriate plant species were built using similar procedures as above and other standard molecular cloning methods (Maniatis *et al.*, 1982). **Table 14** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

[00241] The *sacB* gene was added to the binary vector pDAB3289 as a bacterial negative selection marker to reduce the persistence of *Agrobacterium* associated with transformed plant tissue. SacB is a levan-sucrase enzyme produced by *Bacillus* spp. and is toxic to most Gram negative bacteria when grown in the presence of sucrose (Gay *et al.*, 1983). The *sacB* gene was recovered on a *Hin*d III fragment from plasmid pRE112 (Edwards, *et al.*, 1998) and cloned into the unique *Hin*d III site in pDAB3289.

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Table 14. Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Trans-formed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	-	-	-	Erythromycin	-	pat	CsVMV	Agro binary
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	AAD1 v3	CsVMV	Agro binary
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	sacB	HptII	AtUbi3	Agro binary
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	HptII	AtUbi3	Agro binary
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Aerosol beam
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Agro binary
3403		Cn, R	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	Same as GOI		Whiskers / Gun
3404		Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	pat	OsAct1	Whiskers
3415	1283	Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	AHAS v3	OsAct1	Whiskers
3602	1421	Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Spectinomycin	-	AHAS v3	OsAct1	Agro Superbinary
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	pat	AtUbi11	Agro binary
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	-	-	Erythromycin	-	pat	CsVMV	Agro binary

*A = *Arabidopsis*
T = Tobacco
S = Soybean
Ct = Cotton
R = Rice
Cn = Corn
Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region

(721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region
(3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region

ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase

Example 6 – Transformation into *Arabidopsis* and Selection

[00242] 6.1 – *Arabidopsis thaliana* growth conditions.

[00243] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[00244] Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[00245] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2 – *Agrobacterium* transformation.

[00246] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

[00247] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 μl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[00248] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant

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agitation for 4 hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[00249] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28°C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed Agrobacterium cells, suspended in 10 µl of water, at 100° C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C for 3 minutes, 2) 94° C for 45 seconds, 3) 55° C for 30 seconds, 4) 72° C for 1 minute, for 29 cycles then 1 cycle of 72° C for 10 minutes. The reaction was maintained at 4° C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

[00250] 6.3 – *Arabidopsis* transformation.

[00251] *Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog

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salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044μM benzylamino purine (10 μl/liter of 1 mg/ml stock in DMSO) and 300 μl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

[00252] 6.4 – Selection of transformed plants.

[00253] Freshly harvested T_1 seed (transformed with native [*AAD-1 (v2)*] or plant optimized [*AAD-1 (v3)*] gene) was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00254] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00255] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)* (plant optimized gene) or *AAD-1 (v2)* (native microbial gene) to provide phenoxy auxin herbicide resistance.

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[00256] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of phenoxy herbicides (dichlorprop or 2,4-D). Phenoxy rates initially applied were 12.5 g ae/ha 2,4-D and 50 or 200 g ae/ha dichlorprop. GR_{99} for *Arabidopsis* is about 50 g ae/ha 2,4-D and 200 g ae/ha dichlorprop. Elevated rates were applied in subsequent trials (50, 200, 800, or 3200g ae/ha).

[00257] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of phenoxy herbicides in 200 mM Tris buffer (pH 9.0) to a final pH of ~7-8.

[00258] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether haloxyfop could be effectively degraded *in planta*.

[00259] Although *Arabidopsis*, being a dicot, is not an optimal system for testing ACCase-inhibiting AOPP grass herbicides, *AAD-1 (v3)*-transformed T_1 plants were subjected to elevated rates (400-1600 g ae/ha) of RS-haloxyfop acid (internally synthesized) that do cause growth abnormalities and death of wildtype *Arabidopsis* using the DeVilbiss sprayer as described above Injury ratings were taken 7 and 14 days after treatment. Likewise, T_1 individuals were treated with the pyridyloxyacetate auxin herbicide, fluroxypyr.

[00260] 6.5 – Results of selection of transformed plants.

[00261] The first *Arabidopsis* transformations were conducted using *AAD-1 (v3)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493

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glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (*see* **Table 15**). A small lot of *AAD-1 (v2)* (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

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Table 15: Selection of AAD-1 (v3) (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or plant optimized AAD-2 (v2)-transformed T_1 individual plants using glufosinate and 2,4-D.

Selection agent	Gene	Codon bias	Total seed sown and screened	Number of resistant T_1	Selection rate	Selection rate range	% of selected plants expressing PAT[3]
Glufosinate[1]	*AAD-1 (v2)*	n	84,000	278	0.33%	0.33%	nd[4]
Glufosinate[1]	*AAD-1 (v3)*	p	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D[2]	*AAD-1(v3)*	p	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate[1]	*AAD-2 (v1)*	n	1,301,500	228	0.018%	0.007 to 0.021%	100%
Glufosinate[1]	*AAD-2(v2)*	p	200,000	224	0.11%	0.11%	nd[4]

[1]Glufosinate selection scheme: 280g ai/ha glufosinate applied 5-6 + 10 DAP

[2]2,4-D selection scheme: 50 g ai/ha 2,4-D applied 5-7 + 10-14 DAP

[3]PAT protein expression determined by PAT ELISA strips

[4]nd, not determined

[5]codon bias, n-native microbial gene, p=plant optimized

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[00262] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 16** compares the response of native *AAD-1 (v2)* and plant optimized *AAD-1 (v3)* genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Both genes imparted resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 16** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)* or *AAD-1 (v3)* versus the wildtype or *PAT/Cry1F* transformed controls. Also evident is that tolerance appears to be significantly greater (frequency and overall level of individual response) for the plant optimized sequence *AAD-1 (v3)* versus the native sequence *AAD-1 (v2)* (see **Table 16**). Higher rates of 2,4-D (up to 3,200 g ae/ha) have been applied to additional T_1 individuals expressing AAD-1 (v3). Injury levels tend to be greater and the frequency of highly resistant plants is lower at these elevated rates (6x field rates). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, some individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

[00263] **Table 16.** *AAD-1 v3* (plant optimized), or *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

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Table 16. *AAD-1 v3* (plant optimized), or *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence.

	% Injury			% Injury	
Native AAD-1 (v2) gene					Std
Averages	<20%	20-40%	>40%	Ave	Dev
Untreated control-buffer	20	6	7	25.3	34.7
50 g ae/ha 2,4-D	55	16	9	14.8	22.7
200 g ae/ha 2,4-D	45	11	24	34.1	39.3
800 g ae/ha 2,4-D	11	32	37	52.5	34.2
	% Injury			% Injury	
Native AAD-2 gene				Ave	Std
Averages	<20%	20-40%	>40%		Dev
Untreated control-buffer	4	1	1	25.0	21.7
50 g ae/ha 2,4-D	1	2	11	68.2	30.2
200 g ae/ha 2,4-D	0	3	11	82.7	28.8
800 g ae/ha 2,4-D	0	0	14	99.8	0.8
	% Injury			% Injury	
Rebuilt AAD-1 (v3) gene				Ave	Std
Averages	<20%	20-40%	>40%		Dev
Untreated control-buffer	9	0	0	0.0	0.0
50 g ae/ha 2,4-D	10	1	5	24.3	35.9
200 g ae/ha 2,4-D	11	4	1	14.0	25.9
800 g ae/ha 2,4-D	11	4	1	14.7	26.1
	% Injury			% Injury	
Wildtype				Ave	Std
Averages	<20%	20-40%	>40%		Dev
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.0	0.0
200 g ae/ha 2,4-D	0	0	15	95.1	0.5
800 g ae/ha 2,4-D	0	0	15	100.0	0.0
	% Injury			% Injury	
PAT/Cry1F (transformed control)				Ave	Std
Averages	<20%	20-40%	>40%		Dev
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.7	4.2
200 g ae/ha 2,4-D	0	0	15	97.2	1.7
800 g ae/ha 2,4-D	0	0	15	100.0	0.0

[00264] **Table 17** shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400 – 1,600 g ae/ha (**Table 18**). Normal field use rate for haloxyfop (a grass-specific

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herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some *AAD1 (v3)* transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first *in planta* data that *AAD-1 (v3)* will provide AOPP resistance. No resistance was observed with fluroxypyr (a pryridyloxyacetate auxin) in transformed *Arabidopsis*, consistent with *in vitro* work using heterologously expressed enzyme.

[00265] **Table 17.** T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 17. T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence.

AAD-1 v3	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	7	1	0	5.0	7.6	0-20
50 g ae/ha RS-dichlorprop	7	1	0	3.1	8.8	0-25
200 g ae/ha RS-dichlorprop	4	1	3	40.0	50.1	0-100
800 g ae/ha RS-dichlorprop	0	5	3	51.9	40.0	20-100
PAT/Cry1F	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	0	6	2	38.1	25.3	20-95
50 g ae/ha RS-dichlorprop	0	0	8	80.0	25.3	50-100
200 g ae/ha RS-dichlorprop	0	0	8	98.3	2.2	95-100
800 g ae/ha RS-dichlorprop	0	0	8	100.0	0.0	100
Wildtype	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	3	0	0	13.3	2.9	10-15
50 g ae/ha RS-dichlorprop	0	0	3	53.3	5.8	50-60
200 g ae/ha RS-dichlorprop	0	0	3	95.0	5.0	90-100
800 g ae/ha RS-dichlorprop	0	0	3	100.0	0.0	100

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[00266] **Table 18.** T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T1 is an independent transformation event, one can expect significant variation of individual T1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 18. T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide.

AAD-1 v3	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	3	1	0	6.3	9.5	0-20
800 g ae/ha haloxyfop	1	1	2	46.3	42.7	0-85
1600 g ae/ha haloxyfop	1	0	3	65.0	47.3	0-100
PAT/Cry1F	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	10.0	0.0	10
400 g ae/ha haloxyfop	0	4	0	27.5	5.0	20-30
800 g ae/ha haloxyfop	0	0	4	78.8	6.3	70-85
1600 g ae/ha haloxyfop	0	0	4	47.5	43.5	80-100
Wildtype	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	0	3	0	20.0	0.0	20
800 g ae/ha haloxyfop	0	0	3	73.3	10.4	70-85
1600 g ae/ha haloxyfop	0	0	3	93.3	11.5	80-100

[00267] 6.6 – *AAD-1 (v3)* as a selectable marker.

[00268] The ability to use *AAD-1 (v3)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. T_1 seed

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transformed with *PAT* and *AAD1 (v3)* (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active *PAT* gene.

[00269] Fifty-three out of 70,000 seed planted were identified as resistant to 2,4-D. ELISA was used to screen a subset of 44-individuals from this population for PAT protein expression. Ninety six percent of the individuals were positive indicating the presence of the co-transformed gene, *PAT*. The low number of negative ELISA results (4%) is in line with a 3% error rate in populations of glufosinate-resistant plants (**Table 15**). The efficiency of selection appears to be somewhat less with 2,4-D (0.08%) vs. glufosinate (0.12%); however, the range of selection rates across all experiments would indicate both selection agents are equally good for selecting *Arabidopsis* transformed with *AAD-1 (v3)* or *PAT* genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

[00270] 6.7 – Heritability.

[00271] A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 60% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00272] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line tested: ranging in frequency from one to four out of the eight families tested. These data show *AAD1 (v3)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

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[00273] 6.8 –Additional herbicide resistance attributable to AAD-1 in *Arabidopsis*.

[00274] The ability of *AAD-1 (v3)* to provide resistance to other aryloxyphenoxyalkanoate herbicides in transgenic *Arabidopsis* was determined using a modified *in vitro* plate assay. Seeds from wild-type *Arabidopsis thaliana* as well as *Arabidopsis thaliana* containing the plant optimized AAD-1 (v3) gene (T_4 homozygous plants id = PAAD1.315.064) were sterilized by agitating for 10 min in a 50% bleach solution. These seeds were then rinsed four times with sterile water to remove the bleach.

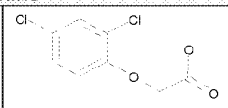
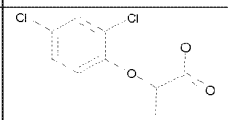
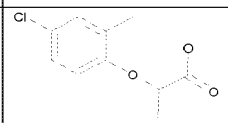
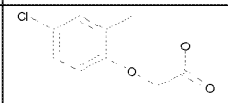
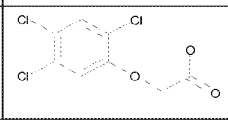
[00275] Dose response assays utilized a nutrient media (see below) supplemented with various rates of test compounds. The test compounds were added to the heated media (55° C) as concentrated solutions in DMSO. Control wells had the appropriate amount of DMSO without any additional compound. The final concentration of DMSO never exceeded 1% (v/v). After thorough mixing, a 6 mL aliquot of the warm media containing the appropriate concentration of compound was added to each well of a 6-well, flat bottom, polystyrene tissue culture tray (Falcon 353046, Becton Dickson and Company, Franklin Lakes, NJ). After the media solidified, approximately 20 to 30 *Arabidopsis* seeds were applied on top of the solidified media and the remaining 2 mL of media was poured over the seeds. The plates were lightly agitated to disperse the seeds, covered and allowed to cool until the media had completely solidified. The plates were incubated for 7 days at 25°C under continual fluorescent lighting (75 $\mu E\ m^{-2}\ s^{-1}$). Nutrient Media Composition was as described in Example 2.2 and in Somerville and Orgen (1982).

[00276] Assessment of growth reduction. The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in **Table 19**. A ratio >2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All

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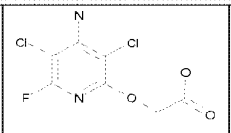
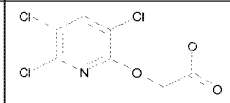
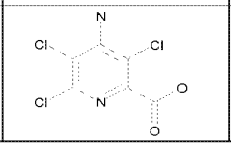
commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported *in planta*. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of *AAD-1*-transformed commercial crops or experimental plant species.

Table 19. *In vitro* plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Phenoxy auxins							
2,4-D		0.2	10	< 0.01	0.04	50	> 4
dichlorprop		2	5	0.01	1.5	2.5	150
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T		1.5	10	<0.01	<0.01	6.67	NA

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Table 19. *In vitro* plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Pyridine auxins							
Fluroxypyr		2	1	0.2	0.2	0.5	1
Triclopyr		0.2	0.04	0.02	0.02	0.2	1
Picloram		1	0.5	0.3	0.15	0.5	0.5

[00277] 6.9 – Foliar applications herbicide resistance in AAD-1 *Arabidopsis* .

[00278] The ability of *AAD-1 (v3)* to provide resistance to other aryloxyphenoxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates described in Example 6.8. T_4 generation *Arabidopsis* seed, homozygous for *AAD-1 (v3)* (line AAD1.01.315.076) was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of phenoxy auxin herbicides: 12.5-1600 g ae/ha 2,4-D dimethylamine salt (DMA) (Riverside Chemicals), 12.5-1600 g ae/ha mecoprop (AH Marks), 50-3200 g ae/ha R-dichlorprop (AH Marks), 8.75-1120 g ae/ha 2,4,5-T (technical grade); pyridyloxyacetates herbicides 50-3200 g ae/ha triclopyr (Dow AgroSciences) and 50-3200 g ae/ha fluroxypyr (Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-1 activity, 2,4-dichlorophenol (DCP, Sigma) (at 50-3200 g ae/ha, technical grade). All applications were formulated in 200 mM Hepes buffer (pH 7.5). Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment and are averaged over two experiments.

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[00279] These results (see **Table 20**) confirm that *AAD-1 (v3)* in *Arabidopsis* provides robust resistance to the phenoxyacetic auxins, phenoxypropionic auxins, but have not shown significant cross resistance to the pyridyloxyacetic auxins tested and corroborates the *in vitro* enzyme and whole plate substrate specificity data. Additionally, there is no effect of the metabolite, 2,4-dichlorphohenol (DCP), on wildtype or transgenic *Arabidopsis*.

Table 20. Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Phenoxypropionic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**PatCry1f - Control**
50 g ae/ha R-Dichlorprop	3	31
200 g ae/ha R-Dichlorprop	3	73
800 g ae/ha R-Dichlorprop	3	89
3200 g ae/ha R-Dichlorprop	3	95
12.5 g ae/ha Mecoprop	3	0
25 g ae/ha Mecoprop	0	2
50 g ae/ha Mecoprop	0	17
100 g ae/ha Mecoprop	0	33
200 g ae/ha Mecoprop	3	62
400 g ae/ha Mecoprop	0	78
800 g ae/ha Mecoprop	0	93
1600 g ae/ha Mecoprop	0	100
Phenoxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Cry1f - Control**
12.5 g ae/ha 2,4-D DMA	0	67
25 g ae/ha 2,4-D DMA	0	78
50 g ae/ha 2,4-D DMA	0	93
100 g ae/ha 2,4-D DMA	0	100
200 g ae/ha 2,4-D DMA	0	100
400 g ae/ha 2,4-D DMA	0	100
800 g ae/ha 2,4-D DMA	0	100
1600 g ae/ha 2,4-D DMA	0	100
8.75 g ae/ha 2,4,5-T	0	0
17.5 g ae/ha 2,4,5-T	3	20
35 g ae/ha 2,4,5-T	0	43
70 g ae/ha 2,4,5-T	3	85
140 g ae/ha 2,4,5-T	0	95
280 g ae/ha 2,4,5-T	0	98
560 g ae/ha 2,4,5-T	17	100
1120 g ae/ha 2,4,5-T	3	100

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Table 20. Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Pyridyloxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Cry1f - Control**
50 g ae/ha Triclopyr	31	36
200 g ae/ha Triclopyr	58	65
800 g ae/ha Triclopyr	74	84
3200 g ae/ha Triclopyr	97	95
50 g ae/ha Fluroxypyr	48	76
200 g ae/ha Fluroxypyr	75	85
800 g ae/ha Fluroxypyr	88	85
3200 g ae/ha Fluroxypyr	95	95
	Inactive DCP metabolite	
50 g ae/ha 2,4-DCP	0	0
200 g ae/ha 2,4-DCP	0	0
800 g ae/ha 2,4-DCP	0	0
3200 g ae/ha 2,4-DCP	0	0

[00280] 6.10 – Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis*.

[00281] An experiment was designed to examine if the level of AAD-1 (v3) expression in *Arabidopsis* varies at different growth stages. A high-tolerance, homozygous, *AAD-1 (v3)* T_4 line (id=PAAD1.01.345.163) was grown in greenhouse. Half of the plants were treated with 800 g ae /ha of 2,4-D (as previously described) while the other half were not treated. Two leaves, the 3rd leaf from the top and the 5th leaf from the bottom, were harvested from 5 plants, both treated and untreated, and analyzed by ELISA and Western Blotting (as described in Example 11) experiments at 4, 10, 14, 20 and 25 DAT. **Figures 8A** and **8B** showed that there was statistically no difference in AAD-1 (v3) expression between young and old leaves. In addition, the herbicide 2,4-D had little impact on the expression level of AAD-1 (v3) protein. The protein levels accumulated in older plants with some significant protein degradation at the later time points.

[00282] In a separate experiment, four different homozygous T_4 lines of *Arabidopsis* displaying different tolerance level to the herbicide 2,4-D were sprayed with various levels (0, 200, 800 and 3200 g/ha) of 2,4-D and their herbicide injury and AAD-1 (v3) expression were examined. Four days after the herbicide treatment, little injury was observed in three of the four lines, even at the highest dose tested (**Figure 9A**). These plants also expressed high

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level of AAD-1 (v3), from 0.1 to 0.25% of (**Figure 9B**). On the contrary, the low tolerance line expressed less than 0.1% of AAD-1 (v3) in TSP and suffered observable injury. More importantly, they recovered from the injury on 14 DAT (**Figure 9A**), indicating that the low level of AAD-1 (v3) expression was able to protect the plants from the serious herbicide damage. All control plants suffered serious injury and died 14 DAT at doses 800 g ae/ha 2,4-D and above.

[00283] 6.11 – Molecular analysis of *AAD-1 (v3) Arabidopsis*.

[00284] Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number and/or Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple *AAD-1 (v3)* homozygous lines to determine stable integration of the plant transformation unit containing *PAT* and *AAD-1 (v3)*. Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[00285] For Southern analysis, a total of 1 µg of DNA was subjected to an overnight digest of Nsi I for pDAB721 to obtain integration data. The samples were run on a large 0.85 % agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of *PAT*. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then

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subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at -80° C.

[00286] Results showed all 2,4-D resistant plants assayed contained *PAT* (and thus by inference, *AAD-1 (v3)*). Copy number analysis showed total inserts ranged from 1 to >10 copies. This correlates, too, with the AAD-1 (V3) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance (>>200 fold) to all commercially available phenoxyacetic and phenoxypropionic acids.

[00287] 6.12 – *Arabidopsis* transformed with molecular stack of *AAD-1 (v3)* and glyphosate resistance gene.

[00288] T_1 *Arabidopsis* seed was produced, as previously described, containing pDAB3230 plasmid (*AAD-1 (v3)* + *EPSPS*) coding for a putative glyphosate resistance trait. T_1 transformants were selected using *AAD-1 (v3)* as the selectable marker as described in example 6.6, except the 2,4-D rate used was 75 g ae/ha. Twenty-four T_1 individually transformed events were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, *AAD-1 (v3)* + *PAT* T_5 homozygous lines (pDAB721-transformed), and *PAT* + *Cry1F* homozygous line (transformed control). Only pDAB3230 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in 200 mM Hepes buffer (pH 7.5). All treatments were replicated 4 or 5 times. Plants were evaluated 7 and 14 days after treatment. I_{50} values were calculated and show >14 fold level of tolerance imparted by *EPSPS* molecularly stacked with *AAD-1 (v3)* (*see* **Figure 10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate + 2,4-D treatments applied in tankmix as described in Example 21 and shown for *AAD-1 (v3)*-transformed corn in Example 8.

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Example 7 – WHISKERS-Mediated Transformation Into Maize, and Use of *AAD-1 (v3)* as a Selectable Marker

[00289] 7.1 – Cloning of *AAD-1 (v3)*.

[00290] The *AAD-1 (v3)* fragment was received on an NcoI/SacI fragment. Construct pDAB4005 was digested with NcoI and SacI and the 5175 bp backbone fragment isolated. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. The correct intermediate plasmid was named pDAB3403. Both pDAB3403 and pDAB8505 (OsAct1/PAT/ZmLip) were digested with NotI. The 3442 bp band from pDAB3403 and the 11017 bp band from pDAB8505 were isolated and purified. The fragments were ligated together, transformed into DH5α, and the resulting plasmids were screened for orientation. The final construct was designated pDAB3404, which contains ZmUbi1/po-aad1/ZmPer5::OsAct1/PAT/ZmLip.

[00291] 7.2 – Callus/suspension initiation.

[00292] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[00293] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.*, 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[00294] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS

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Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L *myo*-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

[00295] 7.3 – Cryopreservation and thawing of suspensions.

[00296] Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C/minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

[00297] For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until

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a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

[00298] 7.4 – Dose response of non-transformed tissue to haloxyfop acid.

[00299] Non-transformed donor Hi-II cell lines were pooled together, WHISKERS-treated without DNA, filtered onto GN6 medium at the rate of 6 ml per filter, and allowed to callus for 2-3 weeks at 28° C. Approximately 200 mg of callused suspension tissue was transferred per treatment to selection media in 60x20 mm plates containing 30, 100 or 300 nM R-haloxyfop acid. Three replicates were used per concentration. A control medium containing 1 mg/L bialaphos (from Herbiace commercial formulation, Meiji Seika, Japan), GN6 (1H), was also included for comparison. Callus was removed after 2 weeks, weighed, and transferred to fresh media of the same concentration for another 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded. Results are shown in **Figure 11**.

[00300] Two separate dose response studies of callus tissue to the phenol degradation products of haloxyfop and cyhalofop, respectively, were also completed to confirm that this end product would not be deleterious to callus growth. Data from a cyhalofop phenol dose response (see **Figure 12)** shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol. Data from a haloxyfop phenol dose response showed that even at 300 nM haloxyfop phenol, growth was equal to or greater than the control lacking haloxyfop phenol (data not shown).

[00301] 7.5 – WHISKERS-mediated transformation using bialaphos selection.

[00302] Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L *myo*-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of GN6 S/M

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liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00303] After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00304] Either 3 or 6 mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C in a dark box that was loosely sealed with a single layer of plastic (<2 mils thick) to minimize evaporation of the individual plates.

[00305] After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (1H) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 1.0 mg/L bialaphos, 2.5 g/L Gelrite, pH 5.8) or GN6D (1H) medium (same as GN6 (1H) except with 8.0 mg/L dicamba and 0.8 mg/L 2,4-D).

[00306] Plates were placed in boxes and cultured as before for an additional week. Two weeks post-transformation, the tissue was embedded by scraping either ½ the cells on the plate or else all cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C) containing 1 mg/L bialaphos. The tissue was broken up and the 3 mL of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (1H) or GN6D (1H) medium. This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for 1 week at 28° C in dark boxes.

[00307] Putatively transformed isolates were typically first visible 5-8 weeks post-transformation. Any potential isolates were removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained

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growth was evident after approximately 2 weeks, an event was deemed to be resistant and was submitted for molecular analysis.

[00308] Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (1H), containing 1 mg/L bialaphos, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week then higher light (40 $\mu Em^{-2}s^{-1}$) for another week before being transferred to regeneration medium, 36 (1H), which was identical to 28 (1H) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

[00309] 7.6 – WHISKERS-mediated transformation using haloxyfop selection.

[00310] DNA delivery parameters for direct selection on "fops" were identical to the bialaphos selection procedure except that 85 µg of pDAB3403 and 85 µg of construct containing a GFP (Green Fluorescent Protein) reporter gene were co-transformed together, and only 3 mL of suspension was filtered onto GN6 medium following the 2-hour recovery.

[00311] After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60x20-mm plates of GN6 medium containing 2 mg/L 2,4-D plus 50, 100, or 200 nM R-haloxyfop acid. Plates were placed in boxes and cultured for one additional week. After one week, the tissue was embedded by scraping all cells from the plate into 3.0 mL of melted GN6 agarose medium containing the same concentration of selection agent as in the previous transfer. All steps afterward were identical to the PAT selection/regeneration protocol except that 100 nM R-haloxyfop acid was included in the regeneration media instead of 1 mg/L bialaphos.

[00312] 7.7 – Results. Multiple experiments testing various levels of haloxyfop and cyhalofop were initiated, and 47 isolates were recovered from direct selection. A subset of the callus events were submitted for screening using PCR and Western analyses. Following these expression data, 21 lead events were submitted for Southern analysis. Results using NcoI, a unique cutter, to obtain integration data following probing with *AAD-1 (v3)*, unequivocally demonstrate stable integration of *AAD-1 (v3)* following Whiskers-mediated transformation coupled with "fop" selection.

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[00313] 7.8 – Quantitative demonstration of *in vitro* tolerance from AAD-1 (v3) expressing callus events from bialaphos selection

[00314] Ninety-seven callus isolates recovered from bialaphos selection were submitted for PAT copy number via Invader analysis and *AAD-1 (v3)* PTU analysis via PCR (see Example 7.10). AAD-1 (v3) protein expression using Western blot/Sandwich ELISA (Example 11) was completed on a subset of the events. A summary is described in **Table 21** below. At least 15 T_0 plants were regenerated from each of these events and sent for spray testing and seed production.

Table 21.

Event	PAT Copy #	PCR for *AAD-1 (v3)* PTU	Callus Western
3404-001	2	+	+
3404-006	2	+	+
3404-013	3	+	+
3404-017	1	+	+
3404-020	3	+	nd
3404-022	2	+	+
3404-025	2	+	+
3404-027	3	+	+
3404-031	1	+	+
3404-033	2	+	nd
3404-036	3	+	+
3404-044	3	+	+
3404-050	3	+	+
3404-053	3	+	+
3404-074	2	+	+
3404-082	2	+	+

[00315] A smaller subset of these events was assessed in dose-response studies in comparison to a non-transformed control. A range of concentrations of haloxyfop (from Gallant Super formulation), up to 400 nM, were tested. Dose response data for one event, 3404-006, was generated using the general methods of Example 7.4, and is shown in **Figure 13**. This demonstrates that event 3404-006 showed no significant reduction in callus growth at haloxyfop concentrations up to 400 nM whereas non-transgenic corn callus tissue growth was inhibited at this rate. These data have not been normalized to account for inherent growth differences that are not related to the expression of the transgene.

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[00316] 7.9 –WHISKERS-mediated transformation using imazethapyr selection.

[00317] The ZmUbi1/*AAD-1 (v3)*/ZmPer5 cassette was removed from pDAB3404 with AscI/SwaI and inserted into pDAB2212 to create the *AAD-1 (v3)* and *AHAS* Whiskers transformation vector pDAB3415, which is also referred to as pDAS1283) Once completed, this construct was transformed into maize via silicon carbide whiskers-mediated transformation as described in Example 7.4 except 2 mL of cells were filtered, followed by a 7-day recovery on GN6 medium followed by selection on media containing 3 µM imazethapyr from Pursuit® DG herbicide. Following Invader analysis, 36 events were identified that contained the AAD-1 (v3) and the AHAS genes.

[00318] Fifty-three corn calli events from these transformations were tested in both ELISA and Western Blotting experiments for their AAD-1 (v3) expression – a subset of the data is shown below. For the events listed in **Table 22,** the expression levels of those detected positive ranged from 90 to over 1000 ppm of total soluble proteins.

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Table 22.		
AAD-1 Event ID Number	**Expression Level (ppm)**	**Western Blot**
1283[1]-001	2	-
1283[1]-002	206	+++
1283[1]-003	1	-
1283[1]-004	90	+++
1283[1]-005	1	-
1283[1]-006	105	+++
1283[1]-007	212	++
1283[1]-008	114	±
1283[1]-009	305	+
1283[1]-010	2	-
1283[1]-011	4	-
1283[1]-012	200	+++
1283[1]-013	134	+++
1283[1]-014	4	-
1283[1]-015	194	+++
1283[1]-016	4	-
1283[1]-017	196	+++
1283[1]-018	3	-
1283[1]-019	178	+
1283[1]-020	260	++
1283[1]-021	144	+++
1283[1]-022	140	+++
1283[1]-023	191	+++
1283[1]-024	392	++
1283[1]-025	368	++
1283[1]-026	14	-
1283[1]-027	1006	++
Neg Control	3	-
Neg Control	3	-
Standard (0.5 µg/mL)		++
Standard (5 µg/mL)		++++

[00319] 7.10– Molecular Analysis: Maize Materials and Methods.

[00320] 7.10.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

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[00321] 7.10.2 – Invader assay analysis. The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis was used to identify the estimated copy of the unknown events.

[00322] 7.10.3 – Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-1 (v3)* PTU were (Forward – ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:25) and (Reverse – CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:26). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR *AAD-1 (v3)* were (Forward – ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse – CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00323] 7.10.4 – Southern blot analysis. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 µg of DNA was subjected to an overnight digestion of Afl II and also EcoRV for pDAB3404, NcoI for pDAB3403, and SpeI for pDAB1421 to obtain integration data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85 % agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC was then set up to

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obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using the above coding region PCR using plasmid pDAB3404. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at -80° C.

Example 8 – *In vivo* tolerance and field tolerance data generated from *PAT*-selected (pDAB3404) *AAD-1 (v3)* events

[00324] 8.1 - Tolerance of T_0 corn plants to AOPP herbicides.

[00325] If more than 15 clone plants per event were successfully regenerated, then extra plants were transferred to the greenhouse for preliminary tolerance screening with postemergence-applied AOPP herbicides on T_0 corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (*i.e.*, plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C under 16 hour light: 8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assure® II (DuPont), Clincher* (Dow AgroSciences), or Gallant Super* (Dow AgroSciences) for quizalofop, cyhalofop, or haloxyfop, respectively. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height, and all sprays contained 1% v/v Agridex crop oil concentrate adjuvant. The number of clones of each event varied from week to week due to the rate of regeneration and acclimation of each event. Overall, an attempt was made to treat representative clones of each event with a range of herbicide doses ranging from 1X

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lethal dose (~1/8 X field dose) up to 8X field doses (64 X lethal dose). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

[00326] AOPP's are generally very potent corn-killing herbicides. Three to four leaf Hi-II corn grown from seed is effectively killed (>95% injury) with 8.8, 62.5, and 4.4 g ae/ha of haloxyfop, cyhalofop, and quizalofop, respectively. Each *AAD-1 (v3)*-transformed line tested survived a minimally lethal dose of each AOPP herbicide tested. In fact, most lines tested survived with no visible injury (14 DAT) even when treated with an 8X field dose (64X lethal dose) of quizalofop. Several individual clones from events "017" and "038," however, did incur significant injury at elevated rates. This could be a function of lower gene expression due to how or where the gene was inserted.

[00327] The high level of AOPP tolerance was demonstrated in most events, even when applications were made to plants just coming out of tissue culture (T_0 stage). Significantly, this tolerance was shown for all three AOPP herbicides and likely will extend to all AOPP herbicides as previously shown for AAD-1 *in vitro*.

[00328] **Figure 14** shows the responses of several *AAD-1 (v3)*–transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied 1 week prior.

[00329] **Table 23** shows data for the responses of selected *AAD-1 (v3)*-transformed T_0 corn events to three AOPP herbicides applied postemergence.

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Table 23. Selected AAD-1 (v3)-transformed T0 corn events response to three AOPP herbicides applied postemergence.

Construct	Event	Clone	Haloxyfop* g ae/ha	Cyhalofop** g ae/ha	Quizalofop*** g ae/ha	% Injury 14 DAT
3404	001	016	8.8			0
3404	001	018		62.5		0
3404	001	017			8.8	0
3404	001	019			8.8	30
3404	001	020			35	0
3404	017	018			35	0
3404	017	019			35	0
3404	017	020			70	0
3404	017	021			70	0
3404	017	022			140	0
3404	017	023			140	30
3404	017	024			280	30
3404	017	025			280	20
3404	022	019	8.8			0
3404	022	020	17.5			0
3404	022	016	17.5			0
3404	022	024		62.5		0
3404	022	018		125		0
3404	022	021			8.8	0
3404	022	017			17.5	0
3404	022	022			35	0
3404	022	023			70	0
3404	033	012			35	0
3404	033	013			70	0
3404	033	014			70	0
3404	033	015			140	0
3404	033	016			280	0
3404	038	016	8.8			0
3404	038	018		62.5		0
3404	038	017			8.8	0
3404	038	019			35	70
3404	038	020			35	80
3404	038	021			70	80
3404	038	022			70	80
			(lethal dose = 8.8 g ae/ha)	(lethal dose = 62.5 g ae/ha)	(lethal dose = 4.4 g ae/ha)	

*Gallant super[#] + 1% COC (v/v)
**Clincher [#] + 1% COC (v/v)
***Assure II + 1% COC (v/v)
[#]Trademark of Dow AgroSciences, LLC

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8.2 – Field tolerance of pDAB3404 T_1 corn plants to quizalofop 2,4-D, and glufosinate herbicides.

[00330] Two field trials were established at field stations in Hawaii and Indiana. Corn seed from inbred T_1 plants were utilized to evaluate sixteen AAD1 event lines for tolerance against quizalofop and 2,4-D. Three non-transformed hybrids were included for comparison purposes. The hybrid Hi-II x 5XH571 is of the same parentage as the *AAD-1 (v3)* event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

[00331] The experimental design was a split-plot with four replications. The main plot was herbicide treatment and the sub-plot was *AAD-1 (v3)* event or comparison hybrid. Plots were one row by 3.7 meters with approximately twenty-five seeds planted in each row. For AAD1 events, seeds from a different lineage within the event were planted in each replicate.

[00332] Glufosinate at 560 g ai/ha was applied to *AAD-1 (v3)* plots at the V2 stage to eliminate non-transformed plants. Experimental treatments included commercial formulations of quizalofop applied at 70 and 140 g ae/ha, 2,4-D (dimethylamine salt) at 560 and 1120 g ae/ha, and an untreated control. Treatments were applied using backpack broadcast boom equipment delivering 187 L/ha carrier volume at 130-200 kpa pressure. Quizalofop treatments were applied at the V3-V4 corn stage and 2,4-D treatments were applied at the V5-V6 stage.

[00333] Quizalofop treated plots were visually assessed for crop injury at one and three weeks after application (WAA) using a 0-100% scale, where 0 equals no injury and 100 equals complete death. 2,4-D treated plots were visually assessed for plant leaning at 2 days after application (DAA) using 0-100% scale where 0 equals no leaning from any plant and 100 equals all plants prone. Additionally, 2,4-D plots were visually assessed at 3-4 WAA for brace root deformation using a 0-10 scale.

[00334] 8.2.1 – Results.

[00335] *AAD-1 (v3)* event response to the highest rates tested of quizalofop and 2,4-D are shown in **Table 24**. These rates represent approximately twice the normal commercial use rates. Non-transformed hybrids were severely injured (80-100%) by quizalofop at 70 g ae/ha including the sethoxydim resistant line, although it displayed slightly better tolerance than the other two hybrids. All *AAD-1 (v3)* events except one lineage of event 3404.001 displayed excellent tolerance to quizalofop at 70 g ae/ha. No visible symptoms were observed on the *AAD-1 (v3)* events except with the events noted above.

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Table 24.

Treatment (rate) =	Quizalofop (140 g ae/ha)		2,4-D amine (1120 g ae/ha)		2,4-D amine (1120 g ae/ha)		Analysis Results			Resistance Segregation Ratios following Liberty Spray		
Evaluation =	% Injury 3 WAA (0-100 scale)		% Leaning 2 DAA (0-100 scale)		Braceroot Deformation 3-4 WAA (0-10 scale)		AAD-1 copy number leaf (Southern analysis)	AAD-1 Leaf Western Blot T_0	AAD-1 Leaf ELISA T_0	T_1 Population Average		T_2 Population Average
Event or Hybrid	IN	HI	IN	HI	IN	HI				IN	HI	HI
3404.001	25	25	0	5	1	2	3	+++	+++	40%	47%	X
3404.006	0	0	0	0	0	0	1	++	+++	33%	26%	X
3404.013	0	0	0	0	0	0	1	+	+++	63%	58%	X
3404.017	0	0	1	0	0	0	2	+/-	++	48%	47%	X
3404.020	0	0	0	0	0	0	2	++	+++	50%	51%	X
3404.022	0	0	1	0	0	0	1	+	++	51%	57%	76%*
3404.025	0	0	0	0	0	0	2	+++	++++	55%	59%	X
3404.027	0	0	3	0	0	0	5	+	++	51%	50%	X
3404.031	0	0	1	0	0	0	1	++	++++	47%	43%	61%*
3404.033	0	0	0	0	0	0	2 or 3	+	++	52%	49%	X
3404.036	0	0	0	0	0	0	4	++	+++	52%	48%	X
3404.044	0	0	1	0	0	1	1	+	+/-	50%	48%	X
3404.050	0	0	0	1	0	1	2	nd	nd	38%	28%	X
3404.053	0	0	1	0	0	0	2	+++	+++	48%	56%	X
3404.074	0	0	0	0	0	0	1	++	+++	53%	52%	73%*
3404.082	0	0	0	0	0	1	3	nd	nd	38%	36%	X
DK493	100	100	20	23	8	9						
HI-II X 5XH571	100	100	13	34	7	9						
CROPLAN 585SR	80	96	11	33	9	9						

* Fits single locus dominant trait segregation as determined by chi square analysis (P>0.05)

[00336] 2,4-D at the 1120 g ae/ha rate caused significant levels (11-33%) of epinastic leaning in the non-transformed hybrids, a normal response when applied beyond the V4 growth stage. Little or no leaning was observed with all *AAD-1 (v3)* events except one lineage of 3404.001 (Indiana location only) where moderate levels (5-13%) of leaning occurred.

[00337] Brace roots of non-transformed hybrids were severely deformed (rating of 9 on a 0-10 scale) by 2,4-D at the 1120 g ae/ha rate. Again, this is a normal response to 2,4-D applied beyond the V4 growth stage. As with the leaning response, little or no brace root injury was observed with all *AAD-1 (v3)* events except one lineage of 3404.001.

[00338] Similar trends occurred with lower tested rates of quizalofop and 2,4-D although at reduced but still significant response levels in the non-transformed hybrids (data not shown).

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[00339] These results indicate that most *AAD-1 (v3)* transformed event lines displayed a high level of resistance to quizalofop and 2,4-D at rates that were lethal or caused severe epinastic malformations to non-transformed corn hybrids. *See also* **Figure 16.**

[00340] 8.2.2- Expected Mendelian segregation ratios on three T_2 events. Plants from individual lineages of each event were randomly self-pollinated in the field. T_2 seed were hand harvested at physiological maturity. Based on single gene copy number (see **Table 24** above) and overall performance in T_1 generation (segregation, herbicide tolerance, and vigor), three events (022, 031, and 074) were chosen for further evaluation in the field. Breeding rows of each event were planted using a precision cone planter each consisting of 2500-3000 seeds. At the V2 growth stage, all *AAD-1 (v3)* lines were sprayed with 140 g ae/ha quizalofop (Assure® II) using a backpack sprayer as previously described. This rate rapidly killed all "null" (untransformed) segregants. Each event had a segregation ratio consistent with Mendelian inheritance of a single locus, dominant gene (3 resistant:1 sensitive, or 75% survival) (see **Table 24**). Homozygotes and hemizygotes from event 74 were identified by zygosity testing (refer to *AAD-1 (v3)* Invader assay description for corn). Hemizygous plants were removed and homozygous *AAD-1 (v3)* plants were crossed with BE1146 corn inbred introgressed and homozygous for glyphosate resistance trait, NK603, creating a homogeneous F_1 hybrid seed that is hemizygous for glyphosate resistance, *AAD-1 (v3)*, and glufosinate resistance.

[00341] 8.3 – Stacking of *AAD-1 (v3)* and *PAT* with glyphosate resistance genes in corn.

[00342] Homozygous T_2 *AAD-1 (v3)/PAT* corn plants were crossed with glyphosate resistant corn plants producing F_1 seed containing *AAD-1 (v3)*, *PAT*, and glyphosate resistance genes as described in the previous example.

[00343] F_1 seeds were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium (Sun Gro Horticulture). The pots were initially subirrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were subirrigated with deionized water.

[00344] Plants were allowed to grow until 2-4 leaves had emerged from the whorl. At this point herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. The plants were sprayed with rates of 2,4-D DMA, glyphosate, glufosinate, and various combinations of the three. All applications were formulated in 200

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mM Hepes buffer (pH 7.5). In spray applications where glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

[00345] At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Results of the study at 14 DAT can be seen in **Table 25.**

Table 25.

	Field Rate	% Injury at 14 DAT	
		Hi II X 5XH751	RR/PAT/AAD1
		Ave	Ave
Untreated control	-	0	0
840 g ae/ha glyphosate	1X	98	0
1680 g ae/ha glyphosate	2X	100	0
3360 g ae/ha glyphosate	4X	100	0
560 g ae/ha 2,4-D DMA	1X	10	0
1120 g ae/ha 2,4-D DMA	2X	14	0
2240 g ae/ha 2,4-D DMA	4X	29	0
470 g ae/ha glufosinate	1X	80	0
940 g ae/ha glufosinate	2X	90	3
1880 g ae/ha glufosinate	4X	96	15
840 g ae/ha glyphosate + 560 g ae/ha 2,4-D DMA	1X + 1X	96	1
1680 g ae/ha glyphosate +1120 g ae/ha 2,4-D DMA	2X + 2X	100	2
3360 g ae/ha glyphosate + 2240 g ae/ha 2,4-D DMA	4X + 4X	100	1
470 g ae/ha glufosinate + 560 g ae/ha 2,4-D DMA	1X + 1X	89	5
940 g ae/ha glufosinate + 1120 g ae/ha 2,4-D DMA	2X + 2X	91	10
1880 g ae/ha glufosinate + 2240 g ae/ha 2,4-D DMA	4X + 4X	97	13
840 g ae/ha glyphosate + 470 g ae/ha glufosinate	1X + 1X	90	5
1680 g ae/ha glyphosate + 940 g ae/ha glufosinate	2X + 2X	98	15
3360 g ae/ha glyphosate + 1880 g ae/ha glufosinate	4X + 4X	100	15

[00346] This study demonstrated that the *AAD-1 (v3)* gene in corn can be stacked with a glyphosate resistance gene and a glufosinate resistance gene to provide robust field-level tolerance to 2,4-D, glyphosate, and glufosinate alone or in tank mix combinations.

[00347] 8.3.1 – Resistance of *AAD-1 (v3)* corn using a tank mix of 2,4-D DMA and quizalofop. T_2BC_1 seeds of hemizygous event number 3404-025.001R/R001 Bulked.001.S058 were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium. The pots were initially sub-irrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were sub-irrigated with de-ionized water.

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[00348] Plants were allowed to grow until V1 stage in the greenhouse. At this point the plants were selected with 560 g ae/ha Assure® II with the addition of 1% Agridex crop oil concentrate in 200 mM Hepes buffer with the research track sprayer set at 187 L/ha. Plants were allowed 4 days to show symptoms of the selection. All plants were uninjured. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 18-in spray height. All applications were formulated in 200 mM Hepes buffer (pH 7.5) with the addition of 1% v/v Agridex.

At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Plants from this particular lineage had 0% injury at 14 DAT for all tank mixed combinations, while the wild-type had 100% injury. These results indicate AAD-1 (v3) not only provides robust field level resistance to 2,4-D and quizalofop individually, but also to exaggerated rates of multiple combinations of the two chemistries. One could logically expect to implement novel weed control measures with combinations of phenoxy auxins and AOPP graminicides in corn (or other crops transformed with AAD-1) not previously enabled by a single gene HTC.

[00349] 8.3.2 - Tolerance of (pDAB3403) T_0 corn plants to quizalofop herbicide. A target of approximately eight T_0 plant clones from each of 17 events were regenerated and transferred to the greenhouse for preliminary tolerance screening with postemergence-applied discriminating rate of quizalofop herbicide applied by track sprayer at 35 g ae/ha (1X field rate, 4X lethal dose) to 3-leaf, greenhouse-adapted T_0 corn plants using track sprayer conditions previously described. Plants were rated as Resistant or Sensitive 7 days after treatment. Control, non-transgenic corn was included with each spray application. Two events, Event 014 and 047, had two or more T_0 clones sensitive to 35 g ae/ha quizalofop, indicating an unexpected level of sensitivity for this event. The 15 other events showed stable integration, protein expression, and the ability to tolerate a 4X lethal dose of quizalofop at the whole plant level.

[00350] 8.3.3 Expression of AAD-1 (v3) with respect to quizalofop tolerance.

[00351] Three different T2 lineages from 3404 transformations that were pre-screened with Liberty® (as described previously) to remove nulls were chosen to compare their tolerance to quizalofop with respect to their AAD-1 (v3) expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT (see **Figure 15.**). The highest tolerance line, event

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3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1X and higher field rates. This data concludes that corn expressing AAD-1 (v3) can be protected from quizalofop injury at the highest level tested (2,240 g/ha), which is 16 times the 1X field dose of 35 g/ha. In addition, the expression level was consistent throughout the period of the experiment.

Example 9 - *Agrobacterium*-Mediated Transformation of Maize with *AAD-1 (v3)*

[00352] 9.1 - Plant material.

[00353] Seeds of a "High II" (*i.e.*, Parent A and B) F_1 cross (Armstrong *et al.*, 1991) are planted directly into 5 gallon-pots containing 95:5 Metro-Mix® 360: Mineral soil. The plants are grown in the greenhouse with a 16 hour photoperiod supplemented by a combination of high pressure sodium and metal halide lamps.

[00354] 9.2 - Tissue source.

[00355] For obtaining immature Hi-II (F2) embryos, controlled sib-pollinations were performed. On the day of pollination, actively shedding tassels are bagged, and fresh pollen is collected and applied carefully onto the silks. Immature embryos were isolated as described in Example 7.2.

[00356] 9.3 - Preparation of a superbinary vector.

[00357] Construction of an *Agrobacterium* construct, pDAB2272, containing the *AAD-1 (v3)* gene in combination with the AHAS selectable marker gene was accomplished by isolating the 3443 base pair NotI fragment from pDAB3404 containing ZmUbi1 v2/ *AAD-1 (v3)*/ZmPer5 v2 and inserting it into the NotI site of pDAB8549. The resulting plasmid contains the ZmUbi1 v2/ *AAD-1 (v3)*/ ZmPer5 v2 and the OsAct1 v2/AHAS v3/ZmLip v1 cassettes flanked by non-identical MAR regions in the direct orientation. This was subsequently transformed into LBA4404/pSB1 to create the superbinary vector, which was named pDAB3602 but was also referred to as pDAS1421.

[00358] 9.4 - Bacterial supply.

[00359] All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Patent No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The

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bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~ $1x10^9$ cfu/ml) and 100 µM acetosyringone added to the solution.

[00360] 9.5 - Infection and cocultivation.

[00361] The immature embryos are isolated directly into a microfuge tube containing 2 ml LS-inf. Mod liquid medium. Each tube, containing ~100 embryos, is vortexed for 3-5 sec. The medium is removed and replaced with fresh liquid medium and the vortex is repeated. The liquid medium is again removed and this time replaced with an *Agrobacterium* solution at the Klett 200 concentration. The *Agrobacterium* and embryo mixture is vortexed for 30 sec. Following a 5 minute incubation at room temperature, the embryos were transferred to LS-As Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 30.0 g/L sucrose, 6 mM L-proline, 0.85 mg/L $AgNO_3$,1, 100 µM acetosyringone, 3.0 g/L Gelrite, pH 5.8) for a 5-day co-cultivation at 25° C.

[00362] 9.6 - Dose response using immature embryos.

[00363] Dose response studies were initiated using immature embryos treated with *Agrobacterium* strain LBA4404 lacking a plasmid as described previously. Once treated, embryos were allowed to co-cultivate for 5 days at 25° C and were then transferred to selection media containing various levels of R-haloxyfop or R-cyhalofop. Embryos were also transferred to media containing 1 mg/L bialaphos and 100 nM imazethapyr as negative controls. Embryos were scored for % embryogenic callus formation after 2 weeks, and then again after 4 weeks. Embryos were tested on R-haloxyfop levels up to 30 nM; however, insufficient reduction of callus formation was seen at the highest levels, so higher concentrations (50 – 100 nM) were used during transformation experiments. Data from embryos grown on cyhalofop-containing media is shown in **Figure 17**.

9.7 – Selection.

[00364] After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then

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moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 µM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the *AHAS* gene is used, based on U.S. Patent No. 5,731,180. Tissue was transferred at biweekly intervals on the same medium until embryogenic colonies were obtained. These colonies were maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies were bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and further analysis.

[00365] 9.8. – Regeneration and seed production.

[00366] For regeneration, the cultures are transferred to 28 "induction" medium and 36 "regeneration" medium as described previously containing either 100 nM R-haloxyfop or 1.5 µM cyhalofop for differentiation of plantlets. When plantlets were established, they were transferred to SHGA tubes to allow for further growth and development of the shoot and roots as described previously. Controlled pollinations for seed production were conducted as described previously.

[00367] 9.9 - Event recovery and analysis particulars; whole plant screening of T_0 corn lineages containing *AAD-1 (v3)* and *AHAS* (pDAS1421).

[00368] Seventy-two *Agrobacterium*-transformed events were selected on various levels of R-haloxyfop acid and R-cyhalofop acid *in vitro*. Twenty-two callus samples were analyzed by Southern blot analysis for stable integration of *AAD-1 (v3)* into the genome as described previously. Ten single copy events, as indicated in **Table 26,** were chosen to be regenerated.

Table 26.

Corn event	*In vitro* Selection agent	Southern Copy # (callus)	Western Blot (AAD-1) T_0 Callus	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop	
	(nM)	AAD-1		Resistant	Sensitive
1421[21]-016	50 Haloxyfop	1	+	8	0
1421[22]-020	100 Haloxyfop	1	++	8	0
1421[22]-022	100 Haloxyfop	1	+	8	0
1421[22]-023	100 Haloxyfop	1	++	8	0
1421[3]-036	100 Haloxyfop	1	++	8	0
1421[4]-031	300 Cyhalofop	1	++	9	0
1421[4]-032	300 Cyhalofop	1	++	8	0
1421[4]-033	300 Cyhalofop	1	++	12	0
1421[4]-034	300 Cyhalofop	1	++	8	0
1421[4]-035	300 Cyhalofop	1	++	6	0
Events with more than 1 copy were not taken to the greenhouse					

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[00370] A minimum six regenerated clonal lineages per event were moved to soil in the greenhouse and screened using a track sprayer as previously described to apply 35 g ae/ha quizalofop when 2-4 new, normal leaves had emerged (see section 8.3.3). Presence of AAD-1 protein on a Western blot correlated perfectly with herbicide resistance in the T_0 generation regardless of which AOPP herbicide was used for selection. There is no negative impact of the second HTC gene (*AHAS*) on the function of the *AAD-1 (v3)*.

Example 10 – Purification of AAD-1 (v1) for Antibody Creation and Biochemical Characterization

[00371] All operations during purification were carried out at 4° C. Frozen or fresh *E. coli* cells from approximately 1 L culture, grown and induced as in Example 3, were re-suspended in 200 ml of extraction buffer containing 20 mM Tris-HCl, 1 mM EDTA, and 2 ml of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The soluble extract was obtained by centrifugation in a GSA rotor (Sorvall) at 12,000 rpm (24,000g) for 20 minutes. The supernatant was then loaded onto a Mono Q ion exchange column (Pharmacia HR 10/10) equilibrated with 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, and the column was washed with same buffer for 10 CV (80 ml). The protein was eluted with 80 ml of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 ml fractions were collected. The fractions containing AAD-1 (v1) were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl, 0.15 M NaCl, and 1 mM DTT, pH 8.0 at a flow rate of 1 ml/min. Purification procedures were analyzed by SDS-PAGE, and protein concentration was determined by Bradford assay using bovine serum albumin as standard.

Example 11 – Recombinant AAD1 Purification and Antibody Production

[00372] Plasmid pDAB3203 containing the *AAD-1 (v1)* gene was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 µL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were

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scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000x g for 20 min to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

[00373] All AAD-1 (v1) purifications were conducted at 4° C as discussed in Example 10, unless otherwise stated. The cell lysate was loaded onto a Mono Q ion exchange column (Pharmacia Cat. #HR 10/10) equilibrated with 20 mM Tris-HCl (pH 8.0) 1 mM EDTA, followed by 80 mL of washing with the same buffer. The proteins were eluted with 80 mL of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 mL fractions were collected. The fractions containing AAD-1 were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl and 1 mM DTT. Protein concentration was determined by Bradford assay using bovine serum albumin as standard.

[00374] Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

[00375] 11.1 – Extracting AAD-1 (v3) from plant leaves.

[00376] Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 µL plant extraction buffer (PBS containing 0.1 % Triton X-100 and 10 mM DTT). The tubes were shaken for 4 min with a bead beater at maximum speed followed by centrifugation for 10 min at 5,000x g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

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[00377] 11.2 – Bradford assay.

[00378] Total soluble protein concentration from plant leaf tissues were determined by Bradford assay using bovine serum albumin (BSA) as standard. Five micro-liter of serially diluted BSA in PBS or plant extract was transferred to 96-well microtiter plate in triplicates. For standards, concentrations were ranged from 2000 to 15.6 µg/mL. The protein assay concentrate was first diluted 5 fold in PBS and 250 µL was added to each well and incubated at room temp for 5 min. Each optical density (OD) was measured at 595 nm using a microplate reader. The protein concentration of each sample was extrapolated from standard curve using the Softmax® Pro (ver. 4.0) (Molecular Devices).

[00379] 11.3 – Enzyme linked immuno-sorbent assay (ELISA).

[00380] The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-1 antibody (0.5 µg/mL) was coated on 96-well microtiter well and incubated at 4° C for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 µL standard AAD-1 of known concentrations or plant extract (see previous section) was incubated in the wells. For standard curve, purified AAD-1 concentrations ranged from 100 to 1.6 ng/mL in triplicates. Plant extracts were diluted 5, 10, 20, and 40 fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-1 antibody-HRP conjugate (0.25 ug/mL) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 µL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-1 in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

[00381] For comparison, each sample was normalized with its TSP concentration and percent expression to TSP was calculated.

[00382] 11.4 – Western blotting analysis.

[00383] Plant extracts or AAD-1 standards (5 and 0.5 µg/mL) were incubated with Laemmli sample buffer at 95° C for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane

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using standard protocol. After blocking in 4% skim milk in PBS, AAD-1 protein was detected by anti-AAD1 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham cat. #RPN 21058).

Example 12 – Tobacco Transformation

[00384] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch *et al.*, 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB721, *AAD-1 (v3)* + *PAT*), grown overnight in a flask on a shaker set at 250 rpm at 28° C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

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[00385] Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$).

[00386] Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The *PAT* gene which was molecularly linked to *AAD-1 (v3)* was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00387] The DNA samples were diluted to 9 ng/µl, then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgCl2 (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates were then incubated at 63° C for 1.5 hours and read on the florometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events (**Table 27**).

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Table 27. Tobacco T0 events transformed with pDAB721 (*AAD-1(v3) + PAT*).

Event	PAT copy number (Southern)	Coding Region PCR for AAD-1	ELISA (µg AAD-1 / ml plant extract)	Relative tolerance T0 spray with 2,4-D *
721(1)1	1	+	0.9	Medium
721(2)1	nd	nd	0.6	Medium
721(2)2	5	+	0.3	Low
721(2)3	3	+	2.6	Medium
721(2)5	5	+	4.1	Variable
721(2)6	3	+	0.5	Variable
721(2)8	5	+	0.3	High
721(2)11	3	+	n/a	High
721(2)12	3	+	4.1	Medium
721(2)13	2	+	0.5	Medium
721(2)14	5	+	0.2	High
721(2)16	4	+	3.2	Medium
721(2)17	3	+	nd	High
721(2)18	5	+	nd	High
721(2)19	>10	+	nd	Low
721(2)20	5	+	nd	Medium
721(2)21	4	+	nd	High
721(2)22	7	+	nd	Medium
721(2)23	>10	+	nd	Variable
721(3)003	3	+	nd	Variable
721(3)008	2	+	nd	High
721(3)012	1	+	nd	High
721(3)4	2	+	0.5	High
721(3)5	9	+	3.3	High
721(3)6	4	+	7.1	Variable
721(3)9	2	+	1	Low
721(3)10	3	+	0.6	High
721(3)11	7	+	6	Low
721(3)13	4	+	0.1	High
721(3)014	2	+	0.1	Medium

nd = not done

Legend:	Relative tolerance*	Injury at 3200 g ae/ha 2,4-D (14DAT)
	Low	>50% injury
	Medium	20-50% injury
	High	<20% injury
	Variable	inconsistent

[00388] Copy number estimations were confirmed by Southern Analysis on several events. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 µgs of DNA was subjected to an overnight digest of NsiI and also HindIII for pDAB721 to obtain integration data. After the overnight digestion an aliquot of ~100 ngs

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was run on a 1% gel to ensure complete digestion. After this assurance the samples were processed using same protocol as in Example 6 section 11.

[00389] All events were also assayed for the presence of the *AAD-1 (v3)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the Coding Region PCR AAD-1 were (RdpAcodF ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:27) and (RdpAcodR CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 30 PCR positive events were regenerated and moved to the greenhouse.

[00390] A representative plant from each of 19 events was assayed for AAD-1 (v3) expression by ELISA methods previously described. All events assayed showed detectable levels of AAD-1 (v3) (**Table 27**). Protein expression varied across events.

[00391] T_0 plants from each of the 30 events were challenged with a wide range of 2,4-D sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 50, 200, 800, or 3200 g ae/ha to representative clones from each event mixed in deionized water. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 800 g ae/ha or less. Some events were uninjured at this rate (equivalent to 1.5X field rate). All events suffered some level temporary auxinic damage 3 DAT when treated with 3200 g ae/ha. Some leaf burning also occurred at this high rate due to the acidity of the spray solution. Future trials at high 2,4-D rates were buffered. Response of T_0 plants treated with 3200 g ae/ha 2,4-D (~6X field rate) was used to discern relative tolerance of each event into "low" (>50% injury 14 DAT), "medium" (20-50% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (**Table 27**).

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[00392] Verification of high 2,4-D tolerance.

[00393] Two to four T_0 individuals surviving high rates of 2,4-D were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. Two *AAD-1 (v3)* tobacco lines (event 721(2)-013.010 and 721(3)-008.005) were chosen from the T_0 generation. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4), followed by selective removal of untransformed nulls in this segregating population with 280 g ai/ha glufosinate (PAT selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided medium and high levels of robustness to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY 160 tobacco. All plants were sprayed with the use of a track sprayer set at 187L/ha. The plants were sprayed from a range of 70-4480 g ae/ha 2,4-D dimethylamine salt (DMA), R-Dichlorprop, and a 50/50 mix of the two herbicides. All applications were formulated in 200mM Hepes buffer (pH 7.5). Each treatment was replicated 4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity. (**Table 28**). No injury was observed at rates below 1X field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by *AAD-1 (v3)*, even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

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Table 28. Segregating AAD-1 T_1 tobacco plants' response to phenoxy auxin herbicides.

Herbicide	KY160 – Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
560 g ae/ha 2,4-D DMA	75	5	0
1120 g ae/ha 2,4-D DMA	80	5	2
2240 g ae/ha 2,4-D DMA	90	5	0
4480 g ae/ha 2,4-D DMA	95	5	5
560 g ae/ha R-dichlorprop	70	5	0
1120 g ae/ha R-dichlorprop	75	5	0
2240 g ae/ha R-dichlorprop	88	5	0
4480 g ae/ha R-dichlorprop	95	10	5
560 g ae/ha 2,4-D DMA/R-dichlorprop	80	5	5
1120 g ae/ha 2,4-D DMA/R-dichlorprop	80	10	10
2240 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15
4480 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15

[00394] A 100 plant progeny test was also conducted on each of the two *AAD-1 (v3)* lines (events 721(2)-013.010 and 721(3)-008.005). The seeds were stratified, sown, and transplanted with respect to the procedure above except null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Both event '013' and '008' segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-1 is heritable as a robust phenoxy auxin resistance gene in multiple species.

[00395] Field level tolerance will be demonstrated by planting T_1 or T_2 seed in the greenhouse, selectively removing the null plants by Liberty selection as previously described, and rearing individual seedlings in 72-well transplant flats (Hummert International) in Metro 360 media according to growing conditions indicated above. Individual plants will be transplanted into the field plots using an industrial vegetable planter. Drip or overhead irrigation will be used to keep plants growing vigorously. Once plants reach 6-12 inches in height, tobacco plants will be sprayed with a broad range of phenoxy auxins and rated as shown above. Environmental stresses are more significant under field conditions; however, based on previous experience with *AAD-1 (v3)*-transformed corn, robust translation of resistance from the greenhouse to the field is expected.

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Example 13 – Soybean Transformation

[00396] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00397] Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee *et al.*, 1989; McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-1 (v3)* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-1 (v3)*.

[00398] 13.1 – Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens*.

[00399] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly

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dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee *et al.*, 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00400] 13.1.1 – *Agrobacterium* preparation. The plasmid, pDAB721, contains the *AAD-1 (v3)* gene under the control of the *Arabidopsis* Ubi10 promoter. This plasmid also carries the *PAT* gene under the control of rice actin promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. This vector can be used in the transformation experiment described below. The construct pDAB721 was mobilized into the *Agrobacterium* strain EHA101S by electroporation.

[00401] *Agrobacterium* cultures harboring pDAB721 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

[00402] 13.1.2 – Plant transformation. Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be rinsed five times with sterile water on hormone-free SHGA medium and allowed to

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germinate for 5 days at 24° C, with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg *et al.* (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100 x 25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee *et al.*, 1988).

[00403] Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* re-suspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES(2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 µM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

[00404] Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin , 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

[00405] Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from10 mg to 100 mg/L. The elongation medium consisted of B5

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medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

[00406] The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

[00407] 13.1.3 – Progeny evaluation. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1 (v3)* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn. Determination of the inheritance of *AAD-1 (v3)* will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

[00408] 13.2 – Transformation Method 2: "No-shake" *Agrobacterium* mediated transformation of non-regenerable soybean suspension cells.

[00409] The DAS Soybean cell suspensions were cultured on a 3d cycle with 10 ml of settled suspension volume transferred to 115 ml of fresh liquid medium. Settled cell volume was determined by allowing the cell suspension to settle for 2 min in the 125-mL flask after vigorous swirling and then drawing cells from the bottom of the flask with a wide bore 10 ml pipette. The flasks were then transferred to orbital shakers at 140 rpm.

[00410] Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 µM acetosyringone (AS) onto a 100x25 sterile Petri plate. EHA105 *Agrobacterium* suspension at a density of 1.2 OD^{650} in a 100 µL volume was added and mixed well. The *Agrobacterium* and suspension cell mixture was swirled well and the plate was transferred to dark growth chamber where the temperature was maintained at 25° C.

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[00411] 13.2.1 – Selection of soybean suspension cells and isolation of transformed colonies. After 4 days of co-cultivation the plate was swirled again to mix the suspension well and a 1.5 ml aliquot was transferred to the selection medium and spread on the gel medium on a 100x15 ml Petri-plate. The selection medium consisted of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose and the medium was amended with glufosinate ammonium at 5 mg/L level. After a 10 min drying in the hood the plates were incubated for 4 weeks in dark at 28° C. Colonies appeared in selection and a total of 11 colonies were transferred to fresh medium from 3 different experiments and maintained for 3-4 months. All the 11 resistant colonies produced calli that were growing on the 5 mg/L glufosinate containing selection medium. The non-transformed suspension cells were sensitive when plated on to 0.5 mg/l glufosinate ammonium medium. However, the transformed colonies were resistant to 5x concentration of glufosinate ammonium and were maintained for up to 4 months.

[00412] Callus events were sampled for analyses when they reached 2 to 3 cm in diameter. At least two of colony isolates, one each from two different experiments, were analyzed for AAD1 protein expression. The ELISA and Western analysis carried out on these two isolates showed positive expression of AAD1 proteins. Both ELISA (**Table 29**) and Western Blotting (**Figure 18**) analysis on two separate soybean calli transformed with *AAD-1 (v3)* gene indicated that the callus cells are expressing AAD-1 (v3) protein. The sandwich ELISA detected 0.0318% and 0.0102% total soluble protein of AAD-1 (v3) in two different callus tissue samples. Due to the sensitivity and cross reactivity of the antiserum, multiple bands were observed in the western blot. However, AAD-1 (v3) specific band was observed in both callus samples but not in the wild type (negative) tissue. Coding region PCR analyses showed the expected size products of the AAD1 and the PAT coding regions in these colonies indicating that they were transformed.

Table 29. PCR and ELISA data for transgenic soybean events.

Event	AAD-1 Coding region PCR	PAT Coding region PCR	T SP (µg/mL)	AAD-1 (ng/mL)	% Expression
1-1	+	+	1995.13	633.89	0.0318%
2-1	+	+	2018.91	205.92	0.0102%
Negative Control	-	-	2074.63	-1.22	-0.0001%

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[00413] 13.3 – Transformation Method 3: aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[00414] Culture of embryogenic soybean callus tissue and subsequent beaming were as described in U.S. Patent No. 6,809,232 (Held *et al.*).

[00415] Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144 and 96E692, were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3295 using linearized DNA at a concentration of approximately 0.2 µg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos. With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light. Embryos that had elongated and produced roots were transferred to tubes containing 1/2 B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

[00416] Variations of media referred to in **Table 30** were tested, *e.g.*, B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/l myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/l myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and 1/2 B5G which contained all ingredients of B5G medium at half strength.

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Table 30. Growth Media for Soybean			
	Ingredients in 1 Liter		
	B1-30	**B3**	**B5G**
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		
Activated charcoal		5 g	
Phytagar	8g	8g	
Gelrite			2g
pH	5.8	5.8	5.8

Example 14 – *AAD-1 (v3)* Enablement in Cotton

[00417] 14.1 – Cotton transformation protocol.

[00418] Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (**Table 31**) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 µE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[00419] Cotyledon segments (~5mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (**Table 31**) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (**Table 31**) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (**Table 31**). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[00420] *Agrobacterium* preparation. Inoculate 35ml of Y media (**Table 31**) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C, while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (**Table 31**) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per mL with a total volume of 40 ml.

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[00421] After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. Callus is then transferred to CG-media (**Table 31**), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (**Table 31**) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

[00422] Larger, well-developed embryos are isolated and transferred to DK media (**Table 31**) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

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Table 31. Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1ml	1 ml	1 ml				
agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

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[00423] 14.2 – Experiment specifics.

[00424] For this experiment 500 cotyledon segments were treated with pDAB721. Of the 500 segments treated, 475 had callus isolated while on selection (95% transformation frequency). The callus was selected on glufosinate-ammonium, due to the inclusion of the *PAT* gene in the construct, since there was already a selection scheme developed. Callus line analysis in the form of PCR and Invader were initiated to determine the insertion patterns and to be sure the gene was present at the callus stage, then callus lines that were embryogenic were sent for Western analysis.

[00425] 14.3 – Callus analysis results.

[00426] The object of the analysis is to eliminate any lines that do not have the complete PTU, show no expression or that have high copy number, so those lines are not regenerated. Of the 475 pDAB721 transformed callus lines, 306 were sent for PCR analysis and Invader assay (**Table 32**). Very few lines were PCR negative. The Invader results are not complete at this time, because some samples had low DNA amounts when extracted, and these have been resubmitted. However, the current Invader data shows a few of the lines submitted have high copy number (copy number of >2) (**Table 32**). Due to the large number of lines that passed analysis, it was necessary to decrease the number of embryogenic callus lines being maintained due to the volume. Ninety lines have been sent for Western analysis, and eight of those were negative. The western analysis showed high expression from the majority of the lines (**Table 32**). Eighty-two embryogenic callus lines are being maintained for plant regeneration based on analysis results (and results pending).

Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western	Line	Copy #	PTU	Western
1	2	pos	***	43	1	pos	*****
2	1	pos	***	44	low DNA	pos	*****
3	1	pos	***	45	1	pos	*****
4	2	pos	***	46	2	pos	*****
5	1	pos	***	47	1	pos	***
6	1	pos	neg	48	1	pos	***
7	1	pos	****	49	3	faint pos	neg
8	1	pos	*****	50	1	pos	****
9	2	pos	*	51	4	pos	neg
10	1	pos	*	52	2	pos	****
11	1	pos	*****	53	1	pos	***
12	1	pos	*	54	1	pos	****

Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western	Line	Copy #	PTU	Western
13	1	pos	*	55	1	pos	neg
14	1	pos	**	56	5	pos	*
15	1	pos	****	57	2	faint pos	neg
16	1	pos	*****	58	8	pos	****
17	1	pos	*	59	2	pos	****
18	1	pos	***	60	5	pos	*****
19	1	pos	**	61	1	pos	**
20	1	pos	*****	62	1	pos	**
21	2	pos	*****	63	1	pos	***
22	1	pos	*****	64	1	pos	***
23	1	pos	*****	65	3	pos	****
24	4	pos	* or neg	66	5	pos	*
25	1	pos	****	67	6	pos	*
26	1	pos	****	68	Low DNA	pos	neg
27	low DNA	pos	*****	69	Low DNA	pos	****
28	low DNA	pos	**	70	low DNA	pos	**
29	low DNA	pos	*****	71	low DNA	faint pos	**
30	17	pos	*	72	low DNA	pos	****
31	low DNA	pos	*****	73	low DNA	neg	***
32	low DNA	pos	*****	74	low DNA	faint pos	***
33	low DNA	pos	****	75	Low DNA	pos	***
34	low DNA	faint pos	*****	76	Low DNA	pos	neg
35	low DNA	pos	****	77	low DNA	faint pos	****
36	low DNA	pos	neg	78	low DNA	pos	****
37	low DNA	pos	****	79	1	pos	**
38	low DNA	neg	*****	80	low DNA	pos	***
39	1	pos	****	81	low DNA	pos	***
40	low DNA	pos	*	82	low DNA	pos	***
41	low DNA	pos	*	83	low DNA	neg	****
42	low DNA	pos	**	84	low DNA	pos	***
				85	low DNA	pos	**
standard	AAD1	5ug/ml	*****	86	low DNA	pos	*
standard	AAD1	0.5ug/ml	**				

[00427] 14.4 – Plant regeneration.

[00428] Two *AAD-1 (v3)* cotton lines have produced plants according to the above protocol that have been sent to the greenhouse. To demonstrate the *AAD-1 (v3)* gene provides resistance to 2,4-D in cotton, both the *AAD-1 (v3)* cotton plant and wild-type cotton plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed at 560 g ae/ha 2,4-DMA formulated in 200 mM Hepes buffer (pH 7.5). The plants were evaluated at 3, 7, and 14 days after treatment. Plants were assigned injury ratings with respect to stunting,

chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Three days after treatment (DAT) the wild-type plant began showing epinasy and received a rating of 15%; in contrast, the *AAD-1 (v3)* plant showed 0% injury. By 7 DAT epinasy continued on the wild-type and the new growth shoots began turning brown. It received a rating of 50% at this time. At 14 DAT the *AAD-1 (v3)* plant was still uninjured, whereas the wild-type was severely stunted, and the new growth areas were brown and shriveled. Thus, the wild-type received a rating of 90% at 14 DAT.

[00429] This study demonstrates that the *AAD-1 (v3)* gene in cotton provides substantial tolerance to 2,4-D up to at least 560 g ae/ha.

Example 15 – *Agrobacterium* Transformation of Other Crops

[00430] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, *see, e.g.,* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.,* Hinchee *et al.*, 1988. For *Agrobacterium*-mediated transformation of sorghum, *see, e.g.,* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see, e.g.,* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see, e.g.,* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see, e.g.,* Hiei *et al.*, 1997.

[00431] The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform *AAD-1 (v3)*, for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta*, and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato (*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Compositae Lactuca sativa*, *perennis*, and *pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), Sorghum (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans

(*Leguminosae Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (*Leguminosae Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Compositae Helianthus annuus*), Squash (*Dicotyledoneae Cucurbita* spp.), Cucumber (*Dicotyledoneae genera*), Tobacco (*Solanaceae Nicotiana* spp.), *Arabidopsis* (*Cruciferae Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, and other families), and Clover (*Leguminosae*). Such plants, with *AAD-1 (v3)* genes, for example, are included in the subject invention.

Example 16 – Stacking *AAD-1 (v3)* with AHAS Herbicide Resistance Gene

[00432] Stacking *AAD-1 (v3)* with an AHAS herbicide resistance gene is described in Example 7.9.

Example 17 – Further Evidence of Surprising Results: *AAD-1 vs. AAD-2*

[00433] 17.1 – *AAD-2 (v1)* initial cloning.

[00434] Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as *AAD-2 (v1)* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00435] The strain of *Bradyrhizobium japonicum* containing the *AAD-2 (v1)* gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site

(RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 (added a Xho I site)].

[00436] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/µl), H_2O 21 µl, Taq polymerase 1 µl (2.5 units/µl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00437] Ten of the resulting white colonies were picked into 3 µl Luria Broth + 1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA GT 3'] (SEQ ID NO:16) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2 (v1)* for internal consistency.

[00438] 17.2 – Completion of *AAD-2 (v1)* binary vector.

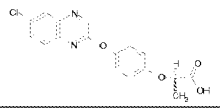
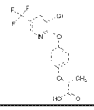
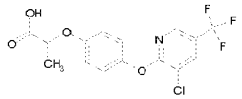
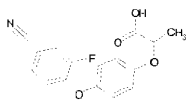
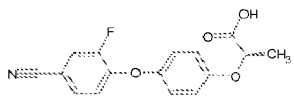
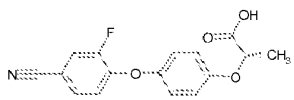
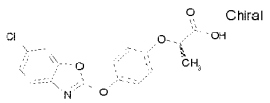
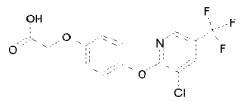
[00439] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:18) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:19) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a

clone with the correct sequence (pDAB716). The AflIII/SacI *AAD-2 (v1)* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI – NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: *AAD-2 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

[00440] 17.3 – Comparison of substrate specificities of AAD-2 (v1) and AAD-1 (v1)

[00441] The activity of an extract from *E. coli* expressing AAD-2 (v1) (pDAB3202) prepared as in Example 11 was tested on four herbicides, 2,4-D, (R,S)-dichlorprop, (R,S)-haloxyfop and (R)-haloxyfop (all at a final concentration of 0.5 mM) using 3 µl (42 µg) of *E. coli* extract per assay with a 15 min assay period. **Figure 22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D = dichlorprop > (R,S)-haloxyfop >> (R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. **Table 33** shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

Table 33. Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 µM Fe^{2+}, 200 µM Na ascorbate, 1 mM α-ketoglutarate using 4 µl AAD1 (32 µg protein) extract or 3 µl AAD2 extract (42 µg protein).

				A510	
STRUCTURE	**Reg ID**	**Compound**	**Enantiomer**	**AAD1**	**AAD2**
	18706	quizalofop	R	0.27	0.01
	8671	haloxyfop	R	0.12	0
	66905	haloxyfop	R,S	0.1	0.3
	14623	cyhalofop	R,S	0.12	0.1
	14603	cyhalofop	R	0.14	0
	7466	cyhalofop	S	0	0.15
 Chiral	11044492	fenoxaprop	R	0.14	0
	43865	haloxyfop-acetate	--	0	0.22

[00442] The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. *See* **Figure 19.** The K_m values for 2,4-D were 97 and 423 µM for AAD-1 (v1) and AAD-2 (v1) respectively and the apparent V_{max} values were 0.11 and 0.86 A_{510} units, respectively (**Table 34**). Because equivalent amounts of enzyme were used in the assays (as determined by SDS-PAGE analysis), it can be concluded that the k_{cat} of AAD-2 (v1) for 2,4-D is almost 8-fold higher than AAD-1 (v1) and the k_{cat}/K_m is 2-

fold higher. Thus AAD-2 (v1) is significantly more efficient at cleaving 2,4-D *in vitro* than AAD-1 (v1). This is in surprising contrast to the *in planta* findings reported below, where plants expressing AAD-1 (v1) are significantly better in conferring 2,4-D resistance relative to AAD-2 (v1).

Table 34. Comparison of the Km and "Vmax" values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	Km (μM) ±SE	Vmax (A510 units)	Km/Vmax (arbitrary units)
AAD-2	2,4-D	423 (±1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (±21)	0.11	1.16

Notes: Assays were performed in MOPS pH 6.75 + 1 mM α-ketoglutarate + 0.1 mM Na ascorbate + 0.1 mM Fe2+ and the released phenols colorimetrically detected using 4-aminoantipyrine/ferricyanide.

[00443] 17.4 – Evaluation of transformed *Arabidopsis*.

[00444] Freshly harvested T_1 seed transformed with a native [*AAD-1 (v2)*], plant optimized [*AAD-1 (v3)*], or native *AAD-2 (v1)* gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00445] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet and then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00446] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and

plants moved to the greenhouse (22±5° C, 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)*, *AAD-1 (v2)*,or *AAD-2 (v1)* to provide phenoxy auxin herbicide resistance.

[00447] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of 2,4-D (50-800 g ae/ha).

[00448] Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO) mixed in 200 mM Tris buffer (pH 9.0).

17.5 – Results of selection of transformed plants.

The first *Arabidopsis* transformations were conducted using *AAD-1 (v3)*. T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (*PAT* gene), equating to a transformation/ selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see **Table 15** in Example 6.5 above). A small lot of native *AAD-1 (v2)* transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency). Surprisingly, *Arabidopsis* transformations using the native *AAD-2(v1)* gene provided a very low transformation frequency when selected for glufosinate tolerance (*PAT* selectable marker function). Approximately 1.3 million seed have been screened and only 228 glufosinate transformants were recovered, equating to a transformation/selection frequency of 0.018% (*see* **Table 15**). Transformation frequency for native *AAD-2 (v1)* was only 6% of that of native *AAD-1 (v2)*. The native *AAD2 (v1)* gene was subsequently synthetically optimized, cloned, and transformed as pDAB3705, into Arabidopsis using methods previously described (see Example 5). The plant optimized *AAD2 (v2)* (SEQ ID NO:29, which encodes SEQ ID NO:30) yielded a normal T_1 *Arabidopsis* selection frequency using Liberty herbicide of approximately 0.11% (*see* **Table 15**).

[00449] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 16** (in

Example 6.5 above) compares the response of *AAD-1 (v2)*, *AAD-1 (v3)*, *AAD-2 (v1)* and *AAD-1(v2)* genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 16** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)*, *AAD-1 (v3)*, *AAD-2 (v1)* or *AAD-2 (v2)* versus the wildtype or *PAT/Cry1F* transformed controls.

[00450] Surprisingly, *AAD-2 (v1)* transformants were far less resistant to 2,4-D than either *AAD-1 (v2)* or *AAD-1 (v3)* genes (**Table 16**) both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) *AAD-1 (v2)*-transformed T_1 plants survived 200 g ae/ha 2,4-D uninjured (population injury average = 34%), and >73% (11 of 15) *AAD-1 (v3)* T_1 plants were uninjured (population injury average = 14%). *See* **Figure 20**. Tolerance improved slightly for plant-optimized *AAD2 (v2)* versus the native gene; however, comparison of both *AAD-1* and *-2* plant optimized genes indicates a significant advantage for *AAD-1 (v3) in planta* (see **Table 16**).

[00451] These results are unexpected given that *in vitro* comparison of native AAD-1 (v2) and AAD-2 (v1) indicated 2,4-D was better degraded by AAD-2 (v1). AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. Little correlation exists between expression level noted on the Western blot and the level of injury from 2,4-D on the same plants. *See* **Figure 21**. No large difference was evident in protein expression level (*in planta)* for the native and plant optimized *AAD-2* genes. These data corroborate earlier findings that make the functional expression of AAD-1 (v3) *in planta* for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected .

Example 18 – Preplant Burndown Applications

[00452] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

[00453] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 – 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (*e.g.*, horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (*see* federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With *AAD-1 (v3)* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will

be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1 (v3)* gene if stably transformed.

Example 19 – In-Crop Use of Phenoxy Auxins Herbicides in *AAD-1 (v3)* Only Transformed Soybeans, Cotton, and Other Dicot Crops

[00454] *AAD-1 (v3)* can enable the use of phenoxy auxin herbicides (*e.g.*, 2,4-D, dichlorprop, MCPA, *et al.*) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560 – 1120 g ae/ha is used. For a complete weed control system, grass weeds must be controlled. A variety of broad spectrum graminicide herbicides, including but not limited to haloxyfop, quizalofop, fenoxaprop, fluazifop, sethoxydim, and clethodim are currently registered for use in most dicot crops which are naturally tolerant to these herbicides. A combination of quizalofop (20 – 100 g ae/ha) plus 2,4-D (420 – 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1 (v3)*-transformed dicot crop (*i.e.*, soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (*see* weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[00455] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/*AAD-1 (v3)* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if

broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

[00456] Further benefits could include tolerance to 2,4-D drift or volatilization as mechanisms for off-site 2,4-D injury to dicot crops; no interval required before planting following 2,4-D application (*see* previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicides) can still be used for the subsequent control of *AAD-1 (v3)*-transformed dicot crop volunteers.

[00457] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1 (v3)* gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - In-Crop Use of Phenoxy Auxins and AOPP Herbicides in *AAD-1 (v3)* Only Transformed Corn, Rice, and Other Monocot Species

[00458] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with *AAD-1 (v3)* would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. *AAD-1 (v3)*-transformed monocot crops would, therefore,

enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560 – 1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, *et al.* could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of *AAD-1 (v3)*-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20 – 100 g ae/ha) plus 2,4-D (420 – 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1 (v3)* transformed monocot crop (*e.g.*, corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[00459] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-1 (v3)* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-

crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

[00460] Those skilled in the art will now also recognize that the above example can be applied to any monocot crop that would be protected by the *AAD-1 (v3)* gene from injury by any AOPP herbicide. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 – *AAD-1 (v3)* Stacked With Glyphosate Tolerance Trait in Any Crop

[00461] The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-1 (v3)* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with*AAD-1 (v3)*, one can selectively

apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (*e.g.*, *Commelina* spp), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica,* 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *AAD-1 (v3)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (preferably 20-100 g ae/ha and more preferably 20-35 g ae/ha), could be for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D.

[00462] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or

independently) with one or more commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 22 - *AAD-1 (v3)* Stacked with Glufosinate Tolerance Trait in Any Crop

[00463] Glufosinate tolerance (*PAT or bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

[00464] By stacking *AAD-1 (v3)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3)*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and

broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

i) Inherently tolerant grass weed species (e.g., *Echinochloa spp* or *Sorghum spp*) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00465] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 23 - *AAD-1 (v3)* Stacked with *AHAS* Trait in Any Crop

[00466] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone

herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking *AAD-1 (v3)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3)*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

   i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

   ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea spp.* can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

   iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00467] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxy auxin herbicides, or AOPP herbicide, alone or in multiple combinations, is enabled by *AAD-1 (v3)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 24 – *AAD-1 (v3)* in Rice

[00468] 24.1 – Media description.

[00469] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26° C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang *et al.* 1996).

[00470] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) consisted of NB

medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/l hygromycin B for 3–4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/l hygromycin B (1/2MSH50).

[00471] 24.2 – Tissue culture development.

[00472] Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3–5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14–17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8–12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then

moved to a growth chamber with a 14-h light period at 30° C and in the dark at 21° C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C for one week prior to storage at 4° C.

[00473] 24.3 – Microprojectile bombardment.

[00474] All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB3403, 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

[00475] 24.4 – Tolerance testing.

[00476] Rice plantlets at the 3-5 leaf stage were sprayed with a 0.3% (v/v) solution of DuPont™ Assure® II containing 1% (v/v) Agridex crop oil concentrate using a DeVilbiss bulb sprayer (model 15-RD glass atomizer). This concentration corresponds to approximately 140 g ae/ha. Each plant was spayed in a fume hood at a distance of 8-12 inches with 6 full squirts of the sprayer directed so that the entire plant was covered with an equal portion of herbicide. Each squirt delivered approximately 100 µl solution to the plantlet. Once sprayed, plantlets were allowed to dry for one hour before being moved out of the fume hood. Rating for sensitivity or resistance was done at 10-14 days after treatment (DAT) and is shown in **Table 35** below.

Table 35.

Sample Name	140 g ae/ha quizalofop
Control	Dead
63-1A	No injury
63-1F	No injury
63-4B	No injury
63-4D	No injury
63-6C	Dead

[00477] 24.5 – Tissue harvesting, DNA isolation and quantification.

[00478] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00479] 24.6 – Southern blot analysis.

[00480] Southern blot analysis was performed with total DNA obtained from the Qiagen DNeasy kit. A total of 2 µg of DNA was subjected to an overnight digest of HindIII for pDAB3403 to obtain integration data. Likewise a total of 2 ug of DNA was subjected to an overnight digest of MfeI to obtain the PTU data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 x SSC was then set-up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80°C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using coding region PCR using plasmid pDAB3404. A total of 100 ng of total DNA was used as template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Southern fragment PCR AAD-1 were (Forward - ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse - GGGCAGGCCTAACTCCACCAA) (SEQ ID NO:32). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute and 45 seconds followed by 72°C for 10 minutes.

[00481] The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60°C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it

RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65°C in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in **Table 36.**

Table 36. Southern Results.

Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049bp
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

[00483] Plants 63-1 A and 63-1 F are not the same event; Plants 63-4 A and 63-4 D are the same event. These events have the expected size PTU's, but they are very complex. This Southern blot PTU data correlates with expression data and the spray data. Sample 63-6 C did not have enough DNA present to perform both Integration and PTU southern blots.

[00484] 24.7 – Western data

[00485] Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in **Table 37.**

Table 37.

Lane	Sample Name	TSP (µg/mL)	ELISA [AAD1] (ng/mL)	ELISA Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	-
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	-
7	AAD1 Standard (0.5 µg/mL)				+++
8	AAD1 Standard (5.0 µg/mL)				+++++

Example 25 – Turf Grass Transformation Procedures

[00486] Genetic transformation, with *AAD-1 (v3)* substituted for the "bar" gene as described below, of creeping bentgrass mediated by Agrobacterium tumefaciens could be achieved through embryogenic callus initiated from seeds (cv. Penn-A-4), as described generally below. *See* "Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation" (Luo *et. al.*, 2004).

[00487] Callus is infected with an *A. tumefaciens* strain (LBA4404) harboring a super-binary vector that contained an herbicide-resistant bar gene driven either by a rice ubiquitin promoter. The overall stable transformation efficiency ranged from 18% to 45%. Southern blot and genetic analysis confirmed transgene integration in the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T_1 generation. All independent transformation events carried one to three copies of the transgene, and a majority (60–65%) contained only a single copy of the foreign gene with no apparent rearrangements.

[00488] 25.1 – Seed preparation for embryogenic callus induction.

[00489] Mature seeds were dehusked with sand paper and surface sterilized in 10% (v/v) Clorox bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-*o*-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytagel. The pH of the medium was adjusted to 5.7 before autoclaving at 120°C for 20 min).

[00490] 25.2 – Embryogenic callus induction.

[00491] The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryogenic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

[00492] 25.3 – *Agrobacterium* infection and co-cultivation.

[00493] One day before agro-infection, the embryogenic callus was divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 µM acetosyringone. A 10-ul aliquot of Agrobacterium (LBA4404) suspension (OD=1.0 at 660 nm) was then applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25°C.

[00494] 25.4 – Resting stage and *Agrobacterium* control.

[00495] For the antibiotic treatment step, the callus was then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

[00496] 25.5 – Selection and potential transgenic colony identification.

[00497] Subsequently, for selection, the callus was moved to callus-induction medium containing 250 mg/l cefotaxime and 10 mg/l phosphinothricin (PPT) for 8 weeks. Antibiotic treatment and the entire selection process were performed at room temperature in the dark. The subculture interval during selection was typically 3 weeks.

[00498] 25.6 – Regeneration of transgenic plants.

[00499] For plant regeneration, the PPT-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime, PPT or hygromycin. These calli were kept in the dark at room temperature for 1 week and then moved into the light for 2–3 weeks to develop shoots. No Albino plants with PPT selection (Hygromycin if used as a selection agent, produces a high level of albino plants).

[00500] 25.7 – Root induction and transfer to greenhouse.

[00501] Small shoots were then separated and transferred to hormone-free regeneration medium containing PPT and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3–5 weeks) were then transferred to soil and grown either in the greenhouse or in the field.

[00502] 25.8 – Vernalization and out-crossing of transgenic plants.

[00503] Transgenic plants were maintained out of doors in a containment nursery (3-6 months) until the winter solstice in December. The vernalized plants were then transferred to the greenhouse and kept at 25°C under a 16/8 h photoperiod and surrounded by non-transgenic wild-type plants that physically isolated them from other pollen sources. The plants started flowering 3-4 weeks after being moved back into the greenhouse. They were out-crossed with the pollen from the surrounding wild type plants. The seeds collected from each individual transgenic plant were germinated in soil at 25°C, and T1 plants were grown in the greenhouse for further analysis.

[00504] 25.9 – Other target grasses.

[00505] Other grasses that can be targeted for AAD-1 transformation according to the subject invention include Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra commutate*), Crabgrass, Creeping bent (*Agrostis stolonifera*), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/sheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*), Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra rubra*), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Timothy, Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysiagrass.

Example 26 – *AAD-1 (v3)* in Canola

[00506] 26.1 – Canola transformation.

[00507] The *AAD-1 (v3)* gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation. The construct contained *AAD-1 (v3)* gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter.

[00508] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[00509] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB721. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00510] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the

segments were placed K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00511] Callus samples from 35 independent events were tested by PCR. All the 35 samples tested positive for the presence of *AAD-1 (v3)*,whereas the non-transformed controls were negative (section on PCR assay). Ten callus samples were confirmed to express the AAD-1 protein as determined by ELISA (section on protein analysis).

[00512] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 3 weeks.

[00513] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00514] The transformed T0 plants were self-pollinated in the greenhouse to obtain T1 seed. The T0 plants and T1 progeny were sprayed with a range of herbicide concentrations to establish the level of protection by the *AAD-1 (v3)* gene.

[00515] 26.2 – "Molecular analysis": canola materials and methods

[00516] 26.2.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green

(Molecular Probes P7589) and read in the floromeler (BioTek) with known standards to obtain the concentration in ng/ul.

[00517] 26.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-1 (v3)* were (Forward – ATGGCTCATG CTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse - CGGGCAGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-1 (v3)* events tested positive. Three negative control samples tested negative.

[00518] 26.3 – ELISA.

[00519] Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in **Table 38.**

Table 38. Expression of AAD1 in Canola calli.

Sample #	Weight (mg)	[TSP] (µg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	-
Control 2	61	2549.60	35.00	13.73	-
Control 3	59	2374.41	22.79	9.60	-

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Claims

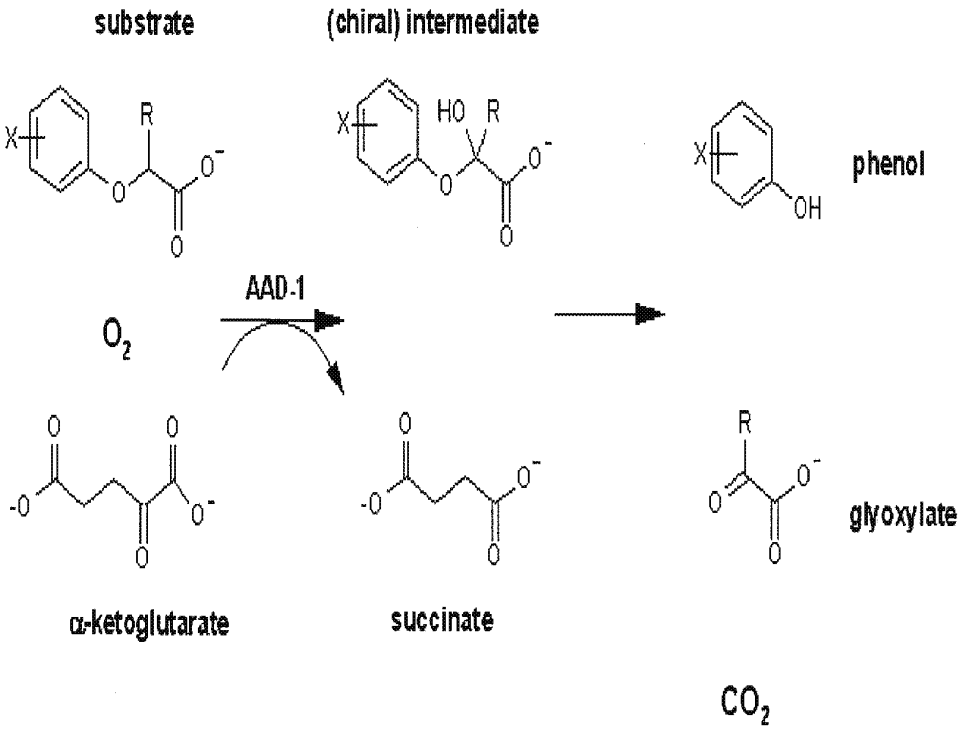
1. An expression cassette comprising a polynucleotide encoding a protein comprising the amino acid sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11, wherein said polynucleotide is operably linked to a heterologous promoter that is functional in a plant cell.
2. The expression cassette of claim 1, wherein said polynucleotide is selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.
3. A plant comprising the expression cassette of claim 1.
4. A plant cell comprising the expression cassette of claim 1.
5. A seed from the plant of claim 3, wherein said seed comprises said expression cassette.
6. A method of protecting a crop plant from damage by an aryloxyalkanoate herbicide, said method comprising growing the plant of claim 3, and applying said herbicide to said plant.
7. A method of selecting the transformed plant cell of claim 4 wherein said method comprises subjecting a plurality of plant cells to transformation with said polynucleotide, then growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells.
8. The expression cassette of claim 1 wherein said promoter is selected from the group consisting of cassava vein mosaic virus promoter (CsVMV), AtUbi10 promoter, AtUbi3 promoter, OsAct1 promoter and ZmUbi1 promoter.
9. The plant of claim 3 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of Bacillus thuringiensis, Photorhabdus, and Xenorhabdus.
10. The plant of claim 3 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.
11. The plant of claim 3, wherein said plant is a monocot.
12. The plant of claim 11, wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, turf grass, oats, sorghum, and pasture grasses.

13. A plant grown from the seed of claim 5.

14. A regenerable part, progeny, or asexual propagate of the plant of claim 3.

Abstract

[00520] The subject invention provides novel plants that are not only resistant to 2,4-D and other phenoxy auxin herbicides, but also to aryloxyphenoxypropionate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention alone or "stacked" together with another herbicide resistance gene, preferably a glyphosate resistance gene, so as to provide broader and more robust weed control, increased treatment flexibility, and improved herbicide resistance management options. More specifically, preferred enzymes and genes for use according to the subject invention are referred to herein as *AAD* (aryloxyalkanoate dioxygenase) genes and proteins. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. This highly novel discovery is the basis of significant herbicide tolerant crop trait opportunities as well as development of selectable marker technology. The subject invention also includes related methods of controlling weeds. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the formation of, and controlling, weeds that are resistant (or naturally more tolerant) to one or more herbicides such as glyphosate.



- Addition of O_2 is *stereospecific*
- Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

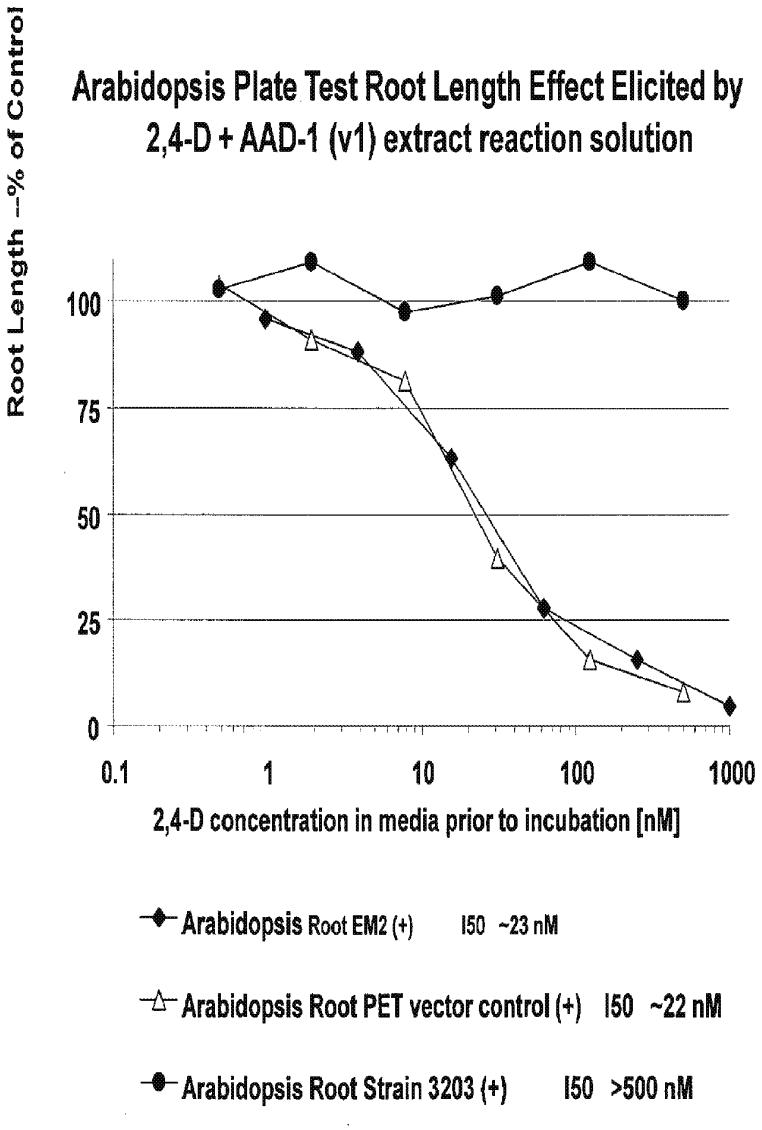

Arabidopsis Plate Test Root Length Effect Elicited by
2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

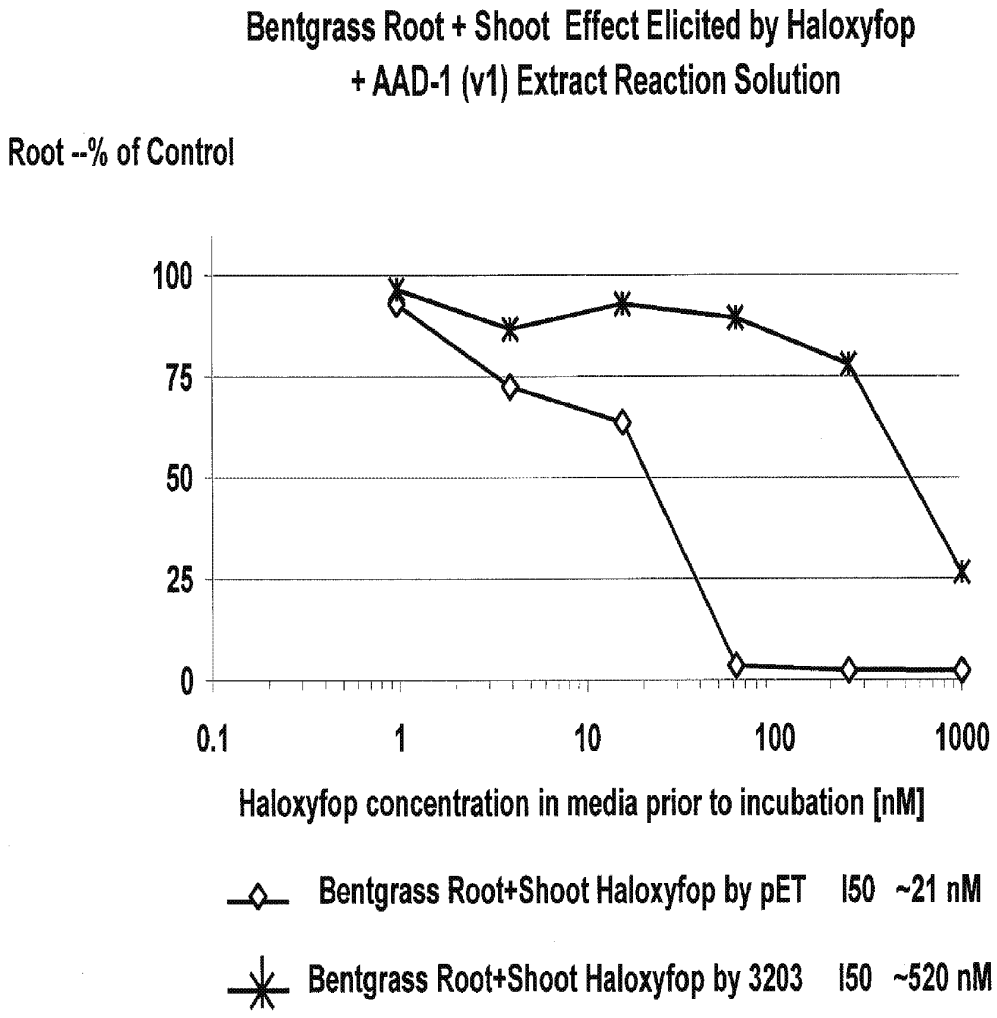

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

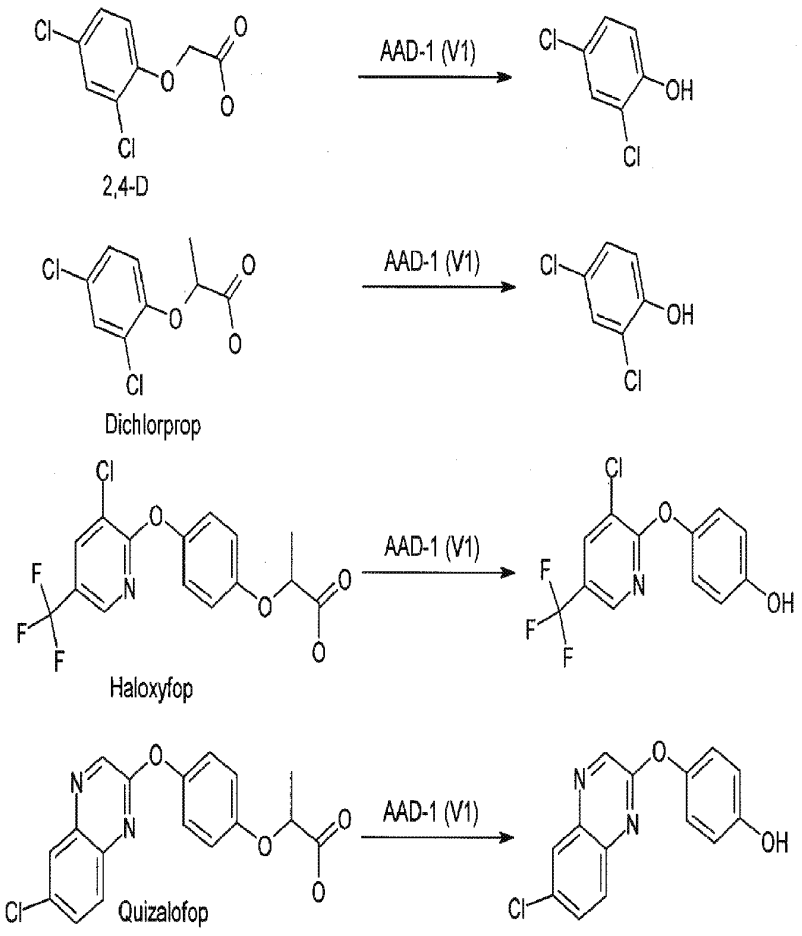

AAD-1 (V1)
2,4-D
AAD-1 (V1)
Dichlorprop
AAD-1 (V1)
Haloxyfop
AAD-1 (V1)
Quizalofop

Fig. 4

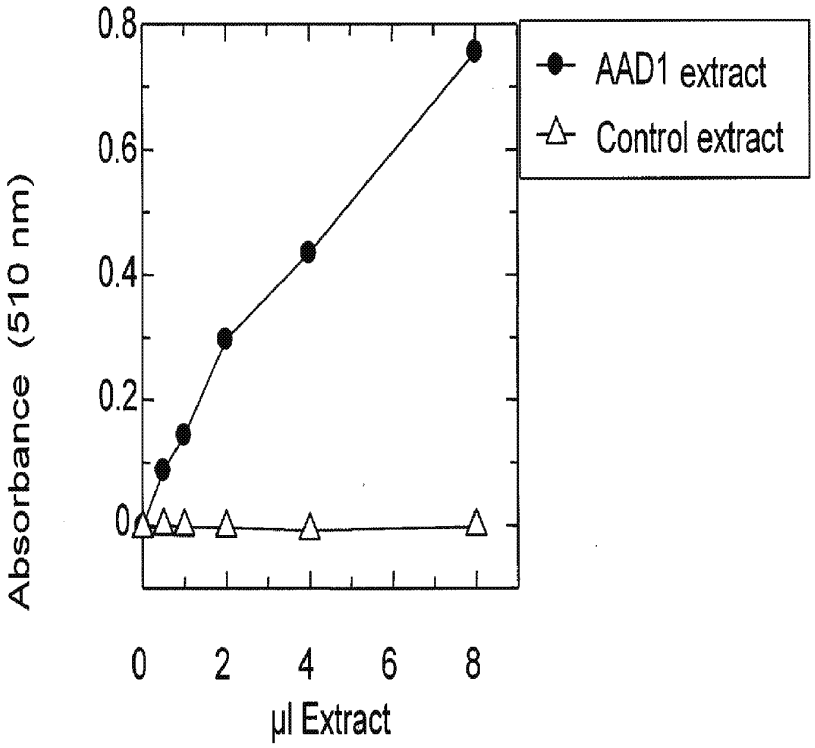

0.8
0.6
0.4
0.2
0
Absorbance (510 nm)
0
2
4
6
8
µl Extract
AAD1 extract
Control extract

Fig. 5

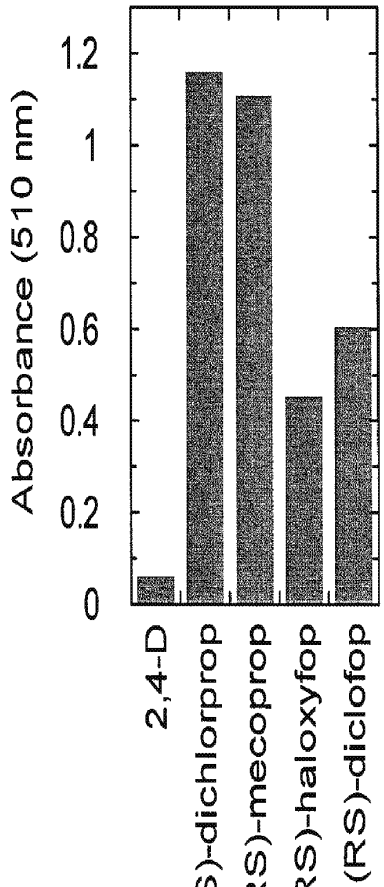

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
2,4-D
(RS)-dichlorprop
(RS)-mecoprop
(RS)-haloxyfop
(RS)-diclofop

Fig. 6A

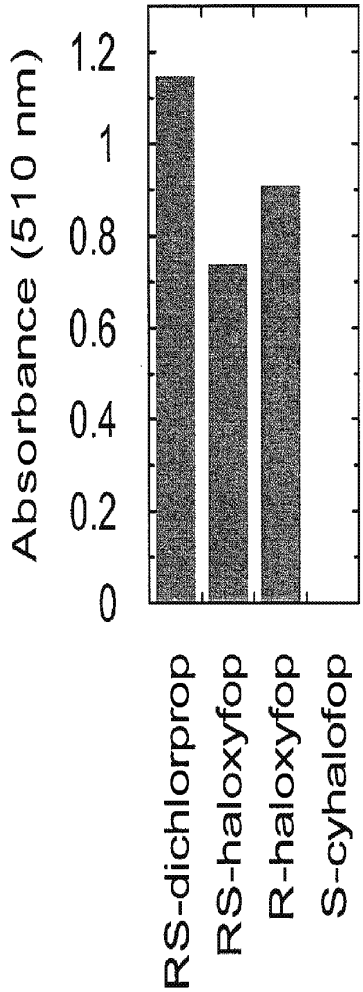

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
RS-dichlorprop
RS-haloxyfop
R-haloxyfop
S-cyhalofop

Fig. 6B

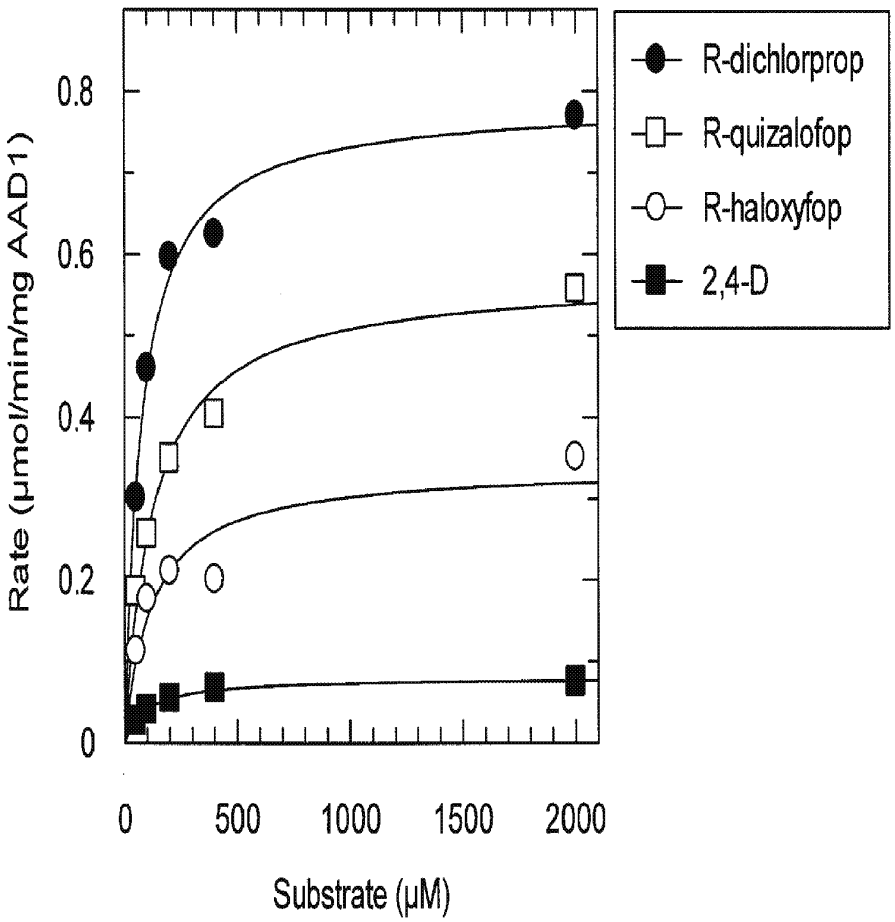

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (µmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (µM)

Fig. 7

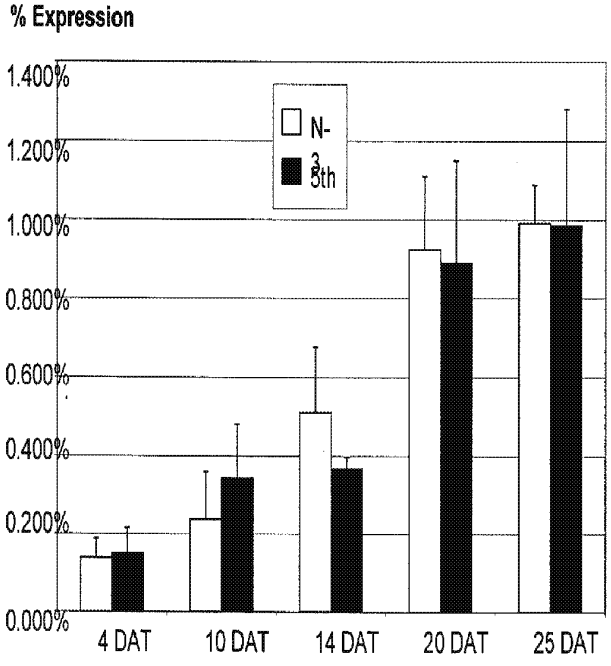

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

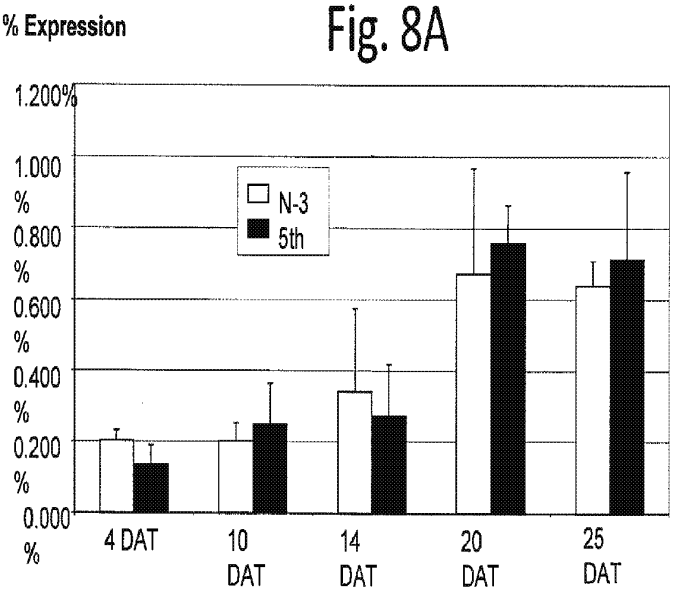

% Expression
1.200%
1.000 %
0.800 %
0.600 %
0.400 %
0.200 %
0.000 %
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

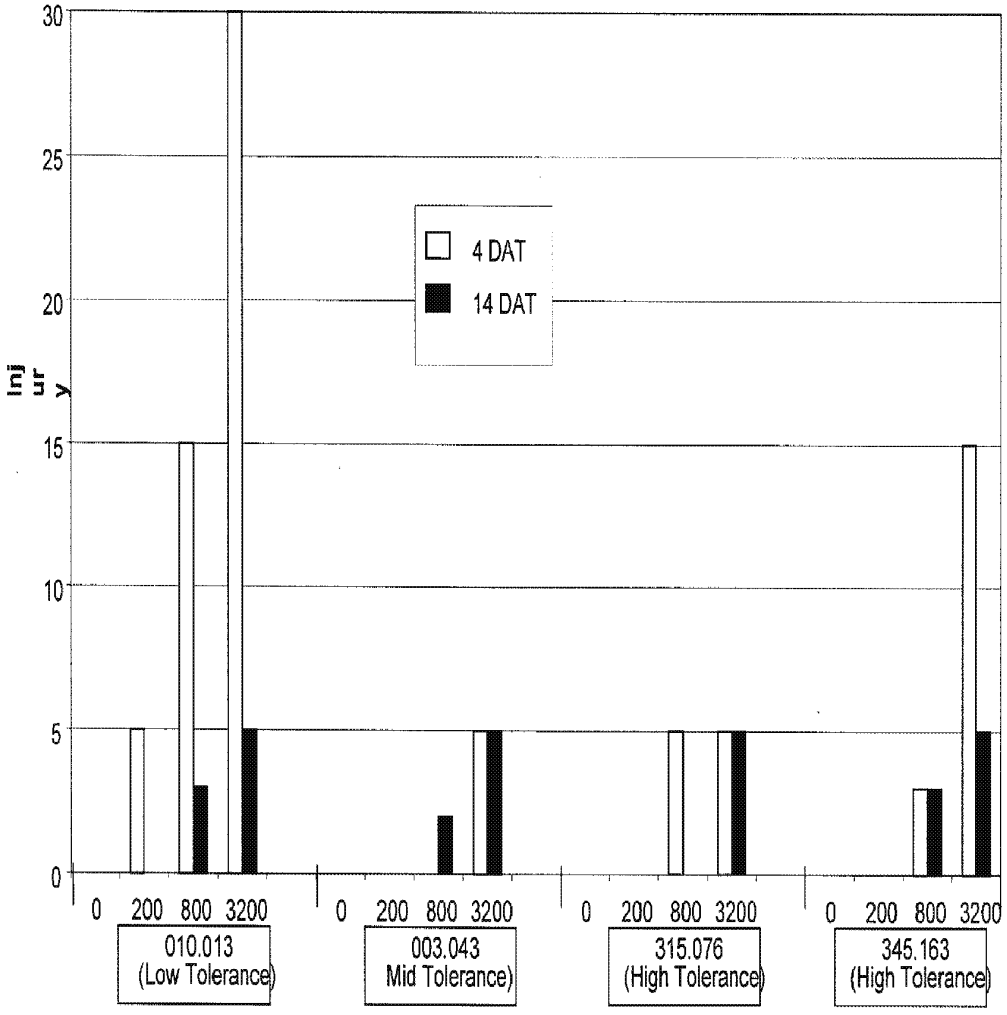

30
25
20
15
10
5
0
Inj ur y
4 DAT
14 DAT
0 200 800 3200
010.013
(Low Tolerance)
0 200 800 3200
003.043
Mid Tolerance)
0 200 800 3200
315.076
(High Tolerance)
0 200 800 3200
345.163
(High Tolerance)

Fig. 9A

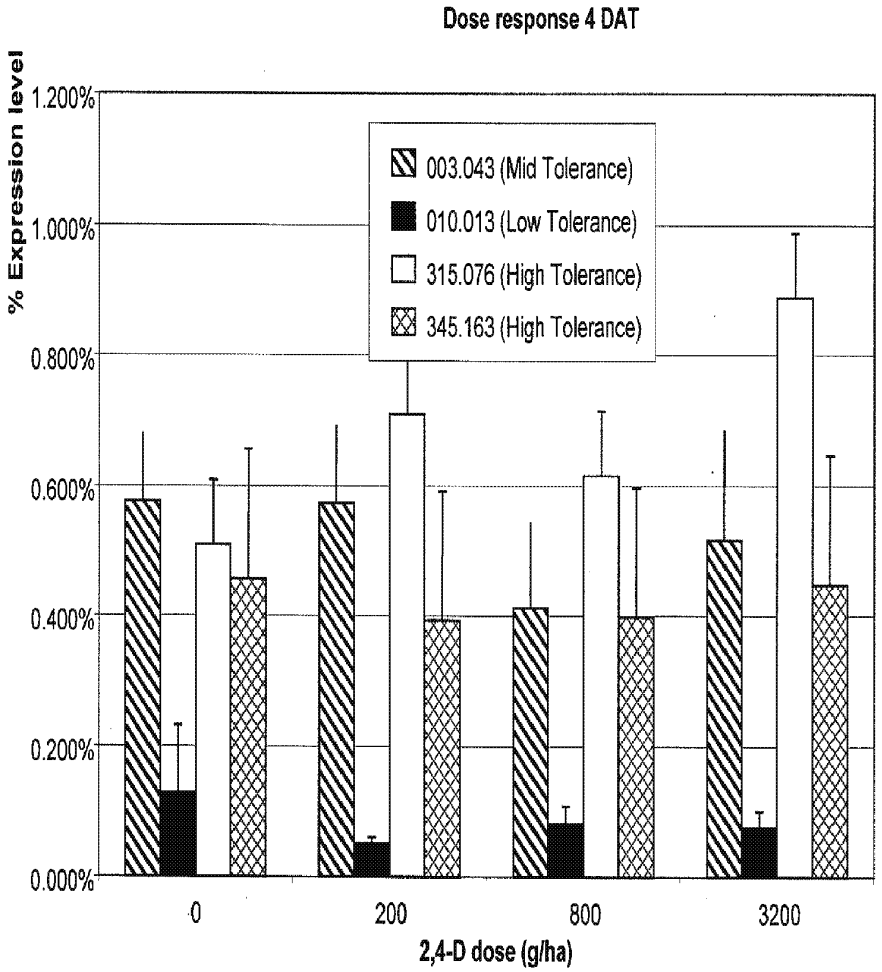

Dose response 4 DAT
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

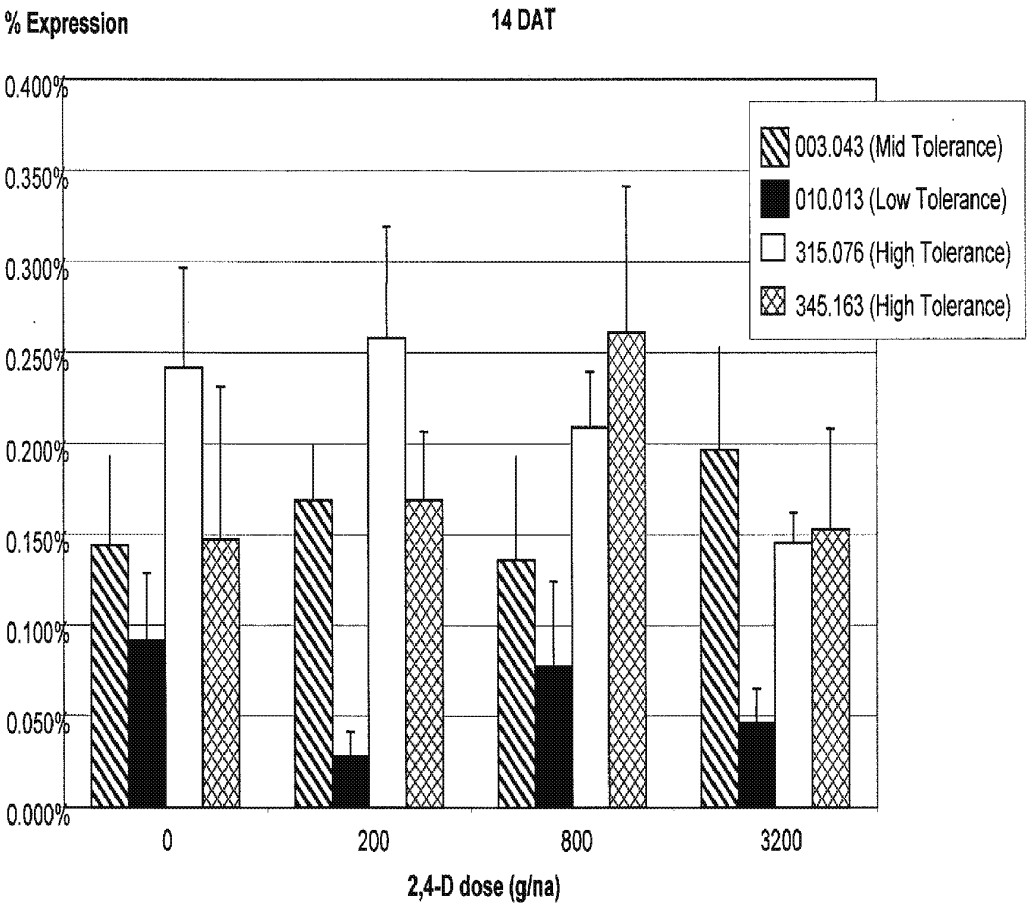

% Expression
14 DAT
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

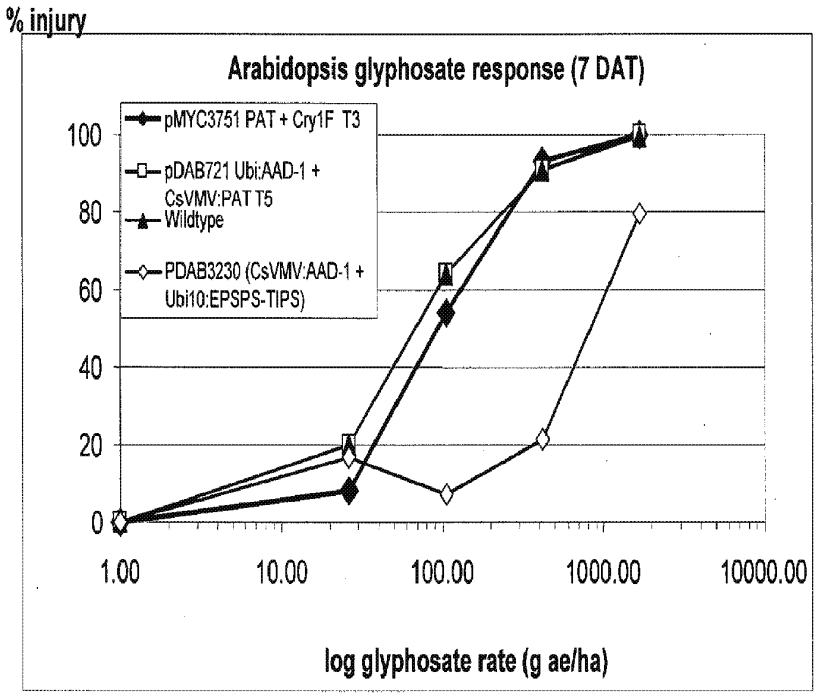

% injury
Arabidopsis glyphosate response (7 DAT)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)

Fig. 10

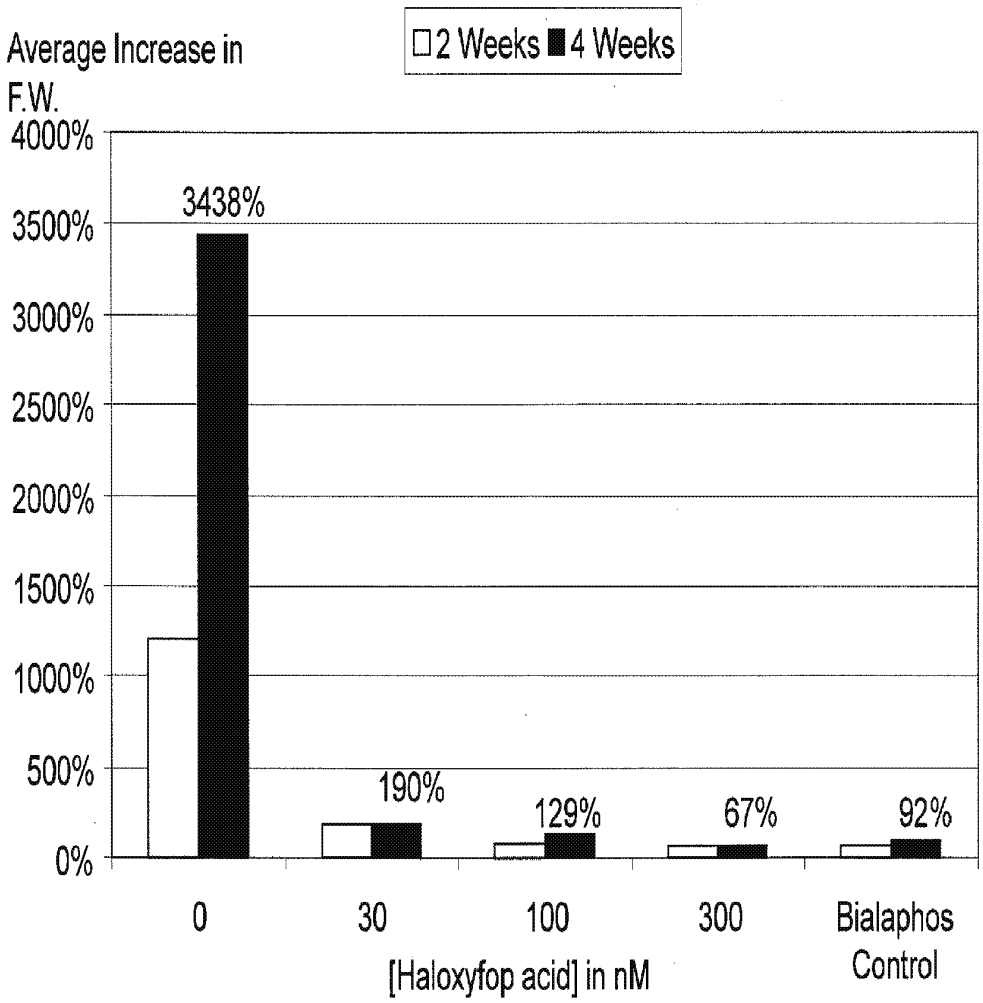

Average Increase in F.W.
2 Weeks
4 Weeks
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

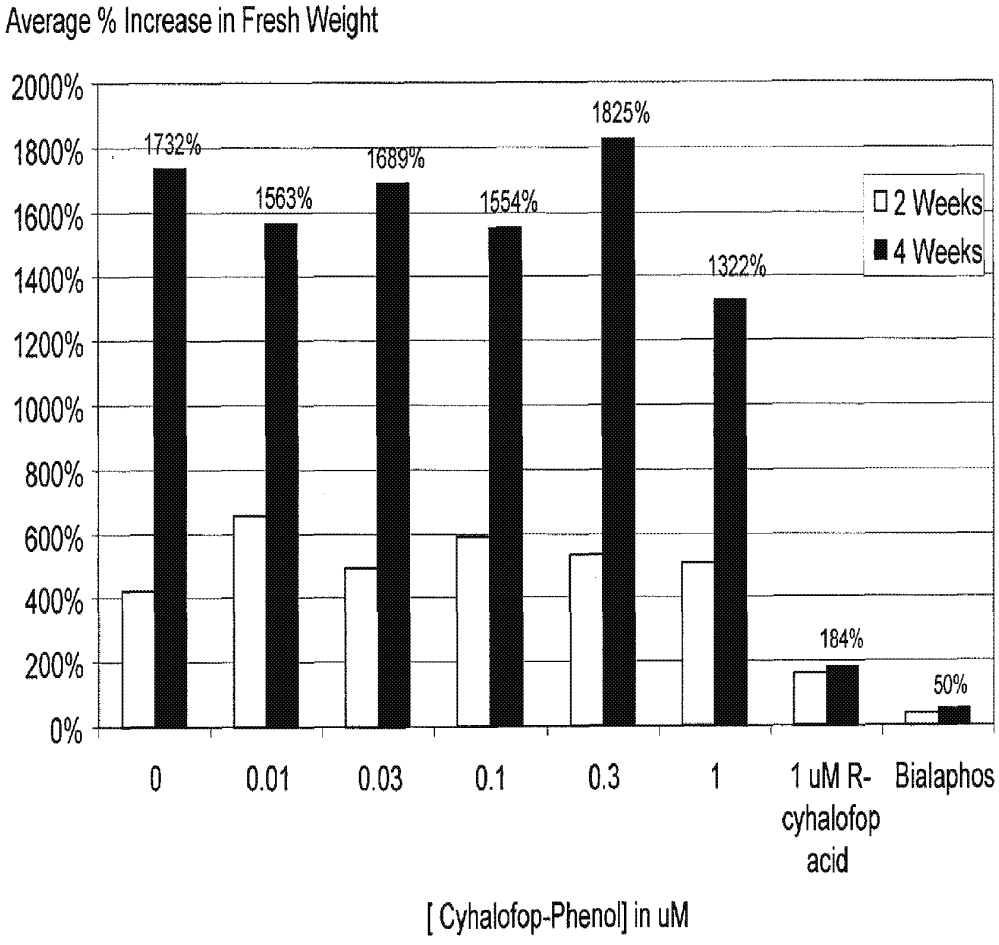

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
2 Weeks
4 Weeks
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

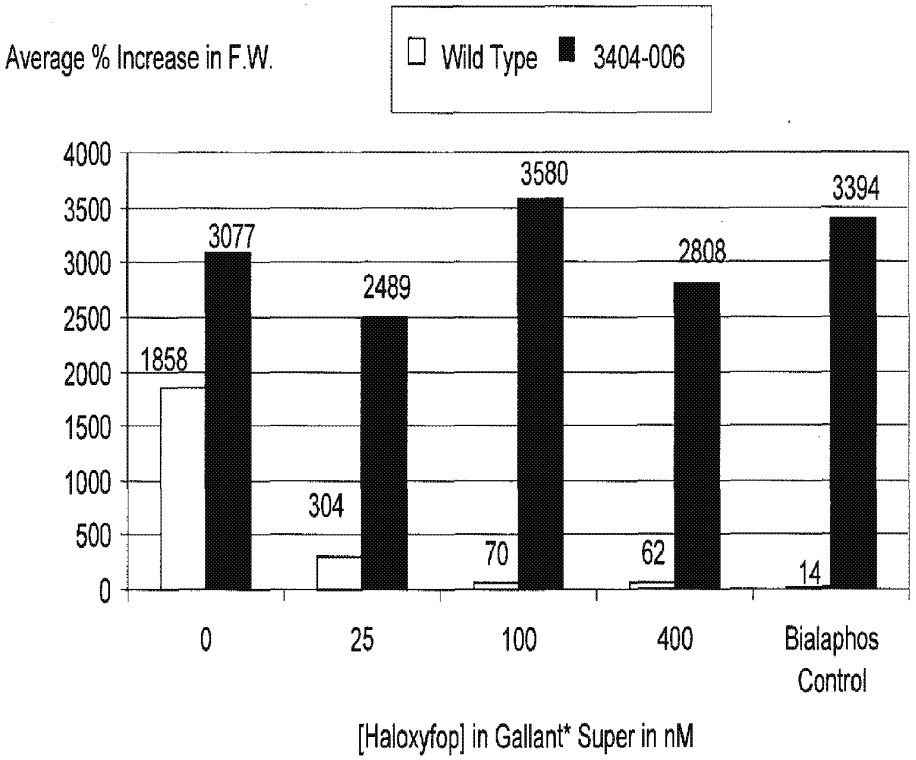

Average % Increase in F.W.
Wild Type
3404-006
4000
3500
3000
2500
2000
1500
1000
500
0
1858
3077
304
2489
70
3580
62
2808
14
3394
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant* Super in nM

Fig. 13

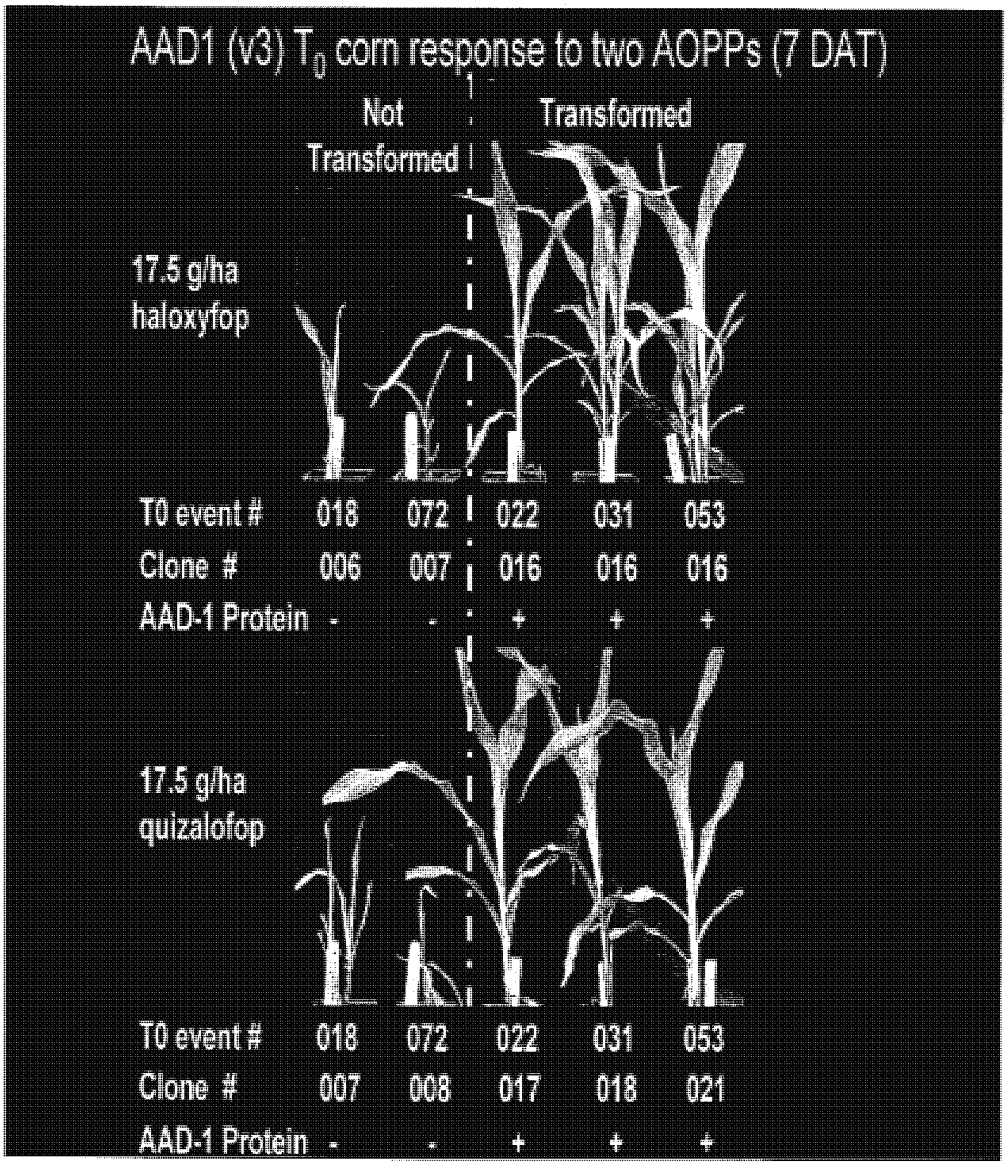

AAD1 (v3) T_0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

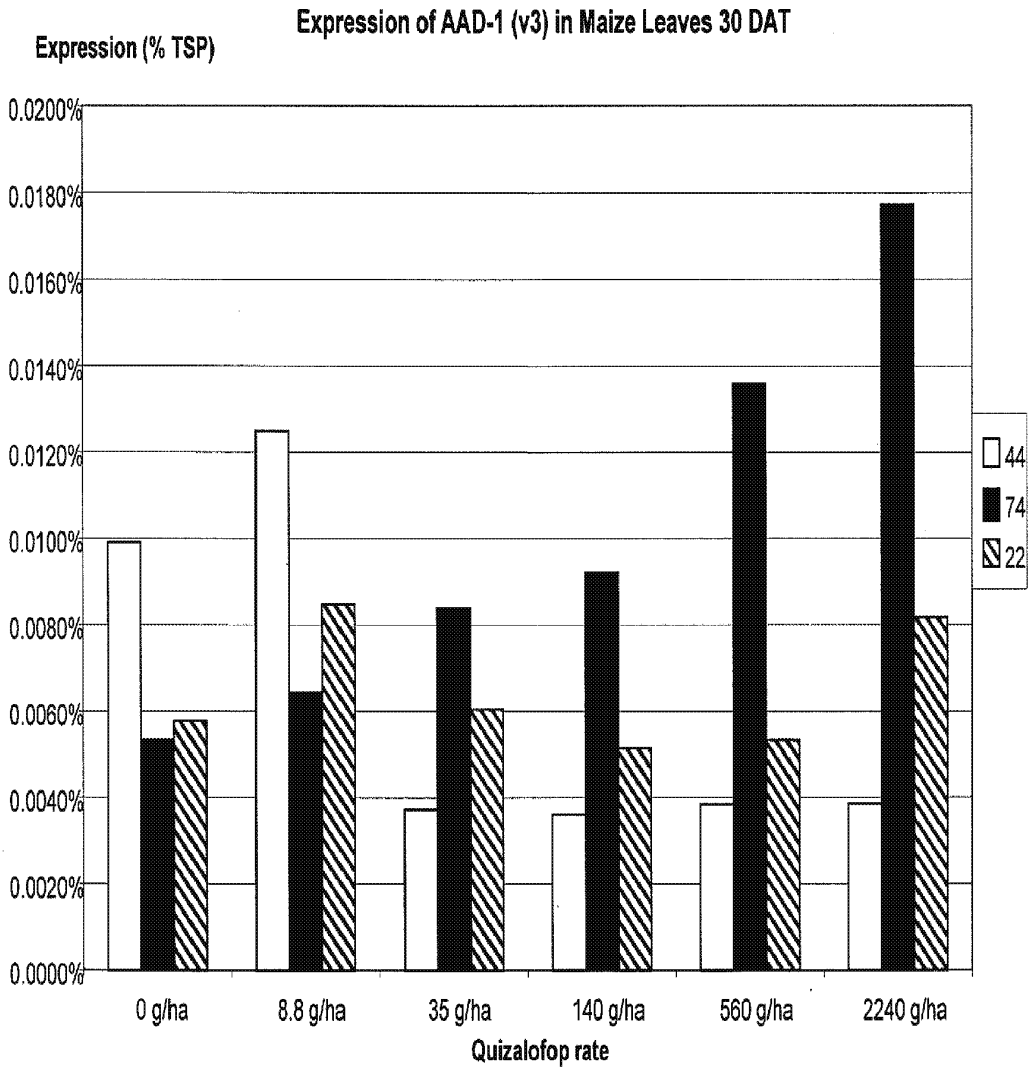

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate
44
74
22

Fig. 15

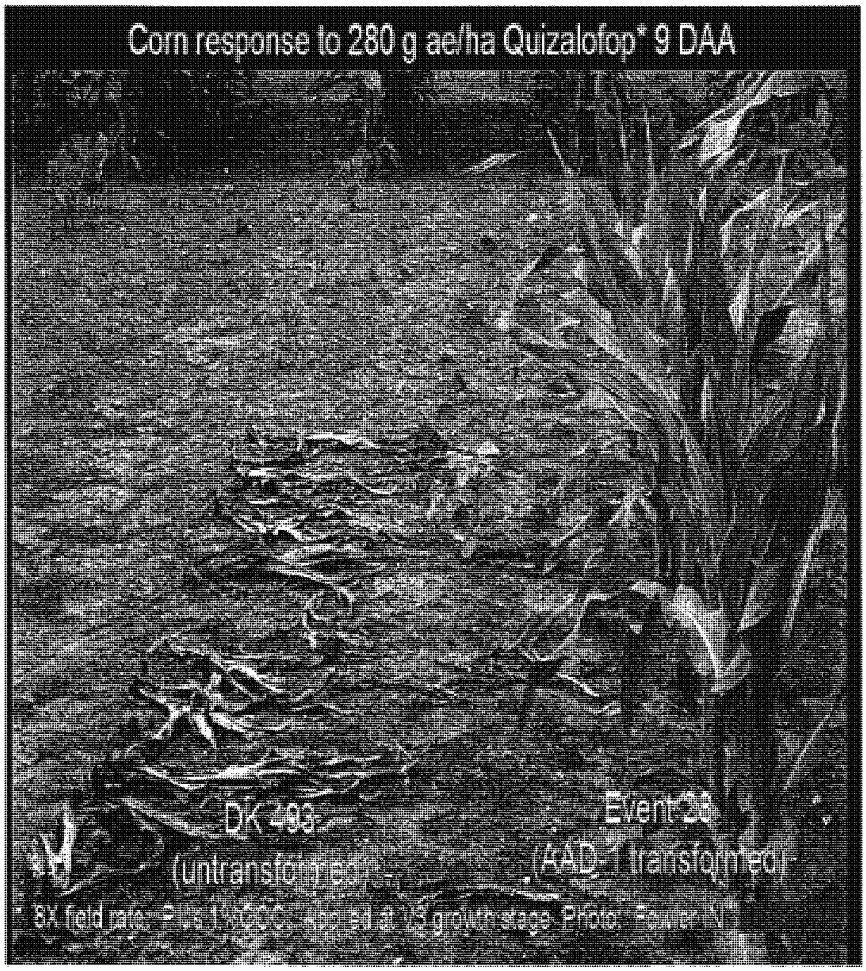

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 493
(untransformed)
Event 3
(AAD-1 transformed)
8X field rate

Fig. 16

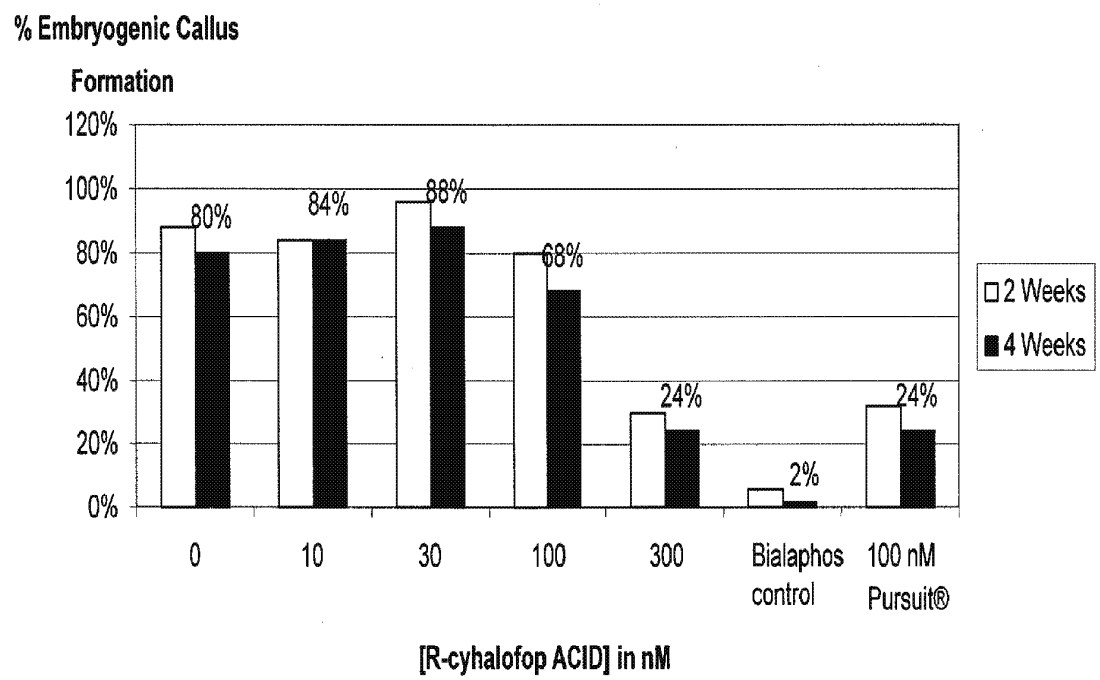
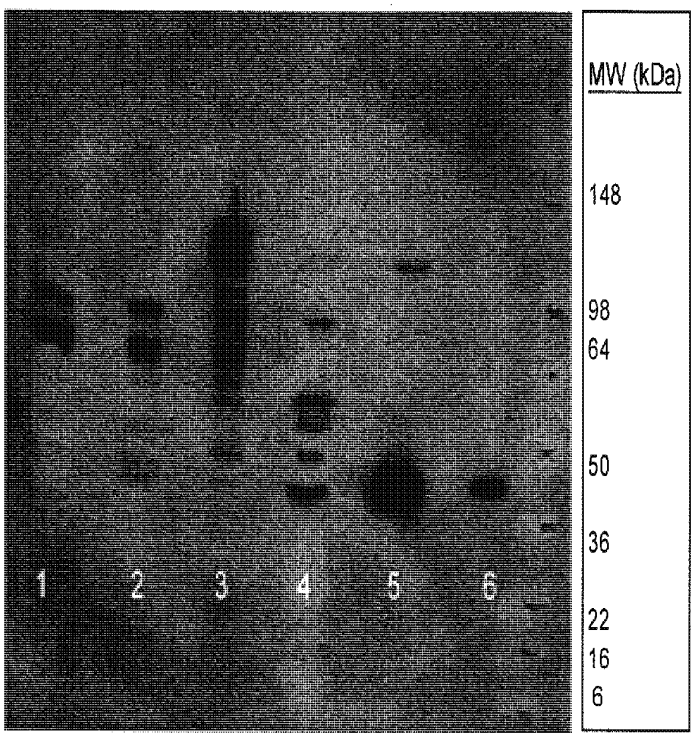

% Embryogenic Callus
Formation
120%
100%
80%
60%
40%
20%
0%
80%
84%
88%
68%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
2 Weeks
4 Weeks
[R-cyhalofop ACID] in nM

Fig. 17



Western

Lane	
1	Calli #1
2	Calli #2
3	Negative
4	AAD1 Corn
5	Standard 5 ug/mL
6	Standard 0.5 ug/mL

Fig. 18

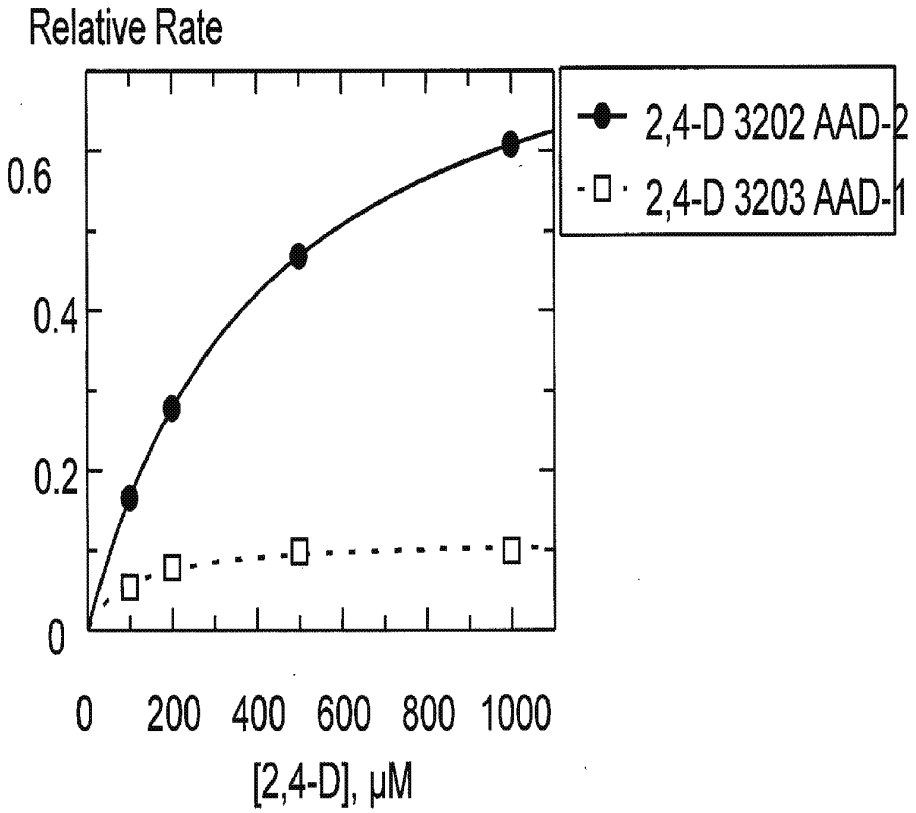

Relative Rate
0.6
0.4
0.2
0
0
200
400
600
800
1000
[2,4-D], µM
2,4-D 3202 AAD-2
2,4-D 3203 AAD-1

Fig. 19

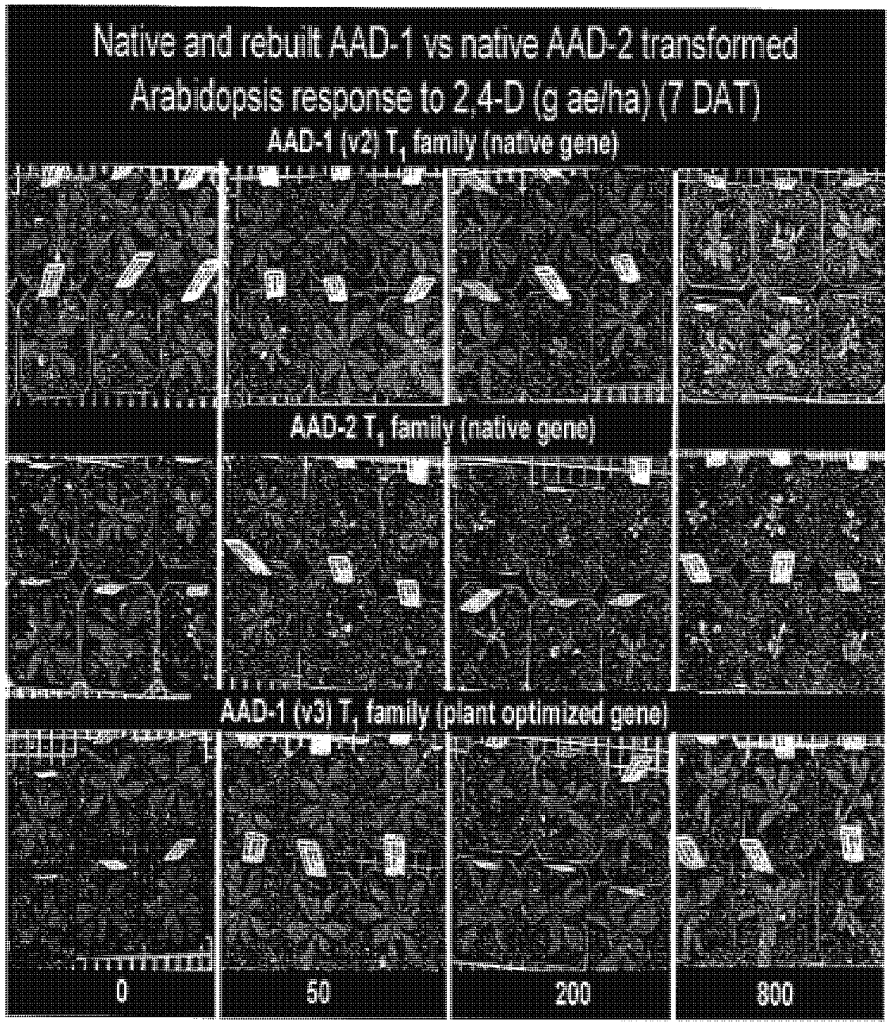

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

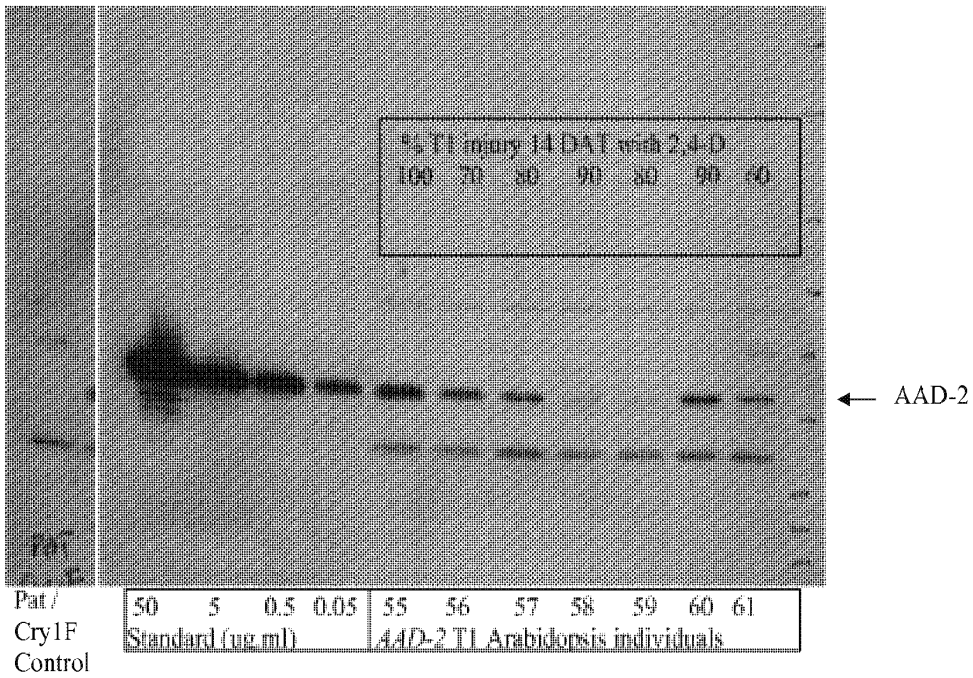


Fig. 21

COR-AAD1-00000219

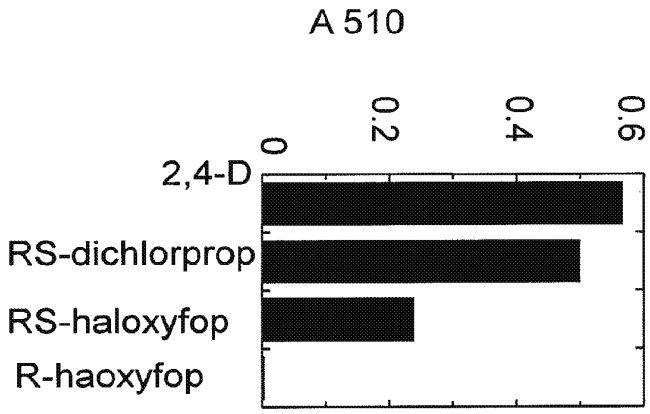


FIG. 22

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If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Anjum, Durreshwar (CGI Federal)
Timestamp: [year=2016; month=10; day=12; hr=11; min=20; sec=18; ms=249;
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Validated By CRFValidator v 1.0.5

Application No: 15288406 Version No: 1.0

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Output Set:

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Finished: 2016-10-07 15:47:07.778
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Total Errors: 0
No. of SeqIDs Defined: 32
Actual SeqID Count: 32

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Input Set:

Output Set:

Started: 2016-10-07 15:47:05.807
Finished: 2016-10-07 15:47:07.778
Elapsed: 0 hr(s) 0 min(s) 1 sec(s) 971 ms
Total Warnings: 29
Total Errors: 0
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Actual SeqID Count: 32

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<140> US 15/288,406
<141> 2016-10-07

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<151> 2015-08-07

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<151> 2008-11-22

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<220>
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<210> 2
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
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<210> 3
<211> 915
<212> DNA

<213> Sphingobium herbicidovorans

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<222> (1)..(18)
<223> Primer linker

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atcgcggtcc agccgctgac cggcgtcctg ggcgccgaga tcaccggcgt cgacctgcgc 120

gagccgctcg acgacagcac ctggaacgaa atcctcgacg cgttccacac ttaccaggtc 180

atctattttc ccggccaggc gatcaccaac gaacagcaca tcgccttcag ccggcgcttc 240

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atccgccgcg aagccaacga aagcgggcgt gtgatcggtg aygactggca caccgacagc 360

accttcctgg acgcaccgcc ggccgccgtg gtgatgcgcg cgatcgacgt gcccgagcat 420

ggcggcgaca ccggttttct gagcatgtac accgcgtggg agacgctgtc gcccaccatg 480

caggccacca tcgaagggtt gaacgtagtg cacagcgcca cgcgtgtgtt cggctcgctc 540

taccaggccc agaaccggcg cttcagcaac accagcgtca aggtgatgga cgtcgacgcg 600

ggcgaccgtg aaaccgtgca ccccctggtg gtgacccatc cgggcagcgg ccgcaagggc 660

ctgtacgtga accaggtcta ttgccagcgc atcgagggca tgaccgatgc cgaaagcaaa 720

ccgctgctgc agttcctgta cgagcatgcg acacggttcg atttcacctg ccgcgtgcgc 780

tggaagaagg accaggtcct ggtctgggac aacctgtgca cgatgcaccg ggccgtaccc 840

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cgctagtaac tcgag 915

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<220>
<223> AAD-1 (v2) primary sequence

<220>

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gcacctggaa cgaaatcctc gacgcgttcc acacttacca ggtcatctat tttcccggcc 180

aggcgatcac caacgaacag cacatcgcct tcagccggcg cttcggcccc gtcgatcccg 240

tgcccctgct caagagcatc gaagggtatc cagaggtgca gatgatccgc cgcgaagcca 300

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ggcgcttcag caacaccagc gtcaaggtga tggacgtcga cgcgggcgac cgtgaaaccg 600

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tgtacgagca tgcgacacgg ttcgatttca cctgccgcgt gcgctggaag aaggaccagg 780

tcctggtctg ggacaacctg tgcacgatgc accgggccgt acccgactac gcgggcaagt 840

tccgctacct gacgcgcacc acggtcggtg gcgtgcgccc ggcgcgctag tgagctc 897

<210> 5
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<212> DNA
<213> Artificial Sequence

<220>
<223> AAD-1 (v3) primary sequence

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<222> (6)..(8)
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cactcactgg tgtccttggt gctgagatca ctggagtgga cttgagggaa ccacttgatg 120

acagcacctg gaatgagata ttggatgcct tccacactta ccaagtcatc tactttcctg 180

gccaagcaat caccaatgag cagcacattg cattctcaag aaggtttgga ccagttgatc 240

cagtgcctct tctcaagagc attgaaggct atccagaggt tcagatgatc cgcagagaag 300

ccaatgagtc tggaagggtg attggtgatg actggcacac agactccact ttccttgatg 360

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accgtcgctt cagcaacacc tcagtcaagg tgatggatgt tgatgctggt gacagagaga 600

cagtccatcc cttggttgtg actcatcctg gctctggaag gaaaggcctt tatgtgaatc 660

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tcctctatga gcatgccacc agatttgact tcacttgccg tgtgaggtgg aagaaagacc 780

aagtccttgt ctgggacaac ttgtgcacca tgcaccgtgc tgttcctgac tatgctggca 840

agttcagata cttgactcgc accacagttg gtggagttag gcctgcccgc tgagtagtta 900

gcttaatcac ctagagctc 919

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> rdpA(ncoI) 5' primer

<400> 6
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<210> 7
<211> 33
<212> DNA

<213> Artificial Sequence

<220>
<223> 3'saci primer

<400> 7
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<210> 8
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> BstEII/ Del NotI 5' primer

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<210> 9
<211> 295
<212> PRT
<213> Sphingobium herbicidovorans

<400> 9

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1 5 10 15

Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
20 25 30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
35 40 45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
50 55 60

Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
65 70 75 80

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
85 90 95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
100 105 110

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala

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Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
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Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
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Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
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Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
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Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
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Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
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Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
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Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
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Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
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Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
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Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
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Val Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile
                85                  90                  95

Arg Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His
            100                 105                 110

Thr Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg
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Ala Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met
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Tyr Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu
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Gly Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr
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Gln Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp
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Val Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His
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Pro Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln
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Arg Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe
225                 230                 235                 240

Leu Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp
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Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
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Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
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Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
65                  70                  75                  80

Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
                85                  90                  95

Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
            100                 105                 110

Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
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Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
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Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
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Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
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Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
            180                 185                 190

Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
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Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
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Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
225                 230                 235                 240

Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met
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Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln Ser
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Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr
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www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/288,406	10/07/2016	1662	3120	14764-258662	34	1

CONFIRMATION NO. 5402

107540
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

FILING RECEIPT

OC000000088538747

Date Mailed: 10/19/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Terry WRIGHT, Westfield, IN;
Justin LIRA, Fishers, IN;
Donald MERLO, Carmel, IN;
Nicole ARNOLD, Indianapolis, IN;

Applicant(s)

Dow AgroSciences LLC, Indianapolis, IN;

Power of Attorney: The patent practitioners associated with Customer Number 107540

Domestic Priority data as claimed by applicant

This application is a CON of 14/820,893 08/07/2015
which is a CON of 12/951,813 11/22/2010 PAT 9127289
which is a CON of 11/587,893 05/22/2008 PAT 7838733
which is a 371 of PCT/US2005/014737 05/02/2005
which claims benefit of 60/567,052 04/30/2004

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

COR-AAD1-00000236
Bayer EX1008 (Part 1 of 9)

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 10/17/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/288,406**

Projected Publication Date: 01/26/2017

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL HERBICIDE RESISTANCE GENES

Preliminary Class

800

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

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Bayer EX1008 (Part 1 of 9)

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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COR-AAD1-00000239
Bayer EX1008 (Part 1 of 9)

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/288,406

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	34 minus 20 =	* 14			OR	x 80 =	1120
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	3120

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
15288406	

Applicant(s) Terry WRIGHT

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
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Total Indep	1		0		0	
Total Depend	33	↵	0	↵	0	↵
Total Claims	34		0		0	

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Document code: WFEE

United States Patent and Trademark Office
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01	FC : 1081	400.00	DA
02	FC : 1202	1,120.00	DA

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Cc: PAIR_eOfficeAction@uspto.gov
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Oct 19, 2016 05:30:07 AM

Dear PAIR Customer:

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UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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Application	Document	Mailroom Date	Attorney Docket No.
15288406	APP.FILE.REC	10/19/2016	14764-258662

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

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Customer No.:	107540
Group:	1663
Confirmation No.:	5402
Application No.:	15/288,406
Invention:	**Novel Herbicide Resistance Genes**
Applicant:	Wright, et al.
Filed:	October 7, 2016
Attorney Docket:	14764-258662
Examiner	KRUSE, David H.

FILED ELECTRONICALLY:

October 20, 2016

TRANSMITTAL OF CORRECTED ADS

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

The captioned utility application was filed electronically on October 7, 2016, with an Application Data Sheet (ADS). Upon review of the file after filing, it was noted that an error appeared in the address of the second named inventor, Justin Lira. The correct address for inventor Lira is -- 8974 Windpointe Pass, Zionsville, IN 46077 -- . A Corrected ADS for the captioned application is submitted herewith to correct the address for inventor Lira. The changes in the corrected ADS are noted via strike-through and underlining.

Applicants believe that no fees are required with this submission. However, if any fees are required, the Commissioner is hereby authorized to charge those fees to the account of Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-258662.

Respectfully submitted,

/John P. Breen/
Registration No. 38,833
Attorney for Applicants

JPB:glt
(317) 261-7940
Indianapolis, Indiana 46204

DMS 4416499v1

CORRECTED ADS

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Terry		WRIGHT	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Westfield	State/Province	IN	Country of Residence	US

Mailing Address of Inventor:

Address 1	14162 Charity Chase Circle		
Address 2			
City	Westfield	State/Province	IN
Postal Code	46074	Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Justin		LIRA	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	~~Fishers~~ Zionsville	State/Province	IN	Country of Residence	US

Mailing Address of Inventor:

Address 1	~~12979 Ensley Drive~~ 8974 Windpointe Pass		
Address 2			
City	~~Fishers~~ Zionsville	State/Province	IN
Postal Code	~~46038~~ 46077	Country	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Donald		MERLO	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

City	Carmel	State/Province	IN	Country of Residence	US

Mailing Address of Inventor:

Address 1	11845 Durbin Drive		
Address 2			
City	Carmel	State/Province	IN
Postal Code	46032	Country	US

Inventor 4 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Nicole		ARNOLD	

Residence Information (Select One) (•) US Residency ( ) Non US Residency ( ) Active US Military Service

City	Indianapolis	State/Province	IN	Country of Residence	US

Mailing Address of Inventor:

Address 1	2518 Chaseway Ct.		
Address 2			
City	Indianapolis	State/Province	IN
Postal Code	46268	Country	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	107540		
Email Address	INDocket@btlaw.com	Add Email	Remove Email

Application Information:

Title of the Invention	NOVEL HERBICIDE RESISTANCE GENES		
Attorney Docket Number	14764-258662	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	24	Suggested Figure for Publication (if any)	

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	107540		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
15/288,406	Continuation of	14/820893	2015-08-07

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Prior Application Status	Patented			Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14/820893	Continuation of	12/951813	2010-11-22	9127289	2015-09-08
Prior Application Status	Patented			Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12/951813	Continuation of	11/587893	2008-05-22	7838733	2010-11-23
Prior Application Status	Expired			Remove	
Application Number	Continuity Type	Prior Application Number		Filing or 371(c) Date (YYYY-MM-DD)	
11/587893	a 371 of international	PCT/US2005/014737		2005-05-02	
Prior Application Status	Expired			Remove	
Application Number	Continuity Type	Prior Application Number		Filing or 371(c) Date (YYYY-MM-DD)	
PCT/US2005/014737	Claims benefit of provisional	60/567052		2004-04-30	

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Dow AgroSciences LLC		
Mailing Address Information For Applicant:			
Address 1	9330 Zionsville Road		
Address 2			
City	Indianapolis	**State/Province**	IN
Country	US	Postal Code	46268
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	14764-258662
	Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/John P. Breen/			Date (YYYY-MM-DD)	~~2016-10-07~~ 2016-10-20
First Name	John	Last Name	Breen	Registration Number	38833

Additional Signature may be generated within this form by selecting the Add button.

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	14764-258662
		Application Number	15/288,406
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

COR-AAD1-00000255
Bayer EX1008 (Part 1 of 9)

Electronic Acknowledgement Receipt

EFS ID:	27269231
Application Number:	15288406
International Application Number:	
Confirmation Number:	5402
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Customer Number:	107540
Filer:	John P. Breen/Garla Twyman
Filer Authorized By:	John P. Breen
Attorney Docket Number:	14764-258662
Receipt Date:	20-OCT-2016
Filing Date:	07-OCT-2016
Time Stamp:	14:16:04
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	Transmittal_of_Corrected_ADS.pdf	7817 c398cce399d1610257f9a4122899a748c7bc4a81	no	2

Warnings:

Information:					
2	Application Data Sheet	ADS.pdf	187186 bd35834e56bca7bcd00ab64fac8f0d88ca0530f9	no	10
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
			Total Files Size (in bytes):	195003	

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/288,406	10/07/2016	Terry WRIGHT	14764-258662

CONFIRMATION NO. 5402

107540
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

PUBLICATION NOTICE

OC000000088753092

Title:NOVEL HERBICIDE RESISTANCE GENES

Publication No.US-2017-0022515-A1
Publication Date:01/26/2017

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

COR-AAD1-00000258
Bayer EX1008 (Part 1 of 9)

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Jan 27, 2017 03:30:16 AM

Dear PAIR Customer:

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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Application	Document	Mailroom Date	Attorney Docket No.
15288406	NTC.PUB	01/26/2017	14764-258662

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If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Please type a plus sign (+) inside this box

INFORMATION DISCLOSURE STATEMENT BY APPLICANT	Complete if Known	
	Application Number:	15/288,406
	Filing Date:	October 7, 2016
	First Named Inventor:	WRIGHT, Terry
	Group Art Unit:	1663
	Confirmation Number:	5402
(Use as many sheets as necessary)	Examiner Name:	KRUSE, David H.
Sheet 1 of 2	Attorney Docket Number:	14764-258662

U.S. PATENT DOCUMENTS

Examiner Initials	Doc. No.	U.S. Patent Document: Application or Patent Number	U.S. Patent Document: Kind Code	Name of Patentee or Applicant	Date of Publication	Filing Date If Appropriate
	A A	5135867		Payne et al.	11-01-1988	
	A B	5273894		Strauch et al.	12-1993	
	A C	5316931		Donson et al.	02-26-1988	
	A D	5500360		Ahlquist et al.	03-07-1985	
	A E	5561236		Leemans et al.	10-1996	
	A F	5589367		Donson et al.	02-26-1988	
	A G	5608147		Kaphammer	03-1997	
	A H	5637489		Strauch et al.	06-1997	
	A I	5646024		Leemans et al.	07-1997	
	A J	5648477		Leemans et al.	07-1997	
	A K	5879903		Strauch et al.	03-1999	
	A L	6153401		Streber et al.	11-2000	
	A M	7112665		Leemans et al.	09-2006	
	A N	7250561		Pallett et al.	07-2007	
	A O	7838733		Wright et al.	11-2010	
	A P	8598413		Cui et al.	12- 2013	
	A Q	9127289		Wright	09-2015	
	A R	2003/0041357		Jepson et al.	02-2003	

FOREIGN PATENT DOCUMENTS

Examiner Initials	Doc. No.	Foreign Patent Document: Office	Foreign Patent Document: Application or Patent Number	Foreign Patent Document: Kind Code	Name of Patentee or Applicant	Date of Publication	Translation: Yes	Translation: No*+
	A S	WO	98/02562		Rhone Poulenc Agrochimie	01-22-1998		
	A T	WO	98/20144		Zeneca Ltd.	05-14-1998		
	A U	WO	2003/034813		US Agriculture	05-01-2003		

OTHER - NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Doc. No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number (s), publisher, city and/or country where published.	Translation: Yes	Translation: No*+
	A V	Genbank Accession No. M16730, Apr. 1993.		
	A W	Horvath, M. et al., Isolation and characterization of a 2-(2,4-dichlorophenoxy) propionic acid-degrading . . . , Appl. Microbiol Biotechnol., 1990, pp. 33, 213-216, (Abstract only).		
	A X	Kohler, Genbank Accession No. Q8KSC8 first published on Oct. 1, 2002 (Oct. 31, 2006).		

EXAMINER		DATE CONSIDERED	

* A concise statement of relevance is being submitted in lieu of a translation. 37 CFR 1.98(a)(3).

\+ An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of relevance under 37 CFR 1.98(a)(3).

Please type a plus sign (+) inside this box +

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary) Sheet 2 of 2	*Complete if Known*	
	Application Number:	15/288,406
	Filing Date:	October 7, 2016
	First Named Inventor:	WRIGHT, Terry
	Group Art Unit:	1663
	Confirmation Number:	5402
	Examiner Name:	KRUSE, David H.
	Attorney Docket Number:	14764-258662

OTHER - NON PATENT LITERATURE DOCUMENTS				
Examiner Initials	Doc. No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number (s), publisher, city and/or country where published.	Translation	
			Yes	No*+
	A Y	Kohler, Sphingomonas herbicidovorans tnpA gene for transposase and rdpA gene for (R)-2-(2,4-dichlorophenoxy) propionate, 2-oxoglutarate dioxygenase, Database EMBI, Accesion No. AJ628859, Mar. 3, 2004, pp. 1-2.		
	A Z	Kohler, "Sphinogomonas herbicidovorans MH: A versitle phenoxyalkanoic acid herbicide degrader," J. Industrial Microbiology & Biotechnology, vol. 23, No. 4-5: 336-340 (Oct. 1999)		
	B A	Lyon, B.R., et al., "Cotton plants transformed with a bacterial degradation gene are protected from accidental spray drift damage by the herbicide 2,4-dichlorophenoxyacetic acid," Transgenic Res., 1993, pp. 2: 166-169.		
	B B	Lyon, B.R., et al., Expression of a bacterial gene in transgenic tobacco confers resistance to the herbicide 2,4-dichlorophenoxyacetic acid, Transgenic Res., 1989, pp. 33: 166-169.		
	B C	Schleinitz et al., Rhodoferax sp. P230 R-2,4-dichlorophenoxypropionate dioxygenase (rdpA) gene, complete eds., Database EMBL, Accession No. AF516751, Aug. 6, 2002, pp. 1-2.		
	B D	Schleinitz, K.M., et al., Sphingobium hervbicidovorans R-2,4-dichlorphenoxypropionate dioxygenase (rdpA) gene, complete eds., Database EMBI., Accesion No. AF516752, Aug. 6, 2002, pp. 1-2.		
	B E	Schleninitz, K.M., Localization and Characterization of Two Novel Genes Encoding Sterospecific Diosygenases Catalyzing 2(2,4-Dichlrophenoxy) propionate cleavage in Delftia acidovorans MCI, Applied and Environmental Microbiology, Sep. 2004, pp. 5357-5365, vol. 70, No. 9.		
	B F	Smejkal, C.W. et al., "Substrate specificity of chlorophenoxyalkanoic acid-degrading bacteria is not dependent upon phylogenetically related *tfdA* gene types," Biol. Fertil., 2001, pp. 33:507-513.		
	B G	Spencer et al., Segregation of transgenes in maize, Plant Molecular Biology, Jan. 1992, pp. 201-210, vol. 18, Issue 2.		
	B H	Streber et al., Analysis, cloning, and high-level expression of 2,4-dichlorophenixyacetic monooxygenase gene tfdA of Alcaligenes eutrophus JMP134, J. Bacteriol., 1987, pp. 169: 2950-2955.		
	C A	Streber et al., Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D, Bio/Technology, 1989, pp. 7:811-816 (Abstract only).		
	C B	van der Meer, Genbank Accession No. AJ628859, NCBI-National Library of Medicine USA, Bethesda, Maryland (Mar. 3, 2004).		
	C C	Westendorf et al., "The two enantiospecific dichlorprop/α- ketoglutarate-dioxygenases from *Delftia acidovorans* MC1 – protein and sequence data of RdpA and SdpA, Microbiol. Res., 2002, pp. 157:317-322 (Abstract only).		
	C D	Westendorf, "Purification and characterization of the enantiospecific dioxygenases from *Delftia acidovorans* MC1 Initiating the Degradation of Phenoxypropionate and Phenoxyacetate Herbicides," Acta Biotechnol., 2003, pp. 23:3-17.		

DMS 11987509v1

EXAMINER		DATE CONSIDERED	

* A concise statement of relevance is being submitted in lieu of a translation. 37 CFR 1.98(a)(3).

\+ An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of relevance under 37 CFR 1.98(a)(3).

Electronic Acknowledgement Receipt

EFS ID:	32500778
Application Number:	15288406
International Application Number:	
Confirmation Number:	5402
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Customer Number:	107540
Filer:	John P. Breen/Mark Sweetin
Filer Authorized By:	John P. Breen
Attorney Docket Number:	14764-258662
Receipt Date:	01-MAY-2018
Filing Date:	07-OCT-2016
Time Stamp:	15:13:18
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		258662_IDS.pdf	54087 567e501916cc93508882bab03e0adf902b4579f2	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal Letter	1	3
	Information Disclosure Statement (IDS) Form (SB08)	4	5
Warnings:			
Information:			
	Total Files Size (in bytes):	54087	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PATENT
Attorney Docket No. 14764-258662
Client Reference: 63364A-US-CNT[3]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:

WRIGHT, Terry

Application No. 15/288,406

Filed: October 7, 2016

Confirmation No. 5402

Group Art Unit: 1663

Examiner: KRUSE, David H.

For: NOVEL HERBICIDE RESISTANCE GENES

INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.97 and 1.98, the references listed on the enclosed Form PTO-1449 and/or Substitute Form PTO-1449 ("Form 1449") are submitted for consideration by the Examiner in the examination of the above-identified patent application.

The full consideration of the references in their entirety by the Examiner is respectfully requested and encouraged. Also, it is respectfully requested that the references be entered into the record of the present application and that the Examiner place his or her initials in the appropriate area on the enclosed Form 1449, thereby indicating the Examiner's consideration of each of the references.

The submission of the references listed on the Form 1449 is for the purpose of providing a complete record and is not a concession that the references listed thereon are prior art to the invention claimed in the patent application. The right is expressly reserved to establish an invention date earlier than the above-identified filing date in order to remove any reference submitted herewith as prior art should it be deemed appropriate to do so.

Further, the submission of the references is not to be taken as a concession that any reference represents art that is relevant or analogous to the claimed invention. Accordingly, the right to argue that any reference is not properly within the scope of prior art relevant to an examination of the claims in the above-identified application is also expressly reserved.

The Information Disclosure Statement is being filed:

☒ **within** any one of the following time periods: (a) within three months of the filing date of a national application other than a continued prosecution application under 37 CFR 1.53(d); (b) within three months of the date of entry of the national stage as set forth in 37 CFR 1.491 of an international application; (c) before the mailing date of a first

Office Action on the merits; or (d) before the mailing of a first Office Action after the filing of a request for continued examination under 37 CFR 1.114.

☐ **after** (a), (b), (c) or (d) above, but before the mailing date of a final action under 37 CFR 1.113, a Notice of Allowance under 37 CFR 1.311, or an action that otherwise closes prosecution in the application, and includes *one* of:

- ☐ the Statement under 37 CFR 1.97(e) (see "Statement under 37 CFR 1.97(e)" below).

or

- ☐ the fee set forth in 37 CFR 1.17(p) (see "Fees" below).

☐ **after** the mailing date of a final action under 37 CFR 1.113 or a Notice of Allowance under 37 CFR 1.311, or an action that otherwise closes prosecution in the application, and on or before payment of the issue fee, and includes the Statement under 37 CFR 1.97(e) (see "Statement under 37 CFR 1.97(e)" below), and the fee set forth in 37 CFR 1.17(p) (see "Fees" below).

☐ **after** the mailing date of a Notice of Allowance under 37 CFR 1.311, and on or before payment of the issue fee, and **within** thirty days of receiving each item of information contained in the Information Disclosure Statement, and includes the Statement under 37 CFR 1.704(d) (see "Statement under 37 CFR 1.704(d)" below), and the fee set forth in 37 CFR 1.17(p) (see "Fees" below).

NOTE: This is for original applications except applications for a design patent, filed on or after May 29, 2000, wherein a paper containing only an Information Disclosure Statement in compliance with 37 CFR 1.97 and 1.98 is being filed.

Copies of the References

☐ Copies of all foreign patents and non-patent literature listed on the enclosed Form 1449 are enclosed herewith.

☐ Attached to each reference not in the English language is a concise explanation of the relevance pursuant to 37 CFR 1.98(a)(3). An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of the relevance pursuant to 37 CFR 1.98(a)(3).

☐ A copy of the international search report is enclosed herewith.

☒ The references listed on the enclosed Form 1449 were previously identified in the parent application(s) of the present application, and copies of the references were furnished at that time. Accordingly, additional copies of the references are not submitted herewith, so as not to burden the file with duplicate copies of references. The Examiner is respectfully requested to carefully review the references in accordance with the requirements set out in the Manual of Patent Examining Procedure. In accordance with 37 CFR 1.98(d), the

COR-AAD1-00000265
Bayer EX1008 (Part 1 of 9)

details of the parent application(s) relied upon for an earlier filing date under 35 USC 120 in which copies of the references were previously furnished are set out below:

U.S. APPLICATIONS		Status *(check one)*		
U.S. APPLICATIONS	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
1. 14/820893	08/07/2015		X	
2. 12/951,813	11/22/2010	X		
3. 11/587,893	05/22/2008	X		

Fees

☒ **No fee** is owed by the applicant(s).
☐ Charge Deposit Account No. 10-0435 in the amount of $0.00.

Authorization to Charge Additional Fees

☒ Please charge any additional required fees or credit any overpayments in connection with this communication to Deposit Account No. 10-0435.

Respectfully submitted,

/John Breen/
John Breen, Reg. No. 38833
BARNES & THORNBURG LLP
Customer No. 23643

Date: May 1, 2018

COR-AAD1-00000266
Bayer EX1008 (Part 1 of 9)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

107540 7590 11/13/2018

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

EXAMINER
KRUSE, DAVID H

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
11/13/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

COR-AAD1-00000267
Bayer EX1008 (Part 1 of 9)

Office Action Summary	Application No. 15/288,406	Applicant(s) WRIGHT et al.	
	Examiner DAVID H KRUSE	Art Unit 1663	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 15-48 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-48 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☑ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on See Continuation Sheet is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

COR-AAD1-00000268
Bayer EX1008 (Part 1 of 9)

Continuation of Application Papers 11): 07 October 2016

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Priority

2. Applicant's claim for the benefit of a prior-filed application under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, 365(c), or 386(c) is acknowledged. Applicant has not complied with one or more conditions for receiving the benefit of an earlier filing date under 35 U.S.C. 120 as follows:

The later-filed application must be an application for a patent for an invention which is also disclosed in the prior application (the parent or original non-provisional application or provisional application). The disclosure of the invention in the parent application and in the later-filed application must be sufficient to comply with the requirements of 35 U.S.C. 112(a) or the first paragraph of pre-AIA 35 U.S.C. 112, except for the best mode requirement. See *Transco Products, Inc. v. Performance Contracting, Inc.*, 38 F.3d 551, 32 USPQ2d 1077 (Fed. Cir. 1994)

The disclosure of the prior-filed application, Application No. 14/820,893, fails to provide adequate support or enablement in the manner provided by 35 U.S.C. 112(a) or pre-AIA 35 U.S.C. 112, first paragraph for one or more claims of this application. The prior filed applications fail to provide written description support for the cited AAD-1 motif recited in the instantly examined claims. The instant specification refers to an α-ketoglutarate-dependent dioxygenase motif on page 17, paragraph 0085, but there is no

description of a specific AAD-1 motif. Consequently the instant claims are given the priority date of the instant application, 7 October 2016.

Specification

3. The specification is objected to as failing to provide proper antecedent basis for the claimed subject matter. See 37 CFR 1.75(d)(1) and MPEP § 608.01(o). Correction of the following is required: the cited AAD-1 motif found in the claims lack prior antecedent basis in the Specification.

Information Disclosure Statement

4. The listing of references on pages 152-158 the specification is not a proper information disclosure statement. 37 CFR 1.98(b) requires a list of all patents, publications, or other information submitted for consideration by the Office, and MPEP § 609.04(a) states, "the list may not be incorporated into the specification but must be submitted in a separate paper." Therefore, unless the references have been cited by the examiner on form PTO-892, they have not been considered.

Double Patenting

5. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46

USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based e-Terminal Disclaimer may be filled out completely online using web-screens. An e-Terminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about e-Terminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

6. Claims 15-48 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-43 of U.S. Patent No. 7,838,733. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '733 Patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

7. Claims 15-48 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 9,127,289. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '289 Patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

8. Claim s 15-48 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-3 and 5-14 of copending Application No. 14/820,893. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the copending Application represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented, but the Examiner notes that copending Application has been allowed.

9. Applicant is put on notice that when/if U.S. Patent Application 14/989,787 is revived after an unintentional abandonment an Obviousness-type Double Patenting will be added to any subsequent Office action. The Examiner notes that said Application was allowed before it went abandoned.

Claim Rejections - 35 USC § 112

10. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

11. Claims 15-48 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicant claims a transgenic plant cell, plant and method of using wherein the plant cell comprises a recombinant polynucleotides encoding an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity having at least 85% sequence identity with instant SEQ ID NO: 9 and an AAD-1 motif of $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$.

Applicant describes the amino acid sequence of the AAD-1 (syn. RdpA) enzyme in instant SEQ ID NO: 9 and two variants thereof in instant SEQ ID Nos. 10 and 11 which are 98.8% and 99.7% identical respectively to said SEQ ID NO: 9.

Applicant does not describe the claimed genus of AAD-1 proteins or the AAD-1 motif (in the specification). The specification describes a motif comprising three conserved histidine residues found in α-ketoglutarate-dependent dioxygenases on page 17, paragraph 0085, $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$.

Hence, it is unclear that Applicant was in possession of the invention as broadly claimed.

Claim Rejections - 35 USC § 103

12. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

13. Claims 15-48 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jepson et al (U.S. Patent Application US 2003/0041357 filed on 23 February 2001) in view of Kohler (Genbank Accession No: Q8KSC8 first published on 1 October 2002).

Jepson *et al* teach a method of controlling at least one weed in an area by applying a phenoxy auxin herbicide said area comprising a transgenic plant comprising a recombinant polynucleotide encoding a protein having aryloxyalkanoate dioxygenase activity (see paragraph 0027 on pages 3-4, especially lines 18-24 on page 4). Jepson *et al* teach said method using a plant transformed with an additional transgene that provides glyphosate, glufosinate, imidazolinone or sulfonylurea herbicide tolerance (page 4, left column, lines 18-27). Jepson *et al* teach applying the phenoxy auxin herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Jepson *et al* teach said method can

be practiced using turf grass or forage grass (page 3, paragraph 0024, last sentence; claim 15). Jepson *et al* teach said method can be practiced using a plant transformed with a transgene that produces tolerance to a pre-emergence herbicide (claim 12).

Jepson *et al* do not teach a transgenic plant comprising a recombinant polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO: 9.

Kohler teaches a polynucleotide that encodes a protein comprising the amino acid sequence of instant SEQ ID NO: 9. Kohler teaches that said protein has R-2,4-dichlorophenoxypropionate dioxygenase activity (i.e. degrades the phenoxy auxin herbicide (R)-dichlorprop).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time of Applicant's invention to modify the teachings of Jepson *et al* to substitute the tfdA herbicide tolerance gene for a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase taught by Kohler. It would have been obvious to combine a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase with another gene for tolerance to additional herbicide tolerances as taught by Jepson et al. Jepson *et al* teach that one would apply one (claim 16) or two (claim 17) herbicides in the method of controlling weeds. Jepson *et al* teach that at the time of Applicant's invention it was desirable to produce plants tolerant to a phenoxy auxin herbicide. Given the high level of skill in the instant art at the time of Applicant's invention, one would have had a reasonable expectation of success.

Claim Rejections - 35 USC § 102

14. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

15. Claim(s) 15-25, 28-31, 45, 47 and 48 is/are rejected under pre-AIA 35 U.S.C. 102(a) and (e) as being anticipated by Ellis *et al* (US 2016/0108422 published 21 April 2016).

Ellis *et al* disclose polynucleotides that exhibit aryloxyalkanoate dioxygenase activity having at least 85% sequence identity with instant SEQ ID NO: 9 at SEQ ID Nos. 22, 25, 28, 31 and 34, for example, (claim 1). Ellis *et al* disclose a transgenic plant cell comprising said polynucleotides at claims 9-12. Ellis *et al* disclose a maize transgenic plant comprising said polynucleotides in Figure 1. Ellis *et al* discloses a method of controlling weeds using said transgenic plant using at least herbicide sleected from AOPP (aryloxyphenoxypropionate) herbicides, phenoxy acid herbicides and pyridinyloxy acid herbicides at claim 20. Hence, Ellis *et al* had previously disclosed the claim limitations.

16. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

17. Claim(s) 15-48 is/are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Wright *et al* (EP 1 740 039 B1).

Wright *et al* disclose a transgenic plant cell comprising instant SEQ ID NO: 9 at claims 1-15 and 46-48, in addition to a second herbicide resistance gene at claims 10-15. Wright *et al* disclose a method of controlling weeds using a plant comprising said transgenic plant cell at claims 16-45. Hence, Wright *et al* had previously disclosed the claimed invention.

Conclusion

18. No claims are allowed.

19. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

Notice of References Cited	Application/Control No. 15/288,406	Applicant(s)/Patent Under Reexamination WRIGHT et al.	
	Examiner DAVID H KRUSE	Art Unit 1663	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20030041357-A1	02-2003	Jepson, Ian	C07K14/415	800/300
*	B	US-20160108422-A1	04-2016	Ellis; Christine M.	A01N57/00	800/278
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N	EP 1 740 039 B1	06-2012	EP	Wright et al	------
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	Kohler, Genbank Accession No: Q8KSC8 first published on 1 October 2002 (Year: 2002)
	V	Hogan et al, The Journal of Biological Chemistry, 275(17): 12400-12409 (Year: 2000)
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/288,406	WRIGHT et al.
	Examiner	Art Unit
	DAVID H KRUSE	1663

CPC - Searched*		
Symbol	Date	Examiner
C12N 9/0069	11/07/2018	/DK/

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventor Name Search	11/07/2018	/DK/
EAST: USPAT, USPGPUB, EPO, JPO, DERWENT	11/07/2018	/DK/
STN: AGRICOLA, BIOSIS, CAPLUS, EMBASE, CABA	11/07/2018	/DK/
Sequence Search of SEQ ID NO: 9	10/30/2018	/DK/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	

COR-AAD1-00000280
Bayer EX1008 (Part 1 of 9)

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number:	15/288,406
Filing Date:	October 7, 2016
First Named Inventor:	WRIGHT, Terry
Group Art Unit:	1663
Confirmation Number:	5402
Examiner Name:	KRUSE, David H.
Attorney Docket Number:	14764-258662

U.S. PATENT DOCUMENTS

Examiner Initials	Doc. No.	U.S. Patent Document: Application or Patent Number	U.S. Patent Document: Kind Code	Name of Patentee or Applicant	Date of Publication	Filing Date If Appropriate
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	AB	5273894		Strauch et al.	12-1993	
	AC	5316931		Donson et al.	02-26-1988	
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Examiner Initials	Doc. No.	Foreign Patent Document: Office	Foreign Patent Document: Application or Patent Number	Foreign Patent Document: Kind Code	Name of Patentee or Applicant	Date of Publication	Translation: Yes	Translation: No*+
	AS	WO	98/02562		Rhone Poulenc Agrochimie	01-22-1998		
	AT	WO	98/20144		Zeneca Ltd.	05-14-1998		
	AU	WO	2003/034813		US Agriculture	05-01-2003		

OTHER - NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Doc. No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number (s), publisher, city and/or country where published.	Translation: Yes	Translation: No*+
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	AX	Kohler, Genbank Accession No. Q8KSC8 first published on Oct. 1, 2002 (Oct. 31, 2006).		

EXAMINER	/DAVID H KRUSE/	DATE CONSIDERED	11/05/2018

* A concise statement of relevance is being submitted in lieu of a translation. 37 CFR 1.98(a)(3).

+ An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of relevance under 37 CFR 1.98(a)(3).

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT	Complete if Known	
	Application Number:	15/288,406
	Filing Date:	October 7, 2016
	First Named Inventor:	WRIGHT, Terry
	Group Art Unit:	1663
	Confirmation Number:	5402
(Use as many sheets as necessary)	Examiner Name:	KRUSE, David H.
Sheet 2 of 2	Attorney Docket Number:	14764-258662

OTHER - NON PATENT LITERATURE DOCUMENTS				
Examiner Initials	Doc. No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number (s), publisher, city and/or country where published.	Translation Yes	Translation No*+
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EXAMINER	/DAVID H KRUSE/	DATE CONSIDERED	11/05/2018

* A concise statement of relevance is being submitted in lieu of a translation. 37 CFR 1.98(a)(3).

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Verified and Acknowledged: /DAVID H KRUSE/ (Examiner's Signature) | (Initials)

Title: NOVEL HERBICIDE RESISTANCE GENES

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CONTINUING DATA

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12951813 is a CON of 11587893 05/22/2008 PAT 7838733

11587893 is a 371 of PCT/US2005/014737 05/02/2005

PCT/US2005/014737 has PRO of 60567052 04/30/2004

FOREIGN APPLICATIONS

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	688	aad-1	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:20
L2	48	aad-1.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:21
L3	14	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:36

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Bayer EX1008 (Part 1 of 9)

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Site-directed Mutagenesis of 2,4-Dichlorophenoxyacetic Acid/α-Ketoglutarate Dioxygenase

IDENTIFICATION OF RESIDUES INVOLVED IN METALLOCENTER FORMATION AND SUBSTRATE BINDING*

(Received for publication, January 18, 2000)

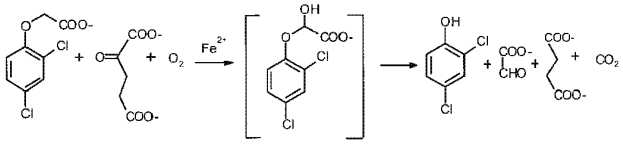
Deborah A. Hogan‡§¶, Sheila R. Smith‖, Eric A. Saari‖, John McCracken‖, and Robert P. Hausinger‡§‡‡**

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2,4-Dichlorophenoxyacetic acid (2,4-D)/α-ketoglutarate (α-KG) dioxygenase (TfdA) is an Fe(II)-dependent enzyme that catalyzes the first step in degradation of the herbicide 2,4-D. The active site structures of a small number of enzymes within the α-KG-dependent dioxygenase superfamily have been characterized and shown to have a similar HXDX$_{50-70}$HX$_{10}$RXS arrangement of residues that make up the binding sites for Fe(II) and α-KG. TfdA does not have obvious homology to the dioxygenases containing the above motif but is related in sequence to eight other enzymes in the superfamily that form a distinct consensus sequence (HX(D/E)X$_{138-207}$HX$_{10}$R/K). Variants of TfdA were created to examine the roles of putative metal-binding residues and the functions of the other seven histidines in this protein. The H167A, H200A, H213A, H245A, and H262A forms of TfdA formed inclusion bodies when overproduced in *Escherichia coli* DH5α; however, these proteins were soluble when fused to the maltose-binding protein (MBP). MBP-TfdA exhibited kinetic parameters similar to the native enzyme. The H8A and H235A variants were catalytically similar to wild-type TfdA. MBP-H213A and H216A TfdA have elevated K_m values for 2,4-D, and the former showed a decreased k_{cat}, suggesting these residues may affect substrate binding or catalysis. The H113A, D115A, MBP-H167A, MBP-H200A, MBP-H245A and MBP-H262A variants of TfdA were inactive. Gel filtration analysis revealed that the latter two proteins were highly aggregated. The remaining four inactive variants were examined in their Cu(II)-substituted forms by EPR and electron spin-echo envelope modulation (ESEEM) spectroscopic methods. Changes in EPR spectra upon addition of substrates indicated that copper was present at the active site in the H113A and D115A variants. ESEEM analysis revealed that two histidines are bound equatorially to the copper in the D115A and MBP-H167A TfdA variants. The experimental data and sequence analysis lead us to conclude that His-113, Asp-115, and His-262 are likely metal ligands in TfdA and that His-213 may aid in catalysis or binding of 2,4-D.

2,4-Dichlorophenoxyacetic acid (2,4-D)[1]/α-ketoglutarate (α-KG) dioxygenase (TfdA) is an Fe(II)- and α-KG-dependent enzyme that catalyzes the first step in degradation of the herbicide 2,4-D. This enzyme couples the oxidative decarboxylation of α-KG to the hydroxylation of a side chain carbon atom. The resultant hemiacetal spontaneously decomposes to form 2,4-dichlorophenol and glyoxalate (1). Mechanistically, TfdA resembles numerous other α-KG-dependent dioxygenases from plants, animals, fungi, and bacteria that catalyze similar hydroxylation reactions at unactivated carbon centers (2, 3).

Members of the α-KG-dependent dioxygenase superfamily are not closely related by their sequences but rather appear to fall into one of three groups of related enzymes or fall into a fourth group of unrelated sequences (4). The best studied α-KG-dependent hydroxylases, including prolyl and lysyl hydroxylase (5) and flavanone hydroxylase (3, 6), have an HXDX$_{\sim 55}$HX$_{10}$RXS motif in common (7, 8), and this motif is present in the more than 20 enzymes, defined here as Group I, within the α-KG dependent dioxygenase superfamily (8, 9). Site-directed mutagenesis studies have confirmed the importance of these residues for activity (8, 10–13), and the crystal structures of two Group I enzymes, isopenicillin *N* synthase (IPNS) and deacetoxycephalosporin C synthase (DAOCS), indicate that these residues comprise the metallocenter and α-KG-binding site (14–17). TfdA is not closely related in sequence to the Group I α-KG-dependent dioxygenases described above but is clearly homologous (25–30% identity) to *Escherichia coli* taurine/α-KG dioxygenase (TauD) (18) and sulfonate/α-KG dioxygenase from *Saccharomyces cerevisiae* (19). Furthermore, PSI-BLAST analyses (20) find additional rela-



SCHEME 1

* This research was supported in part by National Science Foundation Grants DEB9120006 and MCB 9603520 (to R. P. H), National Institutes of Health Grant GM 54065 (to J. M.), and the Michigan State University Agricultural Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: 2,4-D, 2,4-dichlorophenoxyacetic acid; α-KG, α-ketoglutarate; TfdA, 2,4-D/α-KG dioxygenase; TauD, taurine/α-KG dioxygenase; cw-EPR, continuous wave electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; IPNS, isopenicillin *N* synthase; DAOCS, deacetoxycephalosporin synthase; MBP, maltose-binding protein; FT, Fourier transform; MOPS, 4-morpholinepropanesulfonic acid.

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COR-AAD1-00000286
Bayer EX1008 (Part 1 of 9)

TABLE I
Consensus motifs for subgroups within the α-KG-dependent dioxygenase superfamily

Group I (IPNS, DAOCS, and related enzymes)	HXD	X_{50-70}	HX_{10}(R/K)XS
Group II (TfdA, TauD, clavaminate synthase and related enzymes)	HX(D/E)	$X_{138-207}$	HX_{10-13}R
Group III (Phytanoyl-CoA hydroxylase, proline hydroxylase and related enzymes)	HXD	X_{72-101}	HX_{10}(R/K)XS

tionships to γ-butyrobetaine hydroxylase and clavaminate synthase. Alignment of these Group II sequences indicates the conservation of two histidines and one aspartate (His-113, His-262, and Asp 115 in TfdA) as well as an invariant arginine that may be analogous to the α-KG-binding arginine in DAOCS and related enzymes (Table I). A third set of enzyme sequences (Group III) from members of the α-KG-dependent dioxygenase superfamily, including phytanoyl-CoA hydroxylase and proline hydroxylase, exhibit the presence of a third related motif despite the lack of overall sequence similarity to Group I or Group II enzymes.

In this study, we used site-directed mutagenesis methods to examine the roles of potential metal-binding residues in the above motif (His-113, His-262, and Asp-115) and the remaining seven histidines in TfdA. Previously published work showed that TfdA was inactivated by diethylpyrocarbonate, a histidine-selective reagent, and provided evidence consistent with the presence of multiple histidines in the active site (21). Spectroscopic studies of TfdA showed the presence of two equatorially bound imidazole nitrogens as ligands to the active site metal and indicated that one imidazole ligand may be displaced or shifted to an axial position upon substrate binding (22–24). Based on analyses of different TfdA variants, we identify several likely metal ligands and provide evidence that another one or two histidines may aid in substrate binding.

EXPERIMENTAL PROCEDURES

Recombinant Plasmids—All plasmids were constructed from pUS311 (21), a pUC19 derivative that contains the *Ralstonia eutropha* JMP134 *tfdA* gene (Fig. 1). The H8A, D115A, H213A, H216A, H235A, H245A, and H262A TfdA variants were created by direct mutation of *tfdA* in pUS311 by the Stratagene Quickchange System (Stratagene, La Jolla, CA). All mutagenic primers are listed in Table II. Two alternative approaches were used to construct the three remaining variants. Plasmids encoding H113A and H167A TfdA variants were created by CLONTECH mutagenesis of pXHtfdA, a pUC19 plasmid containing the 5′-*Xba*I-*Hin*dII fragment of the *tfdA* gene (Fig. 1). To create the complete gene containing the indicated mutations, the *Xba*I-*Hin*dII fragment was cloned into pHKtfdA, which contains the 3′ end of the *tfdA* gene. pHKtfdA was constructed in two steps. First, the 1.4-kilobase pair *Xba*I-*Sal*I fragment from pUS311, containing the complete *tfdA* gene, was cloned into pBC KS⁻ (Stratagene) cut with *Xba*I and *Xho*I to create pBCtfdA. This step had the benefit of eliminating a *Hin*dII site that interfered with further cloning steps. The 727-base pair *Hin*dII-*Kpn*I fragment of pBCtfdA was subcloned into pBC KS⁻ cut with the same enzymes to give pHKtfdA. Similarly, the gene encoding H200A TfdA was made by mutagenesis of pXXtfdA, a pUC19 plasmid containing the 5′-*Xba*I-*Xho*I fragment of *tfdA*. The altered *Xba*I-*Xho*I fragment was then inserted into pHKtfdA cut with *Xba*I and *Xho*I to give the complete H200A *tfdA* gene. The identity of all final constructs was confirmed by sequence analysis. To insert the genes encoding H167A and H262A variants of TfdA into a plasmid that would allow for isopropyl-1-thio-β-D-galactopyranoside-controlled expression, the *Xba*I-*Sal*I fragments from the corresponding plasmids described above were cloned into pET23a (Novagen) prepared with the same enzymes.

To create the maltose-binding protein (MBP)-TfdA fusion proteins, the wild-type *tfdA* gene was amplified from pUS311 with TfdA-MBPF and TfdA-MBPR primers (Table II) to create an *Xba*I site directly upstream of the GTG start codon of the *tfdA* gene and a *Hin*dIII restriction site 54 base pairs downstream of the stop codon. The polymerase chain reaction product was cloned directly into the pCEM-T vector according to the manufacturer's instructions (Promega, Madison, WI). The *Xba*I-*Hin*dIII fragment was isolated from the resulting plasmid and cloned into the pMAL-c2 vector that had been digested with the same enzymes. The identity of the newly created *malE-tfdA* gene fusion was confirmed by sequencing. Substitution of the mutation-containing internal *Nru*I fragment for the same fragment of the wild-type *malE-tfdA* gene created MBP-fusion forms of altered TfdAs. First, pMAL-*tfdA* was digested with *Nru*I, and the vector fragment was purified and religated to create pMAL-*tfdA*Δ*Nru*I. The resultant plasmid was linearized with *Nru*I and dephosphorylated with calf intestine alkaline phosphatase prior to ligation with the *Nru*I fragments isolated from the previously described mutant genes. Constructs were confirmed by restriction analysis.

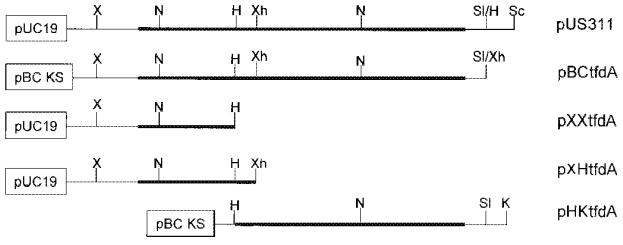


FIG. 1. **Restriction maps of the plasmids used in construction of *tfdA* mutants.** The abbreviations for the restriction enzyme sites are as follows: *H*, *Hin*dII; *X*, *Xba*I; *Xh*, *Xho*I; *Sl*, *Sal*I; *K*, *Kpn*I; *Sc*, *Sac*I; and *N*, *Nru*I. The vector backbone is indicated in the *box* on the *left*. The *tfdA* open reading frame is shown as a *wide, solid line*.

TABLE II
Sequences of mutagenic primers used to create altered tfdA genes

Mutant	Primer sequence[a]
H8A	5′-CGC AAA TCC CCT TGC TCC TCT TTT CGC C-3′
H113A	5′-GTC GCT GGC CCA AGC TGG-3′
D115A	5′-GCA CAG CGC CAG CTC CTT TCA-3′
H167A	5′-GCG TGC CGA GCA GTA CGC ACT G-3′
H200A	5′-GGT TCG AAC CGC CGC CGG CTC-3′
H213A	5′-GCT CGC GGC CGC GCC GAT-3′
H216A	5′-CCT TCG ACG GCG CTC GCG-3′
H235A	5′-GCT TCT CGA GGC GAC ACA G-3′
H245A	5′-GTG TAC CGG GCT CGC TGG AAC-3′
H262A	5′-CGT TCT TGC ACG CGG ACG CAG-3′
Tfda-MBPF	5′-TCT CTA GAG TGA GCG TCG TCG CAA ATC C-3′
Tfda-MBPR	5′-GTC AAG CTT GGT TGC GTA CAT CTT GTG G-3′

[a] The reverse primer used for the creation of all mutants was the complement of the forward primer.

Protein Purification—H8A, H113A, D115A, H216A, H235A, and wild-type TfdA proteins were purified from *E. coli* DH5α cells carrying pUS311 and its mutated derivatives according to a previously described protocol (21). In addition, the non-mutated enzyme and the TfdA variants H167A, H200A, H213A, H245A, and H262A were purified as MBP-TfdA fusion proteins from *E. coli* DH5α by the protocol described in the pMAL Protein Fusion and Purification System Manual (New England Biolabs, Beverly, MA).

Analysis of Kinetic Parameters—Specific activities of the wild-type and variant TfdA proteins were determined by a previously described spectrophotometric assay (21). The typical assay mixture contained 1 mM 2,4-D, 1 mM α-KG, 100 μM $(NH_4)_2Fe(SO_4)_2$, and 100 μM ascorbic acid in 10 mM MOPS buffer (pH 6.75) at 30 °C. The reactions were quenched by the addition of EDTA to a concentration of 5 mM. 2,4-Dichlorophenol was quantified by reaction with 4-aminoantipyrene followed by measurement of the absorbance at 510 nm. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of dichlorophenol·min^{-1}. Protein concentrations were determined using

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the Bio-Rad Protein Assay with bovine serum albumin as a standard. For calculation of the k_{cat} values, the TfdA variants were assumed to have M_r = 31,600 and the MBP-TfdA variants were assigned M_r = 74,500.

The low K_m values for α-KG (~2–5 μM for the wild-type enzyme) precluded use of the 4-aminoantipyrene assay for accurate determination of this value. The alternative method used to measure the K_m values for α-KG quantified the amount of $^{14}CO_2$ liberated from α-[1-^{14}C]KG during the course of the reaction (21).

Native Protein Analysis by Gel Filtration—Size exclusion chromatography was used to estimate the native molecular weights of TfdA, MBP-TfdA, and mutant proteins. The proteins were chromatographed on a Superose 6 gel filtration column (1.0 × 30 cm, Amersham Pharmacia Biotech) in 20 mM Tris buffer (pH 7.5), 1 mM EDTA, and 200 mM NaCl at a flow rate of 0.2 ml·min^{-1}. The elution volumes were compared with those for gel filtration standards (Bio-Rad) including thyroglobulin, 670 kDa; bovine gamma globulin, 158 kDa; chicken ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin B_{12}, 1350 Da.

Spectroscopic Analysis—Proteins for electron paramagnetic resonance (EPR) and electron spin-echo envelope modulation (ESEEM) spectroscopic analyses were exchanged into 25 mM MOPS (pH 6.75) by repeated concentration and dilution in Centricon 30 (Amicon) centrifugal concentrators. The final subunit concentration was 0.5 mM for the non-fusion forms of TfdA and 0.4 mM for the MBP-TfdA proteins. $CuCl_2$ was added to a concentration of 450 and 350 μM, respectively. Buffered solutions of α-KG and 2,4-D were added to final concentrations of 5 mM. Glycerol was present at 40% in all samples.

X-band EPR spectra were obtained at 77 K on a Bruker ESP-300E spectrometer. ESEEM data were collected on a home-built spectrometer; the microwave bridge of this instrument has been previously described in detail (25). Data collection and analyses were controlled by a Power Computing model 200 Power PC using software written with LabView version 5.01 (National Instruments). Electron spin echoes were digitized, averaged, and integrated by a Tektronix model 620B digital oscilloscope interfaced to the spectrometer computer via an IEEE-488 bus. Two four-channel delay and gate generators (Stanford Research Systems model DG535), a Bruker BH-15 magnetic field controller, and a Hewlett-Packard model 8656B radiofrequency synthesizer were also interfaced using IEEE-488 protocol. Data were collected using a reflection cavity that employed a folded microstrip resonator (26). A three-pulse stimulated echo sequence (90-τ-90°-T-90°) was used. ESEEM spectra were generated by Fourier transformation of the time domain data using dead time reconstruction (27). Simulations of the experimental data were performed on a Sun SparcII work station. Simulation programs were written in FORTRAN and based on the density matrix formalism developed by Mims (28). Software for the frequency analysis of the experimental and simulated data was written in Matlab (Mathworks, Natick, MA).

Sequence Comparisons—Related sequences were initially detected by BLAST (29) and PSI-BLAST (20) analyses. Alignments were generated with the CLUSTAL algorithm (30), and the figure was prepared using Genedoc (31).

RESULTS

Production of the Mutant TfdAs—Initially, all of the mutant genes were expressed from their pUC19-based plasmids except for those encoding H113A, H167A, and H200A TfdA, which were in pBC KS$^-$-derived plasmids. By using the standard protocol to produce soluble, wild-type TfdA (growth at 30 °C to early stationary phase), only the H8A, H113A, D115A, H216A, and H235A variants existed as soluble proteins. All of the other TfdA variants were present as inclusion bodies even when grown at lower temperatures (22 °C), in M9 minimal medium, or in LB broth containing 660 mM sorbitol and 2.5 mM betaine (32). In addition, isopropyl-1-thio-β-D-galactopyranoside-controlled production of H167A and H262A proteins from mutant genes cloned into pET23a did not yield soluble samples even when the harvested cell pellets were suspended in buffer containing 20% glycerol to limit protein aggregation.

To overcome the solubility problems for the five TfdA variants, MBP-TfdA fusion proteins were created. Wild-type TfdA and the MBP-TfdA fusion protein had essentially identical k_{cat} and very similar K_m values for α-KG and 2,4-D (Table III). A slight increase in the apparent K_D for Fe(II) may reflect some metal binding capacity of MBP. Since the presence of the fusion protein did not appear to greatly affect the kinetic parameters of wild-type enzyme, similar fusion proteins were created for the H167A, H200A, H213A, H245A, and H262A TfdA variants.

TABLE III
Summary of the kinetic parameters for active TfdA variants

TfdA sample	k_{cat}	K_m αKG	K_m 2,4-D	K_D Fe(II)
	min^{-1}	μM	μM	μM
Wild type	442 ± 33	4.9 ± 0.73	19.3 ± 3.7	2.0 ± 0.5
MBP fusion	411 ± 23	9.9 ± 1.8	33.7 ± 2.5	15.2 ± 5.4
H8A[a]	284 ± 6.9	6.5 ± 1.8	17.7 ± 3.8	1.9 ± 0.7
MBP-H213A	22.1 ± 2.9	8.3 ± 2.9	318 ± 44	27.7 ± 9.8
H216A	474 ± 47	6.05 ± 2.6	52 ± 5	2.4 ± 1.7
H235A	284 ± 25	9.6 ± 1.6	34 ± 8	1.4 ± 1.7

[a] More than 75% of the protein was present as the degradation product. The k_{cat} was estimated from the active, full-length fraction.

Kinetic Analyses of Altered TfdAs—Results from kinetic analyses of the four active mutant proteins are summarized in Table III. H8A TfdA was soluble and active but was rapidly proteolyzed to an inactive form. By electrophoretic comparisons, the cleavage site appeared to be the same as in wild-type TfdA (between Arg-77 and Phe-78) (21). The rate of proteolysis of H8A TfdA was enhanced compared with that seen for the wild-type enzyme despite the presence of EDTA and protease inhibitors in the purification buffer. Because purified H8A TfdA was more than 75% degraded, the catalytic rate constant was calculated with the estimated amount of intact enzyme. These calculations indicate rates and K_m values similar to those for the wild-type enzyme. Similarly, the kinetic parameters for H235A TfdA were comparable to the native enzyme. In contrast, two variants exhibited differences from wild-type enzyme in their kinetic parameters. The H213A MBP-TfdA variant exhibited a 20-fold reduction in k_{cat} and a 10-fold increase in K_m for 2,4-D. In addition, H216A TfdA had a modest (2.5-fold) increase in the K_m for 2,4-D and no change in catalytic rate. The other kinetic parameters for H213A MBP-TfdA and H216A TfdA (K_m for α-KG and K_D for ferrous ion) did not differ significantly from the wild-type values. Six soluble TfdA variants (H113A, D115A, MBP-H167A, MBP-H200A, MBP-H245A, and MBP-H262A) exhibited no activity even when assayed with elevated substrate and cofactor concentrations (10 mM α-KG, 5 mM 2,4-D, and 250 μM Fe(II)).

Evaluation of the Structural Consequences of the Mutations—To assess whether the inactive mutant proteins assumed conformations similar to the wild-type enzyme, their apparent molecular weights were estimated by gel filtration analysis. The observed size of wild-type TfdA was found to be 51 kDa by comparison to protein standards, suggesting that TfdA forms a compact dimer or an elongated monomer. The elution volume for both H113A and D115A corresponded exactly to wild-type TfdA indicating that these proteins are not significantly altered in their quaternary structure. MBP-TfdA eluted both in the void volume (approximately 25% of the protein) and at a position corresponding to 216 kDa (roughly 75% of the protein), suggesting that MBP-TfdA forms at least a dimer. Because each MBP-TfdA subunit is comprised of two domains separated by a 13-amino acid linker, the resultant protein may migrate with a larger apparent molecular weight. MBP-H167A and MBP-H200A samples demonstrated the same two-peak profile as MBP-TfdA but with larger proportions eluting in the void volume. MBP-H245A and MBP-H262A proteins were soluble; however, gel filtration analysis indicated the presence of only highly aggregated material eluting in the void volume. Because these mutant proteins exhibited aberrations in their folding properties, the catalytic role of His-245 and His-262, if any, could not be assessed.

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EPR Spectroscopic Characterization of Variants with Altered Metal Sites—The metallocenter properties for selected TfdA variants were probed by EPR spectroscopy. To circumvent the problems that arise in EPR measurements of integer spin paramagnetic centers, Fe(II) was substituted with cupric ion. Although the Cu(II) form of TfdA is inactive, Cu(II) binds competitively with respect to Fe(II) (K_i = 1–3 μM), and copper-substituted TfdA has been used previously to study the metal coordination environment of this enzyme in the presence and absence of substrates (22–24). Spectral parameters of wild-type Cu(II)-TfdA, Cu(II)-TfdA + α-KG, and Cu(II)-TfdA + α-KG + 2,4-D (Fig. 2*A* and Table IV), agreed well with those reported previously (23, 24). Earlier studies of copper-substituted wild-type TfdA indicated that the metal is bound in a type 2 environment with a mixture of O and N ligands in the equatorial plane. Upon addition of α-KG and 2,4-D to the enzyme, the spectral parameters are altered to a more rhombic signal with accompanying resolution of ligand hyperfine coupling (Fig. 2*A*). These results suggest that binding of the co-substrates to the enzyme leads to a better defined copper site with α-KG binding directly to the metallocenter (23, 24). The small $A_{\parallel}$ (less than 14 mT) for the α-KG- and 2,4-D-bound sample indicates a significant distortion from planarity.

The four inactive mutant forms of TfdA with quaternary structures similar to the corresponding wild-type protein (H113A, D115A, MBP-H167A, and MBP-H200A) were analyzed by EPR spectroscopy to assess the metal coordination environments. No significant differences between the spectra of MBP-TfdA and the non-fusion wild-type TfdA were observed (data not shown). EPR spectra of the copper-substituted samples, in all cases, showed contributions from multiple copper sites indicating a mixture of copper centers, most likely resulting from copper binding in multiple conformations or at sites other than the active site. The presence of alternative copper-binding sites is not surprising in an enzyme with nine histidines.

The EPR spectra for Cu-H113A TfdA alone and in the presence of α-KG and 2,4-D (Fig. 2*B* and Table IV) differ significantly from spectra of wild-type enzyme and show modest changes in Cu(II) g values and hyperfine tensor principal values upon substrate additions. The broadening of the EPR signal in the $g_{\parallel}$ region upon addition of α-KG again suggests a mixture of copper site conformations. Thus, it appears that alteration of His-113 significantly affects the metal binding properties for TfdA such that copper is no longer constrained to a single active site configuration.

The EPR spectrum of D115A-TfdA (Fig. 2*C*) has parameters similar to those observed in the copper-substituted wild-type protein (Table IV), although with less resolution of $A_{\parallel}$. Addition of α-KG (in the presence or absence of 2,4-D) has a dramatic effect on the appearance of the D115A data, enhancing resolution of the Cu(II) hyperfine peaks at $g_{\parallel}$ and the ligand hyperfine structure in the $g_{\perp}$ region (Fig. 2*C*). The appearance of these superhyperfine interactions may result from a subtle shift in the orientation of the principal g tensor such that the imidazole ligands occupy an increasingly equatorial position (33). These results are consistent with the formation of a tighter or more regular copper-binding site upon addition of the co-substrate.

The EPR spectrum for MBP-H167A TfdA (Fig. 2*D*), like that for the H113A variant, is poorly resolved, probably due to binding of copper in multiple configurations instead of formation of one major conformation. Additionally, few significant changes are seen upon addition of either α-KG or 2,4-D. The MBP-H200A EPR spectrum (Fig. 2*E*) is better resolved than that of H113A or MBP-H167A and clearly shows a second set of resonances of lower amplitude with parameters identical to those observed for the copper-substituted wild-type enzyme

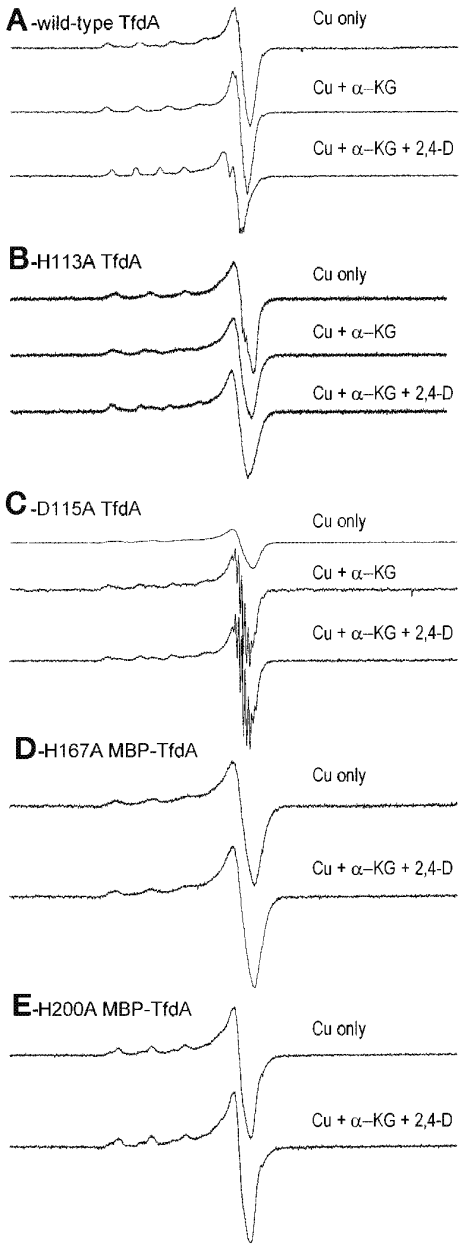


FIG. 2. **X-band CW-EPR spectra of Cu(II)-substituted TfdA variants.** EPR spectra were obtained at 77 K for the following samples (substrate concentrations were 5 mM, when present). *Top to bottom,* wild-type Cu-TfdA in the absence of substrates, with α-KG, and with α-KG + 2,4-D (*A*). H113A Cu-TfdA in the absence of substrates, with α-KG, and with α-KG + 2,4-D (*B*). D115A Cu-TfdA in the absence of substrates, with α-KG, and with α-KG + 2,4-D (*C*). MBP-H167A Cu-TfdA in the absence of substrates, with α-KG, and with α-KG + 2,4-D (*D*). MBP-H200A Cu-TfdA in the absence of substrates and in the presence of α-KG + 2,4-D (*E*). Spectral parameters are listed in Table IV.

(Table IV). The presence of this wild-type signal suggests that His-200 is most likely not a copper-binding ligand in TfdA. As found for H113A and MBP-H167A, addition of α-KG and/or 2,4-D has little effect on the spectrum.

ESEEM Spectroscopic Characterization of Variants with Altered Metal Sites—Pulsed EPR (ESEEM) spectroscopy has proven useful for determining the number of histidines bound to copper in proteins. This approach has been previously used to determine that copper-substituted wild-type TfdA binds cop-

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TABLE IV
Summary of the EPR spectral parameters for Cu(II)-substituted TfdA variants

	$g_\parallel$	$A_\parallel$	$g_\perp$
		mT	
Wild-type TfdA	2.34	16.3	2.07
Wild-type TfdA/α-KG	2.36	15.1	2.07
Wild-type TfdA/α-KG/2,4-D	2.38	12.0	2.09
H113A	2.30	16.6	2.07
H113A/α-KG	2.33	15.8	2.07
H113A/α-KG/2,4-D	2.35	14.0	2.08
D115A	2.30	17.0	2.06
D115A/α-KG	2.30	16.2	2.06
	2.34	16.8	2.06
D115A/α-KG/2,4-D	2.30	16.2	2.06
	2.34	16.8	2.06
MBP-H167A	2.30	17.1	2.07
MBP-H167A/α-KG/2,4-D	2.30	17.1	2.07
MBP-H200A	2.30	17.1	2.07
	2.34	16.1	2.07
MBP-H200A/α-KG/2,4-D	2.30	17.1	2.07
	2.34	16.1	2.07

per in a site with two histidyl residues directly coordinated to the metal. As previously reported (23), three-pulse ESEEM spectra collected in the $g_\parallel$ region for wild-type TfdA exhibit sharp peaks at 0.6, 0.9, and 1.5 MHz and a broad feature at 3.5 MHz (Fig. 3*B*). This signature is typical of imidazole bound in an equatorial position to copper. The additional appearance of narrow combination bands at 2.1, 2.5, and 3.1 MHz in these spectra indicated the presence of at least two such imidazole ligands bound to the copper (34–36). Spectral simulations using the density matrix approach of Mims (28) were used to analyze these data further including determination of the number of histidyl ligands coordinated to copper. The $g_\parallel$ ESEEM of Cu(II)-substituted wild-type TfdA was simulated with magnetic parameters for copper equatorially coordinated to two identical histidines with a simulation program using the angle selection scheme developed for ENDOR analysis (37, 38). For each simulation, a background decay function was applied to allow the amplitudes of the initial 1.5 μs of the simulations to match the data. The resulting simulated data sets (*dashed curves*) are superimposed on the experimental data in Fig. 3. Further analysis of these data suggested that formation of the ternary complex with α-KG and 2,4-D results in the probable loss or reorientation of one of these imidazole ligands, as evidenced by a substantial decrease in the modulation intensity and disappearance of the combination bands (23).

Three-pulse ESEEM patterns for the H113A (*trace A*), MBP-H167A (*trace B*), and D115A (*trace C*) variants of TfdA are shown in Fig. 4. Each data set was collected under identical conditions and normalized so that the integrated echo amplitudes range from zero, determined by shifting the integration window off of the signal at the end of a scan, to one which marks the largest measured echo amplitude. Low frequency modulations indicative of equatorially bound histidyl ligand(s) are observed for all four mutant TfdAs. The ESEEM from H113A (*trace A*) and MBP-H200A (not shown) variants are nearly identical and show modulations that are considerably weaker than those measured for MBP-H167A (*trace B*) and D115A (*trace C*). Each of these data contains a large unmodulated or DC component (well over 80% of the total signal intensity in the case of H113A), contrasting sharply with the ESEEM for copper-substituted wild-type TfdA, where the DC component constituted less than 40% of the total signal intensity (Fig. 3A).

The frequency spectra, obtained by Fourier transformation (FT) of the data shown in Fig. 4, are characterized by major

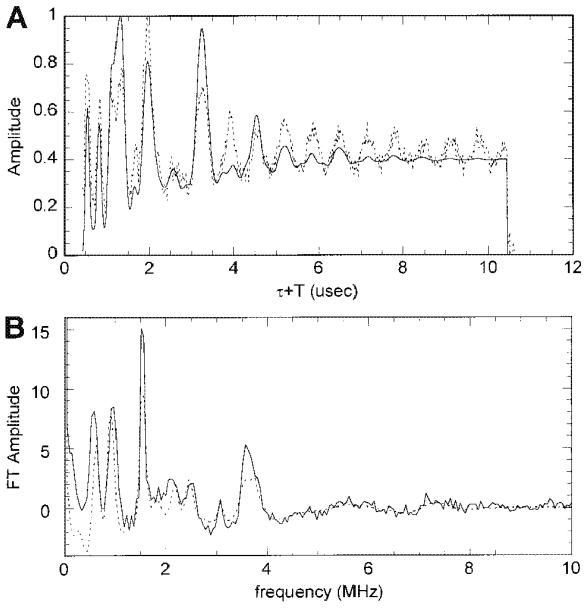


FIG. 3. **Three-pulse ESEEM spectrum and Fourier transformation of Cu(II)-substituted wild-type TfdA with 2-histidine simulation data.** ESEEM spectrum of wild-type Cu(II)-TfdA + α-KG (*solid*) and computer-simulated data for Cu(II) bound by two identical histidines (*dashed*) (*A*) and the corresponding frequency domain spectrum (*B*). Parameters for data collections were magnetic field = 2800 G, τ = 375 ns, T = 50 ns, ν = 8.98 GHz; 4.2 K. Cu(II) hyperfine parameters: g_{xy} = 2.05, g_z = 2.34, $A_{xx} = A_{yy}$ = 20 MHz, A_{zz} = 487 MHz; Superhyperfine parameters: g_N = 0.40347, A_{iso} = 1.7 MHz, radius = 2.75 Å [θ,ϕ] = [2.37,2.07] radians, e^2qQ = 1.60 MHz, η = 0.75, NQI [α,β,γ] = [0.50,1.1,0.1] radians. The simulated data were treated with an exponential decay function to give $y_{ij} = (y_{ij} - 0.17)\cdot\exp((\tau + T)/3500)^{1.5}) + 0.17$. For each simulation, a background decay function was applied to allow the amplitudes of the initial 1.5 μs of the simulations to match the data.

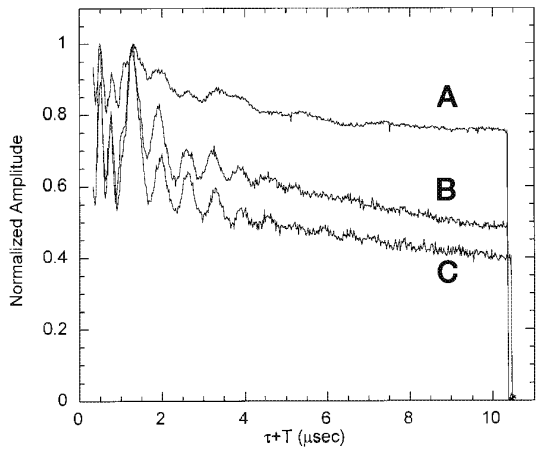


FIG. 4. **Three-pulse ESEEM time domain spectra of TfdA variants.** H113A Cu-TfdA (*A*), magnetic field = 3050 G, τ = 300 ns, T = 40 ns. MBP-H167A Cu-TfdA (*B*), magnetic field = 3100 G, τ = 300 ns, T = 40 ns. D115A Cu-TfdA (*C*), magnetic field = 3050 G, τ = 385 ns, T = 55 ns, ν = 8.8 GHz; 4.2 K.

features at 0.7, 1.5, and 4.0 MHz that are indicative of histidyl imidazole equatorially coordinated to Cu(II) (36). The spectra obtained for D115A and MBP-H167A variants are shown in Figs. 5*B* and 6*B* (*solid lines*), respectively. The resolution in these spectra is poor when compared with similar data obtained for rigid Cu(II) proteins or model complexes (39, 40). In Fig. 5A, the normalized three-pulse ESEEM data for D115A TfdA (*solid line*) are shown with the computer-simulated data

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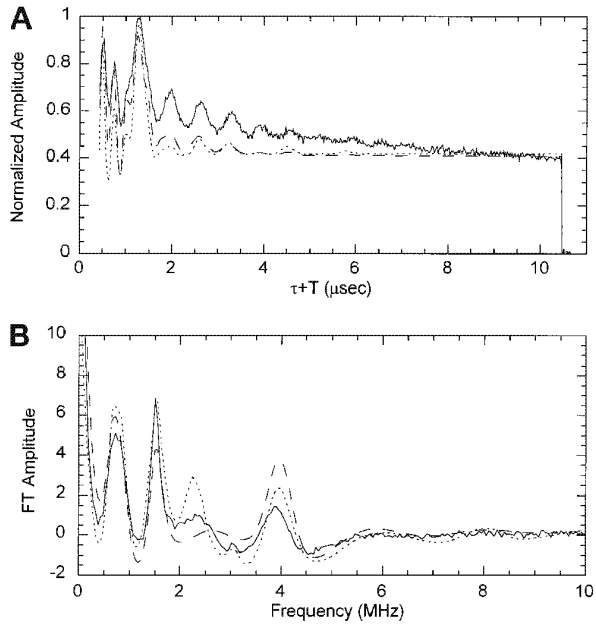


FIG. 5. **Spectral simulation of ESEEM data for D115A-Cu-TfdA.** Experimental (*solid line*), computer-simulated 1-His (*dashed line*), and simulated 2-His (*dotted line*) time domain spectra (*A*) and the corresponding Fourier-transformed data (*B*) for copper-substituted D115A TfdA are shown. Magnetic field = 3050 G, τ = 230 ns, T = 50 ns G, ν = 8.8 GHz, 4.2 K, g_N = 0.40347, e^2qQ = 1.55 MHz, η = 0.87, A_{xx} = 1.6 MHz, A_{yy} = 1.9 MHz, A_{zz} = 2.2 MHz. The simulated data were treated with an exponential decay function to give $y_{ij} = (y_{ij} - 0.05)\cdot\exp((\tau + T)/600)^{0.8}) - 0.05$.

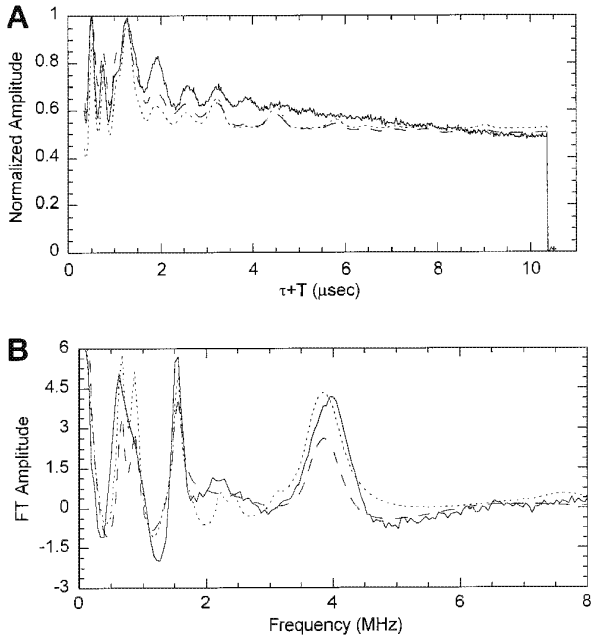


FIG. 6. **Spectral simulation of ESEEM data for MBP-H167A-Cu-TfdA.** Experimental (*solid line*), computer-simulated 1-His (*dashed line*), and 2-His (*dotted line*) time domain spectra (*A*) and the corresponding Fourier-transformed data (*B*) are shown. Magnetic field = 3100 G, τ = 300 ns, T = 40 ns, ν = 8.8 GHz, 4.2 K, g_N = 0.40347, e^2qQ = 1.60 MHz, η = 0.82, A_{xx} = 1.55 MHz, A_{yy} = 1.9 MHz, A_{zz} = 2.0 MHz. The simulated data were treated with an exponential decay function to give $y_{ij} = (y_{ij} - 0.03)\cdot\exp((\tau + T)/350)^{0.5}) - 0.03$.

for one (*dashed line*) and two (*dotted line*) coordinated histidyl ligands obtained using spin Hamiltonian parameters typical for the remote nitrogen of imidazole equatorially bound to Cu(II). To compare the amplitudes of the ESEEM simulations with those obtained experimentally, the simulated ESEEM patterns of Fig. 5*A* were multiplied by background decay functions characterized by an e^{-1} time of 600 ns. This decay time constant is a factor of 6 faster than that used to make the comparison between simulation and experiment for wild-type Cu-TfdA in Fig. 3. Unfortunately, the severity of this decay impairs our ability to distinguish whether 1 or 2 nitrogen nuclei are giving rise to the ESEEM of D115A based on modulation depths.

The Fourier transforms of the experimental and simulated ESEEM functions of Fig. 5*A* are shown in Fig. 5*B*. Although the combination lines are weaker in the data for D115A (*solid line*) than for wild-type TfdA, the combination line at ~3.0 MHz is observed, and its frequency is predicted nicely by the 2-His simulation. The broader line widths of the FT spectra found for D115A TfdA, and the subsequent difficulty in observing the combination lines most likely reflects a distribution of ^{14}N superhyperfine couplings for the Cu(II) sites of the variant. To account for the observed line widths and relative peak amplitudes in our simulations, it was necessary to average several calculated data sets obtained with ^{14}N isotropic hyperfine coupling constants that ranged from 1.6 to 1.8 MHz. This assumption of a distribution of hyperfine couplings results in better agreement between experiment and theory for our ESEEM simulations and is in accord with the poor resolution observed in the cw-EPR spectrum of Cu(II)-D115A TfdA in the absence of substrates.

A parallel analysis was undertaken for the MBP-H167A data, and the results are provided in Fig. 6. The *dash* and *dot* patterns of Fig. 6*A* are simulation results for one and two equatorially bound histidyl ligands, respectively. The broadness of the lines at 0.6 and 0.9 MHz results in these frequencies being unresolved in the experimental data. This effect could be simulated using a higher asymmetry parameter, η, for the nuclear quadrupole interaction but not without significant deficiencies in the prediction of the double quantum peak at 4 MHz. A more satisfactory method of accounting for this coalescence in the simulations was through the averaging of calculation results obtained using a distribution of isotropic hyperfine coupling constants, as in the simulations for D115A. The simulations in Fig. 6 are the result of averaging five such data sets where the ^{14}N isotropic hyperfine coupling ranged from 1.6 to 1.9 MHz. This method resulted in the broadening of the low frequency peaks to the point where they began to overlap, without reducing the magnitude of the double quantum feature at 4 MHz. As in the analysis of D115A, different background decay functions were applied to these simulations to provide the best match to the modulation intensities for the initial 1.5 μs of the ESEEM data (*solid line*). The background decay required to reduce the initial amplitudes of both one and two imidazole simulations was a factor of 10 faster than that used to simulate wild-type Cu(II)-TfdA ESEEM. This problem, combined with the lack of resolved combination peaks in the data, makes it difficult to determine the number of bound histidines. Although the modulation intensities are best accounted for by the one-histidine model, the features of the ESEEM spectrum are better simulated assuming two coupled nitrogens. The ESEEM patterns associated with the H113A and MBP-H200A mutants were too shallow to be consistent with single histidyl coordination, and it is likely that these mutant proteins have multiple Cu(II)-binding sites, some with equatorially coordinated histidine ligand(s) and some without.

The Cu(II)-substituted forms of H113A, D115A, MBP-H167A, and MBP-H200A were also analyzed by ESEEM spec-

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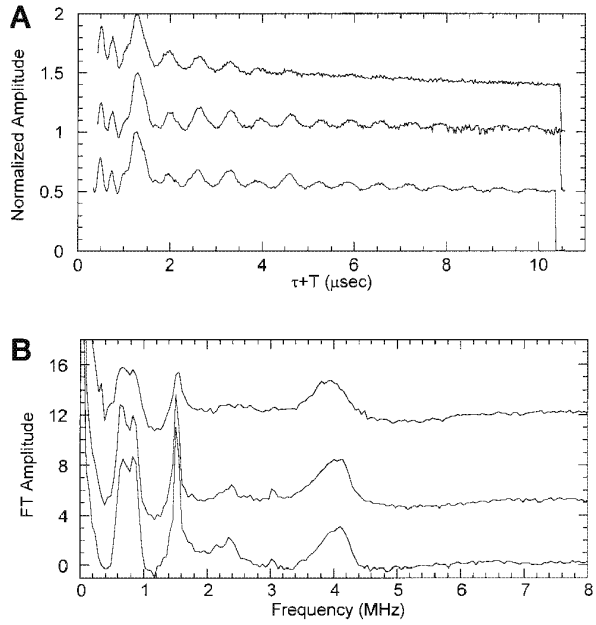


FIG. 7. **Three-pulse ESEEM of D115A TfdA (4.2K).** *Top to bottom,* D115A Cu-TfdA in the absence of substrate, with 5 mM α-KG, and with 5 mM α-KG + 5 mM 2,4-D (*A*) and the corresponding frequency domain spectra (*B*). Conditions for these measurements were identical to those of Fig. 3.

troscopy in the presence of α-KG and 2,4-D. Only slight variations in the line shape of the higher frequency peak at ~4.0 MHz were observed for the three histidine mutants. This result contrasts sharply with those for copper-substituted wild-type TfdA where addition of the co-substrates resulted in data that suggested a shift from two equatorially bound histidyl ligands to only one. The lack of similar changes in the ESEEM of these mutants upon addition of the co-substrates supports the conclusion that alteration of these key histidyl residues significantly effects the metal binding properties of the TfdA enzyme. Three-pulse ESEEM spectra for D115A TfdA in the presence of α-KG and α-KG + 2,4-D (Fig. 7) show that addition of co-substrates leads to improved resolution in the ESEEM spectra without significant changes in the amount of unmodulated intensity. These results mirror the improved resolution observed in the cw-EPR spectra. For the ternary complex with both co-substrates bound to the D115A variant, all three combination lines from the electron spin manifold that gives rise to the intense low frequency peaks are resolved (Fig. 7*B*). Furthermore, the cw-EPR spectra of D115A treated with co-substrates show well resolved ligand hyperfine structure at $g_\perp$. Taken together, these data indicate that two histidyl ligands remain equatorially bound during addition of the co-substrates and support our proposal that the differences between theory and experiment found for the two-histidine simulation of Fig. 5 most likely stem from a distribution of structures present prior to substrate addition.

DISCUSSION

EPR, ESEEM, and extended x-ray absorption fine structure spectroscopies of both the Fe(II)- and Cu(II)-forms of wild-type TfdA had previously indicated that the resting enzyme has two imidazoles and a mixture of bound nitrogens and oxygens as ligands to the metal (22–24). ESEEM and x-ray absorption data indicated that both imidazole ligands are retained upon addition of αKG, whereas addition of 2,4-D leads to the displacement or *g* tensor reorientation of a histidine out of the equatorial plane (22–24). Our present results confirm the importance of multiple histidines and one aspartate for TfdA activity and allow us to assign functions to selected residues.

Mutation of His-113 or Asp-115 to alanine both eliminates activity and alters the structure of Cu(II) metallocenter site. Both variants show EPR spectra consistent with at least two distinct Cu(II)-binding sites. Substrate binding perturbs these spectra giving rise to better resolution of $g_\parallel$ features associated with each metal site suggesting that the copper is likely present at the same binding site as in the wild-type enzyme. His-113 and Asp-115 comprise the HX(D/E) motif that is strictly conserved in enzymes related to TfdA (Fig. 8) and more distantly related α-KG-dependent dioxygenases (Table I) (7). Site-directed mutagenesis studies analogous to those described here demonstrated the importance of this motif in a number of Group I enzymes including aspartyl, lysyl, prolyl, and flavanone hydroxylases (8, 10–13). Furthermore, crystal structures of IPNS and DAOCS established these residues as ligands to the Group I metal centers. Our studies support the proposal that residues in the HX(D/E) motif are ligands to the metal in TfdA and, most likely, other Group II enzymes.

Cu(II)-D115A TfdA ESEEM and EPR spectra (particularly for the case where substrates are bound) indicate the presence of two imidazole ligands at one of the Cu(II) sites. The intensity of the non-modulated echo signal was greater for this variant, as compared with that found for the wild-type enzyme, providing evidence for another copper binding environment that lacks equatorially bound histidines. The absence of the aspartate ligand alters the chemistry that occurs at the active site upon addition of the co-substrates. The geometry of the Cu(II) site shows less dispersion when α-KG and 2,4-D are bound as evidenced by the increased resolution in the EPR spectrum and ESEEM pattern. These observations are consistent with those made for the wild-type Cu(II) TfdA; however, the structural rearrangement triggered by formation of the Cu(II)-αKG-2,4-D ternary complex in wild-type TfdA is not observed for D115A TfdA. Together, these data indicate that Asp-115 is very likely a metal-binding residue and may also be involved in formation of the proper catalytic conformation. Because these observations pertain to the Cu(II) form of TfdA, it is difficult to draw conclusions about the specific electronic role of Asp-115 in the native Fe(II) TfdA based on these observations.

The identity of the second metal-binding histidine, predicted by various spectroscopic studies, is less clear. Spectral simulations of H167A are consistent with the presence of two imidazole nitrogens per copper in this protein. Furthermore, His-167 is replaced with an arginine in the TfdA sequence encoded *tfdA* on pJJB in *Burkholderia cepacia* (GenBank™ accession number AAB47567). Thus, even though this residue most closely matches the spacing observed in the Group I sequences, it is unlikely that His-167 is the third metal ligand to the TfdA iron metallocenter. EPR and ESEEM analyses of H200A TfdA suggest that His-200 is important for creation of the metallocenter. Although His-200 is conserved among the enzymes with high identities to TfdA, such as TauD from *E. coli* and sulfonate/α-KG dioxygenase from yeast, a corresponding histidine is not found in more distantly related Group II enzymes such as γ-butyrobetaine hydroxylase or clavaminate synthase (Fig. 8). Mutational analysis of prolyl 4-hydroxylase (10) provided evidence for a non-ligand histidine at the active site that is important for α-KG binding and for controlling the decarboxylation of α-KG in the absence of substrate. Perhaps His-200 serves a similar function in TfdA, providing an explanation for the changes in active site structure upon its alteration. Sequence comparisons of all Group II enzymes, including several putative dioxygenases with overall similarity to TfdA, reveal that His-262 in TfdA is the only other invariant histidine (Fig.

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FIG. 8. **Sequence comparison of TfdA and other Group II α-KG-dependent dioxygenases.** The accession numbers and sequence identifiers are shown in parentheses for the following Group II sequences: *R. eutropha* 2,4-D/α-KG dioxygenase (TfdA, M16730); *E. coli* taurine/α-KG dioxygenase (TauD, P37610); putative α-KG dioxygenase from sulfate-ester transport gene cluster in *Pseudomonas putida* (AtsK, AF126201); *S. cerevisiae* sulfonate/α-KG dioxygenase (YSD, Q12358); *Erwinia carotovora* carbapenem biosynthesis enzyme C (CarC, AAD38231); *Pseudomonas aeruginosa* pyoverdine biosynthesis enzyme (PvcB, AF002222); *Pseudomonas syringae* SyrI, syringomycin biosynthesis enzyme I (SyrB, AAB63253); *Pseudomonas* sp. γ-butyrobetaine hydroxylase (GBBH, P80193); *Caenorhabditis elegans* γ-butyrobetaine hydroxylase (GBBH_CE, Z66523); *Streptomyces clavuligerus* clavaminate synthase 1 and 2 (CS1, A44241; CS2, L06214). Highly conserved residues (*black boxes*) and residues conserved in more than 75% of the sequences (*dark gray boxes*) are *highlighted*. Amino acid numbers for each sequence are shown at the *right* of each line; mutated TfdA residues are *numbered* above.

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8); thus, we suggest that this is most likely the third metal-binding residue. In support of our assignment of His-262 as a metal ligand, this residue is 10 amino acids away from Arg-273 creating an $HX_{10}R$ motif similar to that in DAOCS and related enzymes (8). This arginine is conserved in the other Group II enzymes (Fig. 8). Together, these data suggest a more general motif for α-KG-dependent dioxygenases, $HX(D/E)X_nHX_{10-13}R$ where X_n varies with the different subgroups within the superfamily (Group I, n = 44–65; Group II n = 123–153; and Group III, n = 74–104) (4).

His-213 and His-216 may also be present at or near the TfdA active site. MBP-H213A and H216A TfdA variants exhibited increased 2,4-D K_m values, and the former enzyme also demonstrated a decreased catalytic rate. These data may indicate that His-213 (perhaps also assisted by His-216) facilitates 2,4-D binding by electrostatic interactions between the imidazole nitrogen and the substrate carboxylate group; however, additional studies are required to test this hypothesis. The importance of the 2,4-D carboxylate for binding to TfdA is indicated by the inability of phenoxyethanol to serve as a substrate (21). Neither His-213 nor His-216 are conserved among other related α-KG-dependent dioxygenases. The divergence of sequences in this region may reflect differences in the residues required to bind substrates unique to each enzyme.

Our results provide no evidence to indicate that His-8, His-235, or His-245 are critical for catalysis. The H8A variant exhibits enhanced protease sensitivity, but the full-length protein possesses near wild-type activity. Similarly, the H235A protein is nearly unaffected in its activity. Neither of these residues is conserved in homologous sequences. As described for the H262A TfdA variant, His-245 appears to be structurally important. Because H262A and H245A derivatives of TfdA exist in non-native, highly aggregated conformations, we were unable to assess directly whether these residues may also play a catalytic role. Although His-245 is not conserved in these sequences, it is in the C-terminal half of the protein which in other α-KG-dependent dioxygenases show a higher level of similarity than the overall sequence comparisons (41). This region might be more constrained for structural reasons.

The $HX(D/E)X_nH$ motif, which is found in all known α-KG dependent dioxygenases, is an example of the more general structural arrangement (2-His-1-carboxylate) found in non-heme Fe(II) enzymes including extradiol dioxygenases and pterindependent hydroxylases (42). It is proposed that these three ligands anchor the iron in the active site of the enzyme while leaving three open coordination sites available for the binding of substrate, cofactor, and oxygen. Several studies suggest that O_2 reacts much more readily with such iron centers after the displacement of solvent molecules by more electronegative substrate molecules (42). In addition, it is the diversity of exogenous ligands that enable non-heme Fe(II)-dependent oxygenases to catalyze such a wide range of reactions.

Our proposed model of the active site of TfdA is shown in Fig. 9. His-113, Asp-115, and a third residue tentatively identified as His-262 are shown as ligands to the metal. As in the crystal structures of both DAOCS and IPNS, water molecules are thought to occupy the three remaining coordination sites in substrate-free enzyme. The model includes a likely binding mode for α-KG based on the DAOCS crystal structure (17). Recent spectroscopic studies of clavaminate synthase (43), TauD (44), and TfdA (24) are consistent with a similar mode of α-KG binding in these enzymes. We have no evidence to suggest direct binding of 2,4-D to the metal center and indicate the presence of a separate 2,4-D-binding site adjacent to the metal in the model. Mutation of His-213 affects both substrate binding and the rate of catalysis and therefore is shown to be present near the active site to indicate potential substrate interactions, but the precise role of the residue is still to be defined. In the absence of a crystal structure, the site-directed mutagenesis, metallocenter spectroscopy, and kinetic analysis studies described here provide the most complete working model of the TfdA active site. This model begins to define the necessary elements for catalysis in Group II α-KG-dependent dioxygenases and finds similarities between these enzymes and other well studied representatives of this superfamily.

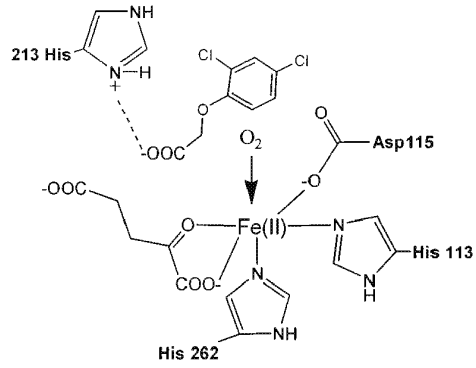


FIG. 9. **Model for the active site of TfdA.** The three residues proposed to ligate the ferrous ion of TfdA are shown along with a possible coordination mode for the substrates, α-KG and 2,4-D. His-213, implicated in catalysis and/or binding of 2,4-D, is also illustrated.

Note Added in Proof—The recently reported structure of clavaminate synthase (45) confirms our proposed roles for residues in the $Hx(D/E)x_{138-207}Hx_{10}$ (R/K) motif for the Group II α-KG-dependent dioxygenases.

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Site-directed Mutagenesis of 2,4-Dichlorophenoxyacetic Acid/α-Ketoglutarate Dioxygenase: IDENTIFICATION OF RESIDUES INVOLVED IN METALLOCENTER FORMATION AND SUBSTRATE BINDING

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Neue Herbizidresistenz-Gene

Nouveaux gènes de résistance aux herbicides

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WO-A-98/02562 WO-A-03/034813

- **DATABASE EMBL [Online] 3 March 2004 (2004-03-03), "Sphingomonas herbicidovorans tnpA gene for transposase and rdpA gene for (R)-2-(2,4-dichlorophenoxy)propionate,2-oxoglutarate dioxygenase" XP002474325 retrieved from EBI accession no. EMBL:AJ628859 Database accession no. AJ628859 & KOHLER H P E: "Sphingomonas herbicidovorans MH: A versatile phenoxyalkanoic acid herbicide degrader" JOURNAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, vol. 23, no. 4-5, October 1999 (1999-10), pages 336-340, ISSN: 1367-5435**
- **DATABASE EMBL [Online] 6 August 2002 (2002-08-06), "Rhodoferax sp. P230 R-2,4-dichlorophenoxypropionate dioxygenase (rdpA) gene, complete cds." XP002474326 retrieved from EBI accession no. EMBL:AF516751 Database accession no. AF516751 & DATABASE EMBL [Online] 6 August 2002 (2002-08-06), "Sphingobium herbicidovorans R-2,4-dichlorophenoxypropionate dioxygenase (rdpA) gene, complete cds." XP002474327 retrieved from EBI accession no. EMBL:AF516752 Database accession no. AF516752 & SCHLEINITZ KATHLEEN M ET AL: "Localization and characterization of two novel genes encoding stereospecific dioxygenases catalyzing 2(2,4-dichlorophenoxy)propionate cleavage in Delftia acidovorans MC1" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 70, no. 9, September 2004 (2004-09), pages 5357-5365, ISSN: 0099-2240**

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Description

Background of the Invention

[0001] Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

[0002] Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant corn, for example, can be sprayed to control weeds without significantly damaging the corn plants.

[0003] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (e.g., wheat, rice, sugar beets, turf, etc.) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; see "isb.vt.edu/cfdocs/fieldtests1.cfm , 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

[0004] Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

[0005] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Ng *et al.,* 2003; Simarmata *et al.,* 2003; Lorraine-Colwill *et al.,* 2003; Sfiligoj, 2004; Miller *et al.,* 2003; Heap, 2005; Murphy *et al.,* 2002; Martin *et al.,* 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past 3-5 years. Resistant weeds include both grass and broadleaf species-*Lolium rigidum, Lolium multiflorum, Eleusine indica, Ambrosia antemisiifolia, Conyza canadensis, Conyza bonariensis,* and *Plantago lanceolata.* Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

[0006] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-diclorophenoxyacetic acid (2,4-D): 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

[0007] 2,4-D is in the phenoxy acid class of herbicides, as are MCPA, mecoprop, and dichlorprop. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known.

[0008] 2,4-D has different levels of selectivity on certain plants (*e.g.,* dicots are more sensitive than grasses). Differential metabolism of 2,4-D by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

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[0009] Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D is currently formulated to have a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

[0010] One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.,* 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA. See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.,* 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.* (1989), Lyon *et al.* (1989), Lyon (1993), and U.S. Patent No. 5,608,147).

[0011] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with α-ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

[0012] One unique example with low homology to *tfdA* (28% amino acid identity) is *rdpA* from *Sphingobium herbicidovorans* (Kohler *et al.,* 1999, Westendorf *et al.,* 2002). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.,* 2003). Although the organisms that degrade phenoxypropionic acid were described some time ago, little progress had been made in characterizing this pathway until recently (Horvath *et al.,* 1990). An additional complication to dichlorprop degradation is the stereospecificity (R *vs.* S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.,* 2003). Heterologous expression of *rdpA* in other microbes, or transformation of this gene into plants, has not heretofore been reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids (*e.g.*, 2,4-D).

[0013] Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

[0014] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxy auxins (such as 2,4-D and dichlorprop), pyridyloxy auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. Discovery of a multifunctional enzyme for the degradation of herbicides covering multiple modes would be both unique and valuable as an HTC trait.

Brief Summary of the Invention

[0015] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidazolinone-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0016] In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, AOPP, and one or more commercially available herbicides (e.g., glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

[0017] Described is also the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and AOPP herbicides, in an enantiospecific manner. No α-ketoglutarate-dependent dioxygenase enzyme has

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previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant HTC trait and selectable marker opportunities.

[0018] There was no prior motivation to produce plants comprising an *AAD-1* gene (preferably an *AAD-1* polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-1 enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides (such as quizalofop, haloxyfop, *et al.).* Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0019] Described is also the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Methods of screening proteins for these activities are disclosed including the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoate auxin herbicides, and aryloxyphenoxypropionate herbicides by a recombinantly expressed AAD-1 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more AOPP, phenoxy auxin, or other aryloxyalkanoate herbicides to plants comprising an *AAD-1* gene. Also provided are methods of using an *AAD-1* gene as a selectable marker for identifying plant cells and whole plants transformed with *AAD-1,* optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. Further described are methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

Brief Description of the Figures

[0020] **Figure 1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

[0021] **Figure 2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

[0022] **Figure 3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

[0023] **Figure 4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

[0024] **Figure 5** shows 2,4-dichlorophenol production by recombinant *AAD-1*.

[0025] **Figures 6A and 6B** show phenol production by recombinant *AAD-1* from various herbicide substrates.

[0026] **Figure 7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

[0027] **Figures 8A and 8B** show that *AAD-1* (*v3*) was expressed equally in *Arabidopsis* leaves of different ages but continued to accumulate throughout the 25 days of experiment. Plants that were not sprayed with the herbicide 2,4-D (panel A) expressed a little more AAD-1 (v3) than those had been sprayed (panel B). Bars represent the mean ± SEM of 5 leaves from 5 different plants, with percent expression of AAD-1 (v3) normalized to total soluble protein. Light bars represent the third young leaves (N-3) collected from the top, dark bars represent the 5th oldest leaves from the bottom.

[0028] **Figures 9A, 9B, and 9C** show injury of *Arabidopsis* plants after 2,4-D treatment. Four different lines were each treated with four different doses of 2,4-D and their injury was graded 4 (panel A) and 14 (panel B) days after treatment. Their expression of AAD-1 (v3) in leaves was also determined using ELISA (panel C). The results were mean ± SEM of five leaves from five different plants received the same treatment.

[0029] **Figure 10** illustrates that pDAB3230-transformed *Arabidopsis (AAD-1 + EPSPS)* shows >14-fold level of glyphosate tolerance 7 DAT vs. wildtype and transformed control *Arabidopsis* lines.

[0030] **Figure 11** shows dose response of callused maize suspensions to R-haloxyfop.

[0031] **Figure 12** shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol.

[0032] **Figure 13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

[0033] **Figure 14** shows the responses of several *AAD-1* (*v3*)-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied as a postemergence spray 1 week prior.

[0034] **Figure 15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

[0035] **Figure 16** shows *AAD-1* (*v3*)-transformed corn tolerant to 8X field rates of quizalofop (Assure II) under field conditions.

[0036] **Figure 17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

[0037] **Figure 18** shows Western Blotting analysis on soybean calli transformed with *AAD-1 (V3)* gene indicating that the callus cells are expressing AAD-1 (v3) protein.

[0038] **Figure 19** shows fitted curves for 2,4-D degradation rates by AAD-2 (v1) vs. AAD-1 (v1).

[0039] **Figure 20** shows the response of *AAD-1 v3* (plant optimized), or *AAD-1 (v2)* (native), *AAD-2 (v1)* (native), or *AAD-2 (v2)* (plant optimized)-transformed T_1 *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot

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represents an individual transformation event within each gene T_1 family.

[0040] **Figure 21** shows western blot analysis of individual native *AAD-2* (*v1*)-transformed T_1 *Arabidopsis* plants. This shows that plants expressing the AAD-2 (v1) protein are suffering severe injury from 200 g ae (acid equivalent)/ha 2,4-D treatments, which normally causes little injury to native *AAD-1* (*v2*) or plant optimized *AAD-1* (*v3*)-transformed *Arabidopsis.* AAD-2 protein is identified on the gel. Several background bands were detected in *AAD-2*-transformed and *Pat*/*CrylF*-transformed samples.

[0041] **Figure 22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D = dichlorprop > (R,S)-haloxyfop >> (R)-haloxyfop.

Brief Description of the Sequences

[0042] **SEQ ID NO:1** is the sequence of a forward primer used to amplify the *rdpA*/*AAD-1* (*v1*) gene.

[0043] **SEQ ID NO:2** is the sequence ofa reverse primer used to amplify the *rdpA*/*AAD-1 (v1)* gene.

[0044] **SEQ ID NO:3** is the nucleotide sequence of *AAD-1 (v1)* from *Sphingobium herbicidovorans.*

[0045] **SEQ ID NO:4** is the nucleic acid sequence of the native *AAD-1* gene with internal NotI restriction site removed. This gene is designated *AAD-1 (v2).* DNA sequencing confirmed that the correct PCR product was generated, but an inadvertent change was made at amino acid #212 from arginine to cysteine.

[0046] **SEQ ID NO:5** is a "plant-optimized" DNA sequence *AAD-1 (v3).* This "gene" encodes SEQ ID NO:11, which is the same as SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations.

[0047] **SEQ ID NO:6** ("rdpA(ncoI)") and **SEQ ID NO:7** ("3'saci") were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[0048] **SEQ ID NO:8** is another PCR primer ("BstEII/Del NotI") that was used with the "3' SacI" primer.

[0049] **SEQ ID NO:9** is the native amino acid sequence encoded by the *AAD-1* (*v1*) gene from *Sphingobium herbicidovorans.*

[0050] **SEQ ID NO:10** is the amino acid sequence encoded by the *AAD-1* (*v2*) DNA sequence of SEQ ID NO:4.

[0051] **SEQ ID NO:11** is the amino acid sequence encoded by the *AAD-1* (*v3*) plant-optimized DNA sequence of SEQ ID NO:5.

[0052] **SEQ ID NO:12** is the DNA sequence of the native *AAD-2* (*v1*) gene.

[0053] **SEQ ID NO:13** is the amino acid sequence of the AAD-2 (v1) protein.

[0054] **SEQ ID NO:14** is a forward primer used to amplify *AAD-2* (*v1*) DNA for cloning.

[0055] **SEQ ID NO:15** is a reverse primer used to amplify *AAD-2* (*v1*) DNA for cloning.

[0056] **SEQ ID NO:16** is the M13 forward primer.

[0057] **SEQ ID NO:17** is the M13 reverse primer.

[0058] **SEQ ID NO:18** is a forward primer used to amplify *AAD-2* (*v1*) DNA for cloning.

[0059] **SEQ ID NO:19** is a reverse primer used to amplify *AAD-2* (*v1*) DNA for cloning.

[0060] **SEQ ID NO:20** is the native soybean EPSPS protein.

[0061] **SEQ ID NO:21** is a doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine).

[0062] **SEQ ID NO:22** is the soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21.

[0063] **SEQ ID NO:23** is primer Pat 5-3.

[0064] **SEQ ID NO:24** is primer Pat 3-3.

[0065] **SEQ ID NO:25** is forward primer AAD-1 PTU.

[0066] **SEQ ID NO:26** is reverse primer AAD-1 PTU.

[0067] **SEQ ID NO:27** is the forward primer for the Coding Region PCR AAD-1.

[0068] **SEQ ID NO:28** is the reverse primer for the Coding Region PCR AAD-1.

[0069] **SEQ ID NO:29** is the *AAD-2 (v2)* nucleotide (plant optimized).

[0070] **SEQ ID NO:30** is the translated AAD-2 (v2) protein sequence.

[0071] **SEQ ID NO:31** is the Southern fragment PCR AAD-1 forward primer.

[0072] **SEQ ID NO:32** is the Southern fragment PCR AAD-1 reverse primer.

Detailed Description of the Invention

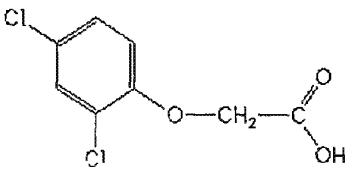
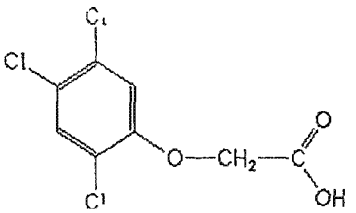
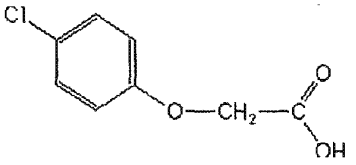
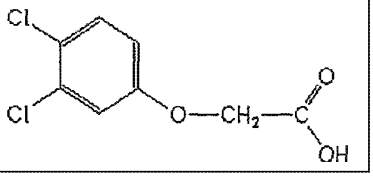
[0073] The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot

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crops would also have greater flexibility in the timing and rate of application. An additional utility of an herbicide tolerance trait for 2,4-D would be its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism and the like. An additional benefit of the *AAD-1* gene is that unlike all *tfdA* homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (*e.g.*, dichlorprop and mecoprop) in addition to achiral phenoxy auxins (*e.g.*, 2,4-D, MCPA, 4-chlorophenoxyacetic acid). *See* **Table 1.** Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the *AAD-1* gene in plants would afford protection to a much wider spectrum of phenoxy auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled protecting from drift or other off-site phenoxy herbicide injury for the full breadth of commercially available phenoxy auxins.

[0074] Table 1. Commercially available phenoxy auxins. Reference to phenoxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta.* Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

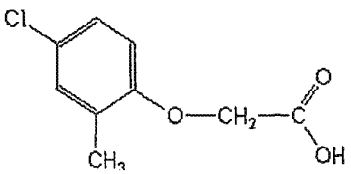
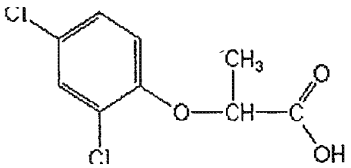
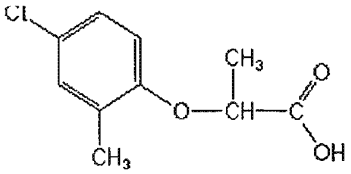
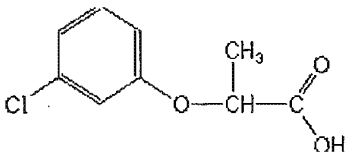
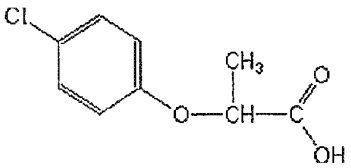
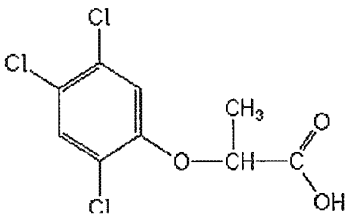
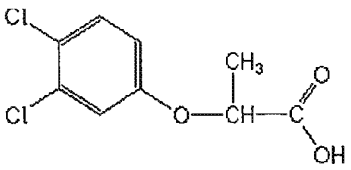
Table 1. Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 - 4000	280 - 1120	
2,4,5-T	93-76-5	25 - 4000	25 - 4000	
4-CPA	122-88-3	25 - 4000	25 - 4000	
3,4-DA	588-22-7	25 - 4000	25 - 4000	

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(continued)

Table 1. Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
MCPA	94-74-6	2S - 4000	125 - 1550	
Dichlorprop	120-36-5	25 - 12000	100 - 2240	
Mecoprop	7085-19-0	25 - 4000	250 - 3360	
Cloprop	101-10-0	25 - 4000	25 - 4000	
4-CPP	3307-39-9	25 - 4000	25 - 4000	
Fenoprop	93-72-1	25 - 4000	25 - 4000	
3,4-DP	3307-41-3	25 - 4000	25 - 4000	

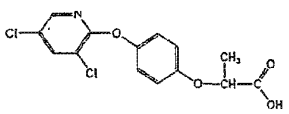
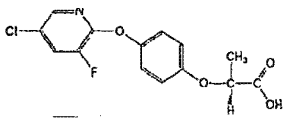
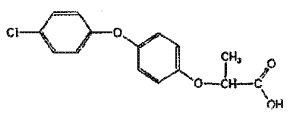
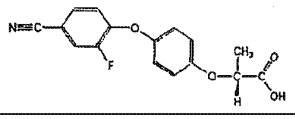
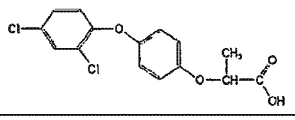
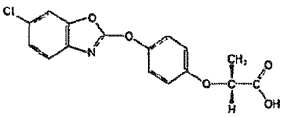
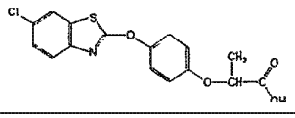
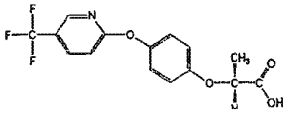
[0075] An additional benefit of the *AAD-1* gene is its unprecedented ability to concomitantly degrade a host of commercial and non-commercial graminicidal compounds of the general class aryloxyphenoxypropionates (AOPPs). *See*

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Table 2. This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing *AAD-1*, where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, oats, sorghum, warm and cool-season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

[0076] **Table 2**. AOPP graminicidal compounds listed by accepted common names. Reference to AOPP herbicides is generally made to the active acid but most are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta.* Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R, S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

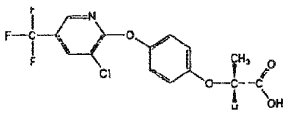
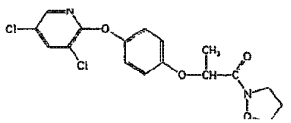
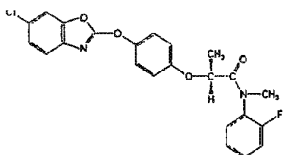
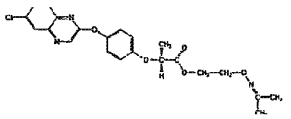
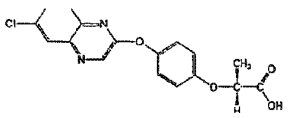
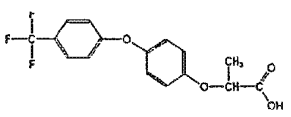
Table 2. AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	
Fenoxaprop	66441-23-4	10-2000	20-200	
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	

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(continued)

Table 2. AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Haloxyfop	69806-40-2	10-2000	20-600	
Isoxapyrifop	87757-18-4	10-2000	30-240	
Metamifop	256412-89-2	10-2000	35-280	
Propaquizafop	111479-05-1	10-2000	30-240	
Quizalofop	76578-14-8	10-2000	20-240	
Trifop	58597-74-4	10-2000	10-2000	

[0077] A single gene *(AAD-1)* has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, **AAD-1** can provide protection *in planta* to AOPP herbicides where natural tolerance also was not sufficient to allow selectivity. Plants containing *AAD-1* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each, phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several AOPP graminicidal compounds may be applied to plants expressing AAD-1 with reduced risk of injury from said herbicides. The rate for each AOPP may range from 10 to 2000 g ae/ha, and more typically from 20-500 g ae/ha for the control of a broad spectrum of monocot weeds. Combinations of these different chemistry classes and herbicides with different modes of action and spectra in the same field (either sequentially or in tank mix combination) shall provide control of most potential weeds for which herbicidal control is desired.

[0078] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking *AAD-1* with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant weed species (either grass weed species with one or more AOPP herbicides, or broadleaf weed species with one or more phenoxy auxins) in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (*e.g.*, 2,4-D) and AOPP herbicide(s) (*e.g.*, quizalofop) selectively in the same crop. Applications of these herbicides could be simultaneously in

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a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from 2 hours to 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0079] It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/*AAD-1* stack could range from 250-2500 g ae/ha; phenoxy auxin herbicide (s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0080] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0081] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant species or resistant weed species could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (*e.g.*, resistant plant or bacterial *EPSPS, GOX, GAT),* glufosinate resistance (*e.g., Pat, bar*), acetolactate synthase (ALS)-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxlphenylpyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (*e.g., psbA*), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (*e.g., PPO-1*), resistance to phenylurea herbicides (*e.g., CYP76B1*), dicamba-degrading enzymes *(see, e.g.,* US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

[0082] Regarding additional herbicides, some additional preferred ALS inhibitors include the triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0083] Additionally, *AAD-1* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0084] Described is the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme from previously known *tfdA* proteins, for example. Even though this enzyme has very low homology to *tfdA,* the genes used can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes.

[0085] More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also AOPP herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0086] Also described is the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed AAD-1 enzyme.

[0087] The proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical and biological assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP (ranging from

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50-100% conversion) *in vitro.* An additional advantage that *AAD-1*-transformed plants provide is that parent herbicide (s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0088] The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an *AAD-1* gene.

[0089] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0090] Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0091] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.,* 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA. See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.,* 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.,* 1989; Lyon *et al., 1989;* Lyon *et al.,* 1993). A large number of *tfdA-type* genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α-ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0092] *RdpA,* from *Sphingobium herbicidovorans* (Westendorf *et al.,* 2002), is one unique example with low homology (28% amino acid identity). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.,* 2003). Although the organism responsible for phenoxypropionic acid degradation has been known for some time, little progress has been made in characterizing this pathway until recently (Horvath *et al.,* 1990). An additional complication to dichlorprop degradation is the stereospecificity (R vs. S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.,* 2003). Heterologous expression of *rdpA* in other microbes or transformation of this gene into plants, heretofore, was not reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids. There was no prior expectation that *rdpA* or *AAD-1* genes could be successfully expressed in plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

[0093] As described in more detail in the Examples below, *rdpA* was cloned from *Sphingobium herbicidovorans* and tested for substrate promiscuity among various herbicide chemical classes. This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and dichlorprop (Westendorf *et al.,* 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of *action. RdpA* has never been expressed in plants, nor was there any motivation to do so because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0094] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides *in vitro.* However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance are the aryloxyphenoxypropionate (AOPP) grass herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities.

[0095] The broad spectrum grass AOPP herbicides are reported herein to be excellent substrates for AAD-1 as well as 2,4-D, dichlorprop, and other phenoxy auxins. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxy auxins) and a range of broad spectrum, highly active grass herbicides (AOPPs).

[0096] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-1). Also disclosed is the identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

[0097] The enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf and grass weeds. *AAD-1* can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits (*e.g.*, glyphosate resistance, glufosinate resistance, imidazolinone resistance, bromoxynil resistance, *et al.),* and insect resistance traits *(Cry1F, Cry1Ab, Cry 34/45, et al.)* for example. Additionally, *AAD-1* can

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serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0098] In addition, the microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis,* corn, tobacco, cotton, soybean, canola, and rice have been transformed with *AAD-1*-containing constructs and have demonstrated high levels of resistance to both the phenoxy auxin and AOPP herbicides. As shown below in Example 6, the exemplified rebuilt gene was more efficacious in conveying herbicide resistance to the plant, as compared to the bacterial gene.

[0099] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-1. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[0100] HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

[0101] Additionally, glyphosate tolerant crops grown worldwide are prevalent. Many times in rotation with other glyphosate tolerant crops, control of glyphosate-resistant volunteers may be difficult in rotational crops. Thus, the use of the subject transgenic traits, stacked or transformed individually into crops, provides a tool for controlling other HTC volunteer crops.

[0102] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[0103] The transgenic expression of the subject *AAD-1* genes is exemplified in, for example, *Arabidopsis,* corn (maize), tobacco, cotton, rice, soybean, and canola. However, the subject invention can be applied to any other desired types of plants. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be applied to multiple other grass and other broadleaf crops. Likewise, 2,4-D can be more positively utilized in grass crops where tolerance to 2,4-D is moderate, and increased tolerance via this trait would provide growers the opportunity to use 2,4-D at more efficacious rates and over a wider application timing without the risk of crop injury.

[0104] Still further, the subject invention uses a single gene that can provide resistance to herbicides that control broadleaf weed (auxins) and grass weeds (AOPPs). This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-1 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

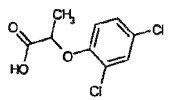
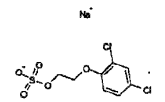
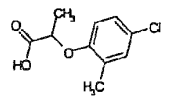
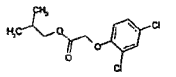
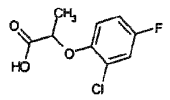
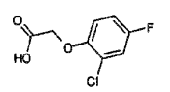
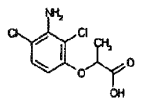
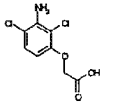
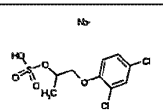
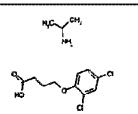
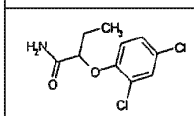
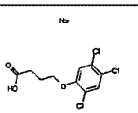
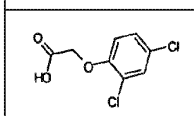
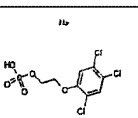
[0105] Separate from, or in addition to, using the subject genes to produce-HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-1 for other phenoxyalkanoic auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

[0106] One gene, referred to herein as *AAD-1* (aryloxyalkanoate dioxygenase), was cloned from *Sphingobium herbicidovorans* (ATCC 700291) by PCR into pET 280-S/S (designated pDAB 3203) and expressed in BL-21 Star *E. coli.* When this gene is overexpressed (by induction of 1mM IPTG and culture lysate combined with the following reaction mix: 112.5 μg/ml 2,4-D, 1 mM Ascorbic acid, 1 mM α-ketoglutarate, 50 μM $Fe(NH_4)_2(SO_4)_2$, the recombinantly produced enzyme degrades 2,4-D into herbicidally inactive DCP (as determined by HPLC, mass spectrometry, colorimetric assay, and *Arabidopsis* plate assay). Additionally, AAD-1 has been demonstrated to convert the following herbicides into their corresponding inactive phenol: dichlorprop, mecoprop, haloxyfop, dichlofop, and others (See **Tables 3 and 4**).

[0107] **Table 3**: Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

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Table 3: Effect of purified AAD-1 (v1) on various herbicidal au xins and auxin an alogs

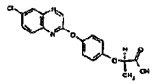
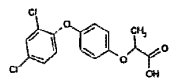
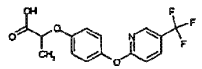
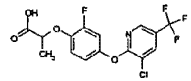
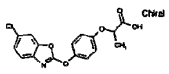
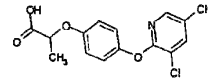
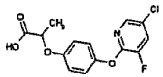
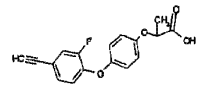
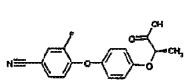
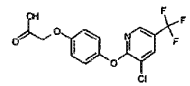
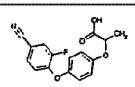
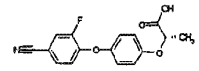
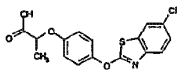
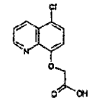
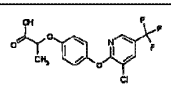
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)		STRUCTURE	ID	Registry ID Compound	AAD1	AAD1 (10x)
	117613	(R, S)-dichlorprop	0.566	2.594			398166	sesone	0.02	0.177
	188874	(R,S)-mecoprop	0.341	2.085			190252		0.008	0.211
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358			124988		0.007	0.058
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676			11263526		0.004	0.069
	188476		0.077	0.687			178577		0.003	0.021
	192132		0.064	0.204			178587		0.003	0.02
	195517	2,4-D	0.034	0.383			188527		0.003	0.036

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[0108] **Table 4:** Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

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Table 4. Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

STRUCTURE	Registry ID	Compound Compound	AAD1	AAD1 (10x)	STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalofop	0.43	2.1		460511	(R,S)-diclofop	0.155	1.663
	67131	(R,S)-fluazifop	0.427	2.17		25646		0.144	1.69
	11044492	(R)-fenoxaprop	0.408	0.597		70222	(R, S)-chlorazifop	0.128	1.584
	34697	(R, S)-clodinofop	0.295	1.98		199608	cyhalofop	0.114	1.26
	14603	(R)-cyhalofop	0.222	1.989		43865	haloxyfop-oxyacetate	0.004	0.053
	14623	(R, S)-cyhalofop	0.215	1.815		7466	(S)-cyhalofop	0.003	0.017
	62942	(R, S)-fenthiaprop	0.199	1.055		204558	Cloquintocet	0	0.001
	66905	haloxyfop	0.172	1.63					

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[0109] Proteins (and source isolates) The present invention uses functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known Herbicide Handbook (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

[0110] Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. Optimized polynucleotide can also be designed based on the protein sequence.

[0111] The subject invention uses classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

[0112] There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Antibodies include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

[0113] One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria. Other members of the orders Firmicutes and Proteobacteria, and specific genera with known *rdpA's,* such as *Sphingobium, Delftia, Rodoferax,* and *Comamonas* for example, can be used as source isolates.

[0114] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[0115] A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

[0116] Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook *et al.,* 1989.

[0117] Polynucleotides and probes. Further provided are nucleotide sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described

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proteins.

[0118] The polynucleotides can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *AAD-1* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

[0119] As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

[0120] Bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

[0121] Further aspects include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

[0122] Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, *etc.*).

[0123] As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) DNA Probes, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

[0124] Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[0125] Hybridization characteristics of a molecule can be used to define polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a

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gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

[0126] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see, e.g.,* Maniatis *et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

$$Tm = 81.5° C + 16.6 \text{ Log}[Na+] + 0.41(\%G+C) - 0.61(\%\text{formamide}) - 600/\text{length of duplex in base pairs.}$$

[0127] Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).
(2) Once at Tm-20° C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

[0128] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

$$Tm (°C) = 2(\text{number T/A base pairs}) + 4(\text{number G/C base pairs})$$

(Suggs *et al.,* 1981).

[0129] Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1x SSPE, 0.1% SDS (low stringency wash).
(2) Once at the hybridization temperature for 15 minutes in 1x SSPE, 0.1% SDS (moderate stringency wash).

[0130] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low:	1 or 2x SSPE, room temperature
Low:	1 or 2x SSPE, 42° C
Moderate:	0.2x or 1x SSPE, 65° C
High:	0.1xSSPE, 65°C.

[0131] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

[0132] PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.,* 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific

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target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus,* the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

[0133] Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template, like, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

[0134] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target pests and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[0135] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[0136] "Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[0137] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, or 297 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[0138] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

[0139] Disclosed herein are proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired

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activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli,* baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, B.t. proteins can be used in a truncated (core protein) form (*see, e.g.,* Höfte *et al.* (1989), and Adang *et al.* (1985)). As used herein, the term "protein" can include functionally active truncations.

[0140] In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

[0141] Certain proteins have been specifically exemplified herein. As these proteins are merely exemplary of the proteins, it should be readily apparent that the variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) have the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

[0142] Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.,* 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.,* 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

[0143] Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 5** provides a listing of examples of amino acids belonging to each class.

Table 5

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[0144] In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

[0145] As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

[0146] Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

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[0147] Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

[0148] Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis,* tobacco, soybeans, cotton, canola, rice, wheat, turf and pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

[0149] In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

[0150] Plant cells transfected with a polynucleotide can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. Also provided is a part, progeny, or asexual propagate of the plant of the invention, wherein said part, progeny or asexual propagate comprises a plurality of cells according to the invention, wherein expression said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

[0151] Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

[0152] A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

[0153] A large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli.* The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley *et al.* (1986); and An *et al.* (1985).

[0154] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria.* The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria.* They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of

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plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[0155] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[0156] In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

[0157] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, *inter alia.* Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate, (*PAT*), glyphosate (*EPSPS*), imazethyapyr (*AHAS*), and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[0158] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Patent Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Patent Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Patent Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Patent No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Patent No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Patent Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Patent Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. *See* U.S. Patent Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Electroporation technology has also been used to transform plants. *See* WO 87/06614 to Boyce Thompson Institute; U.S. Patent Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Patent No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Patent Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[0159] As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, triparental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

[0160] In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al. (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

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[0161] For transformation of plant cells using *Agrobacterium,* explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

[0162] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[0163] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

[0164] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.,* 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli,* the chloramphenicol acetyl transferase gene from Tn9 of *E. coli,* the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria,* and the luciferase genes from firefly *Photinus pyralis.* An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.,* (1987) to identify transformed cells.

[0165] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.,* actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.,* zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[0166] Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[0167] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based

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systems are described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[0168] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1- Method for Identifying Genes That Impart Resistance to 2,4-D *In Planta*

[0169] As a way to identify genes which possess herbicide degrading activities *in planta,* it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (*i.e.,* α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.,* 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤ 50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[0170] PCR and cloning of gene into pET. A single gene (*rdpA*) was identified from the NCBI database (*see* the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 28% amino acid identity to *tfdA* from *Ralstonia eutropha.* Percent identity was determined by first translating both the *rdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[0171] The strain of *Sphingobium herbicidovorans* containing the *rdpA* gene was obtained from ATCC (American Type Culture Collection strain #700291). The lyophilized strain was revived according to ATCC protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 536. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days.

[0172] A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' TCT AGA AGG AGA TAT ACC ATG CAT GCT GCA CTG TCC CCC CTC TCC CAG CG 3' [(SEQ ID NO:1) (added Xba I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' CTC GAG TTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2)(added extra stop codon and Xho I site)].

[0173] Twenty microliter reactions were set up as follows: MasterMix 8 μl, ea. primer 1 μl (50 pmoles/μl), gDNA 2.5 μl, H_2O 7.5 μl. PCR was then carried out under the following conditions: 94° C 45 sec, 52° C 1.5 minute, 72° C 1.5 minute, for 30 cycles, followed by a final cycle of 72° C 5 minute, using Eppendorf's Master Taq kit (Eppendorf cat. #0032 002.250). The resulting ~1kb PCR product was cloned into pCR 2.1 (Invitrogen cat. # K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[0174] Ten of the resulting white colonies were picked into 4 ml Luria Broth + 50 μg/ml Kanamycin (LB K), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Promega Wizard Plus SV kit (Promega cat. #A1460) and following the included protocol. Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward (5' GTA AAA CGA CGG CCA GT 3') (SEQ ID NO:16) and Reverse (5' CAG GAA ACA GCT ATG AC 3') (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence (SEQ ID NO:3), and its corresponding protein (SEQ ID NO:9) was given a new general designation for internal consistency *AAD-1 (v1)* (AryloxyAlkanoate Dioxygenase).

[0175] Using the restriction enzymes corresponding to the sites added with the primer linkers (Xba 1, Xho 1) *AAD-1 (v1)* was cut out of the pCR2.1 vector and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli,* and plated on to Luria Broth + 50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates. To differentiate between *AAD-1 (v1)*:pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37° C for 4 hours with agitation.

[0176] Each culture was then spotted onto LB K plates, which were incubated at 37° C overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3203.

Example 2 - Expression and Testing

[0177] 2.1 -HPLC analysis.

[0178] Plasmid pDAB 3203 was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain

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DR 1878. For expression, plasmid DNA purified from TOP10F' culture using Promega's Wizard kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μl of the cells were plated onto LB S/S agar plates and incubated overnight at 37° C.

[0179] The next morning, all colonies from the entire plate were scraped into 100 mls LB in a 500 ml tri-baffled flask and incubated at 37° C/200 rpm for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C/200 rpm. All 100 ml of culture was centrifuged at 4000 rpm for 20 min. The supernatants were then discarded, and the pellets were resuspended in 10 ml of 50 mM MOPS. These were then subjected to three 45-sec rounds of sonication to lyse the cells. Following this, lysates were centrifuged at 15,000 rpm to remove cell debris. The supernatant was pipetted off and stored at 4° C. To check for recombinant expression, a 20 μl aliquot was run on a 4-20% Tris Glycine gel (Invitrogen cat. #EC60255).

[0180] After expression was confirmed, enzyme activity was tested as follows. First, an aliquot of the cell extract was desalted with a PD-10 cartridge (Amersham cat. #17-0435-01). This was then used for subsequent herbicide enzyme reactions.

[0181] For each reaction, the following were combined: 2,4-D (125 μg/ml), [Ascorbate (1 mM), Ferrous ion (50 μM), α-ketoglutarate (1 mM), in 100 mM MOPS], cell extract (100 μl). This reaction was then incubated at room temp for 30 min., after which the reaction was stopped with the addition of 0.1 N HCl until pH was between 2 and 3. Half of the reaction volume (~500 μl) was set aside for bioassay, the remaining volume was organically extracted using Solid Phase Extraction tubes (Fisher cat. #11-131-6), eluting with 400 μl of Acetonitrile + 0.05% TFA.

[0182] The extracts were then tested on HPLC for loss of the 2,4-D peak or presence of any additional peaks resulting from the degradation or modification of 2,4-D. Conditions for the HPLC were: Luna 10μ C18(2) 250 x 4.6mm (Phenomenex cat. #00G-4253-E0), run at 50% ACN + 0.05% TFA: 50% H_2O + 0.05% TFA to 100% ACN + 0.05% TFA over 5 min.

[0183] 2.2 - Plate test bioassays for herbicide degradation.

[0184] Plant bioassays were used to determine if *in vitro* enzymatic herbicide transformation resulted in a concomitant loss in herbicidal activity. Because of the selective nature of the herbicides being tested (*i.e.,* monocot plants controlled by AOPP herbicides and dicot plants controlled by auxinic herbicides), wildtype *Agrostis palustris* var. Pencross and *Arabidopsis thaliana* var. Columbia were used as monocot and dicot test species, respectively. Each species is amenable to germination and growth in small Petri dishes.

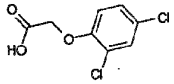
[0185] *Arabidopsis* seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 μL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @ 250 rpm). Bleach solution was decanted inside a sterile hood and rinsed three times with sterile water. Bentgrass seeds were surface sterilized for 20 minutes in a like manner.

[0186] Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4 \cdot 2H_2O$, 10 μM NaCl, 10 nM $CoCl_2 \cdot H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60x15-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR_{50} rate (50% growth reduction) approximately in the center of the range.

[0187] For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed and maintained under sterile conditions in a low light growth chamber (24 h day^{-1}, 100 $\mu E/m^2s^1$, 23° C) for 7 days. Root length or root+shoot length were measured for five randomly chosen *Arabidopsis* and bentgrass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR_{50} determined.

[0188] This bioassay was used to confirm the loss of herbicidal activity as a result of AAD-1 (v1) degradation of the oxyalkanoate side chain from various agronomically relevant herbicides. In several instances, the anticipated phenol product co-eluted with the parent acid on HPLC and the bioassay served as the primary screen for herbicide degradation. **Tables 6 and 7** represent herbicidal substrates tested.

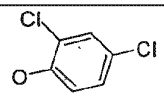
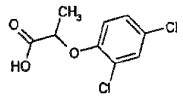
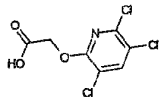
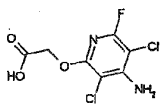
Table 6. *Arabidopsis* plate test bioassay for commercial pyridinyloxyalkanoate auxin substrates. phenoxy and

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
2,4-D	22	17	267	16	

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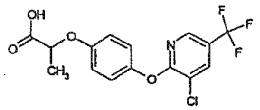
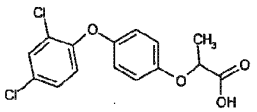
(continued)

Table 6. *Arabidopsis* plate test bioassay for commercial pyridinyloxyalkanoate auxin substrates. phenoxy and

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
DCP	>1000	nd	nd	nd	
Dichlorprop	nd	30	1000	33	
Triclopyr	255	1000	1000	1	
Fluroxypyr	2200	2250	1825	<1	

* Blank vector represents ot cell lysate treatment where E. coli pET vector had no gene insert.
** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥ 2 is considered the threshold for detecting herbicide activity loss with this assay.

Table 7. Bentgrass plate test bioassay for commercial ary ACCase-inhibiting substrates. aryloxyphenoxyalkanoate

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
Haloxyfop-RS	28	21	520	25	
Diclofop-RS	nd	20	130	7	

* Blank vector represents of cell lysate treatment where E. coli pET vector had no gene insert.
** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥ 2 is considered the threshold for detecting herbicide activity loss with this assay.

[0189] 2.3 - HPLC results.

[0190] From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see **Figure 1**) and ferrous ion to bind in the active site. Other experiments in the literature have shown that the addition of ascorbate increased the enzymatic activity by maintaining the iron in the reduced state, thus preventing the enzyme for being degraded. Based on this previous work, initial assays were set up under the assumption that the subject enzyme would work in the same way as other members of this general class of enzyme.

[0191] Surprisingly, the initial HPLC results showed the presence of a new peak at 6.1 minute, in addition to a reduced 2,4-D peak at 5.5 min. This new peak was not present in the control assay. For an initial identification of the peak at 6.1 minutes, a DCP control was run under our assay conditions and predictably this also eluted at 6.1 minutes. The formation of this product was confirmed using a colorimetric assay to detect phenols (see example 3.1) as well as mass spec-

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trometry. As expected, AAD-1 (v1) carries out a similar reaction as other members of this enzyme class. In the bioassay, these same samples were also shown to have an almost complete loss of 2,4-D herbicidal activity in the *Arabidopsis* plate assay (**Figure 2**). Regardless of the specific conditions of the assay (*i.e.*, longer incubations, more enzyme), only 50-75% of the 2,4-D could be degraded to DCP as measured by HPLC. In fact, longer induction of the BL-21 *E. coli* cells with IPTG only resulted in less active enzyme, even though more total recombinant protein was expressed.

[0192] After demonstrating degradation of 2,4-D, additional substrates were tested with similar ring substitutions (*i.e.*, oxyacetates and oxypropionates). The first compounds tested were the pyridine analogs fluroxypyr and triclopyr, which are pyridinyloxyacetates. No enzyme activity was detected on either of these as substrates. Additional tests on various analogs of these two pyridinyloxyacetates with either the fluorine or the amino groups removed also were not degraded. Interestingly however, adding a fluorine to the 5 position of 2,4-D resulted in an almost total loss of enzyme degradation (see next section for additional results).

[0193] ACCase inhibitors, haloxyfop and diclofop, were then tested using the same conditions as with 2,4-D. (The corresponding phenol metabolites co-eluted with the parent compound under the HPLC conditions used.) The bioassay results from these samples showed loss of herbicidal activity against both haloxyfop (**Figure 3**) and diclofop. These results were also confirmed by the colorimetric assay, which was also used to test a wider sampling of these compounds.

[0194] 2.4 - Plate test bioassays for herbicide degradation.

[0195] Bioassay tests results corroborated initial HPLC results that indicated loss of 2,4-D parent following incubation of 2,4-D solutions with unpurified recombinant AAD-1 (v1) extracts (**Figure 2**). Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR_{50} for herbicide+enzyme solution versus the herbicide solution alone served as measure of loss of parent herbicide activity resulting from enzyme activity. A ratio of 2-3 typically correlated with 50-75% loss of parent herbicide activity (**Table 6**). Often a GR_{50} could not be determined following enzyme treatment; *de facto,* no detectable herbicide activity remained.

[0196] The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (v1) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (**Figure 3** and **Table 7**). These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

Example 3 - *In vitro* assay of AAD-1 (v1) activity via colorimetric phenol detection

[0197] 3.1 -AAD-1 (v1) assay.

[0198] AAD-1 (v1) enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (J. Biol. Chem. 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides such as haloxyfop and cyhalofop (see **Figure 4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so were readily detected. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μM phenol.

[0199] Enzyme assays were performed in a total volume of 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 15 minutes of incubation at 25° C, the reaction was terminated by addition of 10 μl 100 mM sodium EDTA. Color was developed by addition of 15 μl pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1N KOH), 1.5 μl 2% 4-aminoantipyrine and 1.5 μl 8% potassium ferricyanide. After 10 to 20 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols. Later assays were made more convenient by consolidating the additions as follows: the reaction was quenched by addition of 30 μl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer and 0.2 % 4-aminoantipyrine, then adding 10 μl 0.8 % potassium ferricyanide.

[0200] 3.2 - Extraction.

[0201] Activity of recombinant AAD-1 (v1) expressed in *Escherichia coli*. *E. coli* cell pellets were resuspended in 0.1 M Tris, pH 7.4 + 1mg/ml lysozyme (5 ml/cells from 250 ml culture; 20 ml/cells from 1 liter) at room temperature. After about 15 minutes with occasional shaking, the suspension was frozen in liquid nitrogen then thawed. DNase was added

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to 0.02 mg/ml final concentration and $MgCl_2$ to 1 mM. After the extract was no longer viscous, the extract was centrifuged for 15 min. The supernatant was passed over a BioRad 10DG column pre-equilibrated with 20 mM MOPS pH 6.75 and the eluant stored in aliquots at -70° C. Assays were either performed with these unpurified desalted extracts or with purified enzymes.

[0202] A cell pellet from a 250 ml culture of induced *E. coli* cells expressing pDAB3203 containing the gene encoding *AAD-1* (*v1*) was extracted and assayed using the previously described protocols. The 2,4-D cleaving activity in the AAD-1 (*v1*) extract was compared to that from *E. coli* cells expressing a vector without *AAD-1* (*v1*) using 1 mM 2,4-D and is shown in **Figure 5.** The amount of 2,4-dichlorophenol formed is clearly proportional to the amount of extract added to the assay whereas the control extract contains no 2,4-D cleaving activity.

[0203] The activity of this extract was tested on four additional herbicides, (R,S)-dichlorprop, (R,S)-mecoprop, (R, S)-haloxyfop and (R,S)-diclofop in comparison to 2,4-D (all at a final concentration of 0.5 mM) using 4 μl of the *E. coli* extract per assay with a 15 min assay period. **Figure 6A** shows that AAD-1 (v1) cleaved all five herbicides to yield a phenol with the relative activity on the substrates being dichlorprop = mecoprop > diclofop > haloxyfop > 2,4-D. Thus AAD-1 (v1) has activity on graminicidal aryloxyphenoxypropionate herbicides as well as phenoxy auxins.

[0204] The AAD-1 (v1) extract was then tested using racemic (R,S)-haloxyfop, the R enantiomer of haloxyfop and the S-enantiomer of cyhalofop (all at 0.5 mM) as potential substrates to ascertain the likely enantiomeric specificity of AAD-1 (v1). The results are shown in **Figure 6B.** The activity of the enzyme on (R)-haloxyfop was equivalent to that on (R, S)-haloxyfop whereas no activity could be seen on the S-enantiomer of cyhalofop indicating that AAD-1 (v1) has R specificity on AOPPs.

Example 4 - Substrate Specificity of AAD-1 (v1)

[0205] 4.1 - Additional substrates of AAD-1 (v1).

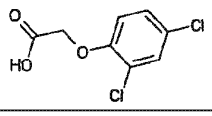
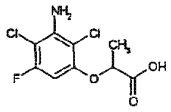
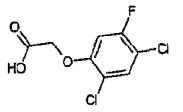
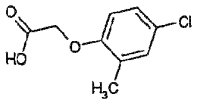
[0206] The substrate specificity of AAD-1 (v1) toward a variety of commercial and experimental herbicides was tested. Purified AAD-1 (v1) was used at either 1 or 10 μg per 160 μl assay and each substrate was tested at 1 mM with an assay time of 15 minutes. **Table 3** shows the A_{510} detected after the action of AAD-1 (v1) on a variety of aryloxyalkanoate auxinic herbicides and auxin analogs. The best substrate tested was dichlorprop, with mecoprop also being efficiently cleaved. Two other phenoxypropionates, the 4-fluoro and 3-amino analogs of dichlorprop, were also acted on effectively by AAD-1 (v1). AAD-1 (v1) produced small amounts of phenol from a variety of phenoxyacetates including 2,4-D. The relative rates on these substrates are better gauged from the assays using the higher amounts (10 μg) of AAD-1 (v1). From these data, 2,4-D is cleaved by AAD-1 (v1), as are two phenoxyalkylsulfonates, X188476 and X398166 (sesone).

[0207] **Table 4** shows data for a variety of AOPP graminicide herbicides as AAD 1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxy-acetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quino-linoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

[0208] Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness **(Table 8).** X11115427 was slightly better as a substrate than 2,4-D (125% of the 2,4-D rate) in contrast to the close analog 3-aminodichlorprop, which is ~7- fold better than 2,4-D as a substrate **(Table 3).** The 5-F substitution appears to decrease the effectiveness of X11115427 as a substrate for AAD-1 (v1). The rates of product formation from 5-F-phenoxyacetate and MCPA were 32% and 55% that of 2,4-D respectively.

[0209] **Table 8:** Effect of AAD-1 (v1) on three substrates relative to 2,4-D. Substrates were assayed as in Table 6 at 1 mM using a crude recombinant AAD-1 (v1) extract from *E. coli.*

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Table 8. Effect of AAD- (v1) on three substrates relative to 2,4-D.				
Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
195517		2,4-D	0.177	100
11115427		(R,S)-3-amino, 5-F-dichlorprop	0.221	125
124987		5-F, 2,4-D	0.056	32
192711		MCPA	0.097	55

[0210] 4.2-Kinetic characterization.

[0211] The K_m and k_{cat} values of purified AAD-1 (v1) (see Example 10) were determined for four herbicide substrates, (R)-dichlorprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 μM Na ascorbate; 200 μM Fe^{2+}; 1 mM α-ketoglutarate; 25° C). The dose response curves were fitted using Grafit (Erithacus Software, UK), and the graphs and derived constants are shown in **Figure 7** and **Table 9** respectively. The K_m values for the four substrates were fairly similar (75 - 125 μM), but the k_{cat} values varied significantly. (R)-dichlorprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichlorprop). These k_{cat} values were consistent with the range of values seen in the substrate specificity tests shown in **Table 3** and **Table 4** as these were performed at high (saturating) substrate concentrations (1 mM).

[0212] **Table 9:** Kinetic Constants for AAD-1 (v1) Substrates. Kinetic constants were derived from the data in Figure 7 using Grafit fitting to the Michaelis-Menten equation.

Table 9. Kinetic Constants for AAD-1 (v1) Substrates.				
Substrate	Km (μM) ± SE	V_{max} (μmol min^{-1} mg^{-1} AAD1) ± SE	k_{cat} (min^{-1})	kcat/K_m (min^{-1} mM^{-1})
R-dichlorprop	75 ± 10	0.79 ± 0.03	26.1	348
R-quizalofop	125 ± 20	0.57 ± 0.03	18.9	151
R-haloxyfop	120 ± 54	0.34 ± 0.04	11.2	94
2,4-D	96 ± 8	0.57 ± 0.00	2.7	28

[0213] The relative K_m and k_{cat} values for dichlorprop and 2,4-D differ significantly from those published for the R-specific dioxygenase from *Delftia acidovorans* by Westendorf *et al.* (2003) (*Acta Biotechnol.* 23: 3-17). The published k_{cat}/K_m value for 2,4-D is 0.6% that of dichlorprop, whereas in our studies, the k_{cat}/K_m value for 2,4-D is 8% that of dichlorprop. Thus, in this study, AAD-1 (v1) is unexpectedly effective at catalyzing the cleavage of 2,4-D. This increases its potential utility for conferring diverse herbicide tolerance traits in transgenic applications.

[0214] 4.3 - Additional substrates for AAD-1 (v1).

[0215] Three additional substrates were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay; X11115427, X124987 and MCPA. The results are shown in **Table 8.** All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness. X11115427 was only slightly better (125%) as a substrate than 2,4-D. This is in contrast to 3-aminodichlorprop which is >7 fold better than 2,4-D as a substrate **(Table 3).** Thus, the 5-F substitution has significantly decreased the effectiveness of X11115427 as a substrate for AAD-1 (v1). A similar pattern is seen with 5-F-2,4-D which is only 32% as effective as a substrate relative to 2,4-D. In this assay, MCPA was also less effective as a substrate of AAD-1 (v1) (55% relative to 2,4-D).

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Example 5 - Optimization of Sequence for Expression in Plants

[0216] 5.1 -Background.

[0217] To obtain high expression of heterologous genes in plants, it may be preferred to reengineer said genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

[0218] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.,* TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.,* polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content; and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[0219] **Table 10** illustrates how high the G+C content is in maize. For the data in **Table 10,** coding regions of the genes were extracted from GenBanlc (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

Table 10: Compilation of G + C contents of protein coding regions of maize genes

Protein Class.sup.a	Range % G + C	Mean % G + C.sup.b
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+-.5.2)

.sup.a Number of genes in class given in parentheses.
.sup.b Standard deviations given in parentheses.
.sup.c Combined groups mean ignored in mean calculation

[0220] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

[0221] In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 11.** After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first,

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second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

Table 11. Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

[0222] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[0223] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled for the gene sequences for the particular plant. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

[0224] 5.2 - Rebuild analysis.

[0225] Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native *AAD-1 (v1)* coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID NO:5) was developed that encodes SEQ ID NO: 11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG

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start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and **Table 12** shows the differences in codon compositions of the native and plant-optimized sequences.

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Table 12. Codon composition comparison of native *AAD-1 (v1)* coding region and Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %	Amino Acid	Codon	Native Gene #		Plant Opt Gene #	Plant Opt Gene %
ALA (A)	GCA	2	9.1	5	22	LEU (L)	CTA	0	0.0	0	0
	GCC	10	45.5	8	35		CTC	5	22.7	7	32
22	GCG	9	40.9	0	0		CTG	16	72.7	0	0
	GCT	1	4.5	10	43	22	CTT	0	0.0	8	36
ARG (R)	AGA	0	0.0	7	30		TTA	0	0.0	0	0
	AGG	0	0.0	7	30		TTG	1	4.5	7	32
	CGA	0	0.0	0	0	LYS (K)	AAA	1	14.3	2	29
23	CGC	16	69.6	5	22	7	AAG	6	85.7	5	71
	CGG	4	17.4	0	0	MET (M)	ATG	8	100	8	100
	CGT	3	13.0	4	17	PHE (F)	TTC	10	76.9	9	69
ASN (N)	AAC	8	100.0	4	50	13	TTT	3	23.1	4	31
8	AAT	0	0.0	4	50	PRO (P)	CCA	1	5.9	8	47
ASP (D)	GAC	15	78.9	10	53		CCC	9	52.9	1	6
19	GAT	4	21.1	9	47	17	CCG	7	41.2	0	0
CYS (C)	TGC	3	100.0	2	67		CCT	0	0.0	8	47
3	TGT	0	0.0	1	33	SER (S)	AGC	11	73.3	4	27
END	TAA	0	0.0	0	0		AGT	0	0.0	0	0
1	TAG	1	100.0	0	0	15	TCA	0	0.0	4	27
	TGA	0	0.0	I	100		TCC	2	13.3	3	20
GLN (Q)	CAA	0	0.0	7	54		TCG	2	13.3	0	0
13	CAG	13	100.0	6	46		TCT	0	0.0	4	27
GLU (E)	GAA	8	50.0	5	31	THR (T)	ACA	1	4.3	6	26
16	GAG	8	50.0	11	69		ACC	16	69.6	9	39
GLY (G)	GGA	0	0.0	6	29	23	ACG	5	21.7	0	0

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(continued)

Table 12. Codon composition comparison of native *AAD-1 (v1)* coding region and Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %	Amino Acid	Codon	Native Gene #		Plant Opt Gene #	Plant Opt Gene %
	GGC	15	71.4	7	33		ACT	1	4.3	8	35
21	GGG	3	14.3	2	10	TRP (W)	TGG	5	**100**	5	**100**
	GGT	3	14.3	6	29	TYR (Y)	TAC	7	70.0	6	60
HIS(H)	CAC	6	60.0	5	50	10	TAT	3	30.0	4	40
10	CAT	4	40.0	5	50	VAL (V)	GTA	2	7.1	0	0
ILE (1)	ATA	0	0.0	3	25		GTC	11	39.3	8	29
12	ATC	12	100.0	5	42	28	GTG	15	53.6	10	36
	ATT	0	0.0	4	33		GTT	0	0.0	10	36
	Totals	148		149			Totals	148		148	

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[0226] 5.3 - Completion of binary vectors.

[0227] 5.3.1 - Rebuilt *AAD-1 (v3).* The plant optimized gene *AAD-1* (*v3*) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ BUD NO:5) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO: 16) and M13 Reverse (SEQ LID NO: 17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-1* (*v3*) DNA sequence. The *AAD-1* (v3) gene was cloned into pDAB726 as an Nco I - Sac I fragment. The resulting construct was designated pDAB720, containing: [AtUbi10 promoter: Nt OSM 5'UTR: *AAD-1* (v3): Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with Not I restriction digests): A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB721, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: *AAD-1 (v3):* Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT:* ORF25/26 3'UTR] was restriction digested (with Bam HI, EcoR I, EcoR V, HinD III, Pac I, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

[0228] 5.3.2 - Native *AAD1 (v1)* and modified *AAD-1 (v2).* The *AAD-1 (v1)* gene (SEQ ID NO:3) was PCR amplified from pDAB3203. During the PCR reaction, alterations were made within the primers to introduce the NcoI and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "rdpA(ncoI)" [CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC] (SEQ ID NO:6) and "3'saci" [GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA] (SEQ ID NO:7) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[0229] The PCR amplicon was ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents.

[0230] Sequence data identified a clone with the correct sequence. During analysis a superfluous NotI restriction site was identified toward the 3' end of *AAD-1 (v1)*. This site was removed to facilitate cloning into pDAB3038. To remove the additional site a PCR reaction was performed with an internal 5' primer. The NotI site was altered by incorporating a new codon for an amino acid to remove the spurious NotI site. This change would alter the arginine at position 212 to a cysteine. The PCR primers "BstEII/Del NotI" [TGG TGG TGA CCC ATC CGG GCA GCG GCT GCA AGG GCC] (SEQ ID NO:8) and "3' saci" (SEQ ID NO:7) were used.

[0231] A PCR reaction was completed using the Fail Safe PCR System (Epicenter) and the resulting fragment was cloned into the pCR 2.1 TOPO TA cloning kit (Invitrogen). Confirmation of the correct PCR product was completed by DNA sequencing, and the "fixed" gene was given the designation *AAD-1 (v2)* (SEQ ID NO:4).

[0232] A sequencing reaction using the M13 Reverse primer (SEQ ID NO:17) and the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents indicated that the correct PCR fragment had been isolated. This construct was digested with the BstEII and SacI enzymes. The resulting fragment was cloned into the pCR2.1 *AAD-1 (v2)* construct (pCR2.1 Delta NotI) and confirmed via restriction enzyme digestion.

[0233] The modified *AAD-1 (v2)* gene was then cloned into pDAB726 as a NcoI/SacI DNA fragment. The resulting construct (pDAB708) was verified with restriction digests. This construct was then cloned into the binary pDAB3038 as a NotI - NotI fragment. The final resulting construct was given the designation pDAB766, containing the [AtUbi10 promoter: Nt OSM5'UTR: *AAD-1 (v2):* Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT:* ORF25/26 3'UTR] and was restriction digested for verification of the correct orientation. The completed construct was then used for transformation into *Agrobacterium.*

[0234] 5.3.3 - Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is presented herein as SEQ ID NO:20. A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is presented herein as SEQ ID N0:21.

[0235] A codon usage table for soybean *(Glycine max)* protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in **Table 13**. Columns D and H of **Table 13** present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of

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GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in **Table 13.** Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

$$\text{Weighted \% of C1} = 1/(\%C1 + \%C2 + \%C3 + \textit{etc.}) \times \%C1 \times 100$$

where C1 is the codon in question, C2, C3, *etc.* represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of **Table 13** (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of **Table 13.** TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Indiana).

Table 13. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A	B	C	D	E	F	G	H
Amino Acid	**Codon**	**Weighted %**	**Soybean %**	**Amino Acid**	**Codon**	**Weighted %**	**Soybean %**
ALA (A)	GCA	33.1	30.3	**LEU (L)**	CTA	**DNU**	**9.1**
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	**DNU***	**8.5**		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	**DNU**	**9.8**
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	**DNU**	**8.2**	**LYS (K)**	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	**DNU**	6.0	**MET (M)**	ATG	100.0	100
	CGT	16.9	14.5	**PHE (F)**	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TAT	50.8	50.8
	AAT	50.0	5 0.0	**PRO (P)**	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	**DNU**	**8.3**
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	**SER (S)**	AGC	16.0	15.1
END	TAA	**DNU**	40.7		AGT	18.2	17.1
	TAG	**DNU**	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	**DNU**	6.1
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	**THR (T)**	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	**DNU**	8.3
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	**TRP(W)**	TGG	100.0	100

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(continued)

Table 13. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A	B	C	D	E	F	G	H
Amino Acid	**Codon**	**Weighted %**	**Soybean %**	**Amino Acid**	**Codon**	**Weighted %**	**Soybean %**
.	GGT	30.4	30.4	**TYR (Y)**	TAC	48.2	48.2
IDS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	**VAL (V)**	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

***DNU** = Do Not Use

[0236] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21.

[0237] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is given as bases 1-1575 of SEQ ID NO:22. Synthesis of a DNA fragment comprising SEQ ID NO:22 was performed by a commercial supplier (PicoScript, Houston TX).

[0238] 5.3.4 - Cloning of additional binary constructs. The completion of pDAB3295 and pDAB3757 incorporated the use of the GateWay Cloning Technology (Invitrogen, cat #11791-043 and cat #12535-019). The GateWay Technology uses lambda phage-based site-specific recombination to insert a gene cassette into a vector. For more information refer to *Gateway Technology: A universal Technology to clone DNA sequence for functional analysis and expression in multiple systems,* © 1999-2003, Invitrogen Corp., 1600 Faraday Ave., Carlsbad, CA 92008 (printed - 2003). All other constructs created for transformation into appropriate plant species were built using similar procedures as above and other standard molecular cloning methods (Maniatis *et al.,* 1982). **Table 14** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

[0239] The *sacB* gene was added to the binary vector pDAB3289 as a bacterial negative selection marker to reduce the persistence of *Agrobacterium* associated with transformed plant tissue. SacB is a levan-sucrase enzyme produced by *Bacillus* spp. and is toxic to most Gram negative bacteria when grown in the presence of sucrose (Gay *et al.,* 1983). The *sacB* gene was recovered on a *Hind* III fragment from plasmid pRE112 (Edwards, *et al.,* 1998) and cloned into the unique *Hind* III site in pDAB3289.

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Table 14. Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Trans-formed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	-	-	-	Erythromycin	-	pat	CsVMV	Agro binary
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	AAD1 v3	CsVMV	Agro binary
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	sacB	HptII	AtUbi3	Agro binary
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	HptII	AtUbi3	Agro binary
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Aerosol beam
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Agro binary
3403		Cn, R	AAD1 v3	ZmUbi1	-	RB7 Mar v2	RB72Mar -	RB72Mar -	Ampicillin	-	Same as GOI		Whiskers / Gun
3404		Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	pat	OsAct1	Whiskers
3415	1283	Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	AHAS v3	OsAct1	Whiskers
3602	1421	Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Spectinomycin	-	AHAS v3	OsAct1	Agro Superbinary
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	pat	AtUbi11	Agro binary

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(continued)

Table 14. Binary constructs used in transformation of various plant species.													
pDAB #	**pDAS #**	**Species * Trans-formed into**	**Gene of interest (GOI)**	**Promoter**	**Feature 1**	**Feature 2**	**GOI 2**	**Promoter**	**Bacterial Selection gene**	**Bacterial Selection gene 2**	**Plant Selection gene**	**Promoter**	**Trxn Method**
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	-	-	Erythromycin	-	pat	CsVMV	Agro binary
*A = *Arabidopsis* CsVMV = Cassava Vein Mosaic Virus Promoter ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter T = Tobacco AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter HptII = hygromycin phosphotransferase S = Soybean RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR) Ct = Cotton Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region R = Rice Cn = Corn (721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region Ca = Canola (3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region													

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Example 6 - Transformation into *Arabidopsis* and Selection

[0240] *6.1-Arabidopsis thaliana* growth conditions.

[0241] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[0242] Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[0243] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2 - *Agrobacterium* transformation.

[0244] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

[0245] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium*. cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 μl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[0246] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10g Bactopeptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[0247] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28°C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed Agrobacterium cells, suspended in 10 μl of water, at 100° C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C for 3 minutes, 2) 94° C for 45 seconds, 3) 55° C for 30 seconds, 4) 72° C for 1 minute, for 29 cycles then 1 cycle of 72° C for 10 minutes. The reaction was maintained at 4° C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

[0248] 6.3 -*Arabidopsis* transformation.

[0249] *Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044μM benzylamino purine (10 μl/liter of 1 mg/ml stock in DMSO) and 300 μl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

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[0250] 6.4 - Selection of transformed plants.

[0251] Freshly harvested T_1 seed (transformed with native [*AAD-1* (*v2*)] or plant optimized [*AAD-1* (*v3*)] gene) was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[0252] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT gene).*

[0253] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)* (plant optimized gene) or *AAD-1 (v2)* (native microbial gene) to provide phenoxy auxin herbicide resistance.

[0254] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of phenoxy herbicides (dichlorprop or 2,4-D). Phenoxy rates initially applied were 12.5 g ae/ha 2,4-D and 50 or 200 g ae/ha dichlorprop. GR_{99} for *Arabidopsis* is about 50 g ae/ha 2,4-D and 200 g ae/ha dichlorprop. Elevated rates were applied in subsequent trials (50, 200, 800, or 3200g ae/ha).

[0255] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of phenoxy herbicides in 200 mM Tris buffer (pH 9.0) to a final pH of ~7-8.

[0256] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether haloxyfop could be effectively degraded *in planta.*

[0257] Although *Arabidopsis,* being a dicot, is not an optimal system for testing ACCase-inhibiting AOPP grass herbicides, *AAD-1 (v3)*-transformed T_1 plants were subjected to elevated rates (400-1600 g ae/ha) of RS-haloxyfop acid (internally synthesized) that do cause growth abnormalities and death of wildtype *Arabidopsis* using the DeVilbiss sprayer as described above Injury ratings were taken 7 and 14 days after treatment. Likewise, T_1 individuals were treated with the pyridyloxyacetate auxin herbicide, fluroxypyr.

[0258] 6.5 - Results of selection of transformed plants.

[0259] The first *Arabidopsis* transformations were conducted using *AAD-1 (v3)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed. seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% *(see* **Table 15**). A small lot of *AAD-1 (v2)* (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

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Table 15: Selection of AAD-1 (v3) (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or plant optimized AAD-2 (v2)-transformed T_1 individual plants using glufosinate and 2,4-D.

Selection agent	Gene	Codon bias	Total seed sown and screened	Number of resistant T_1	Selection rate	Selection rate range	% of selected plants expressing PAT[3]
Glufosinate[1]	*AAD-1 (v2)*	n	84,000	278	0.33%	0.33%	nd[4]
Glufosinate[1]	*AAD-1 (v3)*	p	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D[2]	*AAD-1 (v3)*	p	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate[1]	*AAD-2 (v1)*	n	1,301,500	228	0.018%	0.007 to 0.021%	100%
Glufosinate[1]	*AAD-2(v2)*	p	200,000	224	0.11%	0.11%	nd[4]

[1]Glufosinate selection scheme: 280g ai/ha glufosinate applied 5-6 + 10 DAP
[2]2,4-D selection scheme: 50 g ai/ha 2,4-D applied 5-7 + 10-14 DAP
[3]PAT protein expression determined by PAT ELISA strips
[4]nd, not determined
[5]codon bias, n-native microbial gene, p=plant optimized

[0260] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 16** compares the response of native *AAD-1 (v2)* and plant optimized *AAD-1 (v3)* genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Both genes imparted resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 16** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)* or *AAD-1 (v3)* versus the wildtype or *PAT/CrylF* transformed controls. Also evident is that tolerance appears to be significantly greater (frequency and overall level of individual response) for the plant optimized sequence *AAD-1 (v3)* versus the native sequence *AAD-1 (v2)* (see **Table 16**). Higher rates of 2,4-D (up to 3,200 g ae/ha) have been applied to additional T_1 individuals expressing AAD-1 (v3). Injury levels tend to be greater and the frequency of highly resistant plants is lower at these elevated rates (6x field rates). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, some individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

[0261] **Table 16.** *AAD-1 v3* (plant optimized), or *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

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Table 16. *AAD-1 v3* (plant optimized), or *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence.

	% Injury				% Injury	
Native AAD-1 (v2) gene Averages	<20%	20-40%	>40%		Ave	Std Dev
Untreated control-buffer	20	6	7		25.3	34.7
50 g ae/ha 2,4-D	55	16	9		14.8	22.7
200 g ae/ha 2,4-D	45	11	24		34.1	39.3
800 g ae/ha 2,4-D	11	32	37		52.5	34.2

	% Injury				% Injury	
Native AAD-2 gene Averages	<20%	20-40%	>40%		Ave	Std Dev
Untreated control-buffer	4	1	1		25.0	21.7
50 g ae/ha 2,4-D	1	2	11		68.2	30.2
200 g ae/ha 2,4-D	0	3	11		82.7	28.8
800 g ae/ha 2,4-D	0	0	14		99.8	0.8

	% Injury				% Injury	
Rebuilt AAD-1 (v3) gene Averages	<20%	20-40%	>40%		Ave	Std Dev
Untreated control-buffer	9	0	0		0.0	0.0
50 g ae/ha 2,4-D	10	1	5		24.3	35.9
200 g ae/ha 2,4-D	11	4	1		14.0	25.9
800 g ae/ha 2,4-D	11	4	1		14.7	26.1

	% Injury				% Injury	
Wildtype Averages	<20%	20-40%	>40%		Ave	Std Dev
Untreated control-buffer	11	0	0		0.0	0.0
50 g ae/ha 2,4-D	0	0	15		90.0	0.0

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(continued)

Wildtype Averages	% Injury <20%	% Injury 20-40%	% Injury >40%		% Injury Ave	Std Dev
200 g ae/ha 2,4-D	0	0	15		95.1	0.5
800 g ae/ha 2,4-D	0	0	15		100.0	0.0
PAT/CryIF (transformed control) Averages	% Injury <20%	% Injury 20-40%	% Injury >40%		% Injury Ave	Std Dev
Untreated control-buffer	11	0	0		0.0	0.0
50 g ae/ha 2,4-D	0	0	15		90.7	4.2
200 g ae/ha 2,4-D .	0	0	15		97.2	1.7
800 g ae/ha 2,4-D	0	0	15		100.0	0.0

[0262] **Table 17** shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400 - 1,600 g ae/ha **(Table 18)**. Normal field use rate for haloxyfop (a grass-specific herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some *AAD1 (v3)* transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first *in planta* data that *AAD-1 (v3)* will provide AOPP resistance. No resistance was observed with fluroxypyr (a pyridyloxyacetate auxin) in transformed *Arabidopsis,* consistent with *in vitro* work using heterologously expressed enzyme.

[0263] **Table 17.** T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 17. T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence.

AAD-1 v3 Averages	% Injury <20%	% Injury 20-40%	% Injury >40%		% Injury Ave	Std Dev	Range
Untreated control	3	0	0		0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	7	1	0		5.0	7.6	0-20
50 g ae/ha RS-dichlorprop	7	1	0		3.1	8.8	0-25
200 g ae/ha RS-dichlorprop	4	1	3		40.0	50.1	0-100
800 g ae/ha RS-dichlorprop	0	5	3		51.9	40.0	20-100
PAT/Cry1F Averages	% Injury <20%	% Injury 20-40%	% Injury >40%		% Injury Ave	Std Dev	Range
Untreated control	3	0	0		0.0	0.0	0

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(continued)

PAT/Cry1F Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev	Range
12.5 g ae/ha RS-dichlorprop	0	6	2	38.1	25.3	20-95
50 g ae/ha RS-dichlorprop	0	0	8	80.0	25.3	50-100
200 g ae/ha RS-dichlorprop	0	0	8	98.3	2.2	95-100
800 g ae/ha RS-dichlorprop	0	0	8	100.0	0.0	100
Wildtype Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	3	0	0	13.3	2.9	10-15
50 g ae/ha RS-dichlorprop	0	0	3	53.3	5.8	50-60
200 g ae/ha RS-dichlorprop	0	0	3	95.0	5.0	90-100
800 g ae/ha RS-dichlorprop	0	0	3	100.0	0.0	100

[0264] **Table 18.** T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T1 is an independent transformation event, one can expect significant variation of individual T1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/CrylF*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 18. T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide.

AAD-1 v3 Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	3	1	0	6.3	9.5	0-20
800 g ae/ha haloxyfop	1	1	2	46.3	42.7	0-85
1600 g ae/ha haloxyfop	1	0	3	65.0	47.3	0-100
PAT/Cry1F Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	10.0	0.0	10

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(continued)

PAT/Cry1F Averages	<20%	% Injury 20-40%	>40%		% Injury Ave	Std Dev	Range
400 g ae/ha haloxyfop	0	4	0		27.5	5.0	20-30
800 g ae/ha haloxyfop	0	0	4		78.8	6.3	70-85
1600 g ae/ha haloxyfop	0	0	4		47.5	43.5	80-100

Wildtype Averages	<20%	% Injury 20-40%	>40%		% Injury Ave	Std Dev	Range
Untreated control	3	0	0		0.0	0.0	0
100 g ae/ha haloxyfop	3	0	0		0.0	0.0	0
200 g ae/ha haloxyfop	3	0	0		0.0	0.0	0
400 g ae/ha haloxyfop	0	3	0		20.0	0.0	20
800 g ae/ha haloxyfop	0	0	3		73.3	10.4	70-85
1600 g ae/ha haloxyfop	0	0	3		93.3	11.5	80-100

[0265] 6.6 - *AAD- 1 (v3)* as a selectable marker.

[0266] The ability to use *AAD-1 (v3)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. T_1 seed transformed with *PAT* and *AAD1* (v3) (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active *PAT* gene.

[0267] Fifty-three out of 70,000 seed planted were identified as resistant to 2,4-D. ELISA was used to screen a subset of 44-individuals from this population for PAT protein expression. Ninety six percent of the individuals were positive indicating the presence of the co-transformed gene, *PAT.* The low number of negative ELISA results (4%) is in line with a 3% error rate in populations of glufosinate-resistant plants **(Table 15)**. The efficiency of selection appears to be somewhat less with 2,4-D (0.08%) vs. glufosinate (0.12%); however, the range of selection rates across all experiments would indicate both selection agents are equally good for selecting *Arabidopsis* transformed with *AAD-1* (v3) or *PAT* genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

[0268] 6.7 - Heritability.

[0269] A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 60% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[0270] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line tested: ranging in frequency from one to four out of the eight families tested. These data show *AAD1 (v3)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

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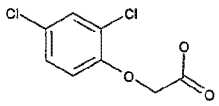
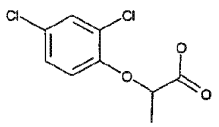
[0271] 6.8 -Additional herbicide resistance attributable to AAD- in *Arabidopsis.*

[0272] The ability of *AAD-1 (v3)* to provide resistance to other aryloxyphenoxyalkanoate herbicides in transgenic *Arabidopsis* was determined using a modified *in vitro* plate assay. Seeds from wild-type *Arabidopsis thaliana* as well as *Arabidopsis thaliana* containing the plant optimized AAD-1 (v3) gene (T_4 homozygous plants id = PAAD1.315.064) were sterilized by agitating for 10 min in a 50% bleach solution. These seeds were then rinsed four times with sterile water to remove the bleach.

[0273] Dose response assays utilized a nutrient media (see below) supplemented with various rates of test compounds. The test compounds were added to the heated media (55° C) as concentrated solutions in DMSO. Control wells had the appropriate amount of DMSO without any additional compound. The final concentration of DMSO never exceeded 1 % (v/v). After thorough mixing, a 6 mL aliquot of the warm media containing the appropriate concentration of compound was added to each well of a 6-well, flat bottom, polystyrene tissue culture tray (Falcon 353046, Becton Dickson and Company, Franklin Lakes, NJ). After the media solidified, approximately 20 to 30 *Arabidopsis* seeds were applied on top of the solidified media and the remaining 2 mL of media was poured over the seeds. The plates were lightly agitated to disperse the seeds, covered and allowed to cool until the media had completely solidified. The plates were incubated for 7 days at 25°C under continual fluorescent lighting (75 μE m^{-2} s^{-1}). Nutrient Media Composition was as described in Example 2.2 and in Somerville and Orgen (1982).

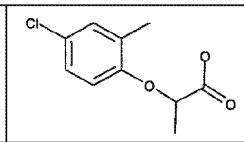
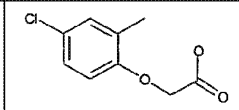
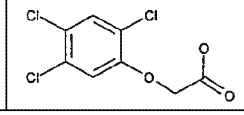
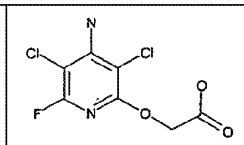
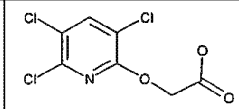
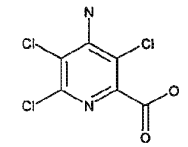
[0274] Assessment of growth reduction. The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis.* The ratios of resistant to sensitive biotypes are included in **Table 19**. A ratio >2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported *in planta.* The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of *AAD-1*-transformed commercial crops or experimental plant species.

Table 19. *In vitro* plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

Compound	Structure	wt ARBTH shoot 150	PAAD1.315.064 T3 ARBTH shoot 150	wt ARBTH root 150	PAAD1.315.064 ARBTH root 150	shoot ratio	root ratio
		ppm					
Phenoxy auxins							
2,4-D		0.2	10	< 0.01	0.04	50	>4
dichlorprop		2	5	0.01	1.5	2.5	150

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(continued)

Phenoxy auxins							
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T		1.5	10	<0.01	<0.01	6.67	NA
Pyridine auxins							
Fluroxypyr		2	1	0.2	0.2	0.5	1
Triclopyr		0.2	0.04	0.02	0.02	0.2	1
Picloram		1	0.5	0.3	0.15	0.5	0.5

[0275] 6.9 - Foliar applications herbicide resistance in AAD-1 *Arabidopsis.*

[0276] The ability of *AAD-1 (v3)* to provide resistance to other aryloxyphenoxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates described in Example 6.8. T_4 generation *Arabidopsis* seed, homozygous for *AAD-1 (v3)* (line AAD1.01.315.076) was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene CrylF was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of phenoxy auxin herbicides: 12.5-1600 g ae/ha 2,4-D dimethylamine salt (DMA) (Riverside Chemicals), 12.5-1600 g ae/ha mecoprop (AH Marks), 50-3200 g ae/ha R-dichlorprop (AH Marks), 8.75-1120 g ae/ha 2,4,5-T (technical grade); pyridyloxyacetates herbicides 50-3200 g ae/ha triclopyr (Dow AgroSciences) and 50-3200 g ae/ha fluroxypyr (Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-1 activity, 2,4-dichlorophenol (DCP, Sigma) (at 50-3200 g ae/ha, technical grade). All applications were formulated in 200 mM Hepes buffer (pH 7.5). Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment and are averaged over two experiments.

[0277] These results (see **Table 20**) confinn that *AAD-1 (v3)* in *Arabidopsis* provides robust resistance to the phenoxyacetic auxins, phenoxypropionic auxins, but have not shown significant cross resistance to the pyridyloxyacetic auxins tested and corroborates *the in vitro* enzyme and whole plate substrate specificity data. Additionally, there is no effect of the metabolite, 2,4-dichlorphohenol (DCP), on wildtype or transgenic *Arabidopsis.*

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Table 20. Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Phenoxypropionic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**PatCry1f - Control**
50 g ae/ha R-Dichlorprop	3	31
200 g ae/ha R-Dichlorprop	3	73
800 g ae/ha R-Dichlorprop	3	89
3200 g ae/ha R-Dichlorprop	3	95
12.5 g ae/ha Mecoprop	3	0
25 g ae/ha Mecoprop	0	2
50 g ae/ha Mecoprop	0	17
100 g ae/ha Mecoprop	0	33
200 g ae/ha Mecoprop	3	62
400 g ae/ha Mecoprop	0	78
800 g ae/ha Mecoprop	0	93
1600 g ae/ha Mecoprop	0	100
Phenoxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Crylf - Control**
12.5 g ae/ha 2,4-D DMA	0	67
25 g ae/ha 2,4-D DMA	0	78
50 g ae/ha 2,4-D DMA	0	93
100 g ae/ha 2,4-D DMA	0	100
200 g ae/ha 2,4-D DMA	0	100
400 g ae/ha 2,4-D DMA	0	100
800 g ae/ha 2,4-D DMA	0	100
1600 g ae/ha 2,4-D DMA	0	100
8.75 g ae/ha 2,4,5-T	0	0
17.5 g ae/ha 2,4,5-T	3	20
35 g ae/ha 2,4,5-T	0	43
70 g ae/ha 2,4,5-T	3	85
140 g ae/ha 2,4,5-T	0	95
280 g ae/ha 2,4,5-T	0	98
560 g ae/ha 2,4,5-T	17	100
1120 g ae/ha 2,4,5-T	3	100
Pyridyloxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Crylf - Control**
50 g ae/ha Triclopyr	31	36
200 g ae/ha Triclopyr	58	65

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(continued)

Pyridyloxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	AAD1.01.315.076.T_4 homozygous AAD1 plants	Pat/Crylf - Control
800 g ae/ha Triclopyr	74	84
3200 g ae/ha Triclopyr	97	95
50 g ae/ha Fluroxypyr	48	76
200 g ae/ha Fluroxypyr	75	85
800 g ae/ha Fluroxypyr	88	85
3200 g ae/ha Fluroxypyr	95	95
	Inactive DCP metabolite	
50 g ae/ha 2,4-DCP	0	0
200 g ae/ha 2,4-DCP	0	0
800 g ae/ha 2,4-DCP	0	0
3200 g ae/ha 2,4-DCP	0	0

[0278] 6.10 - Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis.*

[0279] An experiment was designed to examine if the level of AAD-1 (v3) expression in *Arabidopsis* varies at different growth stages. A high-tolerance, homozygous, *AAD-1 (v3)* T_4 line (id=PAAD1.01.345.163) was grown in greenhouse. Half of the plants were treated with 800 g ae /ha of 2,4-D (as previously described) while the other half were not treated. Two leaves, the 3rd leaf from the top and the 5th leaf from the bottom, were harvested from 5 plants, both treated and untreated, and analyzed by ELISA and Western Blotting (as described in Example 11) experiments at 4, 10, 14, 20 and 25 DAT. **Figures 8A** and **8B** showed that there was statistically no difference in AAD-1 (v3) expression between young and old leaves. In addition, the herbicide 2,4-D had little impact on the expression level of AAD-1 (v3) protein. The protein levels accumulated in older plants with some significant protein degradation at the later time points.

[0280] In a separate experiment, four different homozygous T_4 lines of *Arabidopsis* displaying different tolerance level to the herbicide 2,4-D were sprayed with various levels (0, 200, 800 and 3200 g/ha) of 2,4-D and their herbicide injury and AAD-1 (v3) expression were examined. Four days after the herbicide treatment, little injury was observed in three of the four lines, even at the highest dose tested **(Figure 9A)**. These plants also expressed high level of AAD-1 (v3), from 0.1 to 0.25% of **(Figure 9B)**. On the contrary, the low tolerance line expressed less than 0.1% of AAD-1 (v3) in TSP and suffered observable injury. More importantly, they recovered from the injury on 14 DAT **(Figure 9A)**, indicating that the low level of AAD-1 (v3) expression was able to protect the plants from the serious herbicide damage. All control plants suffered serious injury and died 14 DAT at doses 800 g ae/ha 2,4-D and above.

[0281] 6.11 - Molecular analysis of *AAD-1 (v3) Arabidopsis.*

[0282] Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number and/or Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple *AAD-1 (v3)* homozygous lines to determine stable integration of the plant transformation unit containing *PAT* and *AAD-1 (v3).* Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[0283] For Southern analysis, a total of 1 μg of DNA was subjected to an overnight digest of Nsi I for pDAB13721 to obtain integration data. The samples were run on a large 0.85 % agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATAC-CCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of *PAT.* The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50

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columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at -80° C.

[0284] Results showed all 2,4-D resistant plants assayed contained *PAT* (and thus by inference, *AAD-1 (v3)).* Copy number analysis showed total inserts ranged from 1 to >10 copies. This correlates, too, with the AAD-1 (V3) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance (>>200 fold) to all commercially available phenoxyacetic and phenoxypropionic acids.

[0285] 6.12 - *Arabidopsis* transformed with molecular stack of *AAD-1 (v3)* and glyphosate resistance gene.

[0286] T_1 *Arabidopsis* seed was produced, as previously described, containing pDAB3230 plasmid *(AAD-1 (v3)* + *EPSPS)* coding for a putative glyphosate resistance trait. T_1 transformants were selected using *AAD-1 (v3)* as the selectable marker as described in example 6.6, except the 2,4-D rate used was 75 g ae/ha. Twenty-four T_1 individually transformed events were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, *AAD-1 (v3)* + *PAT* T_5 homozygous lines (pDAB721-transformed), and *PAT* + *CryIF* homozygous line (transformed control). Only pDAB3230 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in 200 mM Hepes buffer (pH 7.5). All treatments were replicated 4 or 5 times. Plants were evaluated 7 and 14 days after treatment. I_{50} values were calculated and show >14 fold level of tolerance imparted by *EPSPS* molecularly stacked with *AAD-1 (v3)* (*see* **Figure 10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate + 2,4-D treatments applied in tankmix as described in Example 21 and shown for *AAD-1 (v3)*-transformed corn in Example 8.

Example 7 - WHISKERS-Mediated Transformation Into Maize, and Use of *AAD-1 (v3)* as a Selectable Marker

[0287] 7.1- Cloning of *AAD-1 (v3).*

[0288] The *AAD-1 (v3)* fragment was received on an NcoI/SacI fragment. Construct pDAB4005 was digested with NcoI and SacI and the 5175 bp backbone fragment isolated. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. The correct intermediate plasmid was named pDAB3403. Both pDAB3403 and pDAB8505 (OsAct1/PAT/ZmLip) were digested with NotI. The 3442 bp band from pDAB3403 and the 11017 bp band from pDAB8505 were isolated and purified. The fragments were ligated together, transformed into DH5α, and the resulting plasmids were screened for orientation. The final construct was designated pDAB3404, which contains ZmUbil/po-aadl/ZmPer5::OsActl/PAT/ZmLip.

[0289] 7.2 - Callus/suspension initiation.

[0290] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[0291] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.,* 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.,* 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[0292] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L *myo*-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

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[0293] 7.3 - Cryopreservation and thawing of suspensions.

[0294] Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C/minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

[0295] For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

[0296] 7.4 - Dose response of non-transformed tissue to haloxyfop acid.

[0297] Non-transformed donor Hi-II cell lines were pooled together, WHISKERS-treated without DNA, filtered onto GN6 medium at the rate of 6 ml per filter, and allowed to callus for 2-3 weeks at 28° C. Approximately 200 mg of callused suspension tissue was transferred per treatment to selection media in 60x20 mm plates containing 30, 100 or 300 nM R-haloxyfop acid. Three replicates were used per concentration. A control medium containing 1 mg/L bialaphos (from Herbiace commercial formulation, Meiji Seika, Japan), GN6 (1H), was also included for comparison. Callus was removed after 2 weeks, weighed, and transferred to fresh media of the same concentration for another 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded. Results are shown in **Figure 11**.

[0298] Two separate dose response studies of callus tissue to the phenol degradation products of haloxyfop and cyhalofop, respectively, were also completed to confirm that this end product would not be deleterious to callus growth. Data from a cyhalofop phenol dose response (see **Figure 12**) shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol. Data from a haloxyfop phenol dose response showed that even at 300 nM haloxyfop phenol, growth was equal to or greater than the control lacking haloxyfop phenol (data not shown).

[0299] 7.5 - WHISKERS-mediated transformation using bialaphos selection.

[0300] Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L *myo*-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

[0301] After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 μg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[0302] Either 3 or 6 mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C in a dark box that was loosely sealed with a single layer of plastic (<2 mils thick) to minimize evaporation of the individual plates.

[0303] After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (1H) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 1.0 mg/L bialaphos; 2.5 g/L Gelrite, pH 5.8) or GN6D (1H) medium (same as GN6 (1H) except with 8.0 mg/L dicamba and 0.8 mg/L 2,4-D).

[0304] Plates were placed in boxes and cultured as before for an additional week. Two weeks post-transformation, the tissue was embedded by scraping either ½ the cells on the plate or else all cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C) containing 1 mg/L bialaphos. The tissue was broken up and the 3 mL

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of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (1H) or GN6D (1H) medium. This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for 1 week at 28° C in dark boxes.

[0305] Putatively transformed isolates were typically first visible 5-8 weeks post-transformation. Any potential isolates were removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained growth was evident after approximately 2 weeks, an event was deemed to be resistant and was submitted for molecular analysis.

[0306] Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (1H), containing 1 mg/L bialaphos, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week then higher light (40 $\mu Em^{-2}s^{-1}$) for another week before being transferred to regeneration medium, 36 (1H), which was identical to 28 (1H) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

[0307] 7.6 - WHISKERS-mediated transformation using haloxyfop selection.

[0308] DNA delivery parameters for direct selection on "fops" were identical to the bialaphos selection procedure except that 85 μg of pDAB3403 and 85 μg of construct containing a GFP (Green Fluorescent Protein) reporter gene were co-transformed together, and only 3 mL of suspension was filtered onto GN6 medium following the 2-hour recovery.

[0309] After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60x20-mm plates of GN6 medium containing 2 mg/L 2,4-D plus 50, 100, or 200 nM R-haloxyfop acid. Plates were placed in boxes and cultured for one additional week. After one week, the tissue was embedded by scraping all cells from the plate into 3.0 mL of melted GN6 agarose medium containing the same concentration of selection agent as in the previous transfer. All steps afterward were identical to the PAT selection/regeneration protocol except that 100 nM R-haloxyfop acid was included in the regeneration media instead of 1 mg/L bialaphos.

[0310] 7.7 - Results. Multiple experiments testing various levels of haloxyfop and cyhalofop were initiated, and 47 isolates were recovered from direct selection. A subset of the callus events were submitted for screening using PCR and Western analyses. Following these expression data, 21 lead events were submitted for Southern analysis. Results using NcoI, a unique cutter, to obtain integration data following probing with *AAD-1 (v3)*, unequivocally demonstrate stable integration of *AAD-1 (v3)* following Whiskers-mediated transformation coupled with "fop" selection.

[0311] 7.8 - Quantitative demonstration of in *vitro* tolerance from AAD-1 (v3) expressing callus events from bialaphos selection

[0312] Ninety-seven callus isolates recovered from bialaphos selection were submitted for PAT copy number via Invader analysis and *AAD-1 (v3)* PTU analysis via PCR (see Example 7.10). AAD-1 (v3) protein expression using Western blot/Sandwich ELISA (Example 11) was completed on a subset of the events. A summary is described in **Table 21** below. At least 15 T0 plants were regenerated from each of these events and sent for spray testing and seed production.

Table 21.

Event	PAT Copy #	PCR for *AAD-1 (v3)* PTU	Callus Western
3404-001	2	+	+
3404-006	2	+	+
3404-013	3	+	+
3404-017	1	+	+
3404-020	3	+	nd
3404-022	2	+	+
3404-025	2	+	+
3404-027	3	+	+
3404-031	1	+	+
3404-033	2	+	nd
3404-036	3	+	+
3404-044	3	+	+

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(continued)

Table 21.			
Event	**PAT Copy #**	**PCR for *AAD-1 (v3)* PTU**	**Callus Western**
3404-050	3	+	+
3404-053	3	+	+
3404-074	2	+	+
3404-082	2	+	+

[0313] A smaller subset of these events was assessed in dose-response studies in comparison to a non-transformed control. A range of concentrations of haloxyfop (from Gallant Super formulation), up to 400 nM, were tested. Dose response data for one event, 3404-006, was generated using the general methods of Example 7.4, and is shown in **Figure 13**. This demonstrates that event 3404-006 showed no significant reduction in callus growth at haloxyfop concentrations up to 400 nM whereas non-transgenic corn callus tissue growth was inhibited at this rate. These data have not been normalized to account for inherent growth differences that are not related to the expression of the transgene.

[0314] 7.9 -WHISKERS-mediated transformation using imazethapyr selection.

[0315] The ZmUbil/*AAD-1 (v3)*/ZmPer5 cassette was removed from pDAB3404 with AscI/SwaI and inserted into pDAB2212 to create the *AAD-1* (v3) and *AHAS* Whiskers transformation vector pDAB3415, which is also referred to as pDAS1283) Once completed, this construct was transformed into maize via silicon carbide whiskers-mediated transformation as described in Example 7.4 except 2 mL of cells were filtered, followed by a 7-day recovery on GN6 medium followed by selection on media containing 3 μM imazethapyr from Pursuit® DG herbicide. Following Invader analysis, 36 events were identified that contained the AAD-1 (v3) and the AHAS genes.

[0316] Fifty-three corn calli events from these transformations were tested in both ELISA and Western Blotting experiments for their AAD-1 (v3) expression - a subset of the data is shown below. For the events listed in **Table 22**, the expression levels of those detected positive ranged from 90 to over 1000 ppm of total soluble proteins.

Table 22.		
AAD-1 Event ID Number	**Expression Level (ppm)**	**Western Blot**
1283[1]-001	2	-
1283[1]-002	206	+++
1283[1]-003	1	-
1283[1]-004	90	+++
1283[1]-005	1	-
1283[1]-006	105	+++
1283[1]-007	212	++
1283[1]-008	114	±
1283[1]-009	305	+
1283[1]-010	2	-
1283[1]-011	4	-
1283[1]-012	200	+++
1283[1]-013	134	+++
1283[1]-014	4	-
1283[1]-015	194	+++
1283[1]-016	4	-
1283[1]-017	196	+++
1283[1]-018	3	-

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(continued)

Table 22.		
AAD-1 Event ID Number	**Expression Level (ppm)**	**Western Blot**
1283[1]-019	178	+
1283[1]-020	260	++
1283[1]-021	144	+++
1283[1]-022	140	+++
1283[1]-023	191	+++
1283[1]-024	392	++
1283[1]-025	368	++
1283[1]-026	14	-
1283[1]-027	1006	++
Neg Control	3	-
Neg Control	3	-
Standard (0.5 μg/mL)		++
Standard (5 μg/mL)		++++

[0317] 7.10- Molecular Analysis: Maize Materials and Methods.

[0318] 7.10.1 - Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μl.

[0319] 7.10.2 - Invader assay analysis. The DNA samples are diluted to 20 ng/μl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 μl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 μl of controls, standards, and 20 ng/μl diluted unknown samples. Each well was overlaid with 15 μl of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis was used to identify the estimated copy of the unknown events.

[0320] 7.10.3 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-1 (v3)* PTU were (Forward - ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:25) and (Reverse - CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:26). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR *AAD-1 (v3)* were (Forward - ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse - CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[0321] 7.10.4 - Southern blot analysis. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digestion of AfI II and also EcoRV for pDAB3404, NcoI for pDAB3403, and SpeI for pDAB1421 to obtain integration data. After the overnight digestion an aliquot of~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85 % agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules.

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The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using the above coding region PCR using plasmid pDAB3404. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer): The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at - 80° C.

Example 8 - *In vivo* tolerance and field tolerance data generated from *PAT* selected (pDAB3404) *AAD-1 (v3)* events

[0322] 8.1 - Tolerance of T_0 corn plants to AOPP herbicides.

[0323] If more than 15 clone plants per event were successfully regenerated, then extra plants were transferred to the greenhouse for preliminary tolerance screening with postemergence-applied AOPP herbicides on T_0 corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (*i.e.,* plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C under 16 hour light: 8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assure® II (DuPont), Clincher* (Dow AgroSciences), or Gallant Super* (Dow AgroSciences) for quizalofop, cyhalofop, or haloxyfop, respectively. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height, and all sprays contained 1% v/v Agridex crop oil concentrate adjuvant. The number of clones of each event varied from week to week due to the rate of regeneration and acclimation of each event. Overall, an attempt was made to treat representative clones of each event with a range of herbicide doses ranging from 1X lethal dose (~1/8 X field dose) up to 8X field doses (64 X lethal dose). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

[0324] AOPP's are generally very potent corn-killing herbicides. Three to four leaf Hi=II corn grown from seed is effectively killed (>95% injury) with 8.8, 62.5, and 4.4 g ae/ha of haloxyfop, cyhalofop, and quizalofop, respectively. Each *AAD-1 (v3)*-transformed line tested survived a minimally lethal dose of each AOPP herbicide tested. In fact, most lines tested survived with no visible injury (14 DAT) even when treated with an 8X field dose (64X lethal dose) of quizalofop. Several individual clones from events "017" and "038," however, did incur significant injury at elevated rates. This could be a function of lower gene expression due to how or where the gene was inserted.

[0325] The high level of AOPP tolerance was demonstrated in most events, even when applications were made to plants just coming out of tissue culture (T_0 stage). Significantly, this tolerance was shown for all three AOPP herbicides and likely will extend to all AOPP herbicides as previously shown for AAD-1 *in vitro.*

[0326] **Figure 14** shows the responses of several *AAD-1 (v3)*-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied 1 week prior.

[0327] **Table 23** shows data for the responses of selected *AAD-1 (v3)*-transformed T_0 corn events to three AOPP herbicides applied postemergence.

Table 23. Selected AAD-1 (v3)-tansformed T0 corn events response to three AOPP herbicides applied postemergence.

Construct	Event	Clone	Haloxyfop* g ae/ha	Cyhalofop** g ae/ha	Quizalofop*** g ae/ha	% Injury 14 DAT
3404	001	016	8.8			0
3404	001	018		62.5		0
3404	001	017			8.8	0
3404	001	019			8.8	30
3404	001	020			35	0
3404	017	018			35	0
3404	017	019			35	0
3404	017	020			70	0

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(continued)

Table 23. Selected AAD-1 (v3)-tansformed T0 corn events response to three AOPP herbicides applied postemergence.

Construct	Event	Clone	Haloxyfop* g ae/ha	Cyhalofop** g ae/ha	Quizalofop*** g ae/ha	% Injury 14 DAT
3404	017	021			70	0
3404	017	022			140	0
3404	017	023			140	30
3404	017	024			280	30
3404	017	025			280	20
3404	022	019	8.8			0
3404	022	020	17.5			0
3404	022	016	17.5			0
3404	022	024		62.5		0
3404	022	018		125		0
3404	022	021			8.8	0
3404	022	017			17.5	0
3404	022	022			35	0
3404	022	023			70	0
3404	033	012			35	0
3404	033	013			70	0
3404	033	014			70	0
3404	033	015			140	0
3404	033	016			280	0
3404	038	016	8.8			0
3404	038	018		62.5		0
3404	038	017			8.8	0
3404	038	019			35	70
3404	038	020			35	80
3404	038	021			70	80
3404	038	022			70	80
*Gallant	super# +1%	COC(v/v)	(lethal dose = 8.8 g ae/ha)	(lethal dose = 62.5 g ae/ha)	(lethal dose = 4.4 g ae/ha)	

*Gallant super# + 1% COC (v/v)
**Clincher # + 1% COC (v/v)
***Assure II+ 1% COC (v/v)
#Trademark of Dow AgroSciences, LLC

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8.2 - Field tolerance of pDAB3404 T_1 corn plants to quizalofop 2,4-D, and glufosinate herbicides.

[0328] Two field trials were established at field stations in Hawaii and Indiana. Corn seed from inbred T_1 plants were utilized to evaluate sixteen AAD1 event lines for tolerance against quizalofop and 2,4-D. Three non-transformed hybrids were included for comparison purposes. The hybrid Hi-II x 5XH571 is of the same parentage as the *AAD-1 (v3)* event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

[0329] The experimental design was a split-plot with four replications. The main plot was herbicide treatment and the sub-plot was *AAD-1(v3)* event or comparison hybrid. Plots were one row by 3.7 meters with approximately twenty-five seeds planted in each row. For AAD1 events, seeds from a different lineage within the event were planted in each replicate.

[0330] Glufosinate at 560 g ai/ha was applied to *AAD-1 (v3)* plots at the V2 stage to eliminate non-transformed plants. Experimental treatments included commercial formulations of quizalofop applied at 70 and 140 g ae/ha, 2,4-D (dimethylamine salt) at 560 and 1120 g ae/ha, and an untreated control. Treatments were applied using backpack broadcast boom equipment delivering 187 L/ha carrier volume at 130-200 kpa pressure. Quizalofop treatments were applied at the V3-V4 corn stage and 2,4-D treatments were applied at the V5-V6 stage.

[0331] Quizalofop treated plots were visually assessed for crop injury at one and three weeks after application (WAA) using a 0-100% scale, where 0 equals no injury and 100 equals complete death. 2,4-D treated plots were visually assessed for plant leaning at 2 days after application (DAA) using 0-100% scale where 0 equals no leaning from any plant and 100 equals all plants prone. Additionally, 2,4-D plots were visually assessed at 3-4 WAA for brace root deformation using a 0-10 scale.

[0332] 8.2.1- Results.

[0333] *AAD-1 (v3)* event response to the highest rates tested of quizalofop and 2,4-D are shown in **Table 24.** These rates represent approximately twice the normal commercial use rates. Non-transformed hybrids were severely injured (80-100%) by quizalofop at 70 g ae/ha including the sethoxydim resistant line, although it displayed slightly better tolerance than the other two hybrids. All *AAD-1 (v3)* events except one lineage of event 3404.001 displayed excellent tolerance to quizalofop at 70 g ae/ha. No visible symptoms were observed on the *AAD-1 (v3)* events except with the events noted above.

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Table 24.

Treatment (rate) = Quizalofop = (140 g ae/ha)			2,4-D (1120 g ae/ha)		2,4-D amine (1120 g ae/ha)		Analysis Results			Resistance Segregation Ratios following Liberty Spray		
Evaluation % Injury 3 WAA (0-100 scale)			% Leaning 2 DAA (0-100 scale)		Braceroot Deformation 3-4 WAA (0-10 scale)		AAD-1 copy number leaf (Southern analysis)	AAD-1 Leaf Western Blot To	AAD-1 Leaf ELISA To	T_1 Population Average		T_2 Population Average
Event or Hybrid	IN	HI	IN	HI	IN	HI				IN	HI	HI
3404.001	25	25	0	5	1	2	3	+++	+++	40%	47%	X
3404.006	0	0	0	0	0	0	1	++	+++	33%	26%	X
3404.013	0	0	0	0	0	0	1	+	+++	63%	58%	X
3404.017	0	0	1	0	0	0	2	+/-	++	48%	47%	X
3404.020	0	0	0	0	0	0	2	++	+++	50%	51%	X
3404.022	0	0	1	0	0	0	1	+	++	51%	57%	76%*
3404.025	0	0	0	0	0	0	2	+++	++++	55%	59%	X
3404.027	0	0	3	0	0	0	5	+	++	51 %	50%	X
3404.031	0	0	1	0	0	0	1	++	++++	47%	43%	61%*
3404.033	0	0	0	0	0	0	2 or 3	+	++	52%	49%	X
3404.036	0	0	0	0	0	0	4	++	+++	52%	48%	X
3404.044	0	0	1	0	0	1	1	+	+/-	50%	48%	X
3404.050	0	0	0	1	0	1	2	nd	nd	38%	28%	X
3404.053	0	0	1	0	0	0	2	+++	+++	48%	56%	X
3404.074	0	0	0	0	0	0	1	++	+++	53%	52%	73%*
3404.082	0	0	0	0	0	1	3	nd	nd	38%	36%	X
DK493 HI-II X	100	100	20	23	8	9						
5XH571 CROPLAN	100	100	13	34	7	9						
585SR	80	96	11	33	9	9						

* Fits single locus dominant trait segregation as determined by chi square analysis (P>0.05)

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[0334] 2,4-D at the 1120 g ae/ha rate caused significant levels (11-33%) of epinastic leaning in the non-transformed hybrids, a normal response when applied beyond the V4 growth stage. Little or no leaning was observed with all *AAD-1 (v3)* events except one lineage of 3404.001 (Indiana location only) where moderate levels (5-13%) of leaning occurred.

[0335] Brace roots of non-transformed hybrids were severely deformed (rating of 9 on a 0-10 scale) by 2,4-D at the 1120 g ae/ha rate. Again, this is a normal response to 2,4-D applied beyond the V4 growth stage. As with the leaning response, little or no brace root injury was observed with all *AAD-1 (v3)* events except one lineage of 3404.001.

[0336] Similar trends occurred with lower tested rates of quizalofop and 2,4-D although at reduced but still significant response levels in the non-transformed hybrids (data not shown).

[0337] These results indicate that most *AAD-1 (v3)* transformed event lines displayed a high level of resistance to quizalofop and 2,4-D at rates that were lethal or caused severe epinastic malformations to non-transformed corn hybrids. *See also* **Figure 16.**

[0338] 8.2.2- Expected Mendelian segregation ratios on three T_2 events. Plants from individual lineages of each event were randomly self-pollinated in the field. T_2 seed were hand harvested at physiological maturity. Based on single gene copy number (see **Table 24** above) and overall performance in T_1 generation (segregation, herbicide tolerance, and vigor), three events (022, 031, and 074) were chosen for further evaluation in the field. Breeding rows of each event were planted using a precision cone planter each consisting of 2500-3000 seeds. At the V2 growth stage, all *AAD-1 (v3)* lines were sprayed with 140 g ae/ha quizalofop (Assure® II) using a backpack sprayer as previously described. This rate rapidly killed all "null" (untransformed) segregants. Each event had a segregation ratio consistent with Mendelian inheritance of a single locus, dominant gene (3 resistant:1 sensitive, or 75% survival) (see **Table 24**). Homozygotes and hemizygotes from event 74 were identified by zygosity testing (refer to *AAD-1 (v3)* Invader assay description for corn). Hemizygous plants were removed and homozygous *AAD-1 (v3)* plants were crossed with BE1146 corn inbred introgressed and homozygous for glyphosate resistance trait, NK603, creating a homogeneous F_1 hybrid seed that is hemizygous for glyphosate resistance, *AAD-1 (v3)*, and glufosinate resistance.

[0339] 8.3 - Stacking of *AAD-1 (v3)* and *PAT* with glyphosate resistance genes in corn.

[0340] Homozygous T_2 *AAD-1 (v3)*/*PAT* corn plants were crossed with glyphosate resistant corn plants producing F_1 seed containing *AAD-1 (v3), PAT,* and glyphosate resistance genes as described in the previous example.

[0341] F_1 seeds were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium (Sun Gro Horticulture). The pots were initially subirrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were subirrigated with deionized water.

[0342] Plants were allowed to grow until 2-4 leaves had emerged from the whorl. At' this point herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. The plants were sprayed with rates of 2,4-D DMA, glyphosate, glufosinate, and various combinations of the three. All applications were formulated in 200 mM Hepes buffer (pH 7.5). In spray applications where glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

[0343] At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Results of the study at 14 DAT can be seen in **Table 25.**

Table 25.

	Field Rate	% Injury at 14 DAT	
		Hi II X 5XH751	RR/PAT/AAD1
		Ave	Ave
Untreated control	-	0	0
840 g ae/ha glyphosate	1X	98	0
1680 g ae/ha glyphosate	2X	100	0
3360 g ae/ha glyphosate	4X	100	0
560 g ae/ha 2,4-D DMA	1X	10	0
1120 g ae/ha 2,4-D DMA	2X	14	0
2240 g ae/ha 2,4-D DMA	4X	29	0
470 g ae/ha glufosinate	1X	80	0
940 g ae/ha glufosinate	2X	90	3

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(continued)

Table 25.

	Field Rate	% Injury at 14 DAT	
		Hi II X 5XH751	RR/PAT/AAD1
		Ave	Ave
1880 g ae/ha glufosinate	4X	96	15
840 g ae/ha glyphosate + 560 g ae/ha 2,4-D DMA	1X + 1X	96	1
1680 g ae/ha glyphosate +1120 g ae/ha 2,4-D DMA	2X + 2X	100	2
3360 g ae/ha glyphosate + 2240 g ae/ha 2,4-D DMA	4X + 4X	100	1
470 g ae/ha glufosinate + 560 g ae/ha 2,4-D DMA	1X + 1X	89	5
940 g ae/ha glufosinate + 1120 g ae/ha 2,4-D DMA	2X + 2X	91	10
1880 g ae/ha glufosinate + 2240 g ae/ha 2,4-D DMA	4X+4X	97	13
840 g ae/ha glyphosate + 470 g ae/ha glufosinate	1X + 1X	90	5
1680 g ae/ha glyphosate + 940 g ae/ha glufosinate	2X+2X	98	15
3360 g ae/ha glyphosate + 1880 g ae/ha glufosinate	4X + 4X	100	15

[0344] This study demonstrated that the *AAD-1 (v3)* gene in corn can be stacked with a glyphosate resistance gene and a glufosinate resistance gene to provide robust field-level tolerance to 2,4-D, glyphosate, and glufosinate alone or in tank mix combinations.

[0345] 8.3.1 - Resistance of *AAD-1 (v3)* corn using a tank mix of 2,4-D DMA and quizalofop. T_2BC_1 seeds of hemizygous event number 3404-025.001R/R001 Bulked.001.5058 were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium. The pots were initially sub-irrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were sub-irrigated with deionized water.

[0346] Plants were allowed to grow until V1 stage in the greenhouse. At this point the plants were selected with 560 g ae/ha Assure® II with the addition of 1% Agridex crop oil concentrate in 200 mM Hepes buffer with the research track sprayer set at 187 L/ha. Plants were allowed 4 days to show symptoms of the selection. All plants were uninjured. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 18-in spray height. All applications were formulated in 200 mM Hepes buffer (pH 7.5) with the addition of 1% v/v Agridex.

At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Plants from this particular lineage had 0% injury at 14 DAT for all tank mixed combinations, while the wild-type had 100% injury. These results indicate AAD-1 (v3) not only provides robust field level resistance to 2,4-D and quizalofop individually, but also to exaggerated rates of multiple combinations of the two chemistries. One could logically expect to implement novel weed control measures with combinations of phenoxy auxins and AOPP graminicides in corn (or other crops transformed with AAD-1) not previously enabled by a single gene HTC.

[0347] 8.3.2 - Tolerance of (pDAB3403) T_0 corn plants to Quizalofop herbicide. A target of approximately eight To plant clones from each of 17 events were regenerated and transferred to the greenhouse for preliminary tolerance screening with postemergence-applied discriminating rate of quizalofop herbicide applied by track sprayer at 35 g ae/ha (1X field rate, 4X lethal dose) to 3-leaf, greenhouse-adapted To corn plants using track sprayer conditions previously described. Plants were rated as Resistant or Sensitive 7 days after treatment. Control, non-transgenic corn was included with each spray application. Two events, Event 014 and 047, had two or more To clones sensitive to 35 g ae/ha quizalofop, indicating an unexpected level of sensitivity for this event. The 15 other events showed stable integration, protein expression, and the ability to tolerate a 4X lethal dose of quizalofop at the whole plant level.

[0348] 8.3.3 Expression of AAD-1 (v3) with respect to quizalofop tolerance.

[0349] Three different T2 lineages from 3404 transformations that were pre-screened with Liberty® (as described previously) to remove nulls were chosen to compare their tolerance to quizalofop with respect to their AAD-1 (v3) expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT (see **Figure 15.**). The highest tolerance line, event 3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1X and higher field rates. This data concludes that corn expressing AAD-1 (v3) can be protected from quizalofop injury at the highest level tested (2,240 g/ha), which is 16 times the 1X field dose of 35 g/ha. In addition, the expression level was consistent

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throughout the period of the experiment.

Examples 9 - *Agrobacterium*-Mediated Transformation of Maize with *AAD-1 (v3)*

[0350] 9.1 - Plant material.

[0351] Seeds of a "High II" (*i.e.,* Parent A and B) F_1 cross (Armstrong *et al.,* 1991) are planted directly into 5 gallon-pots containing 95:5 Metro-Mix® 360: Mineral soil. The plants are grown in the greenhouse with a 16 hour photoperiod supplemented by a combination of high pressure sodium and metal halide lamps.

[0352] 9.2 - Tissue source.

[0353] For obtaining immature Hi-II (F2) embryos, controlled sib-pollinations were performed. On the day of pollination, actively shedding tassels are bagged, and fresh pollen is collected and applied carefully onto the silks. Immature embryos were isolated as described in Example 7.2.

[0354] 9.3 - Preparation of a superbinary vector.

[0355] Construction of an *Agrobacterium* construct, pDAB2272, containing the *AAD-1 (v3)* gene in combination with the AHAS selectable marker gene was accomplished by isolating the 3443 base pair NotI fragment from pDAB3404 containing ZmUbi1 v2/ *AAD-1* (v3)/ZmPer5 v2 and inserting it into the NotI site of pDAB8549. The resulting plasmid contains the ZmUbi1 v2/ *AAD-1* (v3)/ ZmPer5 v2 and the OsActl v2/AHAS v3/ZmLip v1 cassettes flanked by non-identical MAR regions in the direct orientation. This was subsequently transformed into LBA4404/pSB1 to create the superbinary vector, which was named pDAB3602 but was also referred to as pDAS1421.

[0356] 9.4 - Bacterial supply.

[0357] All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Patent No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~$1x10^9$ cfu/ml) and 100 μM actetosyringone added to the solution.

[0358] 9.5 - Infection and cocultivation.

[0359] The immature embryos are isolated directly into a microfuge tube containing 2 ml LS-inf. Mod liquid medium. Each tube, containing ~100 embryos, is vortexed for 3-5 sec. The medium is removed and replaced with fresh liquid medium and the vortex is repeated. The liquid medium is again removed and this time replaced with an *Agrobacterium* solution at the Klett 200 concentration. The *Agrobacterium* and embryo mixture is vortexed for 30 sec. Following a 5 minute incubation at room temperature, the embryos were transferred to LS-As Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 30.0 g/L sucrose, 6 mM L-proline, 0.85 mg/L $AgNO_3$,1, 100 μM actetosyringone, 3.0 g/L Gelrite, pH 5.8) for a 5-day co-cultivation at 25° C.

[0360] 9.6 - Dose response using immature embryos.

[0361] Dose response studies were initiated using immature embryos treated with *Agrobacterium* strain LBA4404 lacking a plasmid as described previously: Once treated, embryos were allowed to co-cultivate for 5 days at 25° C and were then transferred to selection media containing various levels of R-haloxyfop or R-cyhalofop. Embryos were also transferred to media containing 1 mg/L bialaphos and 100 nM imazethapyr as negative controls. Embryos were scored for % embryogenic callus formation after 2 weeks, and then again after 4 weeks. Embryos were tested on R-haloxyfop levels up to 30 nM; however, insufficient reduction of callus formation was seen at the highest levels, so higher concentrations (50 - 100 nM) were used during transformation experiments. Data from embryos grown on cyhalofop-containing media is shown in **Figure 17**.

9.7 - Selection.

[0362] After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the *AHAS* gene is used, based on U.S. Patent No. 5,731,180. Tissue was transferred at biweekly intervals on the same medium until embryogenic colonies were obtained. These colonies were maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies were bulked up by transferring to fresh selection

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medium at 2-week intervals for regeneration and further analysis.

[0363] 9.8. - Regeneration and seed production.

[0364] For regeneration, the cultures are transferred to 28 "induction" medium and 36 "regeneration" medium as described previously containing either 100 nM R-haloxyfop or 1.5 μM cyhalofop for differentiation of plantlets. When plantlets were established, they were transferred to SHGA tubes to allow for further growth and development of the shoot and roots as described previously. Controlled pollinations for seed production were conducted as described previously.

[0365] 9.9 - Event recovery and analysis particulars; whole plant screening of T_0 corn lineages containing *AAD-1 (v3)* and *AHAS* (pDAS 1421).

[0366] Seventy-two Agrobacterium-transformed events were selected on various levels of R-haloxyfop acid and R-cyhalofop acid *in vitro*. Twenty-two callus samples were analyzed by Southern blot analysis for stable integration of *AAD-1 (v3)* into the genome as described previously. Ten single copy events, as indicated in **Table 26,** were chosen to be regenerated.

Table 26.

Corn event	*In vitro* Selection agent	Southern Copy # (callus)	Western Blot (AAD-1) To Callus	Assessment of To lineages resistant to 35 g ae/ha quizalofop	
	(nM)	AAD-1		Resistant	Sensitive
1421 [21]-016	50 Haloxyfop	1	+	8	0
1421 [22]-020	100 Haloxyfop	1	++	8	0
1421[22]-022	100 Haloxyfop	1	+	8	0
1421[22]-023	100 Haloxyfop	1	++	8	0
1421[3]-036	100 Haloxyfop	1	++	8	0
1421[4]-031	300 Cyhalofop	1	++	9	0
1421[4]-032	300 Cyhalofop	1	++	8	0
1421[4]-033	300 Cyhalofop	1	++	12	0
1421[4]-034	300 Cyhalofop	1	++	8	0
1421[4]-035	300 Cyhalofop	1	++	6	0
Events with more than 1 copy were not taken to the greenhouse					

[0367] A minimum six regenerated clonal lineages per event were moved to soil in the greenhouse and screened using a track sprayer as previously described to apply 35 g ae/ha quizalofop when 2-4 new, normal leaves had emerged (see section 8.3.3). Presence of AAD-1 protein on a Western blot correlated perfectly with herbicide resistance in the To generation regardless of which AOPP herbicide was used for selection. There is no negative impact of the second HTC gene (*AHAS*) on the function of the *AAD-1 (v3)*.

Example 10 - Purification of AAD-1 (v1) for Antibody Creation and Biochemical Characterization

[0368] All operations during purification were carried out at 4° C. Frozen or fresh *E. coli* cells from approximately 1 L culture, grown and induced as in Example 3, were re-suspended in 200 ml of extraction buffer containing 20 mM Tris-HCl, 1 mM EDTA, and 2 ml of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The soluble extract was obtained by centrifugation in a GSA rotor (Sorvall) at 12,000 rpm (24,000g) for 20 minutes. The supernatant was then loaded onto a Mono Q ion exchange column (Pharmacia HR 10/10) equilibrated with 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, and the column was washed with same buffer for 10 CV (80 ml). The protein was eluted with 80 ml of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 ml fractions were collected. The fractions containing AAD-1 (v1) were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl, 0.15 M NaCl, and 1 mM DTT, pH 8.0 at a flow rate of 1 ml/min. Purification procedures were analyzed by SDS-PAGE, and protein concentration was determined by Bradford assay using bovine serum albumin as standard.

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Example 11 - Recombinant AAD 1 Purification and Antibody Production

[0369] Plasmid pDAB3203 containing the *AAD-1 (v1)* gene was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000x g for 20 min to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

[0370] All AAD-1 (v1) purifications were conducted at 4° C as discussed in Example 10, unless otherwise stated. The cell lysate was loaded onto a Mono Q ion exchange column (Pharmacia Cat. #HR 10/10) equilibrated with 20 mM Tris-HCl (pH 8.0) 1 mM EDTA, followed by 80 mL of washing with the same buffer. The proteins were eluted with 80 mL of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 mL fractions were collected. The fractions containing AAD-1 were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl and 1 mM DTT. Protein concentration was determined by Bradford assay using bovine serum albumin as standard.

[0371] Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

[0372] 11.1 - Extracting AAD-1 (v3) from plant leaves.

[0373] Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 μL plant extraction buffer (PBS containing 0.1 % Triton X-100 and 10 mM DTT). The tubes were shake for 4 min with a bead beater at maximum speed followed by centrifugation for 10 min at 5,000x g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

[0374] 11.2 - Bradford assay.

[0375] Total soluble protein concentration from plant leaf tissues were determined by Bradford assay using bovine serum albumin (BSA) as standard. Five micro-liter of serially diluted BSA in PBS or plant extract was transferred to 96-well microtiter plate in triplicates. For standards, concentrations were ranged from 2000 to 15.6 μg/mL. The protein assay concentrate was first diluted 5 fold in PBS and 250 μL was added to each well and incubated at room temp for 5 min. Each optical density (OD) was measured at 595 nm using a microplate reader. The protein concentration of each sample was extrapolated from standard curve using the Softmax® Pro (ver. 4.0) (Molecular Devices).

[0376] 11.3 - Enzyme linked immuno-sorbent assay (ELISA).

[0377] The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-1 antibody (0.5 μg/mL) was coated on 96-well microtiter well and incubated at 4° C for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 μL standard AAD-1 of known concentrations or plant extract (see previous section) was incubated in the wells. For standard curve,-purified AAD-1 concentrations ranged from 100 to 1.6 ng/mL in triplicates. Plant extracts were diluted 5, 10, 20, and 40 fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-1 antibody-HRP conjugate (0.25 ug/mL) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 μL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-1 in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

[0378] For comparison, each sample was normalized with its TSP concentration and percent expression to TSP was calculated.

[0379] 11.4 - Western blotting analysis.

[0380] Plant extracts or AAD-1 standards (5 and 0.5 μg/mL) were incubated with Laemmli sample buffer at 95° C for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-1 protein was detected

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by anti-AAD1 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham cat. #RPN 21058).

Example 12 - Tobacco Transformation

[0381] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch *et al.,* 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB721, *AAD-1 (v3) + PAT),* grown overnight in a flask on a shaker set at 250 rpm at 28° C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

[0382] Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$).

[0383] Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The *PAT* gene which was molecularly linked to *AAD-1 (v3)* was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

[0384] The DNA samples were diluted to 9 ng/μl, then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgC12 (Third Wave Technologies). An aliquot of 7.5 μl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 μl of controls, standards, and 20 ng/μl diluted unknown samples. Each well was overlaid with 15 μl of mineral oil (Sigma). The plates were then incubated at 63° C for 1.5 hours and read on the florometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events (**Table 27**).

Table 27. Tobacco T0 events transformed with pDAB721 *(AAD-1(v3) + PAT).*

Event	PAT copy number (Southern)	Coding Region PCR for AAD-1	ELISA (μg AAD-1 / ml plant extract)	Relative tolerance T0 spray with 2,4-D *
721(1)1	1	+	0.9	Medium
721(2)1	nd	nd	0.6	Medium
721(2)2	5	+	0.3	Low
721(2)3	3	+	2.6	Medium
721(2)5	5	+	4.1	Variable

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(continued)

Table 27. Tobacco T0 events transformed with pDAB721 *(AAD-1(v3) + PAT).*

Event	PAT copy number (Southern)	Coding Region PCR for AAD-1	ELISA (μg AAD-1 / ml plant extract)	Relative tolerance T0 spray with 2,4-D *
721(2)6	3	+	0.5	Variable
721(2)8	5	+	0.3	High
721(2)11	3	+	n/a	High
721(2)12	3	+	4.1	Medium
721(2)13	2	+	0.5	Medium
721(2)14	5	+	0.2	High
721(2)16	4	+	.3.2	Medium
721(2)17	3	+	nd	High
721(2)18	5	+	nd	High
721(2)19	>10	+	nd	Low
721(2)20	5	+	nd	Medium
721(2)21	4	+	nd	High
721(2)22	7	+	nd	Medium
721(2)23	>10	+	nd	Variable
721(3)003	3	+	nd	Variable
721(3)008	2	+	nd	High
721(3)012	1	+	nd	High
721(3)4	2	+	0.5	High
721(3)5	9	+	3.3	High
721(3)6	4	+	7.1	Variable
721(3)9	2	+	1	Low
721(3)10	3	+	0.6	High
721(3)11	7	+	6	Low
721(3)13	4	+	0.1	High
721(3)014	2	+	0.1	Medium

nd = not done

Legend:	Relative tolerance*	Injury at 3200 g ae/ha 2,4-D (14DAT)
	Low	>50% injury
	Medium	20-50% injury
	High	<20% injury
	Variable	inconsistent

[0385] Copy number estimations were confirmed by Southern Analysis on several events. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 μgs of DNA was subjected to an overnight digest of NsiI and also HindIII for pDAB721 to obtain integration data. After the overnight digestion an aliquot of ~100 ngs was run on a 1% gel to ensure complete digestion. After this assurance the samples were processed using same protocol as in Example 6 section 11.

[0386] All events were also assayed for the presence of the *AAD-1 (v3)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the Coding Region PCR AAD-1 were (RdpAcodF ATGGCTCA

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TGCTGCCCTCAGCC) (SEQ ID NO:27) and (RdpAcodR CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 30 PCR positive events were regenerated and moved to the greenhouse.

[0387] A representative plant from each of 19 events was assayed for AAD-1 (v3) expression by ELISA methods previously described. All events assayed showed detectable levels of AAD-1 (v3) (**Table 27**). Protein expression varied across events.

[0388] T_0 plants from each of the 30 events were challenged with a wide range of 2,4-D sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 50, 200, 800, or 3200 g ae/ha to representative clones from each event mixed in deionized water. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 800 g ae/ha or less. Some events were uninjured at this rate (equivalent to 1.5X field rate). All events suffered some level temporary auxinic damage 3 DAT when treated with 3200 g ae/ha. Some leaf burning also occurred at this high rate due to the acidity of the spray solution. Future trials at high 2,4-D rates were buffered. Response of T_0 plants treated with 3200 g ae/ha 2,4-D (~6X field rate) was used to discern relative tolerance of each event into "low" (>50% injury 14 DAT), "medium" (20-50% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (**Table 27**).

[0389] Verification of high 2,4-D tolerance.

[0390] Two to four T_0 individuals surviving high rates of 2,4-D were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. Two *AAD-1 (v3)* tobacco lines (event 721(2)-013.010 and 721(3)-008.005) were chosen from the T_0 generation. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4), followed by selective removal of untransformed nulls in this segregating population with 280 g ai/ha glufosinate (PAT selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided medium and high levels of robustness to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY 160 tobacco. All plants were sprayed with the use of a track sprayer set at 187L/ha. The plants were sprayed from a range of 70-4480 g ae/ha 2,4-D dimethylamine salt (DMA), R-Dichlorprop, and a 50/50 mix of the two herbicides. All applications were formulated in 200mM Hepes buffer (pH 7.5). Each treatment was replicated 4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity. (**Table 28**). No injury was observed at rates below 1X field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by *AAD-1 (v3)*, even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

Table 28. Segregating AAD-1 T_1 tobacco plants' response to phenoxy auxin herbicides.

	KY160 - Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
Herbicide	**Average % Injury of Replicates 14 DAT**		
560 g ae/ha 2,4-D DMA	75	5	0
1120 g ae/ha 2,4-D DMA	80	5	2
2240 g ae/ha 2,4-D DMA	90	5	0
4480 g ae/ha 2,4-D DMA	95	5	5
560 g ae/ha R-dichlorprop	70	5	0
1120 g ae/ha R-dichlorprop	75	5	0
2240 g ae/ha R-dichlorprop	88	5	0
4480 g ae/ha R-dichlorprop	95	10	5
560 g ae/ha 2,4-D DMA/R-dichlorprop	80	5	5

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(continued)

Table 28. Segregating AAD-1 T_1 tobacco plants' response to phenoxy auxin herbicides.			
	KY160 - Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
Herbicide	Average % Injury of Replicates 14 DAT		
1120 g ae/ha 2,4-D DMA/R-dichlorprop	80	10	10
2240 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15
4480 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15

[0391] A 100 plant progeny test was also conducted on each of the two *AAD-1 (v3)* lines (events 721(2)-013.010 and 721(3)-008.005). The seeds were stratified, sown, and transplanted with respect to the procedure above except null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Both event '013' and '008' segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-1 is heritable as a robust phenoxy auxin resistance gene in multiple species.

[0392] Field level tolerance will be demonstrated by planting T_1 or T_2 seed in the greenhouse, selectively removing the null plants by Liberty selection as previously described, and rearing individual seedlings in 72-well transplant flats (Hummert International) in Metro 360 media according to growing conditions indicated above. Individual plants will be transplanted into the field plots using an industrial vegetable planter. Drip or overhead irrigation will be used to keep plants growing vigorously. Once plants reach 6-12 inches in height, tobacco plants will be sprayed with a broad range of phenoxy auxins and rated as shown above. Environmental stresses are more significant under field conditions; however, based on previous experience with *AAD-1 (v3)*-transformed corn, robust translation of resistance from the greenhouse to the field is expected.

Example 13 - Soybean Transformation

[0393] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.,* 1995), amino acid modification (Falco *et al.,* 1995), and insect resistance (Parrott *et al.,* 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.,* 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonaryexplants (Hinchee *et al.,* 1988) or zygotic embryos (Chee *et al.,* 1989) have been reported.

[0394] Transformants derived from *Agrobacterium-mediated* transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee *et al.,* 1989; McCabe *et al.,* 1988), cotyledon (Hinchee *et al.,* 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.,* 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-1 (v3)* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-1 (v3).*

[0395] 13.1 - Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens.*

[0396] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.,* 1988) and shoot multiplication from apical meristems (McCabe *et al.,* 1988). In the *A. tumefaciens-based* cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.,* 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the

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infrequent recovery of chimeric plants (Clemente *et al.,* 2000; Olhoft *et al.,* 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.,* 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.,* 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium-based* cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee *et al.,* 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.,* 1999; Zeng *et al.,* 2004).

[0397] 13.1.1 - *Agrobacterium* preparation. The plasmid, pDAB721, contains the *AAD-1 (v3)* gene under the control of the *Arabidopsis* Ubi10 promoter. This plasmid also carries the *PAT* gene under the control of rice actin promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. This vector can be used in the transformation experiment described below. The construct pDAB721 was mobilized into the *Agrobacterium* strain ERA101S by electroporation.

[0398] *Agrobacterium* cultures harboring pDAB721 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

[0399] 13.1.2 - Plant transformation. Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be rinsed five times with sterile water on hormone-free SHGA medium and allowed to germinate for 5 days at 24° C, with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg *et al.* (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100 x 25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee *et al.,* 1988).

[0400] Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* resuspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES(2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 μM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

[0401] Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin , 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

[0402] Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

[0403] The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

[0404] 13.1.3 - Progeny evaluation. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1 (v3)* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for

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glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn. Determination of the inheritance of *AAD-1 (v3)* will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

[0405] 13.2 - Transformation Method 2: "No-shake" *Agrobacterium* mediated transformation of non-regenerable soybean suspension cells.

[0406] The DAS Soybean cell suspensions were cultured on a 3d cycle with 10 ml of settled suspension volume transferred to 115 ml of fresh liquid medium. Settled cell volume was determined by allowing the cell suspension to settle for 2 min in the 125-mL flask after vigorous swirling and then drawing cells from the bottom of the flask with a wide bore 10 ml pipette. The flasks were.then transferred to orbital shakers at 140 rpm.

[0407] Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 μM acetosyringone (AS) onto a 100x25 sterile Petri plate. EHA105 *Agrobacterium* suspension at a density of 1.2 OD^{650} in a 100 μL volume was added and mixed well. The *Agrobacterium* and suspension cell mixture was swirled well and the plate was transferred to dark growth chamber where the temperature was maintained at 25° C.

[0408] 13.2.1 - Selection of soybean suspension cells and isolation of transformed colonies. After 4 days of co-cultivation the plate was swirled again to mix the suspension well and a 1.5 ml aliquot was transferred to the selection medium and spread on the gel medium on a 100x15 ml Petri-plate. The selection medium consisted of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose and the medium was amended with glufosinate ammonium at 5 mg/L level. After a 10 min drying in the hood the plates were incubated for 4 weeks in dark at 28° C. Colonies appeared in selection and a total of 11 colonies were transferred to fresh medium from 3 different experiments and maintained for 3-4 months. All the 11 resistant colonies produced calli that were growing on the 5 mg/L glufosinate containing selection medium. The non-transformed suspension cells were sensitive when plated on to 0.5 mg/l glufosinate ammonium medium. However, the transformed colonies were resistant to 5x concentration of glufosinate ammonium and were maintained for up to 4 months.

[0409] Callus events were sampled for analyses when they reached 2 to 3 cm in diameter. At least two of colony isolates, one each from two different experiments, were analyzed for AAD1 protein expression. The ELISA and Western analysis carried out on these two isolates showed positive expression of AAD1 proteins. Both ELISA (**Table 29**) and Western Blotting **(Figure 18**) analysis on two separate soybean calli transformed with *AAD-1 (v3)* gene indicated that the callus cells are expressing AAD-1 (v3) protein. The sandwich ELISA detected 0.0318% and 0.0102% total soluble protein of AAD-1 (v3) in two different callus tissue samples. Due to the sensitivity and cross reactivity of the antiserum, multiple bands were observed in the western blot. However, AAD-1 (v3) specific band was observed in both callus samples but not in the wild type (negative) tissue. Coding region PCR analyses showed the expected size products of the AAD1 and the PAT coding regions in these colonies indicating that they were transformed.

Table 29. PCR and ELISA data for transgenic soybean events.

Event	AND-1 Coding region PCR	PAT Coding region PCR	T SP (μg/mL)	AND-1 (ng/mL)	% Expression
1-1	+	+	1995.13	633.89	0.0318%
2-1	+	+	2018.91	205.92	0.0102%
Negative Control	-	-	2074.63	-1.22	-0.0001%

[0410] 13.3 - Transformation Method 3: aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[0411] Culture of embryogenic soybean callus tissue and subsequent beaming were as described in U.S. Patent No. 6,809,232 (Held et al.).

[0412] Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144 and 96E692, were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3295 using linearized DNA at a concentration of approximately 0.2 μg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos. With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light.

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Embryos that had elongated and produced roots were transferred to tubes containing 1/2 B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

[0413] Variations of media referred to in **Table 30** were tested, *e.g.,* B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/l myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/l myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and 1/2 B5G which contained all ingredients of B5G medium at half strength.

Table 30. Growth Media for Soybean

	Ingredients in 1 Liter		
	B1-30	**B3**	**B5G**
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		
Activated charcoal		5 g	
Phytagar	8g	8g	
Gelrite			2g
pH	5.8	5.8	5.8

Example 14 - *AAD- 1 (v3)* Enablement in Cotton

[0414] 14.1 - Cotton transformation protocol.

[0415] Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (**Table 31**) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 μE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[0416] Cotyledon segments (~5mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (**Table 31**) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (**Table 31**) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (**Table 31**). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[0417] *Agrobacterium* preparation. Inoculate 35ml of Y media (**Table 31**) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C, while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (**Table 31**) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per mL with a total volume of 40 ml.

[0418] After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. Callus is then transferred to CG-media (**Table 31**), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (**Table 31**) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

[0419] Larger, well-developed embryos are isolated and transferred to DK media (**Table 31**) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

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Table 31. Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1 ml	1 ml	1 ml				
agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

[0420] 14.2 - Experiment specifics.

[0421] For this experiment 500 cotyledon segments were treated with pDAB721. Of the 500 segments treated, 475 had callus isolated while on selection (95% transformation frequency). The callus was selected on glufosinate-ammonium, due to the inclusion of the *PAT* gene in the construct, since there was already a selection scheme developed. Callus line analysis in the form of PCR and Invader were initiated to determine the insertion patterns and to be sure the gene was present at the callus stage, then callus lines that were embryogenic were sent for Western analysis.

[0422] 14.3 - Callus analysis results.

[0423] The object of the analysis is to eliminate any lines that do not have the complete PTU, show no expression or that have high copy number, so those lines are not regenerated. Of the 475 pDAB721 transformed callus lines, 306 were sent for PCR analysis and Invader assay (**Table 32**). Very few lines were PCR negative. The Invader results are not complete at this time, because some samples had low DNA amounts when extracted, and these have been resubmitted. However, the current Invader data shows a few of the lines submitted have high copy number (copy number of >2) (**Table 32**). Due to the large number of lines that passed analysis, it was necessary to decrease the number of embryogenic callus lines being maintained due to the volume. Ninety lines have been sent for Western analysis, and eight of those were negative. The western analysis showed high expression from the majority of the lines (**Table 32**). Eighty-two embryogenic callus lines are being maintained for plant regeneration based on analysis results (and results pending).

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Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western	Line	Copy #	PTU	Western
1	2	pos	***	43	1	pos	*****
2	1	pos	***	44	low DNA	pos	*****
3	1	pos	***	45	1	pos	*****
4	2	pos	***	46	2	pos	*****
5	1	pos	***	47	1	pos	***
6	1	pos	neg	48	1	pos	***
7	1	pos	****	49	3	faint pos	neg
8	1	pos	*****	50	1	pos	****
9	2	pos	*	51	4	pos	neg
10	1	pos	*	52	2	pos	****
11	1	pos	*****	53	1	pos	***
12	1	pos	*	54	1	pos	****
13	1	pos	*	55	1	pos	neg
14	1	pos	**	56	5	pos	*
15	1	pos	****	57	2	faint pos	neg
16	1	pos	*****	58		pos	****
17	1	pos	*	59	2	pos	****
18	1	pos	***	60	5	pos	*****
19	1	pos	**	61	1	pos	**
20	1	pos	*****	62	1	pos	**
21	2	pos	*****	63	1	pos	***
22	1	pos	*****	64	1	pos	***
23	1	pos	*****	65	3	pos	****
24	4	pos	* or neg	66	5	pos	*
25	1	pos	****	67	6	pos	*
26	1	pos	****	68	Low DNA	pos	neg
27	low DNA	pos	*****	69	Low DNA	pos	****
28	low DNA	pos	**	70	low DNA	pos	**
29	low DNA	pos	*****	71	low DNA	faint pos	**
30	17	pos	*	72	low DNA	pos	****
31	low DNA	pos	*****	73	low DNA	neg	***
32	low DNA	pos	*****	74	low DNA	faint pos	***
33	low DNA	pos	****	75	Low DNA	pos	***
34	low DNA	faint pos	*****	76	Low DNA	pos	neg
35	low DNA	pos	****	77	low DNA	faint pos	****
36	low DNA	pos	neg	78	low DNA	pos	****
37	low DNA	pos	****	79	1	pos	**
38	low DNA	neg	*****	80	low DNA	pos	***

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(continued)

Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western	Line	Copy #	PTU	Western
39	1	pos	****	81	low DNA	pos	***
40	low DNA	pos	*	82	low DNA	pos	***
41	low DNA	pos	*	83	low DNA	neg	****
42	low DNA	pos	**	84	low DNA	pos	***
				85	low DNA	pos	**
standard	AAD1	5ug/ml	*****	86	low DNA	pos	*
standard	AAD1	0.5ug/ml	**				

[0424] 14.4 - Plant regeneration.

[0425] Two *AAD-1* (v3) cotton lines have produced plants according to the above protocol that have been sent to the greenhouse. To demonstrate the *AAD-1 (v3)* gene provides resistance to 2,4-D in cotton, both the *AAD-1 (v3)* cotton plant and wild-type cotton plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed at 560 g ae/ha 2,4-DMA formulated in 200 mM Hepes buffer (pH 7.5). The plants were evaluated at 3, 7, and 14 days after treatment. Plants were assigned injury ratings with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Three days after treatment (DAT) the wild-type plant began showing epinasy and received a rating of 15%; in contrast, the *AAD-1 (v3)* plant showed 0% injury. By 7 DAT epinasy continued on the wild-type and the new growth shoots began turning brown. It received a rating of 50% at this time. At 14 DAT the *AAD-1 (v3)* plant was still uninjured, whereas the wild-type was severely stunted, and the new growth areas were brown and shriveled. Thus, the wild-type received a rating of 90% at 14 DAT.

[0426] This study demonstrates that the *AAD-1 (v3)* gene in cotton provides substantial tolerance to 2,4-D up to at least 560 g ae/ha.

Example *15 - Agrobacterium* Transformation of Other Crops

[0427] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, *see, e.g.,* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.,* Hinchee *et al.,* 1988. For *Agrobacterium*-mediated transformation of sorghum, *see, e.g.,* Zhao *et al.,* 2000. For *Agrobacterium*-mediated transformation of barley, *see, e.g.,* Tingay *et al.,* 1997. For *Agrobacterium*-mediated transformation of wheat, *see, e.g., Cheng et al.,* 1997. For *Agrobacterium*-mediated transformation of rice, *see, e.g.,* Hiei *et al.,* 1997.

[0428] The Latin names for these and other plants are given below. It should be clear that these and other (*non-Agrobacterium*) transformation techniques can be used to transform *AAD-1 (v3),* for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta,* and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato (*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa, Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum, sinense, and frutescens*), Lettuce (*Compositae Lactuca sativa, perennis, and pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), Sorghum (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans (*Leguminosae Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (*Leguminosae Pisum, Vigna,* and *Tetragonolobus* spp.), Sunflower (*Compositae Helianthus annuus*), Squash (*Dicotyledoneae Cucurbita* spp.), Cucumber (*Dicotyledoneae genera*), Tobacco (*Solanaceae Nicotiana* spp.), *Arabidopsis* (*Cruciferae Arabidopsis thaliana*), Turfgrass (*Lolium, Agrostis,* and other families), and Clover (*Leguminosae*). Such plants, with *AAD-1 (v3)* genes, for example, are included in the subject invention.

Example 16 - *Stacking AAD-1 (v3)* with AHAS Herbicide Resistance Gene

[0429] Stacking *AAD-1 (v3)* with an AHAS herbicide resistance gene is described in Example 7.9.

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Example 17 - Further Evidence of Surprising Results: *AAD-1 vs. AAD-2*

[0430] *17.1 -AAD-2 (v1)* initial cloning.

[0431] Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA.* This gene is referred to herein as *AAD-2 (v1)* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[0432] The strain of *Bradyrhizobium japonicum* containing the *AAD-2 (v1)* gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(spel) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhol) SEQ ID NO:15 (added a Xho I site)].

[0433] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 μl, ea. primer 1 μl (50 ng/μl), gDNA 1 μl (200 ng/μl), H_2O 21 μl, Taq polymerase 1 μl (2.5 units/μl). Three Fail Safe Buffers-A, B, and C-were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[0434] Ten of the resulting white colonies were picked into 3 μl Luria Broth + 1000 μg/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA GT 3'] (SEQ ID NO:16) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2 (v1)* for internal consistency.

[0435] 17.2 - Completion *of AAD-2 (v1)* binary vector.

[0436] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:18) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:19) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIIII/SacI *AAD-2 (v1)* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2 (v1):* Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI - NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: *AAD-2 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium.*

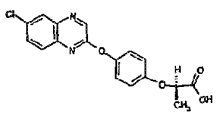
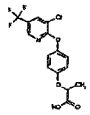
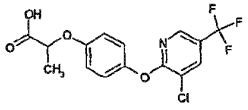
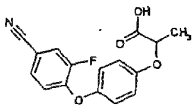
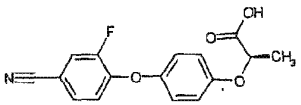
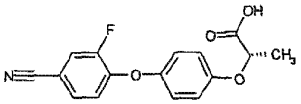
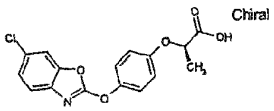
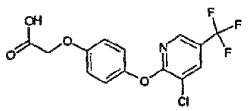
[0437] 17.3 - Comparison of substrate specificities of AAD-2 (v1) and AAD-1 (v1)

[0438] The activity of an extract from *E. coli* expressing And-2 (v1) (pDAB3202) prepared as in Example 11 was tested on four herbicides, 2,4-D, (R,S)-dichlorprop, (R,S)-haloxyfop and (R)-haloxyfop (all at a final concentration of 0.5 mM) using 3 μl (42 μg) of *E. coli* extract per assay with a 15 min assay period. **Figure 22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D = dichlorprop > (R,S)-haloxyfop >> (R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. **Table 33** shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which

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a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

Table 33. Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using 4 μl AAD1 (32 μg protein) extract or 3 μl AAD2 extract (42 μg protein).

				A510	
STRUCTURE	**Reg ID**	**Compound**	**Enantiomer**	**AAD1**	**AAD2**
	18706	quizalofop	R	0.27	0.01
	8671	haloxyfop	R	0.12	0
	66905	haloxyfop	R,S	0.1	0.3
	14623	cyhalofop	R,S	0.12	0.1
	14603	cyhalofop	R	0.14	0
	7466	cyhalofop	S	0	0.15
	11044492	fenoxaprop	R	0.14	0
	43865	haloxyfop-acetate	-	0	0.22

[0439] The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. *See* **Figure 19.** The K_m values for 2,4-D were 97 and 423 μM for AAD-1 (v1) and AAD-2 (v1) respectively and the apparent V_{max} values were 0.11 and 0.86 A_{510} units, respectively **(Table 34).** Because equivalent amounts of enzyme were used in the assays (as determined by SDS-PAGE analysis), it can be concluded that the k_{cat} of AAD-2 (v1) for 2,4-D is almost 8-fold higher than AAD-1 (v1) and the k_{cat}/K_m is 2-fold higher. Thus AAD-2 (v1) is significantly more efficient at cleaving 2,4-D *in vitro* than AAD-1 (v1). This is in surprising contrast to the *in planta* findings reported below, where plants expressing AAD-1 (v1) are significantly better in conferring 2,4-D resistance relative to AAD-2 (v1).

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Table 34. Comparison of the Km and "Vmax" values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	Km (μM) ±SE	Vmax (A510 units)	Km/Vmax (arbitrary units)
AAD-2	2,4-D	423 (±1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (±21)	0.11	1.16

Notes: Assays were performed in MOPS pH 6.75 + 1 mM α-ketoglutarate + 0.1 mM Na ascorbate + 0.1 mM Fe2+ and the released phenols colorimetrically detected using 4-aminoantipyrine/ferricyanide.

[0440] 17.4 - Evaluation *of transformed Arabidopsis.*

[0441] Freshly harvested T_1 seed transformed with a native [*AAD-1 (v2)*], plant optimized *[AAD-1 (v3)],* or native *AAD-2 (v1)* gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[0442] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet and then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[0443] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C, 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3), AAD-1 (v2), or AAD-2 (v1)* to provide phenoxy auxin herbicide resistance.

[0444] Random individual T_1 plants selected for glufosinate resistance above were confinned for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of 2,4-D (50-800 g ae/ha).

[0445] Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO) mixed in 200 mM Tris buffer (pH 9.0).

17.5 - Results of selection of transformed plants.

The first *Arabidopsis* transformations were conducted using *AAD-1 (v3).* T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (*PAT* gene), equating to a transformation/ selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see **Table 15** in Example 6.5 above). A small lot of native *AAD-1 (v2)* transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency). Surprisingly, *Arabidopsis* transformations using the native *AAD-2(v1)* gene provided a very low transformation frequency when selected for glufosinate tolerance (*PAT* selectable marker function). Approximately 1.3 million seed have been screened and only 228 glufosinate transformants were recovered, equating to a transformation/selection frequency of 0.018% *(see* **Table 15**). Transformation frequency for native *AAD-2 (v1)* was only 6% of that of native *AAD-1 (v2)*. The native *AAD2 (v1)* gene was subsequently synthetically optimized, cloned, and transformed as pDAB3705, into Arabidopsis using methods previously described (see Example 5). The plant optimized *AAD2 (v2)* (SEQ ID NO:29, which encodes SEQ ID NO:30) yielded a normal T_1 *Arabidopsis* selection frequency using Liberty herbicide of approximately 0.11 % *(see* **Table 15).**

[0446] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 16** (in Example 6.5 above) compares the response of *AAD-1 (v2)*, *AAD-1 (v3)*, *AAD-2 (v1)* and *AAD-1(v2)* genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Within a

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given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 16** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)*, *AAD-1 (v3)*, *AAD-2 (v1)* or *AAD-2 (v2)* versus the wildtype or *PAT*/*Cry1F* transformed controls.

[0447] Surprisingly, *AAD-2 (v1)* transformants were far less resistant to 2,4-D than either *AAD-1 (v2)* or *AAD-1 (v3)* genes **(Table 16)** both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) *AAD-1 (v2)*-transformed T_1 plants survived 200 g ae/ha 2,4-D uninjured (population injury average = 34%), and >73% (11 of 15) *AAD-1 (v3)* T_1 plants were uninjured (population injury average = 14%). *See* **Figure 20.** Tolerance improved slightly for plant-optimized *AAD2 (v2)* versus the native gene; however, comparison of both *AAD-1* and *-2* plant optimized genes indicates a significant advantage for *AAD-1 (v3) in planta* (see **Table 16**).

[0448] These results are unexpected given that *in vitro* comparison of native AAD-1 (v2) and AAD-2 (v1) indicated 2,4-D was better degraded by AAD-2 (v1). AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. Little correlation exists between expression level noted on the Western blot and the level of injury from 2,4-D on the same plants. *See* **Figure 21.** No large difference was evident in protein expression level (*in planta)* for the native and plant optimized *AAD-2* genes. These data corroborate earlier findings that make the functional expression of AAD-1 (v3) *in planta* for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected.

Example 18 - Preplant Burndown Applications

[0449] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

[0450] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 - 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (*e.g.*, horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting *(see* federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With *AAD-1 (v3)* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1 (v3)* gene if stably transformed.

Example 19 - In-Crop Use of Phenoxy Auxins Herbicides in *AAD-1 (v3)* Only Transformed Soybeans, Cotton, and Other Dicot Crops

[0451] *AAD-1 (v3)* can enable the use of phenoxy auxin herbicides (*e.g.*, 2,4-D, dichlorprop, MCPA, *et al.*) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280

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to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560 - 1120 g ae/ha is used. For a complete weed control system, grass weeds must be controlled. A variety of broad spectrum graminicide herbicides, including but not limited to haloxyfop, quizalofop, fenoxaprop, fluazifop, sethoxydim, and clethodim are currently registered for use in most dicot crops which are naturally tolerant to these herbicides. A combination of quizalofop (20 - 100 g ae/ha) plus 2,4-D (420 - 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1 (v3)*-transformed dicot crop (*i.e.*, soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops *(see* weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[0452] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/*AAD-1 (v3)* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

[0453] Further benefits could include tolerance to 2,4-D drift or volatilization as mechanisms for off-site 2,4-D injury to dicot crops; no interval required before planting following 2,4-D application (*see* previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicides) can still be used for the subsequent control of *AAD-1 (v3)*-transformed dicot crop volunteers.

[0454] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1 (v3)* gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - In-Crop Use of Phenoxy Auxins and AOPP Herbicides in *AAD-1 (v3)* Only Transformed Corn, Rice, and Other Monocot Species

[0455] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with *AAD-1 (v3)* would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. *AAD-1 (v3)*-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560 - 1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, *et al.* could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of *AAD-1 (v3)*-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20 - 100 g ae/ha) plus 2,4-D (420 - 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1 (v3)* transformed monocot crop (*e.g.*, corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[0456] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-1 (v3)* HTC rotation strategy, whether

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one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

[0457] Those skilled in the art will now also recognize that the above example can be applied to any monocot crop that would be protected by the *AAD-1 (v3)* gene from injury by any AOPP herbicide. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 - *AAD-1 (v3)* Stacked With Glyphosate Tolerance Trait in Any Crop

[0458] The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-1 (v3)* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3)*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (*e.g.*, *Commelina* spp), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica*, 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *AAD-1 (v3)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (preferably 20-100 g ae/ha and more preferably 20-35 g ae/ha), could be for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D.

[0459] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels

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compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 22 - *AAD-1 (v3)* Stacked with Glufosinate Tolerance Trait in Any Crop

[0460] Glufosinate tolerance (*PAT or bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

[0461] By stacking *AAD-1 (v3)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3)*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

i) Inherently tolerant grass weed species (e.g., *Echinochloa spp* or *Sorghum spp*) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[0462] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 23 - *AAD- 1 (v3)* Stacked with *AHAS* Trait in Any Crop

[0463] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al*. Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap,

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2004). By stacking *AAD-1 (v3)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3),* one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis*, *Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.
ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea spp. can* also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.
iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[0464] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxy auxin herbicides, or AOPP herbicide, alone or in multiple combinations, is enabled by *AAD-1 (v3)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3),* whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example *24 - AAD-1 (v3)* in Rice

[0465] 24.1 - Media description.

[0466] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25-26° C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang *et al.* 1996).

[0467] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/l hygromycin B for 3-4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/l hygromycin B (1/2MSH50).

[0468] 24.2 - Tissue culture development.

[0469] Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic

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tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C and in the dark at 21° C for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C for one week prior to storage at 4° C.

[0470] 24.3 - Microprojectile bombardment.

[0471] All bombardments were conducted with the Biolistic PDS-1000/He™ system (BioRad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 μl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB3403, 20 μl spermidine (0.1 M) and 50 μl - calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 μl cold 100% ethanol and 8-9 μl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

[0472] 24.4 - Tolerance testing.

[0473] Rice plantlets at the 3-5 leaf stage were sprayed with a 0.3% (v/v) solution of DuPont™ Assure® II containing 1% (v/v) Agridex crop oil concentrate using a DeVilbiss bulb sprayer (model 15-RD glass atomizer). This concentration corresponds to approximately 140 g ae/ha. Each plant was spayed in a fume hood at a distance of 8-12 inches with 6 full squirts of the sprayer directed so that the entire plant was covered with an equal portion of herbicide. Each squirt delivered approximately 100 μl solution to the plantlet. Once sprayed, plantlets were allowed to dry for one hour before being moved out of the fume hood. Rating for sensitivity or resistance was done at 10-14 days after treatment (DAT) and is shown in **Table 35** below.

Table 35.

Sample Name	140 g ae/ha quizalofop
Control	Dead
63-1A	No injury
63-1F	No injury
63-4B	No injury
63-4D	No injury
63-6C	Dead

[0474] 24.5 - Tissue harvesting, DNA isolation and quantification.

[0475] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

[0476] 24.6 - Southern blot analysis.

[0477] Southern blot analysis was performed with total DNA obtained from the Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digest of HindIII for pDAB3403 to obtain integration data. Likewise a total of 2 μg of DNA was subjected to an overnight digest of MfeI to obtain the PTU data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 x SSC was then set-up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer

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the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 micro-joules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80°C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using coding region PCR using plasmid pDAB3404. A total of 100 ng of total DNA was used as template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Southern fragment PCR AAD-1 were (Forward - ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse - GGGCAG-GCCTAACTCCACCAA) (SEQ ID NO:32). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute and 45 seconds followed by 72°C for 10 minutes.

[0478] The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60°C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65°C in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in **Table 36.**

Table 36. Southern Results.

Event	Integration Southern data	PTU Southern data
	Number of bands	**Expected size 3049bp**
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

[0479] Plants 63-1 A and 63-1 F are not the same event; Plants 63-4 A and 63-4 D are the same event. These events have the expected size PTU's, but they are very complex. This Southern blot PTU data correlates with expression data and the spray data. Sample 63-6 C did not have enough DNA present to perform both Integration and PTU southern blots.

[0480] 24.7 - Western data

[0481] Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in **Table 37.**

Table 37.

Lane	Sample Name	TSP (μg/mL)	ELISA [AAD1] (ng/mL)	ELISA Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	-
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	-
7	AAD1 Standard (0.5 μg/mL)				+++
8	AAD1 Standard (5.0 μg/mL)				+++++

Example 25 - Turf Grass Transformation Procedures

[0482] Genetic transformation, with *AAD-1 (v3)* substituted for the "bar" gene as described below, of creeping bentgrass

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mediated by Agrobacterium tumefaciens could be achieved through embryogenic callus initiated from seeds (cv. Penn-A-4), as described generally below. *See* "Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation" (Luo *et. al.*, 2004).

[0483] Callus is infected with an *A. tumefaciens* strain (LBA4404) harboring a super-binary vector that contained an herbicide-resistant bar gene driven either by a rice ubiquitin promoter. The overall stable transformation efficiency ranged from 18% to 45%. Southern blot and genetic analysis confirmed transgene integration in the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T_1 generation. All independent transformation events carried one to three copies of the transgene, and a majority (60-65%) contained only a single copy of the foreign gene with no apparent rearrangements.

[0484] 25.1 - Seed preparation for embryogenic callus induction.

[0485] Mature seeds were dehusked with sand paper and surface sterilized in 10% (v/v) Clorox bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-o-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytagel. The pH of the medium was adjusted to 5.7 before autoclaving at 120°C for 20 min).

[0486] 25.2 - Embryogenic callus induction.

[0487] The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryogenic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

[0488] 25.3 -*Agrobacterium* infection and co-cultivation.

[0489] One day before agro-infection, the embryogenic callus was divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 μM acetosyringone. A 10-ul aliquot of Agrobacterium (LBA4404) suspension (OD=1.0 at 660 nm) was then applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25°C.

[0490] 25.4 - Resting stage and *Agrobacterium* control.

[0491] For the antibiotic treatment step, the callus was then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

[0492] 25.5 - Selection and potential transgenic colony identification.

[0493] Subsequently, for selection, the callus was moved to callus-induction medium containing 250 mg/l cefotaxime and 10 mg/l phosphinothricin (PPT) for 8 weeks. Antibiotic treatment and the entire selection process were performed at room temperature in the dark. The subculture interval during selection was typically 3 weeks.

[0494] 25.6 - Regeneration of transgenic plants.

[0495] For plant regeneration, the PPT-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime, PPT or hygromycin. These calli were kept in the dark at room temperature for 1 week and then moved into the light for 2-3 weeks to develop shoots. No Albino plants with PPT selection (Hygromycin if used as a selection agent, produces a high level of albino plants).

[0496] 25.7 - Root induction and transfer to greenhouse.

[0497] Small shoots were then separated and transferred to hormone-free regeneration medium containing PPT and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3-5 weeks) were then transferred to soil and grown either in the greenhouse or in the field.

[0498] 25.8 - Vernalization and out-crossing of transgenic plants.

[0499] Transgenic plants were maintained out of doors in a containment nursery (3-6 months) until the winter solstice in December. The vernalized plants were then transferred to the greenhouse and kept at 25°C under a 16/8 h photoperiod and surrounded by non-transgenic wild-type plants that physically isolated them from other pollen sources. The plants started flowering 3-4 weeks after being moved back into the greenhouse. They were out-crossed with the pollen from the surrounding wild type plants. The seeds collected from each individual transgenic plant were germinated in soil at 25°C, and T1 plants were grown in the greenhouse for further analysis.

[0500] 25.9 - Other target grasses.

[0501] Other grasses that can be targeted for AAD-1 transformation according to the subject invention include Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra commutate*), Crabgrass, Creeping bent (*Agrostis stolonifera*), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/sheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*), Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra rubra*), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Timothy, Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysia-

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grass.

Example *26 -AAD-1 (v3)* in Canola

[0502] 26.1 - Canola transformation.

[0503] The *AAD-1 (v3)* gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium-mediated* transformation. The construct contained *AAD-1 (v3)* gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter.

[0504] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[0505] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB721. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[0506] After 30 min treatment of the hypocotyl segments with *Agrobacterium,* these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium.* The selection agent Herbiace allowed the growth of the transformed cells.

[0507] Callus samples from 35 independent events were tested by PCR. All the 35 samples tested positive for the presence of *AAD-1* (v3),whereas the non-transformed controls were negative (section on PCR assay). Ten callus samples were confirmed to express the AAD-1 protein as determined by ELISA (section on protein analysis).

[0508] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 3 weeks.

[0509] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSL1(MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[0510] The transformed T0 plants were self-pollinated in the greenhouse to obtain T1 seed. The T0 plants and T1 progeny were sprayed with a range of herbicide concentrations to establish the level of protection by the *AAD-1 (v3)* gene.

[0511] 26.2 - "Molecular analysis": canola materials and methods

[0512] 26.2.1 - Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/ul.

[0513] 26.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-1 (v3)* were (Forward - ATGGCTCATG CTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse. - CGGGCAG-GCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-1* (v3) events tested positive. Three negative control samples tested negative.

[0514] 26.3 - ELISA.

[0515] Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in Table 38.

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Table 38. Expression of AAD1 in Canola calli.

Sample #	Weight (mg)	[TSP] (μg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	-
Control 2	61	2549.60	35.00	13.73	-
Control 3	59	2374.41	22.79	9.60	-

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Bayer EX1008 (Part 1 of 9)

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Ala Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met
    130                 135                 140

Tyr Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu
145                 150                 155                 160

Gly Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr
                165                 170                 175

Gln Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp
            180                 185                 190

Val Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His
        195                 200                 205

Pro Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln
    210                 215                 220

Arg Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe
225                 230                 235                 240
```


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Leu Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp
245 250 255

Lys Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg
260 265 270

Ala Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr
275 280 285

Val Gly Gly Val Arg Pro Ala Arg
290 295

<210> 12
<211> 888
<212> DNA
<213> Bradyrhizobium japonicum USDA 110

<400> 12

atgacgatcg ccatccggca gcttcagacg cattttgtcg gccaggtttc cggcctcgat 60

ttgcgaaagc cgctcacgcc gggcgaggcc cgcgaggtcg agtccgccat ggacaaatac 120

gcggtgctcg ttttccacga ccaggacatc accgacgagc agcagatggc tttcgcgctg 180

aacttcggcc agcgcgagga cgcgcgcggc ggcacggtca ccaaggagaa ggactaccgg 240

ctgcaatccg gcctgaacga cgtctccaat ctcggcaagg acggcaagcc gctggccaag 300

gacagccgca cgcacctgtt caatctcggc aactgcctct ggcactccga cagctcgttc 360

cgtcccattc ccgcaaaatt ctcgctgctg tcggcgcgcg tggtgaaccc gacgggcggc 420

aacaccgaat tcgcggacat gcgcgccgcc tatgacgcgc tcgacgacga gaccaaggcc 480

gaaatcgagg acctcgtctg cgagcactcg ctgatgtatt cgcgcggctc gctcggcttc 540

accgagtaca ccgacgaaga gaagcagatg ttcaagccgg tcctgcaacg cctcgtgcgc 600

acccatccgg tccaccgccg caagtcgctg tatctctcgt cgcatgccgg caagatcgcc 660

agcatgagcg tgccggaggg gcggctgctg ttgcgcgatc tcaacgagca cgcgacgcag 720

ccggaattcg tctacgtcca caaatggaag ctgcatgacc tcgtgatgtg ggacaaccgc 780

cagaccatgc accgcgtccg ccgctacgac cagtcccagc cccgcgacat gcgccgcgcg 840

acggtggcgg ggacggagcc gacggtgcag cagcaggcgg cggagtag 888

<210> 13
<211> 289
<212> PRT
<213> Bradyrhizobium japonicum USDA 110

<400> 13

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```
Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val
1               5                  10                  15

Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu
            20                  25                  30

Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
        35                  40                  45

Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
    50                  55                  60

Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
65                  70                  75                  80

Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
                85                  90                  95

Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
            100                 105                 110

Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
        115                 120                 125

Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
    130                 135                 140

Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
145                 150                 155                 160

Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
                165                 170                 175

Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
            180                 185                 190

Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
        195                 200                 205

Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
    210                 215                 220

Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
225                 230                 235                 240
```


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Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met
245 250 255

Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln Ser
260 265 270

Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr
275 280 285

Val

<210> 14
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> brjap 5'(spel)

<400> 14
actagtaaca aagaaggaga tataccatga cgat 34

<210> 15
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> br jap 3' (xhol)

<400> 15
ttctcgagct atcactccgc cgcctgctgc tgc 33

<210> 16
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 forward primer

<400> 16
gtaaaacgac ggccagt 17

<210> 17
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 reverse primer

<400> 17
caggaaacag ctatgac 17

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<210> 18
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> NcoI of Brady

<400> 18
tataccacat gtcgatcgcc atccggcagc tt 32

<210> 19
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> SacI of Brady

<400> 19
gagctcctat cactccgccg cctgctgctg cac 33

<210> 20
<211> 525
<212> PRT
<213> Glycine max

<400> 20

```
Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1               5                  10                  15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
            20                  25                  30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
        35                  40                  45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
    50                  55                  60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65                  70                  75                  80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
                85                  90                  95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
            100                 105                 110
```


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```
Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350
```


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```
Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525
```

<210> 21
<211> 525
<212> PRT
<213> Artificial sequence

<220>
<223> Doubly mutated Soybean EPSPS protein: threonine 183 converted to isoleucine; proline 187 converted to serine

<400> 21

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Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1 5 10 15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
20 25 30

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Bayer EX1008 (Part 1 of 9)

```
Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
        35                  40                  45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
    50                  55                  60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65                  70                  75                  80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
                85                  90                  95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
            100                 105                 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270
```


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```
Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510
```


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Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
515 520 525

<210> 22
<211> 1604
<212> DNA
<213> Artificial sequence

<220>
<223> Soybean-biased DNA sequence encoding doubly-mutated EPSPS disclosed in SEQ ID NO:21, with added sequence

<220>
<221> misc_feature
<222> (1) .. (1575)
<223> Coding sequence of soybean-biased DNA sequence encoding the EPSPS protien of SEQ ID NO:21

<220>
<221> misc_feature
<222> (1576)..(1578)
<223> Translation terminator tga

<220>
<221> misc_feature
<222> (1579)..(1604)
<223> Sequence included to introduce translation stop codons in all six open reading frames, and to introduce a SacI restriction enzyme recognition site for cloning purposes

<400> 22

atggctcaag tctcccgtgt tcacaatctt gctcagtcaa cccaaatctt tggacattca 60

agcaactcaa acaaactgaa gtctgtgaat tctgtctcac ttcgcccacg cctttgggga 120

gcatccaaga gtcgcatacc aatgcacaag aatgggagtt tcatgggcaa cttcaatgtt 180

gggaaaggca attctggtgt cttcaaagtt tcagcttctg ttgcagccgc agagaaaccc 240

agcacttccc ctgagattgt tcttgaaccc attaaggact tcagtggaac aatcactctg 300

cctggatcaa agagtctttc aaacagaata cttctcttgg cagctctgag tgaaggaacc 360

actgtagttg acaacctttt gtactctgaa gatattcatt acatgttggg tgctctcaga 420

actcttgggt tgagagttga agatgacaag accacaaaac aagccatagt tgaaggatgt 480

ggtgggttgt ttccaacaag caaagaatcc aaagatgaga tcaacttgtt tcttggcaat 540

gctggaattg caatgagaag cctcactgct gcagtagttg cagctggtgg gaatgcaagt 600

tatgtccttg atggtgtccc cagaatgagg gaaaggccca tcggtgacct tgtggctggc 660

ctgaaacagc ttggagcaga tgttgattgc ttcttgggca caaactgccc tccagtgaga 720

gtgaatggga agggaggttt gcctggtgga aaggtcaaac tgagtggatc agtctcttcc 780

cagtatctga ctgccttgct catggctgcc cctctggctt tgggtgatgt ggagattgaa 840

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atagtggaca agttgatttc tgttccatat gtggaaatga ccctcaaact catggagagg 900

tttggagttt ctgttgaaca ttctggcaac tgggatcgtt tccttgtaca tggaggtcag 960

aagtacaaaa gccctggcaa tgcctttgtt gaaggggatg caagctctgc ttcctatctc 1020

ttggctgggg ctgccatcac tggtgggacc atcactgtga atggctgtgg cacctcatcc 1080

cttcaaggtg atgtaaagtt tgcagaggtc ttggagaaaa tgggtgccaa ggtcacctgg 1140

tctgagaaca gtgtaactgt gtctggacct cccagagact tcagtggcag aaaggttctc 1200

cgtggaattg atgtgaacat gaacaagatg ccagatgtgg ccatgaccct cgctgttgta 1260

gccctgtttg caaatggacc aactgcaatc cgtgatgttg cttcatggag ggtgaaggag 1320

acagagagga tgattgccat ttgcacagaa ctccgcaaac ttggtgcaac agttgaagag 1380

ggaccagatt actgtgtgat aaccccacct gagaagctca atgtgacagc cattgacacc 1440

tatgatgacc acagaatggc aatggctttc tcccttgctg cctgtggtga tgtgcctgtg 1500

actatcaaag accctgggtg cacaaggaag acatttccag actactttga agttttggag 1560

aggttgacaa agcactgagt agttagctta atcacctaga gctc 1604

<210> 23
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Primer Pat 5-3

<400> 23
agataccctt ggttggttgc 20

<210> 24
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Primer Pat 3-3

<400> 24
cagatggatc gtttggaagg 20

<210> 25
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> AAD-1 PTU forward primer

<400> 25
ataatgccag cctgttaaac gcc 23

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<210> 26
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> AAD-1 PTU reverse primer

<400> 26
ctcaagcata tgaatgacct cga 23

<210> 27
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Forward primer for Coding Region PCR AAD-1 (RdpAcodF)

<400> 27
atggctcatg ctgccctcag cc 22

<210> 28
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Reverse primer for Coding Region PCR AAD-1 (RdpAcodR)

<400> 28
cgggcaggcc taactccacc aa 22

<210> 29
<211> 932
<212> DNA
<213> Artificial sequence

<220>
<223> AAD-2 v2 plant-optimized nucleotide

<400> 29

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ccatggctac catagcaatc agacagctcc agacccactt tgtgggtcaa gtttctggat     60

tggacctcag aaagccactc actcctggag aagccagaga agttgaatca gctatggaca    120

agtacgcagt tcttgtcttc catgaccaag acatcacaga tgagcaacag atggcctttg    180

ccctcaactt tggtcagagg gaggatgcac gtggtggcac tgtcaccaaa gagaaggatt    240

accgtcttca gtctggcctc aatgatgttt ccaacttggg caaagatgga aagccacttg    300

ccaaggacag ccgcacccat ttgttcaacc ttggaaactg cttgtggcat tctgactcca    360

gcttcagacc aatcccagcc aagttcagcc tcctttctgc tcgtgttgtg aacccaactg    420
```


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gtgggaacac tgagtttgct gacatgagag ctgcctatga tgctcttgac gatgaaacca 480

aagctgagat tgaggacctt gtgtgtgagc actctctcat gtactcaagg ggctcacttg 540

gcttcactga gtacacagat gaagagaagc aaatgttcaa gcccgtcttg cagcgcttgg 600

tccgcacaca ccctgtgcac cgtcgcaaat cactctacct ctccagccat gccggaaaga 660

ttgccagcat gtccgtccct gaagggaggc tcctttgag ggatttgaat gaacatgcta 720

ctcagcctga gttcgtctat gttcacaaat ggaagttgca tgatcttgtg atgtgggaca 780

ataggcaaac catgcacaga gtgaggagat atgaccagtc ccaacccaga gacatgcgcc 840

gtgcaacagt tgctgggacc gagcccacag tgcaacagca agcagcagag tgagtagtta 900

gcttaatcac ctagagctcg gtcaccagat ct 932

<210> 30
<211> 296
<212> PRT
<213> Artificial sequence

<220>
<223> Translated AAD-2 v2 protein

<400> 30

Met Ala Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln
1 5 10 15

Val Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg
20 25 30

Glu Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp
35 40 45

Gln Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly
50 55 60

Gln Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr
65 70 75 80

Arg Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly
85 90 95

Lys Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn
100 105 110

Cys Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe
115 120 125

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Ser Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu
130 135 140

Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys
145 150 155 160

Ala Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg
165 170 175

Gly Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe
180 185 190

Lys Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg
195 200 205

Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
210 215 220

Val Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr
225 230 235 240

Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
245 250 255

Met Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln
260 265 270

Ser Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro
275 280 285

Thr Val Gln Gln Gln Ala Ala Glu
290 295

<210> 31
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Southern fragment PCR AAD-1 forward primer

<400> 31
atggctcatg ctgccctcag cc 22

<210> 32
<211> 21
<212> DNA
<213> Artificial sequence

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<220>
<223> Southern fragment PCR AAD-1 reverse primer

<400> 32
gggcaggcct aactccacca a 21

Claims

1. A transgenic plant cell comprising a polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO:9 or a variant of SEQ ID NO:9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO:9.

2. The cell of claim 1 wherein said plant cell is selected from the group consisting of dicotyledonous cells and monocotyledonous cells.

3. The cell of claim 2 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an *Arabidopsis* cell.

4. The cell of claim 2 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

5. A transgenic plant comprising a plurality of cells according to claim 1, wherein expression of said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide.

6. The plant of claim 5 wherein said herbicide is a phenoxy auxinic herbicide or aryloxyphenoxypropionate.

7. The plant of claim 5 or 6, wherein the plant is also resistant to one or more herbicides selected from the group consisting of glyphosate, imidazolinones, glufosinate, sulfonylureas, bromoxynil and dicamba.

8. The plant of claim 5 wherein said herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, mecoprop, fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, metamifop, cyhalofop, and clodinofop.

9. The plant of claim 5 wherein expression of said polynucleotide renders said plant resistant to both a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

10. The plant of claim 5 wherein said plant further comprises a second herbicide resistance gene.

11. The plant of claim 10, wherein said second herbicide resistance gene renders said plant resistant to glyphosate.

12. The plant of claim 10, wherein said aryloxyalkanoate herbicide is 2,4-D and said second herbicide resistance gene renders said plant resistant to glyphosate.

13. The plant of claim 5 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus,* and *Xenorhabdus.*

14. The plant of claim 5 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

15. The plant of claim 10 wherein said second herbicide resistance gene renders said plant resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, ALS inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), and inhibitors of protoporphyrinogen oxidase (PPO).

16. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 5, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting

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of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

17. The method of claim 16 wherein said herbicide is an R-enantiomer of a chiral phenoxy auxin or an achiral phenoxy auxin.

18. The method of claim 17 wherein said herbicide is selected from the group consisting of R-dichlorprop, R-mecoprop, 2,4-D and MCPA.

19. The method of claim 16 wherein said first herbicide is an aryloxyphenoxypropionate and said plant is a monocot.

20. The method of claim 19 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, turf grass, oats, sorghum, and pasture grasses.

21. The method of claim 16 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

22. The method of claim 21 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

23. The method of claim 22 wherein said method comprises applying a second herbicide.

24. The method of claim 23 wherein said first herbicide and said second herbicide are applied sequentially or concurrently.

25. The method of claim 23 wherein said first herbicide is a phenoxy auxin and second herbicide is an aryloxyphenoxypropionate.

26. The method of claim 16 wherein said method is used to treat or prevent herbicide-resistant weeds.

27. The method of claim 16 wherein said plant is resistant to glyphosate.

28. The method of claim 17 wherein said R-enantiomer is a component of a racemic mixture.

29. The method of claim 23 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

30. The method of claim 29 wherein said second gene is selected from the group consisting of a modified AHAS (acetohydroxyacid synthase), SurA, SurB, Csr1, Csr1-1, Csr1-2, a modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT, PAT (phosphinothricin-N-acetyltransferase), bar, and a dicamba-degrading enzyme.

31. The method of claim 29 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase inhibitors, protoporphyrinogen oxidase inhibitors, and hydroxyphenyl-pyruvate-dioxygenase inhibitors.

32. The method of claim 31 wherein said second herbicide is an acetolactate synthase inhibitor selected from the group consisting of imidazolinones, sulfonyl ureas, and triazolopyrimidine herbicides.

33. The method of claim 32 wherein said second herbicide is an imidazolinone selected from the group consisting of imazamox, imazethapyr, imazaquin, and imazapic.

34. The method of claim 29 wherein said first herbicide is a phenoxy auxin and said second herbicide is selected from the group consisting of glyphosate and glufosinate.

35. The method of claim 34 wherein said phenoxy auxin is 2,4-D and said second herbicide is glyphosate.

36. The method of claim 29 wherein said first herbicide is an aryloxyphenoxypropionate and said second herbicide is glyphosate.

37. The method of claim 36 wherein said first herbicide is selected from the group consisting of quizalofop, haloxyfop,

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and cyhalofop.

38. The method of claim 32 wherein said second herbicide is a sulfonyl urea selected from the group consisting of amidosulfuron, bensulfuron, chlorimuron, chlorsulfuron, cinosulfuron, flupyrsulfuron, foramsulfuron, halosulfuron, nicosulfuron, primisulfuron, prosulfuron, rimsulfuron, sulfometuron, sulfosulfuron, thifensulfuron, triasulfuron, trifloxysulfuron, and triflusulfuron.

39. The method of claim 29 wherein said method further comprises applying a third herbicide.

40. The method of claim 39 wherein said herbicides are 2,4-D; quizalofop; and glufosinate.

41. A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises a cell of claim 1, and wherein said first herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate.

42. The method of claim 41 wherein said first herbicide is an acid, an inorganic salt, an organic salt, an ester, an R-enantiospecific isomer, or a component of a racemic mixture.

43. The method of claim 41 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

44. The method of claim 43 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate

45. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 5, wherein said method comprises applying to at least a portion of said field a first herbicide that is a 2,4-D, wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to glyphosate.

46. A seed comprising a plant cell of claim 1.

47. A plant grown from the seed of claim 46, wherein said plant comprises a transgenic plant cell of claim 1.

48. A part, progeny, or asexual propagate of the plant of claim 5, wherein said part, progeny or asexual propagate comprises a plurality of cells according to claim 1, wherein expression said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide.

49. A method of controlling glyphosate-resistant weeds in a field of glyphosate tolerant crop plants, wherein said plants comprise a transgenic plant cell of claim 1, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

50. The method of claim 49 wherein said herbicide is a phenoxy auxin or an aryloxy phenoxypropionate.

51. The method of claim 50, wherein said phenoxy auxin herbicide is 2,4-D.

52. The method of claim 49 wherein said plants are dicots or monocots.

Patentansprüche

1. Eine transgene Pflanzenzelle, enthaltend ein Polynukleotid, welches ein AAD-1-Protein enthält, enthaltend die Aminosäuresequenz gemäß SEQ ID NO: 9 oder einer Variante von SEQ ID NO: 9, wobei besagte Variante eine Aryloxylalkanoat-Dioxygenaseaktivität, eine Deletion von mindestens einer Aminosäure oder eine konservierte Substitution und mindestens 95% Sequenzidentität mit SEQ ID NO: 9 aufweist.

2. Die Zelle nach Anspruch 1, wobei die besagte Pflanzenzelle ausgewählt ist aus der Gruppe bestehend aus dikotyledonen Zellen und monokotyledonen Zellen.

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3. Die Zelle nach Anspruch 2, wobei besagte Pflanzenzelle dikotyledon ist und ausgewählt ist aus der Gruppe, bestehend aus einer Baumwollzelle, einer Tabakzelle, einer Canolazelle, einer Sojabohnenzelle und einer *Arabidopsis-Zelle.*

4. Die Zelle nach Anspruch 2, wobei besagte Pflanzenzelle eine monokotyledone Zelle ist, ausgewählt aus der Gruppe bestehend aus einer Reiszelle und einer Maiszelle.

5. Eine transgene Pflanze, umfassend eine Mehrzahl von Zellen gemäß Anspruch 1, wobei die Expression des besagten Polynukleotids besagten Zellen Toleranz gegen ein Aryloxylalkanoat-Herbizid verleiht.

6. Die Pflanze nach Anspruch 5, wobei besagtes Herbizid ein PhenoxyauxinHerbizid oder Aryloxyphenoxypropionat ist.

7. Die Pflanze nach Anspruch 5 oder 6, wobei die Pflanze ebenfalls resistent gegen ein oder mehrere Herbizide ausgewählt aus der Gruppe bestehend aus Glyphosat, Imidazolinonen, Glufosinat, Sulfonyl-Harnstoffen, Bromoxynil und Dicamba ist.

8. Die Pflanze nach Anspruch 5, wobei besagtes Herbizid ausgewählt ist aus der Gruppe bestehend aus 2,4-Dichlorphenoxyessigsäure, MCPA, Dichlorprop, Mecoprop, Fluazifop, Haloxyfop, Diclofop, Quizalofop, Fenoxaprop, Metamifop, Cyhalofop und Clodinofop.

9. Die Pflanze nach Anspruch 5, wobei die Expression des besagten Polynukleotids der Pflanze Resistenz sowohl gegen ein PhenoxyauxinHerbizid, als auch ein Aryloxyphenoxypropionat-Herbizid verleiht.

10. Die Pflanze nach Anspruch 5, wobei die besagte Pflanze weiterhin ein zweites Herbizidresistenzgen enthält.

11. Die Pflanze nach Anspruch 10, wobei besagtes zweites Herbizidresistenzgen der Pflanze Resistenz gegen Glyphosphat verleiht.

12. Die Pflanze nach Anspruch 10, wobei das besagte Aryloxylalkanoatherbizid 2,4-D ist und das besagte zweite Herbizidresistenzgen der Pflanze Resistenz gegen Glyphosat verleiht.

13. Die Pflanze nach Anspruch 5, wobei die besagte Pflanze weiterhin ein Insektenresistenzgen enthält, welches aus einem Organismus stammt, der ausgewählt ist aus der Gruppe bestehend aus *Bacillus thuringiensis, Photorhabdus* und *Xenorhabdus.*

14. Die Pflanze nach Anspruch 5, wobei besagte Pflanze weiterhin ein Gen für ein agronomisches Merkmal enthält, welches ausgewählt ist aus der Gruppe bestehend aus Pilzresistenz, Stresstoleranz, erhöhtem Ertrag, verbessertem Ölprofil, verbesserter Faserqualität, Virenresistenz, verzögerter Reife, Kältetoleranz und Salztoleranz.

15. Die Pflanze nach Anspruch 10, wobei das besagte zweite Herbizidresistenzgen der Pflanze Resistenz gegen ein Herbizid verleiht, welches ausgewählt ist aus der Gruppe bestehend aus Glyphosat, Glufosinat, ALS-Inhibitoren, Inhibitoren der 4-Hydroxyphenylpyruvat-Dioxygenase (HPPD) und Inhibitoren der Protoporphyrinogenoxidase (PPO).

16. Ein Verfahren zur Kontrolle wenigstens eines Unkrautes auf einem Feld, wobei das besagte Feld wenigstens eine Pflanze nach Anspruch 5 enthält, wobei das besagte Verfahren die Anwendung eines erstens Herbizids, ausgewählt aus der Gruppe bestehend aus einem Phenoxyauxinherbizid und einem Aryloxyphenoxypropionatherbizid auf wenigstens einem Teil des besagten Feldes beinhaltet.

17. Das Verfahren nach Anspruch 16, wobei das besagte Herbizid ein R-Enantiomer eines chiralen Phenoxyauxins oder eines achiralen Phenoxyauxins ist.

18. Das Verfahren nach Anspruch 17, wobei besagtes Herbizid ausgewählt ist aus der Gruppe bestehend aus R-Dichlorprop, R-Mecoprop, 2,4-D und MCPA.

19. Das Verfahren nach Anspruch 16, wobei das besagte erste Herbizid ein Aryloxyphenoxypropionat und die besagte Pflanze eine Monokotyle ist.

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20. Das Verfahren nach Anspruch 19, wobei besagte Monokotyle ausgewählt ist aus der Gruppe bestehend aus Mais, Reis, Weizen, Gerste, Roggen, Wiesengras, Hafer, Sorghum und Weidegras.

21. Das Verfahren nach Anspruch 16, wobei das besagte erste Herbizid ein Phenoxyauxin und die besagte Pflanze eine Dikotyledone ist.

22. Das Verfahren nach Anspruch 21, wobei besagte Dikotyledone ausgewählt ist aus der Gruppe bestehend aus Baumwolle, Tabak, Canola und Sojabohne.

23. Das Verfahren nach Anspruch 22, wobei das besagte Verfahren die Anwendung eines zweiten Herbizids umfasst.

24. Das Verfahren nach Anspruch 23, wobei das besagte erste Herbizid und das besagte zweite Herbizid aufeinander folgend oder gleichzeitig angewendet werden.

25. Das Verfahren nach Anspruch 23, wobei das erste Herbizid ein Phenoxyauxin und das zweite Herbizid ein Aryloxyphenoxypropionat ist.

26. Das Verfahren nach Anspruch 16, wobei das besagte Verfahren verwendet wird, um Herbizid-resistente Unkräuter zu behandeln oder zu verhindern.

27. Das Verfahren nach Anspruch 16, wobei die besagte Pflanze resistent gegen Glyphosat ist.

28. Das Verfahren nach Anspruch 17, wobei das besagte R-Enantiomer ein Bestandteil eines Racemat-Gemisches ist.

29. Das Verfahren nach Anspruch 23, wobei die besagte Pflanze weiterhin ein zweites Herbizidresistenzgen enthält, welches der Pflanze Resistenz gegen das besagte zweite Herbizid verleiht.

30. Das Verfahren nach Anspruch 29, wobei das besagte zweite Gen ausgewählt ist aus der Gruppe bestehend aus einer modifizierten AHAS (Acetohydroxysäuresynthase), SurA, SurB, Csr1, Csr1-1, Csr1-2, einer modifizierten EPSPS (5-Enolpyruvylshikimat-3-phosphatsynthase), GOX, GAT, PAT (Phosphinothricin-N-acetyltransferase), bar und einem Dicamba-abbauenden Enzym.

31. Das Verfahren nach Anspruch 29, wobei das besagte zweite Herbizid ausgewählt ist aus der Gruppe bestehend aus Glyphosat, Glufosinat, Dicamba, Acetolaktatsynthase-Inhibitoren, Protoporphyrinogenoxidase-Inhibitoren und Hydroxyphenyl-Pyruvat-Dioxygenase-Inhibitoren.

32. Das Verfahren nach Anspruch 31, wobei das besagte zweite Herbizid ein Aetolaktatsynthase-Inhibitor, ausgewählt aus der Gruppe bestehend aus Imidazolinonen, Sulfonyl-Harnstoffen und Triazolopyrimidin-Herbiziden, ist.

33. Das Verfahren nach Anspruch 32, wobei das besagte zweite Herbizid ein Imidazolinon ist, ausgewählt aus der Gruppe bestehend aus Imazamox, Imazethapyr, Imazaquin und Imazapic.

34. Das Verfahren nach Anspruch 29, wobei das besagte erste Herbizid ein Phenoxyauxin ist und das besagte zweite Herbizid ausgewählt ist aus der Gruppe bestehend aus Glyphosat und Glufosinat.

35. Das Verfahren nach Anspruch 34, wobei das besagte Phenoxyauxin 2,4-D und das besagte zweite Herbizid Glyphosat ist.

36. Das Verfahren nach Anspruch 29, wobei das besagte erste Herbizid ein Aryloxyphenoxypropionat und das besagte zweite Herbizid Glyphosat ist.

37. Das Verfahren nach Anspruch 36, wobei das besagte erste Herbizid ausgewählt ist aus der Gruppe bestehend aus Quizalofop, Haloxyfop und Cyhalofop.

38. Das Verfahren nach Anspruch 32, wobei das besagte zweite Herbizid ein Sulfonylharnstoff ist, ausgewählt aus der Gruppe bestehend aus Amidosulfuron, Bensulfuron, Chlorimuron, Chlorsulfuron, Cinosulfuron, Flupyrsulfuron, Foramsulfuron, Halosulfuron, Nicosulfuron, Primisulfuron, Prosulfuron, Rimsulfuron, Sulfometuron, Sulfosulfuron, Thifensulfuron, Triasulfuron, Trifloxysulfuron und Triflusulfuron ist.

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39. Das Verfahren nach Anspruch 29, wobei das besagte Verfahren weiter die Anwendung eines dritten Herbizids beinhaltet.

40. Das Verfahren nach Anspruch 39, wobei die besagten Herbizide 2,4-D, Quizalofop und Glufosinat sind.

41. Ein Verfahren zur Kontrolle von Unkräutern auf einem Feld, wobei das besagte Verfahren die Anwendung eines ersten Herbizides auf dem besagten Feld und die Aussaat eines Samens innerhalb von 14 Tagen nach Anwendung des besagten ersten Herbizides beinhaltet, wobei der besagte Samen eine Zelle nach Anspruch 1 enthält und wobei das besagte erste Herbizid ausgewählt ist aus der Gruppe bestehend aus einem Phenoxyauxin und einem Aryloxyphenoxypropionat.

42. Das Verfahren nach Anspruch 41, wobei das besagte erste Herbizid eine Säure, ein anorganisches Salz, ein organisches Salz, ein Ester, ein R-enantiospezifisches Isomer oder ein Bestandteil eines Racematgemisches ist.

43. Das Verfahren nach Anspruch 41, wobei der besagte Samen ein zweites Gen enthält, welches der besagten Pflanze Resistenz gegen ein zweites Herbizid verleiht und das besagte Verfahren weiterhin die Anwendung des besagten zweiten Herbizids auf dem besagten Feld vor der besagten Aussaat beinhaltet.

44. Das Verfahren nach Anspruch 43, wobei das besagte zweite Herbizid ausgewählt ist aus der Gruppe bestehend aus Glyphosat, Gramoxon und Glufosinat.

45. Ein Verfahren zur Kontrolle wenigstens eines Unkrautes auf einem Feld, wobei das besagte Feld wenigstens eine Pflanze nach Anspruch 5 enthält, wobei das besagte Verfahren die Anwendung eines ersten Herbizids, das ein 2,4-D ist, auf besagtem Feld beinhaltet, wobei die besagte Pflanze weiterhin ein zweites Herbizidresistenzgen enthält, das der besagten Pflanze Resistenz gegen Glyphosat verleiht.

46. Ein Samen, enthaltend eine Pflanzenzelle nach Anspruch 1.

47. Eine aus dem Samen nach Anspruch 46 gezogene Pflanze, wobei die besagte Pflanze eine transgene Pflanzenzelle nach Anspruch 1 enthält.

48. Ein Teil, Nachkomme oder asexuelles Vermehrungsprodukt der Pflanze nach Anspruch 5, wobei der besagte Teil, Nachkomme oder das asexuelle Vermehrungsprodukt eine Mehrzahl von Zellen gemäß Anspruch 1 enthält, wobei die Expression des besagten Polynukleotids den besagten Zellen Toleranz gegen ein Aryloxylalkanoatherbizid verleiht.

49. Ein Verfahren zur Kontrolle von Glyphosat-resistenten Unkräutern auf einem Feld mit Glyphosat-toleranten Nutzpflanzen, wobei die besagten Pflanzen eine transgene Pflanzenzelle nach Anspruch 1 enthalten und das besagte Verfahren die Anwendung eines Aryloxylalkanoatherbizids auf wenigstens einem Teil des besagten Feldes beinhaltet.

50. Das Verfahren nach Anspruch 49, wobei das besagte Herbizid ein Phenoxyauxin oder ein Aryloxyphenoxypropionat ist.

51. Das Verfahren nach Anspruch 50, wobei das besagte Phenoxyauxinherbizid 2,4-D ist.

52. Das Verfahren nach Anspruch 49, wobei die besagten Pflanzen Dikotyledone oder Monokotyledone sind.

Revendications

1. Cellule végétale transgénique comprenant un polynucléotide qui code pour une protéine AAD-1 comprenant la séquence d'acides aminés selon SEQ ID NO: 9 ou une variante de SEQ ID NO: 9, dans laquelle ladite variante a une activité d'aryloxyalcanoate dioxygénase, au moins une délétion ou substitution conservée d'acides aminés, et au moins 95% d'identité de séquence avec SEQ ID NO: 9.

2. Cellule selon la revendication 1, dans laquelle ladite cellule végétale est choisie dans le groupe consistant en cellules dicotylédones et cellules monocotylédones.

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3. Cellule selon la revendication 2, dans laquelle ladite cellule végétale est dicotylédone et choisie dans le groupe consistant en une cellule de coton, une cellule de tabac, une cellule de canola, une cellule de soja, et une cellule d'*Arabidopsis*.

4. Cellule selon la revendication 2, dans laquelle ladite cellule végétale est une cellule monocotylédone choisie dans le groupe consistant en une cellule de riz et une cellule de maïs.

5. Plante transgénique comprenant une pluralité de cellules selon la revendication 1, dans laquelle l'expression dudit polynucléotide rend lesdites cellules tolérantes à un herbicide aryloxyalcanoate.

6. Plante selon la revendication 5, dans laquelle ledit herbicide est un herbicide phénoxy auxinique ou aryloxyphénoxypropionate.

7. Plante selon la revendication 5 ou 6, dans laquelle la plante est également résistante à un ou plusieurs herbicides choisis dans le groupe consistant en glyphosate, imidazolinones, glufoninate, sulfonylurées, bromoxynile et dicamba.

8. Plante selon la revendication 5, dans laquelle ledit herbicide est choisi dans le groupe consistant en acide 2,4-dichlorophénoxyacétique, MCPA, dichlorprop, mécoprop, fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, métamifop, cyhalofop, et clodinofop.

9. Plante selon la revendication 5, dans laquelle l'expression dudit polynucléotide rend ladite plante résistante à la fois à un herbicide phénoxy auxine et à un herbicide aryloxyphénoxypropionate.

10. Plante selon la revendication 5, dans laquelle ladite plante comprend en outre un deuxième gène de résistance aux herbicides.

11. Plante selon la revendication 10, dans laquelle ledit deuxième gène de résistance aux herbicides rend ladite plante résistante au glyphosate.

12. Plante selon la revendication 10, dans laquelle ledit herbicide aryloxyalcanoate est le 2,4-D et ledit deuxième gène de résistance à un herbicide rend ladite plante résistante au glyphosate.

13. Plante selon la revendication 5, dans laquelle ladite plante comprend en outre un gène de résistance aux insectes dérivé d'un organisme choisi dans le groupe consistant en *Bacillus thuringiensis, Photorhabdus,* et *Xenorhabdus.*

14. Plante selon la revendication 5, dans laquelle ladite plante comprend en outre un gène pour un trait agronomique choisi dans le groupe consistant en résistance aux champignons, tolérance au stress, rendement accru, profil en huile amélioré, qualité des fibres améliorée, résistance virale, mûrissement retardé, tolérance au froid et tolérance au sel.

15. Plante selon la revendication 10, dans laquelle ledit deuxième gène de résistance aux herbicides rend ladite plante résistante à un herbicide choisi dans le groupe consistant en glyphosate, glufosinate, inhibiteurs de ALS, inhibiteurs de 4-hydroxyphényl-pyruvate-dioxygénase (HPPD), et inhibiteurs de protoporphyrinogène oxydase (PPO).

16. Procédé pour la maîtrise d'au moins une mauvaise herbe dans un champ, dans lequel ledit champ contient au moins une plante selon la revendication 5, tandis que ledit procédé comprend l'application à au moins une portion dudit champ d'un premier herbicide choisi dans le groupe consistant en un herbicide phénoxy auxine et un herbicide aryloxyphénoxypropionate.

17. Procédé selon la revendication 16, dans lequel ledit herbicide est un R-énantiomère d'une phénoxy auxine chirale ou une phénoxy auxine achirale.

18. Procédé selon la revendication 17, dans lequel ledit herbicide est choisi dans le groupe consistant en R-dichlorprop, R-mécoprop, 2,4-D et MCPA.

19. Procédé selon la revendication 16, dans lequel ledit premier herbicide est un aryloxyphénoxypropionate et ladite plante est une monocotylédone.

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20. Procédé selon la revendication 19, dans lequel ladite monocotylédone est choisie dans le groupe consistant en blé, riz, froment, orge, seigle, herbe pour gazon, avoine, sorgho, et herbes pour pâturage.

21. Procédé selon la revendication 16, dans lequel ledit premier herbicide est une phénoxy auxine et ladite plante est une dicotylédone.

22. Procédé selon la revendication 21, dans lequel ladite dicotylédone est choisie dans le groupe consistant en coton, tabac, canola, et soja.

23. Procédé selon la revendication 22, dans lequel ledit procédé comprend l'application d'un deuxième herbicide.

24. Procédé selon la revendication 23, dans lequel ledit premier herbicide et ledit deuxième herbicide sont appliqués séquentiellement ou concurremment.

25. Procédé selon la revendication 23, dans lequel ledit premier herbicide est une phénoxy auxine et ledit deuxième herbicide est un aryloxyphénoxypropionate.

26. Procédé selon la revendication 16, dans lequel ledit procédé est utilisé pour traiter ou prévenir de mauvaises herbes résistantes aux herbicides.

27. Procédé selon la revendication 16, dans lequel ladite plante est résistante au glyphosate.

28. Procédé selon la revendication 17, dans lequel ledit R-énantiomère est un composant d'un mélange racémique.

29. Procédé selon la revendication 23, dans lequel ladite plante comprend en outre un deuxième gène de résistance aux herbicides qui rend ladite plante résistante audit deuxième herbicide.

30. Procédé selon la revendication 29, dans lequel ledit deuxième gène est choisi dans le groupe consistant en une AHAS (acétohydroxyacide synthase), SurA, SurB, Csr1, Csr1-1, Csr1-2, une EPSPS modifiée (5-énolpyruvylshikimate-3-phosphate synthétase), GOX, GAT, PAT, (phosphinothricine-N-acétyltransférase), bar, et une enzyme dégradant le dicamba.

31. Procédé selon la revendication 29, dans lequel ledit deuxième herbicide est choisi dans le groupe consistant en glyphosate, glufosinate, dicamba, inhibiteurs d'acétolactate synthétase, inhibiteurs de protoporphyrinogène oxydase, et inhibiteurs d'hydroxyphényl-pyruvate dioxygénase.

32. Procédé selon la revendication 31, dans lequel ledit deuxième herbicide est un inhibiteur d'acétolactate synthase choisi dans le groupe consistant en imidazolinones, sulfonyl urées, et herbicides de triazolopyrimidine.

33. Procédé selon la revendication 32, dans lequel ledit deuxième herbicide est une imidazolinone choisie dans le groupe consistant en imazamox, imazethapyr, imazaquin, et imazapic.

34. Procédé selon la revendication 29, dans lequel ledit premier herbicide est une phénoxy auxine et ledit deuxième herbicide est choisi dans le groupe consistant en glyphosate et glufosinate.

35. Procédé selon la revendication 34, dans lequel ladite phénoxy auxine est le 2,4-D et ledit deuxième herbicide est le glyphosate.

36. Procédé selon la revendication 29, dans lequel ledit premier herbicide est un aryloxyphénoxypropionate et ledit deuxième herbicide est le glyphosate.

37. Procédé selon la revendication 36, dans lequel ledit premier herbicide est choisi dans le groupe consistant en quizalofop, heoxyfop, et cyhalofop.

38. Procédé selon la revendication 32, dans lequel ledit deuxième herbicide est une sulfonyl urée choisie dans le groupe consistant en amidosulfuron, bensulfuron, chlorimuron, chlorsulfuron, cinosulfuron, flupyrsulfuron, foramsulfuron, halosulfuron, nicosulfuron, primisulfuron, prosulfuron, rimsulfuron, sulfometuron, sulfosulfuron, thifensulfuron, triasulfuron, trifloxysulfuron, et triflusulfuron.

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39. Procédé selon la revendication 29, dans lequel ledit procédé comprend en outre l'application d'un troisième herbicide.

40. Procédé selon la revendication 39, dans lequel lesdits herbicides sont 2,4-D; quizalofop; et glufosinate.

41. Procédé pour la maîtrise des mauvaises herbes dans un champ, dans lequel ledit procédé comprend l'application d'un premier herbicide audit champ et la plantation d'une graine dans ledit champ dans les 14 jours de l'application du premier herbicide, tandis que ladite graine comprend une cellule selon la revendication 1, et tandis que ledit premier herbicide est choisi dans le groupe consistant une phénoxy auxine et un aryloxyphénoxypropionate.

42. Procédé selon la revendication 41, dans lequel ledit premier herbicide est un acide, un sel inorganique, un sel organique, un ester, un isomère R-énanthiospécifique, ou un composant d'un mélange racémique.

43. Procédé selon la revendication 41, dans lequel ladite graine comprend un deuxième gène qui rend ladite plante résistante à un deuxième herbicide, et ledit procédé comprend en outre l'application dudit deuxième herbicide audit champ avant ladite plantation.

44. Procédé selon la revendication 43, dans lequel ledit deuxième herbicide est choisi dans le groupe consistant en glyphosate, gramoxone, et glufosinate.

45. Procédé pour la maîtrise d'au moins une mauvaise herbe dans un champ, tandis que ledit champ contient au moins une plante selon la revendication 5, tandis que ledit procédé comprend l'application à au moins une portion dudit champ d'un premier herbicide qui est un 2,4-D, tandis que ladite plante comprend en outre un deuxième gène de résistance aux herbicides qui rend ladite plante résistante au glyphosate.

46. Graine comprenant une cellule végétale selon la revendication 1.

47. Plante cultivée à partir de la graine selon la revendication 46, dans laquelle ladite plante comprend une cellule végétale transgénique selon la revendication 1.

48. Partie, descendance ou produit de propagation asexuée de la plante selon la revendication 5, dans lequel ladite partie, ladite descendance ou ledit produit de propagation asexuée comprend une pluralité de cellules selon la revendication 1, tandis que l'expression dudit polynucléotide rend lesdites cellules tolérantes à un herbicide aryloxyalcanoate.

49. Procédé pour la maîtrise de mauvaises herbes résistantes au glyphosate dans un champ de plantes cultivées tolérantes au glyphosate, dans lequel lesdites plantes comprennent une cellule végétale transgénique selon la revendication 1, et ledit procédé comprend l'application d'un herbicide aryloxyalcanoate à au moins une portion dudit champ.

50. Procédé selon la revendication 49, dans lequel ledit herbicide est une phénoxy auxine ou un aryloxy phénoxypropionate.

51. Procédé selon la revendication 50, dans lequel ledit herbicide phénoxy auxine est le 2,4-D.

52. Procédé selon la revendication 49, dans lequel lesdites plantes sont des dicotylédones ou des monocotylédones.

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- Addition of O_2 is *stereospecific*
- Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

COR-AAD1-00000421

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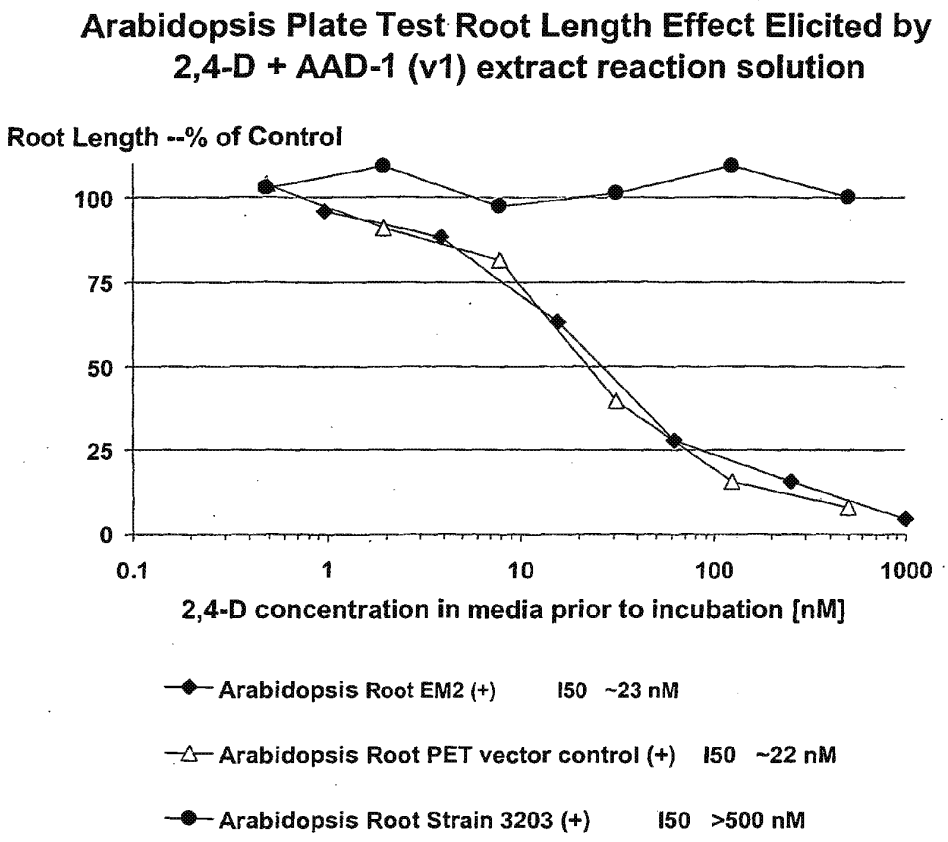


Fig. 2

COR-AAD1-00000422
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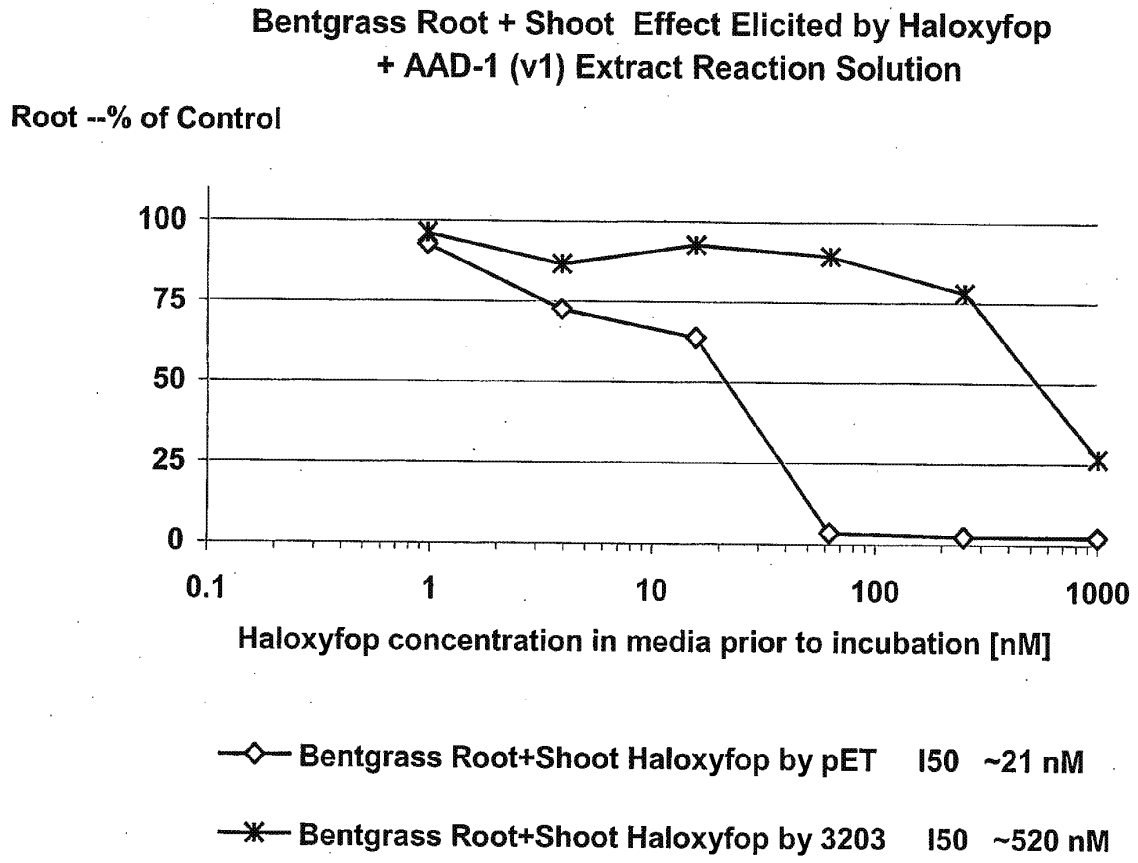


Fig. 3

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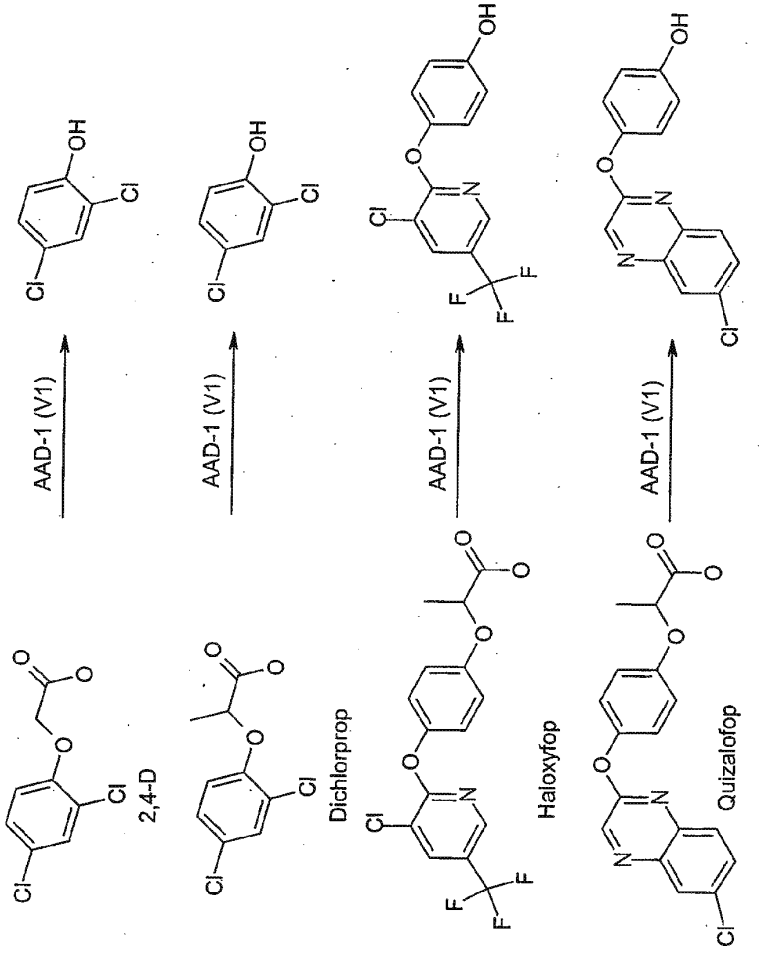


Fig. 4

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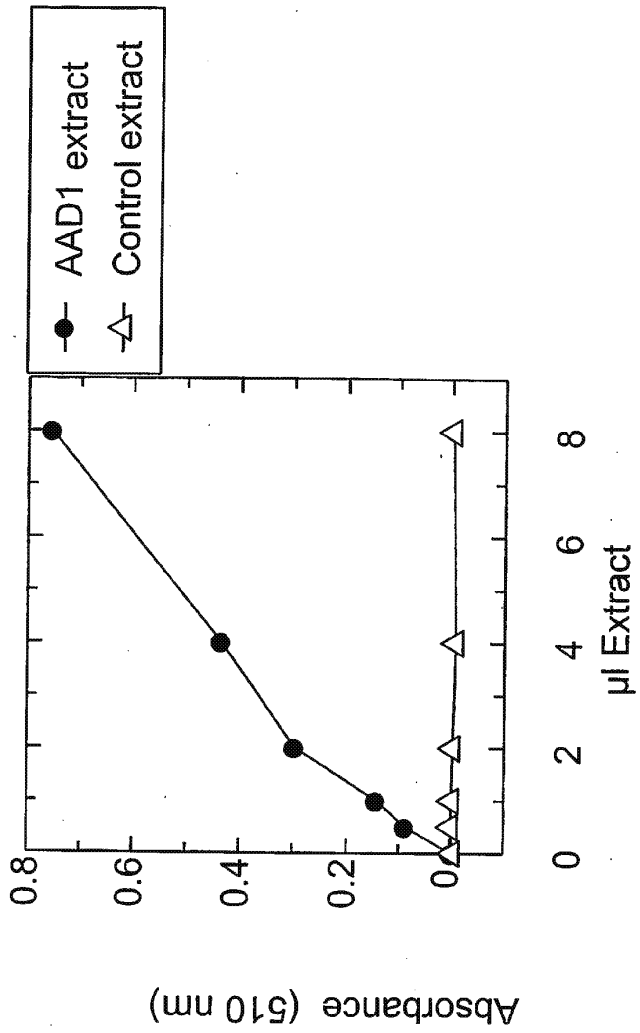


Fig. 5

COR-AAD1-00000425

Fig. 6A

Fig. 6B

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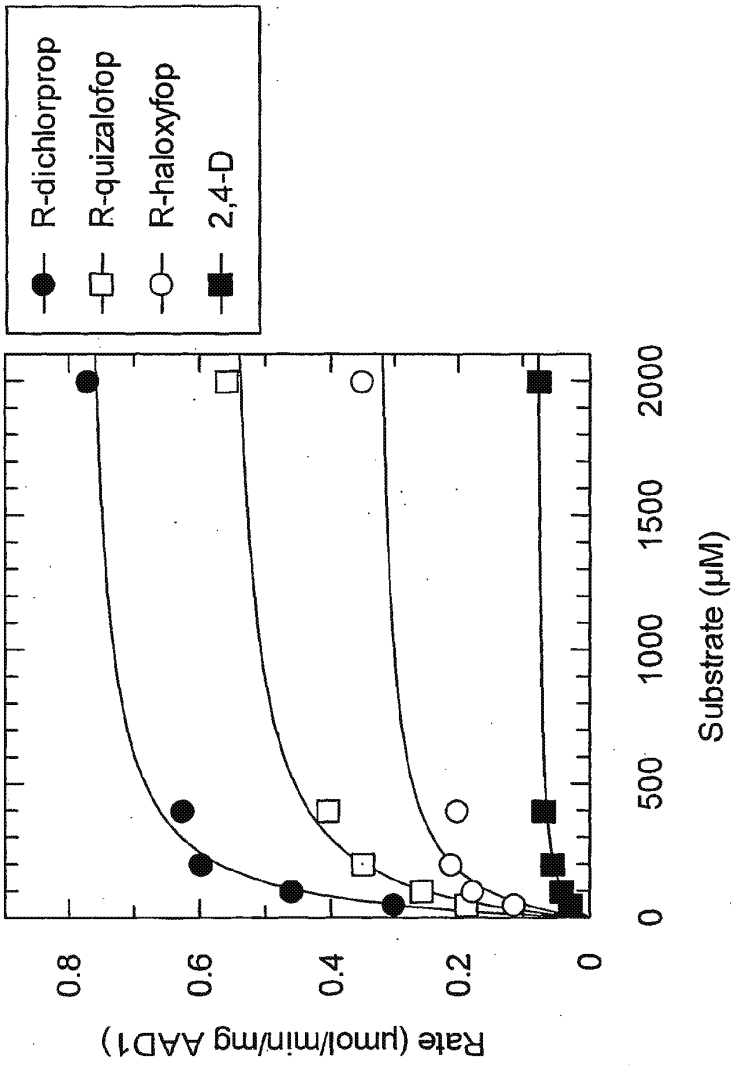


Fig. 7

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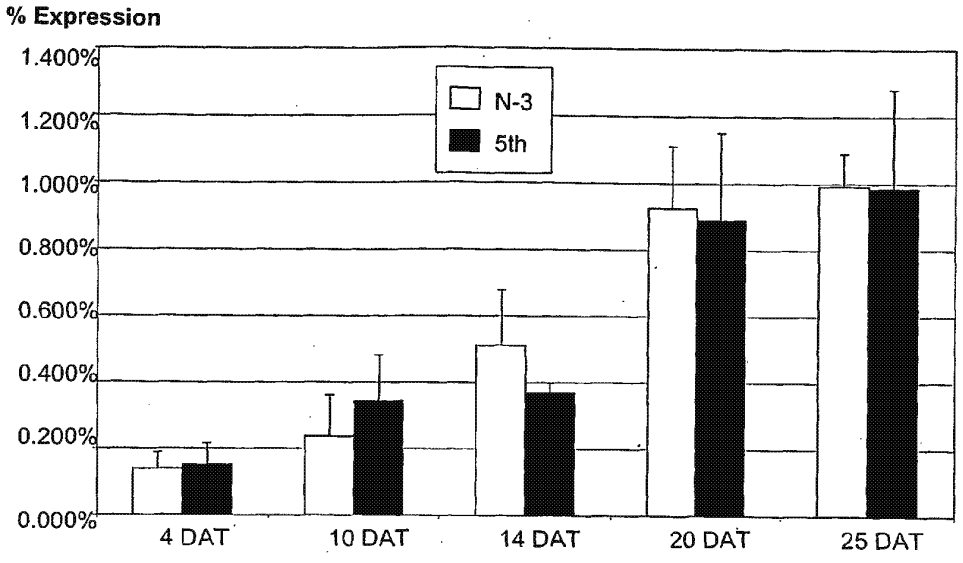


Fig. 8A

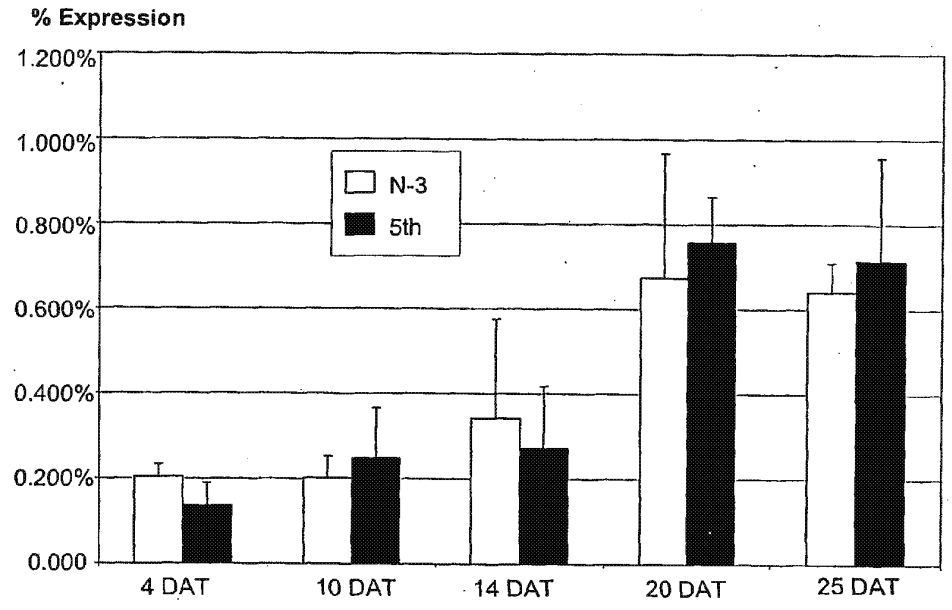


Fig. 8B

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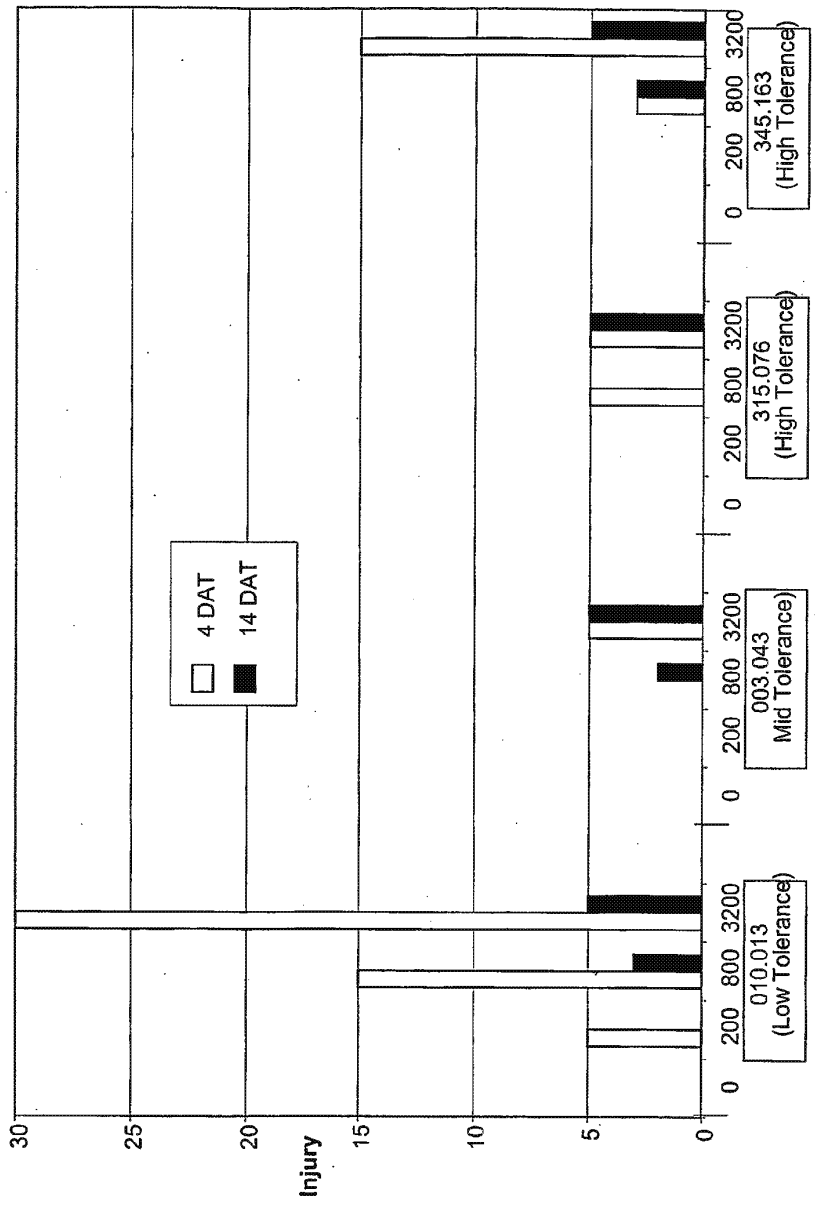


Fig. 9A

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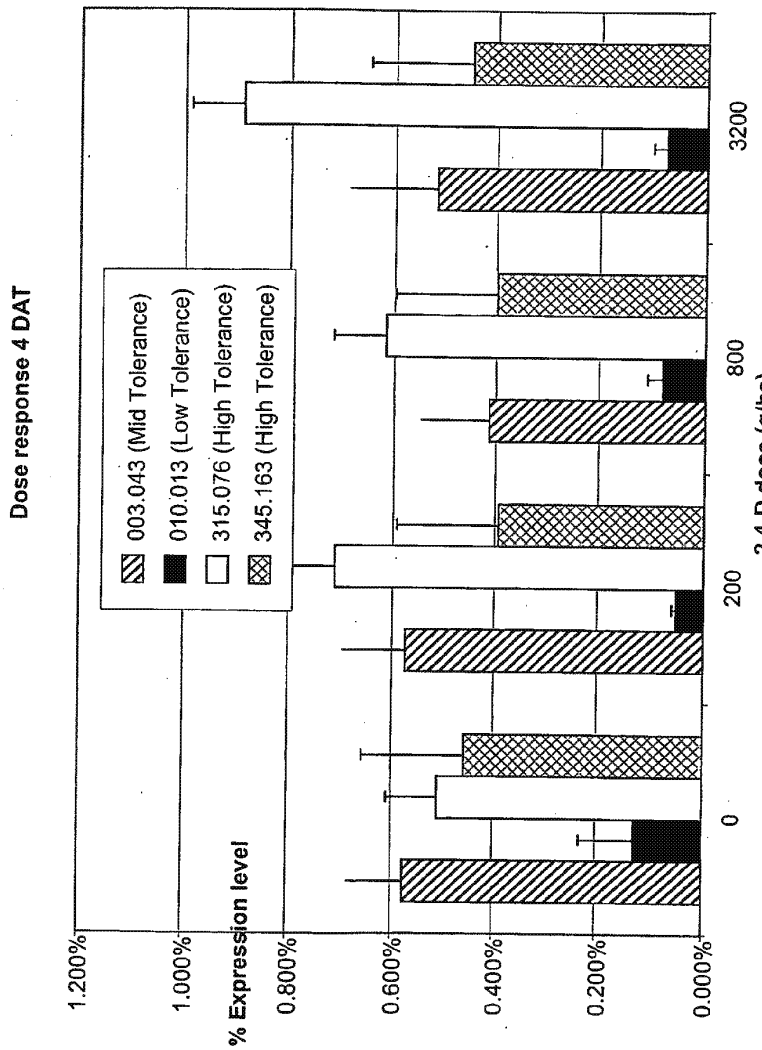


Fig. 9B

COR-AAD1-00000430

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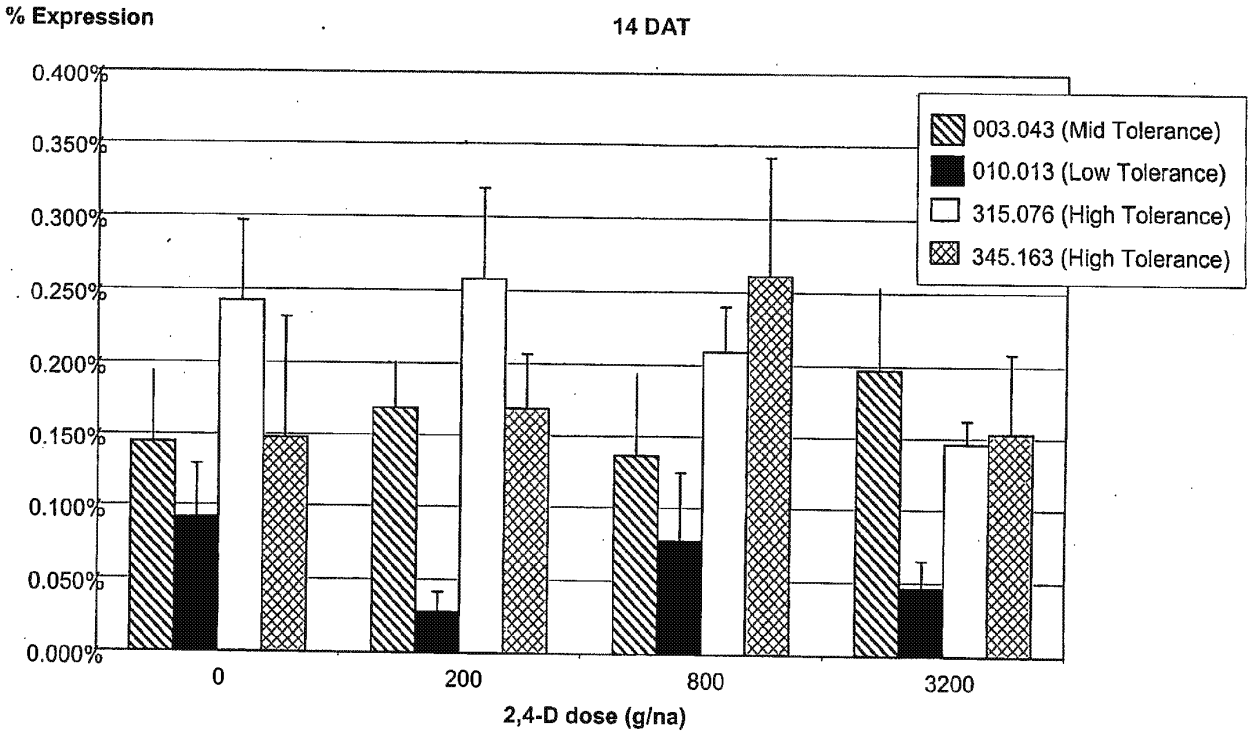


Fig. 9C

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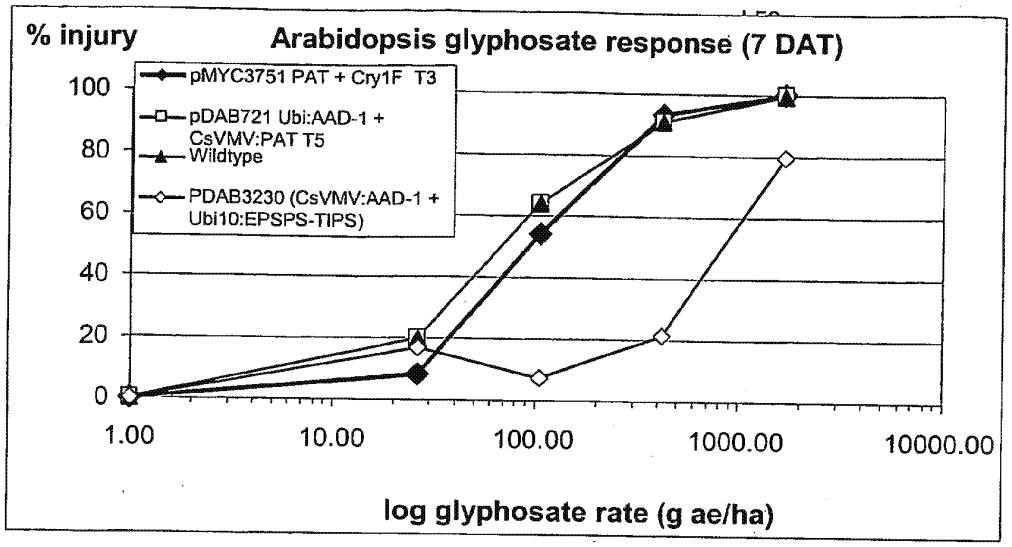


Fig. 10

COR-AAD1-00000432
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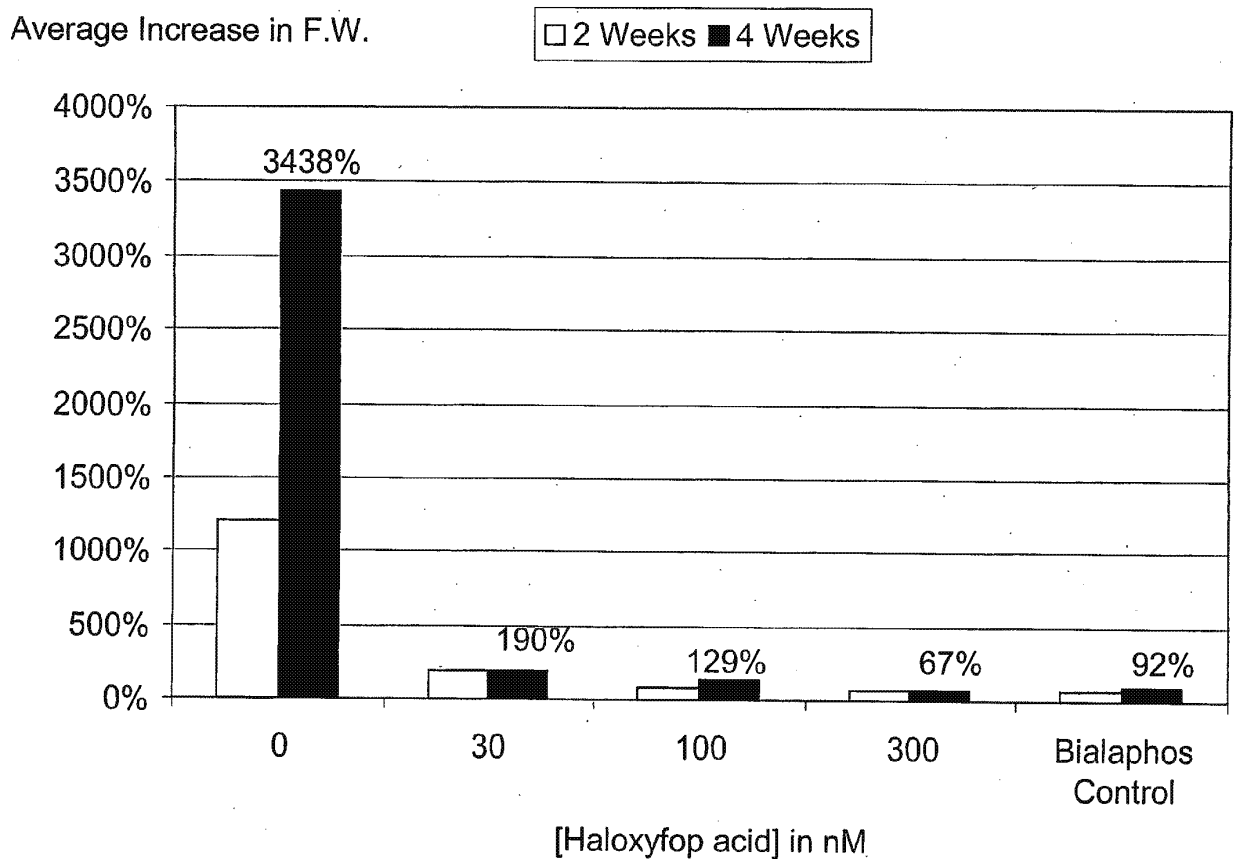


Fig. 11

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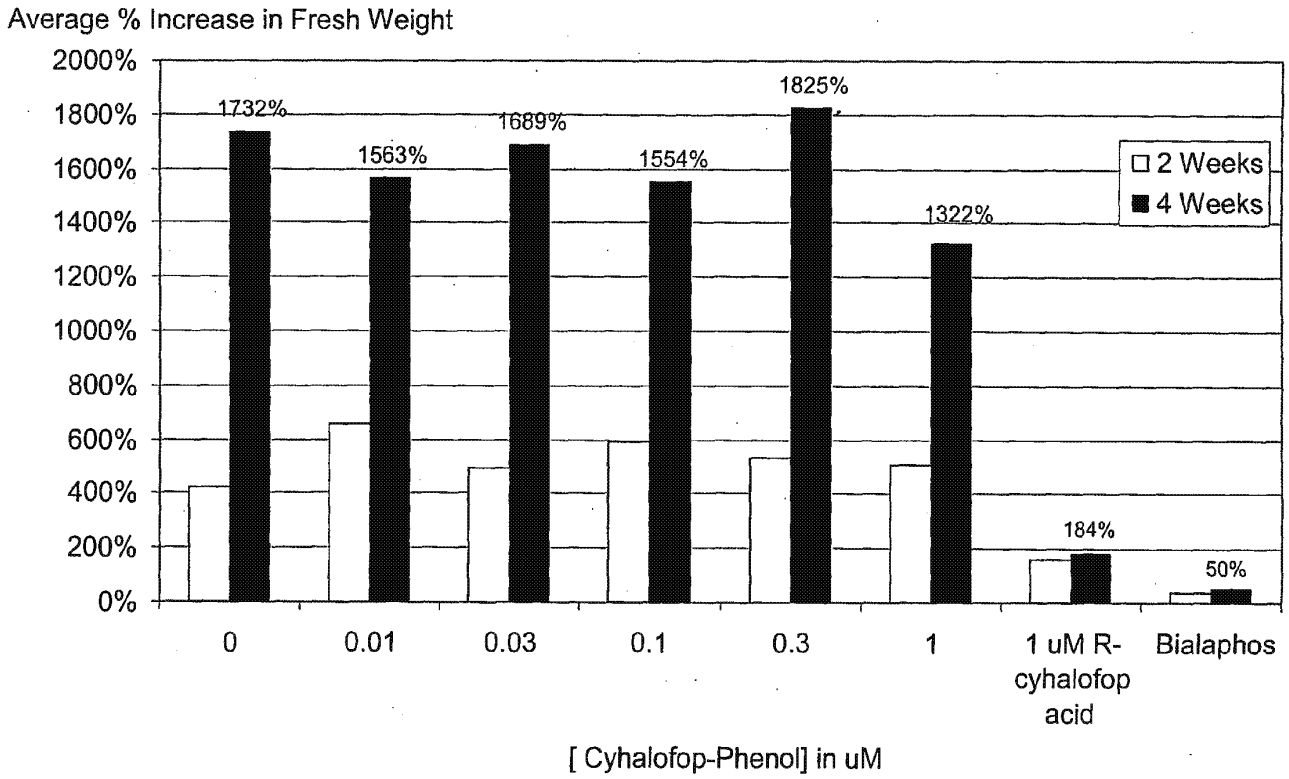


Fig. 12

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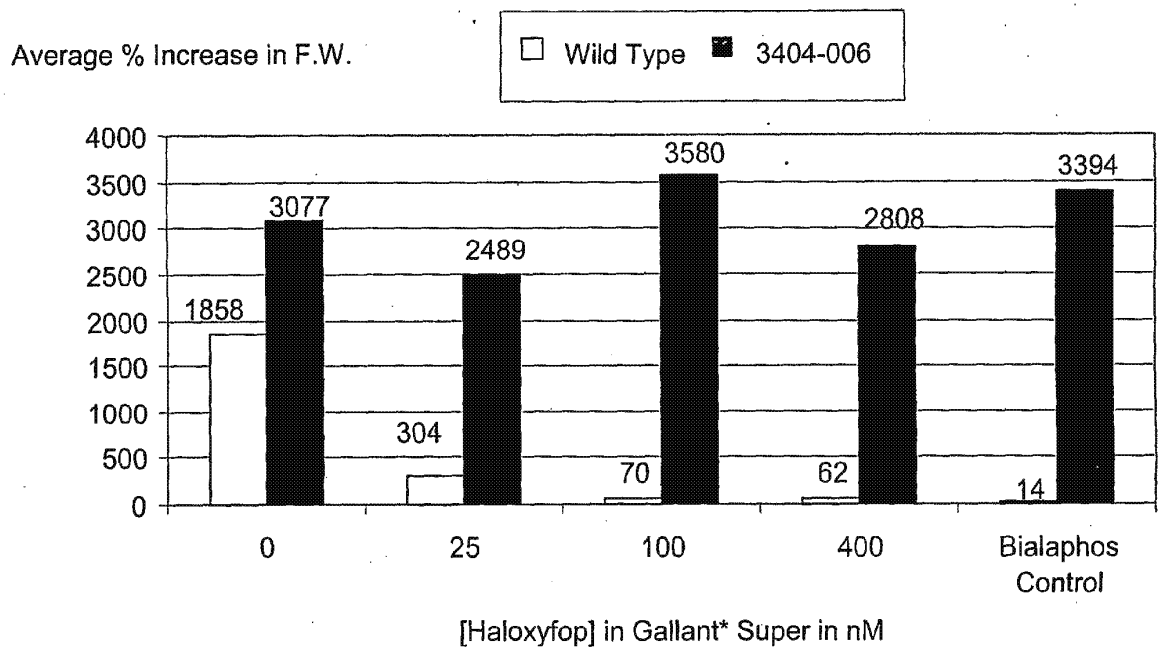


Fig. 13

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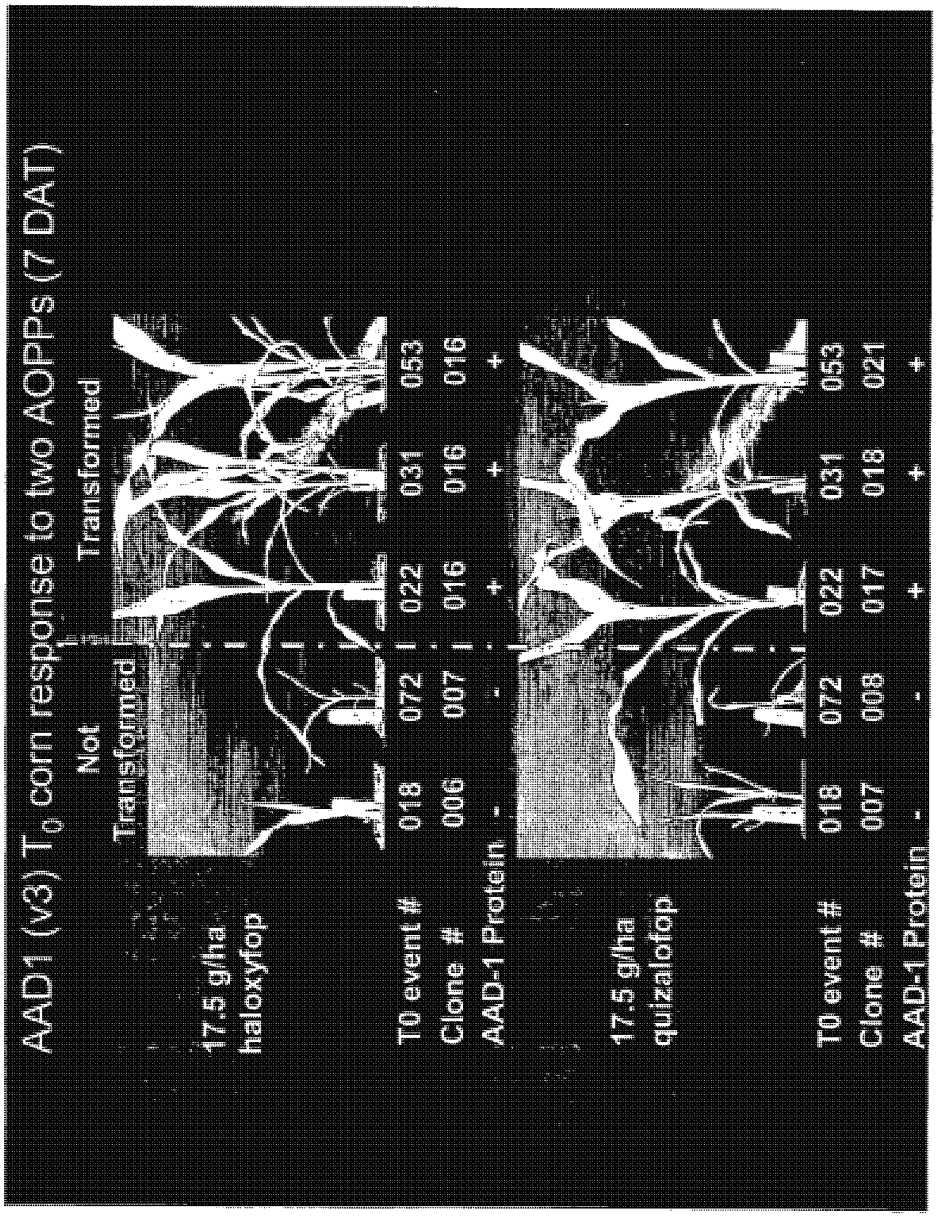


Fig. 14

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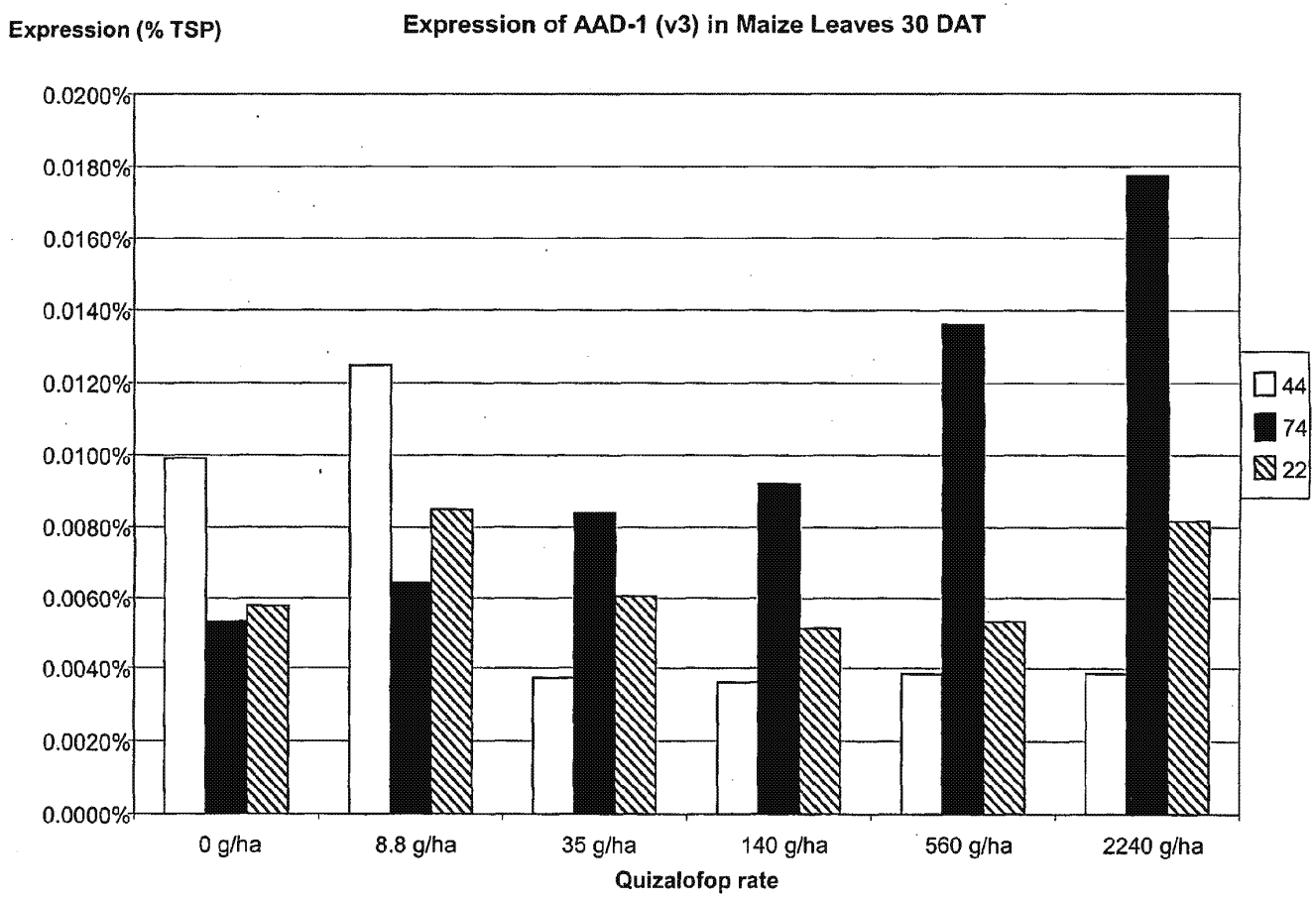


Fig. 15

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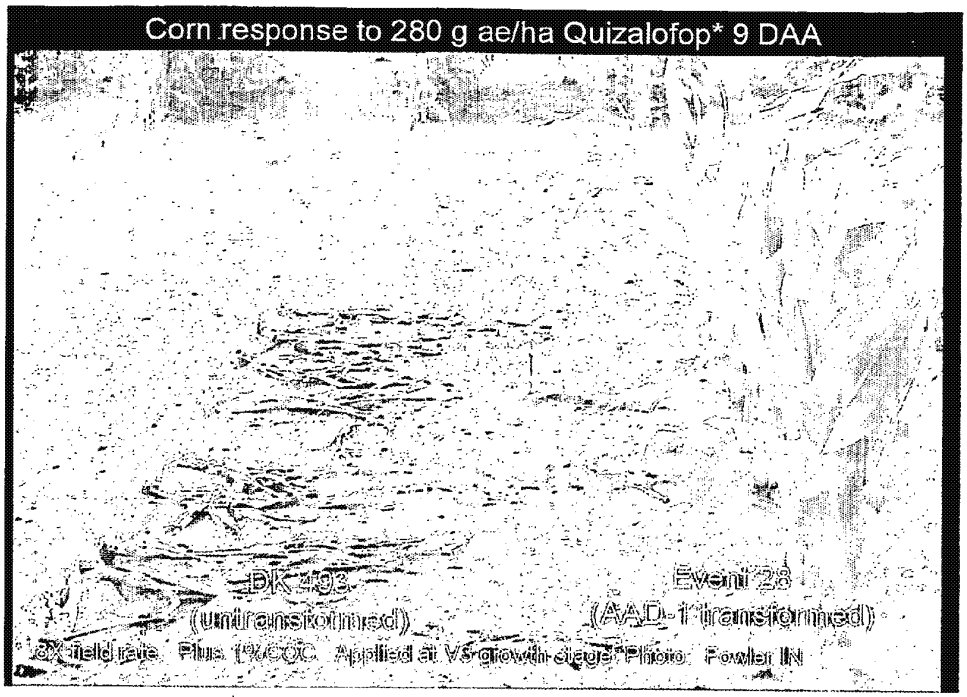


Fig. 16

COR-AAD1-00000438
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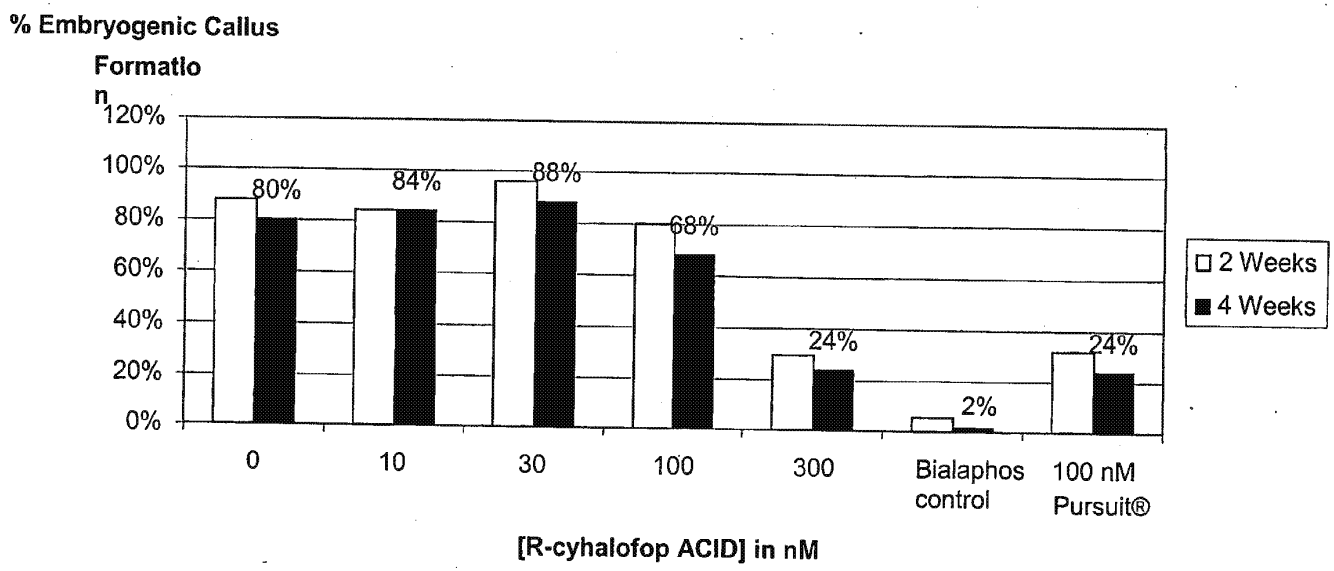
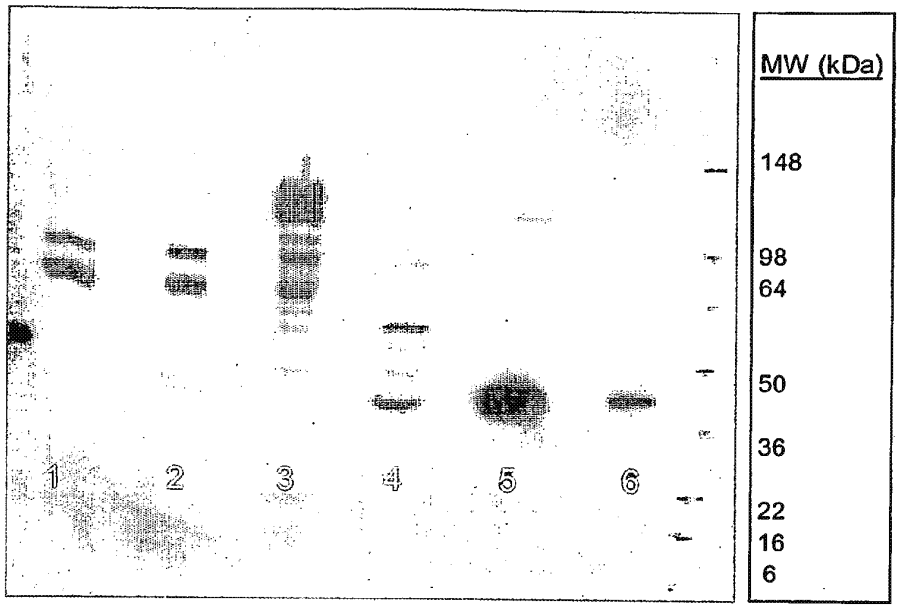


Fig. 17

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Western	
Lane	
1	Calli #1
2	Calli #2
3	Negative
4	AAD1 Corn
5	Standard 5 ug/mL
6	Standard 0.5 ug/mL

Fig. 18

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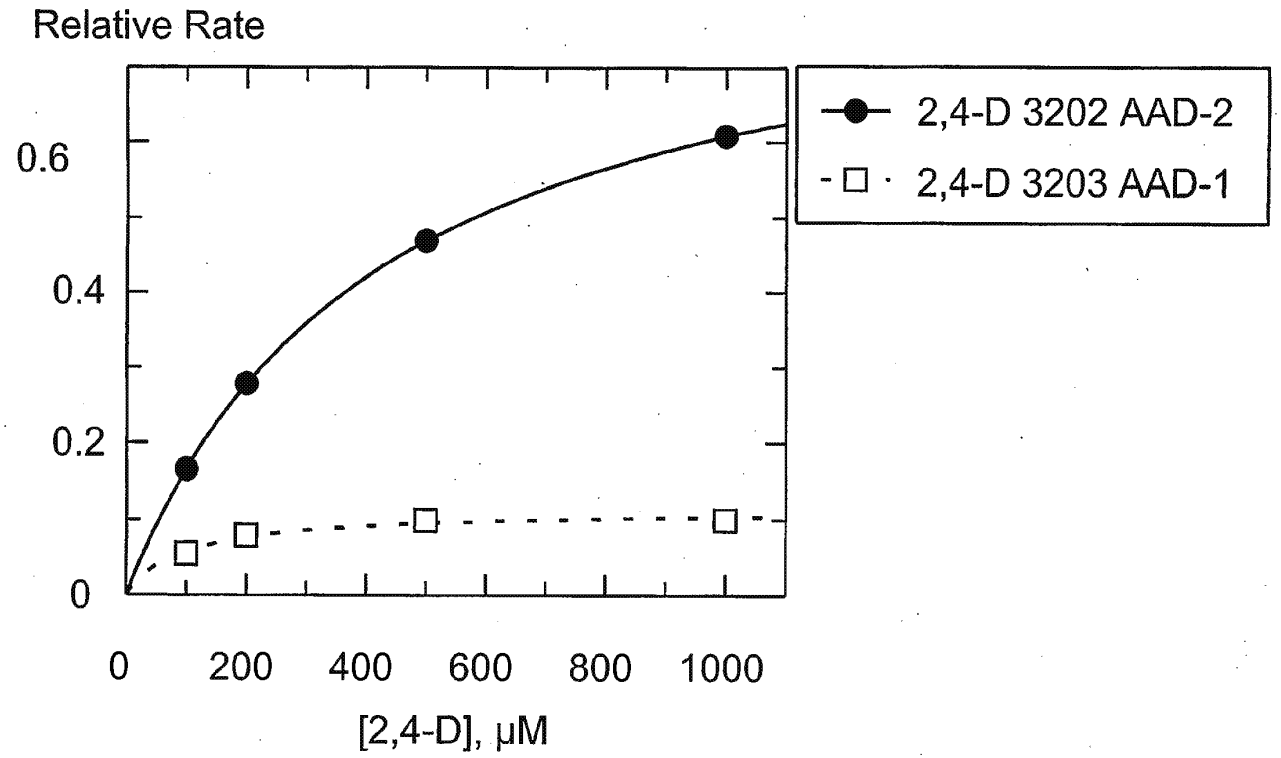


Fig. 19

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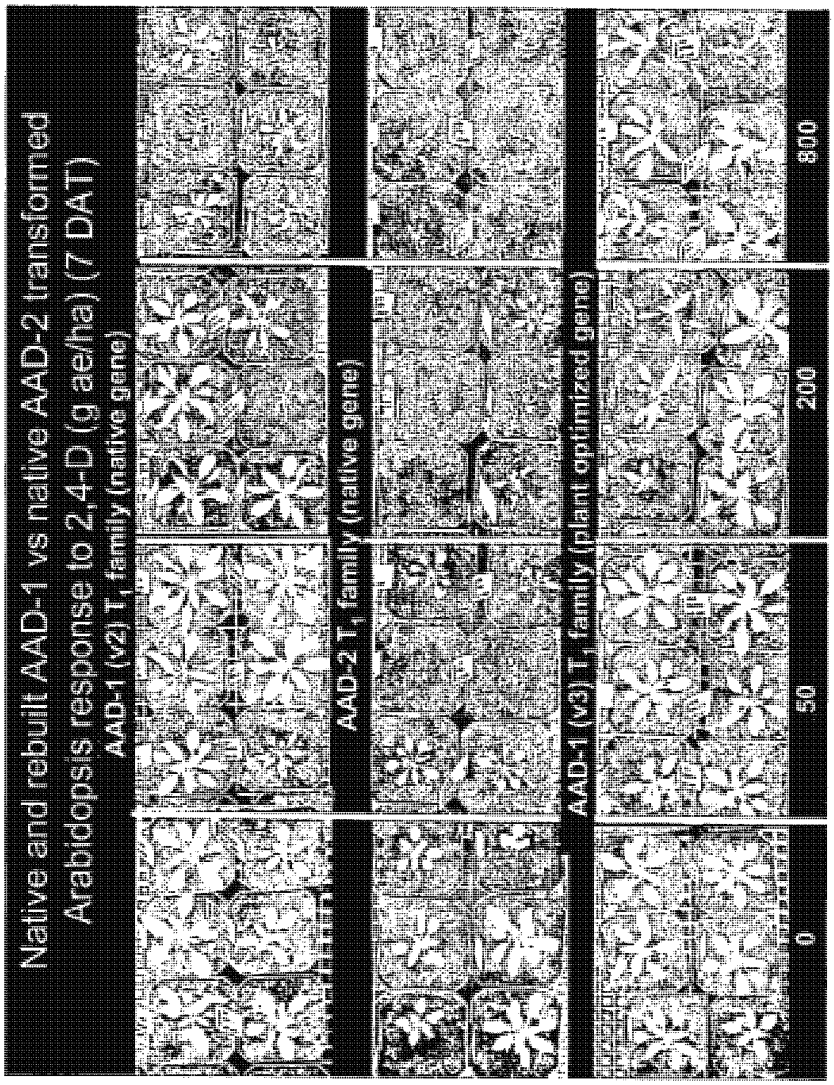


Fig. 20

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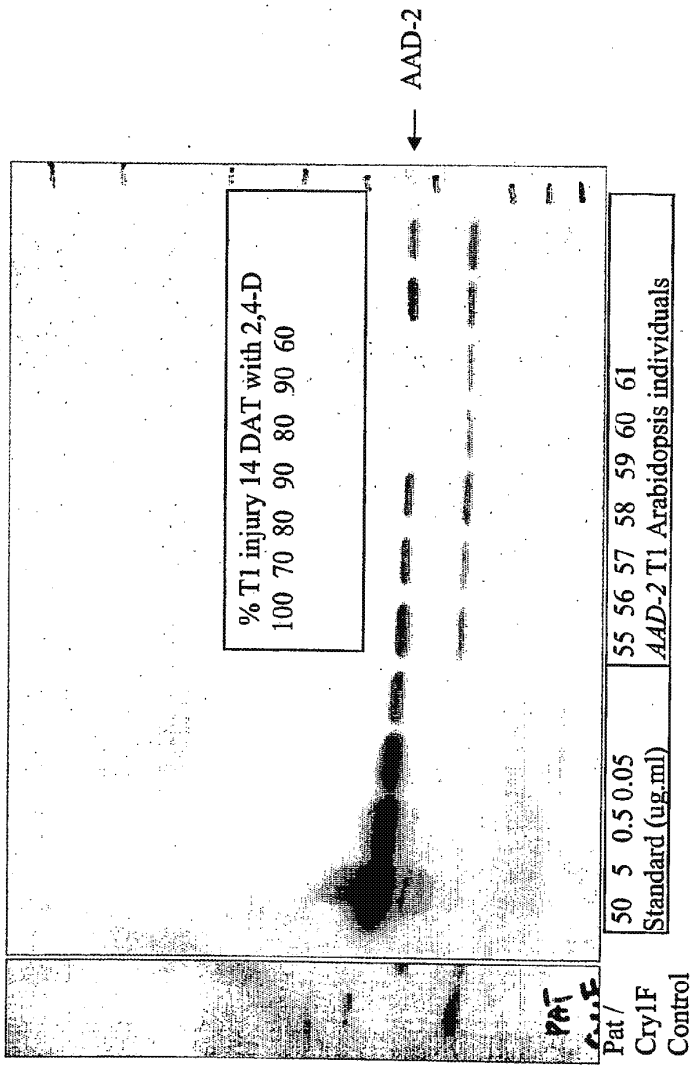


Fig. 21

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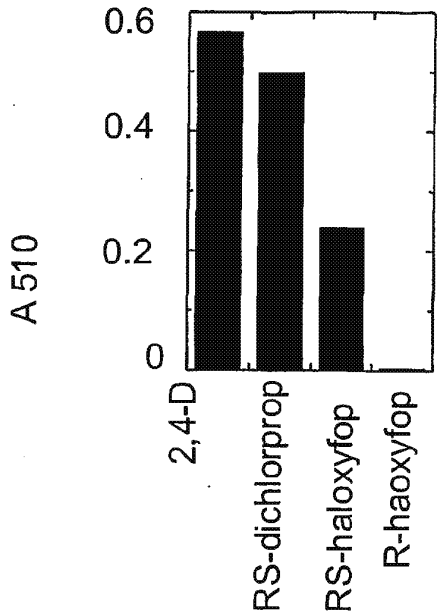


Fig. 22

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REFERENCES CITED IN THE DESCRIPTION

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15288406

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FILE 'AGRICOLA, BIOSIS, CAPLUS, EMBASE, CABA' ENTERED AT 09:11:03 ON 07 NOV 2018

L1 182 S AAD-1
L2 78 S L1 AND DIOXYGENASE
L3 48 DUP REM L2 (30 DUPLICATES REMOVED)

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15288406	CTNF	11/13/2018	14764-258662
	892	11/13/2018	14764-258662
	1449	11/13/2018	14764-258662

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

107540 7590 03/22/2019

Barnes & Thornburg LLP (Dow)
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EXAMINER
KRUSE, DAVID H

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
03/22/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

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COR-AAD1-00000451
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Applicant-Initiated Interview Summary	**Application No.** 15/288,406	**Applicant(s)** WRIGHT et al.	
	Examiner DAVID H KRUSE	**Art Unit** 1663	**AIA (FITF) Status** No

All participants (applicant, applicants representative, PTO personnel):

(1) DAVID H. KRUSE.

(2) John Breen (attorney).

(3) Terry Wright (inventor).

(4) Bill Poirier (company rep).

Date of Interview: 19 March 2019.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☑112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 15.

Identification of prior art discussed: NONE.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The correlation between the AAD-1 motif recited in the claims and the general motif recited in paragraph 0085 on page 17 of the Specification was discussed. The evidence in the Attachment was discussed, specifically that Applicant has modified that required amino acids in the AAD-1 motif, demonstrating that these amino acids are critical for maintaining the claimed function (see claim 15, lines 3 and 4). Applicant pointed out that the motif found in AAD-2 differs from the AAD-1 motif and does not have the claimed function. The Examiner suggested pointing out the correlation between the motif described on page 17 of the Specification and the recited AAD-1 motif found in instant SEQ ID NO: 9. No agreement was reached concerning the "at least 85% sequence identity" limitation in claim 15..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☑ Attachment

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	

COR-AAD1-00000452
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Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

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U.S. Continuation Patent Application Serial No. 15/288,406
Title: **Novel Herbicide Resistance Genes**
Our Matter: [redacted]
Confirmation No. [redacted]
Group 1663

Transmitted are materials to assist with an Examiner's Interview conducted on March 19, 2019 for the above identified matter.

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COR-AAD1-00000454

Bayer EX1008 (Part 1 of 9)

03-19-19;11:23 ;From: To # 2/ 2

AAD-1 Variants Verifying Motif Validity

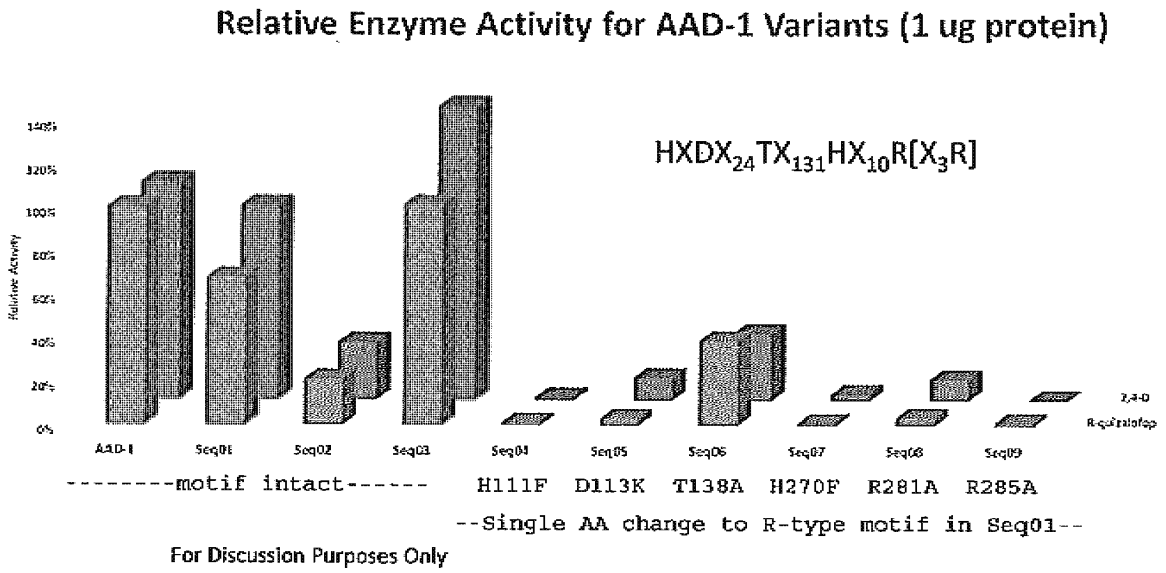
HX(D/E) X_{23-26}(T/S)$X_{114-183}$HX_{10-13}R [PCT 0085] = Group II a-KG (include TauD, TfdA, RdpA, et al)

AAD-1 proteins have novel property of being able to degrade 2,4-D, other phenoxy auxin herbicides, and (R)-AOPP herbicides [0086-0090]. This narrows to specific spacing of key residues HXDX_{24}TX_{131}HX_{10}R[X_3R]

Seq01 - Seq03 = 95% Identity (15 aa changes) to AAD-1 (SEQ ID NO. 9, motif intact)

Remaining Seq's single aa change to motif residues

H111F	Seq04
D113K	Seq05
T138A	Seq06
H270F	Seq07
R281A	Seq08
R285A	Seq09



For Discussion Purposes Only

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In re Application of:

Wright et al.

Serial No.: 15/288,406

Filed: October 7, 2016

For: Novel Herbicide Resistance Genes

Confirmation No.: 5402

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DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. TERRY R. WRIGHT

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I, Terry R. Wright, declare:

1. I am currently a Research Fellow at Dow AgroSciences, LLC, in Indianapolis, Indiana. I am a citizen of the United States of America. I have 28 years of research experience in the fields of agronomy, pesticide science, and plant biotechnology. I received two

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Bachelor of Science degrees (B.S.) in Crop and Soil Science and Agricultural Biochemistry from Michigan State University, a Master of Science (M.S.) degree in Crop Science/Weed Science from Washington State University, and a Doctorate of Philosophy (Ph.D.) from Michigan State University in Herbicide Physiology and Plant Biotechnology. I am a co-inventor on over 35 granted U.S. patents or patent applications, and an author of 15 peer-reviewed scientific journal articles. My research has been in the areas of crop protection and trait discovery and development.

2. I am an inventor for the subject application and am very familiar with the contents of U.S. Patent Application No. 15/288,406 ("the '406 Application") which is a continuation of a national stage filing based on PCT/US2005/014737, published as WO 2005/104737 (hereafter "Wright"). I am an expert in the field of herbicide physiology and herbicide resistance, weed control, and trait discovery and development. I am informed and believe that the claims currently pending in the '406 Application are being amended to reflect those attached hereto as Appendix I.

3. I am informed and believe that in an Office Action of November 13, 2018 in the '406 Application, the U.S. Patent and Trademark Office ("the Office") has rejected claims 15-48 of the '406 Application as allegedly not supported by, or not disclosed in, the specification of the '406 Application.

4. At the time of invention, glyphosate tolerant crops (Roundup Ready) had become the dominant weed control tool in the Americas. Overuse of a single herbicide (glyphosate) was selecting for weeds resistant to glyphosate or shifts of dominant weed species that were naturally less susceptible to glyphosate. The invention resulted from an effort to provide new methods of preventing the development of, and control for, weeds that are resistant (or naturally more tolerant) to herbicides such as glyphosate, by enabling the use of additional modes of action (MOAs) in combination treatments. 2,4-dichlorophenoxyacetic acid (2,4-D), a member of the phenoxy auxin class of herbicides, was identified as an existing commercial herbicide that could provide an additional mode of action to control of glyphosate-resistant-broadleaf weeds in combination with glyphosate. However, key agronomic crops have little or no inherent tolerance to 2,4-D,

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and in order to enable use of mixtures of 2,4-D and glyphosate selectively within these crops, a herbicide tolerance trait would be required.

5. Prior work had identified an enzyme termed TfdA that is involved in the conversion of 2,4-D to herbicidally inactive dichlorophenol, the initial enzymatic step involved in 2,4-D degradation in a number of soil bacteria, in particular from *Cupriavidus necator* (formerly known as *Ralstonia eutrophus*) (as cited in WO 2005/104737 hereafter "Wright" at [0092]) (mentioned in 2, above). TfdA is a member of a broad class of enzymes called Fe(II)/α-ketoglutarate-dependent dioxygenases (α-KG dioxygenases) which catalyze oxygenation reactions on a wide array of biologically important substrates. The current invention sought to identify new, divergent α-KG dioxygenase enzymes that could utilize 2,4-D but having potentially unique substrate specificity.

6. At the time of the present invention, RdpA was an α-KG dioxygenase enzyme identified that had low homology to TfdA but was capable of effectively catalyzing a reaction analogous to that of TfdA. The substrate for RdpA is (R)-dichlorprop, a chiral phenoxypropionate analog of 2,4-D; however, RdpA exhibits very low *in vitro* enzyme activity on 2,4-D. RdpA was enantioselective for (R)-dichlorprop [and other phenoxypropionate auxin herbicides like (R)-mecoprop], with essentially no activity on the (S)-enantiomer. No examination of other herbicidal substrates of RdpA outside of the phenoxy auxin class had previously occurred.

7. As a result of the present invention, it was discovered that RdpA (in the present application, SEQ ID NO:9) could utilize a class of chiral herbicides known as aryloxyphenoxypropionate (AOPP) herbicides as substrates. It was also determined that RdpA exhibited reasonable activity on 2,4-D *in planta*, although chiral phenoxypropionate analogs were clearly preferred substrates. This discovery was significant in that phenoxy herbicides that have an "auxin mimic" MOA almost exclusively control only broadleaf weeds. 2,4-D is the most agronomically important molecule of this class of herbicides and is achiral. With chiral phenoxy auxins, like (R)-dichlorprop, only the (R)-enantiomer is herbicidally active. (S)-enantiomers are not herbicidal, and coincidentally are not substrates for RdpA. The AOPP herbicides have a MOA and spectrum of weed control that is different from, and complementary to, that of

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the phenoxy auxins. The AOPP herbicides' MOA is inhibition of lipid synthesis at the ACCase (Acetyl CoA Carboxylase) enzyme. Additionally, AOPP herbicides control only grassy species, including grass weeds and crops. All commercially-relevant AOPP herbicides have a chiral center, and analogously to the chiral phenoxy auxins such as dichlorprop, only the (R)-enantiomer is herbicidally active and only the (R)-enantiomer is a substrate for RdpA. At the time of invention, no other α-KG dioxygenase enzymes had been reported to be dual MOA α-KG dioxygenases, having the ability to degrade herbicides of two different chemical classes and MOAs. Due to its unique activity and broader chemical substrate range, the enzyme was described in the current patent application as AAD-1 (AryloxyAlkanoate Dioxygenase) (Wright at [0018], [0097]).

8. No prior effort to express RdpA in plants had been reported. For the first time, the present invention demonstrated tolerance to both 2,4-D (and other phenoxy auxins) and AOPP herbicides when expressed in monocot (grassy) crops or dicot (broadleaf) crops. This is highly valuable when used in transgenic crops, enabling use of alternative MOAs in combination with other trait-enabled chemistries (e.g., glyphosate, glufosinate, imidazolinone herbicides, *inter alia*) for weed control and resistance management. This provides tolerance to two different MOAs (individually targeting broadleaf and grass weeds, respectively) with a single gene / enzyme activity. (Wright at [0016-0020])

9. At the time of the present invention, certain sub-groups of α-KG dioxygenases had been identified based on certain structural similarities and enzymatic reactions catalyzed. A motif of the so-called Group-II α-KG dioxygenases (which included TauD, clavaminate synthase, TfdA, *inter alia*) was identified and refined by several investigators and cited in Wright at [0085]: $HX(D/E)\ X_{23-26}(T/S)X_{114-183}HX_{10-13}R$. The subject invention is further described as preferred enzymes and genes for use in degradation of 2,4-D, other phenoxyalkanoic (phenoxy) auxin herbicides, and AOPP herbicides by a recombinantly expressed AAD-1 enzyme. (Wright at [0086-0087])

10. The present specification describes utilizing this type of enzyme (a dual MOA α-KG dioxygenase) to render plants resistant to 2,4-D and AOPP herbicides. (Wright at [0093]). The invention relates to the identification and use of genes encoding an AAD-1 type

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enzyme capable of degrading phenoxy auxin and AOPP herbicides. (Wright at [0098]). The Wright specification describes how one skilled in the art would readily recognize proteins (and genes) of the subject invention (dual MOA degradation) and their use as herbicide resistance proteins (Wright at [0114], [0116], and [0122]). Variant proteins (or equivalent proteins) of the subject invention are described as having amino acid substitutions as long as the proteins retain the desired biological/functional activity (dual MOA herbicide degradation). (Wright at [0135] and [0142]).

11. As stated in Wright at [0142]: "Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having same or similar activity of the exemplified proteins."

12. Example 17 (Wright at [0433]-[0451]) describes another low-identity TfdA homologue, AAD-2, that was two times more efficient in 2,4-D degradation versus AAD-1v1 in *in vitro* assays. However, AAD-2 was unable to degrade (R)-haloxyfop or (R)-quizalofop, or (R)-cyhalofop , all of which belong to the (R)-AOPP class of herbicides. (Wright at [0441] and Table 33). AAD-2, however, did show enzymatic activity on the non-herbicidal, (S)-enantiomer of cyhalofop. AAD-2, therefore, is <u>not an example</u> of dual MOA degradation of herbicides [2,4-D + (R)-AOPP herbicides]. Sequence alignments of AAD-1v1 (SEQ ID NO: 9 of the present invention) and AAD-2 (SEQ ID NO: 13) are presented in Appendix II. The alignments, and subsequent 'mapping' of the defined residues and undefined spacer residues (X_n) demonstrate that each AAD-1 variant [(R)-AOPP specific] and the AAD-2 enzymes [(S)-AOPP specific] were effectively described by the general Group II α-KG dioxygenase motif enzymes motif: HX(D/E) X_{23-26}(T/S)$X_{114-183}$HX_{10-13}R (Wright at [0085]). However, the large undefined residue spacer between the defined T/S and H was clearly different: all examples having dual MOA herbicide degradation activity [(R)-type AOPP + 2,4-D activity] had a 131-amino acid spacer, whereas the (S)-type AOPP + 2,4-D activity (as exemplified by AAD-2) had an analogous spacer between the (T/S) residue and second H reside of the motif that was only 121 amino acids in length. One skilled in the art would recognize the much more

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limited group having the desired functional dual-MOA activity would be defined by the more refined structure of AAD-1 motif $HXDX_{24}TX_{131}$ $HX_{10}R$, which effectively excludes AAD-2.

13. Further in Appendix II, three exemplified AAD-1 variants having the desired functional dual-MOA activity (Examples 3, 4, 5, and 6 in Wright at [0199]-[0288]) are aligned and all demonstrate the AAD-1 motif $HXDX_{24}TX_{131}$ $HX_{10}R$, with defined AAD-1 motif residues highlighted in blue in the aligned AAD-1 sequences. An additional R285 residue in highlighted in gray. At the time of the invention, the analogous arginine in TfdA had been concluded to be involved in 2,4-D carboxylate binding [Dunning Hotopp Biochemistry 41:9787-9794 (2002)], was considered to be potentially involved in a similar role in aryloxyalkanoate herbicide binding in AAD-1, and was present in all examples of AAD-1-type (R)-enzymes, adding an additional four amino acid structure to the AAD-1 motif [in brackets] $HXDX_{24}TX_{131}$ $HX_{10}R[X_3R]$.

14. I was asked by counsel if I could demonstrate the importance of these defined residues in the AAD-1 motif $HXDX_{24}TX_{131}HX_{10}R$ to the desired dual MOA herbicide degradation activity. In addition, I also tested the relevance to that activity of the additional conserved $[X_3R]$ residue. I designed an experiment with one of our junior scientists (Samantha Griffin) to show that the defined amino acids in the AAD-1 motif are real and important. Three variants of AAD-1v1 were created with ~94-95% amino acid identity to AAD-v1 (Seq01-Seq03) and having the AAD-1 motif intact. Six variants of Seq01 were designed, each individually with a single amino acid substitution to one of the defined AAD-1 motif residues (Seq04-Seq09). Alignments of all nine variants and the original AAD-1v1 (SEQ ID NO: 9) are shown in Appendix III. Defined AAD-1 motif residues are highlighted in blue in the AAD-1v1 sequence, and the additional R285 residue in highlighted in gray. Each individual mutation of the defined AAD-1 motif is highlighted in pink in Appendix III. Each variant was expressed in *E. coli* and enzyme activity assessed as described in Appendix III.

15. The relative activity of AAD-1v1 and nine variants (3 with the AAD-1 motif intact, 6 with individual changes to the defined residues of the motif) were compared. Seq01 and Seq03, which preserved the motif, showed on average similar activity to 2,4-D and (R)-

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quizalofop [an (R)-AOPP herbicide)] substrates as AAD-1 (RdpA). Recovery of enzymes Seq02 and Seq05 was low due to a large proportion of expressed protein insolubility. The quantity of enzyme in these samples is likely underestimated (lower than targeted 1 ug protein used in other assays), with the reported values in Appendix III being likely less than expected. When defined amino acids of the motif were mutated, as in Seq04-Seq09, 4 of 5 residues resulted in 90-100% reduced activity. The single-residue change $T_{138}A$ resulted in a sequence that still retained >30 % activity but lost 60-70% of activity against 2,4-D and (R)-quizalofop. This shows both the importance and necessity of each of the defined residues in the AAD-1 motif, $HXDX_{24}TX_{131}HX_{10}R$, as well as the additional conserved [X_3R] residue, for dual MOA activity.

16. For the above reasons, it is evident to me, one skilled in the art, that the specification of the '406 Application supports the refined and narrowed AAD-1 motif, $HXDX_{24}TX_{131}HX_{10}R$ as defining a critical structure of a set of preferred enzymes (and genes encoding them) able to degrade 2,4-D, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxypropionate herbicides [(R)-enantiomers] by recombinantly expressed AAD-1 enzymes. The data also supports the importance of the addition [X_3R] residue for the dual MOA activity. A number of AAD-1 variants were enabled at the time of the invention. Broader examples demonstrating significant variation in the protein structure can be accommodated so long as the defined AAD-1 motif is intact, maintaining the desired dual MOA herbicide tolerance to 2,4-D and (R)-AOPP herbicides.

17. In the foregoing paragraphs, I have referred to the generic motif structure. I note for clarity's sake that the motif of claim 15, and further restricted motif of claim 16, represent the same motif, but denote the undefined amino acid spacers in terms of the residue numbers aligning to SEQ ID NO:9.

18. Interestingly, since the time of the present invention, I am unaware of any new enzyme examples of the AAD-1 dual MOA degradation activity which does not comprise the defined and claimed motif. This indicates to me that enzymes having the dual MOA activity are quite rare.

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19. I hereby declare that all statements made herein are true and that they are based on my own knowledge, information and belief.

Place and Date:

4/10/2019

Indianapolis, Indiana

By:

Terry R. Wright

Research Fellow

Dow AgroSciences LLC

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Appendix I. Amended Claimed Subject Matter

Claims

1-14. (Canceled).

15. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids.

16. The plant cell of claim 15 wherein said AAD-1 motif has the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R(X)_{282-284}R$, wherein $(X)_{282-284}$ represents a sequence of 3 amino acids.

17. (Canceled).

18. The plant cell of claim 15 wherein the AAD-1 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 9.

19. The plant cell of claim 15 wherein said plant cell is dicotyledonous and

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selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an Arabidopsis cell.

20. The plant cell of claim 15 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

21. A transgenic plant comprising a plurality of the plant cell of claim 15, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

22. The plant of claim 21 wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

23. The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

24. The plant of claim 21 wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

25. The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

26. The plant of claim 21 wherein said plant further comprises a second herbicide resistance gene.

27. The plant of claim 26 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

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28. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 21, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide.

29. The method of claim 28 wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

30. The method of claim 28 wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

31. The method of claim 28 wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

32. The method of claim 28 wherein said method comprises applying a second herbicide.

33. The method of claim 32 wherein said first herbicide and said second herbicide are applied sequentially.

34. The method of claim 32 wherein said first herbicide and said second herbicide are applied concurrently.

35. The method of claim 32 wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

36. The method of claim 32 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

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37. The method of claim 32, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

38. The method of claim 32, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

39. The method of claim 32 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

40. The method of claim 39 wherein said second gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

41. The method of claim 40 wherein:

(a) said modified ALS gene is selected from the group consisting of a SurA, SurB, Csr1, Csr1-1, Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT; and,

(c) said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

42. The method of claim 32 wherein said method further comprises applying a third herbicide.

43. The method of claim 42, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

44. The method of claim 43 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

45. A seed comprising a plurality of the plant cell of claim 15.

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46. A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim 45 in said field within 14 days of applying said aryloxyalkanoate herbicide.

47. A plant grown from the seed of claim 45.

48. A part, progeny, or asexual propagate of the plant of claim 47, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

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Appendix II

Alignment of AAD-1v1 (SEQ ID NO. 9 of the present invention) (having α-KG dioxygenase activity on AOPP (R)-enantiomers and 2,4-D or other phenoxy auxins) amino acid sequence with AAD-2 (having α-KG dioxygenase activity on 2,4-D and certain non-herbicidal (S)-enantiomers of AOPP and phenoxypropionate auxin analogs). Defined AAD-1 motif residues are highlighted in blue in the AAD-1v1 sequence, and the additional R285 residue in highlighted in gray. The analogous residues in AAD-2 are also highlighted. The red box identifies a region of the alignment where AAD-2 is 10 amino acids shorter compared to the "X_{131}" amino acid spacer in the AAD-1 motif. Only sequences with precisely 131 amino acid spacer.

```
                1                                                                                 80
AAD-1v1     (1) MHAALSPLSQRFERIAVQPLTGVLGAEITGVDLRRPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFG------
  AAD-2     (1) -------------MTIAIRQLQTHFVGQVSGLDLRKPLTPGEAREVESAMDKYAVLVFHDQDITDEQQMAFALNFGQREDA
                81                                                                               160
AAD-1v1    (76) ---PVDPVPLLKSIEGYPEVQMIRREANESGR---------VIGDDWHVDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMY
  AAD-2    (69) RGGTVTKEKDYRLQSGLNDVSNLGKDGKPLAKDSRTHLFNLGNCLMNSDSSFRPIPAKFSLLSARVVNPTGGKSEFADMR
               161                                                                               240
AAD-1v1   (145) TAWETLSPTMQATIEGLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGRKGLYVNQVYCQR
  AAD-2   (149) AAYDALDDETKAEIEDLVCEHSLMYSRGSLGFTEYTDEE---------KQMFKPVLQRLVRTHPVHRRKSLYLSSHAGK
               241                                                                         317
AAD-1v1   (225) IEGMTDAESKPLLQFLYEHATRFDFTCRVRWKKDQVLVWDNLCTMHEAVPDYAGKRDILTRTTVGGVRPAR------
  AAD-2   (219) IASMSVPEGRLLLRDLNEHATQPEFVYVHKWKLHDLVMWDNRQTMHEVRRYDQSQPDMRRATVAGTEPTVQQQAAE
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Demonstration how the AAD-1 motif having dual MOA activity on (R)-AOPP herbicides and 2,4-D is derived from the general α-KG dioxygenase motif having known activity on 2,4-D and certain non-herbicides like taurine or (S)-AOPP enantiomers like .

General α-KG dioxygenase motif:	X	(R/E)	X_{23-26}	(R /S) $X_{114-383}$	X_{10-13}	(from Wright [0085])
Active on R-AOPP + 2,4-D herbicides						
AAD-1v1	X	R	X_{24}	R X_{131}	X_{10}	(from Wright SEQ ID No.9)
AAD-1v2	X	R	X_{24}	R X_{131}	X_{10}	(from Wright SEQ ID No.10)
AAD-1v3	X	R	X_{24}	R X_{131}	X_{10}	(from Wright SEQ ID No.11)
Active on 2,4-D and non-herbicidal S-AOPPs and not R-AOPPs						
AAD-2	X	R	X_{24}	R X_{121}	X_{10}	(from Wright SEQ ID No.13)

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Demonstration how the three enabled AAD-1 variant enzymes having dual MOA activity on (R)-AOPP herbicides and 2,4-D conforms to the specific AAD-1 motif: $HXDX_{24}TX_{131}HX_{10}R$. AAD-1v1 is the wildtype enzyme. Changes from wildtype are highlighted in light green. Defined AAD-1 motif residues are highlighted in blue in the AAD-1v1 sequence, and the additional R285 residue in highlighted in gray. The analogous arginine in TfdA had been concluded to be involved in 2,4-D carboxylate binding, was expected to play a similar role in aryloxyalkanoate herbicide binding in AAD-1, and was present in all examples.

```
                1                                                                                80
    AAD-1v1     (1) M-HAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDP
    AAD-1v2     (1) M-HAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDP
    AAD-1v3     (1) MHAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDP
AAD-1 motif         --------------------------------------------------------------------------------

                81                                                                              160
    AAD-1v1    (80) VPLLKSIEGYPEVQMIRREANESGRVIGDDWKSTFLDAPPAAVVMRAIDVPEHGGLGFLSMYTAWETLSPTMQATIE
    AAD-1v2    (80) VPLLKSIEGYPEVQMIRREANESGRVIGDDWKSTFLDAPPAAVVMRAIDVPEHGGLGFLSMYTAWETLSPTMQATIE
    AAD-1v3    (81) VPLLKSIEGYPEVQMIRREANESGRVIGDDWKSTFLDAPPAAVVMRAIDVPEHGGLGFLSMYTAWETLSPTMQATIE
AAD-1 motif         ----------------------------HXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
                                               HX<----------X24------------>T<  --------------------

                161                                                                             240
    AAD-1v1   (160) GLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGREGLYVNQVYCQRIEGMTDAESKPLLQF
    AAD-1v2   (160) GLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGREGLYVNQVYCQRIEGMTDAESKPLLQF
    AAD-1v3   (161) GLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGRKGLYVNQVYCQRIEGMTDAESKPLLQF
AAD-1 motif         XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
                    ----------------------------------------X131-----------------------------------

                241                                                                         296
    AAD-1v1   (240) LYEHATRFDFTCRVRWKKDQVLVWDNLCTMRAVPDYAGKFYLTRTTVGGVRPAR
    AAD-1v2   (240) LYEHATRFDFTCRVRWKKDQVLVWDNLCTMRAVPDYAGKFYLTRTTVGGVRPAR
    AAD-1v3   (241) LYEHATRFDFTCRVRWKKDQVLVWDNLCTMRAVPDYAGKFYLTRTTVGGVRPAR
AAD-1 motif         XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXR-----------
                    ------------------------------>H<----X10--->H X3R
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Appendix III

Alignment of AAD-1v1 (SEQ ID NO. 9 of the present invention) (having dual MOA α-KG dioxygenase activity on AOPP (R)-enantiomers and 2,4-D or other phenoxy auxins) amino acid sequence with new AAD-1 variants demonstrating the importance of the defined residues of the AAD-1 motif to the dual MOA α-KG dioxygenase activity. Defined AAD-1 motif residues are highlighted in blue in the AAD-1v1 sequence, and the additional R285 residue in highlighted in gray. Three variants of AAD-1 were created with ~94-95% amino acid identity to AAD-v1 (Seq01-Seq03) and have the AAD-1 motif intact. Six variants of Seq01 were designed, each individually with a single amino acid change to one of the defined AAD-1 motif residues (highlighted in pink). Protein variants were expressed in *E. coli* using a Histidine tag for rapid isolation. Enzyme activity of the nine variants were assayed *in vitro* and compared to the activity of AAD-1v1 under the same conditions described in 'Wright'. Additional changes from the original AAD-1v1 sequence (other than the defined motif residues) are highlighted in light green.

Seq01-Seq03 are 94-95% identical to AAD-1v1 and have the AAD-1 motif intact.

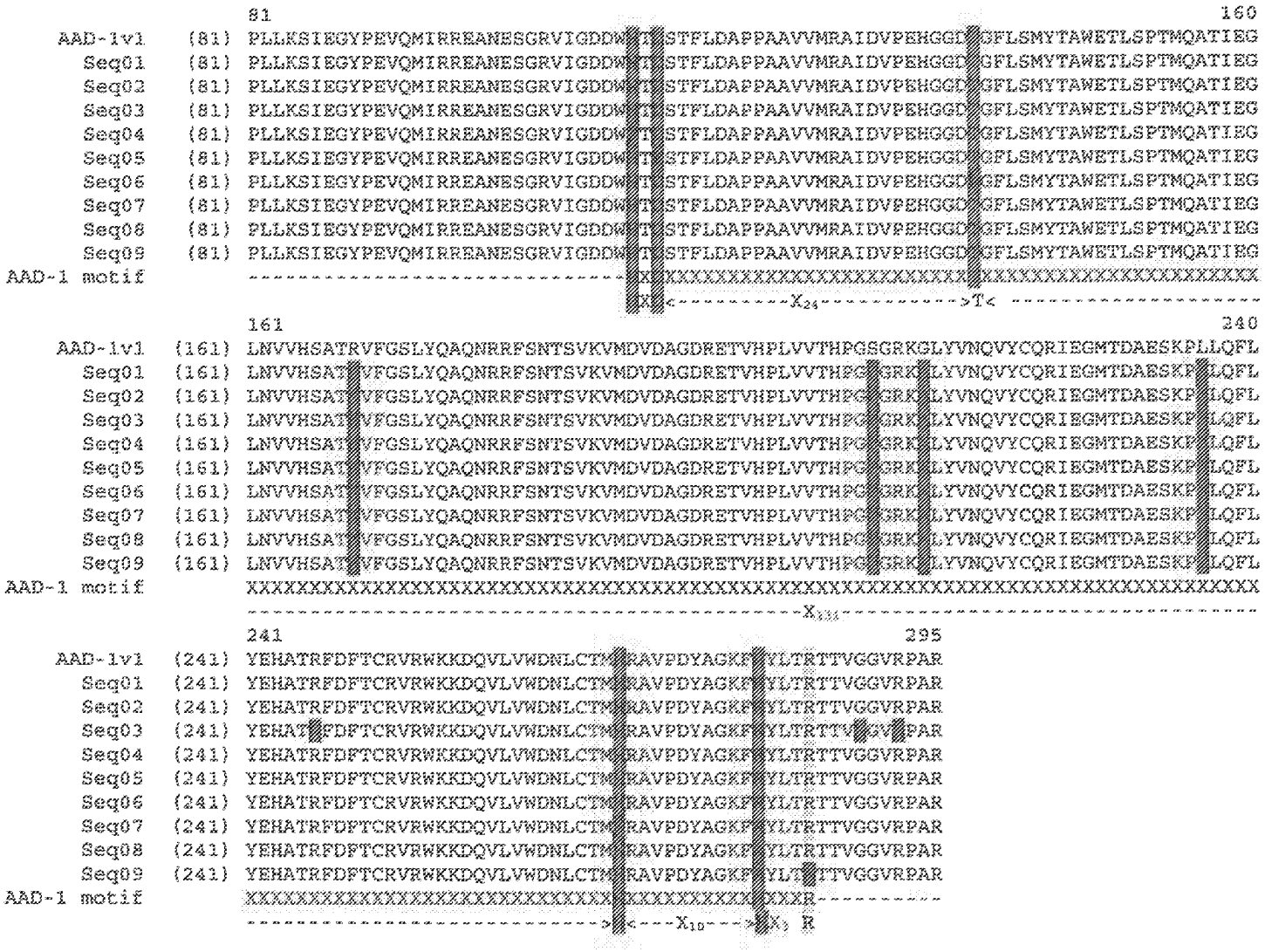
Seq04 to Seq09 begin with Seq01 sequence and individually change one amino acid of the defined AAD-1 motif as follows:

Seq04 $H_{111}F$
Seq05 $D_{113}K$
Seq06 $T_{138}A$
Seq07 $H_{270}F$
Seq08 $R_{281}A$
Seq09 $R_{285}A$

```
                    1                                                                  80
 AAD-1v1     (1) MHAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq01     (1) M  AL PL     I VQPL GVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq02     (1) M  AL PL   ERIAVQPL GVLGA ITGVL REPLDDSTWNE LDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq03     (1) M  AL PL     I VQPL GVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq04     (1) M  AL PL     I VQPL GVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq05     (1) M  AL PL     I VQPL GVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq06     (1) M  AL PL     I VQPL GVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq07     (1) M  AL PL     I VQPL GVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq08     (1) M  AL PL     I VQPL GVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
   Seq09     (1) M  AL PL     I VQPL GVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
AAD-1 motif       ------------------------------------------------------------------------------
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Appendix III-continued



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Appendix III-continued

Comparison of the relative activity of AAD-1v1 and nine variants (3 with the narrow AAD-1 motif intact, 6 with individual changes to the defined residues of the motif). Seq01 and Seq03* showed on average similar activity to 2,4-D and (R)-quizalofop [an (R)-AOPP herbicide] substrates as AAD-1 (RdpA). When defined amino acids of the motif were mutated, 5 of 6 residues resulted in 90-100% reduced activity. One residue $T_{138}A$ still retained >30 % activity but lost 60-70% of activity against 2,4-D and (R)-quizalofop). This shows both the importance and necessity of these residues in the AAD-1 motif for dual MOA herbicide degradation activity.

AAD-1 Variants Verifying Motif Validity

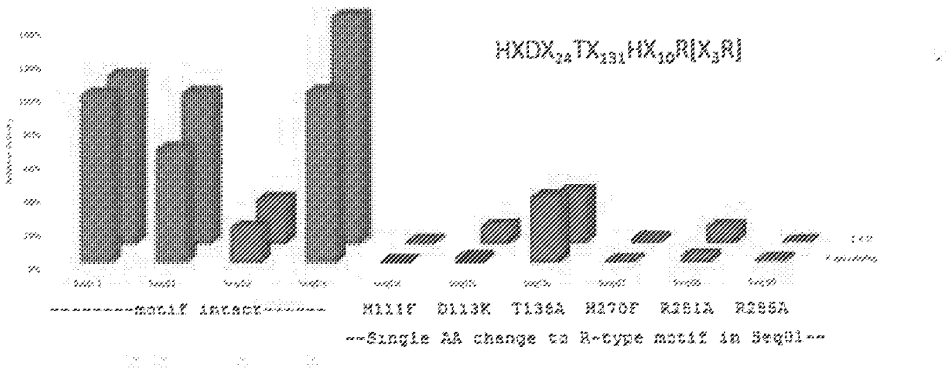
$HX(D/E)\ X_{23-28}(T/S)X_{114-183}HX_{10-13}R$ [PCT 0085] = Group II a-KG (include TauD, TfdA, RdpA, et al)

AAD-1 proteins have novel property of being able to degrade 2,4-D, other phenoxy auxin herbicides, and (R)-AOPP herbicides [0086-0090]. This narrows to specific spacing of key residues $HXDX_{24}TX_{131}HX_{10}R[X_3R]$

Seq01 ~ Seq03 = 94-95% Identity (15-18 aa changes) to AAD-1 (SEQ ID NO. 9, motif intact)

Remaining Seq's single aa change to motif residues

H111F Seq04
D113K Seq05
T138A Seq06
H270F Seq07
R281A Seq08
R285A Seq09



*Recovery of Seq02 and Seq05 was low due to a large proportion of expressed protein insolubility. Quantity of enzyme in these samples is likely an underestimate (lower than targeted 1 ug protein).

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Electronic Patent Application Fee Transmittal

Application Number:	15288406
Filing Date:	07-Oct-2016
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Filer:	John P. Breen/Tanya Horvath
Attorney Docket Number:	14764-258662

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	1252	1	600	600
Miscellaneous:				
		Total in USD ($)		**600**

Electronic Acknowledgement Receipt	
EFS ID:	35711079
Application Number:	15288406
International Application Number:	
Confirmation Number:	5402
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Customer Number:	107540
Filer:	John P. Breen/Tanya Horvath
Filer Authorized By:	John P. Breen
Attorney Docket Number:	14764-258662
Receipt Date:	12-APR-2019
Filing Date:	07-OCT-2016
Time Stamp:	14:43:42
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$600
RAM confirmation Number	041519INTEFSW14443300
Deposit Account	100435
Authorized User	Tanya Horvath
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Amendment/Req. Reconsideration-After Non-Final Reject	63364A_US_CNT320190412_Responseto1stOfficeActionFinalversion.pdf	108488 1b63f33282b25504d5e042bea007866c16bdc363	no	16
Warnings:					
Information:					
2	Oath or Declaration filed	132_Declaration.pdf	691913 c10be69df34008bb988c8165f0782369c6b42863	no	18
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30709 df30acfce5ea5be8cdffe2abcec7558cbb710532	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	831110		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No.:	107540
Group:	1663
Confirmation No.:	5402
Application No.:	15/288,406
Invention:	**Novel Herbicide Resistance Genes**
Inventor:	Terry Wright et al.
Filed:	October 7, 2016
Attorney Docket:	14764-258662
Examiner:	David Kruse

FILED ELECTRONICALLY:

April 12, 2019

AMENDEMENT AND RESPONSE UNDER 37 CFR 1.111

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Sir:

In response to the Office Action mailed November 13, 2018, Applicant requests a two month extension of time, entry of the following amendments and consideration the accompanying remarks. Applicant submits herewith payment of the $600.00 fee (large entity) under 37 CFR 1.17(a) for the requested two month extension, and hereby authorizes the Commissioner to charge any additional fee necessary to affect this filing, to the account of Barnes & Thornburg, Deposit Account No. 10-0435 with reference to our file 14764-258662.

Amendments to the Specification are submitted herewith beginning on page 2.

Amendments to the Claims are submitted herewith beginning on page 3.

Remarks begin on page 8 of this paper.

AMENDMENTS TO THE SPECIFICATION:

Please replace paragraph [0086] on page 17 with the following amended paragraph;

More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also AOPP herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase) genes and proteins. As disclosed herein a peptide of SEQ ID NO: 9 is provided that is capable of degrading 2,4-D, and AOPP herbicides. Mapping the α-ketoglutarate-dependent dioxygenase motif disclosed in the previous paragraph to the sequence of SEQ ID NO: 9 reveals the AAD-1 motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids.

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AMENDMENTS TO THE CLAIMS:

This listing of claims will replace all prior versions, and listings of claims in the application:

Claims:

Claims 1-14. (Canceled).

15. (Currently Amended) A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

~~$(X)_{139\text{-}243}$~~ $\underline{(X)_{139\text{-}269}}$ represents a sequence of 131 amino acids; and

~~$(X)_{245\text{-}254}$~~ $\underline{(X)_{271\text{-}280}}$ represents a sequence of 10 amino acids.

16. (Previously Presented) The plant cell of claim 15 wherein said AAD-1 motif has the general formula of:

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R(X)_{282\text{-}284}R$, wherein $(X)_{282\text{-}284}$ represents a sequence of 3 amino acids.

17. (Canceled).

18. (Previously Presented) The plant cell of claim 15 wherein the AAD-1 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 9.

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19. (Previously Presented) The plant cell of claim 15 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an Arabidopsis cell.

20. (Previously Presented) The plant cell of claim 15 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

21. (Previously Presented) A transgenic plant comprising a plurality of the plant cell of claim 15, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

22. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

23. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

24. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

25. (Currently Amended) The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

26. (Previously Presented) The plant of claim 21 wherein said plant further comprises a second herbicide resistance gene.

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27. (Previously Presented) The plant of claim 26 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

28. (Previously Presented) A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 21, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide.

29. (Previously Presented) The method of claim 28 wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

30. (Previously Presented) The method of claim 28 wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

31. (Currently Amended) The method of claim 28 wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

32. (Previously Presented) The method of claim 28 wherein said method comprises applying a second herbicide.

33. (Previously Presented) The method of claim 32 wherein said first herbicide and said second herbicide are applied sequentially.

34. (Previously Presented) The method of claim 32 wherein said first herbicide and said second herbicide are applied concurrently.

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35. (Previously Presented) The method of claim 32 wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

36. (Previously Presented) The method of claim 32 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

37. (Previously Presented) The method of claim 32, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

38. (Previously Presented) The method of claim 32, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

39. (Previously Presented) The method of claim 32 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

40. (Previously Presented) The method of claim 39 wherein said second gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

41. (Previously Presented) The method of claim 40 wherein:

(a) said modified ALS gene is selected from the group consisting of a SurA, SurB, Csr1, Csr1-1, Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT; and,

(c) said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

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42. (Previously Presented) The method of claim 32 wherein said method further comprises applying a third herbicide.

43. (Previously Presented) The method of claim 42, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

44. (Previously Presented) The method of claim 43 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

45. (Previously Presented) A seed comprising a plurality of the plant cell of claim 15.

46. (Previously Presented) A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim 45 in said field within 14 days of applying said aryloxyalkanoate herbicide.

47. (Previously Presented) A plant grown from the seed of claim 45.

48. (Previously Presented) A part, progeny, or asexual propagate of the plant of claim 47, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

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REMARKS

At the outset, applicant wishes to express their appreciation for the courtesies extended to the undersigned attorney and other participants during an Examiner's Interview conducted on March 19, 2019. Participating in that telephonic Examiner's Interview were inventor Dr. Terry Wright, in house counsel, Phil Poirier and the attorney of record, John Breen. During the teleconference issues relating to written descriptive support of the claimed AAD-1 motif were discussed in conjunction with recent experimental data. Applicant submitted the experimental data (also included with this response) in support of the objective teaching found in paragraph 0085 on page 17 of the specification. The Examiner's interview summary as provided in PTOL-90A, mailed on March 22, 2019 is an accurate summary of the interview.

In the office action mailed on November 13, 2018, the Examiner contends that the disclosure of the parent application, application no. 14/820,893 fails to provide adequate support or enablement to the currently claimed invention. More particularly, the Examiner contends that the parent application fails to provide written description support for the AAD-1 motif recited in the claims. Applicant respectfully traverses the Examiner's conclusion.

As noted by the Examiner, the specification of the current application and the US parent applications each disclose an α-ketoglutarate-dependent dioxygenase motif on page 17, paragraph 0085. The motif described in paragraph 0085 discloses an amino acid sequence comprising a tripeptide of HX(D/E) separated by 23 to 26 amino acids from a (T/S) residue which is separated by 114 to 183 amino acids from a histidine residue which is separated by 10 to 13 amino acids from an arginine residue (i.e., $HX(D/E)X_{23\text{-}26}(T/S)\ X_{114\text{-}183}HX_{10\text{-}13}R$).

As noted in number paragraphs 9 and 10 of the accompanying 37 CFR 1.132 Declaration by Dr. Wright, the present invention is directed to enzymes and genes that have the unique ability to degrade 2,4-D, other phenoxyalkanoic (phenoxy) auxin herbicides, and AOPP herbicides, and the use of such enzymes to render plants resistant to 2,4-D and AOPP herbicides.

Applicant has described the complete amino acid sequence of AryloxyAlkanoate Dioxygenase (AAD-1) proteins (SEQ ID NOs: 9, 10 and 11) that have been found to exhibit activity in degrading 2,4-D as well as AOPP herbicides. Applicant further recognized at the time of the invention that variant peptides of these exemplified species could be prepared that retain the desired dual mode of action (MOA) activity (see paragraphs 10 and 11 of the accompanying Declaration). In analyzing the structure of the identified AAD proteins in light of the known α-ketoglutarate-

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dependent dioxygenase motif, applicant has derived a specific "AAD-1 motif", wherein the first histidine residue of the α-ketoglutarate-dependent dioxygenase motif corresponds to the histidine found at position 111 of SEQ ID NO:9, the (T/S) residue of the α-ketoglutarate-dependent dioxygenase motif corresponds to the threonine residue at position 138 of SEQ ID NO:9, the second histidine residue of the α-ketoglutarate-dependent dioxygenase motif corresponds to the histidine residue at position 270 of SEQ ID NO:9, and the arginine residue of the α-ketoglutarate-dependent dioxygenase motif corresponds to the arginine residue at position 281 of SEQ ID NO:9, thus generating the AAD-1 motif of: $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$.

The spacing found in the AAD-1 motif between the tripeptide $HX_{112}D$ and the threonine at position 138 (24 amino acids); between the threonine at position 138 and the second histidine at position 279 (131 amino acids) and between the second histidine and the arginine at position 281 (10 amino acids) is entirely consistent with the spacing provided in the α-ketoglutarate-dependent dioxygenase motif on page 17, paragraph 0085.

Accordingly, mapping the α-ketoglutarate-dependent dioxygenase motif (as provided in paragraph 0085) onto the sequence of SEQ ID NO: 9, 10 or 11 provides the sequence listed as the AAD-1 motif in current claim 1. To assist the Examiner's review, applicant has provided in Appendix II of the accompanying Declaration an alignment of each of the three disclosed AAD-1 species (v1, v2 and v3) with the AAD-1 motif, as well as an alignment of AAD-1v1 (SEQ ID NO. 9) with AAD-2 (wherein AAD-2 exhibits 2,4-D degradation activity but lacks (R)-AOPP class degradation activity). Since the motif listed in claim 1 was derived from the disclosed α-ketoglutarate-dependent dioxygenase motif as applied to the amino acid sequence of AAD-1 (SEQ ID NO: 9, or its variants SEQ ID NO: 10 and 11), the specification provides complete written descriptive support for the listed AAD-1 motif.

As disclosed in paragraphs 9-12 of the accompanying Declaration by Dr. Wright, one of ordinary skill in the art would recognize the structure of the AAD-1 motif $HXDX_{24}TX_{131}$ $HX_{10}R$, as defining AAD-1 variant enzymes that exhibit a dual MOA α-KG dioxygenase activity. Applicant respectfully submits the parent application provides full written descriptive support for the described AAD-1 motif, and applicant believes that they are fully entitled to the early priority date claimed for the captioned application.

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The Examiner has objected to the specification for failing to provide proper antecedent basis for the term "AAD-1 motif". In accordance with MPEP 608.01(o) applicant has amended to the specification to provide a proper antecedent basis for the term. The amendment of the specification introduces no new matter as the specification is merely amended to recite the structure already present in SEQ ID NO: 9 as a specific example of the AAD-1 motif correlating to the generalized α-ketoglutarate-dependent dioxygenase motif.

Claims 15-48 are rejected for obviousness type double patenting over claims 1-43 of US Patent No 7,838,733. Claims 15-48 are rejected for obviousness type double patenting over claims 1-22 of US Patent No 9,127,289. Claims 15-48 are rejected for obviousness type double patenting over claims 1-3 and 5-14 of co-pending application no 14/820,893 (now US Patent No 10,174,337). Applicants will file the necessary terminal disclaimers to overcome these rejections upon a finding of allowable subject matter.

Claims 15-48 are rejected under 35 USC 112, first paragraph for failing to comply with the written description requirement. Applicant respectfully traverses this rejection.

The fundamental factual inquiry in determining if a claim complies with the written descriptive requirement is to determine whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed. See, e.g., Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. The subject matter of the claim need not be described literally (i.e., using the same terms or *in haec verba*) in order for the disclosure to satisfy the description requirement (MPEP 2163.02).

The original specification of priority document PCT/US2005/014737as filed on May 2, 2005 provides three species of the claimed genus of peptides that exhibit the recited functionality of having aryloxyalkanoate dioxygenase activity, wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide. Additionally, the original specification highlights the key amino acid residues of aryloxyalkanoate dioxygenases

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by providing the structure of a motif comprising the active site of the enzyme. One of ordinary skill in the art would know that the motif provided on page 17 in paragraph [0085] provides a roadmap to identify the corresponding positions in the AAD-1 proteins of SEQ ID NOs; 9, 10 and 11 to allow one to generate a "AAD-1 motif" of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, that is specific to the claimed genus of AAD-1 proteins. Moreover, the claims have been narrowly tailored to be directed to AAD-1 proteins that enzymatically degrade a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide by requiring the claimed peptides to comprise the functional element of the AAD-1 motif.

In support of the objective teachings relating to the structure/function relationship of the AAD-1 genes, applicant submits herewith additional data and a Declaration under 37 CFR 1.132. An expert's Declaration is relevant to the issue of written description when it is direct to specific statements in the originally filed application and used to explain how, in his opinion, a person of ordinary skill in the art would understand those statements (see *Martek Biosciences Corp. v. Nutrinova, Inc.*, 579 F.3d 1363 (Fed. Cir. 2009)).

As detailed in the accompanying 37 CFR 1.132 Declaration, one of ordinary skill in the art upon reading the entire disclosure would recognize that the motif disclosed on page 17 could be used to identify an AAD-1 motif as a means of identifying important residues of the AAD-1 active site. Furthermore, the skilled practitioner would also understand that such amino acids should be maintained in any protein derivatives of SEQ ID NO: 9 to retain the enzymatic activity of the parent protein (see numbered paragraphs 9-12 of the accompanying Declaration).

As described in paragraph 14 of the accompanying Declaration, an experiment was conducted to confirm the objective teachings of the present application that the defined residues in the AAD-1 motif $HXDX_{24}TX_{131}HX_{10}R$ are important to the desired dual MOA herbicide degradation activity. Nine variants of AAD-1v1 (SEQ ID NO: 9) were prepared and tested for enzymatic activity against 2,4-D and (R)-quizalofop. Alignments of all nine variants and the original AAD-1v1 (SEQ ID NO: 9) are shown in Appendix III of the accompanying Declaration. The data presented with the accompanying Declaration (see the bar graph provided in Appendix III and paragraphs 15 and 16 of the accompanying Declaration) confirm the importance of the amino acids at positions 111, 113, 138, 270 and 281 in AAD-1 proteins for maintaining the claimed enzymatic activity. Furthermore, the skilled practitioner readily appreciates that other

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regions of the AAD-1 protein would be more tolerant to modification without loss of the claimed activity.

The Federal Circuit has held that complete or partial structure, other physical and/or chemical properties, or functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics provides evidence that applicant was in possession of the claimed invention (See. *Enzo Biochem*, 323 F.3d at 964, 63 USPQ2d at 1613). In that regard, applicant respectfully submits that they have provided a substantial amount of structure of the members of the claimed genus.

First of all, each peptide of the claimed genus is required to have at least 85% sequence identity with an amino acid of SEQ ID NO: 9. Furthermore, applicant has provided guidance as to where modifications can be made in the AAD-1 sequence requiring all peptides of the claimed genus to comprise the AAD-1 motif. Those skilled in the art are well aware that proteins can be modified while retaining the activity of the original protein, provided that the active site remains intact. Accordingly, the claimed genus is further limited by the requirement that the claimed analogs of SEQ ID NO: 9 comprise the AAD-1 motif and retain enzymatic activity for degrading phenoxy auxin herbicides as well as (R)-aryloxyphenoxypropionate herbicides. As indicated in the accompanying Declaration and data presented in Appendix III, applicant has established a structure function relationship for the desired dual MOA herbicide degradation activity.

Applicant's teachings regarding the AAD-1 motif, in conjunction with the disclosed exemplified species, provide sufficient guidance to those skilled in the art, that one of skill in the art would recognize that applicant was in possession of the necessary common attributes possessed by the each member of the genus.

Therefore, applicant respectfully requests the withdrawal of the rejection of claims 15-48 for failure to comply with the written description requirement.

Claims 15-48 stand rejected under 35 USC 103 as being obvious over the combined teachings of Jepson et al (US 2003/0041357) in view of Kohler (Genbank Accession no Q8KSC8). Jepson is cited as teaching plants that comprise a *tfdA* gene for conferring aryloxyalkanoate dioxygenase activity to those plants, and the use of such plants in methods of weed control using 2,4-D. The Examiner admits that Jepson does not teach AAD-1 (SEQ ID N0:9). Kohler is cited as teaching *Spingomonas herbicidovorans rdpA*, a bacterial gene that

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encodes the peptide of SEQ ID N0:9 of the instant application. The Examiner states that it would have been *prima facie* obvious to substitute the gene of Kohler for the gene of Jepson. Applicants respectfully traverse this rejection.

First of all, applicant respectfully submits that the Examiner fails to provide any rationale for why one of ordinary skill would specifically select a gene encoding a peptide of SEQ ID NO: 9, or a derivative thereof having at least 85% sequence identity to a peptide of SEQ ID NO: 9, to substitute for the *tfdA* gene disclosed in Jepson. Applicant notes there are many other known sequences that share sequence identity with the *tfdA* gene and serve as possible candidates for providing aryloxyalkanoate dioxygenase activity. At the time of applicant's invention it cannot be reasonably said that one would have turned to the specific gene disclosed in Kohler with the expectation that it would be expressed in plants, particularly to convey herbicide tolerance to the plants.

At the time of the invention there was simply no motivation to select a bacterial gene having specific sequence of SEQ ID NO: 9, (or a peptide having at least 85% sequence identity with SEQ ID NO: 9) for combination with a heterologous promoter that is functional in a plant cell for use in producing transgenic plants that are tolerant to damage by an aryloxyalkanoate herbicide.

Example 17 of the subject specification provides data comparing the subject AAD-1 enzyme to another enzyme referred to as AAD-2, wherein AAD-2 has a higher percentage sequence identity with tfdA relative to AAD-1. Accordingly, AAD-2 is a protein that falls outside the scope of the current claims, as AAD-2 does not have at least 85% sequence identity with SEQ ID NO: 9, yet is one with a sequence identity closer to tfdA. Significantly, AAD2 also does not comprise the AAD-1 motif that is present in AAD-1 (see paragraph 12 and Appendix II of the accompanying Declaration).

AAD-2 was one of several genes identified from BLAST search results using tfdA as the reference sequence. The subject application states in Example 17.1:

> Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to tfdA. This gene is referred to herein as AAD-2 (v1) for consistency. Percent identity was determined by first translating both the AAD-2 and tfdA DNA sequences (SEQ ID NO: 12 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID N0:13 and GENBANK Accession No.

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M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

AAD-2 was obtained from *Bradyhizobium japonicum*. Again, AAD-2 is more closely related (about 44%) at the sequence level to tfdA than is AAD-1 (about 27%). However, AAD-2 was surprisingly inactive in plants, while AAD-1 was very active.

Moreover, the subject AAD-1 enzyme was a choice of one of over 100 other possible genes. The first paragraph of Example 1 of the subject application states (emphasis added):

> As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e., α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤ 50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

Thus, the subject AAD-1 enzyme was only 1 of over 100 BLAST search results having some degree of identity with tfdA. As discussed above, there were great numbers of other possible choices that could have been selected.

Furthermore, the subject invention offers surprising advantages that neither Jepson nor Kohler taught. As illustrated by the subject specification, particularly by Table 1 and Table 2, mecoprop and dichlorprop have phenoxy auxin chemistries, while the aryloxyphenoxypropionates or "AOPPs" are in a different class of chemistries. Thus, it is not seen how Kohler would support the reasonable expectation of success to produce a plant that is resistant to aryloxyphenoxypropionates and thus protecting a crop plant from damage by an aryloxyalkanoate herbicide.

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The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (vl) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (Figure 3 and Table 7). **These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins**. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

The above paragraph shows that AAD-1 has surprising advantages that are not contemplated by Kohler, i.e. it acts on two completely different categories of herbicides. This is not taught or suggested by either Jepson or Kohler.

The subject invention relates in part to the identification of an enzyme AAD-1 that is not only able to degrade 2,4-D, but also aryloxyphenoxypropionate (AOPP) herbicides, thus distinguishing the enzyme of the subject invention from previously known tfdA proteins. No alpha-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Accordingly, there was no reason to select the nucleic acid of SEQ ID NO: 9, or derivative thereof, to be operably linked to a plant specific promoter, as there was no reasonable expectation that such a construct would produce the desired effect when transfected into plant cells. In light of this, the withdrawal of the obviousness rejection is respectfully requested.

Claims 15-25, 28-31, 45, 47 and 48 are rejected under 35 USC 102 as being anticipated by Ellis et al (US 2016/0108422). Applicant respectfully traverses this rejection.

The present specification is substantively identical to that of PCT/US2005/014737. The early disclosure of PCT/US2005/014737 provides the sequence of SEQ ID NOs: 9, 10 and 11 and the formulation of the α-ketoglutarate-dependent dioxygenase motif. As noted above those of ordinary skill would understand how to apply the α-ketoglutarate-dependent dioxygenase motif to the AAD-1 proteins sequences of SEQ ID NOs: 9, 10 and 11 to generate the AAD-1 motif listed in the current claims. Therefore, the current claimed invention is entitled to the priority date of May 2, 2005, and Ellis et al is not a proper prior art document.

Withdrawal of the rejection of claims 15-25, 28-31, 45, 47 and 48 as being anticipated by Ellis et al (US 2016/0108422) is respectfully requested.

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Claims 15-48 are rejected under 35 USC 102 as being anticipated by Wright et al (EP 1740039). Applicant respectfully traverses this rejection.

EP 1740039 is a regionalized application of PCT/US2005/014737. Accordingly, EP 1740039 represents the same application as the present application. Therefore, Wright et al (EP 1740039) is not a proper prior art reference to the present application.

Withdrawal of the rejection of claims 15-48 as being anticipated by Wright et al is respectfully requested.

Consideration of the claims and allowance of the application is respectfully requested. If any further discussion of this matter would speed prosecution of this application, the Examiner is invited to call the undersigned at (434) 220-2866.

Respectfully submitted,

/John P. Breen/

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DMS 14341919v1

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/288,406	Filing Date 10/07/2016	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	04/12/2019	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 32	Minus	** 34	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $460 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/GLORIA J TRAMMELL/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

107540 7590 07/12/2019
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

EXAMINER
KRUSE, DAVID H

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
07/12/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

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Bayer EX1008 (Part 1 of 9)

Office Action Summary	Application No. 15/288,406	Applicant(s) WRIGHT et al.	
	Examiner DAVID H KRUSE	Art Unit 1663	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 12 April 2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 15-16 and 18-48 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-16 and 18-48 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

STATUS OF THE APPLICATION

1. The present application is being examined under the pre-AIA first to invent provisions.

2. This Office action is in response to the Amendment and Remarks filed 12 April 2019.

3. The objection to the Specification is withdrawn in view of Applicant's amendment.

4. The Examiner notes that USP Application 14/989,787 has issued as U.S. Patent 10,323,287. After reviewing the issued claims the Examiner has determined that there is no double patenting issue.

5. The Examiner notes that USP Application 14/820,893 has issued as U.S. Patent 10,174,337.

6. The rejection of claims 32, 35, 38 and 46 under 35 U.S.C. 103(a) is withdrawn in view of Applicant's arguments.

Double Patenting

7. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed.

Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based e-Terminal Disclaimer may be filled out completely online using web-screens. An e-Terminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about e-Terminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

8. Claims 15, 15 and 18-48 remain rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-43 of U.S. Patent No. 7,838,733.

Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '733 Patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 10, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

9. Claims 15, 16 and 18-48 remain rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 9,127,289. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '289 Patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 10, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

10. Claims 15, 16 and 18-48 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 10,174,337 (which issued from US Patent Application 14/820,893). Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in

the '337 patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

This rejection has been modified because the Application has issued as a U.S. Patent. Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 10, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

Claim Rejections - 35 USC § 112

11. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

12. Claims 15, 16 and 18-48 remain rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention. This rejection is repeated for the reason of record as set forth in the last Office action mailed 13 November 2018.

Applicant's arguments filed 12 April 2019 have been fully considered but they are not persuasive.

Applicant claims a transgenic plant cell, plant and method of using wherein the plant cell comprises a recombinant polynucleotides encoding an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity having at least 85% sequence identity with instant SEQ ID NO: 9 and an AAD-1 motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$.

Applicant describes the amino acid sequence of the AAD-1 (syn. RdpA) enzyme in instant SEQ ID NO: 9 and two variants thereof in instant SEQ ID Nos. 10 and 11 which are 98.8% and 99.7% identical respectively to said SEQ ID NO: 9.

Applicant does not describe the claimed genus of AAD-1 proteins or the AAD-1 motif (in the specification). The specification describes a motif comprising three conserved histidine residues found in α-ketoglutarate-dependent dioxygenases on page 17, paragraph 0085, $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$.

Hence, it is unclear that Applicant was in possession of the invention as broadly claimed.

Applicant argues that the original specification highlights the key amino acid residues of aryloxyalkanoate dioxygenases by providing the structure of a motif comprising the active site of the enzyme. Applicant argues that one of ordinary skill in the art would know that the motif provided on page 17 in paragraph [0085] provides a roadmap to identify the corresponding positions in the AAD-1 proteins of SEQ ID NOs; 9, 10 and 11 to allow one to generate a "AAD-1 motif" of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, that is specific to the claimed genus of AAD-1 proteins. Applicant argues that the claims have been narrowly tailored to be directed to AAD-1 proteins that

enzymatically degrade a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide by requiring the claimed peptides to comprise the functional element of the AAD-1 motif (page 11, 1st paragraph of the Remarks).

Applicant argues that in support of the objective teachings relating to the structure/function relationship of the AAD-1 genes, Applicant submits herewith additional data and a Declaration under 37 CFR 1.132. Applicant argues that an expert's Declaration is relevant to the issue of written description when it is direct to specific statements in the originally filed application and used to explain how, in his opinion, a person of ordinary skill in the art would understand those statements (see *Martek Biosciences Corp. v. Nutrinova, Inc.*, 579 F.3d 1363 (Fed. Cir. 2009)) (page 11, 2nd paragraph of the Remarks).

Applicant argues that as described in paragraph 14 of the accompanying (Wright) Declaration, an experiment was conducted to confirm the objective teachings of the present application that the defined residues in the AAD-1 motif $HXDX_{24}TX_{13}1HX_{10}R$ are important to the desired dual MOA herbicide degradation activity. Applicant argues that nine variants of AAD-1 (SEQ ID NO: 9) were prepared and tested for enzymatic activity against 2,4-D and (R)-quizalofop. Applicant argues that alignments of all nine variants and the original AAD-1 (SEQ ID NO: 9) are shown in Appendix III of the accompanying Declaration. Applicant argues that the data presented with the accompanying Declaration (see the bar graph provided in Appendix III and paragraphs 15 and 16 of the accompanying Declaration) confirm the importance of the amino acids at positions 111, 113, 138, 270 and 281 in AAD-1 proteins for maintaining the claimed enzymatic activity. Applicant argues that the skilled practitioner readily appreciates that other

regions of the AAD-1 protein would be more tolerant to modification without loss of the claimed activity (page 11, 4th paragraph of the Remarks).

Applicant argues that applicant respectfully submits that they have provided a substantial amount of structure of the members of the claimed genus.

Applicant argues that each peptide of the claimed genus is required to have at least 85% sequence identity with an amino acid of SEQ ID NO: 9. Applicant argues that applicant has provided guidance as to where modifications can be made in the AAD-1 sequence requiring all peptides of the claimed genus to comprise the AAD-1 motif. Applicant argues that those skilled in the art are well aware that proteins can be modified while retaining the activity of the original protein, provided that the active site remains intact. Applicant argues that the claimed genus is further limited by the requirement that the claimed analogs of SEQ ID NO: 9 comprise the AAD-1 motif and retain enzymatic activity for degrading phenoxy auxin herbicides **as well as** (R)-aryloxyphenoxypropionate herbicides (emphasis added by the Examiner). Applicant argues that as indicated in the accompanying Declaration and data presented in Appendix III, applicant has established a structure function relationship for the desired dual MOA herbicide degradation activity.

Applicant argues that the teachings regarding the AAD-1 motif, in conjunction with the disclosed exemplified species, provide sufficient guidance to those skilled in the art, that one of skill in the art would recognize that applicant was in possession of the necessary common attributes possessed by the each member of the genus (page 12 of the Remarks).

Applicant's arguments have been fully considered but are not found to be fully persuasive. The Examiner has reviewed *Martek Biosciences Corp. v. Nutrinova, Inc.*, 579 F.3d 1363 (Fed. Cir. 2009), and acknowledges that that court states that a patent claim is not necessarily invalid for lack of written description just because it is broader than the specific example disclosed (at 1371). But, the written description must allow one of skill in the art to envision the claimed invention based on the text of the application. See *University of Rochester v. G.D. Searle & Co.,* 68 USPQ2d 1424, 1433 (DC WNY 2003) which teaches knowing the "starting point" is not enough; that is little more than a research plan. The court held that the disclosure of screening assays and general classes of compounds was not adequate to describe compounds having the desired activity: without disclosure of which peptides, polynucleotides, or small organic molecules have the desired characteristic, the claims failed to meet the description requirement of § 112.

While the recited AAD-1 motif is similar to other enzymes having a motif comprising three conserved histidine residues found in α-ketoglutarate-dependent dioxygenases the generic motif does not itself define a specific function and Applicant does not describe what structure in the AAD-1 motif provides the specific activity that degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide. While a description of a representative number of species is adequate to represent an entire genus, wherein there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus (see MPEP 2163). Compared to instant SEQ ID NO: 9, the claims encompass (20^{165})-1 variations of just

the AAD-1 motif. Therefore, it is the Examiner's opinion that Applicant has failed to adequately describe the variation within the claimed genus.

Claim Rejections - 35 USC § 103

13. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

14. Claims 15, 16 18-30, 32-34, 36, 37, 39-45, 47 and 48 remain rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jepson *et al* (U.S. Patent Application US 2003/0041357 filed on 23 February 2001) in view of Kohler (Genbank Accession No: Q8KSC8 first published on 1 October 2002). This rejection is repeated for the reason of record as set forth in the last Office action mailed 13 November 2018. Applicant's arguments filed 12 April 2019 have been fully considered but they are not persuasive.

Jepson *et al* teach a method of controlling at least one weed in an area by applying a phenoxy auxin herbicide said area comprising a transgenic plant comprising a recombinant polynucleotide encoding a protein having aryloxyalkanoate dioxygenase activity (see paragraph 0027 on pages 3-4, especially lines 18-24 on page 4). Jepson *et al* teach said method using a plant transformed with an additional transgene that provides glyphosate, glufosinate, imidazolinone or sulfonylurea herbicide tolerance (page 4, left column, lines 18-27). Jepson *et al* teach applying the phenoxy auxin herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Jepson *et al* teach said method can

be practiced using turf grass or forage grass (page 3, paragraph 0024, last sentence; claim 15). Jepson *et al* teach said method can be practiced using a plant transformed with a transgene that produces tolerance to a pre-emergence herbicide (claim 12).

Jepson *et al* do not teach a transgenic plant comprising a recombinant polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO: 9.

Kohler teaches a polynucleotide that encodes a protein comprising the amino acid sequence of instant SEQ ID NO: 9. Kohler teaches that said protein has R-2,4-dichlorophenoxypropionate dioxygenase activity (i.e. degrades the phenoxy auxin herbicide (R)-dichlorprop).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time of Applicant's invention to modify the teachings of Jepson *et al* to substitute the tfdA herbicide tolerance gene for a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase taught by Kohler. It would have been obvious to combine a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase with another gene for tolerance to additional herbicide tolerances as taught by Jepson *et al*. Jepson *et al* teach that one would apply one (claim 16) or two (claim 17) herbicides in the method of controlling weeds. Jepson *et al* teach that at the time of Applicant's invention it was desirable to produce plants tolerant to a phenoxy auxin herbicide. Given the high level of skill in the instant art at the time of Applicant's invention, one would have had a reasonable expectation of success.

Applicant argues that the Examiner fails to provide any rationale for why one of ordinary skill would specifically select a gene encoding a peptide of SEQ ID NO: 9, or a derivative thereof having at least 85% sequence identity to a peptide of SEQ ID NO: 9, to substitute for the *tfdA* gene disclosed in Jepson. Applicant argues that there are many other known sequences that share sequence identity with the *tfdA* gene and serve as possible candidates for providing aryloxyalkanoate dioxygenase activity. Applicant argues that at the time of applicant's invention it cannot be reasonably said that one would have turned to the specific gene disclosed in Kohler with the expectation that it would be expressed in plants, particularly to convey herbicide tolerance to the plants.

Applicant argues that at the time of the invention there was simply no motivation to select a bacterial gene having specific sequence of SEQ ID NO: 9, (or a peptide having at least 85% sequence identity with SEQ ID NO: 9) for combination with a heterologous promoter that is functional in a plant cell for use in producing transgenic plants that are tolerant to damage by an aryloxyalkanoate herbicide.

Applicant argues that Example 17 of the instant specification provides data comparing the subject AAD-1 enzyme to another enzyme referred to as AAD-2, wherein AAD-2 has a higher percentage sequence identity with *tfdA* relative to AAD-1. Applicant argues that, AAD-2 is a protein that falls outside the scope of the current claims, as AAD-2 does not have at least 85% sequence identity with SEQ ID NO: 9, yet is one with a sequence identity closer to *tfdA*. Significantly, AAD2 also does not comprise the AAD-1 motif that is present in AAD-1 (see paragraph 12 and Appendix II of the accompanying Declaration).

Applicant argues that AAD-2 was one of several genes identified from BLAST search results using *tfdA* as the reference sequence (page 13 of the Remarks).

Applicant argues that the subject AAD-1 enzyme was a choice of one of over 100 other possible genes.

Applicant argues that as a way to identify genes which possess herbicide degrading activities in planta, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). Applicant argues that it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e., α-ketoglutarate dioxygenase activity). Applicant argues that this protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Applicant argues that using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. Applicant argues that these range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level.

Applicant argues that traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with <50% homology. Applicant argues that we (the inventors) go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

Applicant argues that the subject AAD-1 enzyme was only 1 of over 100 BLAST search results having some degree of identity with *tfdA*. Applicant argues that there were great numbers of other possible choices that could have been selected.

Applicant argues that the subject invention offers surprising advantages that neither Jepson nor Kohler taught. Applicant argues that as illustrated by the subject specification, particularly by Table 1 and Table 2, mecoprop and dichlorprop have phenoxy auxin chemistries, while the aryloxyphenoxypropionates or "AOPPs" are in a different class of chemistries. Applicant argues that, thus, it is not seen how Kohler would support the reasonable expectation of success to produce a plant that is resistant to aryloxyphenoxypropionates and thus protecting a crop plant from damage by an aryloxyalkanoate herbicide (page 14 of the Remarks).

Applicant argues that the subject invention relates in part to the identification of an enzyme AAD-1 that is not only able to degrade 2,4-D, but also aryloxyphenoxypropionate (AOPP) herbicides, thus distinguishing the enzyme of the subject invention from previously known *tfdA* proteins. Applicant argues that no alpha-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Applicant argues that there was no reason to select the nucleic acid of SEQ ID NO: 9, or derivative thereof, to be operably linked to a plant specific promoter, as there was no reasonable expectation that such a construct would produce the desired effect when transfected into plant cells (page 15, 3rd paragraph of the Remarks).

Applicant's arguments have been fully considered but are not found to be persuasive. The examiner has withdrawn the instant rejection for instant claims 31, 35,

38 and 46 because Kohler, as asserted by Applicant, does not teach that the dioxygenase taught could be capable of degrading an AOPP herbicide. Kohler does specifically teach that the dioxygenase degrades the phenoxy auxin herbicides (R)-dichlorprop and (R)-mecoprop herbicides. The fact that Kohler did not recognize that the dioxygenase could also degrade AOPP herbicides does not mean that the use of the dioxygenase to degrade phenoxy auxin herbicides would not be obvious. Based on the teachings of Jepson *et al*, it would have been obvious to transform a plant cell with a recombinant polynucleotide that encodes a phenoxy auxin herbicide degrading enzyme, to produce a plant from said plant cell and to use the plant in a method to control at least one weed in a field by applying a phenoxy auxin herbicide. The fact that Applicant has recognized another advantage which would flow naturally from following the suggestion of the prior art cannot be the basis for patentability when the differences would otherwise be obvious. See *Ex parte Obiaya*, 227 USPQ 58, 60 (Bd. Pat. App. & Inter. 1985).

The limitations in instant claim 24, for example, do not actually structurally or functionally modify the claimed plant.

Applicant's argument that the subject AAD-1 enzyme was a choice of one of over 100 other possible genes is not persuasive because it would have been routine in the art at the time of Applicant's invention to screen multiple candidate coding sequences (obvious to try), and given the teachings of Kohler where the function had been confirmed there would have been an even greater motivation to try. Nothing in the prior art would suggest that there **would not have been** a reasonable expectation of success

in substituting the *tfdA* gene taught by Jepson *et al* with the gene taught by Kohler. The rejection is maintained for the reasons of record.

Claim Rejections - 35 USC § 102

15. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

16. Claim(s) 15, 16, 18-25, 28-31, 45, 47 and 48 remain rejected under pre-AIA 35 U.S.C. 102(a) and (e) as being anticipated by Ellis *et al* (US 2016/0108422 published 21 April 2016).

Ellis *et al* disclose polynucleotides that exhibit aryloxyalkanoate dioxygenase activity having at least 85% sequence identity with instant SEQ ID NO: 9 at SEQ ID Nos. 22, 25, 28, 31 and 34, for example, (claim 1). Ellis *et al* disclose a transgenic plant cell comprising said polynucleotides at claims 9-12. Ellis *et al* disclose a maize transgenic plant comprising said polynucleotides in Figure 1. Ellis *et al* discloses a method of controlling weeds using said transgenic plant using at least herbicide sleected from AOPP (aryloxyphenoxypropionate) herbicides, phenoxy acid herbicides and pyridinyloxy acid herbicides at claim 20. Hence, Ellis *et al* had previously disclosed the claim limitations.

Applicant argues that the present specification is substantively identical to that of PCT/US2005/014737. The early disclosure of PCT/US2005/014737 provides the sequence of SEQ ID NOs: 9, 10 and 11 and the formulation of the α-ketoglutarate-dependent dioxygenase motif. Applicant argues that as noted above those of ordinary skill would understand how to apply the α-ketoglutarate-dependent dioxygenase motif to the AAD-1 proteins sequences of SEQ ID NOs: 9, 10 and 11 to generate the AAD-1 motif listed in the current claims. Applicant argues that the current claimed invention is entitled to the priority date of May 2, 2005, and Ellis *et al* is not a proper prior art document.

This argument is not found to be persuasive. Each claim is given only one priority date. The Examiner has provided evidence that the instant claims have the priority of the filing date of the instant Application, that being 7 October 2016. Consequently, Ellis *et al* represent prior art under 35 U.S.C. 102(a) and (e).

17. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

18. Claim(s) 15, 16 and 18-48 remain rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Wright *et al* (EP 1 740 039 B1).

Wright *et al* disclose a transgenic plant cell comprising instant SEQ ID NO: 9 at claims 1-15 and 46-48, in addition to a second herbicide resistance gene at claims 10-15. Wright *et al* disclose a method of controlling weeds using a plant comprising said

transgenic plant cell at claims 16-45. Hence, Wright *et al* had previously disclosed the claimed invention.

Applicant argues that EP 1740039 is a regionalized application of PCT/US2005/014737. Accordingly, EP 1740039 represents the same application as the present application. Applicant argues that Wright et al (EP 1740039) is not a proper prior art reference to the present application.

This argument is not found to be persuasive. Each claim is given only one priority date. The Examiner has provided evidence that the instant claims have the priority of the filing date of the instant Application, that being 7 October 2016. Given the fact that Wright *et al* had previously disclosed a species of the instantly claimed invention and thus anticipate the instantly claimed genus.

Conclusion

19. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

20. No claims are allowed.

21. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

Notice of References Cited	Application/Control No. 15/288,406	Applicant(s)/Patent Under Reexamination WRIGHT et al.	
	Examiner DAVID H KRUSE	Art Unit 1663	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-10174337-B2	01-2019	Wright; Terry	C12N15/8274	1/1
	B					
	C					
	D					
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	F					
	G					
	H					
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FOREIGN PATENT DOCUMENTS

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	N					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
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	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

COR-AAD1-00000516
Bayer EX1008 (Part 1 of 9)

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/288,406	WRIGHT et al.
	Examiner	Art Unit
	DAVID H KRUSE	1663

CPC - Searched*		
Symbol	Date	Examiner
C12N 9/0069	11/07/2018	/DK/

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventor Name Search	11/07/2018	/DK/
EAST: USPAT, USPGPUB, EPO, JPO, DERWENT	11/07/2018	/DK/
STN: AGRICOLA, BIOSIS, CAPLUS, EMBASE, CABA	11/07/2018	/DK/
Sequence Search of SEQ ID NO: 9	10/30/2018	/DK/
Update EAST	07/05/2019	/DK/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	

COR-AAD1-00000517
Bayer EX1008 (Part 1 of 9)

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	15	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/07/05 10:38
L2	1	("10323287").PN.	USPAT; USOCR	OR	OFF	2019/07/05 10:39
S1	688	aad-1	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:20
S2	48	aad-1.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:21
S3	14	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:36

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COR-AAD1-00000518
Bayer EX1008 (Part 1 of 9)

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Application	Document	Mailroom Date	Attorney Docket No.
15288406	CTFR	07/12/2019	14764-258662
	892	07/12/2019	14764-258662

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (07-09)
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REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	15288406	Filing Date	2016-10-07	Docket Number (if applicable)	14764-258662	Art Unit	1663
First Named Inventor	Terry WRIGHT et al.			Examiner Name	David KRUSE		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

☐ Other _____

☒ Enclosed

☒ Amendment/Reply

☒ Information Disclosure Statement (IDS)

☐ Affidavit(s)/ Declaration(s)

☒ Other Declaration under 37 C.F.R. 1.132 of Dr. Satish K. Nair

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 100435

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

X Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (07-09)
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Signature of Registered U.S. Patent Practitioner			
Signature	/John P. Breen/	Date (YYYY-MM-DD)	2020-01-10
Name	John P. Breen	Registration Number	38833

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Application No.:	15/288,406
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Applicant:	Terry Wright et al.
Filed:	October 7, 2016
Attorney Docket:	14764-258662
Examiner	David Kruse

FILED ELECTRONICALLY:

January 10, 2020

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

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Alexandria, Virginia 22313-1450

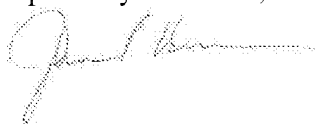
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As this IDS is being filed with an accompanying RCE, applicant believe no fees are required for this filing. However, the Commissioner is hereby authorized to charge any fee necessary to affect this filing to our Deposit Account No. 10-0435, with reference to our Matter No. 14764-258662.

Consideration of the claims, leading to their allowance and passage of the application to issuance, is respectfully requested. If any further discussion of this matter would speed prosecution of this application, the Examiner is invited to call the undersigned at (434) 220-2866.

Respectfully submitted,



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Examiner Initials	Document Number	Issue or Publication Date	Name	Class	Subclass	Filing Date (If Appropriate)

FOREIGN PATENT DOCUMENTS

Examiner Initials	Document Number	Publication Date	Country	Translation Yes	Translation No

OTHER DOCUMENTS

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Localization and Characterization of Two Novel Genes Encoding Stereospecific Dioxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate Cleavage in *Delftia acidovorans* MC1

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Two novel genes, *rdpA* and *sdpA*, encoding the enantiospecific α-ketoglutarate dependent dioxygenases catalyzing *R,S*-dichlorprop cleavage in *Delftia acidovorans* MC1 were identified. Significant similarities to other known genes were not detected, but their deduced amino acid sequences were similar to those of other α-ketoglutarate dioxygenases. RdpA showed 35% identity with TauD of *Pseudomonas aeruginosa*, and SdpA showed 37% identity with TfdA of *Ralstonia eutropha* JMP134. The functionally important amino acid sequence motif HX(D/E)X_{23-26}(T/S)$X_{114-183}$HX_{10-13}R/K, which is highly conserved in group II α-ketoglutarate-dependent dioxygenases, was present in both dichlorprop-cleaving enzymes. Transposon mutagenesis of *rdpA* inactivated *R*-dichlorprop cleavage, indicating that it was a single-copy gene. Both *rdpA* and *sdpA* were located on the plasmid pMC1 that also carries the lower pathway genes. Sequencing of a 25.8-kb fragment showed that the dioxygenase genes were separated by a 13.6-kb region mainly comprising a Tn*501*-like transposon. Furthermore, two copies of a sequence similar to IS*91*-like elements were identified. Hybridization studies comparing the wild-type plasmid and that of the mutant unable to cleave dichlorprop showed that *rdpA* and *sdpA* were deleted, whereas the lower pathway genes were unaffected, and that deletion may be caused by genetic rearrangements of the IS*91*-like elements. Two other dichlorprop-degrading bacterial strains, *Rhodoferax* sp. strain P230 and *Sphingobium herbicidovorans* MH, were shown to carry *rdpA* genes of high similarity to *rdpA* from strain MC1, but *sdpA* was not detected. This suggested that *rdpA* gene products are involved in the degradation of *R*-dichlorprop in these strains.

Chlorinated phenoxyalkanoic acids such as 2,4-dichlorophenoxyacetate (2,4-D), 2-methyl-4-chlorophenoxyacetate (MCPA), 2(2,4-dichlorophenoxy)propionate (dichlorprop), and 2(2-methyl-4-chlorophenoxy)propionate (mecoprop) have been widely used as broad leaf herbicides since their introduction in the 1940s. The ability to degrade the phenoxyacetate herbicides is widespread among bacteria (16). In contrast, breakdown of the structurally very similar chiral phenoxypropionate derivatives is far less widespread, and only few strains able to mineralize these compounds have been described (9, 21, 30, 35, 42, 50).

Degradation of phenoxyalkanoate herbicides is initiated by the etherolytic cleavage of the side chain yielding the respective phenol (Fig. 1). Subsequently, conversion into intermediates of the tricarboxylic acid cycle takes place via the modified *ortho*-pathway (16). Side chain cleaving specificity is crucial and determines the range of substrates that can be mineralized by a given microorganism.

Thus far, two types of 2,4-D-cleaving enzymes have been described. In *Ralstonia eutropha* JMP134, 2,4-D cleavage is catalyzed by a well-studied Fe(II) and α-ketoglutarate (αKG)-dependent dioxygenase, which is encoded by *tfdA* (13, 20, 39, 45, 59). The presence of the functionally essential motif HX(D/E)X_{23-26}(T/S)$X_{114-183}$HX_{10-13}R/K indicates that TfdA is related to several other (referred to as group II) αKG-dependent dioxygenases (18). Recently, a three-dimensional model of the active site was proposed (10) on the basis of predictions made from site-directed mutagenesis studies (20) and secondary structure predictions (10). The identity of the suggested substrate-binding residues was confirmed by site-directed mutagenesis and inactivation studies (8). The *tfdA* genes are widespread among bacteria (17, 31, 33, 36, 39, 42, 47, 53, 54, 55, 56), also among strains unable to degrade 2,4-D (19). Recently, several α-proteobacterial strains were shown to carry *tfdA*α genes, which are *tfdA*-like, showing between 46 and 60% nucleotide identity to the canonical *tfdA* genes and forming a phylogenetic clade distinct from *tfdA* (22, 23). Three other genes, clustered as *cadABC*, were found to encode a second type of 2,4-D-cleaving enzyme, a putative monooxygenase responsible for 2,4-D cleavage in *Bradyrhizobium* sp. strain HW13 (24).

Studies on *Sphingobium* (formerly *Sphingomonas* [48]) *herbicidovorans* MH and *Delftia acidovorans* MC1 indicated that enantiospecific dichlorprop and mecoprop cleavage is catalyzed by Fe(II)- and αKG-dependent dioxygenases (35, 38). The enzymes have been biochemically characterized (34, 38, 57, 58), but no data regarding the genetic background are yet available. Thus far, the dioxygenases have been assumed to be *tfdA* gene products (39), because some dichlorprop- and mecoprop-degrading strains carry *tfdA* genes (36, 39) and cell extracts of *R. eutropha* strain JMP134 showed some specificity

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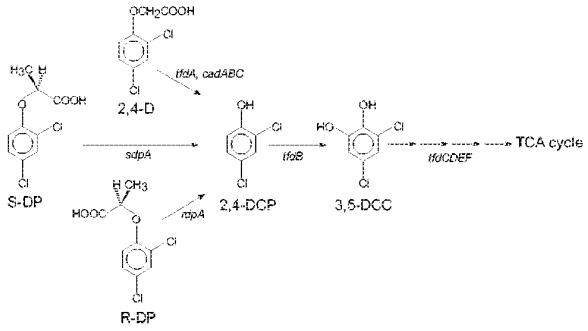


FIG. 1. Modified *ortho*-pathway for the degradation of chlorinated phenoxyalkanoate herbicides. 2,4-D, 2,4-dichlorophenoxyacetate; *R*-DP and *S*-DP, *R*- and *S*-enantiomers of 2(2,4-dichlorophenoxy)propionate, respectively; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; TCA, tricarboxylate.

toward cleaving *S*-dichlorprop (39). However, no activity for *S*-mecoprop or the *R*-enantiomers was detected in strain JMP134, and the organism was unable to grow on phenoxypropionate herbicides. Recently, available N-terminal and internal amino acid sequences of the enantiospecific dioxygenases from *D. acidovorans* MC1 showed only low similarities to TfdA enzymes (57), and *tfdA* genes were not detected in this strain (36). This suggested that genes other than *tfdA* are involved in dichlorprop degradation in strain MC1.

In *D. acidovorans* MC1 the first step in dichlorprop degradation was found to be highly unstable and easily lost under nonselective conditions (36). Loss of dichlorprop-cleaving activity was correlated with a deletion in the plasmid pMC1, and it was therefore hypothesized that the deleted fragment contains the genes encoding dichlorprop cleavage (36).

Based on the recently available peptide sequences of the *R*- and *S*-dichlorprop-cleaving dioxygenases, we investigated the genetic background of dichlorprop cleavage in *D. acidovorans* MC1. Two novel genes encoding the *R*- and *S*-dichlorprop-cleaving dioxygenases in *D. acidovorans* MC1 were identified and localized. The phylogenetic relationship of the *R*,*S*-dichlorprop-cleaving dioxygenases to other α-ketoglutarate-dependent dioxygenases was analyzed. The organization of these genes within the genome of strain MC1, as well as in a mutant deficient in dichlorprop cleavage, was investigated with regard to implications for the instability of this trait.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *D. acidovorans* MC1 and *Rhodoferax* sp. strain P230, isolated from contaminated building rubble (9, 35), *D. acidovorans* MC1-DP$^-$ (a mutant of strain MC1 arisen by spontaneous loss of dichlorprop-cleaving ability), and *S. herbicidovorans* MH, isolated from soil (21), were all grown on MM agar (36) containing 0.5 mM dichlorprop at 30°C. *Escherichia coli* TOP10F′ (pCR2.1; Invitrogen) was used for cloning PCR products. The cosmid library was constructed in *E. coli* XL1-Blue MR (Stratagene). *E. coli* XL1-Blue MRF′ (Stratagene) was used for maintaining pScosPA1. The vector pScosPA1 is a new broad-host-range cosmid identical to pSBC1 (43) except for a gentamicin resistance instead of the tetracycline resistance (E. Mahenthiralingam, unpublished data). A shotgun library of a cosmid was constructed by using the vector pcDNA2.1 (Invitrogen). *E. coli* S17-1 (pAG408::*gfp* [46]) was used in transposon mutagenesis experiments. *E. coli* strains were cultivated on Luria-Bertani (LB) agar at 37°C with the addition of appropriate antibiotics. Overnight cultures were prepared in liquid LB medium containing appropriate antibiotics at 37°C (*E. coli* strains) or 30°C (dichlorprop-degrading strains) on a rotary shaker. Antibiotics and dichlorprop were filter sterilized through a 0.2-μm-pore-size Minisart cellulose acetate filter (Sartorius) and added at a concentration of 100 μg/ml.

Mercury resistance was examined on LB agar plates containing 0.1 mM $HgCl_2$ incubated at 30°C for 48 h. *R. eutropha* JMP134(pJP4) and *R. eutropha* JM222 were used as positive and negative controls, respectively.

Construction of gene libraries. A genomic library of *D. acidovorans* MC1 was constructed by using cosmid pScosPA1, as described elsewhere (43). The average insert size of library clones was 34 kb. A total of 2,592 clones were picked into 96-well plates. After overnight growth 8% (vol/vol) dimethyl formamide was added, and the cosmid library was stored at −80°C.

A shotgun library of cosmid pScos-R5 was constructed in pcDNA2.1 according to the method of Buchrieser et al. (4) by using cosmid DNA isolated as described elsewhere (2, 3).

Transposon mutagenesis. The transposon of the suicide vector pAG408, a miniTn*5* derivative, carries a promoterless *gfp* gene (46) and was used for transposon mutagenesis of strain MC1. Mutagenesis was performed according to standard procedures (40). Overnight cultures of the donor and the recipient grown in LB medium supplemented with antibiotics (donor) were centrifuged, washed, and resuspended in LB medium to a final optical density at 600 nm (OD_{600}) of 1.0. Equal volumes of the donor and the recipient were mixed, and 100 μl of the mixture was plated out on LB agar, followed by incubation at 30°C for 6 h. The bacterial lawn was then washed from the plates with MM medium. Dilution series were prepared in MM and 100 μl of the 10^{-3} to the 10^{-5} dilutions was plated on selective MM agar plates containing 100 μg of gentamicin/ml, 27.8 mM fructose, and 0.5 mM dichlorprop. After incubation at 30°C for 6 days green fluorescing mutants were isolated and tested for dichlorprop degradation by high-pressure liquid chromatography as described elsewhere (60). In mutant clones able to degrade one of the enantiomers but not the other the region flanking the transposon was PCR amplified and sequenced.

DNA amplification. Degenerate primers (Table 1) were designed from N-terminal and internal peptide sequences of the enantiospecific dioxygenases of *D. acidovorans* MC1 (57) for PCR amplification of *rdpA* and *sdpA*. According to preliminary alignments of these peptide sequences with TfdA and TauD, the expected PCR fragment sizes were ca. 350 bp (*R*-dichlorprop dioxygenase gene) and 540 bp (*S*-dichlorprop dioxygenase gene). All primers were synthesized by MWG Biotech. Total genomic DNA used as a template was prepared from colonies grown on LB agar as described elsewhere (25). PCR was performed by using the *Taq* PCR Mastermix kit (Qiagen) and a PTC-200 thermal cycler (MJ Research). First, 100 pmol of each degenerate primer was added to 25 μl of PCR sample. The temperature gradient program involved an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 50 to 70°C for 45 s, and 72°C for 2 min, with a final elongation step of 72°C for 6 min. Amplification of an *rdpA* gene fragment with the primers rdp_fG and rdp_r involved an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 1 min, with a final elongation step of 72°C for 6 min. Amplification of an *sdpA* gene fragment with the primers sdp_fT and sdp_rA was the same as for *rdpA* amplification, except that the annealing step was performed at 60°C and that elongation lasted 90 s.

The *rdpA* genes from *S. herbicidovorans* MH and *Rhodoferax* sp. strain P230 were amplified with primers rdp_forw (5′-atg gta ggt ctc aaA TGC ATG CTG CAC TGT CCC CC-3′; lowercase letters denote a linker sequence) and rdp_rev (5′-atg gta ggt ctc agc gct CTA GCG CGC CGG GCG CAC G-3′). Amplification was the same as for the *rdpA* fragment except for the annealing step being performed at 60°C for 30 s.

Regions flanking Tn*5* transposon insertion sites were amplified by ST-PCR (6). To each sample either Tn5_outF (5′-GGC AGA TCT GAT CAA GAG-3′ [27]) annealing to the I end of the transposon or Tn5_outR (5′-CCG CAC TTG TGT ATA AGA GTC-3′) designed from the O end of the transposon were added as specific primers. Either of the following FP primers (44) was added as a random primer: FP-1, 5′-AGT TCA AGC TTG TCC AGG AAT TC(N)$_7$ GGC CT-3′; FP-2, 5′-AGT TCA AGC TTG TCC AGG AAT TC(N)$_7$ GCG CT-3′; FP-3, 5′-AGT TCA AGC TTG TCC AGG AAT TC(N)$_7$ CCG GT-3′; and FP-4, 5′-AGT TCA AGC TTG TCC AGG AAT TC(N)$_7$ CGC GT-3′.

PCR products for the use as hybridization probes were generated as follows: the *mer* operon was partially amplified with primers mer_f (5′-CAC CAG TTC AGA CAG CAC G-3′) and mer_r (5′-ACC GGT GAA ACC AAG TCC-3′) amplifying a 1,450-bp fragment. The IS*91*-like element was partially amplified by using the primers IS_f (5′-ATG TCA GCC ACT TCC AAG C-3′) and IS_r (5′-GGA GGC AGT ATG CAT TTG G-3′) amplifying a 1,622-bp fragment. The PCR program for both fragments involved an initial denaturation step of 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min, with a final elongation step of 72°C for 6 min. Amplification of *tfdB*, *tfdC*, and

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TABLE 1. Degenerate primers designed for the amplification of *rdpA* and *sdpA*

Peptide or primer	Sequence[a]	Position[b]
RDP_internal sequence 2	W E T L S P T M Q A T I E G L N	162–177
rdp_r	3′-AC GTY CGN TGN TAI CTY CC-5′	
RDP_internal sequence 3	N E I L D A F H T Y Q V I Y F P	43–58
rdp_fA[c]	5′-GCI TTY CAY ACN TAY CA**A** GT-3′	
rdp_fG[c]	5′-GCI TTY CAY ACN TAY CA**G** GT-3′	
SDP_N terminus	M Q T T L Q I T P T G A T L G A	1–16
sdp_fC[c]	5′-ATG CAR ACI ACI CTG CAR AT**C** AC-3′	
sdp_fT[c]	5′-ATG CAR ACI ACI CTG CAR AT**T** AC-3′	
SDP_internal sequence 1	L G H V Q Q A G S A Y I	193–204
sdp_rA[c]	3′-GT**A** CAN GTY GTY CGN CC-5′	
sdp_rG[c]	3′-GT**A** CAN GTY GTY CGN CC-5′	

[a] As described previously (57). IUPAC ambiguity codes: R = A or G, Y = C or T, N = any base, I = inosine.
[b] Alignment is shown in Fig. 2.
[c] To avoid degeneracy near the 3′ end, two pools of primers were designed from some peptides (sequence differences are indicated in boldface).

tfdD gene fragments was performed as described in reference 35. Fragments of *tfdE* and *tfdF* were amplified according to the method of Hoffmann et al. (17).

Sequencing and sequence analysis. For sequencing, PCR products were ligated to pCR2.1 and transformed into *E. coli* TOP10F′ by using the TA cloning kit (Invitrogen). Cycle sequencing was performed by using the ABI Prism BigDye Terminator v2.0 cycle sequencing ready reaction kit (PE Applied Biosystems). M13 primers were used for amplifying and sequencing cloned PCR fragments. After amplification with M13 primers and electrophoretic separation, fragments were extracted with the E.Z.N.A gel extraction kit (Peqlab Biotechnologie). Sequencing reactions (total volume, 10 μl) contained 2 μl of premix, 10 pmol primer, and 10 to 50 ng of template DNA. Primer walking on cosmid DNA was performed with primers designed from the sequences of the *rdpA* and *sdpA* PCR products. Sequencing reactions (total volume, 20 μl) contained 6 μl of premix, 20 pmol of primer, and 500 ng of cosmid DNA. After 25 cycles of 96°C for 30 s, 55°C for 15 s, and 60°C for 4 min, the DNA was precipitated by adding 80 μl of H_2O, 10 μl of 3 M sodium acetate (pH 4.6), and 250 μl of ethanol; washed with 70% ethanol; vacuum dried; and redissolved in 20 μl of template suppression reagent (PE Applied Biosystems). Sequencing from cosmid DNA involved an additional purification step prior to the ethanol precipitation by using Centri-Sep spin columns (Princeton Separations). After denaturation at 90°C/2 min the sample was chilled on ice. Electrophoresis, data collection, and analysis were carried out on an ABI Prism 310 genetic analyzer (PE Applied Biosystems). Both strands were sequenced. Sequencing data were analyzed with the Sequence Navigator software, contigs were assembled by using the Autoassembler software (PE Applied Biosystems), and the BLASTn and BLASTx programs (version 2.2.2. [1]) were used to search for similar sequences in nucleotide and protein sequence databases. Sequences were aligned by using the programs CLUSTAL W (51) or MultAlign (7). Phylogenetic analysis was performed by using the protml tool of the ARB package (29) when the maximum-likelihood method was applied and MEGA2.1 (26) when trees were constructed with the neighbor-joining or maximum-parsimony method.

Recombinant pcDNA2.1 DNA was isolated as described elsewhere (2, 3). A Perkin-Elmer 9700 thermocycler was used for cycle sequencing. Sequencing reactions (total volume, 10 μl) contained ca. 300 ng of template, 1.5 μl of ABI Prism BigDye Terminator v1.1 Cycle Sequencing RR-100 mix, 1.2 μl of 5× sequencing buffer, and 2.4 pmol of primer T7 or M13. The sequencing program involved an initial denaturation step (94°C for 60 s), followed by 70 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reactions were precipitated with 40 μl of 76% ethanol for 30 min, washed with 70% ethanol, and resuspended in 10 μl of 0.3 mM EDTA buffer. Electrophoresis, data collection, and analysis were performed by using an ABI Prism 3700 genetic analyzer (PE Applied Biosystems). Automatic sequence assembly was carried out by using PHRED (11, 12), PHRAP (P. Green, unpublished data), and the Autoassembler software. When gaps were closed between contigs, cosmid DNA was used as a template and custom-made oligonucleotides were added as primers. The sequence was edited by using CONSED (15) and annotated by using Vector NTI Advance (InforMax). Similarity searches were carried out as mentioned above.

DNA-DNA hybridization. Screening of the cosmid library was done by replica plating 12 96-well grids onto positively charged nylon membranes (Roche) that had been placed on selective agar. Cells of strain MC1 (positive control) and *E. coli* XL1-Blue MRF′ (pScosPA1) (negative control), as well as strains MH and P230, were smeared onto the nylon membrane placed on LB agar. After overnight growth, DNA was immobilized onto the membranes according to the manufacturer's guidelines.

Genomic DNA of strain MC1 was isolated as described by Sokol et al. (43). The low-copy plasmid pMC1 was isolated with a Nucleobond AX100 kit (Macherey-Nagel). Plasmid and genomic DNA were PstI digested according to standard procedures (40), separated electrophoretically in 0.8% agarose, blotted onto positively charged nylon membrane according to the manufacturer's guidelines, and cross-linked by UV irradiation for 1 min.

Cosmid and plasmid DNA was isolated from 20 ml of LB medium by using the Nucleobond AX100 kit (Macherey-Nagel). Next, 5 μl of cosmid DNA was applied to positively charged nylon membrane and cross-linked by UV irradiation for 1 min. then, 100 μl of plasmid DNA was PstI or EcoRI/XmaI digested according to standard procedures (40), ethanol precipitated, separated, blotted, and UV cross-linked as described above.

PCR fragments were digoxigenin labeled by using the DIG DNA labeling kit (Roche). For hybridization and immunodetection, the DIG Easy Hyb Wash and Block buffer set and the DIG nucleic acid detection kit (Roche) were used according to the manufacturer's guidelines. Hybridizations were performed at 50°C (*tfdC*, *tfdD*, *tfdE*, and *tfdF* probes), 55°C (*rdpA* probe), 60°C (probes against *tfdB*, the *mer* operon, and the IS*9I* element), or 62°C (*sdpA* probe). Stripping was carried out according to the manufacturer's instructions.

Nucleotide accession numbers. The nucleotide sequences determined in the present study were deposited in the GenBank, EMBL, and DDBJ databases under accession numbers AY327575, AF516751, and AF516752.

RESULTS

Identification, sequencing, and analysis of dichlorprop-cleaving dioxygenase genes in *D. acidovorans* MC1. Degenerate primers were designed from N-terminal and internal peptide sequences of both enantiospecific dioxygenases of *D. acidovorans* MC1. Primers with a degeneracy near the 3′ end proved to be nonspecific; therefore, two pools of primers were synthesized to avoid degeneracy at such positions (Table 1). PCR with the primer pairs rdp_fG-rdp_r (*rdpA*-specific primers) and sdp_fT-sdp_rA (*sdpA*-specific primers) yielded fragments of 339 and 532 bp, respectively, whereas all other primer combinations did not produce specific fragments. The two PCR fragments were cloned and sequenced. The deduced amino acid sequences of both fragments contained sequences at their N and C termini, respectively, that correctly corresponded to the peptide sequences used for designing the prim-

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TABLE 2. Sequence identities of the dichlorprop-cleaving enzymes RdpA and SdpA to other group II αKG-dependent dioxygenases

α-KG dioxygenase[a]	Strain, accession no.	% Identities[b] to	
		RdpA	SdpA
TfdA	*Ralstonia eutropha*, A27082	32/279	37/244
TauD	*Pseudomonas aeruginosa*, NP_252624	35/279	27/258
AtsK	*Pseudomonas putida*, AAD31784	31/296	31/296
YSD	*Saccharomyces cerevisiae*, Q12358	29/279	27/304
PvcB	*Pseudomonas aeruginosa*, AAC21672	20/298	21/277
SyrI	*Pseudomonas syringea*, AAB63253	17/323	18/308
CarC	*Pectobacterium carotovorum*, AAD38231	16/285	17/275
γ-BBH	*Pseudomonas* sp. strain AK-1, P80193	16/309	17/291
CS2	*Streptomyces clavuligerus*, AAA26723	16/208	17/275

[a] TfdA, 2,4-D/αKG dioxygenase; TauD, taurine/αKG dioxygenase; AtsK, putative αKG-dependent alkylsulfatase; YSD, sulfonate/αKG dioxygenase; PvcB, pyoverdine biosynthesis enzyme; SyrI, syringomycin biosynthesis enzyme I; CarC, carbapenem biosynthesis enzyme C; γ-BBH, γ-butyrobetaine hydroxylase; CS2, clavaminate synthase 2.

[b] That is, the percent sequence identity over the total number of amino acids examined.

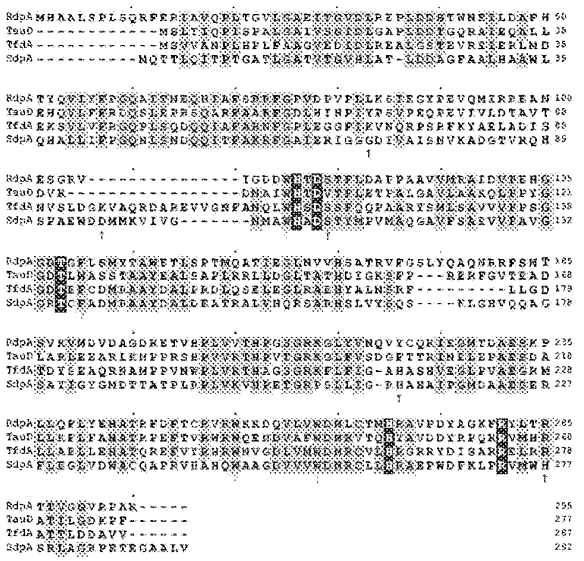


FIG. 2. Sequence alignment of RdpA and SdpA with other group II αKG-dependent dioxygenases. TfdA, *R. eutropha* 2,4-D/αKG dioxygenase (accession no. A27082); TauD, *P. aeruginosa* taurine/αKG dioxygenase (accession no. NP_252624). Conserved Fe(II)- and αKG-binding residues are shaded in black; other conserved regions are shaded in gray. Black arrows indicate 2,4-D binding residues in TfdA; gray arrows indicate residues undergoing posttranslational hydroxylation in TauD or TfdA. Amino acid positions numbers are shown to the right of each line. The alignment was created with CLUSTAL W.

ers. In screens of a genomic cosmid library of strain MC1 10 of 1,152 clones hybridized with the *rdpA* gene probe. Of these 10, 4 hybridized with the *sdpA* gene probe, but PCR with the *sdpA*-specific primers yielded a product with only 1 of these 4 clones, designated pScos-R5. Both dioxygenase genes were then completely sequenced from this clone. The coding regions of the *R*-dichlorprop and *S*-dichlorprop dioxygenase genes were 888 and 879 bp, respectively. No significant sequence similarities to other known genes were found searching nucleotide databases. However, the deduced amino acid sequences of RdpA and SdpA both shared significant similarities to other αKG-dependent dioxygenases ranging between 37 and 16% (Table 2). RdpA and SdpA shared 26% sequence identities. Furthermore, similarities to a number of putative dioxygenases belonging to the genera *Pseudomonas*, *Yersinia*, *Ralstonia*, *Streptomyces*, *Bordetella*, *Bradyrhizobium*, *Azotobacter*, *Shigella*, *Chromobacterium*, *Burkholderia*, and *Mycobacterium* were found and ranged between 31 and 16%.

The amino acid sequences of both dioxygenases were analyzed on the basis of the alignment shown in Fig. 2. It shows that an internal sequence tentatively aligned to positions 257 to 287 of TfdA by Westendorf et al. (57), comprises positions 85 to 131 of RdpA.

Transposon mutagenesis. Of 959 green fluorescing transposon mutants, 2 were found to carry the transposon insertion in *rdpA*. In clone MC1-T270, the insertion was located after bp 627; in clone MC1-T647, the mini-Tn*5* had inserted after bp 814. Both mutants had lost the ability to cleave *R*-dichlorprop but were still able to grow on the *S*-enantiomer.

Organization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1. The 25,768-bp sequence of the genomic fragment from strain MC1 containing *rdpA* and *sdpA* in cosmid pScos-R5 is represented in Fig. 3A. The assembly was verified by PCR of selected regions (data not shown). The *rdpA* and *sdpA* genes were separated by 13.6 kb, which mainly comprised an 8.7-kb putative transposon, organized in a way characteristic for the Tn*501* subgroup of the Tn*3* transposon family (28). It was flanked by 38-bp inverted repeats that were flanked by 5-bp direct repeats generated during insertion. The open reading frames (ORFs) for the putative transposase and resolvase genes *tnpA* and *tnpR* had an outward direction with the ORF for *tnpA* proceeding 5 bp into the inverted repeat. The *res* site, at which cointegrate resolution during transposition takes place, was located upstream of *tnpR*. The remaining part of the transposon was occupied by a mercury resistance gene cluster. The *merR* gene had a direction divergent from the *merTP CADE* operon. A putative *urf2M* was located adjacent to the *mer* operon. The putative Tn*501* transposon showed 81% nucleotide sequence identity to a putative Tn*3* family transposon of *Nitrosomonas europaea* ATCC 19718 (accession no. BX321858.1). The deduced amino acid sequences of the identified ORFs showed between 82 and 97% identity to proteins involved in transposition and mercury resistance (Table 3). The functionality and mobility of this transposon were not tested, but strain MC1 was found to be resistant to 0.1 mM $HgCl_2$. Mercury resistance was not lost when dichlorprop-cleaving activity was lost, but these mutants were still able to grow in the presence of 0.1 mM $HgCl_2$. In Southern hybridizations the *mer* operon of the plasmid was localized on a 4.4-kb fragment of pMC1 (Fig. 4C). This copy was deleted in the plasmid of the mutant MC1-DP$^-$. However, in hybridizations against genomic DNA, another signal at 2.8 kb was detected that was present in the wild-type strain as well as in strain MC1-DP$^-$ (data not shown), suggesting that strain MC1 carries a second copy of the *mer* operon on the chromosome that is not lost when the dichlorprop-cleaving ability is lost. It is possible that the chromosomal copy of the *mer* operon confers mercury resistance to strains MC1 and MC1-DP$^-$, whereas the copy on the plasmid may lack functionality.

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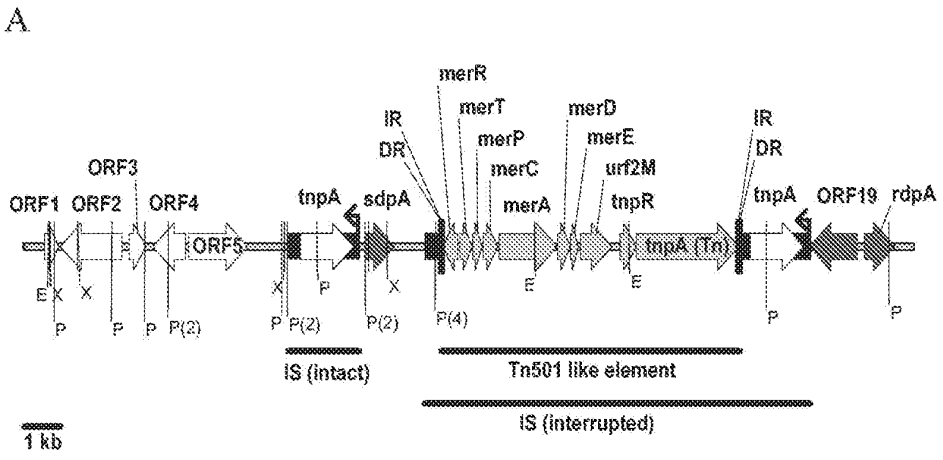


FIG. 3. Organization of *rdpA* and *sdpA* within the genome of strain MC1. (A) Genetic map of the genomic fragment containing *rdpA* and *sdpA*. For ORFs showing a high degree of similarity to other genes (>80% identity of the deduced amino acid sequence) the respective names are given. The gene designations are as follows: *tnpA*/*tnpA*(Tn), putative transposases; *sdpA*, *S*-dichlorprop/α-ketoglutarate dioxygenase; *merR*, putative mercury resistance operon transcriptional regulator; *merT*, *merP* and *merC*, putative mercury ion transport genes; *merA*, putative mercury reductase; *merD*, putative secondary regulator; *merE* and *urf2M*, conserved ORF of unknown function; *tnpR*, putative site-specific resolvase; and *rdpA*, *R*-dichlorprop/α-ketoglutarate dioxygenase. ORF1 through ORF5 and ORF19 show significant similarities to genes encoding the proteins as follows: ORF1, carboxymuconolactone decarboxylase; ORF2, potassium/proton antiporter; ORF3, bacterioferritin; ORF4, LysR type regulator; ORF5, dihydroxyacid dehydratase; and ORF19, MFS transporter. The positions of the following features are indicated: IR, 38-bp inverted repeat flanking the Tn*501*-like transposon, DR, 5-bp direct repeat. Two putative IS*91*-like elements are shown as black boxes; the positions of the *ori*IS are indicated as arrows above the sequence. The right IS*91*-like element is interrupted by a Tn*501*-like transposon. Its left partial sequence is not drawn to scale. Single letters denote the following restriction sites: E, EcoRI; P, PstI; and X, XmaI. In the case of multiple PstI restriction sites within less than 100 bp, only the most left site is given and the number of sites is indicated in brackets. (B) Comparison of the sequence at the right end of the putative IS elements with the IS*91* family *ori*IS consensus sequence. Conserved nucleotides are shown in bold uppercase letters.

Furthermore, two copies of a putative IS element resembling the IS*91*/IS*801* family of insertion elements were identified. Members of this family of IS elements do not contain inverted repeats and do not create duplications of the target sequence (14). Insertion of this family of elements is site specific, and replication utilizes the rolling circle mechanism; therefore, an *ori*IS, the origin of replication, and a *ter*IS, the termination sequence, are found at either end of these IS elements (14). The two copies of the putative IS elements in strain MC1 were essentially identical except for a 9-bp deletion and a 47-bp deletion in the left, intact copy. The element next to ORF19 was interrupted by the Tn*501*-like element (Fig. 3A). The elements were 2,037 bp (intact copy) and 2,084 bp (interrupted copy) and contained an IS*801*-like transposase gene showing 99% sequence identity to the homologues on the atrazine catabolic plasmid pADP-1 (Table 3 [32]) and a transposase associated with chlorobenzene degradation genes in *Ralstonia* sp. strain JS705 (37). At the right end of both elements a sequence was identified, that matched the consensus sequence for origin of replication *ori*IS of this group of insertion elements (49) in all but the first nucleotide (Fig. 3B). Neither the termination sequence *ter*IS nor the tetramers GTTC/CTTG for which IS*91* family elements show site specificity were detected.

ORF19, located upstream of *rdpA* showed significant similarities to aromatic compound major facilitator superfamily (MFS) transporters. A 29% identity to the 2,4-D transporters CadK of *Bradyrhizobium* sp. strain HW13 and TfdK of *Achromobacter xylosoxidans* subsp. *denitrificans* and *R. eutropha* JMP134 was detected. A putative Shine-Dalgarno sequence was found 7 bp upstream of the start codon.

Another five ORFs, ORF1 to ORF5, were identified at the left end side of the insert.

Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of the wild-type plasmid pMC1 with that of the mutant MC1-DP$^-$. Southern hybridizations showed that both *rdpA* and *sdpA* were located on the

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TABLE 3. Localization and predicted function of ORFs in the genomic region of strain MC1 containing *rdpA* and *sdpA*

ORF	Coordinates (bp)	Coding strand[a]	Function of closest match	Source microorganism	% Amino acid identity[b]	No. of amino acids MC1/match[c]	Accession no.
1	574–966	d	Carboxymuconolactone decarboxylase family protein	*Shewanella oneidensis* MR-1	65 (81/124)	130/128	AAN53170.1
2	1006–2799	c	Glutathione-regulated potassium-proton antiporter	*Pseudomonas putida* KT2440	69 (385/556)	597/581	AEO16523.1
3	3018–3494	d	Putative bacterioferritin	*Delftia acidovorans*	70 (112/158)	158/158	CAC83620.1
4	3701–4642	c	Hypothetical protein, similar to LysR-type regulators	*Ralstonia metallidurans*	51 (161/314)	313/360	ZP 00022545.1
5	4745–6438	d	Putative dihydroxy acid dehydratase	*Ralstonia solanacearum*	76 (420/555)	564/557	CAD16119.1
6	7981–9528	d	Putative transposase, similar to IS*801* transposase	*Pseudomonas* sp. strain ADP	99 (512/515)	515/515	AAK50272.1
7	9836–10714	d	2,4-D dioxygenase (TfdA)	*Ralstonia eutropha* JMP134	37 (92/249)	292/287	A27082
8	12107–12541	c	Mercury resistance regulatory protein (MerR)	*Enterobacter aerogenes*	97 (140/144)	144/144	CAA83896.1
9	12613–12963	d	Mercury uptake protein (MerT)	*Alcaligenes* sp.	96 (112/116)	116/116	CAA70196.1
10	12977–13251	d	Mercury uptake protein (MerP)	*Alcaligenes* sp.	94 (86/91)	91/91	CAA70197.1
11	13279–13701	d	Mercury uptake protein (MerC)	*Nitrosomonas europaea* ATCC 19718	86 (121/140)	140/140	CAD84751.1
12	13741–15426	d	Mercury ion reductase (MerA)	*Klebsiella pneumoniae*	88 (494/561)	561/561	AAM44226.1
13	15444–15809	d	Mercury resistance secondary regulatory protein (MerD)	*Pseudomonas* sp. strain ADP	95 (115/121)	121/121	AAK50284.1
14	15806–16042	d	Putative protein (MerE)	*Pseudomonas* sp. strain ADP	91 (71/78)	78/78	AAK50283.1
15	16093–17028	d	Putative protein, similar to Urf2M	*Ralstonia metallidurans*	88 (274/310)	311/329	CAD97548.1
16	17245–17718	d	Resolvase (TnpR)	*Nitrosomonas europaea* ATCC 19718	98 (154/157)	158/186	CAD84747.1
17	17721–20690	d	Tn*3* family transposase (TnpA)	*Nitrosomonas europaea* ATCC 19718	95 (945/989)	989/989	CAD84746.1
18	21024–22571	d	Putative transposase, similar to IS*801* transposase	*Pseudomonas* sp. strain ADP	99 (511/515)	515/515	AAK50727.1
19	22752–24137	c	Putative aromatic compound MFS transporter	*Pseudomonas putida* KT2440	73 (318/433)	461/450	AAN69258.1
20	24330–25217	d	Taurine dioxygenase (TauD)	*Pseudomonas aeruginosa* PA01	35 (100/284)	295/279	NP_252624

[a] c, complementary strand; d, direct strand.
[b] Values in parentheses refer to numbers of identical amino acids over the total number examined.
[c] Number of amino acids in strain MC1 ORF/number in the closest match.

plasmid pMC1 (Fig. 4A). Distinct signals of the expected fragment sizes of 3.5 kb (*rdpA*) and 1.9 kb (*sdpA*) were observed with plasmid DNA (Fig. 4A, lane 1). Much fainter signals of the same fragment sizes were obtained with the genomic DNA, making visible the plasmid content of this. Loss of dichlorprop cleavage in strain MC1-DP$^-$ was associated with a deletion of both dioxygenase genes from pMC1-DP$^-$ (Fig. 4B). Furthermore, the *mer* operon and most of the numerous copies of the IS*91*-like elements were also deleted in the mutant MC1-DP$^-$ (Fig. 4C). In contrast, the lower pathway genes *tfdB-F* remained unaffected (data not shown).

Screening for *rdpA* and *sdpA* in other dichlorprop-degrading bacteria. Southern hybridizations with *rdpA* and *sdpA* probes showed that neither *S. herbicidovorans* MH nor *Rhodoferax* sp. strain P230 carried s*dpA*-like genes, but both strains gave signals with the *rdpA* gene probe (data not shown). The genes were PCR amplified and sequenced. The *rdpA* gene of strain P230 was identical to that of strain MC1; the one of strain MH contained one neutral base substitution.

DISCUSSION

Two novel genes encoding the enantiospecific dichlorprop and mecoprop cleaving dioxygenases RdpA and SdpA in *D. acidovorans* MC1 were identified. The characterization of genes shown to be involved in the cleavage of these chiral herbicides has not previously been reported. To date, *tfdA* genes or genes closely related in sequence to *tfdA* were proposed to be involved in *R*-dichlorprop cleavage in *Alcaligenes denitrificans* (39). This suggestion was based on the observations that a *tfdA* gene is present in *Alcaligenes denitrificans* and that dichlorprop cleavage is catalyzed by an αKG-dependent dioxygenase in this strain as well as in *S. herbicidovorans* MH (38). However, it was not supported by *tfdA* expression studies or amino acid sequences of the *R*-dichlorprop cleaving dioxygenase. The *rdpA* and *sdpA* genes of strain MC1 showed no significant nucleotide similarities to *tfdA*. However, the significant degree of identity of their deduced amino acid sequences to group II αKG-dependent dioxygenases and the distribution of these identities throughout the whole of the primary sequence (Fig. 2) supported the view that RdpA and SdpA are homologous to dioxygenases of this family. The functionally important motif HX(D/E)$X_{23\text{-}26}$(T/S)$X_{114\text{-}183}$H$X_{10\text{-}13}$R/K, which is conserved throughout group II αKG-dependent dioxygenases (18), is present in both RdpA and SdpA. In TfdA of *R. eutropha* it includes the three Fe(II)-binding residues His114, Asp116, and His263, and the two αKG-binding residues Thr141 and Arg274 (Fig. 2). All of these residues were conserved in RdpA and SdpA, representing the putative Fe(II) and αKG-binding residues in these enzymes. Arg278, His214, Ser117, Lys95, and Lys71 of TfdA are known to interact with 2,4-D (8, 10). Ser117 homologues were identified in both dichlorprop-cleaving enzymes. A homologue for Arg278 was present only in RdpA, one for His214 was found in SdpA. The tryptophan residues Trp126, Trp238, and Trp246 undergoing posttranslational hydroxylation in TauD and Trp113 undergoing posttranslational hydroxylation in TfdA form a hydrophobic cluster around the active site (10). In RdpA and SdpA all of these residues were conserved or substituted by other hydrophobic amino acids. The basis of the enantiospecificity of

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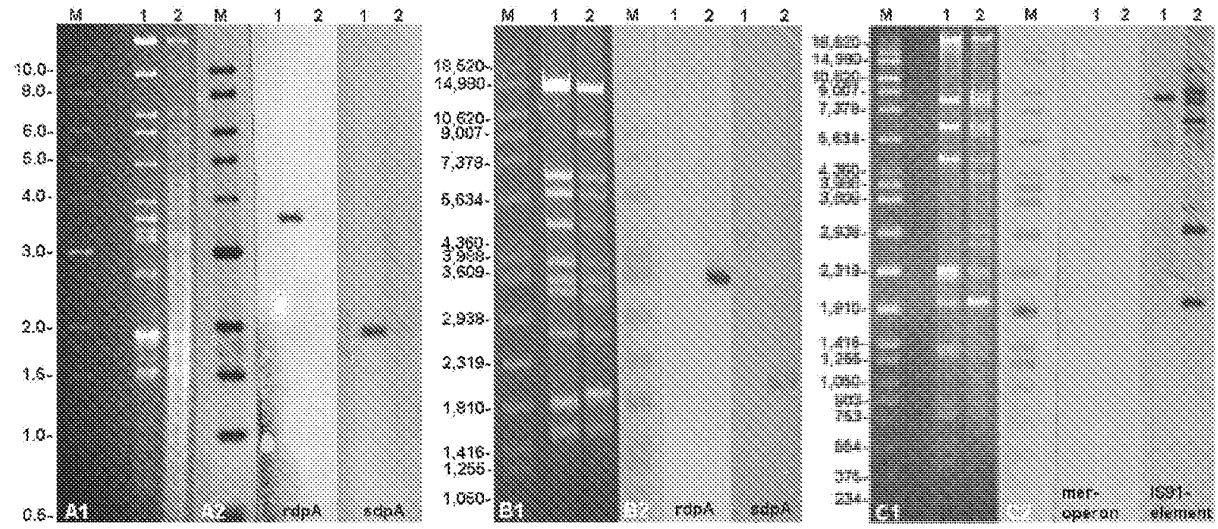


FIG. 4. Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of plasmid pMC1 with pMC1-DP$^-$, the plasmid of mutant MC1-DP$^-$. (A) Localization of *rdpA* and *sdpA* within the genome of strain MC1. Plasmid DNA (lane 1) and genomic DNA (lane 2) was digested with PstI, separated electrophoretically in 0.8% agarose (A1), and hybridized (A2). Gene probes are given at the bottom. (B and C) Comparison of plasmid pMC1 of the wild-type strain of MC1 (lane 2) with pMC1-DP$^-$, the plasmid of the mutant unable to cleave dichlorprop (lane 1). PstI-digested plasmid DNA was separated electrophoretically in 0.8% agarose (B1) and hybridized against *rdpA* and *sdpA* gene probes (B2). EcoRI/XmaI-digested plasmid DNA was separated electrophoretically in 0.8% agarose (C1) and hybridized against probes for the *mer* operon and the IS*91* element (C2).

the dichlorprop-cleaving enzymes is not known. It is known that the tertiary structure of homologous proteins showing at least 20% identity of amino acid sequence is conserved (41). Among group II αKG dioxygenases a high degree of structural conservation was observed between clavaminate synthase and TauD, which share as little as 7.7% primary sequence identity (10). It is therefore likely that 2,4-D and dichlorprop-cleaving enzymes are structurally very similar and that substrate specificity is determined by only a few amino acids. Site-specific mutagenesis studies are needed to reveal the molecular basis of the substrate specificity and stereospecificity of RdpA and SdpA.

Phylogenetic trees calculated by different methods (data not shown) consistently grouped RdpA in one clade with TauD enzymes, but the assignment of SdpA could not be determined precisely. The primary sequence of SdpA most closely resembled that of TfdA and, like this dioxygenase, SdpA is characterized by a relatively broad substrate specificity (34). TfdA has greatest activity with 2,4-D but shows some activity toward *S*-mecoprop (38). SdpA shows greatest activity with the *S*-enantiomers of mecoprop and dichlorprop but has some activity toward 2,4-D (34). These observations suggested that SdpA is most closely related to 2,4-D-cleaving enzymes, which was also consistent with the position of SdpA in trees constructed without an outgroup. However, when PvcB was used as an outgroup SdpA was represented as a sister group of both the TfdA/TfdAα and the RdpA/TauD clades. At present, the database is too small to determine more precisely the phylogenetic relationship between SdpA and other members of the αKG dioxygenase family.

Transposon mutagenesis inactivation of *rdpA* indicated that it was a single-copy gene and gave a further proof of its functionality and role in strain MC1, besides previous characterization of RdpA and SdpA (34, 57, 58).

An *rdpA* gene identical to that of strain MC1 was identified in *Rhodoferax* sp. strain P230, and one nearly identical was found in *S. herbicidovorans* MH, but neither of these strains carried *sdpA* genes, as indicated by Southern hybridizations allowing ca. 20% mismatches. The *rdpA* genes could be involved in *R*-dichlorprop degradation in strains P230 and MH. Further investigations are necessary to study their role to determine whether *rdpA* genes are generally more widely distributed than *sdpA* genes, as these limited data suggest, and to identify the genes encoding the *S*-dichlorprop-cleaving dioxygenases of strains MH and P230.

In *S. herbicidovorans* MH, three transport proteins specific for 2,4-D, *R*-dichlorprop, and *S*-dichlorprop were identified (60). In strain MC1, ORF19 encodes a putative transport protein (Fig. 4 and Table 3). It was in close proximity to *rdpA*, and the significant similarity of the deduced protein sequence to TfdK and CadK suggested an involvement in *R*-dichlorprop transport. No other potential transporters were detected. Likewise, no genes encoding transcriptional regulators of dichlorprop cleavage were identified. This is in accordance with previous studies which found *rdpA* and *sdpA* to be constitutively expressed (36). ORF4, which shows significant similarities to LysR-type transcriptional regulators, probably regulates the expression of ORF5 rather than that of *sdpA* or *rdpA*.

The ability to degrade dichlorprop in *D. acidovorans* MC1 is spontaneously and irreversibly lost at high frequencies under nonselective conditions, whereas the degradation of dichlorophenol is a stable trait (36). This indicated that the ability to cleave the phenoxyalkanoate herbicides is unstable in strain MC1, which would be consistent with recent acquisition of the

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rdpA and *sdpA* genes. The localization and organization of the initial step in dichlorprop degradation imply that its evolution and recruitment took place independently of the lower pathway. Cases and de Lorenzo (5) postulated that degradative pathways for substrates only very recently found in the environment are often poorly regulated, probably reflecting an early step in the optimization of the corresponding metabolic route, and that biodegradative operons evolve from constitutive expression to substrate-responsive and metabolically controlled transcription. The evolution of the dichlorprop cleavage pathway in strain MC1 is an example supporting this hypothesis, since *rdpA* and *sdpA* are constitutively expressed, whereas the consecutive degradation of the intermediate dichlorophenol is inducible (36).

Previously, the loss of genes involved in etherolytic cleavage of dichlorprop was hypothesized to be caused by a deletion within plasmid pMC1, which occurs concomitantly with loss of dichlorprop cleavage (36). In the present study, both dioxygenase genes were shown to be located on the plasmid and to be deleted in the MC1-DP$^-$ mutant, proving this hypothesis to be correct. Moreover, the *mer* operon was deleted, and the copy number of the IS*91*-like element was found to be considerably reduced. This suggests that recombination between IS*91* elements could lead to the deletion of one or both dioxygenase genes and the Tn*501*-like transposon. Although IS*91*-like elements do not cause deletion of regions adjacent to them, a deletion of a genomic fragment flanked by IS*91*-like elements was observed in *Shigella flexneri* (52). In strain MC1, recombination between the interrupted IS element and the intact IS copy downstream of ORF5 would lead to the deletion of the region between the elements, including *sdpA*. Likewise, the IS elements may have played a role in the acquisition of dichlorprop cleavage in strain MC1. In *Ralstonia* sp. strain JS705 the *clc* genes are flanked by an intact and a partial copy of an insertion element highly similar to the ones identified in strain MC1. These IS elements were proposed to have captured the *clc* genes and made possible the gene transfer from *Ralstonia* sp. strain JS745 to strain JS705 (37). Characterization of the region downstream of *rdpA* and comparison of the MC1-DP$^-$ mutant genotype with the MC1 wild-type will further the understanding of processes having lead to the acquisition and causing instability of the degradative trait.

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ERRATUM

Localization and Characterization of Two Novel Genes Encoding Stereospecific Dioxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate Cleavage in *Delftia acidovorans* MC1

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Structural basis for the enantiospecificities of *R*- and *S*-specific phenoxypropionate/α-ketoglutarate dioxygenases

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Abstract

(*R*)- and (*S*)-dichlorprop/α-ketoglutarate dioxygenases (RdpA and SdpA) catalyze the oxidative cleavage of 2-(2,4-dichlorophenoxy)propanoic acid (dichlorprop) and 2-(4-chloro-2-methyl-phenoxy)propanoic acid (mecoprop) to form pyruvate plus the corresponding phenol concurrent with the conversion of α-ketoglutarate (αKG) to succinate plus CO_2. RdpA and SdpA are strictly enantiospecific, converting only the (*R*) or the (*S*) enantiomer, respectively. Homology models were generated for both enzymes on the basis of the structure of the related enzyme TauD (PDB code 1OS7). Docking was used to predict the orientation of the appropriate mecoprop enantiomer in each protein, and the predictions were tested by characterizing the activities of site-directed variants of the enzymes. Mutant proteins that changed at residues predicted to interact with (*R*)- or (*S*)-mecoprop exhibited significantly reduced activity, often accompanied by increased K_m values, consistent with roles for these residues in substrate binding. Four of the designed SdpA variants were (slightly) active with (*R*)-mecoprop. The results of the kinetic investigations are consistent with the identification of key interactions in the structural models and demonstrate that enantiospecificity is coordinated by the interactions of a number of residues in RdpA and SdpA. Most significantly, residues Phe171 in RdpA and Glu69 in SdpA apparently act by hindering the binding of the wrong enantiomer more than the correct one, as judged by the observed decreases in K_m when these side chains are replaced by Ala.

Keywords: dioxygenase; enantiospecificity; mecoprop; site-directed mutagenesis; structural modeling; docking

Supplemental material: see www.proteinscience.org

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; AlkB, alkylation-damaged DNA repair enzyme; AtsK, alkyl sulfatase; ANS, anthocyanidin synthase; CarC, carbapenam synthase; CAS, clavaminate synthase; CSD, Cambridge Structural Database; DAOCS, deacetoxycephalosporin C synthase; dichlorprop, 2-(2,4-dichlorophenoxy)propanoic acid; FIH, factor inhibiting hypoxia-inducible factor; αKG, α-ketoglutarate; mecoprop, 2-(4-chloro-2-methyl-phenoxy)propanoic acid; NTA, nitrilotriacetic acid; PAHX, phytanoyl-coenzyme A 2-hydroxylase; PDB, Protein Data Bank; RdpA, (*R*)-specific dichlorprop/αKG dioxygenase; SdpA, (*S*)-specific dichlorprop/αKG dioxygenase; TfdA, 2,4-D/αKG dioxygenase; TauD, taurine/αKG dioxygenase; TR, tropinone reductase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Phenoxyalkanoic acids are systemic and post-emergence inhibitors of broadleaf weeds and are among the most widely applied herbicides in the world (Worthing and Hance 1991; Ahrens 1994; Donaldson et al. 2002). These synthetic auxins (Åberg 1973; Loos 1975; Ahrens 1994) include 2,4-dichlorophenoxyacetic acid (2,4-D) along with the chiral representatives 2-(2,4-dichlorophenoxy)-propanoic acid (dichlorprop) and 2-(4-chloro-2-methyl-phenoxy)propanoic acid (mecoprop), of which only the (*R*)-enantiomers are herbicidally active (Matell 1953). Microorganisms able to degrade these phenoxyalkanoic acid herbicides have been isolated from different environments, and their degradative pathways have been elucidated (Zipper et al. 1996; Hausinger et al. 1997; Tett et al. 1997;

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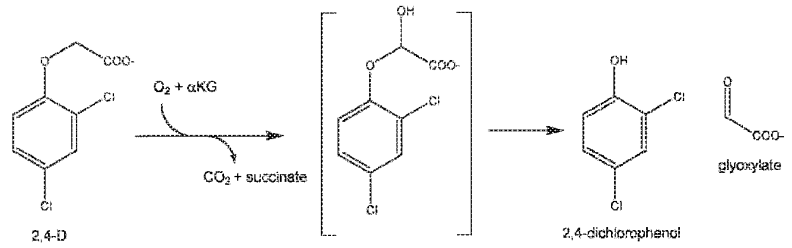
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Müller et al. 1999, 2001). For example, the first step in 2,4-D metabolism is side-chain hydroxylation to form an unstable intermediate that decomposes by elimination of the phenol derivative (Scheme 1).

The 2,4-D hydroxylase (TfdA) from *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134(pJP4) has been intensively studied and shown to require Fe^{II} as a cofactor and α-ketoglutarate (αKG) as a cosubstrate (Fukumori and Hausinger 1993a,b; Saari and Hausinger 1998; Hegg et al. 1999; Hogan et al. 2000; Dunning Hotopp and Hausinger 2002). For mecoprop or dichlorprop metabolism, best studied in the soil bacterium *Sphingomonas herbicidovorans* MH, the enantiomers are separately transported into the cell by distinct uptake systems (Nickel et al. 1997), and enantiomer-specific (*R*)- and (*S*)-dichlorprop/αKG dioxygenases (RdpA and SdpA) catalyze the initial degradation steps as illustrated in Scheme 2 (Nickel et al. 1997; Müller 2004; Müller et al. 2004b). RdpA and SdpA share 30% amino acid sequence identity to each other and 30% and 37% identity, respectively, to TfdA, with no significant gaps in alignment quality, indicating that they are all close structural homologs (Sander and Schneider 1991). The substituted phenol products released from these Fe^{II}/αKG-dependent dioxygenases are subsequently converted to the corresponding catechols and further metabolized by the modified *ortho*-cleavage pathway.

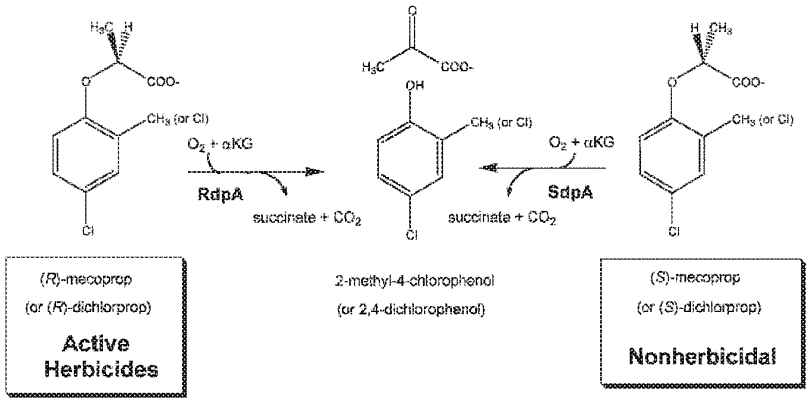
The herbicide-degrading dioxygenases belong to a large family of mononuclear, nonheme Fe^{II} enzymes that catalyze a broad array of reactions (for review, see Hausinger 2004; Clifton et al. 2006) including hydroxylations, epoxidations, desaturations, ring formation, ring expansion, and as only recently discovered, chlorinations (Vaillancourt et al. 2005a,b). Crystal structures have been elucidated for several family members including taurine/αKG dioxygenase (TauD) (Elkins et al. 2002; O'Brien et al. 2003), alkyl sulfatase (AtsK) (Müller et al. 2004a, 2005), clavaminate synthase (CAS) (Zhang et al. 2000), deacetoxycephalosporin C synthase (DAOCS) (Valegård et al. 1998), anthocyanidin synthase (ANS) (Wilmouth et al. 2002), carbapenam synthase (CarC) (Clifton et al. 2003), proline 3-hydroxylase (Clifton et al. 2001), the factor inhibiting hypoxia-inducible factor (FIH) (Dann et al. 2002; Elkins et al. 2003), phytanoyl-coenzyme A 2-hydroxylase (PAHX) (McDonough et al. 2005), and the DNA repair enzyme AlkB (Yu et al. 2006). The structures reveal a common β-jelly roll or double-stranded β-helix fold containing a metal ion-binding motif: His^1-X-Asp/Glu-X_n-His^2 (where *n* varies from 40 to 153 residues). Three water molecules occupy the remaining metal ligand positions in the resting enzyme. Two water molecules are displaced upon binding of the cosubstrate, with the αKG C-2 keto group coordinating opposite the carboxylate side chain and the αKG C-1 carboxyl group binding opposite either His^1 (TauD, AtsK, CAS, and FIH) or opposite His^2 (DAOCS, ANS, CarC, PAHX, and AlkB), with a nearby Arg residue (located 15–22 residues beyond His^2 in the sequence) providing additional stabilization to the C-1 carboxylate in the cases of TauD, AtsK, CAS, CarC, and AlkB. Another Arg residue (located in the sequence about 10 residues beyond His^2) is positioned to form an ion pair with the C-5 carboxylate of αKG in all structures except FIH, where a Lys located elsewhere in the sequence provides stabilization. Unlike other Fe^{II} sites, the αKG-bound metallocenters exhibit a characteristic metal-to-ligand charge-transfer transition (Pavel et al. 1998; Hegg et al. 1999; Ryle et al. 1999; Trewick et al. 2002) conferring a lilac color to this state of the enzymes. The primary substrate (e.g., taurine in the case of TauD) does not bind to the metal center, but the aforementioned crystallographic studies and additional spectroscopic evidence (Ho et al. 2001; Zhou et al. 2001) indicate that substrate binding leads to the loss of the final water molecule, thus creating a site for binding of oxygen. In the case of TauD, oxidative decarboxylation of αKG has been shown to produce an Fe^{IV}-oxo intermediate species that inserts oxygen into the unactivated C–H bond (Price et al. 2003a,b, 2005; Proshlyakov et al. 2004; Riggs-Gelasco et al. 2004; Grzyska et al. 2005).

Sequence alignments highlight several potential key residues of the (*R*)- and (*S*)-dichlorprop/αKG dioxygenases from *S. herbicidovorans* MH (Müller 2004). The His^1-X-Asp/Glu-X_n-His^2 motif of RdpA is comprised of residues His111, Asp113, and His270, while that of SdpA involves His102, Asp104, and His257. Fifteen residues beyond His^2 are residues predicted to interact with the C-1 carboxylate of αKG, Arg285 and His272, respectively.



Scheme 1.

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Scheme 2.

Furthermore, Arg281 and Arg268 of the two proteins are predicted to form salt bridges to the αKG C-5 carboxylate, with additional interactions involving Thr138 of RdpA and Thr129 of SdpA. In contrast to these conserved residues, essentially nothing is known about the phenoxypropanoic acid-binding sites of these proteins, especially with regard to the structural basis of enantiospecificity.

Here, we describe the construction of homology models of SdpA and RdpA from *S. herbicidovorans* MH and the use of docking to identify residues likely to be involved in herbicide binding. Previous homology models have led to successes in elucidating or designing specificity-conferring interactions in ligands. For instance, homology modeling of a cercarial (human parasite) elastase led to the development of an effective elastase inhibitor (Cohen et al. 1991) and to understanding the specificity determinants for ligands binding to a parasite tRNA synthetase versus its human homolog (Sukuru et al. 2006). Here, we test by site-directed mutagenesis and kinetic analysis the residues predicted to be involved in substrate binding, enantiospecificity, or catalysis. The activity experiments are consistent with the key residues identified by modeling being involved in substrate binding. We provide additional evidence that several amino acids are responsible for the enantiospecificity of RdpA and SdpA, demonstrate that the active site of SdpA is less specific than RdpA for its substrate, and discuss the structural implications of these results.

Results

SdpA and RdpA homology models

RdpA and SdpA were aligned with TauD (Supplemental Fig. S1), and homology models were created as described in Materials and Methods (Supplemental Fig. S2) using the TauD structure as a structural template (O'Brien et al. 2003). The two phenoxypropionate-degrading proteins are predicted to contain jelly roll or double-stranded β-helix folds comprised of eight β-strands with connecting loops, as is typical of this enzyme family (Hausinger 2004; Clifton et al. 2006). The homology models contain Fe^{II}-binding sites (His111, Asp113, and His270 in RdpA or His102, Asp104, and His257 in SdpA), as expected from former sequence alignments with other Fe^{II}/αKG-dependent dioxygenases (Müller 2004). The high degree of active-site sequence identity and strong orientation of key side chains by interactions with the Fe^{II} are supportive of the active site being the most conserved and structurally accurate part of the RdpA and SdpA models. Detailed analysis of favored αKG-binding motifs in other members of this enzyme family indicate the iron is chelated by αKG with its keto group positioned opposite Asp113 of RdpA or Asp104 of SdpA and its C-1 carboxylate located so as to interact with His111 and His102, respectively. The positively charged residues Arg285 in RdpA and His272 in SdpA are well positioned to provide additional stabilization of the αKG C-1 carboxylate, and, in each protein, the C-5 carboxylate of αKG forms a salt bridge with Arg268 and Arg281, respectively. Whereas the RdpA structure represents only one subunit of the predicted trimeric protein, SdpA is suggested to be monomeric, based on gel-filtration experiments (Müller 2004).

Docking of substrates into the RdpA and SdpA structures

The natural substrates (*R*)- and (*S*)-mecoprop were docked into the active sites of RdpA and SdpA to gain insight into the basis of enzyme enantiospecificity. First, the cosubstrate αKG was modeled into the active sites of RdpA and SdpA using two distinct conformations, as found in the crystal structures of Fe^{II}/αKG-dependent dioxygenases

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(Clifton et al. 2006). The flat conformation has the five-member ring formed by the metal chelate coplanar with the C-5 carboxylate, whereas the twist conformation has the two planes forming a 90° angle. The resulting four models were energy-minimized and used as targets for substrate docking with the program SLIDE (Zavodszky et al. 2002) with the assumption that the substrate carbon undergoing hydroxylation would be located approximately at the same position relative to the iron center as the key carbon atom of taurine in TauD (Elkins et al. 2002; O'Brien et al. 2003). The mecoprop-docking interactions with RdpA and SdpA were analyzed in detail, and one model of each protein was selected based on the most favorable interactions between enzyme and substrate (see below). These models are illustrated in Figure 1, with the corresponding plots of mecoprop interactions shown in Figure 2.

Binding of (R)-mecoprop to RdpA

The substrate (*R*)-mecoprop consists of a hydrophobic phenoxy ring and a polar propanoic acid, with both components needing to be accommodated and bound by the active site. The αKG conformation leading to the most favorable interactions has αKG in the twist conformation and positions the phenoxy ring of (*R*)-mecoprop as illustrated in Figure 1A (with the corresponding interactions plotted in Fig. 2A). The mecoprop carboxylate interacts with the amide nitrogen of Ser114, the hydroxyl group of Tyr221, and a guanidino nitrogen of Arg285. The Tyr221 hydroxyl group also is predicted to lie near (3.5 Å) the substrate ether oxygen atom and could play a role in directing enantiospecificity. Residues lining the hydrophobic substrate-binding pocket include Val80, Leu83, Ile106, Gly107, and Phe171 (Figs. 1A, 2A), with Val80 and Leu83 being well positioned to interact with the propanoic acid methyl group. The terminal carbon atom (CZ) of the Phe171 side chain is 4.1 Å from the phenoxy group of (*R*)-mecoprop; since LigPlot has a 4.0 Å threshold for hydrophobic interactions, this interaction is missed in Figure 2A.

To directly test the importance of potential substrate-binding residues of RdpA identified by the homology modeling and substrate docking procedures, variant forms of the enzymes were created by site-directed mutagenesis. To eliminate the bulky and polar Tyr221 and Arg285 residues, Y221A and R285A mutants were generated. In the presumed "hydrophobic pocket," Val80, Leu83, Ile106, and Phe171 each were changed to alanine to reduce hydrophobic interactions and thereby decrease the binding

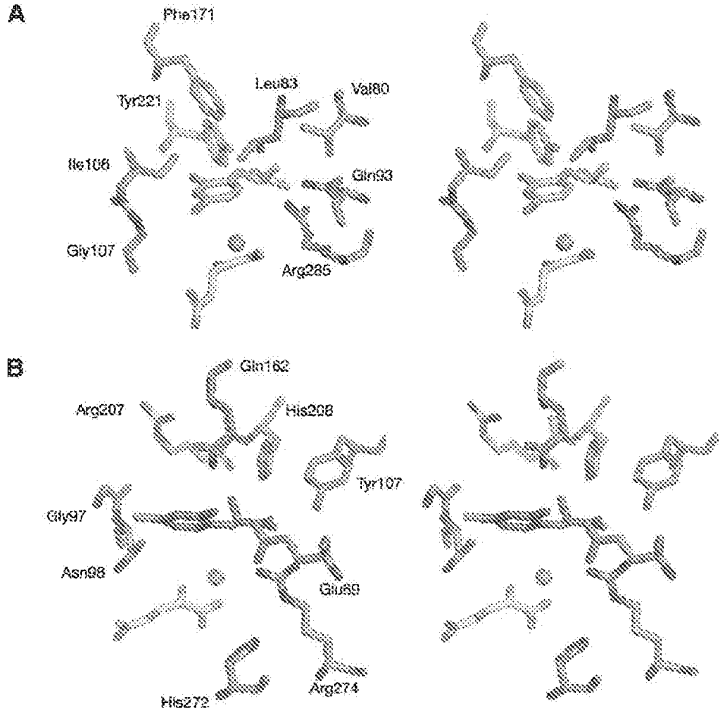


Figure 1. Stereo views of the most favorable models of the active sites of RdpA (*A*) and SdpA (*B*) with bound substrates. Shown are selected protein residues that are predicted to interact with the substrate. For clarity reasons, the Fe^{II} ligands are not depicted. (*R*)- and (*S*)-mecoprop are indicated in light blue and in turquoise, respectively, and αKG in yellow.

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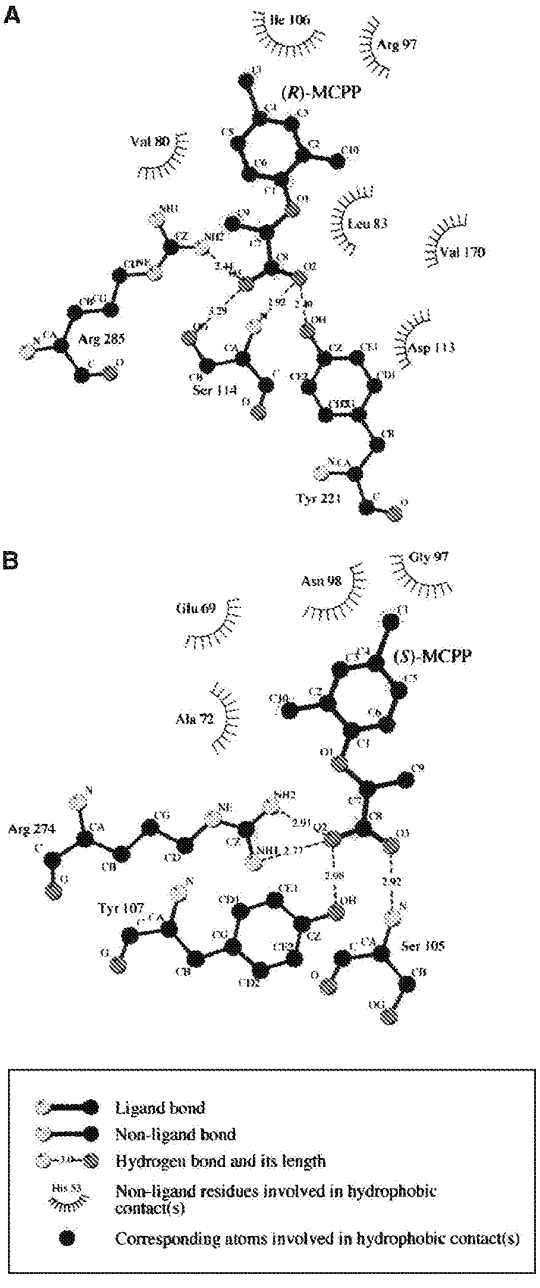


Figure 2. LIGPLOT diagrams of the active site models of RdpA (*A*) and SdpA (*B*). Shown are the most important interactions between the substrate and the active site according to the model.

affinity of (*R*)-mecoprop to the active site. Replacing Gly107—positioned near and coplanar with the phenoxy ring—with a bulkier hydrophobic residue is expected to hinder substrate binding, so this residue was mutated to Ile and Asn in the double mutants I106G/G107I and I106G/G107N, respectively.

The specific activities of the RdpA variants were tested using (*R*)-mecoprop and the racemic mixture (Table 1). The (*S*) enantiomer is not sold commercially and was available in very limited supply, so the RdpA variants were not tested with this compound. When the RdpA variants were examined using 4 mM (*R*)-mecoprop, the V80A and F171A variants exhibited ~60% of wild-type enzyme activity; I106A had ~30% of that activity and L83A, Y221A, and R285A were ~10% active. The activities of the double mutants I106G/G107I and I106G/G107N were further reduced compared with that of I106A, consistent with a bulkier side chain at position 107 presenting steric hindrance to substrate binding in a reactive conformation.

The RdpA variants retaining at least 10% of wild-type enzyme activity were subjected to more detailed kinetic characterization (Table 2). The maximal concentration of substrates that could be tested was 4 mM due to solubility limitations; therefore, K_m values higher than 800 μM are only approximations and possess large errors. The K_m values of V80A and R285A RdpA variants were at least fivefold increased over that of the wild-type enzyme, whereas that of the I106A RdpA variant was more than 20-fold greater, supporting the described docking orientation of the model. The effect on the R285A variant can be understood in terms of decreased interaction with the substrate carboxylate, while the changes observed for the V80A and I106A variants are likely to arise from loss of hydrophobic interactions. The F171A protein had a threefold lower K_m, indicating either that this residue does not interact specifically with the phenoxy ring of (*R*)-mecoprop or that F171 is actually slightly hindering (*R*)-mecoprop binding. The calculated k_{cat} of all mutant proteins was similar to the wild-type value with the exception of the R285A mutant enzyme. Arg285 is postulated to interact with both the mecoprop carboxylate and the C-1 carboxylate of αKG, so it could directly influence catalysis.

Binding of (S)-mecoprop to SdpA

The most favorable orientation of (*S*)-mecoprop was obtained with αKG in the twist conformation in SdpA and (*S*)-mecoprop bound as illustrated in Figure 1B (with the corresponding ligand interactions shown in Fig. 2B). In this model, the substrate carboxylate interacts with the amide nitrogen of Ser105, the hydroxyl group of Tyr107, and the guanidino nitrogens of Arg274. Additional active site residues near the polar carboxylate include His272 and His208. Also of interest, the substrate ether oxygen atom is predicted to lie within 3.4 Å of the two carboxyl oxygens of Glu69. If protonated or bridged by the proton of a bound water molecule, the Glu69 carboxyl group could confer specificity to the (*S*) enantiomer by making a hydrogen bond with the ether oxygen (a prediction not borne out by experimental results, vide infra). Residues predicted to be

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Table 1. *Activity measurements of native and variant forms of RdpA and SdpA*

RdpA Sample	Hypothesis probed[a]	(*R*)-mecoprop (4 mM) Specific activity (U/mg protein)	(%)	(*R*)-mecoprop (2 mM) Specific activity (U/mg protein)	(%)	(*R,S*)-mecoprop (4 mM) Specific activity (U/mg protein)	(%)
Wild type	—	7.11 ± 1.34	100	5.60 ± 0.26	100	6.46 ± 0.44	100
V80A[b]	I	4.3 ± 2.1	60.5	2.06 ± 0.48	36.8	2.7 ± 0.5	41.8
L83A[c]	I, II	0.37 ± 0.09	5.2	0.24 ± 0.06	4.3	0.27 ± 0.02	4.2
Q93A[b]	II	4.3 ± 0.7	60.5	3.27 ± 0.23	58.3	2.27 ± 0.15	35.2
I106A[c]	I, II	2.14 ± 0.025	30.1	1.41 ± 0.29	25.2	1.10 ± 0.11	17.0
F171A[c]	I, II	4.83 ± 0.31	67.9	4.75 ± 0.22	84.8	4.59 ± 0.35	71.1
F171Q[c]	II	5.2 ± 0.4	73.1	4.86 ± 0.52	86.8	4.49 ± 0.31	69.5
Y221A[b]	I	0.37 ± 0.17	5.2	0.43 ± 0.02	7.7	0.31 ± 0.01	4.8
R285A[c]	I, II	1.1 ± 0.1	14.8	0.92 ± 0.06	16.4	0.80 ± 0.06	12.4
I106G/G107I[d]	I, II	0.05 ± 0.03	0.7	0.04 ± 0.03	0.7	0.05 ± 0.05	0.8
I106G/G107N[d]	I, II	0.62 ± 0.06	8.7	0.56 ± 0.05	10.0	0.53 ± 0.04	8.2

SdpA Sample	Hypothesis probed[a]	(*S*)-mecoprop (4 mM) Specific activity (U/mg protein)	(%)	(*S*)-mecoprop (2 mM) Specific activity (U/mg protein)	(%)	(*R,S*)-mecoprop (4 mM) Specific activity (U/mg protein)	(%)	(*R*)-mecoprop (4 mM) Specific activity (U/mg protein)
Wild type	—	25.5 ± 1.7	100.0	28.96 ± 3.6	100	23.5 ± 0.74	100.0	0
E69A[d]	I, II	7.85 ± 1.65	30.8	6.6 ± 1.3	22.8	5.54 ± 1.24	23.6	0.51 ± 0.22
Q162F[e]	I, II	1.22 ± 0.3	4.8	0.87 ± 0.15	3.0	0.53 ± 0.08	2.3	0
R207A[e]	I, II	4.4 ± 0.57	17.3	3.13 ± 0.52	10.8	2.08 ± 0.3	8.9	0.1 ± 0.02
R207V[e]	II	0.71 ± 0.07	2.8	0.52 ± 0.05	1.8	0.35 ± 0.04	1.5	0.01 ± 0.004
H208A[d]	I, II	0.35 ± 0.1	1.4	0.27 ± 0.07	0.9	0.14 ± 0.05	0.6	0
H272A[d]	I	0.3 ± 0.16	1.2	0.27 ± 0.16	0.9	0.25 ± 0.09	1.1	0
R274A[d]	I	0.01 ± 0.01	0.0	0.01 ± 0.01	0.0	0	0.0	0
G97N/N98G[d]	II	0.07 ± 0.03	0.3	0.06 ± 0.01	0.2	0.02 ± 0.03	0.1	0
G97I/N98G[d]	II	0.96 ± 0.27	3.8	0.93 ± 0.26	3.2	0.83 ± 0.26	3.5	0.01 ± 0.01

[a] The letters indicate whether the mutant was used to probe (I) the predicted binding mode of the substrate or (II) the enantiospecificity of the enzyme.
[b] Values were determined by measuring activities of samples isolated from three independent cell cultures.
[c] Values were determined by triplicate measurements of a single sample.
[d] Values were determined by measuring activities of samples isolated from two independent cell cultures.
[e] Values were determined by triplicate (for (*R,S*)-mecoprop) or duplicate (for (*S*)- and (*R*)-mecoprop) measurements of a single sample.

in contact with the hydrophobic ring of (*S*)-mecoprop include Ala71, Ala72, Leu82, Val84, Gly97, Asn98, Gln162, and Arg207 (with the latter four residues depicted in Fig. 1B). π-Cation interactions between positively charged side chains like Arg207 and aromatic side groups like that in mecoprop can contribute very favorably to ligand binding (Mitchell et al. 1994).

To experimentally test the importance of several of these residues for the binding of (*S*)-mecoprop in the active site of SdpA, a series of mutant proteins was constructed. Glu69, His208, His272, and Arg274 each were changed to alanine to eliminate the bulky and charged side chains proposed to interact with the substrate carboxylate. The extended side chain of Arg207 was eliminated in the R207A SdpA variant, and Gln162 was substituted with the corresponding hydrophobic residue found in RdpA to generate the Q162F variant.

The specific activities of the SdpA variants were tested using the (*S*) enantiomer, the racemic mixture, and the (*R*) enantiomer of mecoprop (Table 1). The activity of all SdpA mutants was strongly reduced. The most active SdpA variants were the E69A and R207A proteins, while all other variants exhibited <5% of the wild-type enzyme activity. No activity was detected in the R274A sample. E69A, R207A, and H208A were subjected to detailed kinetic characterization (Table 2). When analyzed with (*S*)-mecoprop, the R207A and H208A samples had 15- and 18-fold increases, respectively, in their K_m values compared with the wild-type enzyme. The H208A result is compatible with an ionic interaction between the mecoprop carboxylate group and His208. A similar interaction might be possible with the highly flexible Arg207 residue, or this side chain may participate in a π-cation interaction with the aromatic group of the substrate, in either case accounting for the large K_m change in the R207A variant. In contrast to these results, the E69A variant exhibited a lower K_m than the native SdpA, arguing against the ether oxygen having a significant role in substrate binding. With (*S*)-mecoprop, the k_{cat} was reduced fourfold for the E69A and R207A SdpA mutants compared with wild-type enzyme, and even greater reductions were noted for the H208A variant.

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Table 2. *Kinetic parameters of selected variant forms of RdpA and SdpA*

RdpA Variant	(*R*)-mecoprop Range (μM)	(*R*)-mecoprop K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}*\mu M^{-1}$)
Wild type	100–4000	380 ± 28	252 ± 5	0.66
V80A[a]	200–4000	2300 ± 1100	248 ± 56	0.11
Q93A[a]	200–4000	2800 ± 400	260 ± 21	0.09
I106A[b]	500–4000	8900 ± 2100	238 ± 42	0.03
F171A[b]	200–4000	108 ± 7	175 ± 2	1.62
F171Q[b]	62.5–4000	438 ± 34	203 ± 4	0.46
R285A[a]	200–4000	1550 ± 220	52 ± 4	0.03

	(*S*)-mecoprop				(*R,S*)-mecoprop			
SdpA Variant	Range (μM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}*\mu M^{-1}$)	Range (μM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}*\mu M^{-1}$)
Wild type	31–4000	161 ± 20	1010 ± 44	6.24	31–4000	234 ± 18	899 ± 24	3.85
E69A[c]	31–4000	91 ± 14	241 ± 8	2.65	222–4000	181 ± 38	194 ± 8.4	1.1
R207A[d]	250–4000	2500 ± 400	238 ± 20	0.1	250–4000	2780 ± 370	117 ± 8.4	0.04
H208A[c]	500–4000	2900 ± 960	21 ± 4	0.007	N.D.			
G97I/N98G[c]	250–4000	1100 ± 270	42 ± 4.4	0.04	N.D.			

N.D., not determined.
[a] Values were determined by measuring activities of samples isolated from three independent cell cultures.
[b] Values were determined by triplicate measurements of a single sample.
[c] Values were determined by measuring activities of samples isolated from two independent cell cultures.
[d] Values were determined by triplicate (for (*R,S*)-mecoprop) or duplicate (for (*S*)- and (*R*)-mecoprop) measurements of a single sample.

Residues determining enantiospecificity in RdpA and SdpA

A direct comparison of the active sites in the RdpA and SdpA models is shown in Figure 3. The different substrate enantiomers are predicted to bind with remarkably similar geometries in the two predicted structures, with the carboxyl groups and aromatic rings nearly overlapping but at distinct angles. RdpA residues Leu83, Gln93, Ile95 (omitted for clarity), Ile106, Phe171, and Arg285 (shown in Fig. 1A) could hinder binding of (*S*)-mecoprop; the corresponding residues in SdpA are Ala72, Leu82, Val84, Gly97, Gln162, and His272, respectively. Similarly, SdpA residues Glu69, Asn98, Ser161, His208, and Arg207 could restrict binding of (*R*)-mecoprop; Val80, Gly107, Val170, Tyr221, and Val220 occupy these positions in RdpA. Ala72, Gly97, and Gln162 are predicted to allow access by the (*S*) enantiomer. In general, side-chain interactions suggest that SdpA is less specific than RdpA for its substrate. This finding is compatible with activity measurements (Table 1), such as the activity with 2 mM (*S*)-mecoprop versus that with 4 mM of the racemate, that show inhibition of SdpA activity by (*R*)-mecoprop. However, RdpA remains completely active toward the (*R*) enantiomer in the presence of (*S*)-mecoprop (Müller 2004).

Site-directed variants of RdpA and SdpA, created and purified as described earlier, were used to test a subset of the above-mentioned residues for their importance in controlling enantioselectivity. Single substitutions included the L83A, Q93A, I106A, F171A, F171Q, and R285A variants of RdpA along with the E69A, Q162F, R207A, R207V, and H208A variants of SdpA. In addition, to test the possibility that Ile106/Gly107 (large residue/small residue) in RdpA versus Gly97/Asn98 (small residue/large residue) in SdpA confers some measure of enantiospecificity to the enzymes, the I106G/G107I and I106G/G107N double variants of RdpA along with G97N/N98G and G97I/N98G double variants of SdpA were generated. Each enzyme was tested for activity using its enantiomer, the racemate, and in the case of the SdpA mutants, with (*R*)-mecoprop, too (Tables 1, 2).

Among the RdpA variants were several that show evidence of inhibition by the (*S*) enantiomer. For example, the Q93A variant retains 60% of wild-type enzyme activity, but this dropped to 35% when the racemate was provided, suggesting that Gln93 prevents the incorrect enantiomer from binding to and inhibiting the enzyme. Significant decreases in activity also were apparent when comparing rates using the racemic mixture of mecoprop versus the (*R*)-enantiomer with the Y221A and R285A variants; these results suggest that Tyr221 and Arg285 contribute to enantiospecificity. These variant proteins exhibited ~5% to ~15% of the wild-type enzyme activity. Less dramatic differences were observed using the I106A, F171A, F171Q, and I106G/G107N variants, but in each case the activity was reduced in the presence of the incorrect enantiomer. The I106A, F171A, F171Q, and R285A proteins were subjected to detailed kinetic

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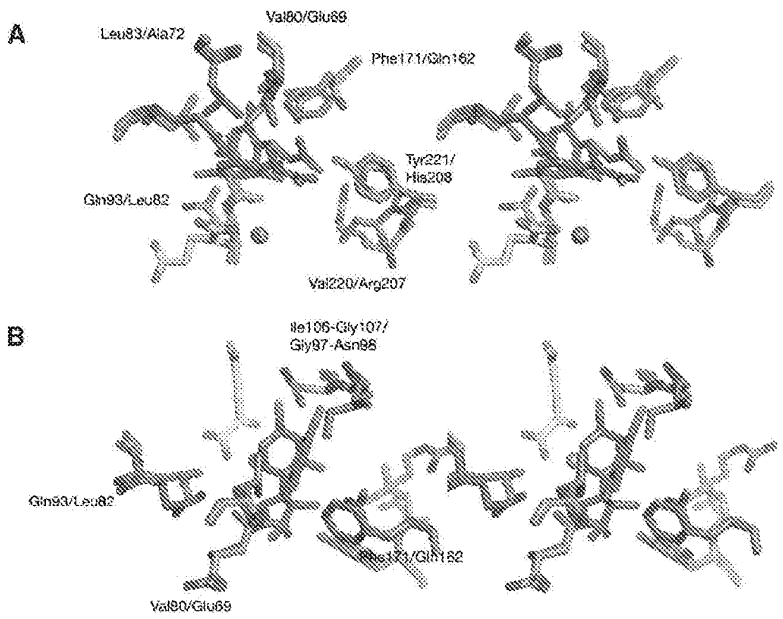


Figure 3. Stereo views of the superimposition of the substrate-bound active site models of RdpA and SdpA. Shown are selected residues predicted to be involved in dictating enantiospecificity. Amino acid residues of RdpA are depicted in pink; residues of SdpA, in green. (*R*)-mecoprop is shown in purple; (*S*)-mecoprop, in turquoise. (*A*) View with the focus on the propanoic acid moiety and its binding to the active sites. (*B*) Same as in *A* but rotated to focus on the phenoxy ring and its interaction with the amino acid residues Ile106/Gly107 in RdpA and Gly97/Asn98 in SdpA.

analysis with (*R*)-mecoprop (Table 2). With the exception of the R285A variant (which exhibited ~20% of the wild-type enzyme k_{cat}), the proteins retained >70% of the wild-type enzyme k_{cat}. The I106A and R285A variants exhibited 23- and fourfold increases in K_m; the F171Q form exhibited a K_m similar to the wild-type enzyme, and the F171A mutant had a threefold lower K_m. The latter result is consistent with Phe171 hindering binding of the correct (*R*) enantiomer while helping to exclude the (*S*) enantiomer from the RdpA active site, so that the expanded active site in the F171A variant more readily binds its substrate.

Significantly, four SdpA variants (E69A, R207A, R207V, and G97I/N98G) showed slight activity with the opposite enantiomer, (*R*)-mecoprop (Table 1). These results suggest that Glu69, Arg207, and Gly97/Asn98 contribute to determining the enantiomeric specificity, as predicted by docking. They are compatible both with the postulated interaction of Glu69 with the (*S*)-mecoprop ether oxygen and with steric constraints on (*R*)-mecoprop binding imposed by Glu69, Arg207, and Asn98. Kinetic analyses of the E69A variant (Table 2) argue against a positive, stabilizing interaction between Glu69 and the substrate ether atom, because the K_m of this variant is less than that of the wild-type enzyme. These results are most consistent with Glu69 discriminating against binding by the opposite enantiomer while slightly hindering binding of the correct enantiomer. The apparent K_m of wild-type SdpA was approximately doubled when examined using the racemic mixture, as expected from earlier work (Müller 2004). Similar results were obtained for the E69A variant, while the K_m was essentially unchanged for the R207A protein when comparing the single enantiomer versus the racemate. The R207A K_m results are consistent with both substrate enantiomers binding to the active site with similar affinities. This point was directly demonstrated by assaying the R207A enzyme with (*R*)-mecoprop, yielding a K_m of 4.0 ± 1.3 mM and a k_{cat} of ~0.16 min^{-1}. For comparison, the K_m of E69A SdpA for (*R*)-mecoprop was determined to be 5.4 ± 1.7 mM with a k_{cat} of 65 ± 15 min^{-1}. The k_{cat} was further reduced for the E69A and H208A variants when using the racemic mixture, consistent with binding and inhibition by the (*R*) enantiomer. The low activities of the H208A and G97I/N98G proteins precluded a similar analysis.

Discussion

On the basis of the modeled RdpA and SdpA structures, we identified several amino acid residues that are likely to interact with (*R*)- and (*S*)-mecoprop, including several that are likely to contribute to the enantiospecificities of

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the two enzymes. The structural modeling performed here incorporated small-scale conformational changes in optimizing the protein–ligand complex through flexible docking using SLIDE and energy minimization of the structures. However, many enzymes, including selected representatives of the Fe^{II}/αKG dioxygenases (Clifton et al. 2006), are known to undergo larger-scale conformational changes upon ligand binding. The problem then becomes one of predicting protein conformational change as well as the orientation of ligand binding, a prodigious task for which no generally proven method exists. Success has been shown on subproblems for some complexes, e.g., predicting relative degrees of flexibility within a protein and how this flexibility redistributes upon complex formation (Gohlke et al. 2004), predicting large-scale conformational change between two protein states (Tama et al. 2004), evaluating conformational ensembles of the ligand and protein structures that are consistent with binding (Zavodszky et al. 2004), and predicting ligand binding by docking into a series of experimentally known enzyme conformations that approximate larger-scale protein flexibility (Lorber and Shoichet 1998). Molecular dynamics simulation of the protein–ligand complex is another option. However, it is generally difficult to access substantially different ligand orientations and protein conformations in molecular dynamics simulations. In our case, the situation is further complicated given the trimeric state of RdpA and the possible influence of subunit interfaces on active-site conformations. Therefore, the accuracy of homology modeling and prediction of (*R*)- and (*S*)-mecoprop interactions with the RdpA and SdpA enzymes may be limited by not anticipating medium- to large-scale protein conformational change. The predictive value of this modeling—identifying residues likely to be responsible for recognizing the (*R*) versus (*S*) enantiomer of mecoprop—can be assessed by consistency with the binding effects of mutations of these residues. Site-specific variants of RdpA and SdpA were created, their activities measured, and, in selected cases, detailed kinetic experiments were performed. The results confirm the residues involved in substrate or cosubstrate binding and provide useful insights into the structural basis of enantiomeric specificity.

Basis of the substrate-binding specificity of RdpA and SdpA

(*R*)- and (*S*)-mecoprop docked to the predicted structures of RdpA and SdpA, respectively, indicate that the mecoprop carboxylates and phenoxy rings are similarly positioned in the two proteins, with the substrate methyl group oriented in opposite configurations. The activity and kinetic data for R285A and Y221A variants of RdpA are compatible with the (*R*)-mecoprop carboxylate interacting with both the guanidino group of Arg285 and the hydroxyl group of Tyr221, where Tyr221 also forms a potential hydrogen bond to the ether oxygen atom. Similarly, activity and kinetic measurements of the R274A and H208A proteins are consistent with SdpA using the corresponding residues Arg274 and His208 to bind the carboxylate of (*S*)-mecoprop. Comparable Arg residues are conserved in many Fe^{II}/αKG-dependent dioxygenases, where they play a dual role of binding the primary substrate and the C1-carboxyl group of αKG (Valegård et al. 1998; Elkins et al. 2002; O'Brien et al. 2003; Hausinger 2004; Müller et al. 2004a). Such a dual role is predicted for Arg285 of RdpA, whereas Arg274 and His272 split these functions in SdpA.

Examination of the mutant forms of RdpA and SdpA also provided insights into the specificity for the distinct mecoprop enantiomers. For RdpA variants, Gln93, Ile106, and Phe171 are proposed to sterically exclude binding by the incorrect enantiomer, whereas the Q93A variant yields results consistent with inhibition by (*S*)-mecoprop. Similarly, the results with the SdpA mutants are consistent with Glu69, Asn98, and Arg207 and His208 acting to prevent binding of the (*R*) enantiomer. In particular, the E69A, R207A, and G97I/N98G variants exhibited activity with the incorrect substrate, (*R*)-mecoprop. Hydrophobic interactions are also likely to be critical for substrate discrimination. For example, Val80 and Leu83 in RdpA are predicted to be well positioned to interact with the propanoic acid methyl group, whereas Glu69 and Ala72 occupy these positions in SdpA. We conclude that several residues in each protein play a role in dictating the substrate enantiospecificity.

Comparison of the proposed RdpA- and SdpA-substrate interactions with 2,4-D binding by TfdA

RdpA and SdpA are 30% and 37% identical to TfdA, for which a homology model was previously constructed and its 2,4-D substrate docked (Elkins et al. 2002). Predicted interactions in theTfdA model previously were tested by site-directed mutagenesis and kinetic analyses (Hogan et al. 2000; Dunning Hotopp and Hausinger 2002), similar to what is described here for RdpA and SdpA. In the case of TfdA, the substrate carboxylate was suggested to interact with Arg278, His214, and Lys71. While the TfdA Arg and His residues have direct counterparts in the mecoprop-degrading enzymes (Arg285 and Tyr221 in RdpA; Arg274 and His208 in SdpA), there is no indication of a carboxylate-stabilizing Lys in RdpA or SdpA. Also of interest, Lys71 and/or Lys95 of TfdA was suggested to interact with the ether oxygen atom of 2,4-D. Again, a Lys does not appear to function in this manner in the mecoprop-degrading enzymes; however, Tyr221 of RdpA is reasonably well positioned for this role.

TfdA is slightly active with (*S*)-dichlorprop, but not with (*R*)-dichlorprop (Saari et al. 1999). Based on secondary

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structure alignment, TfdA residues Ala109 and Asn110 correspond to the Gly97/Asn98 (small residue/large residue) pair in SdpA, versus Ile106/Gly107 in RdpA. Evidence described earlier is consistent with this pair of RdpA and SdpA residues contributing to the enantiospecificity of these enzymes. The presence of (*S*)-dichlorprop-degrading activity in TfdA supports this proposal. 2,4-D converting TfdAs from other strains also show slight activities with phenoxypropanoic acids. Like TfdA from *C. necator* JMP134(pJP4), TfdA from *Burkholderia cepacia* strain RASC cleaves the ether bond of (*S*)-dichlorprop (Saari et al. 1999), whereas TfdA from *Alcaligenes denitrificans* exclusively converts the (*R*) enantiomer (Tett et al. 1997). Interestingly, PCR-derived DNA fragments reveal that these enzymes share 86% amino acid sequence identity in the corresponding region of the proteins (Saari et al. 1999); if the entire proteins exhibit this level of identity, the enantiospecificity is likely to be conferred by a few key amino acid residues.

Conclusion and relationship to other enantiospecific enzymes

The stereocenter-recognition-model of Sundaresan and Abrol (2002, 2005), much like the simpler three-point interaction model (Davankov 1997), generalizes the interactions between a chiral substrate and an enzyme or a receptor by postulating that at least three points of interaction are needed to distinguish between enantiomers with one chiral center. For the enantiospecific binding of mecoprop to RdpA and SdpA, the active site could be expected to interact with the carboxylic acid, either the ether oxygen atom or the phenoxy ring, and the methyl group. The predicted substrate interactions and kinetic results for RdpA and its variants are consistent with such mecoprop interactions. That is, several protein side chains are predicted to position the carboxylate, and Tyr221 likely forms a hydrogen bond to the ether oxygen, while several residues, notably the Ile106/Gly107 pair, position the phenoxy ring, and Val80 and Leu83 interact with the methyl group. In contrast, the SdpA model displays only two clear interactions with (*S*)-mecoprop: Tyr107 and Arg274 side chains position the carboxylate, and several, including the Gly97Asn98 pair, position the phenoxy ring, but the methyl group exhibits little interaction with the active site. Therefore, the active site of SdpA appears to be inherently less enantiospecific for (*S*)-mecoprop and more easily binds the opposite (*R*) enantiomer. The observed inhibition of SdpA by the (*R*) enantiomer and the reactivity of certain SdpA variants with (*R*)-mecoprop support this hypothesis.

In general, enantioselectivity is known to arise from both stabilization of the preferred substrate and relative destabilization of binding by the other enantiomer (Sundaresan and Abrol 2002, 2005). For example, tropinone reductase (TR)-I and TR-II reduce tropinone in a stereoselective manner to produce tropine and Ψ-tropine. Tropinone is differently oriented in the active sites of the two enzymes, with both proteins providing stabilizing interactions and TR-I acting to prevent the inappropriate binding mode by a repulsion interaction involving the positively charged nitrogen of tropinone and a positively charged histidine residue (Nakajima et al. 1998). RdpA and SdpA are proposed to conform to this general pattern by using both positive and negative interactions to enhance binding by the appropriate enantiomer.

Materials and methods

Recombinant plasmids

Mutagenesis of *rdpA* and *sdpA* within pMec15 and pMec19 was carried out by using the Stratagene QuikChange System (Stratagene) and the mutagenic primers listed in Supplemental Table S1. Each mutation was confirmed by sequence analysis (Davis Sequencing). These constructs produce "wild-type" and variant enzymes as His_6-tagged fusion proteins.

Enzyme purification

Escherichia coli C41(DE3) (Miroux and Walker 1996) cells harboring pMec15, pMec151, pMec152, pMec153, pMec154, pMec155, pMec156, pMec157, pMec158, pMec160, and pMec161 were used to purify His_6-tagged wild-type RdpA and its V80A, Q93A, Y221A, R285A, I106G/G107I, I106G/G107N, L83A, I106A, F171A, and F171Q variants, respectively. Similarly, *E. coli* BL21(DE3) (Novagen) cells harboring pMec19, pMec191, pMec192, pMec193, pMec194, pMec195, pMec196, pMec197, and pMec198 were used to purify His_6-tagged wild-type SdpA and its E69A, H208A, H272A, H274A, G97N/N98G, G98I/N98G, Q162F, R207A, and R207V variants, respectively. The cells were grown with constant shaking in Erlenmeyer flasks containing terrific broth supplemented with 200 μg/mL of ampicillin. Starter cultures grown overnight at 37°C were used to inoculate (0.5% [v/v]) medium for growth at room temperature. Upon reaching an OD_{600} between 0.3 and 0.7, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the cultures were grown for an additional 16–18 h. To overexpress wild-type and variant forms of His_6-tagged RdpA in *E. coli* C41(DE3) cells, it was important to fill flasks to 50% of their volume with medium and that the shaking be kept at moderate levels to minimize aeration and decrease growth rate. The cells were harvested by centrifugation at 6000*g* for 10 min at 4°C. To prepare cell extracts, each 1 g of the cell pellet was resuspended in 2.5 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole at pH 8), and the cells were disrupted by sonication with cooling on ice between pulses. The disrupted cells were centrifuged at 130,000*g* for 60 min at 4°C, and the clarified cell extracts were stored in aliquots at –80°C. RdpA and SdpA accounted for up to 23% and 33% of the protein in the respective soluble cell extracts.

His_6-RdpA and its variants were purified by a one-step procedure carried out at room temperature. Up to 100 mg of protein was loaded onto a Ni-bound NTA-agarose column (5 × 2.5 cm; Invitrogen) in lysis buffer. Unbound proteins were removed from

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the column with five column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole at pH 8). His_6-RdpA and its variants were released from the resin with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 200 mM imidazole at a pH adjusted to 8) containing 20% glycerol to increase stability. His_6-SdpA and its mutants were purified as described for the His_6-RdpA, except that no glycerol was included in the elution buffer (see representative purifications in Supplemental Fig. S3). Purified proteins were stored on ice for up to 4 d.

Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out according to established procedures (Laemmli 1970).

Assays

Typical assays contained 100 μM $(NH_4)_2Fe(SO_4)_2$, 1 mM ascorbic acid, 1 mM αKG, and the indicated amount of substrate in 100 mM imidazole buffer (pH 6.75). All assays were carried out at 30°C. To determine kinetic parameters for phenoxypropanoic acids, the coupled continuous enzyme assay was used as previously described (Müller 2004). Units of activity were defined as micromoles of product produced per min per mg protein. Values of k_{cat} were calculated using M_r 34,950 for the His_6-RdpA variants and 33,460 for the His_6-SdpA variants.

Structural modeling

Homology models of RdpA and SdpA were generated on the basis of the structure of the related taurine/αKG dioxygenase (TauD) from *E. coli* (O'Brien et al. 2003). With 33% and 29% identity to RdpA and SdpA and no significant gaps in the alignment, TauD (PDB code 1OS7, chain A) meets the criterion of at least 25% identity over at least 80 residues (Sander and Schneider 1991) to be an accurate structural template for modeling. Multiple alignments for the dichlorprop hydroxylase structures were obtained with the Bioinfo Meta Server (http://bioinfo.pl/Meta), and one was selected based on optimal agreement with predicted secondary structures. Side chains of RdpA and SdpA were reconstructed with the MMTSB Tool Set (Feig et al. 2000, 2004). Nonconserved loops and missing residues were added with Modeller (Fiser et al. 2000, 2002). The iron was placed into the active site according to the TauD structure, and the side chains in the predicted binding sites were adjusted using Insight II (Accelrys) to have similar conformations to the corresponding side chains from TauD, since conserved residues in catalytic sites tend to be highly structurally conserved. Catalytic sites are even more strongly conserved in structure when they involve a metal center, which strongly orients its ligating side chains. In particular, residues His111, Asp113, and His270 in RdpA and His102, Asp104, and His257 in SdpA were repositioned to bind the Fe^{II} metallocenter as they do in TauD, and αKG was positioned with its C-1 carboxylate plus C-2 carbonyl group chelating the metal and its C-5 carboxylate forming an ion pair with Arg268 or Arg281, respectively. The conformation of the free αKG (ID code COTPAC) was obtained from the Cambridge Structural Database (CSD).

To model αKG interactions with RdpA and SdpA as accurately as possible, multiple bound αKG conformations were retrieved from crystal structures of other αKG-dependent dioxygenases (PDB codes 1OS7, 1GY9, 1GQW, 1OII, 1OIJ, 1OIK, 1NX4, and 1NX8). This analysis revealed two distinct conformations: The flat conformation has the five-member ring formed by chelating the Fe^{II} nearly coplanar with the C-5 carboxylate, whereas the twist conformation has the two planes at a 90° angle. Both conformations could be accommodated by RdpA and SdpA, and four models (RdpA and SdpA with the flat and the twist αKG conformation) were built and energy-minimized in the context of the protein structure, with an implicit aqueous solvent (Generalized Born with solvent accessibility correction) using the MMTSB tool set and the CHARMM22 force field (Brooks et al. 1983; Feig et al. 2004).

Docking of substrates

The energy-minimized structures with each of the αKG conformations were used as targets for docking with the program SLIDE (Schnecke and Kuhn 2000; Zavodszky et al. 2002), which models the small-scale protein and ligand side-chain rotations that are ubiquitous in induced fit between proteins and their ligands (Zavodszky and Kuhn 2005). The free (*R*)-mecoprop conformation was obtained from CSD. Alternative low-energy conformations for both the (*R*) and the (*S*) enantiomer, reflecting the range of conformations likely to be energetically accessible, were generated from connectivity information using Omega v1.8.b3 (OpenEye Scientific Software). The energy window allowed for the conformers was 20 kcal/mol, ensuring a very thorough search of the ligands' conformations. Nine conformers each of (*R*)- and (*S*)-mecoprop and of the CSD structure were generated, and all 27 conformers were docked into each of the four models (RdpA with flat and twisted αKG conformations, and likewise for SdpA) with SLIDE using the default parameter set (Schnecke and Kuhn 2000; Zavodszky et al. 2002). To identify the most likely binding orientation, the dockings of mecoprop into RdpA and SdpA were filtered using the following geometric criteria for catalysis: The distance between the Fe^{II} and the C7 carbon atom of mecoprop should be ~4 Å, and the C7-H4 bond should orient toward the Fe^{II}. In addition, based on the interactions observed between enzymes of this class and their substrates, the carboxylate group of mecoprop is expected to bind in the same region and make similar interactions as the taurine sulfate group bound to TauD, anchoring the substrate for catalysis. Among the (*R*)- mecoprop dockings satisfying these criteria, the one with the most favorable interactions positioned the mecoprop phenoxy ring into the enlarged hydrophobic pocket formed by the replacement of bulky Tyr73 in TauD by Leu83 in RdpA, and the propanoic acid methyl group in the hydrophobic pocket created by replacing His70 of TauD with Val80. This comprised the favored RdpA-*(R)*-mecoprop model. The (*S*)-mecoprop dockings satisfying the above geometric criteria were very similar to each other, and the one with optimal protein-substrate hydrogen bonds was selected.

Figures 1, and 3, and Supplemental Figure S2 of RdpA and SdpA structures and active sites were produced using the program PyMOL (DeLano Scientific LLC). Ligand interactions were depicted using LIGPLOT v.4.4.2 (Wallace et al. 1995).

Electronic supplemental material

Coordinate files in PDB format containing the atomic coordinates of the modeled RdpA and SdpA structures in complex with *(R)*- and *(S)*-mecoprop and a document file containing a table, three figures, and their legends.

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Probing the 2,4-Dichlorophenoxyacetate/α-Ketoglutarate Dioxygenase Substrate-Binding Site by Site-Directed Mutagenesis and Mechanism-Based Inactivation†

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ABSTRACT: TfdA is an Fe(II)- and α-ketoglutarate- (αKG-) dependent dioxygenase that hydroxylates the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) producing a hemiacetal that spontaneously decomposes to 2,4-dichlorophenol and glyoxylate. On the basis of a recently published TfdA structural model [Elkins et al. (2002) *Biochemistry 41*, 5185−5192], His214, Lys71, Arg278, and the backbone amide of Ser117 are suggested to bind the 2,4-D carboxylate; Lys95 and possibly Lys71 are hypothesized to interact with the 2,4-D ether atom; and Arg274 and Thr141 are suspected to bind αKG. TfdA variants with substitutions at these and other positions were purified and characterized in order to explore the roles of these residues in catalysis. The K71L, K71Q, K95L, K95Q, R274Q, R274L, and R278Q variants exhibited significantly increased 2,4-D K_m, αKG K_m, and αKG K_d values, consistent with their proposed roles in substrate binding. A protease-sensitive site was successfully eliminated in the R78Q variant, which also exhibited decreased affinity for 2,4-D. In contrast, the Y81F, Y126F, T141V, Y169F, and Y244F variants showed only modest changes in their kinetics. An observed 4-fold lower K_m of the K95L variant compared to wild-type protein with the alternative substrate 2,4-dichlorocinnamic acid provided additional evidence for an interaction between Lys95 and the 2,4-D ether atom. Phenylpropiolic acid was identified as a mechanism-based inactivator of the enzyme [K_i = 38.1 ± 6.0 μM and $k(\text{inact})_{max}$ = 2.3 ± 0.1 min^{-1}]. This acetylenic compound covalently modifies a peptide (166-AEHYALNSR-174) that is predicted to form one side of the substrate-binding pocket. The K95L variant of TfdA was not inactivated by phenylpropiolic acid, providing added support that Lys95 is present at the active site. These results support the identity of suspected substrate-binding residues derived from structural modeling studies and extend our understanding of the oxidative chemistry carried out by TfdA.

α-Ketoglutarate- (αKG-)[1] dependent dioxygenases are mononuclear non-heme Fe(II) enzymes that couple the oxidative decarboxylation of αKG to substrate oxidation (*1*, *2*). Depending on the specific enzyme, the primary substrate may be a protein side chain, a cellular metabolite, or a compound taken up from the environment (*3*). Most commonly, substrate oxidation involves hydroxylation at an unactivated carbon atom, but desaturations, epoxidations, or ring formation or expansion reactions have also been characterized. On the basis of crystal structures of several family members (*4*−*9*) and site-directed mutagenesis studies of the corresponding genes (e.g., ref *10*), Fe(II) is known to be ligated by two histidines and a carboxylic acid-containing side chain, leaving three coordination sites for binding of solvent. Upon binding, αKG displaces two water molecules and chelates the metal via the C-1 carboxylate and C-2 keto group, while the C-5 carboxylate forms a salt bridge with an arginine or lysine side chain. When substrate binds, the final water is displaced, leaving an open coordination site that is available for oxygen activation (*1*, *2*). A "jellyroll" protein architecture serves as the platform for these residues (*4*−*9*), which form a conserved $HX(D/E)X_nHX_{(\sim 10)}(R/K)$ motif (*10*).

2,4-Dichlorophenoxyacetic acid (2,4-D)/αKG dioxygenase (TfdA) is a representative family member that hydroxylates the herbicide 2,4-D, producing a hemiacetal that spontaneously decomposes to 2,4-dichlorophenol and glyoxylate (Scheme 1). Previous site-directed mutagenesis studies revealed the roles of His114, Asp116, and His263 in binding iron and suggested that His214 functions in 2,4-D binding or catalysis (*10*). Recently, a model of the three-dimensional structure of TfdA (Figure 1) was developed on the basis of the crystal structures of two homologous enzymes, taurine/

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[1] Abbreviations: αKG, α-ketoglutarate; CS, clavaminate synthase; DAOCS, deacetoxycephalosporin C synthase; 2,4-D, 2,4-dichlorophenoxyacetic acid; EDTA, ethylenediaminetetraacetic acid; FAB, fast atom bombardment; MALDI, matrix-assisted laser desorption−ionization; PPA, phenylpropiolic acid; TauD, taurine/αKG dioxygenase; TFA, trifluoroacetic acid; TfdA, 2,4-D/αKG dioxygenase; Tris, tris(hydroxymethyl)aminomethane.

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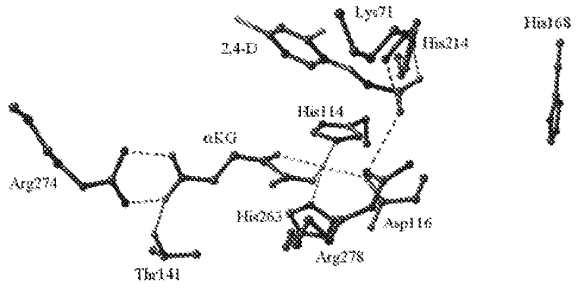
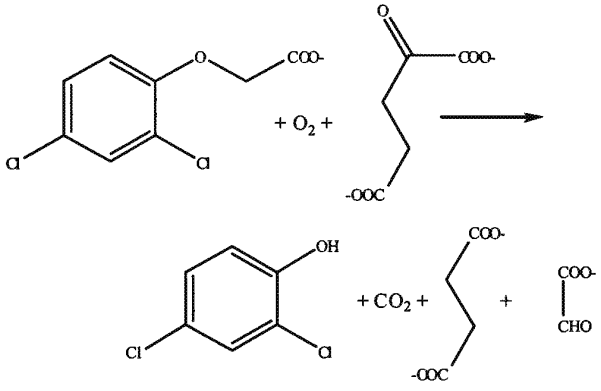


FIGURE 1: Diagram illustrating the predicted positions of active site residues in TfdA. The crystal structures of TauD and CS were previously used to model the homologous protein TfdA (PDB accession number 1GQX) (*9*). The residues believed to be involved in substrate binding, and for which variants were made in these studies, are illustrated. Not shown is Lys95, which is found on a loop that could not be convincingly modeled; however, several possible models positioned this residue where it could interact with the 2,4-D ether atom.

Scheme 1



α-ketoglutarate dioxygenase (TauD) (*9*) and clavaminate synthase 1 (CS) (*6*). The TfdA model placed His114, Asp116, and His263 together in a geometry consistent with a metal-binding site, located His214 nearby at the suspected 2,4-D binding site, and revealed that a protease-sensitive site involving Arg78 was located on the surface of the protein. Furthermore, the model newly identified a set of additional residues likely to be involved in 2,4-D and αKG binding.

The studies described here utilize site-directed mutagenesis and mechanism-based inactivation approaches to better define the residues at the substrate-binding site of TfdA. The TfdA model is tested by characterizing selected mutant proteins substituted at the proposed substrate-binding residues. Additional TfdA mutants are characterized in order to assess the effects of altering a residue at the protease-sensitive Arg78 site and to explore the importance of tyrosine residues located near the metal center. Finally, phenylpropiolic acid (PPA) is shown to be a mechanism-based, irreversible inactivator of TfdA that covalently attaches to the protein, thus revealing an additional sequence located at the active site.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The amino acid residue numbering scheme used here is based on that derived from the gene sequence rather than that of the purified protein and differs by the addition of the amino-terminal methionine residue compared to that used previously (*10*, *11*). TfdA variants were created using the Stratagene QuikChange mutagenesis system and pUS311 (*12*) as the starting plasmid. This pUC19 derivative contains the *Ralstonia eutropha* JMP134 (pJP4) *tfdA* gene (*13*).

Protein Purification Methods. Protein concentrations were determined by using the Bio-Rad assay and bovine serum albumin as the standard. Wild-type and all variant forms of TfdA except Y126F protein were purified from *Escherichia coli* DH5α as previously described (*14*). Y126F TfdA was temperature sensitive so the protein was purified at 4 °C and with alterations to the last two chromatographic steps. Specifically, the active fractions eluting from the DEAE-Sepharose column were pooled, adjusted to 1 M ammonium sulfate, loaded onto a phenyl-Sepharose column (2.5 cm × 19 cm) equilibrated with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.7) containing the same concentration of this salt, and chromatographed with a 400 mL linear gradient from 1 to 0 M ammonium sulfate. The active fractions were dialyzed to remove the ammonium sulfate and then chromatographed on a Q-Sepharose column (2.5 cm × 19 cm) using TE buffer and a 400 mL linear gradient to 200 mM NaCl in TE.

Kinetic Analyses. Typical activity measurements used the 4-aminoantipyrene assay (*15*) for detecting 2,4-dichlorophenol. Oxygen consumption and 2,4-dichlorocinnamic acid oxidation were monitored using previously described oxygen electrode and HPLC methods, respectively (*14*). As previously documented (*16*), TfdA activity decreases over time by a combination of irreversible inactivation [possibly associated with an enzymatic self-hydroxylation process (*11*)] and ascorbate-reversible inactivation (presumably due to metal oxidation). For the wild-type enzyme and some of its variants, the inactivation rate is insignificant over the course of a typical assay and linear kinetics were assumed. For several variant proteins, however, the activity was observed to decrease over the assay time course; thus, progress curves were analyzed by fitting the data to eq 1 where P_t is the

$$P_t = V_i(1 - e^{-k(\text{inact})t})k(\text{inact})^{-1} \quad (1)$$

accumulated product at time t, V_i is the initial velocity, and k(inact) is the inactivation rate constant (*16*). In cases where high concentrations of substrate were found to decrease the k_{cat}, the data were analyzed for competitive substrate inhibition (measured as K_{si}) by fitting to eq 2. The K_d values

$$k = \frac{k_{cat}[S]}{K_m + [S] + [S]^2/K_{si}} \quad (2)$$

associated with binding of Fe(II) and αKG to TfdA and its variants were measured by using intrinsic tryptophan fluorescence and fitting to eq 3 as previously described (*17*). In

$$\Delta F = \Delta F_{max}\,(K_d + [L_T] + [E_T] - \sqrt{(K_d + [L_T] + [E_T])^2 - 4[L_T][E_T]})/2[E_T] \quad (3)$$

this equation, the observed changes in fluorescence (ΔF) are related to the maximal fluorescence change (ΔF_{max}) extrapolated to infinite concentration of titrant. On the basis of the known total concentrations of enzyme subunit ([E_T]) and

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ligand ([L_T]), the K_d and ΔF_{max} were calculated. Curve fits were computed using KaleidaGraph for Windows by Abelbeck Software.

Kinetics of TfdA Inactivation by Phenylpropiolic Acid. The inactivation of TfdA by PPA was studied in 10 mM imidazole, 1 mM αKG, 5 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, and 20 μM ascorbic acid containing varying concentrations of the inactivator at 30 °C. Stock solutions of all reagents were made fresh prior to each set of experiments. Aliquots were withdrawn at the indicated time intervals and diluted 100-fold into fresh assay buffer containing 1 mM 2,4-D, and residual activities were determined as in the standard 4-aminoantipyrene assay procedure. These data were plotted according to log(residual activity) versus time in order to obtain the inactivation rates, *k*(inact), and further analyzed by fitting to the equation:

$$k(\text{inact}) = \frac{k(\text{inact})_{max}[\text{I}]}{K_i + [\text{I}]} \quad (4)$$

where [I] is the inactivator concentration, $k(\text{inact})_{max}$ is the inactivation rate at saturating [I], and K_i is the [I] that gives rise to half-maximal rates of inactivation.

Large-Scale Inactivation of TfdA by PPA Followed by Alkylation and Protease Digestion. TfdA (1−5 mg) was inactivated by PPA in 10 mM imidazole buffer (pH 6.8) containing 1 mM αKG, 1 mM PPA, 5 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, and 20 μM ascorbic acid at 30 °C. Inactivation was complete by 2 min as confirmed by using the 4-aminoantipyrene assay. PPA-treated protein was dialyzed against water, lyophilized, and redissolved in 6 M guanidine hydrochloride, 100 mM Tris (pH 8), and 10 mM EDTA. Using darkened vials, the sample was degassed, and dithiothreitol was added at 10-fold excess over the cysteines. After 30 min at 50 °C, iodoacetate was added in 2-fold excess to dithiothreitol. The alkylation reaction was quenched with excess β-mercaptoethanol, and the sample was dialyzed into 50 mM ammonium bicarbonate (pH 7.8). TPCK-treated trypsin (1% w/w, Sigma) was added twice with incubation at 37 °C for 1 h after each addition. Digestion was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (*18*) and matrix-assisted laser desorption−ionization (MALDI) mass spectrometry (Michigan State University Mass Spectrometry Facility).

The MALDI mass spectra were measured using a Voyager STR time-of-flight mass spectrometer (Perceptive Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm, 3 ns pulse). Spectra were measured in the linear mode of operation. The accelerating potential of the Voyager instrument was +25 kV. The MALDI spectra represent the accumulation of 50−100 laser shots, fired at the sample. The sample matrix α-cyano-4-hydroxycinnamic acid was dissolved in water/acetonitrile (1:1 v/v) to give a saturated solution at room temperature. To prepare the sample, 0.5 μL of the peptide solution (1−10 pmol/μL of 0.1% trifluoroacetic acid) was added to 0.5 μL of matrix and applied on a stainless steel target. The solution was left to dry at room temperature, and the sample plate was inserted into the mass spectrometer and analyzed. Time to mass conversion was achieved by internal or external calibration using bradykinin and adrenocorticotropic hormone (18−39).

Table 1: 2,4-D-Related Kinetics of Variant TfdA Proteins[a]

variant	range (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} mM^{-1})	K_{si}[b] (mM)
wild type[c]	5−1000	1020 ± 90	20.0 ± 5.4	51000	
K71L[d,e]	100−10000	256 ± 48	370 ± 170	692	16 ± 11
K71Q[d,e]	100−10000	2093 ± 150	1100 ± 300	1900	
R78Q	125−5000	452 ± 21	1500 ± 200	301	
Y81F	5−1000	346 ± 12	33.2 ± 3.3	10400	
K95L[d,e]	25−1000	893 ± 41	78.5 ± 12	11400	
K95Q	10−1000	733 ± 22	39.5 ± 5.2	18600	
Y126F	5−1000	576 ± 26	31.9 ± 5.1	18100	
T141V	5−1000	707 ± 19	21.6 ± 2.2	32700	
Y169F	5−1000	444 ± 10	42.9 ± 5.1	10300	
Y244F	5−1000	512 ± 32	30.8 ± 4.0	16600	
R274Q[e,f]	250−10000	208 ± 16	1600 ± 30	131	39 ± 18
R274L	250−10000	296 ± 176	2500 ± 1300	118	9.9 ± 7.4
R278Q[e,f]	250−10000	256 ± 17	4200 ± 200	160	11 ± 8

[a] The kinetics were linear during the time course of the assay, and 1 mM αKG was utilized, except where noted. [b] Substrate inhibition constant calculated according to eq 2. [c] Previously published (*12*). [d] Values were determined using 10 mM αKG. [e] Reaction progress was nonlinear during the course of the assay, and data were analyzed by using eq 1. [f] Values were determined using 2 mM αKG.

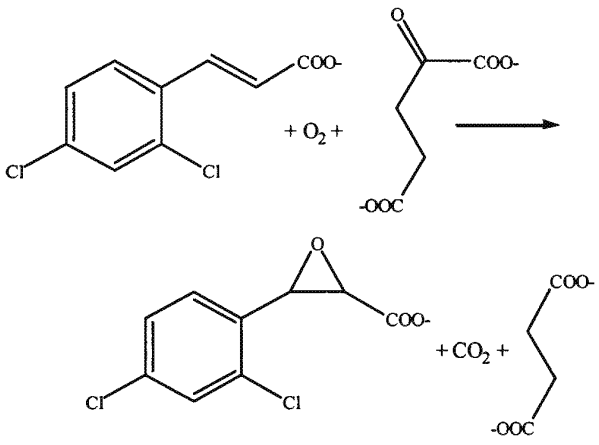
Separation and Characterization of TfdA Peptides. The tryptic digests of control and modified proteins were chromatographed on a pep-RPC (HR10/10) (Pharmacia) column by using a linear 200 mL gradient from 0.1% trifluoroacetic acid (TFA) in water to methanol containing 0.1% TFA while monitoring the absorption at 280 nm. All peptides absorbing at this wavelength were characterized by MALDI mass spectrometry (Michigan State University Mass Spectrometry Facility). In addition, the aromatic species uniquely identified in the PPA-treated sample was subjected to fast atom bombardment (FAB) mass spectrometry. FAB mass spectra were obtained using a JEOL HX-110 double-focusing mass spectrometer (JEOL USA, Peabody, MA) operating in the positive ion mode. Ions were produced by bombardment with a beam of Xe atoms (6 keV). The accelerating voltage was 10 kV, and the resolution was set at 1000. The samples were scanned from *m/z* 50 to *m/z* 1500. Spectra are derived from averaging five to seven scans.

RESULTS

Site-Directed Mutagenesis of Proposed 2,4-D Ligands. R278Q, K71L, K71Q, K95L, and K95Q TfdA variants were purified and characterized to assess the roles of these residues in catalysis and 2,4-D binding. Iron binding to these proteins was not affected on the basis of K_d determinations using previously described (*17*) fluorescence techniques (data not shown). Kinetic analyses revealed only modest changes in k_{cat}, with an approximately 2-fold increase in the rate for the K71Q mutant and 4−5-fold decreases for the K71L and R278Q variants (Table 1). The most striking changes observed for these proteins involved K_m, ranging from a 2-fold increase in the case of K95Q to a 200-fold increase for R278Q (Table 1). The large increases in K_m result in corresponding decreases in the catalytic efficiency (k_{cat}/K_m) of these variants (Table 1). The observed changes in 2,4-D K_m are likely to correlate with the K_d changes; however, the values need not be related. Direct determination of the 2,4-D K_d by the fluorescence quenching approach was not possible because 2,4-D, along with all other TfdA substrates, absorb at the fluorescence excitation wavelength and interfere with

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Scheme 2



the analysis. Nevertheless, the steady-state kinetic results are consistent with a role for Arg278, Lys71, and perhaps Lys95 in substrate binding.

To further examine the roles of these suspected 2,4-D binding residues, the variant proteins were incubated with the alternative substrate 2,4-dichlorocinnamic acid. This compound resembles 2,4-D but possesses a carbon−carbon double bond in the side chain rather than the ether oxygen. Wild-type TfdA converts 2,4-dichlorocinnamic acid to the epoxide, 2,4-dichlorophenylglycidic acid (Scheme 2), with a K_m of 190 ± 56 μM and a k_{cat} of 52.5 ± 5.1 min^{-1} (*14*). No detectable epoxidation activity was observed for the K71L, K71Q, and R278Q variants of TfdA in the presence of 1 mM 2,4-dichlorocinnamic acid (higher concentrations could not be tested due to its limited solubility in water). In contrast, both the K95Q and K95L variants were shown to oxidize the aliphatic substrate. Whereas the K95Q variant had an estimated K_m >500 μM for this compound, K95L TfdA had an approximate 4-fold decrease in the K_m and similar k_{cat} for 2,4-dichlorocinnamic acid (K_m = 56.5 ± 14.0 μM and k_{cat} = 41.3 ± 3.2 min^{-1}) when compared to wild-type enzyme. These results are consistent with participation of Lys95 in 2,4-D binding but not in 2,4-dichlorocinnamic acid binding.

Site-Directed Mutagenesis of Proposed αKG Ligands. T141V, R274L, and R274Q variants of TfdA were purified and characterized to assess the roles of these residues in catalysis and αKG binding (Tables 1 and 2). None of the variants exhibited significant differences in iron K_d (not shown), and the substitutions had only modest effects on k_{cat}. T141V also had no significant change in its αKG K_m, αKG K_d, or 2,4-D K_m, suggesting that it has at most a minor role in binding to αKG. In contrast, R274L and R274Q were found to have ~1000-fold increases in the αKG K_m and K_d (Table 2), thus confirming the critical role for Arg274 at the active site. To examine whether the reduced activity of R274Q TfdA could be overcome by use of a different cosubstrate, several alternative α-keto acids were examined. The enzyme used α-ketocaproate and α-ketoisovalerate with K_m values of 1.7 ± 0.5 mM and 0.70 ± 0.09 mM and k_{cat} values of 365 ± 31 min^{-1} and 22.4 ± 2.8 min^{-1}, respectively.

Arg78. To eliminate a protease-sensitive site in TfdA (*12*), the R78Q variant was constructed and characterized. In contrast to the situation for wild-type protein, no significant proteolysis product was observed. Although the TfdA model suggests that Arg78 is not located near the active site, the R78Q variant exhibited a nearly 100-fold increase in the 2,4-D K_m. This variant was unaffected in the iron K_d and, unlike variants of the active site 2,4-D binding residues, did not have an increase in the αKG K_d or αKG K_m. This lack of effect on αKG binding is consistent with the residue not being in the active site.

Site-Directed Mutagenesis of Tyrosines Located near the Active Site. Tyrosine mutants of TfdA were of interest because a tyrosine radical has been detected in TauD (a 30% identical protein) and considered as a possible catalytically relevant intermediate (M. J. Ryle, A. Liu, R. B. Muthukumaran, R. Y. N. Ho, J. McCracken, L. Que, Jr., and R. P. Hausinger, unpublished observations). To assess the importance of the four TfdA tyrosines predicted to lie closest to the active site (Tyr81, Tyr126, Tyr169, and Tyr244), each was individually substituted with phenylalanine, and the variant enzymes were characterized. All four variants were only slightly affected when compared to wild-type enzyme in terms of iron K_d, αKG K_d, αKG K_m, and 2,4-D K_m (Tables 1 and 2), suggesting that these tyrosines are not involved in catalysis or substrate binding. The Y81F, Y168F, and Y244F proteins were purified by previously described procedures, but Y126F TfdA had the unique characteristic of rapidly precipitating unless maintained at low temperature. This lability was not evident during the typical time course of the assay but was observed during the initial purification steps at room temperature. The lack of temperature sensitivity during catalysis may imply that the presence of one or more of the substrates stabilizes the protein during activity measurements.

Inactivation of TfdA by PPA. As an alternative approach to examine residues at the substrate-binding site, PPA, an acetylenic analogue of 2,4-D, was examined as a possible mechanism-based inactivator. PPA appeared to strongly inhibit or inactivate the enzyme on the basis of the absence of oxygen consumption after addition of 2,4-D to protein previously incubated with iron, αKG, ascorbate, and this compound. Kinetic analysis of enzyme inactivation revealed a first-order loss of activity (Figure 2, panel A) that was dependent on the concentration of PPA and required αKG and ferrous ions. Consistent with binding of the inactivator to the active site, high concentrations of 2,4-D protected the enzyme against inactivation. Saturation kinetics were observed for the loss of activity, allowing the calculation of K_i = 38.1 ± 6.0 μM and $k(\text{inact})_{max}$ = 2.3 ± 0.1 min^{-1} (Figure 2, panel B). Inactivation of TfdA by PPA was not reversible by dialysis against 10 mM imidazole (pH 6.8) for 72 h, consistent with it being an irreversible, mechanism-based inactivator.

In contrast to wild-type TfdA that was inactivated within 1 min, no inactivation of the K95L variant was observed for up to 4 min at 1 mM PPA. The K71L, K71Q, and R278Q variants were not examined by this approach since they have significantly increased 2,4-D K_m values and would likely bind the inactivator poorly. As a control, the Y81F variant was found to be inactivated by PPA at the same rate as wild-type enzyme (data not shown). The lack of inactivation in the K95L mutant was not due to a lack of binding since PPA is a competitive inhibitor with respect to 2,4-D with a $K_i \approx 60$ μM (Figure 3).

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Table 2: αKG-Related Kinetics of Variant TfdA Proteins[a]

variant	range (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} mM^{-1})	K_d (μM)	K_{si}[b] (mM)
wild type		643 ± 44[c]	3.2 ± 0.6[c]	201000[c]	3.4 ± 0.4	
K71L[d,e]	2500−10^6	112 ± 10	11700 ± 3300	9.6	2100 ± 400	
K71Q[d,e]	2500−10^6	>1900	>20000		4800 ± 300	
R78Q	5−1000	403 ± 45	<40	>10100	9.9 ± 0.7	
Y81F	5−1000	285 ± 21	<10	>28500	8.9 ± 0.7	
K95L[d]	5−1000	461 ± 51	<35	>13200	10.0 ± 1.1	
K95Q	5−1000	301 ± 5	<10	>20100	14.9 ± 2.6	
Y126F	5−1000	435 ± 3	<20	>21800	14.8 ± 1.0	
T141V	5−1000	560 ± 48	<20	>28000	10.9 ± 1.2	
Y169F	5−1000	454 ± 14	<10	>45000	11.1 ± 2.2	
Y244F	5−1000	512 ± 32	<10	>51200	16.6 ± 3.7	
R274Q[d,f]	500−10^6	263 ± 72	8200 ± 3900	32	2300 ± 200	44 ± 24
R274L	250−10^6	271 ± 68	4800 ± 2700	56	9000 ± 600	116 ± 92
R278Q[d,f]	500−10^6	224 ± 12	4100 ± 500	53	1350 ± 50	133 ± 24

[a] The kinetics were linear during the time course of the assay and utilized 1 mM 2,4-D, except where noted. [b] Substrate inhibition constant calculated according to eq 2. [c] Data from Fukumori et al. (*12*). [d] Reaction progress was nonlinear during the course of the assay, and data were analyzed by using eq 1. [e] Determined using 10 mM 2,4-D. [f] Determined using 2 mM 2,4-D.

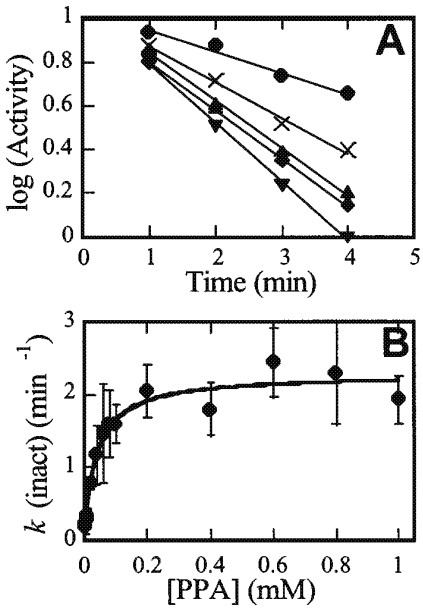


FIGURE 2: TfdA inactivation kinetics in the presence of PPA. (A) First-order loss of activity over time for 2 μM (●), 4 μM (×), 6 μM (◆), 8 μM (▲), and 10 μM (▼) PPA. (B) Inactivation rates versus the concentration of PPA, fit to eq 4.

PPA-treated and untreated samples of wild-type TfdA were digested with trypsin and compared by using MALDI mass spectrometry and reverse-phase chromatography. No differences were observed between the direct MALDI analyses of digestions of modified and unmodified proteins (data not shown), even though 92% of the peptides with molecular masses between 1100 and 2600 Da were able to be identified. In an extension of these studies, the tryptic digests were subjected to reverse-phase column chromatography while monitoring the absorbance at 280 nm (allowing detection of peptides containing Trp, Tyr, and the PPA aromatic ring). Comparison of the chromatography profiles revealed a novel peak in the PPA-treated sample (labeled * in Figure 4, bottom) compared to the profile of untreated sample (Figure 4, top). In addition, a diminution in intensity and change in shape typically was observed for peak 1 of the PPA-treated sample (changes in this feature were more apparent in other preparations). Each of the 280 nm absorbing peaks was collected from the chromatography of the modified protein and analyzed by MALDI mass spectrometry (Table 3). The peptide molecular weights revealed by the MALDI analyses indicated that peak 1 contained two Tyr-containing peptides and showed that all four Trp and all seven Tyr in TfdA were accounted for. Notably, the purified peptide containing Lys95 (peak 5) gave a very weak MALDI signal, and this peak suppression was consistent with the inability to detect this peptide during MALDI analysis of the whole digest peptide mixture. The sample in the fraction corresponding to the novel 280 nm absorbing peak also exhibited MALDI suppression problems, so that no novel mass was observed in the PPA-treated protein compared to the nontreated control. By contrast, FAB mass spectrometric analysis consistently revealed masses at 1177 and 1222 Da in both positive and negative ion modes. A mass of 1222 Da is consistent with the addition of phenylpropiolic acid (146 Da) and oxygen, and loss of a hydrogen atom, to the peptide corresponding to 166-AEHYALNSR-174 (1061 Da). A mass of 1177 could reasonably result from the decarboxylation of this same modified peptide (α-keto acids are known to be readily decarboxylated). Notably, the nonmodified form of this peptide elutes in peak 1, which is altered in appearance between the treated and nontreated samples. The small difference in intensities of peak 1 in the two samples, accounting for less than 20% absorption loss, suggests that modification of this peptide is substoichiometric. N-Terminal sequencing of the fraction containing the novel peak only revealed the sequence of the contaminating peptide from peak 5, suggesting that the modified peptide is blocked close to the N-terminus.

DISCUSSION

On the basis of TfdA structural modeling studies, interactions with the 2,4-D carboxylic acid were suggested to involve Arg278, Lys71, His214 (Figure 1), and the backbone amide of Ser117 (not shown) (*9*). The model also indicated that a loop comprising residues 86−111 made up one face of the 2,4-D binding pocket; however, a consensus structure for this region could not be obtained, and these 25 residues were omitted from the final model. Notably, several possible structures for this loop placed Lys95 where it could assist

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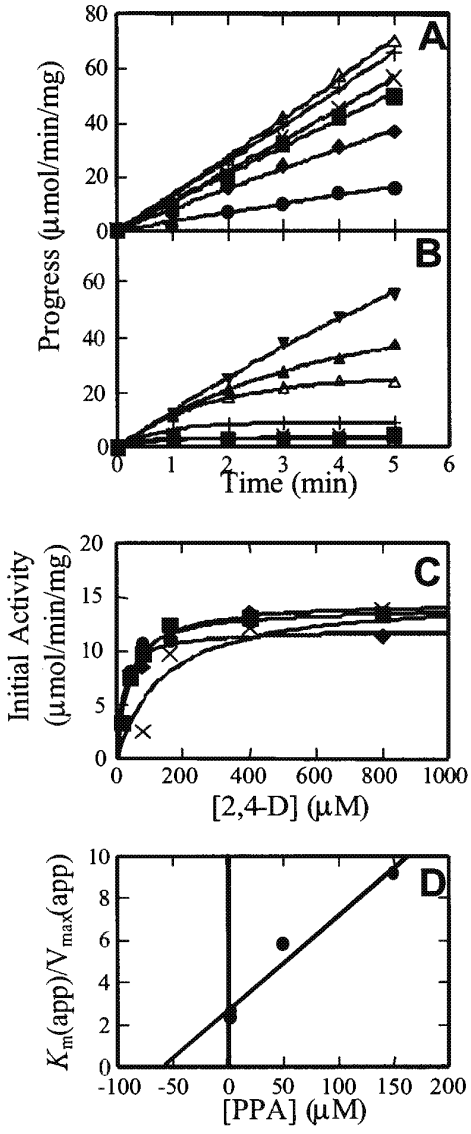


FIGURE 3: PPA competitive inhibition kinetics for K95L TfdA. Panels A and B depict progress curves of the K95L variant enzyme incubated with 2.5 μM and 150 μM PPA, respectively, with varying 2,4-D concentrations [16 μM (●), 40 μM (◆), 80 μM (■), 160 μM (×), 400 μM (+), 800 μM (△), 1600 μM (▲), and 4000 μM (▼)]. (C) The initial reaction velocities of the above and additional progress curves [utilizing 1.25 μM (●), 2.5 μM (■), 50 μM (◆), and 150 μM (×) PPA] were plotted versus the concentration of 2,4-D. (D) The K_m of PPA for the K95L protein was deduced by replotting the apparent K_m/V_{max} as a function of PPA concentration.

in binding 2,4-D via interactions with the carboxylate or the ether moiety. In an effort to test this model, we used a site-directed mutagenesis approach. Previous studies had provided evidence for the participation of His214 in binding 2,4-D (*10*), supporting at least that portion of the model. The current investigation focused on Arg278, Lys71, and Lys95 variants of TfdA and demonstrated that proteins altered at each of these positions exhibit decreased affinity for 2,4-D. Additional studies with a Lys95 variant and the alternative substrate 2,4-dichlorocinnamic acid supported the participation of this residue in binding 2,4-D. Whereas the K71L, K71Q, and R278Q variants of TfdA did not metabolize this substrate, presumably due to large increases in 2,4-dichlorocinnamic acid K_m similar to their increased 2,4-D K_m, the K95L variant exhibited higher catalytic efficiency for the aliphatic substrate than found with wild-type enzyme. These results imply that changing the basic lysine to the more hydrophobic leucine at the active site enhances the substrate preference for the more aliphatic side chain of 2,4-dichlorocinnamic acid, consistent with Lys95 having a role in binding the ether of 2,4-D. We conclude that Lys71 and Arg278 have important roles in binding the carboxylate of 2,4-D and that Lys95 interacts with the 2,4-D ether oxygen, as predicted by the TfdA structural model. Residues homologous to Arg278 are conserved in several other αKG-dependent dioxygenases (see sequence comparison in ref 9), whereas Lys71 and Lys95 are not conserved. Given the diversity of substrate structures used by these enzymes, the lack of substrate-binding site conservation is not unexpected.

Many of the αKG-dependent dioxygenases have a conserved arginine and threonine/serine motif, the residues of which are shown or hypothesized to interact with the C-5 carboxylate of αKG (*5*−*10*). In TfdA, this motif is comprised of Arg274 and Thr141 (*10*) as predicted by our computer-

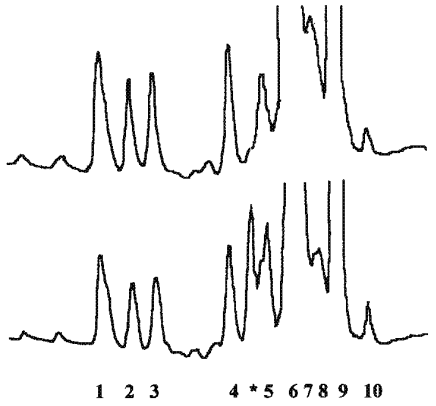


FIGURE 4: Peptide purification and identification. Tryptic digests of (top) untreated and (bottom) PPA-treated TfdA were chromatographed on a reverse-phase column while the absorption was monitored at 280 nm. The asterisk indicates a peak observed only in the treated sample.

Table 3: MALDI Mass Spectrometric Analysis of PPA-Treated TfdA Tryptic Peptides[a]

peak no.	molecular weight	sequence
1	1061.3	166-AEH**Y**ALNSR-174
	881.2	267-R**Y**DISAR-273 and/or 268-**Y**DISARR-274
2	714.0	241-EFV**Y**R-245
3	877.2	148-AA**Y**DALPR-155
4	1515.1	175-FLLGDTD**Y**SEAQR-187
5	1595.1	81-**Y**AELADISNVSLDGK-95
6	1394.4	188-NAMPPVN**W**PLVR-199
7	2589.8	103-EVVGNFANQL**W**HSDSSFQQPAAR-125
8	2420.2	126-**Y**SMLSAVVVPPSGGDTEFCDMR-147
9	1505.2	248-**W**NVGDLVM**W**DNR-259
10	2234.0	unidentified
	1525.1	unidentified

[a] Each of the 280 nm absorbing peaks collected from the chromatograph of Figure 4 for PPA-modified protein was examined by MALDI mass spectrometry. The molecular masses of the major MALDI species are indicated, along with the sequences corresponding to these masses. Aromatic residues are highlighted in bold.

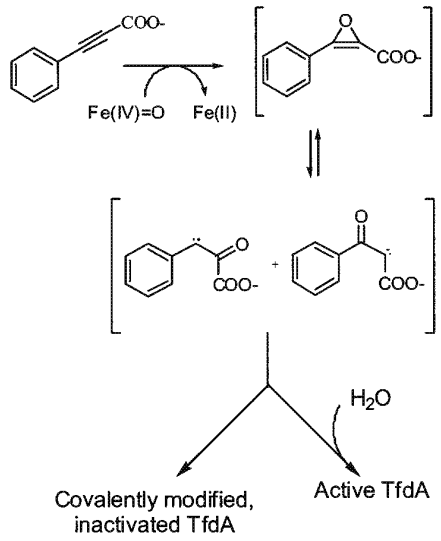
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generated structural model (*9*) (Figure 1). Mutagenesis studies were carried out to test whether these residues are critical to αKG binding. Arg274 was shown to play a substantial role in binding αKG, whereas Thr141 has only a minor, if any, role in interacting with αKG. Codons encoding the analogous threonine residue have not been mutated in other family subgroup members; however, the corresponding arginine residues have been substituted in several related enzymes. For example, conversion of this arginine in deacetoxycephalosporin C synthase (DAOCS, another αKG-dependent dioxygenase) to a glutamine was reported not to affect the K_m of its primary substrate (*19*). The loss of activity arising from conversion of the corresponding arginine to glutamine in DAOCS (and phytanoyl-CoA hydroxylase, another family member) was found to be reversed by using longer chain length α-keto acids, a phenomenon termed cosubstrate rescue (*19*, *20*). In particular, α-ketocaproate and α-ketoisovalerate restored activity to greater than 100% of the αKG-dependent wild-type activity in the R258Q mutant of DAOCS. Cosubstrate rescue was not observed with the R274Q variant of TfdA using either α-ketocaproate or α-ketoisovalerate. The reasons for the differences in behavior of the arginine mutants in TfdA versus DAOCS or phytanoyl-CoA hydroxylase are not clear.

In addition to mutating codons encoding the residues hypothesized from the model to be involved in substrate binding, Arg78 was converted to a glutamine in order to eliminate a protease-sensitive site in TfdA (*12*) and the requirement for several tyrosine residues was examined. Our successful removal of the protease-sensitive site might be beneficial in future crystallography efforts, since preliminary attempts to crystallize TfdA have been stymied, in part, by the heterogeneity of the sample due to the presence of the proteolysis product. Although this arginine residue is predicted to lie on the protein surface distant from the active site, a significant increase in 2,4-D K_m was observed for the R78Q variant. A hypothetical role of Arg78 is to act as a gate for 2,4-D entry into the active site. This role also has been hypothesized for His217, since the alanine variant (H217A) of this residue has an increased 2,4-D K_m but is not in the active site (*10*). Possibly related to these findings, the crystal structure of the αKG-dependent dioxygenase anthocyanidin synthase reveals two bound substrate molecules with one binding site proposed to assist in transfer of substrate to the active site (*8*). Our mutagenesis studies involving four tyrosine residues located near the TfdA active site rule out any essential role for these residues.

Lysines and arginines are expected to have multiple hydrogen-bonding partners because of their large size and positively charged side chains. Substitution of these residues could reasonably have long-range effects. One such effect observed here is the significantly increased αKG K_m and αKG K_d of the 2,4-D-binding site mutants (Table 2), possibly resulting from repositioning of various active site residues, shifting of the protein backbone, and/or disruption of secondary structure elements. Complementary results were observed with the R274L and R274Q mutations involving the αKG-binding site; i.e., the 2,4-D K_m increased ~100-fold (Table 1). Such interactions between two substrate-binding sites are not unexpected but have not been documented in prior mutagenesis studies within this enzyme family.

Scheme 3: Theoretical Mechanism of TfdA Inactivation by PPA, Based on the Inactivation Mechanism Determined for the Thymine Hydroxylation Interaction with an Acetylenic Substrate Analogue[a]



[a] References *21*–*23*.

PPA was demonstrated to be a mechanism-based inactivator of TfdA. A reasonable mechanism for how PPA reacts with TfdA is illustrated in Scheme 3. Initial oxidation of PPA by TfdA likely produces a highly reactive oxirene intermediate that rearranges to form either of two carbene intermediates. Analogous oxidative chemistry was established for thymine hydroxylase, an αKG-dependent dioxygenase that is inactivated upon treatment with an acetylenic analogue of thymine (*21*–*23*). In addition, cytochrome P450 enzymes are known to catalyze this type of reaction with acetylenic compounds (*24*). Low-energy interconversions between oxirene and carbenes on adjacent carbon atoms are well documented (*25*, *26*). Since carbenes are known to be able to react with amino acid side chains, a covalent linkage could reasonably form between oxidized PPA and a TfdA active site residue. Alternatively, carbenes may react with solvent to spare the enzyme and release an aromatic product. To explore this model further, two approaches were used: PPA-dependent inactivation kinetics were analyzed using the previously prepared K95L variant and peptide studies were carried out on PPA-inactivated TfdA.

Modeling and site-directed mutagenesis studies indicate that Lys95 is likely to be positioned near the 2,4-D ether oxygen and thus may lie close to the suspected oxirene intermediate formed from PPA acid. The K95L variant of TfdA was found to be resistant to inactivation by PPA. A reasonable explanation for the observed lack of inactivation is that the carbene intermediate formed by the enzyme reacts with water (Scheme 3), as previously described for inactivation of thymine hydroxylase by an acetylenic substrate analogue (*21*–*23*). For example, replacement of Lys95 by Leu may result in greater solvent access to the active site. Arguing against this scenario, however, K95L TfdA did not release detectable quantities of a new aromatic product arising from PPA when analyzed by HPLC, and turnover of PPA by the variant enzyme was indiscernible on the basis of oxygen consumption. While the mechanism by which

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K95L TfdA resists inactivation by PPA remains unclear, these results are consistent with the placement of Lys95 at the active site.

MALDI mass spectrometric studies of tryptic peptides derived from PPA-treated wild-type TfdA are consistent with a covalent linkage formed between PPA and the 166-AEHYALNSR-174 peptide. Portions of this peptide are predicted to lie close to the 2,4-D binding site (e.g., the Ser173 side chain hydroxyl is located only 5.4 Å from the 2,4-D carboxyl group) according to the TfdA model. Substitution of Tyr169 by Phe produced no effect on activity or binding of 2,4-D or αKG (Tables 1 and 2). In contrast, previous studies analyzing the H168A TfdA variant (as a maltose binding protein fusion) resulted in an inactive enzyme (*10*). The mutagenesis and PPA-dependent inactivation results are consistent with the indicated peptide being present at the active site with His168 playing a role in catalysis. Of interest, a basic residue is conserved at this position in all sequenced 2,4-D/αKG dioxygenases (*10*). These PPA reactivity studies extend our understanding of the range of chemistry available to TfdA by indicating the enzyme's ability to oxidize an acetylenic compound.

ACKNOWLEDGMENT

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REMARKS

Amendments to the Claims

Applicant has added new claim 49 directed to a transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein. More particularly, the claim requires the AAD-1 protein to comprise an amino acid sequence that is 90% identical to the 171 amino acid AAD-1 motif sequence of SEQ ID NO: 9. Exemplary support for new claim 49 is found in numbered paragraphs [0085]-[0087], [0050]-[0052] and [0142].

Priority Claim

The Examiner contends that the prior-filed application (USSN: 14/820,893) fails to provide adequate support or enablement for the claimed invention. More particularly, the Examiner asserts that the prior application failed to provide written description support for the AAD-1 motif recited in the instant claimed invention, and thus that the current claimed subject matter is only entitled to the filing date of the present application. Applicant respectfully traverses the Examiner's findings.

To be entitled to an earlier priority date or filing date under 35 U.S.C. 119, 120, 365, or 386, each claim limitation must be expressly, implicitly, or inherently supported in the originally filed disclosure (MPEP §2163(II)(3)(b)). To establish inherency, the extrinsic evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. *Yeda Research and Dev. Co. v. Abbott GMBH & Co.*, 837 F.3d 1341, 120 USPQ2d 1299 (Fed. Cir. 2016). The issue of whether a patent specification adequately describes the subject matter claimed is a question of fact. *In re Alton*, 76 F.3d 1168, 1174 (Fed. Cir. 1996).

Applicant notes the Examiner's recognition that the specification discloses a general α–ketoglutarate-dependent dioxygenase motif $(HX(D/E)(X)_{23\text{-}26}(T/S)(X)_{114\text{-}183}H(X)_{10\text{-}13}R$, wherein 23 to 26 amino acids separate the D/E amino acid from the T/S amino acid; 114 to 183 amino acids separate the T/S amino acid from the histidine residue; and 10 to 13 amino acid separate the histidine residue from the arginine residue) that is shared by each member of an α–ketoglutarate-dependent dioxygenase family of proteins. This α–ketoglutarate-dependent dioxygenase motif and the protein sequences of SEQ ID NOs: 9, 10 and 11 were all described in the priority documents back to at least international application PCT/US2005/014737 as filed on May 2, 2005.

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Applicant has described at least three members of this family of α–ketoglutarate-dependent dioxygenases (i.e., SEQ ID NOs: 9, 10 and 11) that have been found to exhibit activities beyond those of the standard α–ketoglutarate-dependent dioxygenases. In particular, applicant described and reduced to practice a group of α–ketoglutarate-dependent dioxygenases (collectively termed AAD-1) that are dual mode of action α–ketoglutarate-dependent dioxygenases, not only capable of degrading 2,4-D, but also AOPP herbicides (hereinafter "dual MOA activity"). Furthermore, the specification discloses in paragraph [0085] that the AAD-1 proteins are members of the α–ketoglutarate-dependent dioxygenase family of proteins, all of which contain the disclosed general α–ketoglutarate-dependent dioxygenase motif: $(HX(D/E)(X)_{23\text{-}26}(T/S)(X)_{114\text{-}183}H(X)_{10\text{-}13}R)$. In paragraph [0142] applicant states: "Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having same or similar activity as the exemplified proteins. The disclosure provided in paragraphs [0085] and [0142] of the present application is also found in the parent application, USSN: 14/820,893 and in priority application PCT/US2005/014737 at the identical location of the respective specifications. Applicant clearly envisioned such variant proteins to be part of the described invention.

Based on the guidance in the specification, the working examples that demonstrate activity and general knowledge in the art for α–ketoglutarate-dependent dioxygenases, those skilled in the art would readily understand that proteins having a similar activity to the exemplified AAD-1 proteins of SEQ ID NOs: 9, 10 and 11 would need to retain the known active site of those peptides. The skilled practitioner would know how to readily align the amino acid sequences of SEQ ID NO: 9, 10 and 11 with the disclosed general α–ketoglutarate-dependent dioxygenase motif $(HX(D/E)(X)_{23\text{-}26}(T/S)(X)_{114\text{-}183}H(X)_{10\text{-}13}R)$ for analysis and identification of the five specific amino acids (H, D/E, T/S, H and R) that correlate to those of the general α–ketoglutarate-dependent dioxygenase motif $(HX(D/E)(X)_{23\text{-}26}(T/S)(X)_{114\text{-}183}H(X)_{10\text{-}13}R)$. Due to the variable spacing between four of the amino acids of the general α–ketoglutarate-dependent dioxygenase motif (i.e., between D/E and T/S, between T/S and H, and between H and R) one would logically start with the initial designated tripeptide "HXD/E" of the general motif when aligning and analyzing the amino acid sequences of SEQ ID NO: 9, 10 and 11 to identify the amino acids of the AAD-1 proteins that correlate with the designated amino acids of the general α–ketoglutarate-dependent dioxygenase motif. Specifically, SEQ ID NO: 9 (as well as SEQ ID NOs 10 and 11) contains only a single instance of the initial

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tripeptide "HX(D/E)" of the generalized α–ketoglutarate-dependent dioxygenase motif, located at amino acid positions 111-113 of SEQ ID NO: 9. Starting with an alignment of the disclosed general α–ketoglutarate-dependent dioxygenase motif at position 111 of SEQ ID NO: 9, and complying with the spacing requirements between the individual amino acids of the disclosed general α–ketoglutarate-dependent dioxygenase motif, identifies the specific locations of the remaining amino acids in SEQ ID NO: 9 that correlate to the remaining amino acids of the general α–ketoglutarate-dependent dioxygenase motif. Specifically, the five amino acids at H_{111}, D_{113}, T_{137}, H_{270}, and R_{281} of SEQ ID NO: 9 correlate with the five amino acids designated in the general α–ketoglutarate-dependent dioxygenase motif $(HX(D/E)(X)_{23-26}(T/S)(X)_{114-183}H(X)_{10-13}R)$, thus identifying the AAD-1 motif.

Thus the well-known general α–ketoglutarate-dependent dioxygenase motif disclosed in paragraph [0085] of the application provides a roadmap to the specific locations of the corresponding amino acids of the AAD-1 protein, revealing a corresponding motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, that applicant has designated as the AAD-1 motif. This disclosed AAD-1 motif is embodied in the disclosed AAD-1 sequences of SEQ ID NOs: 9, 10 and 11.

> As stated in *Yeda Research and Dev. Co. v. Abbott GMBH & Co.*, 837 F.3d 1341, at 1345:
>
> Under the doctrine of inherent disclosure, when a specification describes an invention that has certain undisclosed yet inherent properties, that specification serves as adequate written description to support a subsequent patent application that explicitly recites the invention's inherent properties. *See* Kennecott, 835 F.2d at 1423.

Applicant respectfully submits they are merely explicitly reciting the AAD-1 motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ that is embodied in the disclosed AAD-1 sequences of SEQ ID NOs: 9, 10 and 11 as revealed by mapping the general α–ketoglutarate-dependent dioxygenase motif onto the disclosed AAD-1 sequences.

With this response, Applicant submits a Declaration by Dr. Satish K. Nair, a recognized expert in the arts of enzymology and structural biology, who is familiar with the literature related to the Fe/α-ketoglutarate-dependent dioxygenases (this Declaration will be hereinafter termed the "Nair Declaration"). Expert opinion testimony such as this is "entitled to consideration and some weight" where it goes to a matter of fact. M.P.E.P 716.01(c)(III). The Federal Circuit has explicitly recognized that evidence going to the factual question here presented, which is whether the patent specification adequately describes the subject matter claimed, should be considered even if it is

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styled as opinion evidence. *Alton*, 76 F.3d at 1175 n.10 ("[W]e are aware of no reason why opinion evidence relating to a fact issue should not be considered by an examiner.").

Dr. Nair states that the general α–ketoglutarate-dependent dioxygenase motif, together with the remainder of the disclosure in the specification and the knowledge of one of skill in the relevant art, established that Applicant had at the time of filing identified the AAD-1 motif responsible for the dual MOA activity:

> In addition to three exemplified AAD-1 homologues that exhibit dual MOA Fe/α-KG dioxygenase activity, applicant's analysis of the structures of the tfdA gene homologues ~~known at the time of the invention~~ with the amino acid sequences of SEQ ID NO: 9, 10 and 11 revealed conserved amino acids relevant to the dual herbicide MOA Fe/α-KG dioxygenase activity. Specifically, the '406 application described a general α–ketoglutarate-dependent dioxygenase motif in paragraph [0085] of the '406 application. **I believe that, on reviewing the specific residues of SEQ ID NOS:9, 10, and 11 aligned with that general α–ketoglutarate-dependent dioxygenase motif, a skilled practitioner would readily envision a disclosed AAD-1 motif in the form of $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, where the X terms designate an unknown amino acid at the corresponding position of SEQ ID NO: 9.**

Nair Declaration, Paragraph 9 (emphasis added). This is in addition to the previously-submitted Declaration of Dr. Terry Wright, who stated, based on his knowledge as one of skill in the art, that

> it is evident to me, one skilled in the art, that the specification of the '406 Application supports the refined and narrowed AAD-1 motif, HXDX24TX131HX10R[1] as defining a critical structure of a set of preferred enzymes (and genes encoding them) able to degrade 2,4-D, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxypropionate herbicides [(R)-enantiomers] by recombinantly expressed AAD-1 enzymes.

Wright Declaration, Paragraph 16.

The Federal Circuit found sufficient disclosure to satisfy a priority claim in an analogous situation in *Yeda Research and Development Company v. Abbott GMBH & Company*. In that case, the patentee had disclosed in the priority application certain structural features of the claimed protein, in the form of a partial N-terminal amino acid sequence (nine of the fifteen claimed amino acids of the unique full N-terminal sequence). The patentee had also disclosed the function of the protein, in the form of its specific binding to a target protein. The court affirmed the finding that these facts supported the priority claim for a claimed protein detailing the full N-terminal amino acid sequence. *See Yeda,* 837 F. 3d 1341, 1345 (Fed. Cir. 2016). Like the

[1] This is simply an alternate way of representing the AAD-1 motif, substituting for the designations set forth in Claim 15, wherein the subscript designates the total number of intervening amino acids. See Wright Declaration at para. 12.

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patentee in *Yeda*, Applicant has explicitly disclosed at least part of the structure of the claimed genus of proteins, in this case the generalized motif, and has at least implicitly disclosed the full AAD-1 motif. Furthermore, as in Yeda, the function of the protein, in this case the dual MOA activity, was also explicitly disclosed. Accordingly, the priority claim should be accepted here.

The Examiner states that he "has provided evidence that the instant claims have the priority of the filing date of the instant Application." Office Action of July 12, 2019 at page 17. However, the only statements made by the Examiner relating to this issue do not offer conclusive objective evidence of a lack of Section 112 support for the claim limitations in the specification. In the Office Action issued on November 13, 2018, the Examiner simply stated that "[t]he instant specification refers to an α-ketoglutarate-dependent dioxygenase motif on page 17, paragraph 0085, but there is no description of a specific AAD-1 motif." Office Action of November 13, 2018 at 2-3. In other words, the Examiner admitted the explicit disclosure of the α-ketoglutarate-dependent dioxygenase motif, but failed to find a similarly explicit disclosure of the AAD-1 motif, and appears solely on that basis to have found that the claims were not entitled to the priority filing date. With respect, the standard implicitly applied by the Examiner is incorrect. A verbatim description of all limitations in the specification is not required to satisfy Section 112. See *Alton*, 76 F.3d at 1175 ("[If] a person of ordinary skill in the art would have understood the inventor to have been in possession of the claimed invention at the time of filing, *even if every nuance of the claims is not explicitly described in the specification*, then the adequate written description requirement is met.") (emphasis added).

In response to the expert declaration of Dr. Wright, the Examiner simply responded that the argument based on the Wright declaration "is not found to be persuasive. Each claim is given only one priority date." Office Action of July 12, 2019 at page 17. Applicant respectfully submits that the Examiner appears to have essentially failed to give due weight to the Wright declaration. With the present response, Applicant has now provided two separate expert declarations, in which each expert reviews the same factual background considered by the Examiner, but reaches the opposite factual conclusion. Each expert finds that a person of ordinary skill in the art would have understood the inventor to have been in possession of the claimed AAD-1 motif at the time of filing.

Applicant respectfully submits in light of the proffered evidence that upon consideration of the teaching of the specification as a whole, one of ordinary skill would readily understand that the prior-filed application (USSN: 14/820,893) provides full written descriptive support for the AAD-1 motif recited in the instant claimed invention. Accordingly, Applicant respectfully submits that the

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currently claimed invention is entitled to the benefit of the early priority date, and herby requests acknowledgement by the Examiner of the early priority date of the present claims.

Double Patenting Rejections

Claims 15, 16 and 18-48 are rejected for obviousness type double patenting over claims 1-43 of US Patent No 7,838,733. Claims 15, 16 and 18-48 are rejected for obviousness type double patenting over claims 1-22 of US Patent No 9,127,289. Claims 15, 16 and 18-48 are rejected for obviousness type double patenting over claims 1-13 of US Patent No 10,174,337. Applicant will file the necessary terminal disclaimers to overcome these rejections upon a finding of allowable subject matter.

Written Description Rejection

Claims 15, 16 and 18-48 are rejected under 35 USC 112, first paragraph for failing to comply with the written description requirement. Applicant respectfully traverses this rejection.

The Law Relating to Written Description of Sequence-Based Inventions

The fundamental factual inquiry in determining if a claim complies with the written descriptive requirement is to determine whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed. See, e.g., *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. The subject matter of the claim need not be described literally (i.e., using the same terms or *in haec verba*) in order for the disclosure to satisfy the description requirement (MPEP §2163.02).

The framework for assessing the written description requirement for biological sequences was laid down by the Federal Circuit in 1997 in *Regents of the University of California v. Eli Lilly and Company*. In that case, which related to nucleic acid sequences, the Federal Circuit articulated the still-current test for such sequences: a description of a genus of such sequences "may be achieved by means of a recitation of a representative number of [species], defined by nucleotide sequence, falling within the scope of the genus ***or*** of a recitation

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of *structural features common to the members of the genus*, which features constitute a substantial portion of the genus." *Regents of the Univ. of Cal. v. Eli Lilly & Co.*, 119 F.3d 1559, 1568 (Fed. Cir. 1997) (emphasis added). Five years later, in *Enzo Biochem, Inc. v. Gen-Probe Incorporated,* the Federal Circuit adopted the then-current Patent Office guidelines relating to assessing whether descriptions of genetic material met the written description requirement. In relevant part, the test adopted provided that the written description requirement was met by disclosing, e.g., "complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." *Enzo Biochem, Inc. v. Gen-Probe Inc.,* 323 F.3d 956, 964 (Fed. Cir. 2002). Eight years later, the Federal Circuit explicitly rejected the contention that written description in such cases could only be satisfied by a recitation of all sequences contained within a claimed genus: "The doctrine never created a heightened requirement to provide a nucleotide-by-nucleotide recitation of the entire genus of claimed genetic material; it has always expressly permitted the disclosure of *structural features common to the members of the genus*." *Ariad Pharms., Inc. v. Eli Lilly & Co.,* 598 F.3d 1336, 1352 (Fed. Cir. 2010) (en banc) (emphasis added) (citing *Eli Lilly*, 119 F.3d at 1569). Under the current state of the law, then, disclosure of a common structure providing a claimed function can be sufficient to satisfy the written description requirement for a genus of claimed sequences, whether or not a representative number of species are expressly disclosed.

Indeed, the Federal Circuit has recently confirmed that even a relatively small number of disclosed species can suffice, where a correlation between structure and function is established. In *Ajinomoto Co., Inc. v. International Trade Commission*, which was decided only a few months ago, the validity of claim 9 of US Patent No. 7,666,655 was challenged relating to the claim element: "replacing the native promoter which precedes the DNA on the chromosome of the bacterium with a more potent promoter." Appellee asserted that the claim lacked adequate written description support for the genus of "more potent promoter[s]" recited in claims 9 and 15 and, by incorporation, in claim 20. In particular, the '655 patent only disclosed ***four*** examples of "potent promoters": the "PL promoter of lambda phage," the "lac promoter," the "trp promoter," and the "trc promoter." However, the '655 specification also made reference to the general knowledge of the skilled practitioner by citing a 1986 article by Deuschle et al. as disclosing examples of potent promoters and methods for the evaluation of the strength of promoters. The

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article was cited by the Court as evidence supporting a finding that "enhancing promoter activity was well-known" and that a skilled artisan "would have been able to identify more potent promoters by employing common tools for measuring RNA transcription."

Furthermore, the court held that common structural features existed for the genus members due to the well-known link between consensus sequences and promoter strength, in that promoters having fewer departures from a "consensus sequence in promoter are generally stronger than promoters with more departures from such a sequence." It was thus the case that even with a disclosed number of species in the low single digits, the written description requirement was satisfied because there was a "correlation between structure and function" (i.e., the known link between promoter consensus sequences and promoter strength). *Ajinomoto Co., Inc. v. International Trade Commission,* 932 F.3d 1342, 1359-1360 (2019).

The courts are much more likely to find a lack of written description support where there are no working examples disclosed. In *Boston Scientific Corp. v. Johnson & Johnson*, 647 F.3d 1353 (Fed. Cir. 2011), the specification contained "no examples of macrocyclic lactone analogs of rapamycin" (the claimed genus) and essentially "no guidance on how to properly determine whether a compound is a macrocyclic lactone analog of rapamycin." 647 F.3d at 1364. In *Abbvie Deutschland GmbH & Co., KGv.Janssen Biotech, Inc.*, 759 F.3d 1285 (Fed. Cir. 2014) there was "no evidence to show any described antibody to be structurally similar to, and thus representative of," an antibody accused of coming within the claim, nor was there "evidence to show whether one of skill in the art could make predictable changes to the described antibodies to arrive at other types of antibodies such as" the accused antibody. 759 F.3d at 1301. And in *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559 (Fed. Cir. 1997) the specification described "a process for obtaining human insulin-encoding cDNA" (such cDNA required by the claim at issue) but not any "sequence information indicating which nucleotides constitute human cDNA" or "further information in the patent pertaining to that cDNA's relevant structural or physical characteristics." 119 F.3d 1559, 1567 (Fed. Cir. 1997). Here, by contrast, and similar to the fact pattern of *Ajinomoto Co., Inc.,* the present specification provides a number of actual working embodiments coupled with a required functional activity (dual MOA activity) and structural elements relating to that functional activity. Specifically, the present disclosure provides structural information relating to the active site of the class of α–ketoglutarate-dependent dioxygenases that exhibit dual MOA activity.

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Application of the "Structural Features Common to Members of the Genus" Test

Consistent with the above legal background, and particularly with the holding in *Ajinomoto Co., Inc. v. International Trade Commission*, Applicant submits that they have provided sufficient correlation between the provided structure and the recited function that one of ordinary skill would appreciate that applicant was in possession of the claimed genus as currently claimed in the present invention.

As noted previously, both of Applicant's declaratory witnesses have provided evidence that, in their view as experts skilled in the relevant art, Applicant's originally-filed specification, in view of the knowledge of those of skill in the relevant art, provides written description support for the claimed genus. *See* Wright Declaration at para. 16; Nair Declaration at para. 13. Both witness declarations emphasize the importance of the AAD-1 motif as the structure that provides the dual MOA activity that is common to members of the genus, and assert that one of skill in the art would recognize the AAD-1 motif as disclosed in the originally-filed specification. *See* Wright Declaration at para. 12 ("One skilled in the art would recognize the much more limited group having the desired functional dual-MOA activity would be defined by the more refined structure of [the] AAD-1 motif"); Nair Declaration at paras. 10-11 ("Specifically, the comparison of the AAD-1 enzymes and AAD-2 reveal while the two proteins retain all five active site residues, there is a difference in the 131 amino acid ($(X)_{139-269}$) interval in the AAD-1 motif $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ vs the spacing in AAD-2 which is a 2,4-D-degrading Fe/α-KG dioxygenase that cannot also catalyze (R)-AOPP herbicides . . . Based on the disclosure contained in the '406 Application, those skilled in the art would have compared and recognized the importance of the sequence differences between AAD-1 variants [(R)-specific dioxygenase] having dual herbicide MOA Fe/α-KG dioxygenase activity and (S)-specific dioxygenases not having dual herbicide MOA Fe/α-KG dioxygenase activity…"). To assist the Examiner in evaluating this declaratory evidence, Applicant provides further information as to the knowledge of those of skill in the art at the time of filing below.

Those of Skill at the Time of Filing Had Extensive Knowledge of the Family of Enzymes

As noted in *Ajinomoto*, support for a claimed genus is not limited to information contained within the specification but also includes knowledge of protein structure, such as consensus sequences, that are well-known to those of skill in the art. Applicant respectfully submits that those skilled in the art are well familiar with the structure of α-ketoglutarate-

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dependent dioxygenases. As described on page 1357, 1st full paragraph, of Müller et al, Protein Science (2006), 15:1356–1368, "The 2,4-D hydroxylase (TfdA) from *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134(pJP4) has been intensively studied..." Earlier published articles (e.g., Hotopp et al, Biochemistry 2002, 41, 9787-9794; see abstract) reference a published TfdA structural model and describe the identity of substrate-binding residues in tfdA. Copies of Müller et al and Hotopp et al are provided herewith. Specifically, Hotopp states in the paragraph bridging pages 9787 and 9788:

> Previous site-directed mutagenesis studies revealed the roles of His114, Asp116, and His263 in binding iron and suggested that His214 functions in 2,4-D binding or catalysis (*10*). Recently, a model of the three-dimensional structure of TfdA (Figure 1) was developed on the basis of the crystal structures of two homologous enzymes, taurine/ α-ketoglutarate dioxygenase (TauD) (*9*) and clavaminate synthase 1 (CS) (*6*). The TfdA model placed His114, Asp116, and His263 together in a geometry consistent with a metal-binding site, located His214 nearby at the suspected 2,4-D binding site, and revealed that a protease-sensitive site involving Arg78 was located on the surface of the protein. Furthermore, the model newly identified a set of additional residues likely to be involved in 2,4-D and αKG binding. The studies described here utilize site-directed mutagenesis and mechanism-based inactivation approaches to better define the residues at the substrate-binding site of TfdA.

The AAD-1 proteins of the present invention bind to the same phenoxy auxin herbicide substrates as the tfdA protein and presumably retain similar structural domains as the homologous tfdA proteins.

As noted in paragraph [0012] of the specification, a large number of tfdA-type genes that encode proteins capable of degrading 2,4-D have been identified and their sequences deposited into the Genbank database. Although the specification does not disclose the sequence of these tfdA-type genes, they were known to those skilled in the art as they were deposited in the Genbank database prior to the present invention. Information which is known in the art need not be described in detail in the specification. See, e.g., *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d1367, 1379-80, 231 USPQ 81, 90 (Fed. Cir. 1986).

Exhibit A (attached hereto) presents an alignment of 16 tfdA-type proteins, demonstrating a significant level of conserved amino acids shared between the aligned sequences of these homologous proteins. Those skilled in the art appreciate that conserved amino acids typically designate regions of a peptide that are important to the proteins structure and function.

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Accordingly, sequence alignments of those disclosed proteins provide further information, based on conserved amino acids of homologous proteins, regarding the structural elements required in a protein capable of degrading 2,4-D. The skilled practitioner would know not to make modifications to amino acids that are conserved, and thus applicant's required functional limitations effectively add these structural elements to the claimed genus of proteins. This information, in combination with applicant's description of the AAD-1 motif (defining the key structural elements of the enzyme active site), provides a detailed description of the structural elements of the claimed genus members. Thus one of ordinary skill would appreciate that the specification in conjunction with common knowledge of the well-studied tfdA homologs provide extensive guidance to prepare derivatives of SEQ ID NO: 9 that could easily be tested to confirm their ability to exhibit the required dual activity of degrading phenoxy auxin herbicides and (R)-aryloxyphenoxypropionate herbicides.

As noted above, comparison of the amino acid sequences of the α-ketoglutarate-dependent dioxygenases in Exhibit A (including AAD-1 and tfdA) reveals numerous regions of homology that have been conserved between the aligned α-ketoglutarate-dependent dioxygenases. As noted in Müller et al, Protein Science (2006), 15:1356–1368, at page 1358, first full paragraph, such information can be used to elucidate or design functional analogs of proteins:

> Here, we describe the construction of homology models of SdpA and RdpA from *S. herbicidovorans* MH and the use of docking to identify residues likely to be involved in herbicide binding. Previous homology models have led to successes in elucidating or designing specificity-conferring interactions in ligands. For instance, homology modeling of a cercarial (human parasite) elastase led to the development of an effective elastase inhibitor (Cohen et al. 1991) and to understanding the specificity determinants for ligands binding to a parasite tRNA synthetase versus its human homolog (Sukuru et al. 2006).

Furthermore, one skilled in the art appreciates that regions of conservation between homologous proteins suggests a structure/function role for such sequences. Accordingly, as homologous amino acid sequences were known at the time of the invention, they provide guidance to regions where the AAD-1 sequence of SEQ ID NO: 9, 10 or 11 should be retained. Accordingly, similar to the fact pattern described in *Ajinomoto Co., Inc. v. International Trade Commission*, 932 F.3d 1342 (2019), a skilled artisan could make relatively predictable changes

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to the native AAD-1 amino acid sequence in non-critical areas of the protein (based on the alignment data of known homologs, see Exhibit A) to arrive at protein variants that retain the ability to enzymatically degrade phenoxy auxin herbicides and (R)-aryloxyphenoxypropionate herbicides.

Applicant respectfully submits that, at the time of the invention, a large amount of information was known regarding the structure/function of tfdA and related α-ketoglutarate-dependent dioxygenases. Applicant has used such information and further described a subset of α-ketoglutarate-dependent dioxygenases that retain the ability to enzymatically degrade phenoxy auxin herbicides and, due to the presence of the AAD-1 specific motif, also have the ability to degrade (R)-aryloxyphenoxypropionate herbicides.

The AAD-1 Motif Was Disclosed in the Specification

Applicant respectfully submits that they have identified the structural elements associated with the unique enzymatic activity of the claimed genus of proteins. In accordance with *Ariad Pharmaceuticals, Inc. v. Eli Lilly and Co.* (F.3d 1336, 1341) (Fed. Cir. 2010), functional claim language can meet the written description requirement when the art has established a correlation between structure and function. Applicant has submitted further evidence validating the importance of the AAD-1 motif disclosed in the original specification for providing the unique dual modes of activity of the genus of proteins of the claimed invention.

The original specification of priority document PCT/US2005/014737 as filed on May 2, 2005 provides the complete sequence of three species of the claimed genus of peptides that exhibit the recited functionality of having aryloxyalkanoate dioxygenase activity, wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide. In analyzing the structure of the identified AAD-1 proteins in light of the known α-ketoglutarate-dependent dioxygenase motif, the skilled practitioner can readily identify a specific "AAD-1 motif", wherein the first histidine residue of the general α-ketoglutarate-dependent dioxygenase motif corresponds to the histidine found at position 111 of SEQ ID NO:9, the(D/E) residue of the general α-ketoglutarate-dependent dioxygenase motif corresponds to the aspartic acid residue at position 113 of SEQ ID NO:9, the (T/S) residue of the general α-ketoglutarate-dependent dioxygenase motif corresponds to the threonine residue at position 138 of SEQ ID NO:9, the second histidine residue of the general α-ketoglutarate-dependent dioxygenase motif corresponds to the histidine residue at position 270

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of SEQ ID NO:9, and the arginine residue of the general α-ketoglutarate-dependent dioxygenase motif corresponds to the arginine residue at position 281 of SEQ ID NO:9, thus establishing the AAD-1 motif of: $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$.

The motif disclosed in paragraph [0085] of the specification highlights important amino acids comprising the active site of α-ketoglutarate-dependent dioxygenases. As noted above, one of ordinary skill in the art would know that the motif provided in paragraph [0085] provides a roadmap to identify the corresponding positions in the AAD-1 proteins of SEQ ID NOs; 9, 10 and 11 thus identifying the specific "AAD-1 motif" of $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$. This motif provides structural elements of the active site of the unique AAD-1 proteins of the present invention that enzymatically degrade phenoxy auxin herbicides and (R)-aryloxyphenoxypropionate herbicides.

Evidence Confirms the Importance of the AAD-1 Motif

In support of the objective teachings relating to the structure/function relationship of the AAD-1 genes, applicant previously submitted on April 12, 2019 a Declaration under 37 CFR § 1.132 by Dr. Wright (the Wright Declaration) with accompanying experimental data. An expert's Declaration is relevant to the issue of written description when it is direct to specific statements in the originally filed application and used to explain how, in his opinion, a person of ordinary skill in the art would understand those statements (see *Martek Biosciences Corp. v. Nutrinova, Inc.*, 579 F.3d 1363 (Fed. Cir. 2009)).

As detailed in the previously submitted Wright Declaration, one of ordinary skill in the art upon reading the entire disclosure would recognize that the motif disclosed in paragraph [0085] could be used to identify an AAD-1 motif as a means of identifying important residues of the AAD-1 active site. Furthermore, the skilled practitioner would also understand that such amino acids should be maintained in any protein derivatives of SEQ ID NO: 9 to retain the enzymatic activity of the parent protein (see numbered paragraphs 9-12 of the Wright Declaration).

As described in paragraph 14 of the Wright Declaration, an experiment was conducted to confirm the objective teachings of the present application that the defined residues in the AAD-1 motif $HXDX_{24}TX_{131}HX_{10}R$[2] are important to the desired dual mode of action herbicide

[2] This is simply an alternate way of representing the AAD-1 motif, substituting for the designations set forth in Claim 15, wherein the subscript designates the total number of intervening amino acids. *See* Wright Declaration at para. 12.

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degradation activity. Nine variants of AAD-1v1 (SEQ ID NO: 9) were prepared and tested for enzymatic activity against 2,4-D and (R)-quizalofop. Alignments of all nine variants and the original AAD-1v1 (SEQ ID NO: 9) are shown in Appendix III of the accompanying Wright Declaration. The data presented with the Wright Declaration (e.g., see the bar graph provided in Appendix III and paragraphs 15 and 16 of the accompanying Wright Declaration) confirm the importance of the amino acids at positions 111, 113, 138, 270 and 281 in AAD-1 proteins for maintaining the claimed dual enzymatic activity.

Furthermore, extensive study of tfdA and other αKG dioxygenases has revealed that 5 identified residues in the substrate-capping region are important in coordination of the common co-substrate (2KG) and the Fe(II) ion in an architecture that sets them up for reaction with molecular oxygen and the target substrate. *See* Nair Declaration at paras. 10-11. As revealed by sequence alignments, the spacing and composition of the intervening amino acid residues distinguish the substrate accommodation of the claimed genus of protein variants. The difference of the 131 amino acid spacing ($(X)_{139-269}$ in the AAD-1 motif) becomes evident with alignment of R vs S-type αKG-dioxygenases that have activity on 2,4-D; as the R-AOPP activity has only been seen where the 131 amino acid spacing exists.

The Prior Patent Office Guidelines Confirm that Written Description is Present

In further support of applicant's contention that the presently claimed invention complies with the written description requirements of 35 USC 112, applicant directs the Examiner to Example 11B of the March 25, 2008 revised written description training materials. Although these guidelines have been archived and have been deemed outdated, Applicant notes that the primary reason for the archiving of the 2008 guidelines appears to be related to the 2017 *Amgen v. Sanofi* decision, which concerned adequate written description for claims drawn to antibodies. *Amgen v. Sanofi,* 872 F.3d 1367 (Fed. Cir. 2017). Applicant respectfully submits that the legal analysis associated with the subject matter of Example 11B has not been impacted by recent case law and remains valid for analysis of written description for amino acid sequences. Applicant notes that Example 11B is particularly relevant to the present invention as the example relates to written descriptive support for the following hypothetical claim:

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> An isolated nucleic acid that encodes a polypeptide with at least 85% amino acid sequence identity to SEQ ID NO: 2; wherein the polypeptide has activity X.

As noted in the 2008 guidelines, for this example, the specification identified two domains responsible for activity Y, i.e., a binding domain and catalytic domain. The specification also predicted that conservative mutations in these domains will result in a protein having activity Y. Although all conservative amino acid substitutions in these domains will not necessarily result in a protein having activity Y, those of ordinary skill in the art would expect that many of these conservative substitutions would result in a protein having the required activity. The 2008 guidelines go on to state that

> amino acid substitutions outside of the two identified functional domains are unlikely to greatly affect activity Y. Thus, a correlation exists between the function of the claimed protein and the structure of the disclosed binding and catalytic domains.

The 2008 guidelines continue with the analysis stating that, consequently, there is information about which nucleic acids can vary from SEQ ID NO: 1 in the claimed genus of nucleic acids and still encode a polypeptide having activity Y. Based on the applicant's disclosure and the knowledge within the art, those of ordinary skill in the art would conclude that the applicant was in possession of the claimed genus of nucleic acids based on the disclosure of the single species of SEQ ID NO: 1 that encodes a protein of SEQ ID NO: 2.

The fact pattern of Example 11B of the 2008 written description guidelines is closely aligned with the claimed subject matter of the present invention. Specifically, the present invention relates to transgenic plant cells comprising a nucleic acid sequence selected from a similar genus of nucleic acid molecules and encoding a similar scope of specified proteins. The amino acid sequence of a protein along with knowledge of the genetic code puts an inventor in possession of the genus of nucleic acids capable of encoding the protein. See MPEP §2106(I)(A); *In re Bell*, 991 F.2d 781, 26 USPQ2d 1529 (Fed. Cir. 1993).

Similar to Example 11B of the March 25, 2008 revised written description training materials, applicant has provided the complete amino acid sequence of three working embodiments and has provided the structure of the active site (the AAD-1 motif) of the members of the claimed genus of proteins. Similar to Example 11B of the 2008 written description guidelines, applicant has described the functional activity all the protein variants must exhibit

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(the ability to enzymatically degrade a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide). In addition, the claimed genus of the present application is limited to peptide variants having at least 85% sequence identity to the exemplified protein and that exhibit the specified activity. Thus the claimed subject matter is similar in scope, with a similar level of written description support, as the hypothetical claimed genus of Example 11B of the 2008 revised written description training materials that was found to meet the written description requirements of 35 USC 112. Accordingly, consistent with the 2008 written description guidelines, the currently claimed subject matter should be found to meet the written description requirements of 35 USC 112. Applicant also notes that the analysis of Example 11B of the March 25, 2008 revised written description training materials is entirely consistent with the recent holding in *Ajinomoto Co., Inc. v. International Trade Commission.*

Written Description Requirement: Conclusion

The Federal Circuit has held that complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics provides evidence that applicant was in possession of the claimed invention. *See Enzo Biochem, Inc. v. Gen–Probe Inc.*, 323 F.3d 956 at 964. Specifically, in *Ariad* the *en banc* court reaffirmed that the written description doctrine has "always expressly permitted the disclosure of structural features common to the members of the genus." *Ariad Pharmaceuticals,* 598 F.3d at 1352. Applicant respectfully submits that they have provided structure of the members of the claimed genus sufficient to satisfy the written description test.

First of all, each peptide of the claimed genus is required to have at least 85% sequence identity with an amino acid of SEQ ID NO: 9. Furthermore, applicant has provided guidance as to where modifications can be made in the AAD-1 sequence by requiring all peptides of the claimed genus to comprise the AAD-1 motif. Additional structure is also provided by the extensive knowledge of homologous αKG dioxygenases derived from analysis and alignments of those amino acids sequences. Those skilled in the art are well aware that proteins can be modified while retaining the activity of the original protein, provided that the active site remains intact. Accordingly, the claimed genus is further limited by the requirement that the claimed analogs of SEQ ID NO: 9 comprise the AAD-1 motif and retain enzymatic activity for degrading phenoxy auxin herbicides and (R)-aryloxyphenoxypropionate herbicides.

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An adequate written description of the invention may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention. See, e.g., *Purdue Pharma L.P. v. Faulding Inc.*, 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed.Cir. 2000). Moreover, both declarants have provided evidence that a skilled practitioner would readily appreciate that applicant was in possession of the invention as currently claimed at the time of filing the priority application. *See* Nair Declaration at paragraph 13, Wright Declaration at paragraph 16.

Applicant respectfully submits that the structural data provided by the working embodiments of the present invention and the numerous previously disclosed tfdA-type proteins, in conjunction with applicant's disclosed AAD-1 motif, provides sufficient description of the structure of the functional elements of the claimed subject matter to demonstrate applicants were in possession of the claimed invention. Therefore, applicant respectfully requests the withdrawal of the rejection of claims 15, 16 and 18-48 for failure to comply with the written description requirement.

Obviousness Rejection

Claims 15, 16, 18-30, 32-34, 36, 37, 39-45, 47 and 48 stand rejected under 35 USC 103 as being obvious over the combined teachings of Jepson et al (US 2003/0041357) in view of Kohler (Genbank Accession no Q8KSC8). Jepson is cited as teaching plants that comprise a *tfdA* gene for conferring aryloxyalkanoate dioxygenase activity to those plants, and the use of such plants in methods of weed control using 2,4-D. The Examiner admits that Jepson does not teach AAD-1 (SEQ ID NO:9). Kohler is cited as teaching *Spingomonas herbicidovorans rdpA*, a bacterial gene that encodes the peptide of SEQ ID NO:9 of the instant application. The Examiner states that it would have been *prima facie* obvious to substitute the gene of Kohler for the gene of Jepson. Applicant respectfully traverses this rejection.

First of all, applicant respectfully submits that the Examiner fails to provide any rationale for why one of ordinary skill would specifically select a gene encoding a peptide of SEQ ID NO: 9, or a derivative thereof having at least 85% sequence identity to a peptide of SEQ ID NO: 9, to substitute for the *tfdA* gene disclosed in Jepson. Applicant notes there are many other known sequences that share sequence identity with the *tfdA* gene and serve as possible candidates for providing aryloxyalkanoate dioxygenase activity. At the time of applicant's

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invention it cannot be reasonably said that one would have turned to the specific gene disclosed in Kohler with the expectation that it would be expressed in plants, particularly to convey herbicide tolerance to the plants.

At the time of the invention there was simply no motivation to select a bacterial gene having specific sequence of SEQ ID NO: 9, (or a peptide having at least 85% sequence identity with SEQ ID NO: 9) for combination with a heterologous promoter that is functional in a plant cell for use in producing transgenic plants that are tolerant to damage by an aryloxyalkanoate herbicide. The Examiner contends that in spite of the lack of teaching regarding AAD-1's ability to degrade AOPP herbicides, it was still obvious to prepare a transgenic plant comprising the sequence of SEQ ID NO: 9 to achieve a plant tolerant to phenoxy auxin herbicide with a reasonable expectation of success.

First of all applicant notes that not all known α-ketoglutarate-dependent dioxygenases exhibit the ability to degrade phenoxy auxin herbicides when inserted into plant cells. Furthermore, the Examiner fails to identify any rationale for why one would select the rdpA protein (SEQ ID NO: 9) from the many other available tdfA-type proteins and instead relies on an improper obvious to try standard to compensate for the inadequacies of the prior art teachings.

The Federal Circuit has differentiated between proper and improper applications of "obvious to try" in *In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1681 (Fed. Cir.). More particularly, two classes of improper obvious to try were exemplified as follows: (1) when what would have been "obvious to try" would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful; and (2) when what was "obvious to try" was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it.

The Examiner has cited Kohler as teaching a polynucleotide that encodes a protein of SEQ ID NO: 9 and that such a protein has R-2,4-dicholorophenoxypropiaonate dioxygenase activity. However, there was no teaching provided by the prior art to indicate that the rdpA bacterial gene would provide tolerance to phenoxy auxin herbicides upon introduction into plant cells. Due to the unpredictability of the activity of bacterial genes in plant systems, contrary to

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the Examiner's assertions, there was no reasonable expectation of success based on the combined teachings of Jepson et al and Kohler that a plant transformed with the rdpA gene would produce a plant having tolerance to a phenoxy auxin herbicide.

The unpredictability associated with bacterial activity relative to activity in plants is highlighted in the present specification as detailed with the results obtained with AAD-2. Example 17 of the subject specification provides data comparing the subject AAD-1 enzyme to another enzyme referred to as AAD-2, wherein AAD-2 has a higher percentage sequence identity with tfdA relative to AAD-1. Accordingly, AAD-2 is a protein that falls outside the scope of the current claims, as AAD-2 does not have at least 85% sequence identity with SEQ ID NO: 9, yet is one with a sequence identity closer to tfdA. Significantly, AAD2 also does not comprise the AAD-1 motif that is present in AAD-1 (see paragraph 12 and Appendix II of the Wright Declaration).

AAD-2 was one of several genes identified from BLAST search results using tfdA as the reference sequence. The subject application states in Example 17.1:

> Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to tfdA. This gene is referred to herein as AAD-2 (v1) for consistency. Percent identity was determined by first translating both the AAD-2 and tfdA DNA sequences (SEQ ID NO: 12 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

AAD-2 was obtained from *Bradyhizobium japonicum*. Again, AAD-2 is more closely related (about 44%) at the sequence level to tfdA than is AAD-1 (about 27%). However, AAD-2 was surprisingly inactive in plants, while AAD-1 was very active.

Jepson et. al., along with the many other teachings of bacterial α–ketoglutarate-dependent dioxygenases, merely provide an invitation to experiment with no guidance as to which of the many tdfA-type genes would be successful in plants, even with regard to providing tolerance to phenoxy auxin herbicides. Accordingly, the cited references fail to establish a *prima fascia* case of obviousness.

Furthermore, applicant, in selecting the rdpA gene for investigation, proceeded contrary to the conventional wisdom by selecting a tdfA-type gene that had a low sequence identity to

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tfdA. Proceeding contrary to conventional wisdom is a secondary consideration that would rebut arguments that the present invention is obvious.

The subject AAD-1 enzyme was a choice of one of over 100 other possible genes. The first paragraph of Example 1 of the subject application states (emphasis added):

> As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e., α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤ 50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

Thus, the subject AAD-1 enzyme was only 1 of over 100 BLAST search results having some degree of identity with tfdA. As discussed above, there were great numbers of other possible choices that could have been selected. As noted in paragraphs [0012] and[0013] of the specification, many of those possible choices had amino acid sequence similar to tfdA (>85% amino acid identity) and have similar *in vitro* enzymatic properties to tfdA. Typically one would select a peptide homolog with high sequence identity if one wanted to obtain a homolog that retained the activity of the parent protein (i.e., activity against phenoxy auxin herbicides). However, applicant selected the rdpA gene from *Sphingobium herbicidovorans* for analysis, even though the protein exhibited low homology to tfdA (28% amino acid identity).

Furthermore, the subject invention offers surprising advantages that neither Jepson nor Kohler taught. As illustrated by the subject specification, particularly by Table 1 and Table 2, mecoprop and dichlorprop have phenoxy auxin chemistries, while the aryloxyphenoxypropionates or "AOPPs" are in a different class of chemistries. Thus, it is not seen how Kohler would support the reasonable expectation of success to produce a plant that is

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resistant to aryloxyphenoxypropionates and thus protecting a crop plant from damage by an aryloxyalkanoate herbicide.

The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (vl) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (Figure 3 and Table 7 of the present specification). **These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins**. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

The above paragraph shows that AAD-1 has surprising advantages that are not contemplated by Kohler, (i.e., it acts on two completely different modes of action herbicides). This is not taught or suggested by either Jepson or Kohler.

The subject invention relates in part to the identification of an enzyme AAD-1 that is not only able to degrade 2,4-D, but also aryloxyphenoxypropionate (AOPP) herbicides, thus distinguishing the enzyme of the subject invention from previously known tfdA proteins. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Accordingly, there was no reason to select the nucleic acid of SEQ ID NO: 9, or derivatives thereof that retain the ability to degrade aryloxyphenoxypropionate (AOPP) herbicides, to be operably linked to a plant specific promoter, as there was no reasonable expectation that such a construct would produce the desired effect when transfected into plant cells. This unexpected property is a further secondary consideration that rebuts any arguments that the present invention is obvious.

Accordingly, applicant respectfully requests the withdrawal of the rejection of claims 15, 16, 18-30, 32-34, 36, 37, 39-45, 47 and 48 as being obvious over the combined teachings of Jepson et al in view of Kohler.

Novelty Rejections

Claims 15, 16 and 18-25, 28-31, 45, 47 and 48 are rejected under 35 USC § 102 as being anticipated by Ellis et al (US 2016/0108422). Applicant respectfully traverses this rejection.

The present specification is substantively identical to that of PCT/US2005/014737. As discussed on pages 8-13 of this response, applicant submits the currently claimed subject matter is entitled to the priority date of PCT/US2005/014737. The early disclosure of PCT/US2005/014737

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provides the sequence of SEQ ID NOs: 9, 10 and 11 and the formulation of the α-ketoglutarate-dependent dioxygenase motif. As noted above those of ordinary skill would understand how to apply the α-ketoglutarate-dependent dioxygenase motif to the AAD-1 proteins sequences of SEQ ID NOs: 9, 10 and 11 to generate the AAD-1 motif listed in the current claims. Therefore, the current claimed invention is entitled to the priority date of May 2, 2005, and Ellis et al is not a proper prior art document.

Withdrawal of the rejection of claims 15, 16 and 18-25, 28-31, 45, 47 and 48 as being anticipated by Ellis et al (US 2016/0108422) is respectfully requested.

Claims 15, 16 and 18-48 are rejected under 35 USC 102 as being anticipated by Wright et. al., (EP 1740039). Applicant respectfully traverses this rejection.

EP 1740039 is a regionalized application of PCT/US2005/014737. As discussed on pages 8-13 of this response, applicant submits the currently claimed subject matter is entitled to the priority date of PCT/US2005/014737. Accordingly, EP 1740039 represents the same application as the present application. Therefore, Wright et. al., (EP 1740039) is not a proper prior art reference to the present application.

Withdrawal of the rejection of claims 15, 16 and 18-48 as being anticipated by Wright et. al., is respectfully requested.

Consideration of the claims and allowance of the application is respectfully requested. If any further discussion of this matter would speed prosecution of this application, the Examiner is invited to call the undersigned at (434) 220-2866.

Respectfully submitted,

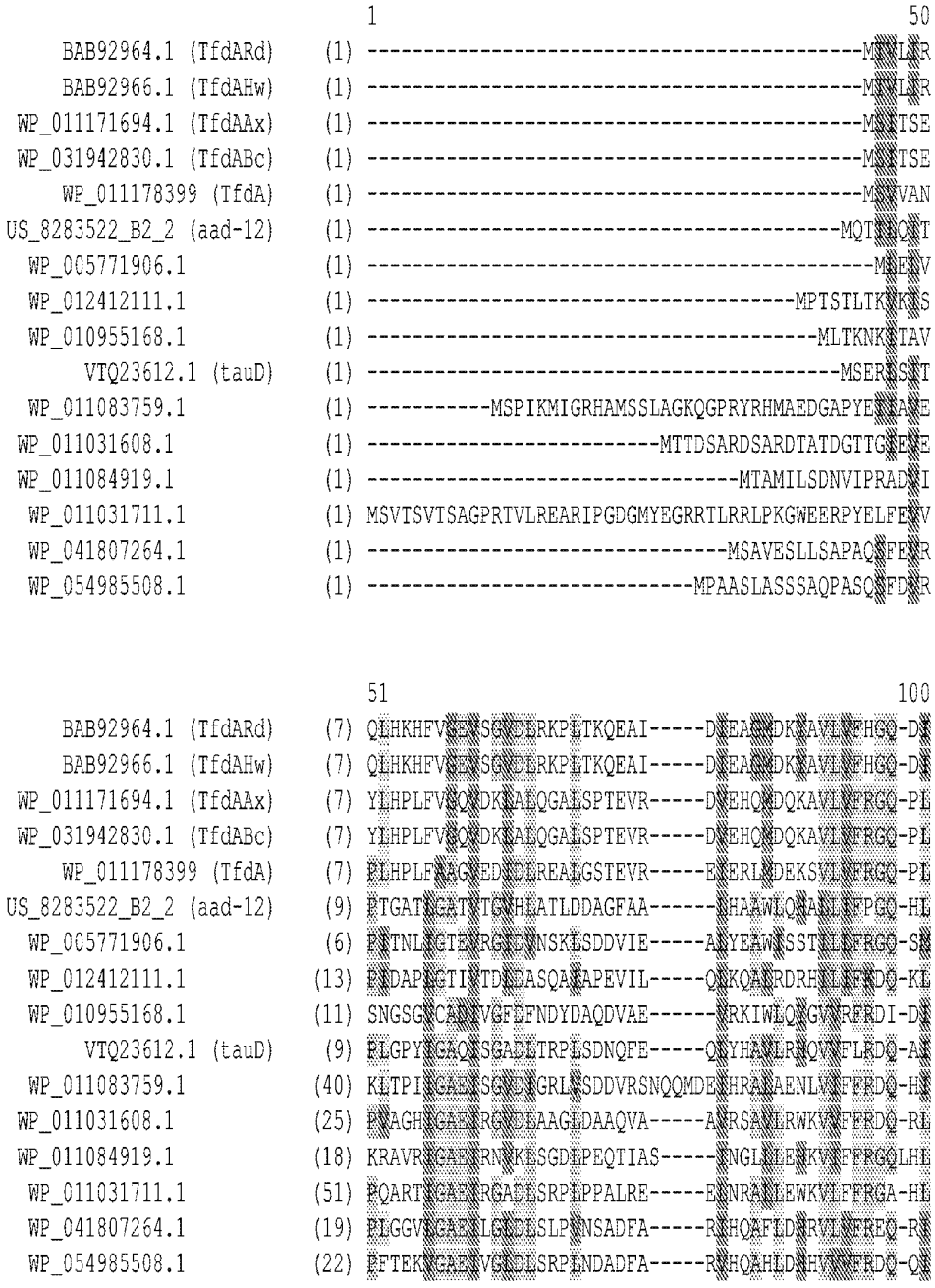
/John P. Breen/

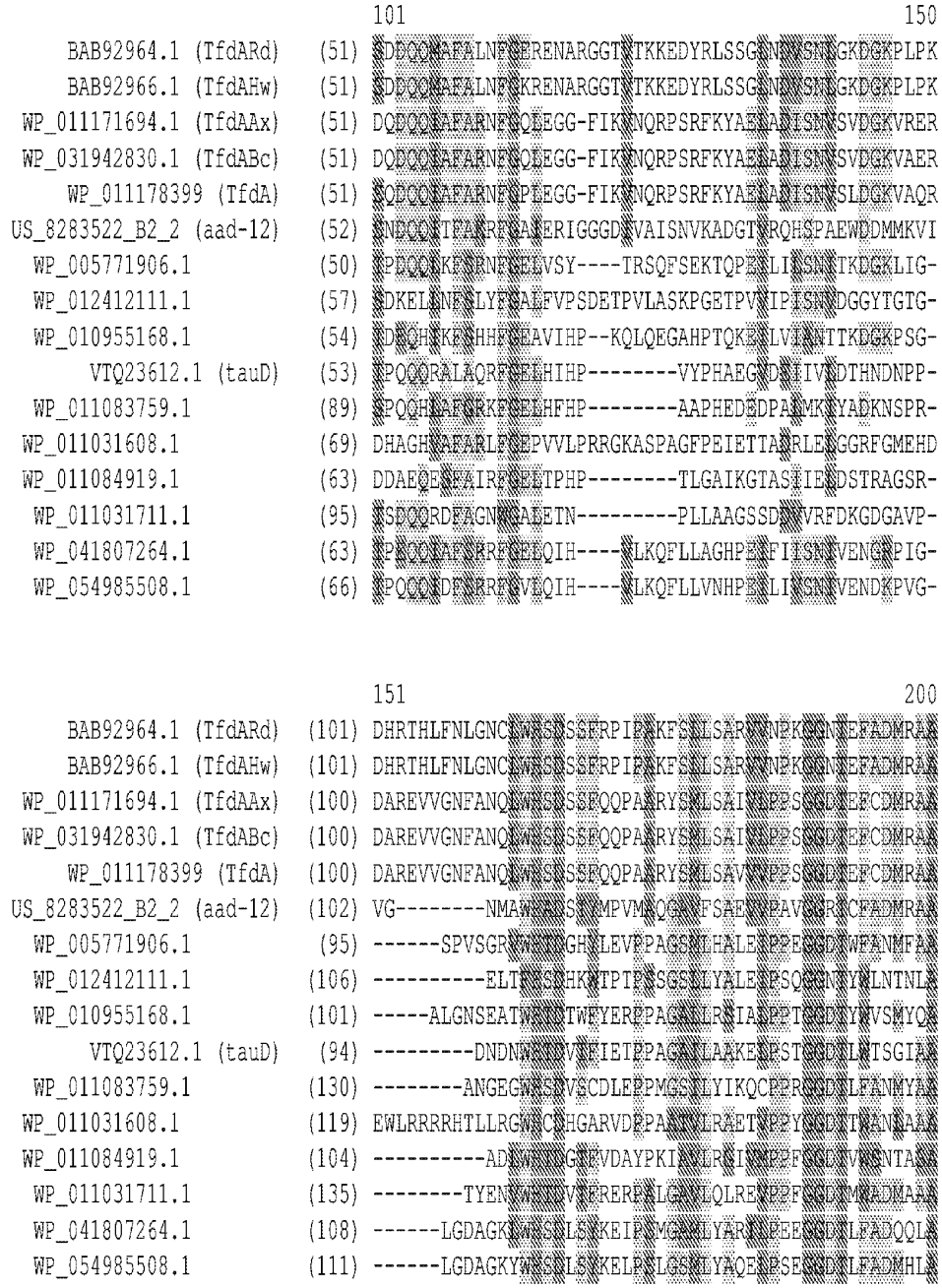
John P. Breen
Registration No. 38,833
Attorney for Applicants

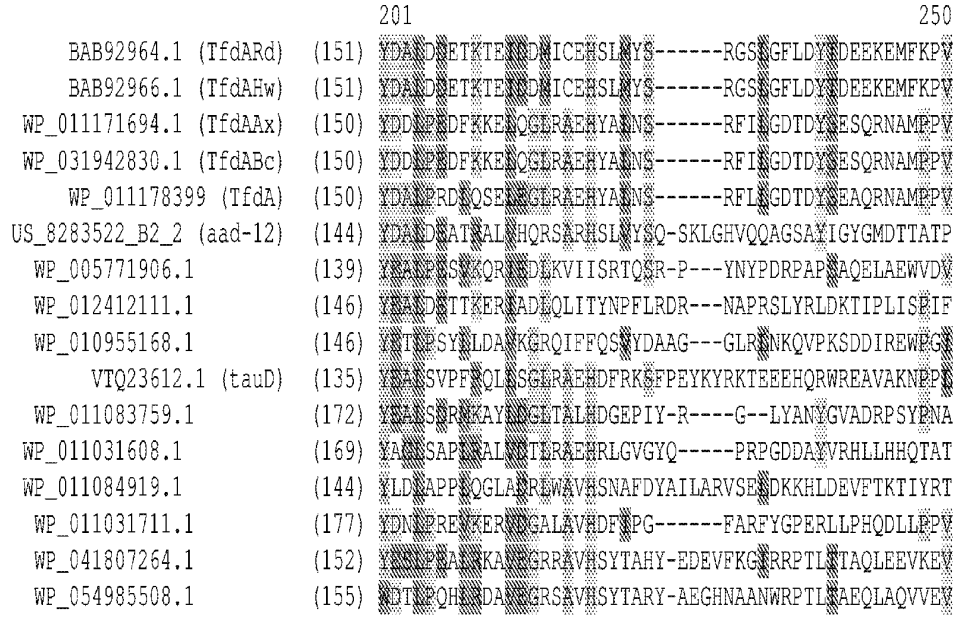
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Indianapolis, Indiana 46204

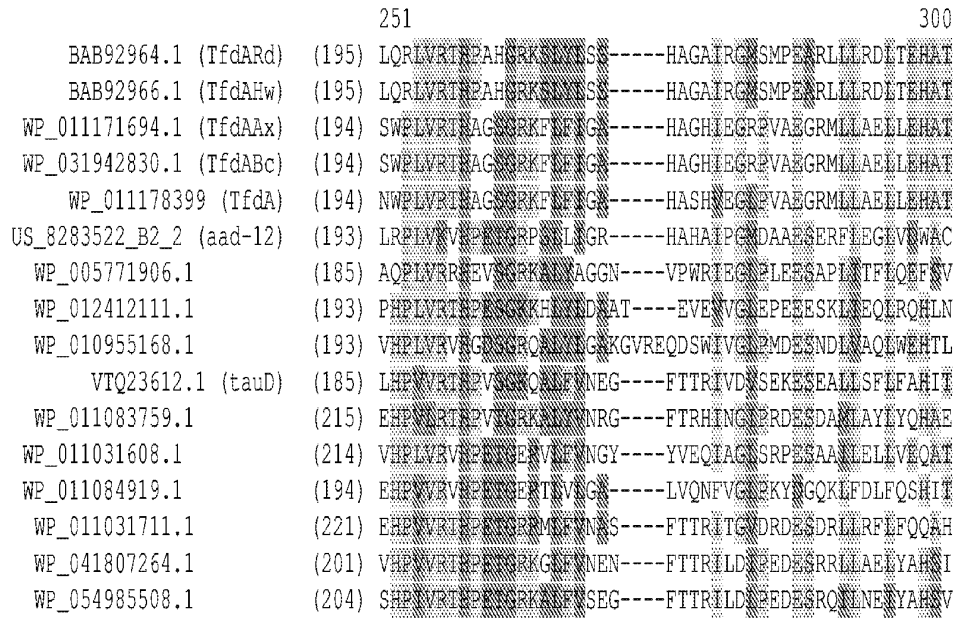
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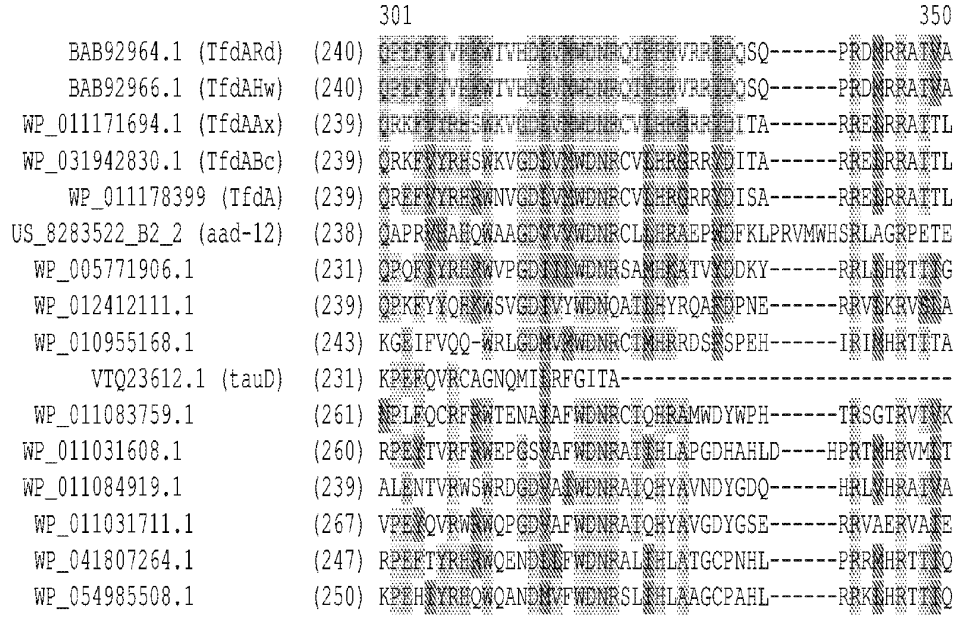
Exhibit A: Alignment of tfdA-type proteins

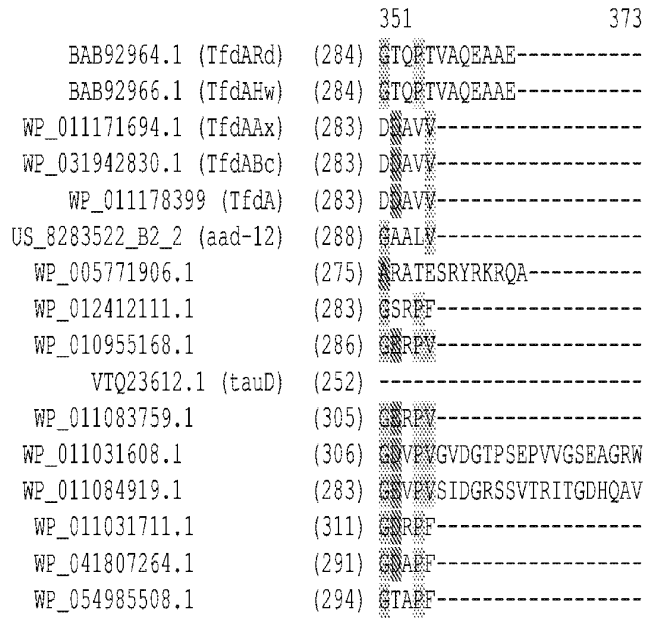



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WP_031942830.1 (TfdABc) (51)
WP_011178399 (TfdA) (51)
US_8283522_B2_2 (aad-12) (52)
WP_005771906.1 (50)
WP_012412111.1 (57)
WP_010955168.1 (54)
VTQ23612.1 (tauD) (53)
WP_011083759.1 (89)
WP_011031608.1 (69)
WP_011084919.1 (63)
WP_011031711.1 (95)
WP_041807264.1 (63)
WP_054985508.1 (66)
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WP_011031608.1 (119)
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WP_011031711.1 (135)
WP_041807264.1 (108)
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WP_011031608.1 (169) YAGLSAPSRALSTLRAEHRLGVGYQ-----PRPGDDAYVRHLLHHQTAT
WP_011084919.1 (144) YLDLAPPSQGLASRLWAVHSNAFDYAILARVSESDKKHLDEVFTKTIYRT
WP_011031711.1 (177) YDNLPRESKERSTGALAVHDFTPG------FARFYGPERLLPHQDLLPPV
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251
300
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WP_011084919.1 (194) EHPVVRVNPETGERTIVGG-----LVQNFVGLPKYSGQKLFDLFQSHIT
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WP_041807264.1 (201) VHPVVRINPETGPKGLFVNEN----FTTRILDLPEDESRRLLAELYAHSI
WP_054985508.1 (204) SHPIVRINPETGRKALFVSEG----FTTRILDLPEDESRQLLNELYAHSV


301
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WP_011031711.1 (311)
WP_041807264.1 (291)
WP_054985508.1 (294)

AMENDMENTS TO THE CLAIMS:

This listing of claims will replace all prior versions, and listings of claims in the application:

Claims 1-14. (Canceled).

15. (Previously Presented) A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

$(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and

$(X)_{271\text{-}280}$ represents a sequence of 10 amino acids.

16. (Previously Presented) The plant cell of claim 15 wherein said AAD-1 motif has the general formula of:

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R(X)_{282\text{-}284}R$, wherein $(X)_{282\text{-}284}$ represents a sequence of 3 amino acids.

17. (Canceled).

18. (Previously Presented) The plant cell of claim 15 wherein the AAD-1 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 9.

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19. (Previously Presented) The plant cell of claim 15 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an Arabidopsis cell.

20. (Previously Presented) The plant cell of claim 15 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

21. (Previously Presented) A transgenic plant comprising a plurality of the plant cell of claim 15, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

22. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

23. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

24. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

25. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

26. (Previously Presented) The plant of claim 21 wherein said plant further comprises a second herbicide resistance gene.

27. (Previously Presented) The plant of claim 26 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group

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consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

28. (Previously Presented) A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 21, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide.

29. (Previously Presented) The method of claim 28 wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

30. (Previously Presented) The method of claim 28 wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

31. (Previously Presented) The method of claim 28 wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

32. (Previously Presented) The method of claim 28 wherein said method comprises applying a second herbicide.

33. (Previously Presented) The method of claim 32 wherein said first herbicide and said second herbicide are applied sequentially.

34. (Previously Presented) The method of claim 32 wherein said first herbicide and said second herbicide are applied concurrently.

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35. (Previously Presented) The method of claim 32 wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

36. (Previously Presented) The method of claim 32 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

37. (Previously Presented) The method of claim 32, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

38. (Previously Presented) The method of claim 32, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

39. (Previously Presented) The method of claim 32 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

40. (Previously Presented) The method of claim 39 wherein said second gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

41. (Previously Presented) The method of claim 40 wherein:

(a) said modified ALS gene is selected from the group consisting of a SurA, SurB, Csr1, Csr1-1, Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT; and,

(c) said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

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42. (Previously Presented) The method of claim 32 wherein said method further comprises applying a third herbicide.

43. (Previously Presented) The method of claim 42, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

44. (Previously Presented) The method of claim 43 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

45. (Previously Presented) A seed comprising a plurality of the plant cell of claim 15.

46. (Previously Presented) A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim 45 in said field within 14 days of applying said aryloxyalkanoate herbicide.

47. (Previously Presented) A plant grown from the seed of claim 45.

48. (Previously Presented) A part, progeny, or asexual propagate of the plant of claim 47, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

49. (New) An (R)-aryloxyphenoxypropionate herbicide tolerant transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

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X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

$(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and

$(X)_{271\text{-}280}$ represents a sequence of 10 amino acids, wherein said motif has 90% sequence identity with corresponding amino acids of position 111 to 281 of SEQ ID NO: 9.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No.:	107540
Group:	1663
Confirmation No.:	5402
Application No.:	15/288,406
Invention:	**Novel Herbicide Resistance Genes**
Inventor:	Terry Wright et al.
Filed:	October 7, 2016
Attorney Docket:	14764-258662
Examiner:	David Kruse

FILED ELECTRONICALLY:

January 10, 2020

AMENDMENT AND RESPONSE ACCOMPANYING RCE

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Sir:

In response to the final Office Action mailed July 12, 2019, Applicant transmits herewith an RCE, and requests a three month extension of time, entry of the following claim amendments, and consideration of the accompanying remarks. Applicant submits herewith payment of the $ 1,400.00 fee under 37 CFR § 1.17(a) for the requested three month extension and the 1,300.00 fee for the RCE request (large entity), and hereby authorizes the Commissioner to charge any additional fee necessary to affect this filing, to the account of Barnes & Thornburg, Deposit Account No. 10-0435 with reference to our file 14764-258662.

Amendments to the Claims are submitted herewith beginning on page 2.

Remarks begin on page 8 of this paper.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Wright et al.

Serial No.: 15/288,406

Filed: October 7, 2016

For: Novel Herbicide Resistance Genes

Confirmation No.: 5402

Examiner: David H. Kruse

Group Art Unit: 1663

Attorney Docket No.: 14764-258662
(63364A-US-CNT[3])

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. SATISH K. NAIR

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Satish K. Nair, declare:

1. I am currently employed at the University of Illinois and hold the following positions: I.C. Gunsalus Professor and Interim Head in the Department of Biochemistry in the School of Molecular and Cell Biology, Professor in the Carl R. Woese Institute for Genomic Biology,

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Professor, Director of the Center for Biophysics and Quantitative Biology, and Affiliate Professor with the Department of Chemistry. I am a citizen of the United States of America. I have 30 years of research experience in the fields of biochemistry, microbiology, and structural biology. I received a Bachelor of Science degree in Chemistry (with Honors) from Brown University, a Doctor of Philosophy degree in Chemistry from the University of Pennsylvania, and conducted Postdoctoral research in Molecular Biophysics at Rockefeller University. In addition to the appointments listed above I have received the follow awards of recognition: I.C. Gunsalus Endowed Chair. I am an author of 119 peer-reviewed scientific journal articles. My research has been in the areas of drug discovery, enzymology, microbial physiology, host-pathogen interactions, and protein structure. My *curriculum vitae* is attached to this declaration as Exhibit XX.

I am not affiliated with the assignee, Dow AgroSciences LLC, Indianapolis, IN and do not have any financial interest in, or directly related, to the subject matter of US Patent Application no: 15/288,406 ("the '406 Application") which is a continuation of a national stage filing based on PCT/US2005/014737, published as WO 2005/104737. However, I have conducted investigative research on certain proteins discovered by Dow AgroSciences (now Corteva Agriscience) and my laboratory has received research funding from Corteva Agriscience in connection with this research.

2. I have conducted research in the field of enzymology and structural biology, among other scientific disciplines mentioned above. I have worked with a number of different classes of metalloenzymes, including the Fe/α-ketoglutarate-dependent dioxygenases (Fe/α-KG dioxygenases), and am very familiar with the relevant literature cited in paragraph [0085] of the '406 application. I have been asked by applicant to provide my opinion regarding the scope of the subject matter disclosed in the '406 Application.

3. I am familiar with the subject matter disclosed in the '406 application, as I have reviewed the '406 patent application specification, its accompanying drawings and the pending claims. I understand that this application claimed priority to U.S. Provisional Patent Application No. 60/567052, filed on April 30, 2004 (the "priority date"). I have also reviewed and am familiar with the 37 CFR Declaration of Dr. Terry R. Wright, and the accompanying data, submitted to the USPTO for the '406 application, on April 4, 2019.

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4. At the time of the priority date, use of a single herbicide (glyphosate) in glyphosate tolerant soybean crops was resulting in selection of weeds resistant to glyphosate. 2,4-dichlorophenoxyacetic acid (2,4-D), a member of the phenoxy auxin class of herbicides, was an existing commercial herbicide that could provide an additional mode of action to control glyphosate-resistant weeds in combination with glyphosate. However, key agronomic crops such as soybeans had little or no inherent tolerance to 2,4-D. Therefore, in order to enable use of 2,4-D over such crops, an herbicide tolerance trait was needed.

5. Prior work had identified an enzyme termed TfdA from *Ralstonia eutropha* (classified under multiple other names in the scientific literature, such as *Ralstonia eutropha,* and *Cupriavidus necator*) that is involved in the conversion of 2,4-D to herbicidally inactive dichlorophenol. As described in paragraph [0012] of the '406 application, a large number of *tfdA*-type homologous genes that encode proteins capable of degrading 2,4-D had been identified from the environment and their sequences had been disclosed in GenBank deposits at the time of the priority date. The known *tfdA*-type homologous genes encoding proteins capable of degrading 2,4-D represented a large pool of genes that could be used to transform plant lines to generate 2,4-D tolerant crops. However, the scientific literature failed to provide any particular guidance as to which of the many homologous genes would be effective when introduced into plants. Applicant chose to focus on genes that had relatively low homology to the tfdA gene.

6. Comparison of the *tfdA* gene homologues known at the time of the priority date revealed conserved structural elements required for 2,4-D degradation (among other substrates) functionality. A motif of the so-called Group-II α-KG dioxygenases (which included TauD, clavaminate synthase, TfdA, *inter alia*) was identified and refined by several investigators and cited at paragraph [0085] of the '406 application as: $HX(D/E)\ X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$ (see also, Schleinitz et al., Appl. Env. Microbiol. 70:5357-5365, 2004).

7. Based on my understanding of the '406 application and my knowledge of the relevant art, I understand that applicant has discovered and described homologues of the known *tfdA* gene homologues that have activity in the degradation of 2,4-D, other phenoxyalkanoic (phenoxy) auxin herbicides, and AOPP herbicides (inhibitors of lipid biosynthesis), which were

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previously not known. Enzymes having such dual modes of action (MOA) are described in the '406 application as AAD-1 (AryloxyAlkanoate Dioxygenase) enzymes. Applicant has demonstrated that a recombinantly expressed AAD-1 enzyme confers tolerance to both a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide when expressed in monocot (grassy) crops or dicot (broadleaf) crops. (see the '406 application at paragraphs [0086-0087] [0098-0099]).

8. At the time of the priority date, no other Fe/α-KG dioxygenase enzymes had been reported to exhibit dual herbicide MOA Fe/α-KG dioxygenase activity, having the ability to degrade herbicides of two different chemical classes and different MOAs. (See the '406 application at [0018], [0097]). Applicant also described the stereospecific utilization of (R)-aryloxyphenoxypropionate [(R)-AOPP] herbicide substrates of AAD-1, as well as variants of SEQ ID NO: 9 that exhibit dual MOA Fe/α-KG dioxygenase activity, and further compared the peptide of SEQ ID NO: 9 and variants thereof to an (S)-enantiomer-specific TfdA homologue, designated as AAD-2. (see the '406 application at [0433]-[0451]). Such comparisons assisted applicant in identifying the elements of AAD-1 that accounted for the protein's unique ability to exhibit dual herbicide MOA Fe/α-KG dioxygenase activity.

9. In addition to three exemplified AAD-1 homologues that exhibit dual MOA Fe/α-KG dioxygenase activity, applicant's analysis of the structures of the tfdA gene homologues with the amino acid sequences of SEQ ID NO: 9, 10 and 11 revealed conserved amino acids relevant to the dual herbicide MOA Fe/α-KG dioxygenase activity. Specifically, the '406 application described a general α–ketoglutarate-dependent dioxygenase motif in paragraph [0085] of the '406 application. I believe that, on reviewing the specific amino acid residues of SEQ ID NOS: 9, 10, and 11 aligned with that general α–ketoglutarate-dependent dioxygenase motif, a skilled practitioner would readily envision a disclosed AAD-1 motif in the form of $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, where the X terms designate an unknown amino acid at the corresponding position of SEQ ID NO: 9. The AAD-1 motif recited in pending claim 1 is readily apparent based on the disclosure of the '406 application and the prevailing knowledge in the art at the time of the priority date. The skilled practitioner would readily appreciate the disclosed AAD-1 motif is disclosed in the '406 application because the specification discloses a general α–ketoglutarate-dependent dioxygenase motif $(HX(D/E)(X)_{23\text{-}26}(T/S)(X)_{114\text{-}183}H(X)_{10\text{-}13}R)$ that defines key amino acids

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of the active sites of α–ketoglutarate-dependent dioxygenases as well as the spacing between the amino acids (i.e., 23 to 26 amino acids separate the D/E amino acid from the T/S amino acid; 114 to 183 amino acids separate the T/S amino acid from the histidine residue; and 10 to 13 amino acid separate the histidine residue from the arginine residue). Furthermore the skilled practitioner has the knowledge that this general α–ketoglutarate-dependent dioxygenase motif can be aligned with the AAD-1 proteins (SEQ ID Nos. 9, 10 and 11) to identify the specific locations of the corresponding amino acids, and the specific spacing between those key amino acids, in the AAD-1 proteins. As there is a clear single maximal alignment of the general motif with the AAD-1 proteins (SEQ ID NO: 9 only contains a single instance of the tripeptide HX(D/E)), the specific AAD-1 motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ is readily apparent and further validated by additional knowledge in the art at the time of the priority date as well as subsequent experimental data validating the objective truth of the original disclosure.

10. In particular, I have reviewed the data submitted to the USPTO on April 4, 2019 and believe that consistent with the disclosure of the '406 application, applicant has confirmed that the "AAD-1 motif" as claimed is required for the dual herbicide MOA activity. When amino acid sequence alignments of the AAD-1 amino acid sequences with 2,4-D-degrading Fe/α-KG dioxygenases that cannot also catalyze (R)-AOPP herbicides are compared, a distinguishing characteristic of these AAD-1 variants is apparent, characterized by the five critical active site residues (H, D, T, H, and R) and the spacing between those 5 residues (separated by undefined X_n spacer amino acids). Specifically, the comparison of the AAD-1 enzymes and AAD-2 reveal while the two proteins retain all five active site residues, there is a difference in the 131 amino acid ($(X)_{139-269}$) interval in the AAD-1 motif $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ vs. the spacing in AAD-2, which is a 2,4-D-degrading Fe/α-KG dioxygenase that cannot also catalyze (R)-AOPP herbicides.

11. My lab, in conjunction with Dow AgroSciences LLC employees, has investigated the biochemistry and crystal structures of AAD-1 and AAD-2 specifically, among other dioxygenase enzymes. The results were published in the Proceedings of the National Academy of Science journal (Chekan et al., PNAS 166:13299-13304, 2019). Comparison of the sequence alignments of AAD-1 (an (R)-specific enantiomer enzyme) and (S)-specific-enantiomer dioxygenases (including AAD-2, TfdA, and another homologue, AAD-12)

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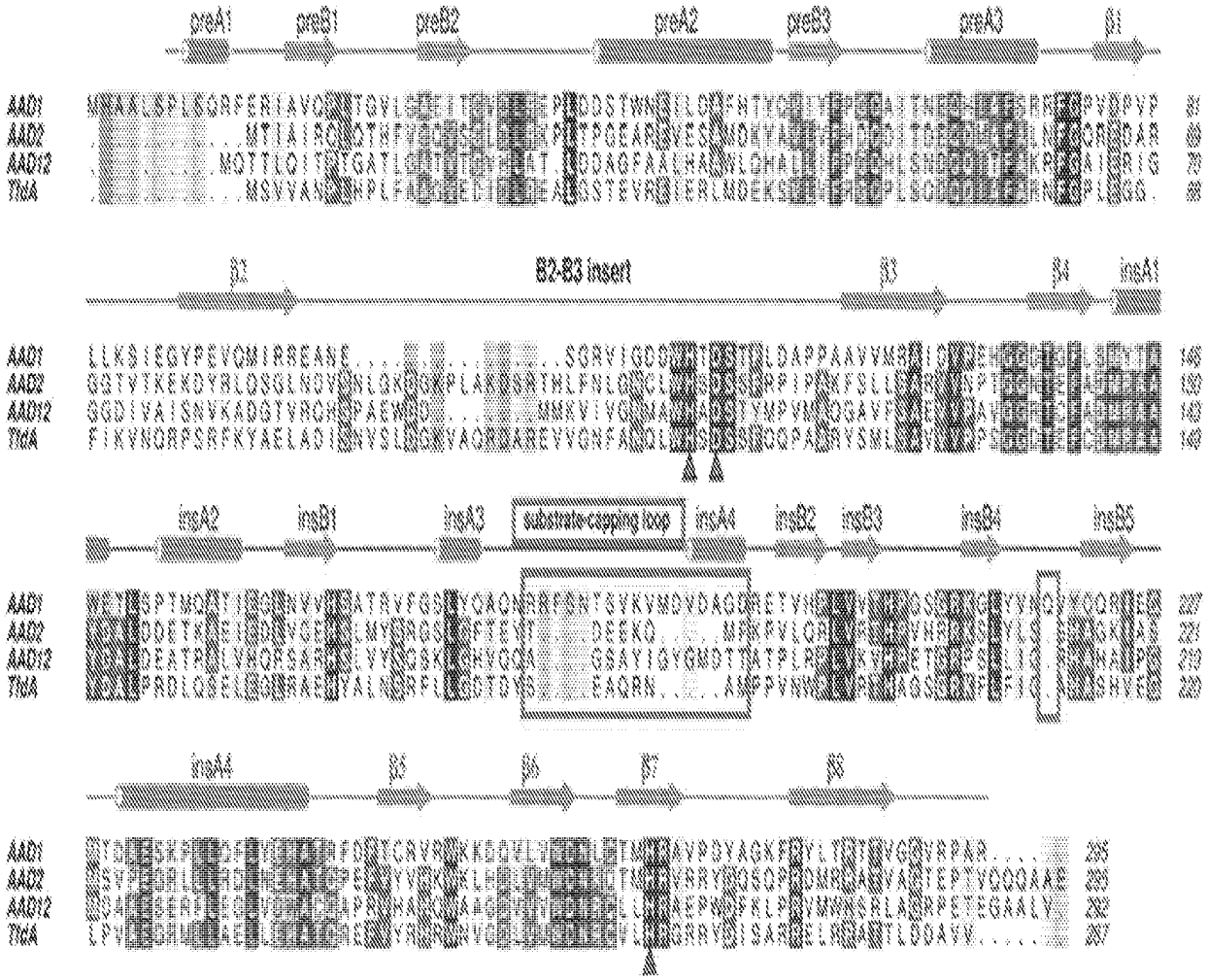
confirmed a divergence in the length and identity of residues which mapped to the substrate-capping loop as identified by comparison of substrate-bound crystal structures. Based on the disclosure contained in the '406 Application, those skilled in the art would have compared and recognized the importance of the sequence differences between AAD-1 variants [(R)-specific dioxygenase] having dual herbicide MOA Fe/α-KG dioxygenase activity and (S)-specific dioxygenases not having dual herbicide MOA Fe/α-KG dioxygenase activity even without crystallography information. However, the crystallography data further validates the importance of the spacing between the critical active site amino acids as defined in the AAD-1 motif of the claimed invention. As recited in Chekan et al.:

> A second distinguishing structural feature of AAD-2 is its shorter substrate-capping loop in comparison with that in AAD-1. In general, each of the (*S*)-specific AADs has a substrate-capping loop that is shortened by 4 to 10 residues in the region surrounding and including Ser183 through Asp193 (*SI Appendix*, Fig. 10). In AAD-1 structure, residues in the extended substrate-capping loop are in van der Waals contact with the substrate, and Phe182 in the loop is positioned for hydrophobic interactions with the phenoxypropionate ring in the substrate tunnel. Changes in both the length and identity of residues in this loop may result in differences in enantioselectivity among AADs.

(Chekan et al., 2019)

Figure 10 from the PNAS paper is disclosed below showing the sequence alignment of the AAD1, AAD2, AAD12, and TfdA proteins and the noted variation in the length of the substrate-capping loop.

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12. Those skilled in the art appreciate that variant proteins (and genes) of the subject invention, having amino acid modifications, can be prepared that retain the desired biological/functional activity of the parent protein (i.e., dual herbicide MOA Fe/α-KG dioxygenase activity degradation). Applicant clearly intended such variant proteins to be part of the invention as the specification states in paragraph [00142]:

> Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins.

Applicant provided an extensive description in paragraphs [00135]-[00145] of the types of modifications that could be made to the exemplified species of SEQ ID NO: 9, 10 and 11 to generate the further variant peptides envisioned by the applicant and encompassed by the invention. Such variant peptides were limited to peptides having activity equivalent or similar to an exemplified peptide.

13. I believe that the skilled practitioner using the description of the claimed subject matter provided in the specification, including the described three exemplified embodiments and the identification of the structure of the active site (i.e., the AAD-1 motif $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$), in conjunction with what was previously known regarding the structure of the *tdfA* homologues, would readily appreciate that the disclosure provided in the '406 Application establishes that the applicant was in possession of the invention as currently claimed.

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14. I hereby declare that all statements made herein are true and that they are based on my own knowledge, information and belief.

Place and Date: Jan 6, 2020 (Urbana, IL)

By:

Dr. Satish K. Nair
Professor, Dept. of Biochemistry, University of Illinois

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Appendix I. Amended Claimed Subject Matter

Claims

1-14. (Canceled).

15. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

$(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and

$(X)_{271\text{-}280}$ represents a sequence of 10 amino acids.

16. The plant cell of claim 15 wherein said AAD-1 motif has the general formula of:

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R(X)_{282\text{-}284}R$, wherein $(X)_{282\text{-}284}$ represents a sequence of 3 amino acids.

17. (Canceled).

18. The plant cell of claim 15 wherein the AAD-1 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 9.

19. The plant cell of claim 15 wherein said plant cell is dicotyledonous and

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selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an Arabidopsis cell.

20. The plant cell of claim 15 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

21. A transgenic plant comprising a plurality of the plant cell of claim 15, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

22. The plant of claim 21 wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

23. The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

24. The plant of claim 21 wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

25. The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

26. The plant of claim 21 wherein said plant further comprises a second herbicide resistance gene.

27. The plant of claim 26 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

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28. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 21, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide.

29. The method of claim 28 wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

30. The method of claim 28 wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

31. The method of claim 28 wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

32. The method of claim 28 wherein said method comprises applying a second herbicide.

33. The method of claim 32 wherein said first herbicide and said second herbicide are applied sequentially.

34. The method of claim 32 wherein said first herbicide and said second herbicide are applied concurrently.

35. The method of claim 32 wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

36. The method of claim 32 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

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37. The method of claim 32, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

38. The method of claim 32, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

39. The method of claim 32 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

40. The method of claim 39 wherein said second gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

41. The method of claim 40 wherein:

(a) said modified ALS gene is selected from the group consisting of a SurA, SurB, Csr1, Csr1-1, Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT; and,

(c) said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

42. The method of claim 32 wherein said method further comprises applying a third herbicide.

43. The method of claim 42, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

44. The method of claim 43 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

45. A seed comprising a plurality of the plant cell of claim 15.

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46. A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim 45 in said field within 14 days of applying said aryloxyalkanoate herbicide.

47. A plant grown from the seed of claim 45.

48. A part, progeny, or asexual propagate of the plant of claim 47, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

49. An (R)-aryloxyphenoxypropionate herbicide tolerant transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids, wherein said motif has 90% sequence identity with corresponding amino acids of position 111 to 281 of SEQ ID NO: 9.

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Appendix II

Alignment of AAD-1v1 amino acid sequence (SEQ ID NO. 9 of the present invention) (having α-KG dioxygenase activity on AOPP (R)-enantiomers and 2,4-D or other phenoxy auxins) with AAD-2 (having α-KG dioxygenase activity on 2,4-D and certain non-herbicidal (S)-enantiomers of AOPP and phenoxypropionate auxin analogs). Defined AAD-1 motif residues are highlighted in blue in the AAD-1v1 sequence, and the additional R285 residue in highlighted in gray. The analogous residues in AAD-2 are also highlighted. The red box identifies a region of the alignment where AAD-2 is 10 amino acids shorter compared to the "X_{131}" amino acid spacer in the AAD-1 motif. Only sequences with the indicated 131 amino acid spacer and the identified motif amino acids have been found to exhibit the due activity on AOPP (R)-enantiomers and 2,4-D.

```
                1                                                                80
AAD-1v1    (1)  MHAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFG-----
  AAD-2    (1)  ------------MTIAIRQLQTHFVGQVSGLDLRKPLTPGEAREVESAMDKYAVLVFHDQDITDEQQMAFALNFGQREDA
               81                                                              160
AAD-1v1   (76)  ---PVDPVPLLKSIEGYPEVQMIRREANESGR--------VIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMY
  AAD-2   (69)  RGGTVTKEKDYRLQSGLNDVSNLGKDGKPLAKDSRTHLFNLGNCLWHSDSSFRPIPAKFSLLSARVVNPTGGNTEFADMR
               161                                                             240
AAD-1v1  (145)  TAWETLSPTMQATIEGLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGRKGLYVNQVYCQR
  AAD-2  (149)  AAYDALDDETKAEIEDLVCEHSLMYSRGSLGFTEYTDEE---------KQMFKPVLQRLVRTHPVHRRKSLYLS-SHAGK
               241                                                          317
AAD-1v1  (225)  IEGMTDAESKPLLQFLYEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR------
  AAD-2  (219)  IASMSVPEGRLLLRDLNEHATQPEFVYVHKWKLHDLVMWDNRQTMHRVRRYDQSQPRDMRRATVAGTEPTVQQQAAE
```

Demonstration how the AAD-1 motif having dual MOA activity on (R)-AOPP herbicides and 2,4-D is derived from the general α-KG dioxygenase motif having known activity on 2,4-D and certain non-herbicides like taurine or (S)-AOPP enantiomers like .

General α-KG dioxygenase motif:	H	X	(D/E)	X_{23-26}	(T /S)	$X_{114-183}$	H X_{10-13}	R (from '406 application [0085])
Active on R-AOPP + 2,4-D herbicides								
AAD-1v1	H	X	D	X_{24}	T	X_{131}	H X_{10}	R (from '406 application SEQ ID No.9)
AAD-1v2	H	X	D	X_{24}	T	X_{131}	H X_{10}	R (from '406 application SEQ ID No.10)
AAD-1v3	H	X	D	X_{24}	T	X_{131}	H X_{10}	R (from '406 application SEQ ID No.11)
Active on 2,4-D and non-herbicidal S-AOPPs and not R-AOPPs								
AAD-2	H	X	D	X_{24}	T	X_{121}	H X_{10}	R (from '406 application SEQ ID No.13)

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Demonstration how the three enabled AAD-1 variant enzymes having dual MOA activity on (R)-AOPP herbicides and 2,4-D conforms to the specific AAD-1 motif: $HXDX_{24}TX_{131}HX_{10}R$. AAD-1v1 is the wildtype enzyme. Changes from wildtype are highlighted in light green. Defined AAD-1 motif residues are highlighted in blue in the AAD-1v1 sequence, and the additional R285 residue in highlighted in gray. The analogous arginine in TfdA had been concluded to be involved in 2,4-D carboxylate binding, was expected to play a similar role in aryloxyalkanoate herbicide binding in AAD-1, and was present in all examples.

```
                1                                                                    80
    AAD-1v1    (1) M-HAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDP
    AAD-1v2    (1) M-AAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDP
    AAD-1v3    (1) MAHAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDP
AAD-1 motif        --------------------------------------------------------------------------------

                81                                                                  160
    AAD-1v1   (80) VPLLKSIEGYPEVQMIRREANESGRVIGDDWHXDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQAIIE
    AAD-1v2   (80) VPLLKSIEGYPEVQMIRREANESGRVIGDDWHXDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQAIIE
    AAD-1v3   (81) VPLLKSIEGYPEVQMIRREANESGRVIGDDWHXDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQAIIE
AAD-1 motif        --------------------------------HXDXXXXXXXXXXXXXXXXXXXXXXXTXXXXXXXXXXXXXXXXXXXXX
                                                   HXD<---------X24----------->T< --------------------

                161                                                                 240
    AAD-1v1  (160) GLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGRKGLYVNQVYCQRIEGMTDAESKPLLQF
    AAD-1v2  (160) GLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGCKGLYVNQVYCQRIEGMTDAESKPLLQF
    AAD-1v3  (161) GLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGRKGLYVNQVYCQRIEGMTDAESKPLLQF
AAD-1 motif        XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
                   -------------------------------------------X131---------------------------------

                241                                                         296
    AAD-1v1  (240) LYEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
    AAD-1v2  (240) LYEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
    AAD-1v3  (241) LYEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
AAD-1 motif        XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXHXXXXXXXXXXRXXXR----------
                   ------------------------------>H<---X10--->R X3R
```


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Appendix III

Alignment of AAD-1v1 (SEQ ID NO. 9 of the present invention) (having dual MOA α-KG dioxygenase activity on AOPP (R)-enantiomers and 2,4-D or other phenoxy auxins) amino acid sequence with new AAD-1 variants demonstrating the importance of the defined residues of the AAD-1 motif to the dual MOA α-KG dioxygenase activity. Defined AAD-1 motif residues are highlighted in blue in the AAD-1v1 sequence, and the additional R285 residue in highlighted in gray. Three variants of AAD-1 were created with ~94-95% amino acid identity to AAD-v1 (Seq01-Seq03) and have the AAD-1 motif intact. Six variants of Seq01 were designed, each individually with a single amino acid change to one of the defined AAD-1 motif residues (highlighted in pink). Protein variants were expressed in *E. coli* using a Histidine tag for rapid isolation. Enzyme activity of the nine variants were assayed *in vitro* and compared to the activity of AAD-1v1 under the same conditions described in 'Wright'. Additional changes from the original AAD-1v1 sequence (other than the defined motif residues) are highlighted in light green.

Seq01-Seq03 are 94-95% identical to AAD-1v1 and have the AAD-1 motif intact.

Seq04 to Seq09 begin with Seq01 sequence and individually change one amino acid of the defined AAD-1 motif as follows:

Seq04 $H_{111}F$
Seq05 $D_{113}K$
Seq06 $T_{139}A$
Seq07 $H_{270}F$
Seq08 $R_{281}A$
Seq09 $R_{285}A$

```
                1                                                                    80
AAD-1v1    (1) MHAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq01      (1) MAGALTPLTNKYDKIGVQPLSGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq02      (1) MAGALTPLTNKYERIAVQPLSGVLGADITGVDIREPLDDSTWNELLDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq03      (1) MAGALTPLTNKYDKIGVQPLSGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq04      (1) MAGALTPLTNKYDKIGVQPLSGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq05      (1) MAGALTPLTNKYDKIGVQPLSGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq06      (1) MAGALTPLTNKYDKIGVQPLSGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq07      (1) MAGALTPLTNKYDKIGVQPLSGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq08      (1) MAGALTPLTNKYDKIGVQPLSGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
Seq09      (1) MAGALTPLTNKYDKIGVQPLSGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFGPVDPV
AAD-1 motif    ----------------------------------------------------------------------------------
```


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Appendix III-continued

```
                   81                                                                              160
     AAD-1v1  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
       Seq01  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
       Seq02  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
       Seq03  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
       Seq04  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWATDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
       Seq05  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTASTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
       Seq06  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDAGFLSMYTAWETLSPTMQATIEG
       Seq07  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
       Seq08  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
       Seq09  (81) PLLKSIEGYPEVQMIRREANESGRVIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQATIEG
 AAD-1 motif       ------------------------------HXDXXXXXXXXXXXXXXXXXXXXXXXTXXXXXXXXXXXXXXXXXXXXXXX
                                                 HXD<---------X24----------->T< --------------------
                   161                                                                             240
     AAD-1v1 (161) LNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGSGRKGLYVNQVYCQRIEGMTDAESKPLLQFL
       Seq01 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
       Seq02 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
       Seq03 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
       Seq04 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
       Seq05 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
       Seq06 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
       Seq07 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
       Seq08 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
       Seq09 (161) LNVVHSATKVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVTHPGTGRKALYVNQVYCQRIEGMTDAESKPILQFL
 AAD-1 motif       XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
                   ------------------------------------------X131---------------------------------
                   241                                                   295
     AAD-1v1 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
       Seq01 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
       Seq02 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
       Seq03 (241) YEHATKFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVAGVKPAR
       Seq04 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
       Seq05 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
       Seq06 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR
       Seq07 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMARAVPDYAGKFRYLTRTTVGGVRPAR
       Seq08 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFAYLTRTTVGGVRPAR
       Seq09 (241) YEHATRFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTATTVGGVRPAR
 AAD-1 motif       XXXXXXXXXXXXXXXXXXXXXXXXXXXXXHXXXXXXXXXXRXXXR----------
                   ---------------------------->H<---X10--->RX3 R
```


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Appendix III-continued

Comparison of the relative activity of AAD-1v1 and nine variants (3 with the narrow AAD-1 motif intact, 6 with individual changes to the defined residues of the motif). Seq01 and Seq03* showed on average similar activity to 2,4-D and (R)-quizalofop [an (R)-AOPP herbicide] substrates as AAD-1 (RdpA). When defined amino acids of the motif were mutated, 5 of 6 residues resulted in 90-100% reduced activity. One residue $T_{138}A$ still retained >30 % activity but lost 60-70% of activity against 2,4-D and (R)-quizalofop). This shows both the importance and necessity of these residues in the AAD-1 motif for dual MOA herbicide degradation activity.

AAD-1 Variants Verifying Motif Validity

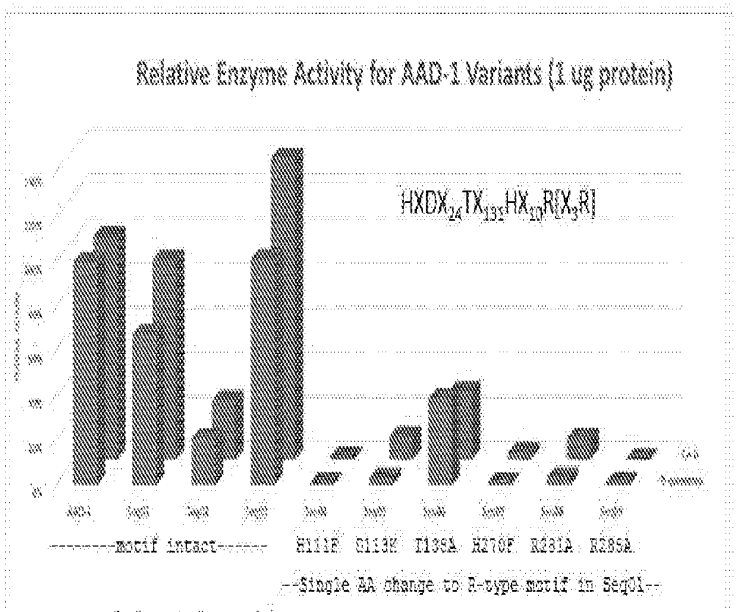
$HX(D/E)X_{(3-28)}(T/S)X_{(114-183)}HX_{(10-13)}R$ [PCT 0085] = Group II a-KG (include TauD, TfdA, RdpA, et al)

AAD-1 proteins have novel property of being able to degrade 2,4-D, other phenoxy auxin herbicides, and (R)-AOPP herbicides [0086-0090]. This narrows to specific spacing of key residues $HXDX_{24}TX_{131}HX_{10}R[X_3R]$

Seq01 - Seq03 = 94-95% Identity (15-18 aa changes) to AAD-1 (SEQ ID NO. 3, motif intact)

Remaining Seq's single aa change to motif residues

H111F Seq04
D113K Seq05
T138A Seq06
H270F Seq07
R281A Seq08
R285A Seq09



*Recovery of Seq02 and Seq05 was low due to a large proportion of expressed protein insolubility. Quantity of enzyme in these samples is likely an underestimate (lower than targeted 1 ug protein).

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EXHIBIT XX

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Interim Head & I.C. Gunsalus Chair, Department of Biochemistry
Director, Center for Biophysics & Computational Biology
Member, Institute for Genomic Biology
Phone: 217-333-0641 Email: snair@illinois.edu

Education

1989	Sc.B.	Brown University	Chemistry (with Honors)
1994	Ph.D.	University of Pennsylvania	Chemistry (PI: David W. Christianson)
1999	Postdoctoral	Rockefeller University	Biophysics (PI: Stephen K. Burley)
1999-2001	Research Associate (w. S.K. Burley)		Rockefeller University New York, NY

Positions

2001-2007	Assistant Professor	Department of Biochemistry, Biophysics & Computational Biology University of Illinois
2007-2012	Associate Professor	Department of Biochemistry, Biophysics & Computational Biology
2012-2017	Professor	Department of Biochemistry Biophysics & Computational Biology
2016-current	Director	Biophysics & Computational Biology
2017-current	I.C. Gunsalus Chair	Department of Biochemistry
2019-current	Interim Head	Department of Biochemistry

Other Professional Employment

2008-current	Affiliate Member	Department of Chemistry, UIUC
2012-current	Member	Institute for Genomic Biology, UIUC
2019-current	Member	Cancer Center at Illinois

Extramural funding (current)

Current

NIH RO1 AI117210 2016-2021 $2,489,205
PI: Satish K. Nair, Ph.D.
Exploring Peptide Conjugates as Trojan Horse Systems for Drug Design and Discovery.

NIH RO1 GM131347 2019-2023 $1,207,592
PI: Satish K. Nair, Ph.D.
Structural Biological Studies of Thiopeptide Biosynthesis and Engineering.

Sfunga Therapeutics 2019-2021 $410,354 (Nair project costs)
PI: Martin D. Burke, P.I. (role: Project P.I.)
Structural Studies of Polyene Glycosyltransferases
Project PI- Satish K. Nair, Ph.D.

Pending

NIH S10 OD028700 (Impact Score of 14) $1,400,000
PI: Satish K. Nair, Ph.D.
Cryo-TEM and Micro Electron Diffraction Instrument

NIH RO1 GM079038 2020-2024
PI: Satish K. Nair, Ph.D.
Mechanistic Studies of Lantibiotic Biosynthetic and Tailoring Enzymes. (Competitive renewal).

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Honors and Service:

1996-1999	Leukemia Society of America Fellowship
2007	NSF, Genes and Genome Systems Cluster, Ad hoc
2008	NSF, Genes and Genome Systems Cluster, Ad hoc
2008	NIH, Macromolecular Structure and Function B Study Section, Ad hoc
2009	NIH, Special Panel ZAT SM1, Ad hoc
2009	NIH, Special Panel ZRG1 AARR-C 22, Ad hoc
2009	U.S. Army, American Institute for Biological Sciences, Ad hoc
2010	NIH, Special Panel ZRG1 AARR-H (HIV/AIDS), Ad hoc
2010	NSF, Catalysis and Biocatalysis Cluster Ad hoc
2010	MRC, U.K., Programme Grant referee
2010	SIMB, Session chair; Enzyme Mechanism
2011	NIH, Macromolecular Structure and Function B Study Section, Ad hoc
2011	SIMB, Session chair; Enzyme Mechanism
2012	NIH, Special Panel ZRG1 AARR-H (Fogerty Centers HIV/AIDS), Ad hoc
2012	SIMB, Session chair; Enzyme Mechanism
2013	NIH, NIAID Special Emphasis (Infectious Diseases), Ad hoc
2014	NIH, Macromolecular Structure and Function A Study Section, Mail-in
2016	NIH, DP5 Pathways to Independence, Mail-in
2017	NIH, Macromolecular Structure and Function A Study Section, Ad hoc
2018	NIH, Prokaryotic Cell and Molecular Biology Study Section, Ad hoc
2018	NSF, CLP Enzyme Chemistry Panel
2018	NIH, P20 Special Emphasis Panel, Center for Biomedical Research Excellence
2020	Gordon Research Conference, Vice Chair (Enzymes, Coenzymes & Pathways)
2021	Gordon Research Conference, Chair (Enzymes, Coenzymes & Pathways)
2019-2023	NIH, Macromolecular Structure and Function A Study Section (Standing Member)

Patents

Publication number: WO201130509-A1. Zhao, H., Sulliva, R.P., Bae, D., and Nair, S.K. (Oct 2011) Mutants of L-arbainitol 4-dehydrogenase from *Neurospora crassa*.

Publication number: US8357518-B2. Han, Y., Dodd, D., Nair, S.K., Mackiem, R. and Cann, I.K. (Jan. 2013) Thermostable enzymes for the hydrolysis of mannan-containing polysaccharides.

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Publications (chronological)

[Graduate Research]

1. Alexander, R.S., Nair, S.K., and Christianson, D.W. (1991) "Engineering the Hydrophobic Pocket of Carbonic Anhydrase II" **Biochemistry** *30*, 11068-11072.

2. Nair, S.K., Calderone, T.L., Christianson, D.W., and Fierke, C.A. (1991) "Altering the Mouth of a Hydrophobic Pocket: Structure and Kinetics of Human Carbonic Anhydrase II Mutants at Residue Val-121" **J. Biol. Chem.** *266*, 17320-17325.

3. Nair, S.K., and Christianson, D.W. (1991) "Structural Properties of Human Carbonic Anhydrase II at pH 9.5" **Biochem. Biophys. Res. Commun.** *181*, 9455-9458.

4. Nair, S.K., and Christianson, D.W. (1991) "Unexpected pH-Dependent Conformation of His-64, the Proton Shuttle of Carbonic Anhydrase II" **J. Am.Chem. Soc.** *113*, 9455-9458.

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5. Tweedy, N.B., Nair, S.K., Paterno, S.A., Fierke, C.A., and Christianson, D.W. (1993) "Structure and Energetics of a Non-Proline cis-Peptidyl Linkage in a Pro-202→Ala Carbonic Anhydrase II variant" **Biochemistry** *32*, 10944-10949.

6. Nair, S.K., and Christianson, D.W. (1993) "Crystallographic Studies of Azide Binding to Human Carbonic Anhydrase II" **Eur. J. Biochem.** *213*, 507-515.

7. Nair, S.K., and Christianson, D.W. (1993) "Structural Consequences of a Hydrophilic Amino Acid Substitution in the Hydrophobic Pocket of Human Carbonic Anhydrase II" **Biochemistry** *32*, 4506-4514.

8. Nair, S.K., Ludwig, P.A., and Christianson, D.W. (1994) "Two-Site Binding of Phenol in the Active Site of Human Carbonic Anhydrase II: Structural Implications for Substrate Association" **J. Am. Chem. Soc.** *116*, 3659-3660.

9. Ippolito, J.A., Nair, S.K., Alexander, R.S., Kiefer, L.L., Fierke, C.A., and Christianson, D.W. (1995) "Structure of His94→Asp carbonic anhydrase II in a new crystalline form reveals a partially occupied zinc binding site" **Protein Eng.** *8*, 975-980.

10. Nair, S.K., Krebs, J.F., Christianson, D.W., and Fierke, C.A. (1995) "Structural Basis of Inhibitor Affinity to Variants of Human Carbonic Anhydrase II" **Biochemistry** *34*, 3981-3989.

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107. Ongpipattanakul, C., and Nair, S.K. (2018) "Molecular Basis for Autocatalytic Backbone N-Methylation in RiPP Natural Product Biosynthesis." **ACS Chem. Bio.** *13*, 2989-99.

108. Estrada, P., Morita, M., Hao, Y., Schmidt, E.W.*, and Nair, S.K.* (2018) "A Single Amino Acid Switch Alters the Isoprene Donor Specificity in RiPP Prenyltransferases." **J. Am. Chem. Soc.** *140*, 8124-27. (*Corresponding author)

109. Bobeica, S.C., Dong, S., Huo, L., Mazo, N., McLaughlin, M.H., Jimenez-Oses, G., Nair, S.K.*, and van der Donk, W.A.* (2019) "Insights into AMS/PCAT transporters from biochemical and structural characterization of a double Glycine motif protease" **eLife** doi: 10.755/eLife.42305. (*Corresponding author)

110. Nguyen, K., DeSieno, M.A., Bae, B., Johanes, T.W., Cobb, R.E., Zhao, H.* and Nair, S.K.* (2019) "Characterization of the flavin monooxygenase involved in the biosynthesis of the antimalarial FR-9000098" **Org. Biomol. Chem.** *17*, 1506-18. (*Corresponding author)

111. Petronikolou, N., Ortega, M., Borisova, S., Nair, S.K.* and Metcalf, W.W.* (2019) "Molecular basis of Bacillus subtitles ATCC6623 self-resistance to the phosphono-oligopeptide antibiotic rhizocticin" **ACS Chem. Bio.** *14*, 742-50. (*Corresponding author)

112. Dong, S., Kulikovsky, A., Zukher, I., Estrada, P., Dubiley, S., Severinov, K.* and Nair, S.K.* (2019) "Biosynthesis of the RiPP Trojan horse nucleotide antibiotic microcin C is directed by the N-formyl of the peptide precursor" **Chem. Sci.** *10*, 2391-95. (*Corresponding author)

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113. Dong, S., Liu, A., Mahanta, N, Mitchell, D.A.,* and Nair, S.K.* (2019) "Mechanistic basis for ribosomal peptide backbone modification." **ACS. Central Sci.** *5*, 842-51. (*Corresponding author)

114. Chekan, J.R., Ongpipattanakul, C., Wright, T.R., Zhang, B., Bollinger, J.M., Rajakovich, L.J., Krebs, C., Cicchillo, R.M., and Nair, S.K. (2019) "Molecular Basis for Enentioselective Herbicide Degradation Imparted by Aryloxyalanoate Dioxygenases (AADs) in Transgenic Plants." **Proc. Nat'l Acad. Sci USA.** *116*, 13299-13304.

115. Bothwell, I.R., Cogan, D.P., Kim, T., Reinhardt, C.J., van der Donk, W.A.,* and Nair, S.K.* (2019) "Characterization of glutamyl-tRNA-dependent dehydratases using nonreactive substrate mimics." **Proc. Nat'l Acad. Sci USA.** *116*, 17245-17250. (*Corresponding author)

116. Chekan, J.R., Ongpipattanakul, C., and Nair, S.K. (2019) "Steric complementarity directs sequence promiscuous leader binding in RiPP biosynthesis." **Proc. Nat'l Acad. Sci USA.** *116*, 24049-55.

Former Graduate Students

John Paul Yu 2001-2006 M.D./Ph.D. completed Feb 2006
"Structure and function of Lantibiotic Biosynthesis and Immunity"
(Assistant Professor: Department of Radiology, University of Wisconsin, USA)

Michael Lasker 2001-2006 M.D./Ph.D. completed April 2006
"Structural and Biochemical Studies of the Innate Immune Pathway"
(Surgeon: University of California at Davis Medical Center)

Yaozhong Zou 2003-2008 Ph.D. completed June 2008
"Structural Studies of Enzymes Involved in Drug Resistance and Antibiotic Biosynthesis"
(Postdoc with Brian Kobilka, HHMI/Stanford; Permanent address: Pfizer Pharmaceuticals)

Brian Bae 2005-2009 Ph.D. completed Dec. 2009
"Structural Studies of Archaeal DNA Replication."
(Postdoc with Seth Darst, Rockefeller University)

Zhi Li 2008-2012 Ph.D. completed Dec. 2012
"Structural Studies on the Biosynthesis and Biological Functions of Natural Products."
(Postdoc with Hening Lin, HHMI/Cornell; Permanent address: Bristol-Myers Squibb)

Vinayak Agarwal 2007-2012 Ph.D. completed April 2012
"Structural studies of enzymes involved in antibiotic resistance and phosphonate degradation"
(Helen Hay-Whitney postdoc with Brad Moore, UCSD; Asst. Prof: Georgia Tech 2017-on)

Neha Garg 2008-2013 Ph.D. completed April 2013
"Exploring and Understanding Lantibiotic Biosynthesis"
(Postdoc with Pieter Dorrestein, UCSD; Asst. Prof: Georgia Tech 2017-on)

Yue Hao 2010-2015 Ph.D. completed Nov 2015
"Studies of Tailoring Enzymes Involved in Ribosomal Peptide Synthesis"
(Staff Scientist: Amgen Pharmaceuticals, Cambridge MA)

Jonathan Chekan 2012-2016 Ph.D. completed Mar 2016
"Biochemical and structural studies on proteins involved in the production of natural products and degradation of polysaccharides"
(Life Sciences Research Foundation postdoc with Brad Moore, UCSD, Scripps)

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David Park 2012-2016 Ph.D. completed Dec 2015
"Structural and Biochemical Studies of Enzymes Involved in NP Biosynthesis and Cellular Respiration"
(Visiting Instructor of Biology, Ferrum College, VA).

Nektaria Petronikolou 2013-2018 Ph.D. completed April 2018
"Biochemical Studies of Enzymes Involved in the Biosynthesis of Value-Added Products"
(Postdoc with Danica Fujimori, UCSF).

Iti Kapoor 2013-2018 Ph.D. completed Dec 2018
"Biochemical and Synthetic Chemical Approaches to Studying the Biosynthesis and Regulation of Bacterial Secondary Metabolites"
(Postdoc with Ted Jardetzky, Stanford University).

Dillon P. Cogan 2013-2019 Ph.D. completed Dec 2018
"Mechanistic Interrogations of Ribosomal and Non-ribosomal Natural Product Enzymes"
(Postdoc with Chaitan Khosla, Stanford University).

Former Postdoctoral Fellows

Houjin Zhang, PhD	2006-2009	Professor, Huazhong Univ. of Sci and Tech, PRC
Jui-Hui Chen, PhD	2009-2012	Scientist, Academia Sinica, PRC
Tom Graham, MD/PhD	2008-2010	Dermatologist, Carle Hospital, USA
Tiit Lukk, PhD	2010-2013	Asst. Prof., Tallinn Univ. of Technology, Estonia
Jaigeeth Devershetty, PhD	2012-2014	Researcher, NASA Institute for Astrobiology, USA
Shangwen Lou, Ph.D.	2016-2019	Research Assistant, Lanzhou University
Shi-Hui Dong, PhD	2014-2019	Associate Professor, Lanzhou University

Current students

Paola Estrada	2014-2019	Herbert R. Carter Fellow (Defense date: Dec 2019).
Chayanid Ongpipattanakul	2016-present	
Andrea Garcia-Hernandez	2017-present	Westcott Fellow
Josephine Watson	2019-present	

Current Postdoctoral Fellow

Yiwu Zheng	2019-present
Zing-fei Pei, Ph.D.	2019-present
Daniel Catlin	2019-present

Summary of Service

Thesis Committees:

Department of Biochemistry	127
Department of Chemistry	57
Center for Biophysics and Quant. Biology	42
Department of Cell & Dev. Biology	3
Department of Microbiology	6
Department of Animal Sciences	2
Department of Physics	3
Department of Chemical Engineering	3
Tutorial mentor in Biophysics:	32

Department and Program:

Biophysics Admissions Committee	2007-2011
Biophysics Executive Committee (Elected to service)	2009-2013

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MCB Courses and Curriculum Committee	2008-2014
Biochemistry Admissions Committee (Chair)	2010-2015
Biochemistry Awards Committee	2008-2015
MCB Executive Committee (Elected to service)	2010-2014
Biophysics Qualifying Exam Committee	2011-2013
Biochemistry Strategic Planning Committee	2012-2014
Director of Graduate Studies, Biochemistry	2017-2019
Biochemistry Rutter Chair Search Committee	2014-present
MCB Executive Committee	2017-present
Director of Biophysics	2016-present
Interim Head of Biochemistry	2019-present
Biophysics Admissions Committee (Chair)	2015-present
Biochemistry Admissions Committee (Chair	2018-present

Campus:

LS-CAT Executive Committee Board	2007-present
College of LAS Committee on Academic Standards	2015-2018
College of LAS Awards Committee	2017-2019

Campus Outreach and Diversity

Campus level mentor for the Alfred P. Sloan Center of Exemplary Mentoring (Biophysics)
Campus level guide for UIUC/ASPIRE program (Biophysics).
Student mentor for future Medical Research Investigators (COM-UIUC).
Summer Research Opportunities Program- Faculty Mentor to V. Quarles (Dillard University) 2008.
Summer REU- Faculty mentor to L. Badmus (University of Alabama) in 2013.
Current thesis advisor for three URM students: A. Hernandez-Garcia, J. Watson and P. Estrada.

Training Grants

Chemical Biology Training Grant Executive Committee (2007-present).

Teaching activities

Spring 2019
Biochemistry 455: Current Topics in Biochemistry
Biophysics 595A: Biophysics Seminars
Spring 2018
Biochemistry 455: Current Topics in Biochemistry
Biophysics 595A: Biophysics Seminars
Spring 2017
Biochemistry 455: Current Topics in Biochemistry
Biophysics 595A: Biophysics Seminars
Spring 2016
Biochemistry 455: Current Topics in Biochemistry
Biophysics 595A: Biophysics Seminars
Spring 2015
Biochemistry 354: Biochemical and Physical Basis for Life
Fall 2014
Biochemistry 354: Biochemical and Physical Basis for Life
Spring 2014
Biochemistry 354: Biochemical and Physical Basis for Life
Spring 2013
Biochemistry 354: Biochemical and Physical Basis for Life
Fall 2012
Biochemistry 354: Biochemical and Physical Basis for Life

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Spring 2012
Biochemistry 354: Biochemical and Physical Basis for Life
Spring 2007, 2008, 2009, 2010, 2011
Biochemistry 440 Introduction to Physical Chemistry I
Fall 2007, 2008, 2009, 2010, 2011, 2012
Biochemistry 446 Introduction to Physical Chemistry II
Spring 2003, Spring 2004, Spring 2005, Spring 2006:
Biophysics 420 "Introduction to Molecular Biophysics"

Undergraduate research. Served as research advisor for 78 total undergraduate students starting from 2005, of whom the majority have written senior theses and graduated with Honors (or better).

COR-AAD1-00000622
Bayer EX1008 (Part 1 of 9)

Electronic Patent Application Fee Transmittal

Application Number:	15288406
Filing Date:	07-Oct-2016
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Filer:	John P. Breen/Garla Twyman
Attorney Docket Number:	14764-258662

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
RCE- 1ST REQUEST	1801	1	1300	1300
		Total in USD ($)		**2700**

Electronic Acknowledgement Receipt	
EFS ID:	38261572
Application Number:	15288406
International Application Number:	
Confirmation Number:	5402
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Customer Number:	107540
Filer:	John P. Breen/Garla Twyman
Filer Authorized By:	John P. Breen
Attorney Docket Number:	14764-258662
Receipt Date:	10-JAN-2020
Filing Date:	07-OCT-2016
Time Stamp:	16:21:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$2700
RAM confirmation Number	E202010G22118196
Deposit Account	100435
Authorized User	Garla Twyman
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	RCE.pdf	697661 7d702861783c616a772b01217d5ae49e42c5cb11	no	3
Warnings:					
Information:					
2	Transmittal Letter	Supplemental_IDS.pdf	36844 17cf6af78e538bf1a31ab37ad5e40c1202bac562	no	3
Warnings:					
Information:					
3	Non Patent Literature	Schleinitzl.pdf	922592 a7bd109ea4bdf3dc085dcbbd51587b4eaff23c93	no	10
Warnings:					
Information:					
4	Non Patent Literature	Mueller.pdf	478391 fa8f05e1b77142b67597cdd54ed11842fe6c0e5f	no	13
Warnings:					
Information:					
5	Non Patent Literature	Hotopp.pdf	115410 0fb51aac40cdebfa64c161b26258e4318320c6a21	no	8
Warnings:					
Information:					
6		Amendment_and_Response_Accompanying_RCE.pdf	226690 e6366e2dca4c64bfdae30f396d9e3a06194c8d35	yes	33
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	

	Applicant Arguments/Remarks Made in an Amendment		8	33	
	Claims		2	7	
	Amendment Submitted/Entered with Filing of CPA/RCE		1	1	
Warnings:					
Information:					
7	Affidavit-traversing rejectns or objectns rule 132	Declaration_under_37_C_F_R_1_132-NAIR.pdf	815488 e093990a0b9f8e0da8377f18db76a67a4a8f3f18	no	33
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	32423 57dc8502f19523aed7ee891df213e239b2d6e0a2	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	3325499		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/288,406	Filing Date 10/07/2016	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	01/10/2020	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 34	Minus	** 34	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $460 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	SLIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/HELENA L PAYTON/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

107540 7590 03/10/2020
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

EXAMINER
KRUSE, DAVID H

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
03/10/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

COR-AAD1-00000629
Bayer EX1008 (Part 1 of 9)

Office Action Summary	Application No. 15/288,406	Applicant(s) WRIGHT et al.	
	Examiner DAVID H KRUSE	Art Unit 1663	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 10 January 2020.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 15-16 and 18-49 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-16 and 18-49 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

COR-AAD1-00000630
Bayer EX1008 (Part 1 of 9)

STATUS OF THE APPLICATION

Continued Examination Under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 10 January 2020 has been entered.

2. The rejection under 35 U.S.C. 102(a) and (e) over Elliss *et al* and 102(b) over Wright *et al* are withdrawn in view of Applicant's arguments.

Double Patenting

3. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper time-wise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based e-Terminal Disclaimer may be filled out completely online using web-screens. An e-Terminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about e-Terminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

4. Claims 15, 16 and 18-48 remain and claim 49 is rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-43 of U.S. Patent No. 7,838,733. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '733 Patent represents a

subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 13, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

5. Claims 15, 16 and 18-48 remain and claim 49 is rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 9,127,289. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '289 Patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 13, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

6. Claims 15, 16 and 18-48 remain and claim 49 is rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 10,174,337 (which issued from US Patent Application 14/820,893). Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '337 patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

This rejection has been modified because the Application has issued as a U.S. Patent. Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 13, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

Claim Rejections - 35 USC § 112

7. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

8. Claims 15, 16 and 18-48 remain and claim 49 is rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicant claims a transgenic plant cell, plant and method of using wherein the plant cell comprises a recombinant polynucleotides encoding an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity having at least 85% sequence identity

with instant SEQ ID NO: 9 and an AAD-1 motif of HX112D(X)114-137T(X)139-269H(X)271-280R. New claim 49 claims wherein said motif has 95% sequence identity with corresponding amino acid positions 11-281 of SEQ ID NO: 9.

Applicant describes the amino acid sequence of the AAD-1 (syn. RdpA) enzyme in instant SEQ ID NO: 9 and two variants thereof in instant SEQ ID Nos. 10 and 11 which are 98.8% and 99.7% identical respectively to said SEQ ID NO: 9.

Applicant does not describe the claimed genus of AAD-1 proteins or the AAD-1 motif (in the specification). The specification describes a motif comprising three conserved histidine residues found in α-ketoglutarate-dependent dioxygenases on page 17, paragraph 0085, $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$.

Hence, it is unclear that Applicant was in possession of the invention as broadly claimed.

Applicant argues that one skilled in the art would recognize the much more limited group having the desired functional dual-MOA activity would be defined by the more refined structure of [the] AAD-1 motif. Applicant argues that the comparison of the AAD-1 enzymes and AAD-2 reveal while the two proteins retain all five active site residues, there is a difference in the 131 amino acid ($(X)_{139-269}$) interval in the AAD-1 motif $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ vs the spacing in AAD-2 which is a 2,4-D-degrading Fe/α-KG dioxygenase that cannot also catalyze (R)-AOPP herbicides. Applicant argues that those skilled in the art would have compared and recognized the importance of the sequence differences between AAD-1 variants [(R)-specific dioxygenase] having dual herbicide MOA Fe/α-KG dioxygenase activity and (S)-specific

dioxygenases not having dual herbicide MOA Fe/α-KG dioxygenase activity..." (page 16, 2nd paragraph of the Remarks).A

Applicant argues that support for a claimed genus is not limited to information contained within the specification but also includes knowledge of protein structure, such as consensus sequences, that are well-known to those of skill in the art (page 16, 3rd paragraph of the Remarks). Applicant argues that the AAD-1 proteins of the present invention bind to the same phenoxy auxin herbicide substrates as the tfdA protein and presumably retain similar structural domains as the homologous tfdA proteins (page 17, 1st paragraph of the Remarks).

Applicant argues that the skilled practitioner would know not to make modifications to amino acids that are conserved, and thus applicant's required functional limitations effectively add these structural elements to the claimed genus of proteins. Applicant argues that this information, in combination with applicant's description of the AAD-1 motif (defining the key structural elements of the enzyme active site), provides a detailed description of the structural elements of the claimed genus members (page 18, 1st paragraph of the Remarks). Applicant argues that one skilled in the art appreciates that regions of conservation between homologous proteins suggests a structure/function role for such sequences (page 18, 3rd paragraph of the Remarks).

Applicant argues that the motif disclosed in paragraph [0085] of the specification highlights important amino acids comprising the active site of a-ketoglutarate-dependent dioxygenases. Applicant argues that one of ordinary skill in the art would know that the motif provided in paragraph [0085] provides a roadmap to identify the corresponding positions in the AAD-1 proteins of SEQ ID NOs: 9, 10 and 11 thus identifying the

specific "AAD-1 motif" of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$. Applicant argues that this motif provides structural elements of the active site of the unique AAD-1 proteins of the present invention that enzymatically degrade phenoxy auxin herbicides and (R)-aryloxyphenoxypropionate herbicides (page 20, 2nd paragraph of the Remarks).

Applicant argues that the difference of the 131 amino acid spacing $(X)_{139-269}$ in the AAD-1 motif) becomes evident with alignment of R vs S-type αKG-dioxygenases that have activity on 2,4-D; as the R-AOPP activity has only been seen where the 131 amino acid spacing exists (page 21, 2nd paragraph of the Remarks). Applicant argues that the fact pattern of Example 11B (claim 2) of the 2008 written description guidelines is closely aligned with the claimed subject matter of the present invention (page 22, 3rd paragraph of the Remarks).

Applicant's arguments have been considered but are not found to be persuasive. Applicant never specifically identifies the 131 amino acid spacing $(X)_{139-269}$ in the AAD-1 motif, or what structural feature therein defines the R-AOPP activity. While the prior art recognized a structure function relationship for the α-ketoglutarate-dependent dioxygenases motif $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$, the prior art does not describe a structure function relationship for specific enzymatic activity without actual sequence information. The α-ketoglutarate-dependent dioxygenases define a large family of enzymes with diverse enzymatic activities. Further, applicant does not claim conservative substitutions as exemplified in the 2008 WD guidelines. The decision in *AbbVie Deutschland GmbH & Co. v. Janssen Biotech, Inc.* 111 USPQ2d 1780 (Fed. Cir. 2014) seems to be germane to the instant case. In *Abbvie*, an analogy is drawn between a claimed genus and a plot of land (see pages 1789-1791). Using this analogy,

the Court offered that "[I]f the disclosed species only abide in a corner of the genus, one has not described the genus sufficiently to show that the inventor invented, or had possession of, the genus. He only described a portion of it." Similar to the analogy drawn in *Abbvie*, in the case of *Ariad Pharmaceuticals, Inc. v. Eli Lilly & Co.,* 94 USPQ2d 1161 (Fed. Cir. 2010), the court (at page 1171) offered: [M]erely drawing a fence around the outer limits of purposed genus is not an adequate substitute for describing a variety of materials constituting the genus and showing that one has invented a genus and not just a species. Applicant has only described a very limited "corner" of the claimed genus.

Claim Rejections - 35 USC § 103

9. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

10. Claims 15, 16 18-30, 32-34, 36, 37, 39-45, 47 and 48 remain and claim 49 is rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jepson *et al* (U.S. Patent Application US 2003/0041357 filed on 23 February 2001) in view of Kohler (Genbank Accession No: Q8KSC8 first published on 1 October 2002).

Jepson *et al* teach a method of controlling at least one weed in an area by applying a phenoxy auxin herbicide said area comprising a transgenic plant comprising a recombinant polynucleotide encoding a protein having aryloxyalkanoate dioxygenase activity (see paragraph 0027 on pages 3-4, especially lines 18-24 on page 4). Jepson *et*

al teaches one of skill in the art can use mutagenized or modified variants thereof (page 2, paragraph 0012). Jepson *et al* teach said method using a plant transformed with an additional transgene that provides glyphosate, glufosinate, imidazolinone or sulfonylurea herbicide tolerance (page 4, left column, lines 18-27). Jepson *et al* teach applying the phenoxy auxin herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Jepson *et al* teach said method can be practiced using turf grass or forage grass (page 3, paragraph 0024, last sentence; claim 15). Jepson *et al* teach said method can be practiced using a plant transformed with a transgene that produces tolerance to a pre-emergence herbicide (claim 12).

Jepson *et al* do not teach a transgenic plant comprising a recombinant polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO: 9.

Kohler teaches a polynucleotide that encodes a protein comprising the amino acid sequence of instant SEQ ID NO: 9. Kohler teaches that said protein has R-2,4-dichlorophenoxypropionate dioxygenase activity (i.e. degrades the phenoxy auxin herbicide (R)-dichlorprop).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time of Applicant's invention to modify the teachings of Jepson *et al* to substitute the *tfdA* herbicide tolerance gene for a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase taught by Kohler. It would have been obvious to combine a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase with another gene for tolerance to additional herbicide tolerances as taught by Jepson *et al*. Jepson *et al* teach that one would apply one (claim 16) or two (claim 17) herbicides in the method of controlling

weeds. Jepson *et al* teach that at the time of Applicant's invention it was desirable to produce plants tolerant to a phenoxy auxin herbicide. Given the high level of skill in the instant art at the time of Applicant's invention, one would have had a reasonable expectation of success.

Applicant argues that the Examiner fails to provide any rational for why one of ordinary skill would specifically select a gene encoding a peptide of SEQ ID NO; 9 to substitute for the *tfdA* gene disclosed in Jepson (page 24, 4th paragraph of the Remarks). Applicant's argument is not found persuasive because substitution of equivalents having the same or similar function would have been obvious to one of ordinary skill in the art at the time of Applicant's invention. The Examiner expressed that the reason would be for herbicide tolerance of similar function.

Applicant argues that it cannot be reasonably said that one would have turned to the specific gene disclosed in Kohler with the expectation that it would be expressed in plants, particularly to convey herbicide tolerance to the plants (page 25, 1st paragraph of the Remarks). Applicant's argument is not found persuasive because there is no requirement for an absolute expectation of success, just a reasonable expectation. Jepson *et al* had taught that a bacterial R-2,4-dichlorophenoxypropionate dioxygenase enzymes can be used to produce a transgenic plant resistant to the herbicide 2,4-D.

Applicant argues that not all known α-ketoglutarate-dependent dioxygenases exhibit the ability to degrade phenoxy auxin herbicides when inserted into plant cells (page 25, 3rd paragraph of the Remarks). Applicant argues that there was no teaching provided by the prior art to indicate that the rdpA bacterial gene would provide tolerance to phenoxy auxin herbicides upon introduction into plant cells (page 25, 5th paragraph of

the Remarks). Applicant's arguments are not found persuasive because Applicant is arguing that the claims are not anticipated by the prior art, the instant rejection is directed to an issue of obviousness. Many of the genes used to produce herbicide tolerant transgenic plants at the time of Applicant's invention encode bacterial enzymes, including the *tfdA* taught by Jepson *et al*. Further, given the generally high level of skill in the art at the time of Applicant's invention it would have been obvious to try genes encoding other bacterial genes that degrade herbicides of agricultural importance such as 2,4-D.

Applicant argues that Applicant's experience expressing the AAD-2 enzyme in a plant not providing herbicide tolerance is evidence of unpredictability (page 26 of the Remarks). This argument is not persuasive because it does not provide evidence that it would not have been obvious to try the enzyme taught by Kohler, just that there would not have been an **absolute** expectation of success, just that one of ordinary skill in the art would have had to **try** by making the transgenic plant.

Applicant argues that the subject AAD-1 enzyme was only 1 of over 100 BLAST search results having some degree of identity with tfdA (page 27 of the Remark). This argument is not found persuasive because transforming plant with transgenes encoding 100 potential enzymes would have been routine in the art at the time of Applicant's invention, and further Kohler had taught a function that would be useful for producing a transgenic plant as suggested by Jepson *et al*.

Conclusion

11. No claims are allowed.

12. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/288,406	WRIGHT et al.
	Examiner	Art Unit
	DAVID H KRUSE	1663

CPC - Searched*		
Symbol	Date	Examiner
C12N 9/0069	11/07/2018	/DK/

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventor Name Search	11/07/2018	/DK/
EAST: USPAT, USPGPUB, EPO, JPO, DERWENT	11/07/2018	/DK/
STN: AGRICOLA, BIOSIS, CAPLUS, EMBASE, CABA	11/07/2018	/DK/
Sequence Search of SEQ ID NO: 9	10/30/2018	/DK/
Update EAST	07/05/2019	/DK/
Update EAST	03/04/2020	/DK/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	

COR-AAD1-00000643
Bayer EX1008 (Part 1 of 9)

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	54	aad-1.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/03/04 12:46
L2	16	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/03/04 12:46
L3	859	aad-1	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/03/04 12:46
S1	688	aad-1	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:20
S2	48	aad-1.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:21
S3	14	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:36
S4	15	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/07/05 10:38
S5	1	("10323287").PN.	USPAT; USOCR	OR	OFF	2019/07/05 10:39
S6	1	("10174337").PN.	USPAT; USOCR	OR	OFF	2019/07/05 12:38

3/4/2020 12:47:38 PM
C:\Users\dkruse\Documents\EAST\Workspaces\15288406.wsp

COR-AAD1-00000644
Bayer EX1008 (Part 1 of 9)

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No.:	107540
Group:	1663
Confirmation No.:	5402
Application No.:	15/288,406
Invention:	**NOVEL HERBICIDE RESISTANCE GENES**
Applicant:	Terry Wright et al.
Filed:	October 7, 2016
Attorney Docket:	14764-258662
Examiner	David Kruse

FILED ELECTRONICALLY:

January 10, 2020

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

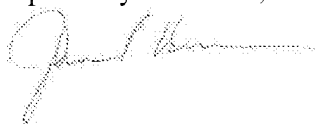
Sir:

This Statement is filed pursuant to 37 C.F.R. § 1.56. No representation is intended that a complete search has been made of the prior art or that no better art references than the references cited in this IDS are available. The filing of this IDS shall not be construed to be an admission that the information cited in the IDS is, or is considered to be, material to patentability as defined in §1.56(b). Pursuant to 37 C.F.R. § 1.98(a)(2)(ii), copies of cited U.S. patents and patent publications are not provided herewith; a copy of each cited foreign and non-patent reference is provided herewith for review by the Examiner.

As this IDS is being filed with an accompanying RCE, applicant believe no fees are required for this filing. However, the Commissioner is hereby authorized to charge any fee necessary to affect this filing to our Deposit Account No. 10-0435, with reference to our Matter No. 14764-258662.

Consideration of the claims, leading to their allowance and passage of the application to issuance, is respectfully requested. If any further discussion of this matter would speed prosecution of this application, the Examiner is invited to call the undersigned at (434) 220-2866.

Respectfully submitted,



John P. Breen
Registration No. 38,833
Attorney for Applicants

JPB:glt
(317) 261-7940
Indianapolis, Indiana 46204

COR-AAD1-00000646
Bayer EX1008 (Part 1 of 9)

SHEET 1 of 1

Form PTO-1449 (Modified)	Atty. Docket No.	Serial No.
INFORMATION DISCLOSURE STATEMENT	14764-258662	15/288,406
	Applicant(s) Terry Wright et al.	
	Filing Date October 7, 2016	Art Unit 1663

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K/

U.S. PATENT DOCUMENTS

Examiner Initials	Document Number	Issue or Publication Date	Name	Class	Subclass	Filing Date (If Appropriate)

FOREIGN PATENT DOCUMENTS

Examiner Initials	Document Number	Publication Date	Country	Translation Yes	Translation No

OTHER DOCUMENTS

	Schleinitz et al., Appl. Env. Microbiol. 70:5357-5365, 2004
	Müller et al, Protein Science (2006), 15:1356–1368
	Hotopp et al, Biochemistry 2002, 41, 9787-9794

EXAMINER: /DAVID H KRUSE/	DATE CONSIDERED: 03/03/2020

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Mar 10, 2020 03:49:09 AM

Dear PAIR Customer:

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Application	Document	Mailroom Date	Attorney Docket No.
15288406	CTNF	03/10/2020	14764-258662
	1449	03/10/2020	14764-258662

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

107540 7590 08/31/2020
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

EXAMINER
KRUSE, DAVID H

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
08/31/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

COR-AAD1-00000649
Bayer EX1008 (Part 1 of 9)

Applicant-Initiated Interview Summary	**Application No.** 15/288,406	**Applicant(s)** WRIGHT et al.	
	Examiner DAVID H KRUSE	**Art Unit** 1663	**AIA (FITF) Status** No

All participants (applicant, applicants representative, PTO personnel):

(1) DAVID H. KRUSE.

(2) John Breen (attorney).

(3) Terry Wright (inventor).

(4) Bill Poirier (company rep).

Date of Interview: 26 August 2020.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☑ Yes ☐ No.
If Yes, brief description: Graph of enzymatic activity comparison of variants of the AAD1 enzyme (see attached).

Issues Discussed ☐101 ☑112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 15.

Identification of prior art discussed: Hotopp 2002.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicant presented the attached exhibit demonstrating that the ADD1 enzyme could be modified down to 82% identical to instant SEQ ID NO: 9 and retain dioxygenase activity in the presence of both 2,4-D and R-quizalofop. The Inventor expressed that the illustrated variants have multiple modifications throughout the polypeptide sequence (but not the specific amino acids in the recited AAD-1 motif) and that the variants maintained the recited activity in instant claim 15 (for example). The Inventor asserted that the critical structural feature of a-ketoglutarate-dependent dioxygenases were well understood at the time of the invention (Hotopp 2002) and thus the Specification had adequately described the claimed genus in instant claim 15..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☑ Attachment

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	

COR-AAD1-00000650
Bayer EX1008 (Part 1 of 9)

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

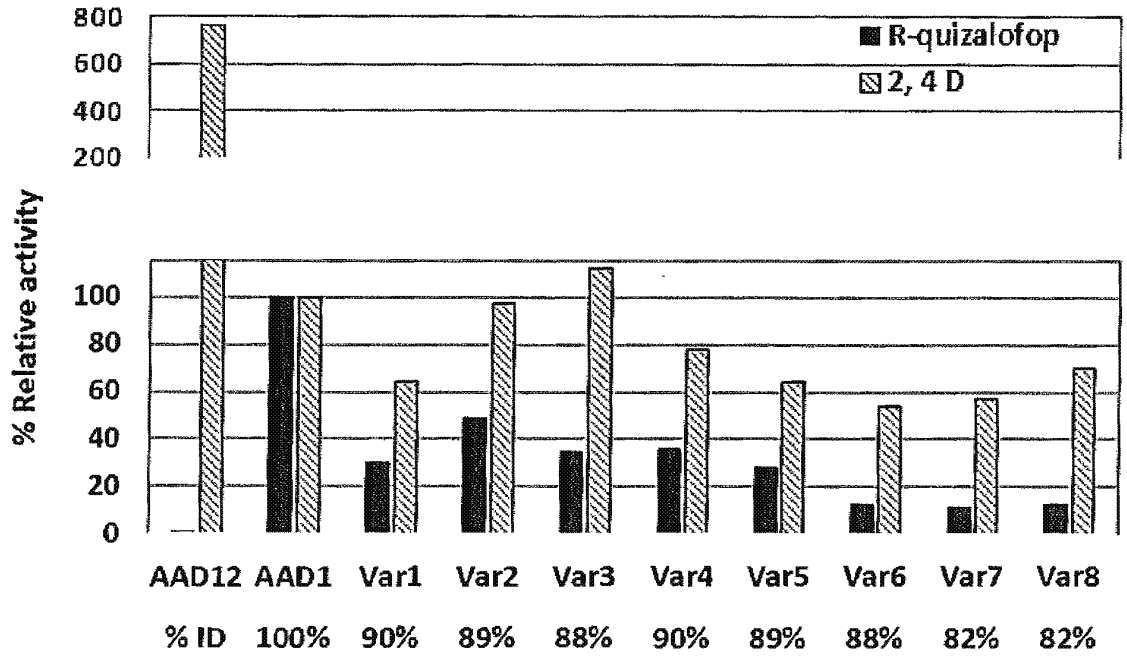
09-25-20;14:59 ;From: To:16712730799 ;4342202866 # 2/ 2

For Discussion Purposes Only

For purposes of discussion during an Examiner Interview on August 26, 2020 at 10 AM

[redacted]

Dual MOA retained for variants with AAD-1 motif



PAGE 2/2 * RCVD AT 8/25/2020 3:44:20 PM [Eastern Daylight Time] * SVR:IW-PTOFAX-001/19 * DNIS:2730799 * CSID:4342202866 * ANI:4342202866 * DURATION (mm-ss):00-46

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Aug 31, 2020 03:41:54 AM

Dear PAIR Customer:

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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Application	Document	Mailroom Date	Attorney Docket No.
15288406	INTV.SUM.APP	08/31/2020	14764-258662
	OA.APPENDIX	08/31/2020	14764-258662

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Thank you for prompt attention to this notice,

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No.:	107540
Group:	1663
Confirmation No.:	5402
Application No.:	15/288,406
Invention:	**Novel Herbicide Resistance Genes**
Inventor:	Terry Wright et al.
Filed:	October 7, 2016
Attorney Docket:	14764-258662
Examiner:	David Kruse

FILED ELECTRONICALLY:

September 9, 2020

AMENDMENT AND RESPONSE UNDER 37 CFR 1.111

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

In response to the non-final Office Action mailed March 10, 2020, Applicant requests consideration of the accompanying remarks and submits herewith a petition for a three-month extension of time. Applicant has submitted payment of the $1,400.00 fee under 37 CFR § 1.17(a) for the requested three-month extension of time, and hereby authorizes the Commissioner to charge any additional fee necessary to effect this filing, to the account of Barnes & Thornburg, Deposit Account No. 10-0435 with reference to our file 14764-258662.

A listing of the pending claims is submitted herewith beginning on page 2.

Remarks begin on page 8 of this paper.

BARNES & THORNBURG LLP

11 South Meridian Street
Indianapolis, Indiana 46204
(317) 236-1313
(317) 231-7433 Fax

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Wright et al.

Serial No.: 15/288,406

Filed: October 7, 2016

For: Novel Herbicide Resistance Genes

Confirmation No.: 5402

Examiner: David H. Kruse

Group Art Unit: 1663

Attorney Docket No.: 14764-258662
(63364A-US-CNT[3])

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. TERRY R. WRIGHT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Terry R. Wright, declare:

1. I am currently a Distinguished Laureate at Corteva Agriscience (formerly Dow AgroSciences, LLC), in Indianapolis, Indiana. I am a citizen of the United States of America. I have 29 years of research experience in the fields of agronomy, pesticide

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science, and plant biotechnology. I received two Bachelor of Science degrees (B.S.) in Crop and Soil Science and Agricultural Biochemistry from Michigan State University, a Master of Science (M.S.) degree in Crop Science/Weed Science from Washington State University, and a Doctorate of Philosophy (Ph.D.) from Michigan State University in Herbicide Physiology and Plant Biotechnology. I am a co-inventor on over 40 granted U.S. patents, and an author of 17 peer-reviewed scientific journal articles. My research has been in the areas of crop protection and trait discovery and development. I am an expert in the field of herbicide physiology and herbicide resistance, weed control, and trait discovery and development.

2. I am an inventor and am very familiar with the contents of the subject application, U.S. Patent Application No. 15/288,406 ("the '406 Application"). I am informed and believe that the claims currently pending in the '406 Application reflect those attached hereto as Appendix I. I have reviewed and understand those claims.

3. I understand that in an Office Action of March 10, 2020 in the '406 Application, the Examiner has rejected claims 15, 16, and 18-48 and new claim 49 of the '406 Application as allegedly failing to comply with the written description requirement (Office Action at point 8). I participated in an interview with the Examiner on Wednesday, August 26, 2020.

4. I previously provided a declaration under 37 C.F.R. §1.132 dated April 10, 2019 in support of patentability of the pending claims, which I will refer to as "Wright-I". I am providing the present declaration in further support of the '406 application. Specifically, this declaration presents confirmatory data that variants of AAD-1 encompassed by the claims that retain the claimed structural motif also retain the claimed dual herbicide mode of action (2,4-D and R-AOPP) enzymatic activity (the "dual MOA"). This dual MOA was even demonstrated with a percent sequence identity *lower* than the 85% minimum identity currently being claimed. I also address the Examiner's question about whether a person of ordinary skill in the art in 2004 would have been able to envision the claimed AAD-1 variants. As I will discuss in further detail below, I believe that a person of skill

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in the art in 2004, based on the specification and the background knowledge of α-ketoglutarate-dependent ("α-KG") dioxygenases in the art, would have envisioned the claimed AAD-1 variants by their common structure (i.e., the active site "motif").

I. The Active-Site Motif and Structure-Function Relationship Disclosed in our Patent Application in 2004

5. Our application discloses the general structural motif of the active site of Group-II α-KG dioxygenases, HX(D/E) X_{23-26}(T/S)$X_{114-183}$HX_{10-13}R (see specification at [0085]), which include TauD and TfdA, *inter alia*. In this general motif representation, the subscripts indicate ranges of numbers of amino acids possible within the general motif.

6. AAD-1 is also a Group-II α-KG dioxygenase, which follows the known, general structural motif. We described the AAD-1 gene and its amino acid sequence, along with two additional variant amino acid sequences having the same dual MOA, in our patent application. The amino acid sequences are designated SEQ ID NOS: 9-11.

7. When aligning the AAD-1 variant sequences in the present application with reference to the general motif, the five highly conserved residues (H, D, T, H, R) of the general active site motif and their specific spacings are readily apparent to skilled artisans, as shown in Wright-I at Appendix II. These residues along with their specific spacings, which are within the ranges of the general active site motif of paragraph [0085], constitute the more specific "AAD-1 motif," which is claimed as "HX_{112}D$(X)_{114-137}$T$(X)_{139-269}$H$(X)_{271-280}$R." Note that, in this representation of the claimed motif, the subscripts indicate amino acid positions in reference to SEQ ID NO: 9, rather than numbers of amino acids.

8. As described in the specification, the general structural motif is the known active site of Group II α-KG dioxygenases, conferring their enzymatic activity (specification at [0085]). That active site, with specific spacing compared to the general motif, is disclosed in SEQ ID NOS: 9-11. These three dual MOA sequences share the same spacing between the conserved residues of the general structural motif, but different from other α-KG dioxygenases without the dual MOA (Wright-I at Appendix II, see also attached Appendix II). A skilled artisan would thus have readily envisioned the α-KG

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dioxygenases encompassed by the claims from their common active site structure (i.e., the AAD-1 motif), which is consistent with the known general structural motif of enzymes in the Group II α-KG dioxygenase family.

9. Moreover, as explained below, in reading the specification, a person skilled in the art would have understood how to make such alignments and confirm the common catalytic structure within the disclosed sequences for AAD-1 and its variants.

II. Background Knowledge in the Art

10. Around the time of our patent application filing in 2004, researchers had already grouped enzymes in the α-KG dioxygenase family according to refined motifs into various subfamilies based on consensus sequences (Table 1, Hogan, J. Biol. Chem. 275:12400-12409 (2000) ("Hogan") (reproduced below)). The concept that different sequence motifs in the α-KG dioxygenase family led to different substrates and their respective activities, was accordingly understood by skilled artisans studying these enzymes.

TABLE 1
Consensus motifs for subgroups within the α-KG-dependent dioxygenase superfamily

Group I (IPNS, DAOCS, and related enzymes)	HXD	X_{90-26}	HX_{10}(R/K)(S/T)S
Group II (TfdA, TauD, clavaminate synthase and related enzymes)	HX(D/E)	$X_{135-207}$	$HX_{10-13}R$
Group III (Phytanoyl-CoA hydroxylase, proline hydroxylase and related enzymes)	HXD	X_{72-101}	HX_{10}(R/K)(S/T)S

11. Skilled artisans were also utilizing homology models with crystal structures of enzymes with similar catalytic activities to provide structural information for proteins without known crystal structures. The Group II subfamily, for example, had been investigated with respect to sequence identity and divergence of the sub-family members. It was known that α-KG-dependent dioxygenases sequences that retain 2,4-D-degrading (TfdA) activity range from about 78-85% amino acid identity to the *Cupriavidus necator* TfdA among beta-and gamma-proteobacteria to about 44-45% amino acid identity among alpha-proteobacteria, while maintaining the intact active site structural motif (see attached Appendix II and Appendix III). This wide divergence indicates that enzymes belonging to this subgroup can exhibit significant structural plasticity (as shown by lower sequence identity) while retaining enzymatic activity.

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12. Consistent with this structure-function observation, Hogan et al. had used site-directed mutagenesis studies to confirm the importance of some key residues identified in the common structural motif of Group II α-KG dioxygenase enzymes (e.g., Hogan Table II). Based on the crystal structure of TauD (a member of the Group II sub-family), Hogan made a number of mutations in the related TfdA sequence to confirm the function of key Fe-coordinating residues (H_{113}, D_{115}, H_{262}). Hogan also showed through sequence alignment the commonality of the structural motif among members of the superfamily (Hogan at Fig. 8).

13. Using similar methods as Hogan, Hotopp et al. further used the TfdA structural model to confirm the role of other residues in TfdA's function (Biochem. 41:9787-9794, (2002) ("Hotopp 2002")). Hotopp identified two additional residues (T_{141} and R_{274}) responsible for binding the co-substrate (α -ketoglutarate) for each member of this class of enzyme. Thus, Hogan and Hotopp's work demonstrates that prior to our application filing in 2004, researchers studying Group II α-KG dioxygenase proteins employed the necessary tools and assays to understand the structure-function relationships of these enzymes. As Hogan and Hotopp disclosed, skilled artisans also understood that the five highly conserved residues (H, D, T, H, R) of the general active site motif "**H**X(**D**/E) X_{23-26}(**T**/S)$X_{114-183}$**H**X_{10-13}**R**" (specification at [0085]) were important for the observed α-KG dioxygenase activity.

14. Based on the surprising discovery described in our specification of both the function (dual MOA) and the structure of the catalytic center motif, researchers would have recognized that the AAD-1 motif represents a specific motif among the Group-II α-KG dioxygenases. Since 2,4-D specific or S-dichlorprop dioxygenases like SdpA and others that do not degrade R-AOPP herbicides do not have the AAD-1 motif (see attached Appendix II), one of ordinary skill in the art, based on our application and the available knowledge in the art, would have readily appreciated that the claimed AAD-1 variants form a specific group within the Class-II α-KG-dependent dioxygenases. (See also Wright-I at paragraph 12.)

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15. In summary, in view of the background knowledge in the art described above, skilled artisans would have readily understood from our application that the activity of the claimed AAD-1 variants was attributable to their common catalytic structural motif, and that as long as the catalytic structural motif was retained, activity would be retained similar to other Group II proteins that vary widely in sequence identity and were known to retain enzymatic activity. Thus, one of ordinary skill in the art would have readily envisioned the claimed AAD-1 variants through their common motif structure.

III. Further Confirmation of Structure-Function Relationship for the Claimed AAD-1 Variants

16. In the Wright-I declaration, I submitted evidence further supporting the importance of the conserved residues in the AAD-1 motif. As I showed then, mutation of each individual conserved residue of the AAD-1 motif substantially reduced the dual MOA, showing the importance of the defined residues in the AAD-1 motif. (See Wright-I declaration at 14-15, Appendix III).

17. To further confirm our previous demonstration, I designed further confirmatory assays to verify that the exemplified AAD-1 variants can tolerate a wide range of amino acid substitutions while retaining both their dual MOA (e.g., R-quizalofop and 2,4-D degrading activity) and the claimed AAD-1 motif. Six variants with around 88-90% amino acid identity to AAD-1 (SEQ ID NO: 9) and two variants at 82% amino acid identity to SEQ ID NO: 9 were evaluated.

18. One of Corteva's research scientists, under my guidance, conducted the assays to confirm the dual MOA of the AAD-1 variants as described above. Highly conserved residues constituting the AAD-1 motif were retained in the variants tested. Amino acid changes were made throughout the protein sequence. The following variants were created and tested:

Name	% identity to AAD-1v1	Notes
Var 1	90	Contains AAD-1 motif
Var 2	89	Contains AAD-1 motif

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Var 3	88	Contains AAD-1 motif
Var 4	90	Contains AAD-1 motif
Var 5	89	Contains AAD-1 motif
Var 6	88	Contains AAD-1 motif
Var 7	82	Contains AAD-1 motif
Var 8	82	Contains AAD-1 motif
AAD-1	100	Used as a positive dual MOA control, Contains AAD-1 motif
AAD-12	25	Does not contain the AAD-1 motif and was used as a negative control for R-AOPP activity; does retain activity for 2,4-D.

19. Each of the eight variants was expressed in *E. coli* and the enzyme activity was assessed as described in the attached Appendix IV.

20. Appendix IV shows the relative activity of the AAD-1 and AAD-12 controls in comparison to the eight variants, all of which retained an intact AAD-1 motif, and which ranged from 82 to 90% amino acid identity to AAD-1 (SEQ ID NO:9). The 2,4-D activity of the eight variants ranged from 54 to 112% of that of AAD-1 under the same conditions (Appendix IV data graph). When tested with R-quizalofop, the enzyme activity ranged from 11 to 49% relative to wildtype AAD-1. Hence, the variant AAD-1 enzymes retained activity against both 2,4-D and R-quizalofop (an R-AOPP herbicide) when the AAD-1 motif was retained, even at a level of identity to AAD-1 (SEQ ID NO: 9) that was *below* the claimed 85%.

21. My previous declaration (Wright-I) confirmed the importance of identified residues in the AAD-1 motif for the claimed dual MOA activity. The data I discussed in this declaration further confirms that, consistent with the expectations of the skilled practitioner based on the known plasticity of tfdA homologs (i.e., retaining activity across a wide range of sequence variation outside of the conserved structural motif), AAD-1 variants having a sequence identity at least as low as 82% to the AAD-1 sequence (SEQ ID NO: 9) could be prepared that still retain the dual MOA (the ability to enzymatically degrade a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide).

22. I hereby declare that all statements herein made of my own knowledge are true, and all statements herein made on information and belief are believed to be true; these statements are made with the knowledge that willful false statements and the like so made

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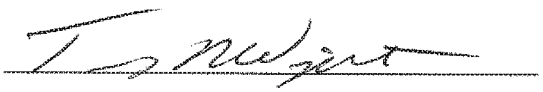
are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Place and Date:

9/9/2020

Indianapolis, Indiana

By:



Terry R. Wright

Distinguished Laureate

Corteva Agriscience

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Appendix I. Previously Presented Claimed Subject Matter

Claims 1-14. (Canceled).

15. (Previously Presented) A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids.

16. (Previously Presented) The plant cell of claim 15 wherein said AAD-1 motif has the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R(X)_{282-284}R$, wherein $(X)_{282-284}$ represents a sequence of 3 amino acids.

17. (Canceled).

18. (Previously Presented) The plant cell of claim 15 wherein the AAD-1 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 9.

19. (Previously Presented) The plant cell of claim 15 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola

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cell, a soybean cell, and an Arabidopsis cell.

20. (Previously Presented) The plant cell of claim 15 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

21. (Previously Presented) A transgenic plant comprising a plurality of the plant cell of claim 15, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

22. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

23. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

24. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

25. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

26. (Previously Presented) The plant of claim 21 wherein said plant further comprises a second herbicide resistance gene.

27. (Previously Presented) The plant of claim 26 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

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28. (Previously Presented) A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 21, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide.

29. (Previously Presented) The method of claim 28 wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

30. (Previously Presented) The method of claim 28 wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

31. (Previously Presented) The method of claim 28 wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

32. (Previously Presented) The method of claim 28 wherein said method comprises applying a second herbicide.

33. (Previously Presented) The method of claim 32 wherein said first herbicide and said second herbicide are applied sequentially.

34. (Previously Presented) The method of claim 32 wherein said first herbicide and said second herbicide are applied concurrently.

35. (Previously Presented) The method of claim 32 wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

36. (Previously Presented) The method of claim 32 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate

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synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

37. (Previously Presented) The method of claim 32, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

38. (Previously Presented) The method of claim 32, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

39. (Previously Presented) The method of claim 32 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

40. (Previously Presented) The method of claim 39 wherein said second gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

41. (Previously Presented) The method of claim 40 wherein:

(a) said modified ALS gene is selected from the group consisting of a SurA, SurB, Csr1, Csr1-1, Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT; and,

(c) said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

42. (Previously Presented) The method of claim 32 wherein said method further comprises applying a third herbicide.

43. (Previously Presented) The method of claim 42, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

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44. (Previously Presented) The method of claim 43 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

45. (Previously Presented) A seed comprising a plurality of the plant cell of claim 15.

46. (Previously Presented) A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim 45 in said field within 14 days of applying said aryloxyalkanoate herbicide.

47. (Previously Presented) A plant grown from the seed of claim 45.

48. (Previously Presented) A part, progeny, or asexual propagate of the plant of claim 47, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

49. (Previously Presented) An (R)-aryloxyphenoxypropionate herbicide tolerant transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids, wherein said motif has 90% sequence identity with corresponding amino acids of position 111 to 281 of SEQ ID NO: 9.

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Appendix II

α-KG dioxygenase general motif residues by amino acid location and intervening spacer for representative Group-II dioxygenases.

	Location	Spacer	Location	Spacer	Location	Spacer	Location	Spacer	Location	GenBank	Reference
Consensus-->	H	X	D	X	T	X	H	X	R		
tfdA Cn	114	1	116	24	141	121	263	10	274	AAA21983.1	1
tfdA Bc	114	1	116	24	141	121	263	10	274	AAK81681.1	2
tfdA RASC	114	1	116	24	141	121	263	10	274	Q45423.1	3, 4
tfdA Ad	114	1	116	24	141	121	263	10	274	AAS49436	5
tfdA P4a	114	1	116	24	141	121	263	10	274	AAM76772.1	6
tfdA pIJB	115	1	117	24	142	121	264	10	275	AAB47567.1	7
tfdA RD5-C2	115	1	117	24	142	121	264	10	275	BAB92964.1	4
tfdA HWK12	115	1	117	24	142	121	264	10	275	BAB92965.1	4
tfdA HW13	115	1	117	24	142	121	264	10	275	BAB92966.1	4
tfdA Bj	115	1	117	24	142	121	264	10	275	WP_011084309.1	8
sdpA MC1	108	1	110	24	135	126	262	10	273	P83309.2	9
sdpA MH	102	1	104	24	129	127	257	10	268	Q700X4.1	10
sdpA P230	102	1	104	24	129	127	257	10	268	ABD67501.1	11
AAD1v1 , RdpA (SEQ ID NO: 9)	111	1	113	24	138	131	270	10	281	Q8KSC8.2	9, 10, 12
AAD1 v2 (SEQ ID NO: 10)	111	1	113	24	138	131	270	10	281	-	12
AAD1 v3 (SEQ ID NO: 11)	112	1	114	24	139	131	271	10	282	-	12

References

1 J. Bacteriol. 169: 2950-2955 (1987)
2 Microbios 105: 43-63 (2001)
3 Appl. Environ. Microbiol. 62:2464-2469 (1996)
4 Appl. Environ. Microbiol. 68:3449-3454 (2002)
5 J. Bacteriol. 186:7161-7174 (2004)
6 Acta Biotechnol. 21, 321-331 (2001)
7 Appl. Environ. Microbiol. 70:2110-2118 (2004)
8 DNA Res. 9:225-256 (2002)
9 Appl. Environ. Microbiol. 70:5357-5365 (2004)
10 Appl. Environ. Microbiol. 70:6066-6075 (2004)
11 Microbiol. Res. 156:121--131 (2001)
12 WO 2005/104737

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Appendix III

Percent identity of known complete coding sequences of 2,4-D-active degrading α-KG dioxygenases compared to TfdA (*Cupriavidus necator*).

		GenBank	Reference
	% ID*		
	tfdA Cn		
tfdA Cn	100	AAA21983.1	1
tfdA Bc	85	AAK81681.1	2
tfdA RASC	78	Q45423.1	3, 4
tfdA Ad	84	AAS49436	5
tfdA P4a	85	AAM76772.1	6
tfdA pJ8	80	AAB47567.1	7
tfdA RD5-C2	45	BAB92964.1	4
tfdA HWK12	45	BAB92965.1	4
tfdA HW13	45	BAB92966.1	4
tfdA Bj	44	WP_011084309.1	8
sdpA MC1	31	P83309.2	9
sdpA MH	35	Q700X4.1	10
sdpA P230	35	ABD67501.1	11
AAD1v1 , RdpA (SEQ ID NO: 9)	28	Q8KSC8.2	9, 10, 12
AAD1 v2 (SEQ ID NO: 10)	28		12
AAD1 v3 (SEQ ID NO: 11)	28		12

* % identity of known complete coding sequences of 2,4-D-active degrading a-KG dioxygenases comparted to TfdA (Cupriavidus necator)

References

1 J. Bacteriol. 169: 2950-2955 (1987)
2 Microbios 105: 43-63 (2001)
3 Appl. Environ. Microbiol. 62:2464-2469 (1996)
4 Appl. Environ. Microbiol. 68:3449-3454 (2002)
5 J. Bacteriol. 186:7161-7174 (2004)
6 Acta Biotechnol. 21, 321-331 (2001)
7 Appl. Environ. Microbiol. 70:2110-2118 (2004)
8 DNA Res. 9:225-256 (2002)
9 Appl. Environ. Microbiol. 70:5357-5365 (2004)
10 Appl. Environ. Microbiol. 70:6066-6075 (2004)
11 Microbiol. Res. 156:121--131 (2001)
12 WO 2005/104737

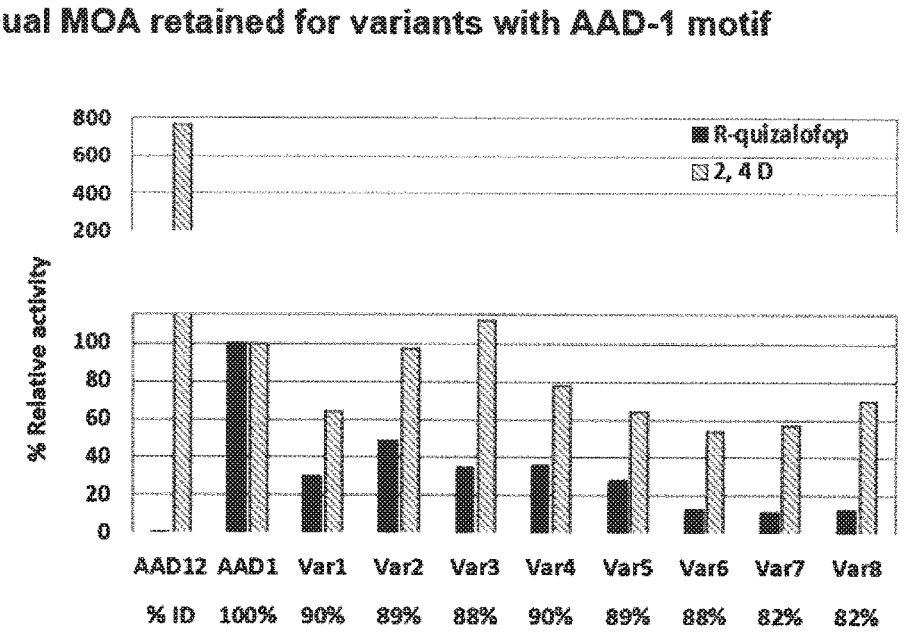
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Appendix IV

Comparison of the relative activity of AAD-1v1 and eight AAD-1 variants created with either 90, 89, 88, or 82% amino acid identity to AAD-v1 and have the AAD-1 motif intact. Protein variants were expressed in *E. coli* using a Histidine tag for rapid isolation. Enzyme activity of the eight variants were assayed *in vitro* and compared to the activity of AAD-1v1 under the same conditions described in the specification. A total of two experiments were run with two separate batches of expressed protein and replicated 1-3 times per experiment, depending on yield, and averaged across experiments (Var7 was tested in one experiment only). Enzyme variants were tested between 2 and 7.5ug / assay, depending on yield using R-quizalofop and 2,4-D as individual substrates for each MOA. AAD-1 and AAD-12 standards were tested at the same enzyme concentration so enzyme activity could be normalized to % relative activity vs AAD-1, as previously reported.

Hatched bars represent the % relative activity of each variant compared to wildtype AAD-1v1 under the same conditions tested with 2,4-D. [Note: a 'split graph' is used to present all data in the same graphic]. The eight variants ranged from 54 to 112% activity relative to AAD1v1.

Solid bars represent the % relative activity of each variant compared to wildtype AAD-1v1 under the same conditions tested with R-quizalofop. The eight variants ranged from 11 to 49% activity relative to AAD1v1. [Note: AAD-12 enzyme had nearly undetectable activity (<1%) relative to AAD-1v1, consistent as a negative control for R-AOPP activity].



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Electronic Patent Application Fee Transmittal

Application Number:	15288406
Filing Date:	07-Oct-2016
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Filer:	John P. Breen/Garla Twyman
Attorney Docket Number:	14764-258662

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
		Total in USD ($)		**1400**

Electronic Acknowledgement Receipt	
EFS ID:	40509909
Application Number:	15288406
International Application Number:	
Confirmation Number:	5402
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Customer Number:	107540
Filer:	John P. Breen/Garla Twyman
Filer Authorized By:	John P. Breen
Attorney Docket Number:	14764-258662
Receipt Date:	09-SEP-2020
Filing Date:	07-OCT-2016
Time Stamp:	17:13:48
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1400
RAM confirmation Number	E202099H14336958
Deposit Account	100435
Authorized User	Garla Twyman
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Amendment_and_Response_under_37_C_F_R_1_111.pdf	341684 2cb07b12a5ea4e0bce0ec0d62a11ac538af9c964	yes	19

Multipart Description/PDF files in .zip description

Document Description	Start	End
Applicant Arguments/Remarks Made in an Amendment	8	19
Claims	2	7
Amendment/Req. Reconsideration-After Non-Final Reject	1	1

Warnings:					
Information:					
2	Affidavit-traversing rejectns or objectns rule 132	Declaration_under_37_C_F_R_1_132-WRIGHT.pdf	1166688 47c61ac620b7db82b868a6dd703e350200ce944f	no	16
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30698 e3380d465d9265b1eb857ded5b529dc6740f42c5	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1539070		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

REMARKS

Claims 15, 16, and 18-49 are currently pending and under examination. No claims are amended by this response.

Applicant acknowledges the withdrawal of the rejection of the claims under 35 USC 102(a) over Ellis et al. and 35 USC 102(b) over Wright and the acceptance of Applicant's claim of priority to prior-filed application (USSN: 14/820,893). Applicant respectfully traverses the rejections set forth in the Office Action dated March 10, 2020. In support of this response, Applicant respectfully submits a second declaration of Dr. Terry Wright (hereinafter "Wright-II") under 37 CFR 1.132.

Interview Summary

Applicant wishes to thank the Examiner for the courtesies extended to Applicant's representatives and the undersigned attorney of record during an Examiner's Interview conducted on August 26, 2020. The interview summary prepared by the Examiner and mailed on August 31, 2020 represents an accurate summary of that discussion. However, we note the fourth participant was Phil Poirier rather than the listed "Bill Poirier".

Claim Rejections

Double Patenting Rejections

Claims 15, 16 and 18-49 are rejected for obviousness type double patenting over claims 1-43 of US Patent No. 7,838,733. Claims 15, 16 and 18-49 are rejected for obviousness type double patenting over claims 1-22 of US Patent No. 9,127,289. Claims 15, 16 and 18-49 are rejected for obviousness type double patenting over claims 1-13 of US Patent No. 10,174,337. Applicant will file the necessary terminal disclaimers to overcome these rejections upon a finding of allowable subject matter.

The Written Description Rejection Should Be Withdrawn

Claims 15, 16 and 18-49 are rejected under 35 USC 112, first paragraph, for failing to comply with the written description requirement. Applicant respectfully traverses this rejection, as the Examiner has not met his burden of establishing applicant's failure to comply with the written description requirement of 35 USC 112. As set forth below, under a proper common-

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structure analysis, the specification adequately describes the claimed structural motif such that as of the filing date a skilled artisan would have recognized that the inventors were in possession of the claimed subgenus.

The Examiner Incorrectly Analogizes to Representative-Species Cases

To support a genus or sub-genus claim, a patent specification may disclose "*either* a representative number of species falling within the scope of the claims or structural features common to the members of the genus." *Ajinomoto Co., Inc. v. International Trade Commission*, 932 F.3d 1342, 1358-59 (2019) (quoting *Ariad Pharm., Inc. v. Eli Lilly & Co.*, 598 F.3d 1336, 1350 (Fed. Cir. 2010) (en banc)). In the Office Action, the Examiner incorrectly relies on representative-species cases to reject the claims, overlooking the common structure disclosed in Applicant's specification and expressly recited in Applicant's claims.

For example, the Examiner relies on *AbbVie*, in which the claims did not recite any structure for the claimed antibodies. Office Action at 8-9; *AbbVie Deutschland GmbH & Co. v. Janssen Biotech, Inc.*, 759 F.3d 1285, 1292 (Fed. Cir. 2014) ("The claims … at issue in these appeals define the claimed antibodies by their function, *i.e.*, IL-12 binding and neutralizing characteristics, rather than by structure."). In *AbbVie*, the patentee's expert further conceded that the specifications of the asserted patents did "not disclose structural features common to the members of the claimed genus." *Id.* at 1299. *AbbVie* thus did not involve a common-structure analysis and its facts bear little resemblance to Applicant's claims, which expressly recite the structural motif $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ that is common to members of the claimed subgenus as described in the specification and a specific sequence identity of at least 85% to SEQ ID NO: 9.

The Examiner also relies on *Ariad*'s discussion of a "variety of materials constituting the genus," i.e., representative species. Office Action at 9; *Ariad*, 598 F.3d at 1350 (requiring "a variety of species that accomplish the result"). In *Ariad*, similar to *AbbVie*, common structure was not at issue. The *Ariad* claims, for example, contained no structural limitations. *Ariad*, 598 F.3d at 1340-41. The specification in *Ariad* also disclosed no structural features common to the members of the genus that had been shown to provide any NF-κB activity. *Id.* at 1358-59. *Ariad* instead focused on an analysis of representative species. *Id.* ("The '516 patent discloses no working or even prophetic examples of methods that reduce NF-κB activity, and no completed

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syntheses of any of the molecules prophesized to be capable of reducing NF-κB activity."). *Ariad* is thus also inapposite to Applicant's claims.

In contrast, the Office Action fails to distinguish or address Applicant's reliance on *Ajinomoto*, in which the Federal Circuit held that common structural features supported claims to a genus. There the court made reference to known consensus sequences that provided structure to the claimed functional limitation, analogous to Applicant's claimed AAD-1 motif, and found that this in combination with the background knowledge of a skilled artisan conferred adequate written description support. *Ajinomoto*, 932 F.3d at 1359-60 (distinguishing *AbbVie*). In particular, the ITC concluded that a skilled artisan would have recognized that promoters having fewer departures from a "consensus sequence in a promoter" were "generally stronger than promoters with more departures from such a sequence," and the Federal Circuit affirmed. *Id.* Rejecting arguments for a heightened written-description standard involving common structure, the *Ajinomoto* Court emphasized that the law "does not require a perfect correspondence between the members of the genus and the asserted common structural feature." *Id.* Instead, the Court has "spoken more modestly of a '*correlation* between structure and function.'" (citation omitted) (emphasis in original)).

Here, as detailed below, *Ajinomoto* is closely analogous and supports withdrawal of the written-description rejection.

The Specification Discloses Common Structure Supporting Applicant's Subgenus Claims

Applicant's specification describes the common structure of the catalytic core of α-ketoglutarate-dependent dioxygenases. Indeed, as noted earlier, the specification describes the structural motif $HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$ that characterizes the α-ketoglutarate-dependent dioxygenase family. Specification at ¶ [0085]; Office Action at 6. The specification further discloses that this motif "comprises the active site" of the claimed dioxygenases, as the histidine residues "coordinate [the] Fe^{2+} ion in the active site that is essential for catalytic activity." Specification at ¶ [0085].

The art also recognized the common structure and importance of the general structural motif as the active site for α-ketoglutarate-dependent dioxygenases, including for the "Group II" subfamily encompassing TfdA and AAD-1, *inter alia*. *See, e.g.*, Hogan et al., *J Biol Chem*, 2000; 275(17): 12400-12409 at Table 1. Hogan, which is cited in the specification at paragraph [0085], describes the general motif of α-ketoglutarate-dependent dioxygenases and shows the

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importance of residues within that motif for the catalytic activity of TfdA via site-directed mutagenesis studies. *See, e.g.*, Hogan at 12406 (disclosing that site-directed mutagenesis studies "support the proposal that the residues in the HX(D/E) motif are ligands to the metal I TfdA and, most likely, other Group II enzymes"). Hogan initially identified His-113, Asp-115, and His-262, deemed a "consensus sequence," as active site residues for TfdA. *Id.* By 2002, the same research group then identified two more residues, Thr-141 and Arg-274, as also part of the active site. *See* Hotopp et al., *Biochem.* 41:9787-9794, (2002). These five residues correspond to Applicant's disclosed general structural motif for the common catalytic core: **H**X(**D**/E)$X_{23\text{-}26}$(**T**/S)$X_{114\text{-}183}$**H**$X_{10\text{-}13}$**R**. Wright-II at ¶¶ 12, 13; *see Ajinomoto*, 932 F.3d at 1359-60 (finding adequate written description based on a known promoter "consensus sequence" and other supporting background knowledge in the art).

The specification further describes, as the active site, the specific AAD-1 motif recited in the claims: "$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$." Indeed, the specification discloses (1) the sequence of AAD-1 (SEQ ID NO: 9), which has the conserved α-ketoglutarate-dependent dioxygenase center motif (i.e., structure), (2) the classification of AAD-1 within the same family as TfdA, (3) the conserved catalytic center across this subfamily of dioxygenases, and (4) the additional sequences of AAD-1 variants sharing the common motif and common properties. Specification at ¶ [0085]; *see also id.* at SEQ ID NOs: 9-11. The specification specifically discloses this active-site motif, which is identifiable in SEQ ID NOs: 9-11.

A skilled artisan would have recognized that the $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$ motif in the AAD-1 sequence is the catalytic core in view of its relation to the disclosed "active site" motif in paragraph [0085] of the specification, as well as based on background knowledge in the art in which researchers had investigated and characterized other α-ketoglutarate-dependent dioxygenases. Wright-II at ¶¶ 8, 11-15. Hogan, for example, investigated TfdA, which is in the same family as AAD-1 (*see* Specification at ¶ [0085]), by comparing it to other enzymes in the family and showing the conservation of the common active-site structure. Hogan at Fig. 8. As Dr. Nair explains, a skilled artisan would have readily identified the $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$ motif in the AAD-1 sequence as the active site through similar sequence alignment. *See* Nair Declaration at ¶ 9; Wright-II at ¶ 8-9, 15.

The Office Action thus errs in requiring "actual sequence information" to satisfy the written-description requirement where Applicant's specification clearly describes the common

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structural motif conferring the active site to the claimed dioxygenases—just as the art had previously recognized for other α-ketoglutarate-dependent dioxygenases within the same subfamily. Office Action at 6; Hogan at abstract; *Ajinomoto*, 932 F.3d at 1358-59 (section 112 requires only common structure *or* a representative number of species). Because the claimed common structure of the $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ motif corresponds to the recognized active site of dioxygenases, a skilled artisan would have readily appreciated that the disclosed AAD-1 common structural motif confers the specific activity of the claimed dioxygenases, including the phenoxy auxin and R-AOPP degrading activity. Wright-II at ¶¶ 7-8; *Ajinomoto*, 932 F.3d at 1359-60 (affirming adequate written description where "a skilled artisan could identify more [embodiments] given the well-known link between consensus sequence and [activity]" (internal quotation marks omitted)).

Furthermore, a skilled artisan would have appreciated that Applicant was in possession of the full scope encompassed by the current claims. As noted at paragraphs 10-13 of the Wright-II Declaration, the family of α-KG dioxygenases were a well-studied class of enzymes by 2004. Many members of this class of proteins were fully sequenced, and those skilled in the art recognized that members of this class varied widely in their respective sequence identity to one another (ranging down to about 78-85% amino acid identity to the Cupriavidus necator TfdA among beta-and gamma-proteobacteria to about 44-45% amino acid identity among alpha-proteobacteria) while retaining functional activity against phenoxy auxin herbicides (e.g. the ability to metabolize 2,4 D). Wright-II at ¶ 11. Furthermore, the crystal structure of TauD (a member of the Group II sub-family) had been obtained, providing additional data on the structure of Group II α-KG dioxygenases, and this crystal structure had been used to make mutations in TfdA, another Group II family member. Wright-II at ¶¶ 12-13. Accordingly, based on the general knowledge of the skilled practitioner at the time of filing the application, in combination with applicant's disclosed structure of the exemplified embodiments and the AAD-1 motif, one of ordinary skill would readily have been able to envision the structure of variants that meet the limitations of the claimed invention and would thus understand that applicant was in possession of the invention as claimed. Wright-II at ¶¶ 14-15.

Accordingly, Applicant respectfully requests withdrawal of the written-description rejection in view of the specification's specific disclosure of common structure, supported by the high level of background knowledge in the art. *Ajinomoto*, 932 F.3d at 1359-60.

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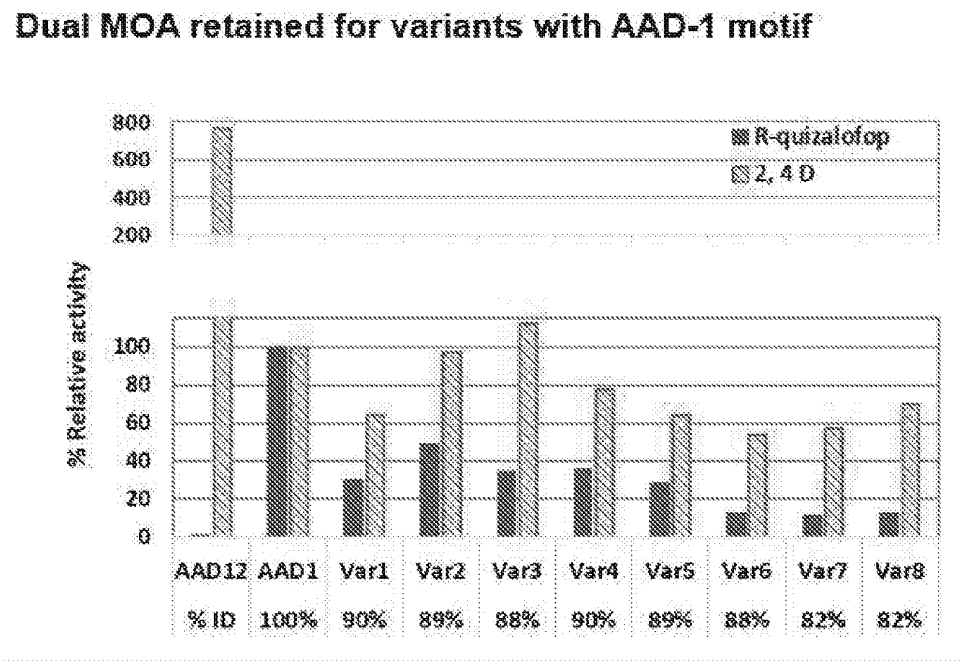
Additional Evidence Confirms the Importance of the Claimed Structural Motif

In addition to the 2019 Wright Declaration (hereinafter "Wright-I") previously submitted, Applicant's Wright-II Declaration describes development of additional variants confirming the importance of the claimed structural motif described in the specification. Expert declarations are relevant to the issue of written description when they are directed to specific statements in the originally filed application and used to explain how, in the declarant's opinion, a person of ordinary skill in the art would understand those statements (see *Martek Biosciences Corp. v. Nutrinova, Inc.*, 579 F.3d 1363 (Fed. Cir. 2009)). Here, Dr. Wright's declarations further confirm that "a skilled artisan could identify more [embodiments] given the well-known link between consensus sequence and [activity]." *Ajinomoto*, 932 F.3d at 1359-60.

As detailed in Dr. Wright's 2019 declaration, mutations to the conserved residues of the common catalytic AAD-1 structural motif reduced or abolished enzymatic activity. Wright-I declaration at ¶¶ 14-16. By contrast, variants that maintained the intact common structural motif retained enzymatic activity. *Id.*

Dr. Wright's 2020 declaration further details variants maintaining the claimed AAD-1 structural motif that have as low as 82% sequence identity to SEQ ID NO: 9. These variants, encompassing the claimed minimum of 85% sequence identity, continue to exhibit both 2,4-D and R-AOPP enzymatic activity. Wright-II at ¶ 20-21, Appendix IV. Indeed, as shown below, variants having 90% (vars. 1, 4), 88% (vars. 3, 6), and 82% (vars. 7, 8) sequence identity to AAD-1 (SEQ ID NO: 9) were tested and found to exhibit 2,4-D and R-AOPP degrading activity.

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Wright-II at ¶ 20, Appendix IV (explaining that AAD-1 served as a positive control for 2,4-D and R-AOPP activity, while AAD-12 served as a positive control for 2,4-D activity and a negative control for R-AOPP activity). Accordingly, the additional data provided in Dr. Wright's declarations further confirm the importance of the claimed structural motif and the plasticity of the remaining sequence to modification, consistent with other related TfdA-like homologs.

For all of the above reasons, Applicant respectfully requests withdrawal of the written description rejection.

The Obviousness Rejection Should Be Withdrawn

Claims 15, 16, 18-30, 32-34, 36, 37, 39-45, 47, 48 and 49 stand rejected under 35 USC 103 as obvious based on Jepson et al. (US 2003/0041357) in view of Kohler (GenBank Accession No. Q8KSC8). Jepson is cited as teaching plants that comprise a *tfdA* gene for conferring aryloxyalkanoate dioxygenase activity to those plants, and the use of such plants in methods of weed control using 2,4-D. The Examiner acknowledges that Jepson does not teach AAD-1 (SEQ ID NO: 9). Kohler is cited as teaching *Spingomonas herbicidovorans rdpA*, a bacterial gene that encodes the peptide of SEQ ID NO: 9 of the instant application. The Examiner states that it would have been *prima facie* obvious to substitute the gene of Kohler for the *tdfA* gene of Jepson. Applicant respectfully traverses this rejection.

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The Office Action Fails to Establish Motivation to Combine

Under 35 USC 103, the Office bears the burden of establishing that "a skilled artisan would have had reason to combine the teaching of the prior art references to achieve the claimed invention." *In re Cyclobenzaprine Hydrochloride Extended-Release Capsule Patent Litig.*, 676 F.3d 1063, 1068-69 (Fed. Cir. 2012). To establish motivation, courts recognize an "obvious to try" rationale under only limited circumstances, i.e., when "there are a finite number of identified, predictable solutions." *KSR Int'l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 1742 (2007). Where one of ordinary skill would need "to try each of numerous possible choices until one possibly arrived at a successful result," an obvious-to-try rationale is legally deficient and cannot establish motivation. *In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988). When analyzing patent claims for obviousness, the fact finder must avoid "even a hint of hindsight." *Cheese Sys., Inc. v. Tetra Pak Cheese & Powder Sys., Inc.*, 725 F.3d 1341, 1352 (Fed. Cir. 2013).

Here, the Office Action errs by adopting an unsupported obvious-to-try rationale. Office Action at 11. Applicant respectfully submits that the Examiner fails to provide any objective reasoning for why one of ordinary skill would have specifically selected Kohler's *RdpA* gene to substitute for the *tfdA* gene disclosed in Jepson. The Office Action fails to acknowledge that there were many other known sequences that shared sequence identity with the *tfdA* gene and served as possible candidates for providing aryloxyalkanoate dioxygenase activity. For example, as the specification states, identifying the *RdpA* (AAD-1) enzyme required mining an extensive database that, even under default search options, provide more than 100 other genes as potential selections:

> As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e., α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤ 50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid

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> conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

Specification at ¶ [0171] (emphases added).

Absent hindsight, a person of ordinary skill in the art would have had no reason or motivation to select Kohler's *RdpA* gene from among the many potential polynucleotides encoding herbicidal proteins. Indeed, a person of ordinary skill in the art would have known that *RdpA* had *low activity* for degrading 2,4-D *in vitro*, and thus Kohler's teachings would not have been viewed as suitable for combination with Jepson's. Wright-I at ¶ 6; Nair Declaration at ¶ 5; A. Westendorff et al., *Purification and Characterisation of the Enantiospecific Dioxygenases from Delftia Acidovorans MC1 Initiating the Degradation of Phenoxypropionate and Phenoxyacetate Herbicides*, 23 ACTA BIOTECH. 3, 10 (2003) (Table 2 disclosing only 0.6% activity against 2,4-D); *see also* K. Nickel et al., *Involvement of Two α-Ketoglutarate-Dependent Dioxygenases in Enantioselective Degradation of (R)- and (S)-Mecoprop by Sphingomonas Herbicidovorans MH*, 179 J. BACTERIOLOGY 6674, 6677 (1997) (Table 2 disclosing only 15% activity against 2,4-D).

Moreover, by selecting the *RdpA* gene, Applicant proceeded contrary to conventional wisdom by selecting a gene that had a low sequence identity to *tfdA*—i.e., the gene that the Office cites and relies upon in Jepson to substitute with Kohler's *RdpA* gene. *In re Hedges*, 783 F.3d 1038, 1041 (Fed. Cir. 1986) (an applicant "proceed[ing] contrary to the accepted wisdom" is "strong evidence of nonobviousness"). As noted in paragraphs [0012], [0013], and [0171] of the specification, many of the possible choices of herbicide-encoding genes have amino acid sequences similar to *tfdA* (>85% amino acid identity) and have similar *in vitro* enzymatic properties to *tfdA*. Conventional wisdom held that one would have selected a peptide homolog with high sequence identity to *tfdA* if one wanted to obtain a homolog that retained the activity of the parent protein (i.e., activity against phenoxy auxin herbicides). Applicant, however, selected the *RdpA* gene from *Sphingobium herbicidovorans*, even though the protein exhibited very low homology to tfdA (28% amino acid identity).

Against Applicant's evidence showing that (1) a skilled artisan would have been confronted with "numerous possible choices" with no guidance as to which would be successful (*see O'Farrell*, 853 F.2d at 903), and that (2) conventional wisdom would have directed one

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away from selecting the *RdpA* gene with low sequence homology to the *tdfA* gene (*see Hedges*, 783 F.3d at 1041), the Office Action asserts only conclusory, unsupported arguments for its obvious-to-try rationale. The Office Action, for example, asserts that skilled artisans would have routinely "transform[ed] plant[s] with transgenes encoding 100 potential enzymes," apparently without regard for their amino acid sequence, enzymatic activity, and anticipated properties. Office Action at 12. Notably, the Office Action cites *no evidence* in support of its sweeping argument, which essentially dispenses with the motivation-to-combine requirement altogether. *KSR*, 127 S. Ct. at 1741 ("[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." (citation omitted)).

The Office Action also incorrectly asserts that a motivation to combine Jepson and Kohler would have existed based on the alleged rationale of a "substitution of equivalents having the same or similar function." Office Action at 11. As described above, however, a person of ordinary skill would have known that *RdpA* had as low as 0.6% activity for degrading 2,4-D *(* Westendorff *et al*, *supra*), and thus would not have viewed the *RdpA* gene as an "equivalent" for substitution with the *tdfA* gene as the Office Action contends. The Office Action—by casting a wide net of potential genes for indiscriminate investigation and disregarding the entirety of the prior art disclosures concerning the *RdpA* gene for what they "fairly suggest[ ] to one skilled in the art"—improperly relies on hindsight. *Hedges*, 783 F.3d at 1041.

Accordingly, the Office Action does not establish any motivation to combine Jepson and Kohler, and thus fails to set forth a *prima facie* case of obviousness.

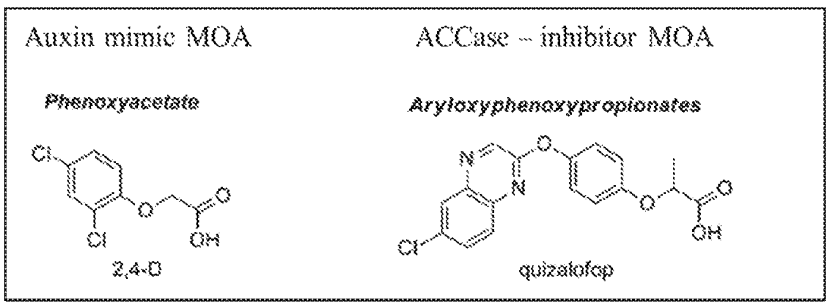
Applicant's Unexpected Results Demonstrate Nonobviousness

The Office Action additionally errs in disregarding Applicant's undisputed evidence of unexpected results. *See* January 10, 2020 Amendment and Response at 28.

Objective evidence of nonobviousness "must always" be considered when evaluating obviousness. *Cyclobenzaprine*, 676 F.3d at 1075-76. Objective evidence, such as unexpected results, "may often be the most probative and cogent evidence of the record … [and] may often establish that an invention appearing to have been obvious in light of the prior art was not." *Id.* (citing *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1538-39 (Fed. Cir. 1983)). Objective evidence must be considered as "*part* of the whole obviousness analysis" and cannot be treated as an "afterthought." *Leo Pharm. Prods., Ltd. v. Rea*, 726 F.3d 1346, 1357-58 (Fed. Cir. 2013).

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Here, it is undisputed that Applicant's transgenic plant cell expressing an AAD-1 protein is not only able to degrade 2,4D, but also AOPP herbicides, exhibiting a dual activity that was entirely unexpected and surprising in view of the cited art. Wright-I at ¶¶ 7-8; Nair Declaration at ¶ 8. Indeed, until Applicant's invention, no α-ketoglutarate-dependent dioxygenase enzyme had been reported to have any ability to degrade two herbicides of different chemical classes and modes of action. In addition, phenoxy auxin class herbicides and the AOPP class of herbicides are structurally quite distinct (see e.g., phenoxyacetate and quizalofop, *shown below*):



There was no report of any examination of the AAD-1 enzyme for activity on any herbicidal substrate outside of the phenoxy auxin class. Wright-I at ¶ 6.

Thus, while there was no motivation to even select an AAD-1 enzyme for herbicide tolerance given its little or no reported 2,4-D activity (Wright-I at ¶ 6), there was also no reasonable expectation that combining the cited art would successfully achieve the claimed activity against a different herbicide class – R-AOPP herbicides. The Office Action has not established any rational basis, absent hindsight, for a reasonable expectation of success given Applicant's surprising results.

Applicant's unexpected results are more than sufficient to establish nonobviousness. In *Albrecht*, for example, the CCPA reversed the Office's obviousness rejection where the applicant's claimed compounds—similar to Applicant's claims here—unexpectedly achieved a *dual activity* of exhibiting both antiviral and anesthetic properties, whereas structurally similar prior art compounds only exhibited anesthetic properties. *In re Albrecht,* 514 F.2d 1389, 1395-96 (CCPA 1975). The Court concluded that the fact that a claimed invention "possesses a certain advantageous activity which is not in fact possessed by a prior art compound *is itself evidence of the nonobviousness of the subject matter as a whole*." *Id.* (emphasis added). Here, as in *Albrecht*, Applicant's claimed invention achieves a dual activity "totally dissimilar" to any prior art analogs, which compels a finding of nonobviousness. *Id.*

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Moreover, as the Federal Circuit has held, "when an applicant demonstrates substantially improved results … and states that the results were unexpected, this should suffice to establish unexpected results in the absence of evidence to the contrary." *In re Soni*, 54 F.3d 746, 751 (Fed. Cir. 1995) (reversing Office's obviousness rejection where the Office did not meaningfully contest Applicant's unexpected results). Here, the Office Action does not rebut or address Applicant's asserted unexpected results. *See* January 10, 2020 Amendment and Response at 28. Thus, Applicant's unexpected results stand uncontroverted. As such, the obviousness rejection should be withdrawn. *Soni*, 54 F.3d at 751.

Conclusion

Reconsideration of the claims and allowance of the application is respectfully requested. If any further discussion of this matter would speed prosecution of this application, the Examiner is invited to call the undersigned at (434) 220-2866.

Respectfully submitted,

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LISTING OF CLAIMS:

This listing of claims is provided for convenience. No amendments are presented by this response.

Claims 1-14. (Canceled).

15. (Previously Presented) A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids.

16. (Previously Presented) The plant cell of claim 15 wherein said AAD-1 motif has the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R(X)_{282-284}R$, wherein $(X)_{282-284}$ represents a sequence of 3 amino acids.

17. (Canceled).

18. (Previously Presented) The plant cell of claim 15 wherein the AAD-1 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 9.

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19. (Previously Presented) The plant cell of claim 15 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an Arabidopsis cell.

20. (Previously Presented) The plant cell of claim 15 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

21. (Previously Presented) A transgenic plant comprising a plurality of the plant cell of claim 15, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

22. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

23. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

24. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

25. (Previously Presented) The plant of claim 21 wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

26. (Previously Presented) The plant of claim 21 wherein said plant further comprises a second herbicide resistance gene.

27. (Previously Presented) The plant of claim 26 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group

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consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

28. (Previously Presented) A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 21, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide.

29. (Previously Presented) The method of claim 28 wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

30. (Previously Presented) The method of claim 28 wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

31. (Previously Presented) The method of claim 28 wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

32. (Previously Presented) The method of claim 28 wherein said method comprises applying a second herbicide.

33. (Previously Presented) The method of claim 32 wherein said first herbicide and said second herbicide are applied sequentially.

34. (Previously Presented) The method of claim 32 wherein said first herbicide and said second herbicide are applied concurrently.

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35. (Previously Presented) The method of claim 32 wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

36. (Previously Presented) The method of claim 32 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

37. (Previously Presented) The method of claim 32, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

38. (Previously Presented) The method of claim 32, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

39. (Previously Presented) The method of claim 32 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

40. (Previously Presented) The method of claim 39 wherein said second gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

41. (Previously Presented) The method of claim 40 wherein:

(a) said modified ALS gene is selected from the group consisting of a SurA, SurB, Csr1, Csr1-1, Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT; and,

(c) said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

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42. (Previously Presented) The method of claim 32 wherein said method further comprises applying a third herbicide.

43. (Previously Presented) The method of claim 42, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

44. (Previously Presented) The method of claim 43 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

45. (Previously Presented) A seed comprising a plurality of the plant cell of claim 15.

46. (Previously Presented) A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim 45 in said field within 14 days of applying said aryloxyalkanoate herbicide.

47. (Previously Presented) A plant grown from the seed of claim 45.

48. (Previously Presented) A part, progeny, or asexual propagate of the plant of claim 47, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

49. (Previously Presented) An (R)-aryloxyphenoxypropionate herbicide tolerant transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

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X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids, wherein said motif has 90% sequence identity with corresponding amino acids of position 111 to 281 of SEQ ID NO: 9.

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	Filing Date	2016-10-07
	First Named Inventor	Terry Wright
	Art Unit	1663
	Examiner Name	KRUSE, DAVID H
	Attorney Docket Number	14764-258662

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	1	STREBER et al., Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene tfdA of Alcaligenes eutrophus JMP134, Journal of Bacteriology (1987), 169(7), 2950-5.	
	2	Poh et al., 2,4-Dichlorophenoxyacetate / α-ketoglutarate dioxygenases from Burkholderia cepacia 2a and Ralstonia eutropha JMP134, Microbios 105 43-63 2001.	
	3	SUWA et al., Characterization of a chromosomally encoded 2,4-dichlorophenoxyacetic acid/ketoglutarate dioxygenase from Burkholderia sp. strain RASC, Applied and Environmental Microbiology (1996), 62(7), 2464-2469.	
	4	ITOH et al., tfdA-like genes in 2,4-dichlorophenoxyacetic acid-degrading bacteria belonging to the Bradyrhizobium-Agromonas-Nitrobacter-Afipia cluster in Proteobacteria, Applied and Environmental Microbiology (2002), 68(7), 3449-3454.	
	5	Vedler et al. The Completely Sequenced Plasmid pEST4011 Contains a Novel IncP1 Backbone and a Catabolic Transposon Harboring tfd Genes for 2,4-Dichlorophenoxyacetic Acid Degradation, JOURNAL OF BACTERIOLOGY, Vol. 186, No. 21, Nov. 2004, p. 7161–7174.	
	6	HOFFMANN et al., Development and application of PCR primers for the detection of the tfd genes in Delftia acidovorans P4a involved in the degradation of 2,4-D, Acta Biotechnologica (2001), 21(4), 321-331.	
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	10	Muller et al., Genetic Analysis of Phenoxyalkanoic Acid Degradation in Sphingomonas herbicidovorans MH; APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2004, Vol. 70, No. 10, p. 6066–6075.	
	11	MULLER et al., Physiological and genetic characteristics of two bacterial strains utilizing phenoxypropionate and phenoxyacetate herbicides, Microbiological Research (2001), 156(2), 121-131.	

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☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Breen/	Date (YYYY-MM-DD)	2020-09-10
Name/Print	John P. Breen	Registration Number	38833

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Filing Date:	07-Oct-2016
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Filer:	John P. Breen/Garla Twyman
Attorney Docket Number:	14764-258662

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

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Analysis, Cloning, and High-Level Expression of 2,4-Dichlorophenoxyacetate Monooxygenase Gene *tfdA* of *Alcaligenes eutrophus* JMP134

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Plasmid pJP4 of *Alcaligenes eutrophus* JMP134 contains all genes for the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). Five of these genes, *tfdB*, *tfdC*, *tfdD*, *tfdE*, and *tfdF*, have recently been localized and cloned (R. H. Don, A. J. Weightman, H.-J. Knackmuss, and K. N. Timmis, J. Bacteriol. 161:85–90, 1985). Gene *tfdA*, which codes for the 2,4-D monooxygenase, has now been found by mutagenesis with transposon Tn*5*. A 3-kilobase fragment of pJP4 cloned in a broad-host-range vector could complement the 2,4-D-negative phenotype of two mutants which lacked 2,4-D monooxygenase activity. The cloned *tfdA* gene was also transferred to *A. eutrophus* JMP222, which is a cured derivative of JMP134. The recombinant strain could utilize phenoxyacetic acid as a sole source of carbon and energy. *Pseudomonas* sp. strain B13, containing the cloned *tfdA*, was able to degrade phenoxyacetic acid and 4-chlorophenoxyacetic acid. Gene *tfdA* was subcloned and analyzed by deletions. Expression of 2,4-D monooxygenase in *Escherichia coli* containing a 1.4-kilobase subfragment was demonstrated by radioisotopic enzyme assay, and a protein of 32,000-dalton molecular mass was detected by labeling experiments. A 2-kilobase subfragment containing *tfdA* has been sequenced. Sequence analysis revealed an open reading frame of 861 bases which was identified as the coding region of *tfdA* by insertion mutagenesis.

Bacteria which degrade halogenated aromatic compounds are of general importance for the detoxification of pesticides and herbicides in nature (17). The extensive use of chlorinated phenoxyalcanoic acid herbicides in agriculture has induced the rapid evolution and dissemination of specific degradative pathways for these compounds in soil and water bacteria. 2,4-Dichlorophenoxyacetic acid (2,4-D) especially is known to be metabolized by organisms belonging to a variety of different bacterial genera, such as *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Corynebacterium*, and *Pseudomonas* (4, 5, 7, 11–13, 16, 33, 35–37).

Alcaligenes eutrophus JMP134 is one of the best-characterized organisms among this group. It harbors a conjugative plasmid, pJP4, of about 80 kilobase (kb) size, which carries genes essential for the degradation of 2,4-D, 2-methyl-4-chlorophenoxyacetic acid, and 3-chlorobenzoic acid (3-CB) (7). Recently this plasmid has been isolated and characterized physically (8). Five genes involved in the degradation of 2,4-D and 3-CB have already been localized by transposon mutagenesis and cloned in *Escherichia coli*. Functions have been assigned to four of them by biochemical studies (9): *tfdB*, *tfdC*, *tfdD*, and *tfdE* encode 2,4-dichlorophenol hydroxylase, dichlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and chlorodienelactone hydrolase, respectively. Transposon insertion mutation of gene *tfdA* encoding the first enzyme in 2,4-D degradation, which is generally designated as a monooxygenase (9, 35), could not be found in this experiment. Another research group, however, reported the expression of the ability to remove the acetate side chain from 2,4-D by an *E. coli* strain containing a cloned 21-kb *Hin*dIII fragment from pJP4 (1). Characterization of gene *tfdA* would complete our knowledge about the degradative functions in *A. eutrophus* JMP134 and would make it available for genetic engineering studies involving manipulation of microbial organisms to broaden their degrading capacity, or involving the potential use of *tfdA* as a gene specifying herbicide resistance for plants.

We have now isolated transposon mutants of *A. eutrophus* JMP134 inactivated in 2,4-D monooxygenase and have localized gene *tfdA* by cloning and deletion analysis. We have shown the expression of the cloned 2,4-D monooxygenase in different strains, detected the corresponding protein by specific labeling, and determined the nucleotide sequence of a 2-kb fragment containing the *tfdA* coding region.

MATERIALS AND METHODS

Strains and plasmids. *A. eutrophus* JMP134 (7) contains plasmid pJP4 and degrades 2,4-D (Tfd^+) and 3-CB ($3cb^+$). Strain JMP222 is a streptomycin-resistant, pJP4-negative derivative of strain JMP134 and is Tfd^- and $3cb^-$ (7). *Pseudomonas* sp. strain B13 (10) degrades 3-CB and 4-chlorophenol (29). *E. coli* LE392 (27) was used for cloning experiments. *E. coli* HB101 (6) was the recipient for the conjugative transfer of mutated pJP4 derivatives. *E. coli* S17-1 (32) contains a chromosomally integrated RP4 derivative which is able to mobilize broad-host-range plasmids. Strain K38 (=C600)(2) containing plasmid pGP1-2 (34) can express T7 RNA polymerase controlled by a heat-sensitive lambda repressor, and plasmids pT7-5 and pT7-6 (S. Tabor, unpublished data) carry the corresponding T7 promotor in front of a multiple cloning site. Plasmids pVK101 (22), pGSS33 (30) and pKT231 (3) are mobilizable broad-host-range vectors. pSUP2021 is a mobilizable plasmid carrying transposon Tn*5* (specifies kanamycin resistance). For sequencing we used M13tg130 and M13tg131 (21). pDOC37 (D.

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O'Connor, unpublished data) was the source of the omega fragment (15).

Media and cultural conditions. *A. eutrophus* strains were grown with aeration at 30°C. PYE (7) was used as complete medium; liquid PYE medium was supplemented with fructose (10 mM) when cells were grown for preparative plasmid isolation. Minimal medium contained (per liter): K_2HPO_4, 1.6 g; KH_2PO_4, 0.4 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; and $FeSO_4 \cdot 7H_2O$, 0.01 g. Carbon sources were added to the following final concentrations: 2,4-D, 1 mM; 3-CB, 2 mM; 4-chlorophenoxyacetic acid, 1 mM; phenoxyacetic acid (PAA), 4 mM; fructose, 10 mM; and sodium pyruvate, 15 mM. Matings on solid medium were done on PYE agar plates (7). *E. coli* strains were grown in LB medium (25) at 37°C if not otherwise specified. Antibiotics were incorporated at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; streptomycin, 25 μg/ml; and spectinomycin, 80 μg/ml.

Conjugative crosses. For transposon mutagenesis, donor and recipient cells were grown in liquid culture to the late exponential phase and mixed in a 1:1 ratio relative to their optical density. Samples of 1.5 ml of the mixture were spread onto PYE plates and incubated overnight at 30°C. Plate surfaces were then washed with 0.7% NaCl solution, the cell suspension was diluted, and samples were spread onto selective plates.

For complementation tests, mutant strains were grown overnight in liquid culture and spread onto 2,4-D minimal plates, and donor strains were streaked onto plate surfaces. Conjugation and selection were simultaneously done by incubation at 30°C for 14 days.

For all other conjugations, cells were grown in liquid culture overnight, mixed, and spotted onto PYE plates. After incubation at 30°C for 4 to 6 h, cells were suspended in 0.7% NaCl solution, diluted, and spread onto selective plates.

Transposon mutagenesis. The mobilizable transposon carrier plasmid pSUP2021 (32) was transferred by conjugation from the mobilizing donor strain *E. coli* S17-1 (32) to *A. eutrophus* JMP134. Transconjugants were selected from mating mixture on fructose minimal medium containing 380 μg of kanamycin per ml and subsequently screened for loss of 2,4-D degradative ability by replica plating on minimal medium containing 2,4-D as the single carbon source. The absence of degradative functions was also verified by testing on 2,4-D indicator medium (24).

Oxygen uptake assays. *A. eutrophus* wild-type or mutant strains were grown overnight in 250 ml of minimal medium containing 15 mM sodium pyruvate. For induction of 2,4-D-degradative genes, the same medium was used with addition of 3-CB to a final concentration of 1 mM (9). Cells were harvested by centrifugation at 4°C, washed three times in cold minimal medium without carbon source, and finally suspended in a portion of minimal medium to an optical density at 420 nm (A_{420}) of 30.

For assay of 2,4-D monooxygenase or 2,4-dichlorophenol hydroxylase activity, 1 volume of cell suspension was mixed with 9 volumes of minimal medium saturated with oxygen to yield an A_{420} of 3, and background level of oxygen uptake was determined for 10 min. Then substrate was added to a final concentration of 1 mM 2,4-D, 1 mM PAA, or 0.2 mM 2,4-dichlorophenol, and linear decrease of oxygen concentration was measured over 20 min.

Preparative plasmid isolation. Plasmid pJP4 and transposon Tn*5* containing derivatives of pJP4 were isolated from *A. eutrophus* by the procedure of Hansen and Olsen (18). Plasmids from *E. coli* were prepared by the alkaline lysis method described by Maniatis et al. (25).

Radioisotopic 2,4-D assay. For the assay of 2,4-D monooxygenase in *E. coli*, cells of strain K38 containing both pGP1-2 and the pT7 recombinant plasmid were induced for overproduction of enzyme. They were grown at 30°C in 20 ml of LB medium under ampicillin and kanamycin selection. At an A_{590} of 1.0 the temperature was raised to 42°C for 25 min, rifampin was added to a final concentration of 100 μg/ml, and cells were shaken for 2 h at 37°C. Harvesting, washing, and incubation followed the protocol given by Amy et al. (1) with the following modifications: 20 ml of cell suspension was incubated with 0.1 μCi of ^{14}C-labeled 2,4-D in a 250-ml Warburg flask, which was closed with a tapered glass stopper and a sérum stopper. A 0.5-ml volume of beta-phenylethylamine was placed in the central vial, and 2 ml of 1 N H_2SO_4 was injected into the surrounding cell suspension. After 1 h of gentle shaking, the trapped $^{14}CO_2$ was eluted from the central vial with 5 ml of Rotiszint 22 (Roth GmbH & Co. Chemische Fabrik, Karlsruhe, Federal Republic of Germany) and counted for radioactivity.

Radioactive labeling of plasmid-encoded proteins. The following protocol was received from Stan Tabor (Harvard Medical School, Boston, Mass.). Cells of *E. coli* K38 containing both pGP1-2 and the pT7-recombinant plasmid were grown in LB medium under ampicillin and kanamycin selection. At an A_{590} of 0.5, 0.2 ml of cells was centrifuged, washed in 5 ml of M9 medium (25), recentrifuged, and suspended in 1.0 ml of M9 medium supplemented with 20 μg of thiamine per ml and 18 proteinogenic amino acids (each 0.01%, without cysteine and methionine). The cells were shaken at 30°C for 60 min, and then the temperature was increased to 42°C. After 15 min, 10 μl of a rifampin stock solution (20 mg/ml in methanol) was added to a final concentration of 200 μg/ml, and the cells were left at 42°C for 10 min. Thereafter the temperature was decreased to 30°C for 20 min, and samples were then pulsed with 10 μCi of L-[^{35}S]methionine (Amersham; cell labeling grade) for 5 min at room temperature and centrifuged. The cell pellet was suspended in 120 μl of cracking buffer (60 mM Tris hydrochloride, pH 6.8, 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue). The samples were heated to 95°C for 3 min and loaded onto an SDS-polyacrylamide gel as described by Laemmli (23).

RESULTS

Transposon mutants of pJP4. Of 1,000 kanamycin-resistant clones which had Tn*5* stably integrated in their genome, we isolated 8 mutants that were unable to grow on 2,4-D. All of these strains carried the transposon on plasmid pJP4, as determined by conjugative transfer of kanamycin resistance to *E. coli* HB101 and *A. eutrophus* JMP222. Four 2,4-D$^-$ mutants were still able to grow on 3-CB, thus belonging to mutant class A, B, or F, as described by Don et al. (9). To determine the specific enzyme defects, oxygen uptake of these strains was measured during incubation of whole cells with 2,4-D and 2,4-dichlorophenol as substrates. Two mutant strains were deficient in 2,4-D monooxygenase activity, whereas their 2,4-dichlorophenol hydroxylase was still active when 3-CB was used as an inducer. This fact assigns these strains to mutant class A.

Restriction mapping of pJP4 derivatives of *tfdA* mutants showed that in addition to insertion of Tn*5*, large deletions or rearrangements had occurred which involved *Eco*RI fragments C, E, G, H, and I. Because of their complexity they could not be physically characterized further.

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Cloning of *tfdA*. We first cloned the 21-kilobase *Hin*dIII fragment of pJP4 in pVK101. A restriction map of the fragment is shown in Fig. 1. The recombinant plasmid pVJH21 was transferred by direct conjugation on selective medium to *tfdA*-defective transposon mutants, using *E. coli* S17-1 as a mobilizing donor strain. Colonies appeared in mating experiments on 2,4-D medium after 14 days and showed wild-type growth. The system was further used to test subcloned fragments for complementation or marker rescue of Tn*5* mutants. *Sac*I fragments from pVJH21 were shotgun-ligated with the broad-host-range vector plasmid pGSS33, and *E. coli* LE392 was transformed with the DNA. Recombinant plasmids were isolated, identified, brought into strain S17-1 by transformation, and subsequently transferred to the transposon mutants. Plasmids such as pGJS3 containing a 3-kilobase *Sac*I fragment of pJP4/pVJH21 were able to restore 2,4-D metabolism in both mutants.

Expression in *A. eutrophus* JMP222. Strain JMP222 is able to grow on phenol as a carbon source by using chromosomally located genes for a meta-cleavage pathway, but cannot grow on PAA. On the other hand, we know from oxygen uptake assays with induced cultures of strain JMP134 that the *tfdA*-encoded 2,4-D monooxygenase can also convert unsubstituted PAA to phenol. Thus, growth on PAA was chosen as a test for expression of *tfdA* in strain JMP222. The 3-kb *Sac*I fragment was subcloned by using the broad-host-range vector pKT231, and hybrid plasmids pKJS31 and pKJS32 (inserts in opposite orientations) were subsequently mobilized from *E. coli* S17-1 to *A. eutrophus* JMP222. Colonies growing on PAA (Paa$^+$) appeared after 3 to 4 days; no difference was observed in the behavior of clones carrying the different hybrid plasmids. All tested Paa$^+$ colonies were kanamycin resistant, whereas from colonies selected on kanamycin several had lost the ability to grow on PAA.

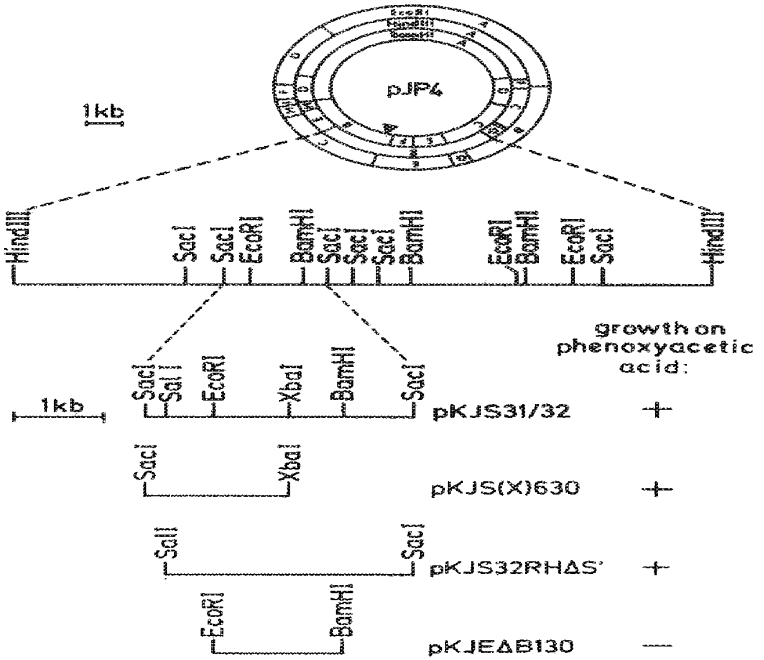


FIG. 1. Cloning and deletion analysis of *tfdA*. Hybrid plasmids containing fragments of pJP4 cloned in broad-host-range vector pKT231 were transferred from *E. coli* S17-1 to *A. eutrophus* JMP222 by conjugation. Transconjugants carrying an intact *tfdA* gene were able to grow on PAA as a carbon source. The localization of *tfdA* on pJP4 is indicated by the solid triangle.

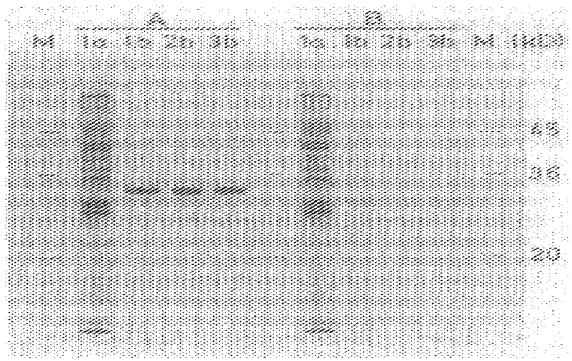


FIG. 2. Expression of gene *tfdA* by T7 RNA polymerase. Three DNA fragments able to confer PAA degradation on *A. eutrophus* JMP222 (1, 2.8-kb *Sac*I-*Sal*I; 2, 2.1-kb *Bam*HI-*Sal*I; 3, 1.4-kb *Xba*I-*Sal*I) were cloned in T7 promoter plasmids pT7-5 (A) and pT7-6 (B), yielding constructions with both orientations of the insert in relation to the T7 promoter. Strains of *E.coli* K38 carrying a heat-inducible T7 RNA polymerase gene on plasmid pGP1-2 and one of the hybrid pT7 plasmids were used for specific labeling of plasmid-encoded proteins: gene expression was induced by heat, the host RNA polymerase was inhibited with rifampin, and cells were pulsed with L-[^{35}S]methionine as described in Materials and Methods (lanes b). For labeling of total cell protein, samples were treated omitting both heat induction and rifampin addition (lanes a). Proteins were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and revealed by autoradiography. Transcription from the *Xba*I to the *Sal*I site resulted in expression of a 32,000-dalton protein (panel A), whereas no protein was expressed from opposite orientations (panel B). M, Molecular weight markers (transferred from Coomassie blue-stained gel).

Expression in *Pseudomonas* sp. strain B13. As the side-chain cleavage enzyme encoded by *tfdA* accepts differently substituted and unsubstituted PAAs as substrates, we supposed that introduction of *tfdA* into *Pseudomonas* sp. strain B13, which is able to degrade 4-chlorophenol (29), could extend the degradative capacity of strain B13 to 4-chlorophenoxyacetic acid. Plasmids pKJS31 and pKJS32 were transferred by conjugation from *E. coli* S17-1 to *Pseudomonas* sp. strain B13. As expected, transconjugants were able to grow on 4-chlorophenoxyacetic acid, and, in addition, they could also use PAA as a growth substrate.

Deletion analysis. We used the growth of strain JMP222 on PAA as an appropriate system to test constructions with several deletions for the expression of *tfdA*. Small DNA fragments were deleted between unique restriction sites on pKT231 and the *Sac*I insert. Up to the *Xba*I site from one

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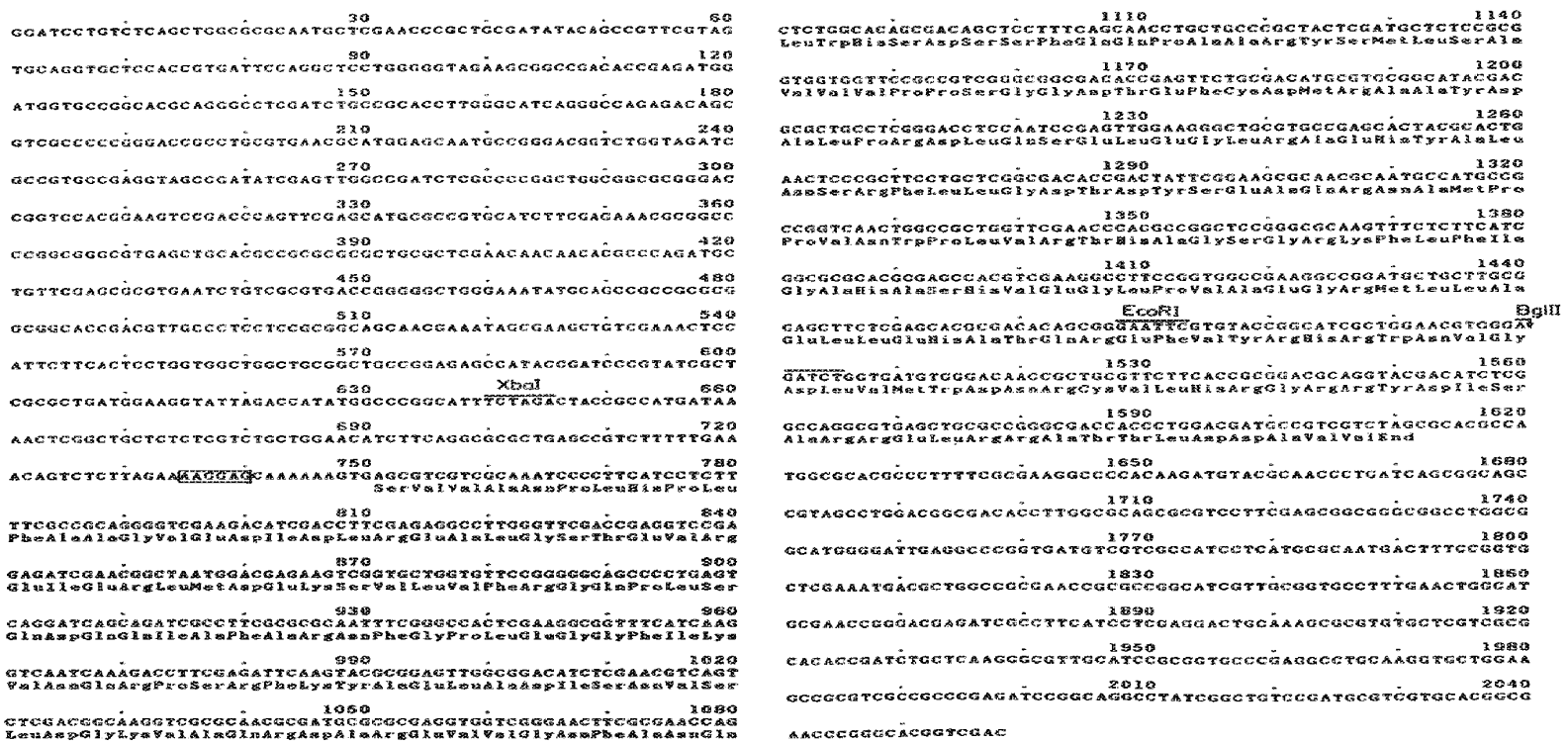


FIG. 3. Nucleotide sequence of the 2.06-kb *Bam*HI-*Sal*I fragment of pJP4 encoding the 2,4-D monooxygenase gene *tfdA*. The coding strand of the DNA is presented in the 5′-to-3′ direction along with the deduced amino acid sequence of the only possible open reading frame. The *Bgl*II cleavage site where the omega fragment was inserted is indicated at nucleotide 1500. A putative Shine-Dalgarno sequence in front of the coding region is enclosed in a box.

end and to the *Sal*I site from the other end, deletions could be made without loss of degradative activity (Fig. 1), whereas deletion of the smaller *Eco*RI-*Sac*I fragment resulted in inactivation of *tfdA*.

Specific labeling of *tfdA* gene product. All fragments able to confer PAA degradation on strain JMP222 were subcloned from pKT231 in T7 RNA polymerase-promoter plasmids pT7-5 and pT7-6, yielding constructions with both orientations of the insert in relation to the promoter. Plasmid-encoded proteins were labeled with L-[^{35}S]methionine, separated by SDS-polyacrylamide gel electrophoresis, and revealed by autoradiography. Figure 2 shows the expression of a single protein from all constructions in which the T7 RNA polymerase reads from the *Xba*I to the *Sal*I restriction site. The protein molecular weight was determined to be about 32,000 by comparison with standard protein markers.

Expression of 2,4-D monooxygenase in *E. coli*. pTJS′X535 is a hybrid plasmid of pT7-5 and the *Sal*I-*Xba*I fragment of pJP4 (Fig. 1). *E. coli* K38, containing both pGP1-2 and pTJS′X535, was able to release $^{14}CO_2$ from 2,4-D labeled in position 2 of the acetate side chain. Kinetic assays show that 70% of total trapped radioactivity is released within the first hour of incubation, thus indicating a high level of expression of 2,4-D monooxygenase from the T7 RNA promoter.

Nucleotide sequence of the cloned *tfdA* DNA. The 2.8-kb *Sac*I-*Sal*I fragment shown in Fig. 1 was subcloned in replicative forms of both phages M13tg130 and M13tg131. To create a series of deletions for primed dideoxynucleotide sequencing, unidirectional digestion with exonuclease III was carried out by the method of Henikoff (20). For this purpose, recombinants from M13tg130 were cleaved by *Sal*I and *Pst*I, whereas recombinants from M13tg131 were cleaved by *Sac*I and *Bam*HI. Clones containing plasmids with deletions were analyzed by restriction endonuclease cleavage of replicative forms, and appropriate deletions were chosen for sequencing. The nucleotide sequence was determined by the method of Sanger et al. (28).

Translation of the sequence shown in Fig. 3, from the *Xba*I to the *Sal*I site where *tfdA* gene activity is expressed, reveals one possible open reading frame which corresponds

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in its length to a protein size of 32,000 as determined from electrophoresis of labeled *tfdA* gene product.

Insertion mutagenesis of cloned *tfdA*. As 2,4-D monooxygenase activity was directly correlated with expression of a protein of 32,000 molecular weight and with presence of an intact open reading frame of 861 base pairs, we assumed that insertion of transcriptional and translational stop signals at a *Bgl*II site situated within this reading frame would result in expression of a truncated protein and in inactivation of 2,4-D monooxygenase. We excised the omega fragment from pDOC37 with *Bam*HI and inserted it into a *Bgl*II-cleaved pTJS'X535. Specific labeling by the method of Tabor and Richardson (34) as described above revealed a protein of 29,000 molecular weight (Fig. 4), which matches the predicted size calculated from a shortened open reading frame of 768 base pairs. No enzymatically active 2,4-D monooxygenase was expressed from the omega-mutagenized plasmid, as shown by radioisotopic 2,4-D assay (Table 1).

DISCUSSION

In this report we describe the cloning and characterization of gene *tfdA* from *A. eutrophus* JMP134, which encodes the first enzymatic step in the degradation of 2,4-D. The identity of the cloned gene was confirmed by the following criteria: (i) complementation of *tfdA*-defective transposon mutants; (ii) expression of PAA degradative capacity in *A. eutrophus* JMP222; and (iii) expression of 2,4-D side chain cleavage in *E. coli*.

We have identified the protein encoded by *tfdA*, and we have determined the nucleotide sequence of the cloned gene. One possible open reading frame was found, which starts at base 748 with a GTG codon and ends at base 1608 in front of a TAG stop codon. The translational start was confirmed by determination of the first 16 N-terminal amino acids of the purified protein (data not shown). Upstream of base 748, a Shine-Dalgarno sequence can be identified (base 735). Estimation of a molecular weight of 31,000 to 33,000 for the *tfdA* gene product by SDS-polyacrylamide gel electrophoresis is in agreement with the molecular weight of 32,171 predicted from the 861-nucleotide open reading frame. Functional correlation of the nucleotide sequence and enzyme activity has been shown by insertion mutagenesis.

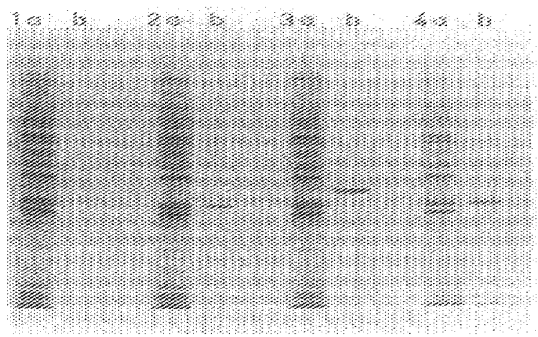


FIG. 4. Omega mutagenesis of gene *tfdA*. The omega fragment, which carries transcriptional and translational stop signals, was inserted at a *Bgl*II site situated within the coding region of *tfdA* on plasmid pTJS'X535. Gene expression was induced using the T7 RNA polymerase-promoter system. Protein was labeled with L-[^{35}S]methionine, loaded onto a 12.5% SDS-polyacrylamide gel, and revealed by autoradiography. No protein is expressed from the vector plasmid pT7-5 (lanes 1). A truncated *tfdA* gene product is expressed from both omega-mutagenized plasmids pTJS'X535omega (lanes 2 and 4), whereas a full-length protein is expressed from pTJS'X535 (lanes 3). Samples were labeled after heat induction and addition of rifampin (lanes b) or omitting induction and rifampin addition (lanes a).

TABLE 1. Correlation of 2,4-D monooxygenase activity, protein length, protein length, and open reading frames in a *tfdA*-pT7 hybrid plasmid and its insertion derivative

Plasmid	Enzyme activity[a] (kcpm)	Mol mass of protein[b] (daltons)	Open reading frame[c] (base pairs)
pT7-5	0.3		
pTJS'X535	70	32,000	861
pTJS'X535omega	0.4	29,000	768

[a] Measured release of $^{14}CO_2$.
[b] Determined by SDS-polyacrylamide gel electrophoresis.
[c] From GTG (base 748) to first in-frame stop.

It is now of interest to elucidate the regulation of 2,4-D degradation in strain JMP134. The promoter which is obviously present on the cloned *tfdA* fragment could serve as a tool for the search of a regulatory gene. Sequence analysis shows an AT-rich area upstream of the coding region, and insertion of a DNA fragment into the *Xba*I site results in poor expression of *tfdA* if a foreign promoter is not provided on the insert (data not shown). Promoter structures can therefore be expected around that area.

Interestingly, gene *tfdA* is located at a distance of 13 kb from the gene cluster encoding the hydroxylation and *meta*-cleavage of 2,4-dichlorophenol. A similar separation of catabolic genes into upper and lower pathway gene clusters has also been observed on the NAH (naphthalene degradation) plasmid (39) and the TOL (toluene degradation) plasmid (14). This finding provides additional support for the "module" theory, which postulates an evolution of degradative pathways by sequential assembly of distinct parts of the pathway (14). Together with conjugation, this provides gram-negative bacteria with an enormous flexibility for the evolution of new degradative functions.

Another trait which gene *tfdA* has in common with other catabolic genes is the broad substrate specificity of the encoded enzyme. It is able to use 2,4-D, 2-methyl-4-chlorophenoxyacetic acid, 4-chlorophenoxyacetic acid, and PAA as substrates. A variety of related compounds which have not yet been tested may perhaps be metabolized as well. Similar broad specificities have been demonstrated for xylene oxidase (19, 26). We can expect therefore that the cloned *tfdA* gene will be useful in the construction of novel pathways. Potential uses of gene *tfdA* may be seen not only in the field of bacterial degradative functions, but also in genetic engineering of plants. The metabolism of 2,4-D by some plant species has already been reported (31, 38). We achieved tolerance of a *Nicotiana silvestris* haploid cell suspension culture against 2,4-D by adapting it to higher concentrations of the synthetic growth hormone (40). As gene *tfdA* specifies the side chain cleavage of 2,4-D, the capacity of plant cells to detoxify chlorinated PAA herbicides could be increased by the transformation of cells with *tfdA*, followed by expression of an enzymatically active protein. Thus, another bacterial gene would be available, in

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addition to the prevalently used neomycin phosphotransferase gene, to function as a selectable marker gene for plant genetic experiments, and perhaps transformed cells can be regenerated to whole, herbicide-resistant plants.

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2,4-Dichlorophenoxyacetate/α-ketoglutarate dioxygenases from *Burkholderia cepacia* 2a and *Ralstonia eutropha* JMP134

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Key words: 2,4-D/α-ketoglutarate dioxygenase, *B. cepacia*, *R. eutropha*

Abstract

2,4-Dichlorophenoxyacetate (2,4-D)/α-ketoglutarate (α-KG) dioxygenase has been purified to apparent homogeneity from *Burkholderia cepacia* strain 2a, which utilizes 2,4-D as sole carbon source. The enzyme required ferrous ions, and was a homodimer composed of subunits having an M_r of ~32,000. The reaction catalysed consumed one mol each of 2,4-D, α-KG and dioxygen, with the production of one mol each of succinate, 2,4-dichlorophenol and glyoxylate. Maximum activity was exhibited at pH 7.8 and 25°C, and reactivity was enhanced by the presence of ascorbate and cysteine. Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+} and Co^{2+} were inhibitory, and chemical modification of the dioxygenase revealed that thiol groups were essential for activity. The enzyme was active towards other substituted phenoxyacetates, but reacted most rapidly with 2,4-D. The apparent Michaelis constants for 2,4-D and α-KG were 109 and 8.9 μM, respectively. The properties of this enzyme are compared with those of the 2,4-D/α-KG dioxygenase from *Ralstonia eutropha* JMP134, which exhibits a differing N-terminal amino-acid sequence, and a different temperature 'optimum', pH optimum, substrate specificity and sensitivity to thiol-binding reagents.

Introduction

The degradation of chlorinated phenoxyacetate herbicides, in particular 2,4-dichlorophenoxyacetate (2,4-D) and 2-methyl-4-chlorophenoxyacetate (MCPA), has been the subject of investigation for more than 50 years. Loos *et al.* (1967a,b,c) and Evans *et al.* (1971) demonstrated that 2,4-D was metabolized *via* 2,4-dichlorophenol (2,4-DCP) and 3,5-dichlorocatechol prior to 1:2-ring fission and conversion to products metabolized by the tricarboxylic acid cycle. Ether-cleavage activity, which is responsible for the removal of the methylenecarboxy group from 2,4-D to produce the corresponding phenol, is the first step in the degradation.

Cell-free oxygen-dependent ether-cleavage activity was first demonstrated in a 2,4-D-degrading strain of *Arthrobacter* (Tiedje and Alexander, 1969). Helling *et al.* (1968) showed that glyoxylate was the initial product formed by removal of the methylenecarboxy side-chain in the conversion of [^{18}O]-ether-labelled phenoxyacetate to [^{18}O]-phenol. The degradation of 2,4-D has since been intensively investigated in *Ralstonia eutropha* (= *Alcaligenes eutrophus*) JMP134

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(Don *et al.*, 1985). The gene encoding the ether-cleavage enzyme, *tfdA*, cloned from plasmid pJP4 of *R. eutropha* JMP134, expressed the same activity in *Escherichia coli* (Streber *et al.*, 1987). Fukumori and Hausinger (1993a,b) purified the product of the *tfdA* gene expressed in *E. coli* pUS311. Concomitantly, with the dissimilation of 2,4-D to 2,4-DCP, the enzyme converted α-ketoglutarate (α-KG) to succinate and carbon dioxide:

$$2,4\text{-D} + O_2 + \alpha\text{-KG} \rightarrow 2,4\text{-DCP} + \text{succinate} + CO_2 + \text{glyoxylate}$$

The requirement of the enzyme for ferrous ions and stimulation of activity by ascorbate indicated that the enzyme was an α-KG-dependent dioxygenase (Abbott and Udenfriend, 1974). The purified enzyme, designated 2,4-D/α-KG dioxygenase, was reported by Fukumori and Hausinger (1993b) to be a homodimer of M_r 50,000 composed of M_r 32,000.

In this paper, we report the purification and characterization of the corresponding enzyme from *Burkholderia cepacia* 2a, and compare it with 2,4-D/α-KG dioxygenase extracted directly from *R. eutropha* JMP134. The 2,4-DCP hydroxylase from *B. cepacia* 2a (EC 1.14.13.20) was described in an earlier paper (Beadle and Smith, 1982).

Materials and methods

Chemicals

Most chemicals were of analytical reagent grade and were obtained from the Sigma-Aldrich Company (Poole, Dorset) or BDH Chemical Company (Poole, Dorset). 2,4-Dichlorophenoxyacetic acid and 2,4-dichlorophenol were purchased from ICN Biomedicals Ltd (Oxford). Bacto-tryptone, Bacto-yeast extract and agar were supplied by Difco Laboratories Ltd (West Molesey, Surrey).

Organisms

Ralstonia eutropha JMP134 (pJP4) (Don and Pemberton, 1981) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen. *Burkholderia cepacia* 2a (pIJB) was isolated in 1965 by one of us (ARWS) from Bangor (North Wales) garden soil, using an enrichment technique with 2,4-D as the sole carbon source. Strain 2a was initially misidentified at NCIMB, Aberdeen, as *Acinetobacter* sp. (Beadle and Smith, 1982), on the basis of apparent nonmotility and a weakly-positive oxidase reaction. However, the organism was consistently observed by us to be both motile and strongly oxidase-positive, and re-examination by Mrs A. J. Baxter at NCIMB revealed that strain 2a was *Pseudomonas cepacia* (= *B. cepacia*; Yabuuchi *et al.*, 1992).

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Strains were cultured and maintained as described previously by Beadle and Smith (1982). The characteristics of organism 2a are as follows.

Cell morphology

Strain 2a was a Gram-negative, nonsporing, motile coccobacillus. Phase contrast microscopy showed that the coccobacilli occurred singly, with some in pairs, after growth in nutrient broth containing 0.75% agar for 2 h at 30°C.

Colony morphology

On yeast-mannitol agar at 3 days, colonies of strain 2a were 3 mm in diameter, white, round, entire, convex, shiny, opaque mucoid, and spreading. Growth occurred on nutrient agar and in nutrient broth at 30°C but not at 37, 41 or 45°C.

Biochemistry

B. cepacia were catalase and oxidase (Kovacs)-positive within 10 s, and glucose was attacked oxidatively. The API rapid test (3 days at 30°C) showed that the bacterium was cytochrome oxidase-positive; NO_3^- was not reduced, nor indole produced, and arginine dihydrolase was absent. Aesculin and gelatin were not hydrolysed; no acid was produced from glucose; and urease and β-galactosidase were produced. Glucose, mannose, mannitol, arabinose, N-acetyl-glucosamine and gluconate were assimilated, but not maltose. Caprate, adipate, malate and citrate were assimilated poorly, and phenylacetate was not assimilated. Results for nitrate reduction, acid from glucose, and urease production were not confirmed in 7-day tests.

Further tests

After 7 days at 30°C, L-arginine, betaine, acetate, citrate (Koser's), glucose and fructose were utilized. Allantoin and tartrate were rendered alkaline, but not acetamide. Phenylalanine was not deaminated, 3-ketolactose not produced, and urease was absent. Arginine, lysine and ornithine were not decarboxylated (Moeller). Nitrate was reduced to nitrite but not converted to nitrogen. Deoxyribonuclease was absent, whereas Tween 80 was hydrolysed, but not gelatin, casein or starch. Weak growth occurred at 41°C in yeast-mannitol broth.

Except for the nonutilization of phenylacetate and the absence of ornithine and lysine decarboxylases, the results indicated identification as *B. cepacia*.

Bulk cultures

B. cepacia 2a was grown in 2,4-D-minimal salts medium (Beadle and Smith, 1982) in a 15 litre fermenter (Chemap AG, Volketswil,

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Switzerland) at 25°C with stirring. Air was supplied at the rate required to maintain pO_2 at 30% saturation. The pH was maintained at 6.5 by the automatic addition of 0.5 M Na_2CO_3. Four portions of substrate each equivalent to 2 g 2,4-D (1 of medium)$^{-1}$ were supplied during growth. *R. eutropha* JMP134 was grown under similar conditions except that the medium pH was 7.0 and the temperature was 30°C. Cells were harvested by centrifugation whilst 2,4-D was still present, and washed once in 50 mM sodium phosphate buffer at pH 6.5. Cell paste (yield ~1 g l^{-1}) was stored at −20°C.

Purification of the 2,4-D/α-KG dioxygenases

Thawed cell paste of either strain (5 g) was suspended in 5 ml of 50 mM citric acid-Na_2HPO_4 buffer at pH 6.5, containing 5 mM cysteine hydrochloride, and subjected to sonic irradiation at 20 kHz for 10 min at 0°C. All subsequent operations were carried out at 4°C. The crude extract was centrifuged at 30,000 x *g* for 30 min to remove unbroken cells and cell debris.

Buffers for the purification procedure detailed below were of pH 7.8 for *B. cepacia* 2a and 7.4 for *R. eutropha* JMP134. The supernatant was applied directly to a Q-Sepharose HP column (1.6 x 10 cm; Amersham Pharmacia Biotech Ltd) equilibrated with 20 mM Tris-HCl buffer. Occasionally a DEAE-Sepharose Fast Flow column (Amersham Pharmacia Biotech Ltd) was used instead. The column was washed with 20 mM Tris-HCl buffer, at a flow rate of 60 ml h^{-1}, then eluted at the same rate with a NaCl gradient (0.0–0.4 M in 20 mM Tris-HCl buffer; total volume 400 ml). Fractions with dioxygenase activity were pooled and concentrated in Macrosep 30k centrifugal concentrators (Filton Technology Co., Northborough, Massachusetts, U.S.A.) by centrifugation at 5,000 x *g*, until the total volume was reduced to ~5 ml. The concentrated preparation was layered onto a Sephacryl S-200 High Resolution column (1.6 cm i.d. x 65 cm; Amersham Pharmacia Biotech Ltd) and eluted with 20 mM Tris-HCl at a flow rate of 65 ml h^{-1}. Pooled active fractions were concentrated by centrifugation as above. The purified enzyme was mixed with glycerol (final concentration, 20% v/v), and stored in liquid nitrogen.

Assay of 2,4-D/α-KG dioxygenase activity

Activity was routinely assayed by measurement of the phenol produced by the enzyme-catalysed oxidation of the substrate, using a modification of the colorimetric method of Lacoste *et al.* (1959). For dioxygenase freed of 2,4-DCP hydroxylase, 2,4-D was used as the substrate, and the accumulation of 2,4-DCP was measured. For crude dioxygenase preparations, phenoxyacetate was the substrate, since

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the liberated phenol was not oxidized by 2,4-DCP hydroxylase (Beadle and Smith, 1982). The standard assay system routinely contained, in a total volume of 200–500 µl, ascorbate, ferrous sulphate, α-KG and phenoxyacetate or 2,4-D, each at 1 mM, together with 1–5 mM cysteine hydrochloride in 100 mM Tris-HCl buffer at pH 7.8 (*B. cepacia* strain 2a) or 7.4 (*R. eutropha* strain JMP134). Incubation was performed in a shaking water-bath at 25°C, and reactions were initiated by addition of the enzyme. Samples of reaction mixtures were rapidly diluted at intervals into 0.5 ml 0.27 M boric acid/0.18 M NaOH buffer containing 20 mM EDTA to halt the reaction. Then 25 µl of 0.3% (w/v) 4-aminophenazone was added, followed by 50 µl of 1% (w/v) potassium ferricyanide, and the resultant colour was read at 505 nm. Blanks contained no substrate, and standard curves were prepared using authentic 2,4-DCP or phenol. Enzyme activity was expressed on the basis of either 2,4-D or phenoxyacetate consumption. The 2,4-D unit is defined as that amount which at 25°C and pH 7.8 (for *B. cepacia* strain 2a) or 30°C and pH 7.4 (for *R. eutropha* strain JMP134) converts 1 µmol 2,4-D into 2,4-DCP per min; the phenoxyacetate unit as that amount of enzyme which converts 1 nmol phenoxyacetate into phenol per min.

Enzyme activity was also measured by following oxygen uptake at 25°C in a Clark oxygen electrode (Rank Bros., Bottisham, Cambridge, England) using the same assay mixture in a total volume of 2.0 ml.

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Determination of protein

Protein was routinely assessed by the method of Bradford (1976), using Coomassie protein assay reagent (Pierce Ltd, Chester) with bovine serum albumin (fraction V) as the reference standard. To determine protein in chromatographic fractions, microtitre plates were used with the bicinchoninic acid protein assay reagent kit (Pierce Ltd, Chester) (Brown *et al.*, 1989), using a bovine serum albumin standard.

Determination of M_r

Relative molecular masses of the native enzymes were determined by gel filtration on a Sephacryl S-200 High Resolution column, as described above. The column was calibrated with the following markers: cytochrome c, M_r 12,400; carbonic anhydrase, M_r 29,000; bovine serum albumin, M_r 66,000; β-amylase, M_r 200,000. The subunit M_r of the enzyme was determined by sodium dodecyl sulphate-polyacrylamide (12% w/v) gel electrophoresis (Laemmli, 1970) using the following proteins as M_r standards: bovine albumin, M_r 66,000; egg albumin, M_r 45,000; glyceraldehyde-3-phosphate dehydrogenase, M_r 36,000; carbonic anhydrase, M_r 29,000; trypsinogen, M_r 24,000; trypsin inhibitor, M_r 20,100; α-lactalbumin, M_r 14,200.

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N-terminal sequencing of 2,4-D/α-KG dioxygenase of *B. cepacia* 2a

The 2,4-D/α-KG dioxygenase of *B. cepacia* was further resolved from minor contaminant proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The single major band was recovered by electro-transfer onto a Problott (PVDF) membrane (Perkin-Elmer Corp.), dried, and submitted for N-terminal amino-acid sequencing by automated Edman degradation, to the Department of Biochemistry, University of Cambridge.

Determination of glyoxylate and succinate

Glyoxylate was determined by the method of Paget and Berger (1938), in which glycerate, oxalate, citrate, malonate, glycollate and 2,4-D produced no colour (Gamar and Gaunt, 1971). In the present investigation, α-KG and succinate were also tested and gave no reaction.

Glyoxylate was assayed by high performance chromatography (HPLC), using a C_{18} reverse-phase column (Spherisorb ODS2, 5 μm; 15 cm x 46 mm) at room temperature, and a Kontron HPLC 332 UV detector operated at 210 nm. Sodium phosphate-HCl buffer (20 mM) at pH 2.8, was used as the mobile phase at a flow rate of 0.5 ml min^{-1}.

Succinate was determined by the same procedure, except that the mobile phase was 100 mM phosphoric acid (0.5 ml min^{-1}). The identity of succinate was confirmed by mass spectrometry.

Results

Growth on 2,4-D-salts medium

Provided that pO_2 did not exceed 30% saturation, cells of both organisms grew satisfactorily with a doubling time of 7 h, and if harvested before substrate depletion, yielded active extracts.

2,4-D/α-KG dioxygenase activity in crude cell-free extracts of *B. cepacia*

In the presence of dioxygen, unsupplemented extracts of *B. cepacia* 2a rapidly converted phenoxyacetate to phenol over a period of 10–15 min, after which the reaction terminated before complete substrate consumption, due to exhaustion of the endogenous cofactors. Activity was regenerated in both nondialysed and dialysed extracts by the addition of α-KG, ferrous ions, ascorbate and cysteine.

Purification

A representative scheme for the purification of 2,4-D/α-KG dioxygenase from *B. cepacia* 2a is summarized in Table 1. After 55-fold purification, the preparation contained 20% of the activity of the crude cell-extract. On sodium dodecyl sulphate-polyacrylamide gels, the purified enzyme gave a single prominent

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Table 1 Representative purification table for 2,4-D/α-KG dioxygenase from *B. cepacia* strain 2a

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (%)	Purification (-fold)
(1) Cell-free extract	9.6	119	1,435	12.1	100	1
(2) DEAE-Sepharose	5.0	2.0	448	221	31.2	18.3
(3) Sephacryl S-200	1.2	0.44	291	662	20.3	54.8

One unit is defined as the amount of enzyme required to convert 1 nmol of phenoxyacetate into phenol per min at 25°C.

band. The dioxygenase from *R. eutropha* strain JMP134 was purified by the same procedure as above, in similar yield.

Determination of M_r

The relative molecular mass of the native dioxygenase from *B. cepacia* was estimated by gel filtration to be 64,900; dioxygenase from *R. eutropha* gave M_r 66,500 (Figure 1). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the purified enzymes from *B. cepacia* and *R. eutropha* both yielded single bands of M_r 31,900 (Figure 2), consistent with both enzymes existing as homodimers.

N-terminal sequence of dioxygenase from *B. cepacia* 2a

The N-terminal sequence of the first twenty amino acids of *B. cepacia* strain 2a dioxygenase was as follows:

B. cepacia 2a S-I-T-S-E-Y-L-H-P-L-F-V-G-Q-V-D-K-L-A-L-Q-
R. eutropha JMP134 M-S-V-V-A-N-P-L-H-P-L-F-A-A-G-V-E-D-I-D-L-R-

Comparison with the corresponding sequence from *R. eutropha* JMP134 [inferred from the nucleotide sequence of *tfdA* (Streber *et al.*, 1987) and shown above] revealed several differences between the two.

Cofactor requirements for dioxygenase activity

The activity of purified *B. cepacia* dioxygenase towards 2,4-D was dependent on α-KG and required dioxygen (Table 2). Ferrous ion (0.5 mM) was essential for activity (Figure 3a), and ascorbate at 0.6 mM markedly stimulated the activity of the purified enzyme (Figure 3b). This effect was seen to a lesser extent with cysteine

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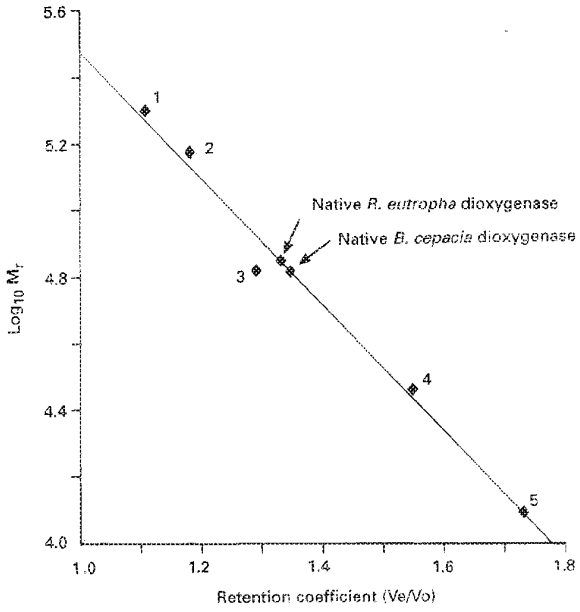


Figure 1 Relative molecular mass determinations of *Burkholderia cepacia* strain 2a and *Ralstonia eutropha* strain JMP134 dioxygenases by gel filtration. Plot of the retention coefficient V_e/V_o *versus* log M_r values for protein markers on a Sephacryl S-200 column (65 x 1.6 cm i.d.). All protein solutions were diluted to A_{260} = 0.50, and 0.5 ml portions were loaded onto the column. Elution was monitored spectrophotometrically at 260 nm. The M_r of marker proteins were: 1, β-amylase, 200 k; 2, alcohol dehydrogenase, 150 k; 3, bovine serum albumin, 66 k; 4, carbonic anhydrase, 29 k; 5, cytochrome c, 12.4 k; $y = -1.89x + 7.36$; $R^2 = 0.98$.

(Figure 3c). In their absence, activity was rapidly lost. Dithiothreitol and glutathione (Table 3) was stimulatory, indicating a requirement for thiol groups.

In contrast, the concentration of ferrous ions required to support maximal activity of *R. eutropha* dioxygenase was 0.25 mM (Figure 3a). Cysteine (Figures 3b and 3c) was not stimulatory, but ascorbate was required to support reactivity beyond 10 min. Figure 4 shows the progress of 2,4-D consumption by partially-purified dioxygenase preparations from both organisms. *R. eutropha* dioxygenase remained active under the conditions of incubation for more than 30 min, but the activity of *B. cepacia* dioxygenase was lost by 6 min. The instability of *B. cepacia* dioxygenase was demonstrated by its rapid inactivation (within minutes) at 4°C, at all pH values between 6 and 10, whereas the enzyme from *R. eutropha* retained >60% of its initial activity for 20 h when stored under the same conditions.

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Effect of pH on enzyme activity

The pH dependence of *B. cepacia* dioxygenase activity was determined in sodium phosphate and Tris-HCl buffers (Figure 5). Optimum pH for the purified enzyme was ~7.8, and catalytic activity was slightly higher in Tris-HCl buffer. The pH optimum for *R. eutropha* dioxygenase in imidazole and Tris-HCl buffers was 7.4.

Effect of temperature on enzyme activity

The maximal activity of purified *B. cepacia* dioxygenase was achieved at 28°C, although 77% of the maximal activity was still evident at 10°C (Figure 6). *R. eutropha* dioxygenase displayed maximal activity at 35°C.

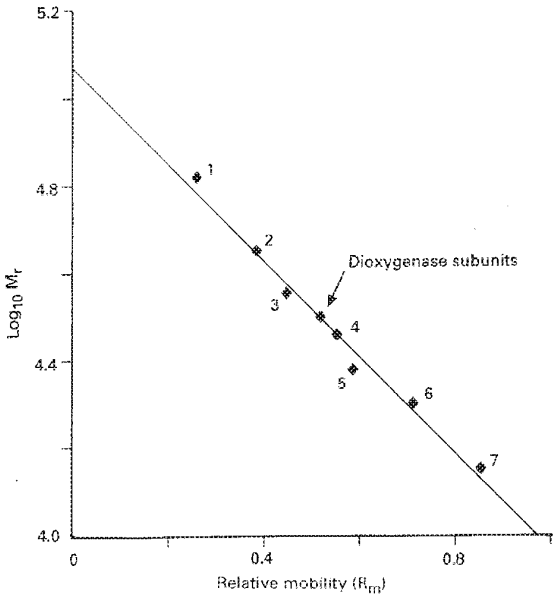


Figure 2 Relative molecular mass determinations of *Burkholderia cepacia* 2a and *Ralstonia eutropha* JMP134 dioxygenases by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Plot of relative mobility (R_m) *versus* log M_r values of protein standards derived by electrophoresis. Samples were denatured in loading buffer containing 2-mercaptoethanol and sodium dodecyl sulphate heated to 95°C for 5 min, cooled on ice and analysed on a 12% polyacrylamide gel. The M_r of marker proteins were: 1, bovine serum albumin, 66 k; 2, egg albumin, 45 k; 3, glyceraldehyde dehydrogenase (rabbit), 36 k; 4, carbonic anhydrase, 29 k; 5, trypsinogen, 24 k; 6, trypsin inhibitor, 20.1 k; 7, α-lactalbumin, 14.2 k; $y = -1.10x + 5.07$; $R^2 = 0.98$.

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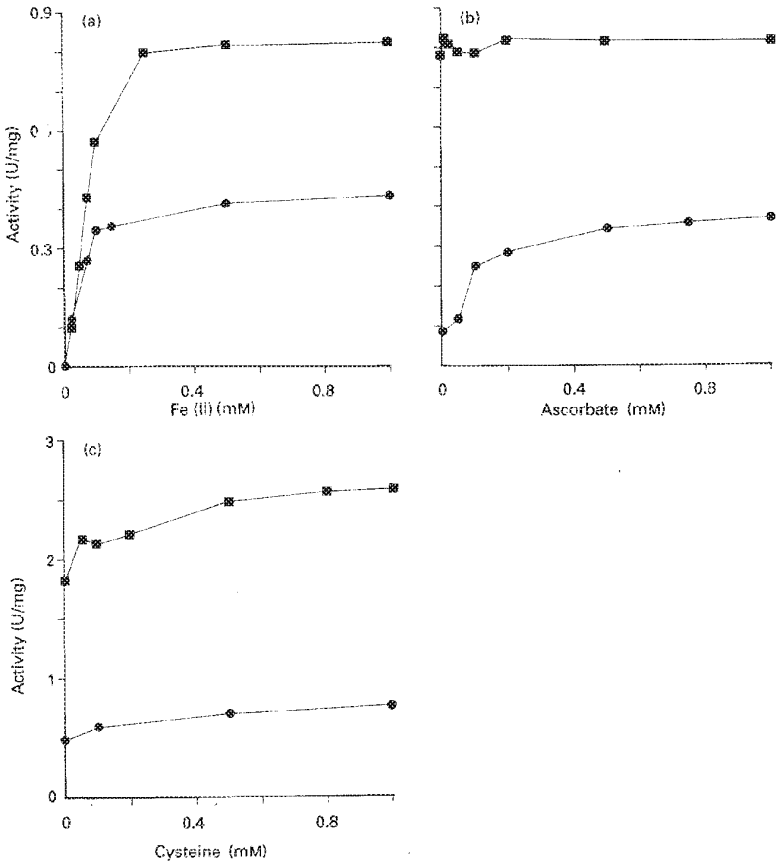


Figure 3 Effect of ferrous ions, ascorbate and cysteine on the activity of *B. cepacia* 2a and *R. eutropha* JMP134 dioxygenases. Enzyme activity was determined by assay of the 2,4-DCP produced during incubation at 25°C or 30°C respectively. Partially-purified *B. cepacia* dioxygenase (260 µg) or purified *R. eutropha* dioxygenase (17 µg) was added to initiate the reaction in incubation mixtures containing 2,4-D, α-KG and ferrous sulphate (all 1 mM, except where the concentration of a particular component was varied) in 100 mM Tris-HCl buffer, pH 7.8 or 7.3 respectively, in a total volume of 800 or 200 µl respectively. For *B. cepacia* enzyme, portions (200 µl) were assayed at 1 min intervals; a single assay was performed for *R. eutropha* enzyme after 2 min. (a) 0.0–1.0 mM ferrous ion; (b) 0.0–1.0 mM ascorbate; (c) 0.0–1.0 mM cysteine. *B. cepacia* dioxygenase (●); *R. eutropha* dioxygenase (■).

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Table 2 Cofactor and oxygen requirements of purified *B. cepacia* strain 2a dioxygenase

Addition (1 mM)	Atmosphere	2,4-Dichlorophenol produced (nmol)
None	Air	3.2
α-KG	Air	4.0
Fe_2SO_4	Air	10.2
α-KG + Fe_2SO_4	Air	228.0
α-KG + Fe_2SO_4	Nitrogen	20.4

Enzyme activity was determined at 25°C by assay of the 2,4-DCP produced after incubation with 2,4-D for 30 min. The assay mixture contained 3.7 μg purified enzyme, added to initiate the reaction; 200 nmol 2,4-D, and 100 mM Tris-HCl buffer, pH 7.8, in a total volume of 200 μl. Oxygen-free conditions were achieved by displacing the air from the reaction mixture with nitrogen *via* a hypodermic needle inserted through a rubber seal at the mouth of the reaction tube.

Effects of metal ions and chelating agents on the activity of *B. cepacia* dioxygenase

The effects of several cations, *viz* Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+} and Zn^{2+} (all at 1.0 mM) were investigated. The enzyme was pre-treated on ice for 60 min with each ion, before activity was determined at 25°C. Mn^{2+}, Zn^{2+} and Cu^{2+} were strongly inhibitory, whilst Fe^{3+} and Co^{2+} gave moderate inhibition (Table 4), possibly due to the formation of mercaptides by combination with essential sulphydryl groups on the enzyme, and/or by competing for binding with essential ferrous ions. As expected, presumably due to chelation of ferrous ions, the activity was eliminated by 60 min pretreatment on ice with EDTA or 8-hydroxyquinoline (Table 4).

Chemical modification of *B. cepacia* dioxygenase

Histidine-selective reagent diethylpyrocarbonate, and trinitrobenzene-sulphonate (selective for lysine) both inhibited the enzyme (Table 5). Iodoacetamide, N-ethylmaleimide and *p*-chloromercuribenzoate were also inhibitory (Table 5). Iodoacetamide and N-ethylmaleimide alkylate cysteinyl-SH groups, and *p*-chloromercuribenzoate formed mercaptides with thiols. In general, these results provided further evidence that *B. cepacia* dioxygenase contained essential cysteinyl thiol group(s).

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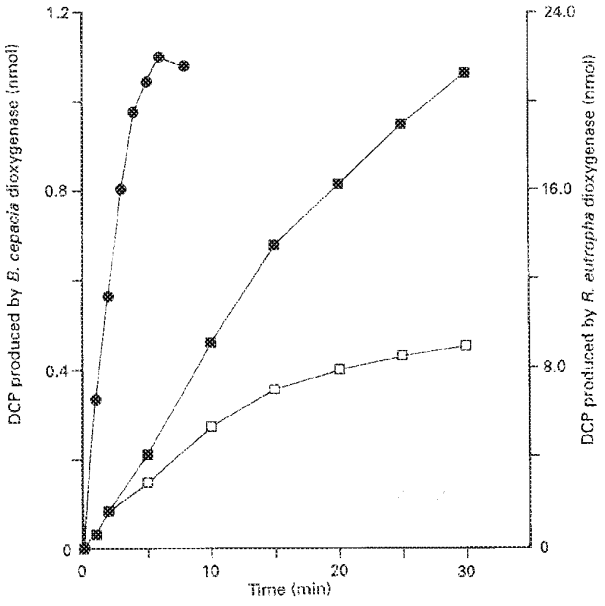


Figure 4 Progress of 2,4-D consumption by *Burkholderia cepacia* 2a and *Ralstonia eutropha* JMP134 dioxygenases. Mixtures contained 2,4-D, α-KG and ferrous sulphate (all 1 mM) in 50 mM Tris-HCl buffer at pH 7.8 or 7.4, respectively. *B. cepacia* mixtures contained additionally 5 mM cysteine. To initiate the reaction, partially-purified *B. cepacia* or *R. eutropha* dioxygenase (112 or 109 µg) was added to the mixtures at 25 or 30°C, respectively, to yield a final volume of 1.6 ml. Activity was determined by assaying 200 µl portions at 1 or 5 min intervals, respectively, for the 2,4-DCP produced. *B. cepacia* dioxygenase in the presence of 1 mM ascorbate (●); *R. eutropha* dioxygenase in the presence (■) and absence (□) of 1 mM ascorbate.

Table 3 Effects of thiol compounds on the activity of *B. cepacia* strain 2a dioxygenase

Addition (1 mM)	Activity (U mg^{-1})
None	0.27
Glutathione	0.33
Dithiothreitol	1.10
Cysteine	1.12

Enzyme activity was determined by assay of the 2,4-DCP produced after incubation at 25°C for 5 min. The incubation mixture contained 3.7 µg purified enzyme (added to initiate the reaction); 2,4-D, α-KG and ferrous sulphate (all 1 mM) and 100 mM Tris-HCl buffer, pH 7.8, in a total volume of 200 µl. One unit is defined as the amount of enzyme required to convert 1 µmol of 2,4-D into 2,4-DCP per min.

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Product identification and stoichiometry of the *B. cepacia* dioxygenase reaction

With crude cell-free extract, phenol was produced from phenoxyacetate and accumulated in the reaction mixture. The identification of phenol was based on (a) its production with the reagent of Lacoste *et al.* (1959) of a coloured product of λ_{max} = 505 nm, identical with the product generated from authentic phenol; and (b) the UV spectrum of the extracted phenol (λ_{max} = 270 nm, shifting at alkaline pH to 288 nm), which was identical with that of authentic phenol. The stoichiometry of the reaction in the presence of purified enzyme indicated that 1.01 and 1.03 mol of 2,4-DCP were generated from 1.0 mol of 2,4-D and α-KG supplied, respectively (mean of two determinations each). Measurements of oxygen consumption gave a value of 0.96 mol O_2 per mol of 2,4-D metabolized (mean of four determinations).

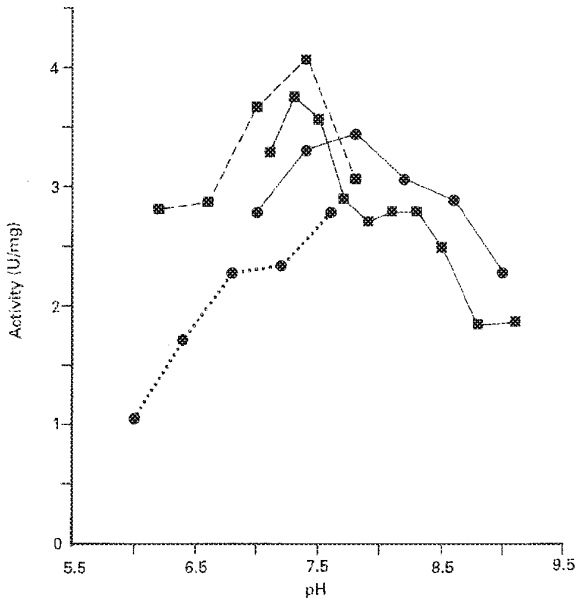


Figure 5 Activities of *B. cepacia* 2a and *R. eutropha* JMP134 dioxygenases as a function of pH. Mixtures contained, in the buffer indicated, 2,4-D, α-KG, ascorbate and ferrous sulphate (all 1 mM). *B. cepacia* mixtures contained additionally 5 mM cysteine. To initiate the reaction, purified *B. cepacia* dioxygenase (3.7 µg) or partially-purified *R. eutropha* enzyme (5.5 µg) was added to the mixtures at 25 or 30°C, respectively, to yield a final volume of 200 µl. Activity was determined by assay of the 2,4-DCP produced by *B. cepacia* dioxygenase incubated for 2 min (●) or by *R. eutropha* enzyme for 10 min (■). Buffers used: pH 6.0 to 7.6, 100 mM Na_2HPO_4 - NaH_2PO_4 (. . .); pH 6.2 to 7.8, 50 mM imidazole (– – –); pH 7.1 to 9.1, 100 mM Tris-HCl (——).

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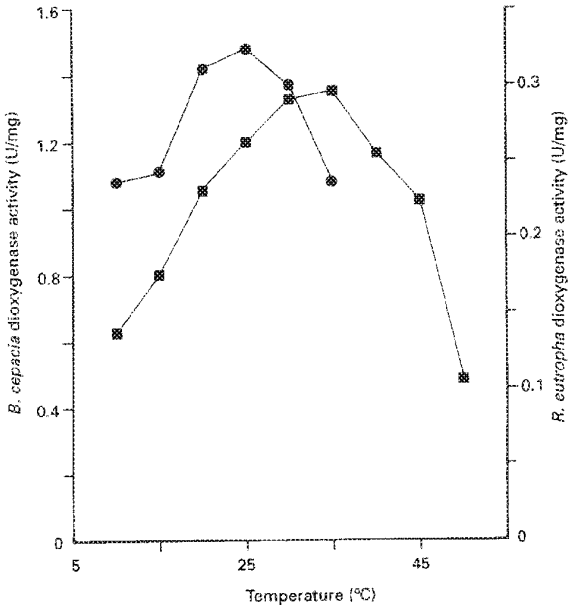


Figure 6 Effect of temperature on the activities of *B. cepacia* 2a and *R. eutropha* JMP134 dioxygenases. The mixtures contained 2,4-D, α-KG, ascorbate and $FeSO_4$ (all 1 mM) in 50 mM Tris-HCl buffer (pH 7.8), pre-equilibrated at temperatures in the range 10–50°C. For *B. cepacia* dioxygenase (●), mixtures also contained 5 mM cysteine. Partially-purified enzyme (64 μg) was added to initiate the reaction, yielding a final volume of 800 μl. Aliquots (200 μl) were withdrawn at 30 s intervals over 2 min for assay of the 2,4-DCP produced. For *R. eutropha* dioxygenase (■), partially-purified enzyme (32.6 μg) was added to initiate the reaction, yielding a final volume of 200 μl. Reactions were terminated at 2 min for assay of the 2,4-DCP produced. One unit is defined as the amount of enzyme required to convert 1 μmol of 2,4-D to 2,4-DCP per min.

The product of the reaction arising from the side-chain was identified as glyoxylate on the basis of (a) analysis by high performance chromatography (HPLC), which revealed a peak of the same retention time as authentic glyoxylate, which was absent from a control incubation mixture from which 2,4-D was omitted; (b) the colour reaction with the reagent of Paget and Berger (1938), which is specific for glyoxylate (Gamar and Gaunt, 1971). However, the pink colour generated was unstable due to interference from ferrous ions present in the reaction mixture, which rapidly turned the colour green, therefore λ_{max} could not be determined. Succinate was also identified as a reaction product by HPLC-mass spectrometry (MS), and 0.86 and 0.93 mol were recovered per mol of 2,4-D and α-KG supplied respectively (one determination each).

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The above results revealed that the reaction catalysed by the enzyme from *B. cepacia* 2a was the same as that catalysed by the 2,4-D/α-KG dioxygenase of *R. eutropha* JMP134, in which α-KG was oxidatively converted to succinate, concomitantly with the conversion of 2,4-D to 2,4-DCP and glyoxylate.

Substrate specificity of *B. cepacia* dioxygenase

A range of substituted phenoxyacetates was investigated as potential substrates for the enzyme (Table 6). The enzyme showed wide specificity. Maximum activity was obtained with 2,4-D. The activities towards other substituted phenoxyacetates were less than 40% of that towards 2,4-D. The lowest rates of reaction were seen towards 2-phenoxypropionate, and 2- and 4-(2,4-dichlorophenoxy)butyrate, suggesting that the nature of the -alkanoate group had more influence on enzyme activity than did the ring substituents. The activity towards 2,6-dichlorophenoxyacetate was the lowest of all the chlorinated phenoxyacetates, presumably due to steric effects exerted by the proximity of the two chlorine atoms *ortho-* to the ether bond.

Table 4 Effects of metal ions and chelating agents on the activities of *B. cepacia* strain 2a and *R. eutropha* strain JMP134 dioxygenases

Addition (1 mM)	2,4-DCP produced (controls = 100): *B. cepacia*	*R. eutropha*
None	100	100
$CoCl_2$	88	24
$CuSO_4$	2	6
$FeCl_3$	58	4
$MgSO_4$	93	89
$MnSO_4$	0	19
$ZnSO_4$	0	12
EDTA	0	72
8-Hydroxyquinoline	0	99

For *B. cepacia* 2a, enzyme (3.7 μg) was mixed with the ions specified and held on ice for 60 min before adding to 2,4-D, α-KG, ascorbate, ferrous sulphate (all 1 mM), 5 mM cysteine, in 100 mM Tris-HCl buffer, pH 7.8, in a total volume of 200 μl. Activity was determined by assay of the 2,4-DCP produced after incubation at 25°C for 5 min. For *R. eutropha* JMP134, enzyme (5.5 μg of protein) was mixed with the compounds specified and held on ice for 60 min before adding to 2,4-D, α-KG, ascorbate, ferrous sulphate (all 1 mM), 5 mM cysteine, in 100 mM Tris-HCl buffer, pH 7.3, in a total volume of 200 μl. Activity was determined by assay of the 2,4-DCP produced after incubation at 30°C for 5 min.

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Table 5 Chemical modification of *B. cepacia* strain 2a dioxygenase

Addition	Activity (U mg^{-1})
None	4.10
Diethylpyrocarbonate (5 mM)	0.29
Trinitrobenzenesulphonate (2.5% w/v)	0.24
Iodoacetamide (5 mM)	1.16
Iodoacetamide (50 mM)	0.08
N-ethylmaleimide (5 mM)	1.07
N-ethylmaleimide (50 mM)	1.02
p-Chloromercuribenzoate (5 mM)	0.32
p-Chloromercuribenzoate (50 mM)	0.17

Enzyme (3.7 µg protein) was treated with the compounds indicated and held on ice for 60 min before addition to α-KG, ascorbate and ferrous sulphate (all 1 mM), 200 nmol 2,4-D, in 100 mM Tris-HCl buffer, pH 7.8, in a total volume of 200 µl. Activity was determined by assay of the 2,4-DCP produced after incubation at 25°C for 5 min. One unit is defined as the amount of enzyme required to convert 1 µmol of 2,4-D into 2,4-DCP per min.

Kinetic studies of *B. cepacia* dioxygenase

Initial reaction rates were determined at 25°C. Reactions were initiated by addition of the enzyme, and portions of reaction mixtures were assayed for 2,4-DCP at 30 s intervals. Initial rates of reaction were calculated from a minimum of two consecutive point determinations made over the initial 1.5 min. The apparent Michaelis constants (K_m), obtained from Lineweaver-Burke plots, were 107 µM for 2,4-D (in the presence of 1 mM α-KG), and 8.7 µM for α-KG (in the presence of 1 mM 2,4-D).

Discussion

An α-KG- and ferrous ion-dependent dioxygenase, similar to that of *R. eutropha* JMP134 described by Fukumori and Hausinger (1993a,b), has been purified from *B. cepacia* strain 2a. Studies on the cofactor requirements and the reaction products of the purified enzyme have shown that it likewise belongs to a group of α-KG-dependent dioxygenases (Abbott and Udenfriend, 1974), typical characteristics of which are specific requirements for ferrous ions and

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α-KG, and stimulation of activity by ascorbate. For example, prolyl hydroxylase, which converts the prolyl residues of collagen to hydroxyprolyl groups, bears a ferrous atom at its active site, and as shown using $^{18}O_2$, the hydroxyl-oxygen originates in dioxygen. The other oxygen atom emerges in succinate, which is formed from α-KG, the obligatory cosubstrate in this reaction. Prolyl hydroxylase is relatively stable in the presence of ascorbate, but is rapidly inactivated in its absence; 90% of the enzyme is inactivated in approximately 1 min (Myllylä *et al.*, 1978).

Inactivation is thought to occur by a side-reaction of the enzyme in which α-KG is decarboxylated in the absence of the proline ring, leaving the metal at the active centre in an oxidized state which

Table 6 Specificity of *B. cepacia* strain 2a dioxygenase

Substrate	Yield of phenol (nmol ml^{-1})	Relative activity
2,4-D	383	100
3,4-Dichlorophenoxyacetate	149	39
2,3-Dichlorophenoxyacetate	115	30
2,3,4,6-Tetrachlorophenoxyacetate	107	28
2-Chlorophenoxyacetate	103	27
2,5-Dichlorophenoxyacetate	100	26
4-Chlorophenoxyacetate	88	23
3-Chlorophenoxyacetate	84	22
MCPA	73	19
2,4-Trichlorophenoxyacetate	69	18
Phenoxyacetate	61	16
2,6-Dichlorophenoxyacetate	46	12
2-Phenoxypropionate	38	10
2-(2,4-Dichlorophenoxy)butyrate	31	8
4-(2,4-Dichlorophenoxy)butyrate	15	4

Activity was determined by assay of the corresponding phenol produced after incubation at 25°C for 5 min. The assay mixture contained 3.7 μg enzyme protein (added to initiate the reaction); substrate, α-KG, ascorbate and ferrous sulphate (all 1 mM); and 5 mM cysteine hydrochloride, in 100 mM Tris-HCl buffer, pH 7.8, in a total volume of 200 μl. For each phenoxyalkanoate derivative, the corresponding authentic phenol was used as the calibration standard.

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Table 7 Comparison of the 2,4-D/α-KG dioxygenases of *Burkholderia cepacia* 2a, *Ralstonia eutropha* JMP134 and *R. eutropha* enzyme cloned into *E. coli* pUS311

	B. cepacia	*R. eutropha*[b]	*R. eutropha* enzyme cloned in *E. coli* pUS311[c]
M_r (native enzyme)	64.9	66.5	50.0 ± 2.5
M_r (subunit)	31.9	31.6–32.1	32.1
Oligomer type	Homodimer	Homodimer	Homodimer
Optimum pH	7.8	7.4	6.5–7.0
Temperature optimum (°C)	28	35	
Minimal cofactor requirements:			
Fe^{2+} (mM)	0.4	0.05	0.05–0.075
Cysteine (mM)	0.5	0	0
Ascorbate (mM)	0.5	0	<0.05
O_2 stoichiometry (mol/mol 2,4-D)	0.95		
Chemical modification:			
Diethylpyrocarbonate	+		+
Trinitrobenzene-sulphonate	+		+
Iodoacetamide	+		–
N-ethylmaleimide	+		–
Inhibition:			
EDTA (1 mM)	100%	29%	
EDTA (100 mM)		100%	
Substrate specificity	Broad[a]		Broad[a]
K_m for 2,4-D (μM)	107.0		17.5
K_m for α-KG (μM)	8.7		3.2

+, inhibited; –, no effect. [a]Substrate preference towards substituted phenoxyacetates differs from that of the other enzyme. [b]Data in this study. [c]Data from Fukumori and Hausinger (1993a,b).

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subsequently must be reduced by ascorbate to be returned to the catalytically active form (Counts *et al.*, 1978). The stoichiometry of oxygen uptake, cofactor requirement, reaction products, and effect of ascorbate on the purified dioxygenase from *B. cepacia* strain 2a are all consistent with these features.

After a 55-fold purification by a simple two-step procedure, the enzyme appeared by gel electrophoresis to be homogeneous. Relative molecular mass determinations carried out under denaturing and non-denaturing conditions revealed that the enzyme was a homodimer with a monomer M_r of 31,900, which is the same as for the subunit of *R. eutropha* JMP134 dioxygenase. However, the M_r of the native (dimeric) dioxygenase from *R. eutropha* JMP134 has been reported to be 50,000 (Fukumori and Hausinger, 1993a,b), which differs from the values for *B. cepacia* dioxygenase (M_r 64,900) and the *R. eutropha* enzyme (M_r 66,500) obtained during the present investigation.

The inhibitory effects of diethylpyrocarbonate, trinitrobenzene-sulphonate, iodoacetamide, N-ethylmaleimide and *p*-chloromercuri-benzoate on enzyme activity were consistent with the presence of essential cysteine residues, which was corroborated by the protective effects of thiol compounds. Unlike *B. cepacia* strain 2a dioxygenase, the enzyme from *R. eutropha* JMP134 was unaffected by iodoacetamide or N-ethylmaleimide, hence ruling out the presence of accessible thiol groups on this protein. Therefore, although the two enzymes have similar subunit M_r, their primary structures differ, as indicated by the differences between the N-terminal amino-acid sequences of the two proteins. The nucleotide sequence of the *B. cepacia* dioxygenase gene and the deduced primary structure of the enzyme are the subjects of a further paper.

The optimum pH of the purified dioxygenase from *B. cepacia* 2a was found to be 7.8 in Tris-HCl buffer, higher than the value obtained in the present investigation (7.4) for the enzyme from *R. eutropha* JMP134. Although the highest reaction rate for the *B. cepacia* enzyme was obtained at 25°C, the activity at 10°C was still, unexpectedly, 77% of that value. The effect of temperature on the activity of the cloned dioxygenase from *E. coli* pUS311 was not reported by Fukumori and Hausinger (1993b), though the enzyme appears to be sensitive to thermal inactivation, since it remains stable *in vitro* only up to 30°C. At 37°C the formation of aggregates in *E. coli* is recorded, and activity is completely lost within 5 min at 40°C. In our hands, the dioxygenase extracted from cells of *R. eutropha* JMP134 displayed maximal activity at 35°C, and became rapidly inactivated only above 45°C.

Broad substrate specificity was revealed for the purified dioxygenase from *B. cepacia* 2a. Notably, the enzyme is capable of metabolizing

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phenoxyacetate, 2,5-, 2,6- and 3,4-dichloro-, 2,4,5-trichloro-, and 2,3,4,6-tetrachlorophenoxyacetate, although the phenols produced are not substrates of the next enzyme in the catabolic pathway, dichlorophenol hydroxylase (Beadle and Smith, 1982). The 2,4-D/α-KG dioxygenase from this organism therefore has a broader substrate specificity than the corresponding hydroxylase, and the specificity of the latter appears to limit the range of phenoxyacetates that *B. cepacia* 2a can utilize. For instance, 2,4,5-trichlorophenoxyacetate is converted to 2,4,5-trichlorophenol by the dioxygenase, although it is a recalcitrant herbicide which cannot be degraded by most microorganisms, including *B. cepacia* 2a. The obstacle to further dissimilation exists at the second step instead of at the very beginning of the pathway. The dioxygenase of *R. eutropha* JMP134 has a similar, broad substrate range, but the relative catalytic efficiencies differ. For example, 2,4,5-trichlorophenoxyacetate is the best substrate among substituted phenoxyacetates other than 2,4-D, but is a poor substrate for *B. cepacia* dioxygenase. Conversely, the best dichlorinated substrate for the enzyme from *B. cepacia strain* 2a, 3,4-dichlorophenoxy-acetate, is a poor substrate for the dioxygenase from *R. eutropha* JMP134; K_m is 12.5-fold higher than for 2,4-D.

In summary, studies on the purified 2,4-D/α-KG dioxygenase of *B. cepacia* 2a indicate that it is a typical α-KG-dependent dioxygenase, of same type as that of *R. eutropha* JMP134, though with a different N-terminal amino-acid sequence. Both are homodimers with similar subunit molecular weights, but their maximal rates of reaction are exhibited at different temperatures, and they display different pH optima, substrate specificities and sensitivities to thiol-binding reagents. A comparison of the two enzymes is summarized in Table 7.

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Characterization of a Chromosomally Encoded 2,4-Dichlorophenoxyacetic Acid/α-Ketoglutarate Dioxygenase from *Burkholderia* sp. Strain RASC

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The findings of previous studies indicate that the genes required for metabolism of the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) are typically encoded on broad-host-range plasmids. However, characterization of plasmid-cured strains of *Burkholderia* sp. strain RASC, as well as mutants obtained by transposon mutagenesis, suggested that the 2,4-D catabolic genes were located on the chromosome of this strain. Mutants of *Burkholderia* strain RASC unable to degrade 2,4-D (2,4-D$^-$ strains) were obtained by insertional inactivation with Tn*5*. One such mutant (d1) was shown to have Tn*5* inserted in $tfdA_{RASC}$, which encodes 2,4-D/α-ketoglutarate dioxygenase. This is the first reported example of a chromosomally encoded *tfdA*. The $tfdA_{RASC}$ gene was cloned from a library of wild-type *Burkholderia* strain RASC DNA and shown to express 2,4-D/α-ketoglutarate dioxygenase activity in *Escherichia coli*. The DNA sequence of the gene was determined and shown to be similar, although not identical, to those of isofunctional genes from other bacteria. Moreover, the gene product ($TfdA_{RASC}$) was purified and shown to be similar in molecular weight, amino-terminal sequence, and reaction mechanism to the canonical TfdA of *Alcaligenes eutrophus* JMP134. The data presented here indicate that *tfdA* genes can be found on the chromosome of some bacterial species and suggest that these catabolic genes are rather mobile and may be transferred by means other than conjugation.

Synthetic chlorinated organic compounds have been used extensively as herbicides and pesticides over the last five decades. Certain of these compounds, such as 2,4-dichlorophenoxyacetic acid (2,4-D), are subject to rapid biological degradation in natural environments (26, 38, 42, 43), suggesting that catabolic pathways specific for their degradation have evolved and been disseminated among various microbial populations.

The best-described pathway for 2,4-D degradation, both genetically and biochemically, is the plasmid-encoded pathway of the saprophytic soil bacterium *Alcaligenes eutrophus* JMP134 that was isolated in Australia (39). This organism harbors an 88-kb self-conjugable plasmid (pJP4), which encodes all of the structural and regulatory genes needed to convert 2,4-D to 2-chloromaleylacetic acid, namely, *tfdA* through *tfdF*, *tfdR*, and *tfdS* (11, 12, 20, 28–30, 37). This intermediate is subsequently reduced by the chromosomal *mar* gene product to β-ketoadipate, which is further metabolized by chromosomally encoded gene products to ultimately yield CO_2 (32).

Members of other bacterial genera, such as *Pseudomonas*, *Sphingomonas*, *Bordetella*, *Acinetobacter*, *Arthrobacter*, *Xanthobacter*, and *Corynebacterium*, have been shown to mineralize 2,4-D (9, 10, 14, 18, 24–26, 31, 34). The diversity of 2,4-D-degrading bacteria has recently been assessed by polyphasic taxonomic characterization of isolated bacteria (46), by hybridization of 2,4-D catabolic genes from isolated bacteria (3, 4, 17, 46), and by hybridization analysis of total soil bacterial community DNA (22, 25, 26). These studies have shown that there is substantial phylogenetic diversity among 2,4-D-degrading populations and significant genetic diversity among the 2,4-D-catabolic genes they carry. Studies in our group have focused on determining the genetic and functional diversity of TfdA (2,4-D/α-ketoglutarate dioxygenase) and assessing the biogeographical and among-species distribution of this enzymatic activity (15–17, 24, 27, 36, 46, 47).

Earlier experiments in this laboratory showed that *Burkholderia* strain RASC contained a single 11-kb plasmid, pRASC, and that this plasmid had no detectable homology to the pJP4 plasmid (46). The lack of homology and the small size of pRASC suggested that at least some genes known to be involved in 2,4-D degradation might be located on the chromosome. Here, we show that none of the genes involved in 2,4-D degradation by *Burkholderia* strain RASC are encoded on pRASC. We also report the cloning and DNA sequence analysis of $tfdA_{RASC}$, show that the gene is located on the chromosome of strain RASC, and biochemically characterize its product, 2,4-D/α-ketoglutarate dioxygenase.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. *Burkholderia* strain RASC was originally isolated in Oregon from return activated sludge on the basis of its ability to degrade 2,4-D (2) and was kindly provided by P. Amy. Strains of *Escherichia coli* were grown on Luria-Bertani (LB) broth or LB agar medium (40) at 37°C except when otherwise specified. Full-strength peptone-tryptone-yeast extract-glucose (PTYG) medium contained (per liter) 2.5 g of peptone (Difco Laboratories, Detroit, Mich.), 2.5 g of tryptone (Difco), 5.0 g of yeast extract (Difco), 5.0 g of glucose, 0.3 g of $MgSO_4 \cdot 7H_2O$, and 0.035 g of $CaCl_2$. PTYG was used at 1/10 strength (PTYG/10) throughout these experiments. MMO basal salts medium (MMO) was used as described previously (44), except that nitrilotriacetic acid was omitted since it appeared to serve as a carbon source for the strain and the medium was supplemented with 2,4-D and/or Casamino Acids (Difco) as indicated below. For solid media, 1.5% (wt/vol) Bacto Agar (Difco) was added. PIA/2 medium consists of half-strength (22.5 g/liter) *Pseudomonas* isolation agar (Difco) supplemented with 6.8 g of Bacto Agar per liter (to account for halving

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the recipe) and 20 ml of glycerol per liter. All cultures of strain RASC were incubated at 30°C. Broth cultures were incubated with shaking at 150 rpm on a rotary platform shaker.

Restriction endonucleases were purchased from GIBCO-BRL, Gaithersburg, Md., and used as specified by the manufacturer.

Tn*5* mutagenesis. Kanamycin-resistant Tn*5* transposon mutants of strain RASC were selected following conjugation with the Tn5 suicide vector pRL1062a (48), which can replicate only in *E. coli*. Triparental matings between strain RASC, the donor strain *E. coli* DH5α(pRL1062a), and *E. coli* DH5α(pRK2013), which carried a helper plasmid, were performed as described previously (8), and the strains were plated onto PIA/2 agar containing 50 μg of kanamycin per ml and incubated for 4 days at 30°C. The PIA/2 agar contains Irgasan (Ciba-Geigy, Basel, Switzerland), which selects against both *E. coli* strains, while the kanamycin selects for Tn5-containing derivatives of strain RASC.

To obtain insertion mutants bearing Tn5 specifically on pRASC and not the chromosome, a strategy involving both conjugation and transformation techniques was used. As before, the mating suspensions were plated on PIA/2 agar containing 50 μg of kanamycin per ml. Following incubation, all of the kanamycin-resistant colonies on the plates were collected by suspension and subsequent washing with TE buffer (10 mM Tris [pH 7.0], 1 mM EDTA). Plasmids were extracted from this suspension by the method of Hirsch et al. (21). A fresh preparation of strain RASC was transformed with this plasmid preparation by electroporation at 2.5 kV for 5 ms with a gene pulser (Bio-Rad, Hercules, Calif.) as specified by the manufacturer and subsequently plated on PIA/2 agar containing 50 μg of kanamycin per ml. Since wild-type pRASC and pRASC::Tn*5* derivative plasmids have the same origin of replication, they are incompatible with each other. Hence, growth on this medium forces segregation of wild-type pRASC and selects for cells harboring pRASC::Tn*5*. Kanamycin-resistant colonies were purified twice on this selective medium to ensure segregation of wild-type pRASC.

Plasmid curing. Strain RASC was cured of its plasmid (pRASC) by physical loss via electroporation. This was accomplished by using a Tn5 insertion derivative of pRASC (pRASC::Tn5) to facilitate monitoring of this cryptic plasmid. Electrocompetent cells of strain RASC/pRASC::Tn5 were prepared as specified by the manufacturer and then diluted to about 10^5 cells per ml with sterile distilled water. This cell suspension was subjected to electroporation at 2.5 kV for about 5.5 ms and then plated onto PTYG/10 agar. Several hundred colonies were screened, and several clones that were sensitive to kanamycin and presumably cured of the plasmid were identified. The loss of pRASC from these strains was confirmed by Southern blot hybridization of *Eco*RI-digested total DNA preparation of strain RASC as described previously (40), with ^{35}S-labeled pRASC DNA (Genius; Boeringer Mannheim, Indianapolis, Ind.) as a probe.

2,4-D biodegradation assays. Cultures to be tested were incubated overnight in PTYG/10 broth; then 0.1 ml was transferred to 3.5 ml of MMO supplemented with 500 mg of 2,4-D per ml and 0.3% (wt/vol) Casamino Acids and incubated with shaking at 30°C. After 3 days, a 1-ml sample of culture was taken and the cells were pelleted by centrifugation in a microcentrifuge (Sorvall, MC 12V; Dupont) at 14,000 rpm for 3 min. The supernatant was filtered (pore size, 0.45 μm), and the concentration of 2,4-D was determined by high-pressure liquid chromatography as described previously (22). When Tn5 insertion mutants were being analyzed, kanamycin (50 μg/ml) was added to the culture and test media.

To quantitate the ability to completely mineralize 2,4-D, a single drop (ca. 40 μl) of overnight culture in PTYG/10 broth was transferred to 5 ml of MMO supplemented with 100 mg of 2,4-D per ml, 0.1% (wt/vol) Casamino Acids, and 0.1 mCi of [U-*ring*-^{14}C]2,4-D (Sigma, St. Louis, Mo.) in a 25-ml glass bottle. The bottle was tightly closed with a double-layer rubber stopper from which a dipper containing a filter paper wick was hung. After 1 week of incubation at room temperature, 0.5 ml of 2 N KOH was added to the filter paper wick and 0.5 ml of 2 N HCl was added to the culture fluid by injection through the stopper. The amount of $^{14}CO_2$ absorbed on the KOH-saturated wick was determined by liquid scintillation counting.

Screening for 2,4-D$^-$ mutants. 2,4-D$^-$ mutants were obtained by screening clones with chromosomal Tn5 insertions for the inability to incorporate [U-*ring*-^{14}C]2,4-D into cell biomass on solid media (13). Briefly, dilutions of bacterial suspensions subjected to Tn5 mutagenesis were spread onto nitrocellulose filters resting on PIA/2 supplemented with 50 μg of kanamycin per ml and incubated overnight at 30°C. Replica filters were made from these master filters, laid onto PTYG/10 agar supplemented with 125 μg of Irgasan per ml, 50 μg of kanamycin per ml, and 500 μg of 2,4-D per ml including 0.5 mCi of [U-*ring*-^{14}C]2,4-D, and incubated for 2 days. The filters were transferred onto an agar plate, containing only 40 mM sodium phosphate buffer (pH 6.8), for 1 h to diffuse unincorporated label from the filter and then allowed to dry. Dried filters were exposed to X-ray film, which was subsequently developed and then matched to the master filters to identify colonies that had failed to incorporate ^{14}C into their cell biomass. These colonies were presumptive 2,4-D$^-$ mutants. Presumptive evidence that Tn5 had inserted into $tfdA_{RASC}$ was obtained by demonstrating that the transformation of a mutant with pYS11 complemented the mutation and restored the ability of mutants to metabolize 2,4-D. This plasmid (pYS11) contains a 1.2-kb *Sph*I fragment with the *tfdA* gene from pJP4 that had been subcloned from pUS311 (15).

Cloning of $tfdA_{RASC}$. The modified Tn5 transposon used for mutagenesis of strain RASC has the *oriV* origin of replication (48). Consequently, DNA containing $tfdA_{RASC}$::Tn5 could be cloned by partial digestion of mutant strain d1 genomic DNA with *Sau*3AI followed by self-ligation and transformation into *E. coli*. The circularized fragments with Tn5 were propagated in *E. coli* cells as plasmids and conferred kanamycin resistance. A 12.3-kb plasmid (pS18) from one of the transformants was chosen for further study. A 1.0-kb *Xba*I-*Pst*I DNA fragment from pS18 that flanked the transposon hybridized at low stringency with *tfdA*. This fragment was subcloned into pGEM3Zf(+) (Promega, Madison, Wis.) to generate pYS15 and was used as a probe to identify the wild-type $tfdA_{RASC}$ gene.

Genomic DNA from a plasmid-cured derivative of RASC was purified (5) and completely digested with *Eco*RI. Since pYS15 hybridized to a single 7.2-kb *Eco*RI fragment of genomic DNA of wild-type strain RASC, DNA fragments of about 7 kb were gel purified on a 1% low-melting-temperature agarose gel (SeaPlaque agarose; FMC, Rockland, Maine) and ligated into pGEM3Zf(+). The ligated DNA was transformed into *E. coli* DH5α by electroporation to create a library of subclones, which were then screened by colony hybridization (40) with the 1.0-kb *Xba*I-*Pst*I fragment as a probe. Eight colonies hybridized strongly with the probe (data not shown), and plasmid DNA was purified from these and analyzed by restriction digestion with *Eco*RI. One plasmid, pYC6, which contained a 7.2-kb *Eco*RI fragment was selected for further analysis. Templates for DNA sequencing were obtained by using exonuclease III (Erase-a-Base; Promega) to produce nested deletions of pYC6 DNA that were subcloned into plasmid pBluescriptII KS(+) or SK(+) (Stratagene, La Jolla, Calif.). DNA sequencing was performed at the Michigan State University DNA Sequencing Facility with an ABI Robotic Catalyst and 373A DNA sequencer.

Purification of $TfdA_{RASC}$. The $TfdA_{RASC}$ enzyme was purified essentially as described for TfdA (16) with minor modifications. Cultures (2 liters) of strain RASC in PTYG/10 supplemented with 2,4-D (2.5 mM) were incubated with shaking to near the end of the exponential growth phase and then harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. The cell pellets were resuspended in 25 ml of 20 mM phosphate buffer (pH 7.2) and stored at −20°C prior to use. The cell suspension was adjusted to a final concentration of 1 mM phenylmethylsulfonyl fluoride and 0.1 mM EDTA, and the cells were disrupted by passage through a precooled (4°C) French pressure cell at 120 MPa. The cell lysate was cleared of debris by centrifugation at 100,000 × *g* for 30 min at 4°C. The resulting cell extracts were adjusted to 20% glycerol and 0.1 mM dithiothreitol; then, $(NH_4)_2SO_4$ was added to 50% saturation. Precipitated proteins were collected by centrifugation (10,000 × *g* for 10 min at 4°C), dissolved in buffer A (20 mM Tris-HCl [pH 7.26], 20% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA), and dialyzed against buffer A at 4°C. The dialyzed sample was applied to a column of DEAE-Sepharose (2.5 by 30 cm, equilibrated in buffer A) and eluted with a 200-ml linear salt gradient to 0.5 M NaCl in buffer A with a flow rate of 2.5 ml min^{-1} at 4°C. The fractions containing enzyme activity (eluting at approximately 0.1 M NaCl) were pooled, dialyzed at 4°C against buffer A, and applied to a MonoQ HR 10/10 column (Pharmacia-LKB Biotechnology Inc., Piscataway, N.J.) equilibrated in buffer A at room temperature. A 100-ml linear salt gradient to 0.2 M NaCl in buffer A was used to elute the enzyme at 1 ml min^{-1}. Active fractions were combined, and the protein solution was concentrated with a Centricon 30 microconcentrator (Amicon Corp., Danvers, Mass.). This sample was chromatographed on a Superose 12 column (1.0 by 30 cm) in buffer A at 1 ml min^{-1} at room temperature.

$TfdA_{RASC}$ enzyme assay and characterization. The 2,4-D/α-ketoglutarate dioxygenase activity of the $TfdA_{RASC}$ enzyme was assayed at 30°C as previously described for TfdA (15, 16). One unit of activity is defined as the amount of enzyme that forms 1 μmol of 2,4-dichlorophenol per min under these conditions. Kinetic parameters were calculated as previously described (16), and protein determinations were made by the method of Lowry et al. (35) with bovine serum albumin as a standard.

The subunit molecular weight of $TfdA_{RASC}$ was determined by denaturing gel electrophoresis by the method of Laemmli (33) with a 4.5% acrylamide stacking gel and a 12% running gel. To visualize the polypeptides, gels were stained with Coomassie brilliant blue (7). The amino-terminal sequence of the enzyme was determined by automated Edman degradation with a model 477A sequencer (Applied Biosystems, Foster City, Calif.) once the denatured proteins were transferred to a polyvinylidene difluoride membrane.

Preparation of cell extracts of subclones. *E. coli* cells carrying plasmid subclones were cultured in LB broth containing ampicillin (100 μg/ml) at 37°C with shaking. At the end of the exponential growth phase, the cells were harvested by centrifugation (6,000 × *g* for 10 min at 4°C), resuspended in 25 ml of 20 mM phosphate buffer (pH 7.2), and stored at −20°C prior to use. Suspended cells were disrupted by passage through a precooled (4°C) French pressure cell at 120 MPa, and the lysates were cleared by centrifugation at 100,000 × *g* for 30 min at 4°C. $TfdA_{RASC}$ activity was assayed as described above.

Nucleotide sequence accession number. The sequence of the $tfdA_{RASC}$ gene was deposited with GenBank under accession number U25717.

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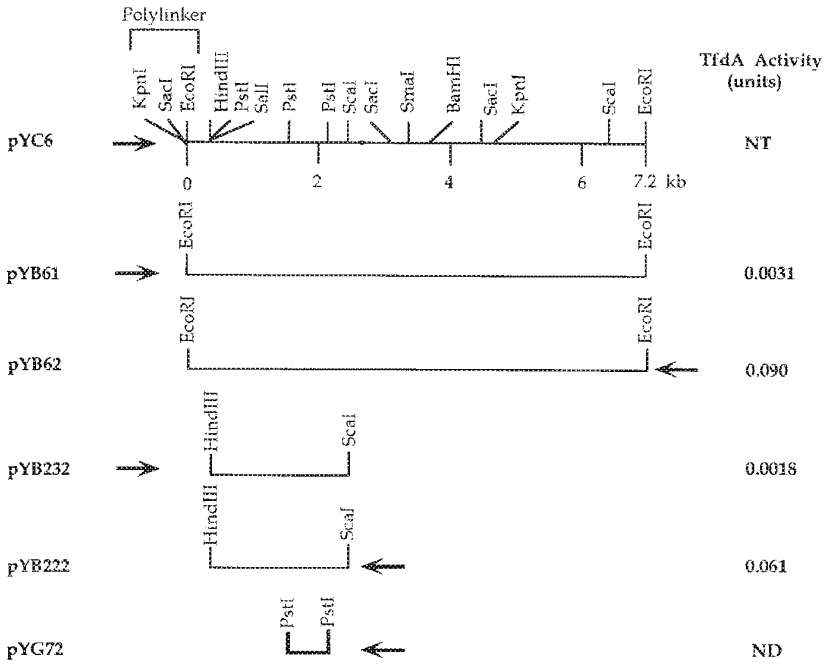


FIG. 1. Subcloning of the 2,4-D/α-ketoglutarate dioxygenase gene of *Burkholderia* sp. strain RASC. The location of the *lac* promoter from the vector in each recombinant plasmid is indicated by an arrow. The 2,4-D/α-ketoglutarate dioxygenase specific activities in *E. coli* JM109 containing the indicated plasmids were measured as described in Materials and Methods. NT, not tested; ND, not done.

RESULTS AND DISCUSSION

2,4-D degradation by plasmid insertion mutants and plasmid-cured derivatives of strain RASC. Strain RASC harbors an 11-kb plasmid (pRASC) which has no apparent homology to the 88-kb self-transmissible plasmid pJP4 encoding the 2,4-D degradative genes in *A. eutrophus* JMP134 (46). Tn*5* insertion derivatives of this plasmid were examined to determine whether pRASC encodes genes involved in 2,4-D degradation. Fifty independent pRASC insertion mutants were cultivated at 30°C for 3 days in MMO minimal salts medium supplemented with 0.3% (wt/vol) Casamino Acids and 500 μg of 2,4-D per ml, and the concentration of 2,4-D remaining in the culture fluid was determined. Each of the 50 mutants completely degraded the 2,4-D. Restriction analyses of the mutant plasmids with *Eco*RI confirmed that Tn*5* insertion into pRASC was not site specific. Each of the 28 plasmids examined was 19 kb, which was consistent with insertion of an 8-kb fragment with the transposon into the plasmid, and there were 25 clearly different restriction patterns, indicating that Tn*5* had inserted at random locations in pRASC (data not shown).

Further evidence that the genes responsible for 2,4-D degradation in strain RASC are not plasmid encoded was obtained by analysis of plasmid-free derivatives of this strain. Five pRASC-free isolates were obtained as described above, and all were able to degrade 2,4-D as evidenced by the evolution of $^{14}CO_2$ from radiolabelled 2,4-D at rates comparable to that observed with the wild-type strain (data not shown).

Several alternative plasmid isolation protocols, including those specialized for isolation of large plasmids (6, 19, 23) and pulsed-field gel electrophoresis following in-well lysis (41), were performed in attempts to detect large plasmids or episomes that might reside in strain RASC. Despite repeated attempts, we were never able to obtain evidence for the existence of extrachromosomal replicons other than pRASC. These results, the plasmid insertion data, and the data from plasmid-cured strains indicate that no genes essential for 2,4-D degradation are plasmid encoded in *Burkholderia* sp. strain RASC.

Isolation and characterization of 2,4-D$^-$ mutants. Approximately 4,000 Tn*5* insertion mutants were screened for the inability to incorporate [^{14}C]2,4-D into biomass (data not shown). Of these, two putative 2,4-D$^-$ mutants were obtained and further characterized. The ability of mutant d1 to degrade 2,4-D was restored when it was transformed with pYS11, which contains the entire *tfdA* gene from pJP4 (data not shown). This result suggests that the Tn*5* insertion in d1 interrupted a gene that was isofunctional to *tfdA*. These data indicate that the $tfdA_{RASC}$ gene is encoded on the chromosome. Preliminary data (27, 49) suggest that other genes required for the catabolism of 2,4-D, including *tfdB* and *tfdC*, are also chromosomally encoded in this strain. This is in contrast to the plasmid-encoded degradative pathways of *A. eutrophus* JMP134 and other strains (46).

Cloning of $tfdA_{RASC}$. The wild-type $tfdA_{RASC}$ gene was cloned from a library of genomic DNAs of strain RASC by using as a probe a 1.0-kb *Xba*I-*Pst*I DNA fragment from the transposon-gene junction composed mostly of DNA that flanked the transposon insertion site in mutant d1. Eight colonies that hybridized strongly with the probe (data not shown) were obtained, and one of these contained a plasmid (pYC6) with a 7.2-kb *Eco*RI fragment. Several subclones of this DNA were made in pBluescriptII KS(+) (Fig. 1). Plasmids pYB61 and pYB62 were subclones of the entire 7.2-kb *Eco*RI fragment that differed in orientation with respect to the *lac* promoter of pBluescriptII KS(+). Both of these plasmids expressed 2,4-D/α-ketoglutarate dioxygenase activity in *E. coli*

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JM109, suggesting that the cloned fragment encodes the native promoter region for the $tfdA_{RASC}$ gene and that this promoter functions in *E. coli*. However, *E. coli* containing pYB62 had nearly 30-fold-greater activity than did *E. coli* containing pYB61 (Fig. 1), indicating that the native promoter is much weaker than the *lac* promoter in *E. coli*. Additional smaller subclones were constructed in each orientation and assayed for 2,4-D/α-ketoglutarate dioxygenase activity, ultimately resulting in a 2.1-kb *Hin*dIII-*Sca*I fragment that still retained activity (Fig. 1). The forward orientation of this subclone, pYB222, also had approximately 30-fold more activity than did the reverse orientation, pYB232. Plasmid pYG72, containing a 1-kb *Pst*I subclone (Fig. 1), had no measurable activity, suggesting that one or both *Pst*I sites interrupt the $tfdA_{RASC}$ gene or that the gene was entirely contained on the *Hin*dIII-*Pst*I fragment. The pYB232 subclone was selected for DNA sequence analysis.

DNA and amino acid sequence analysis of $tfdA_{RASC}$. The *Hin*dIII-*Pst*I, *Pst*I-*Pst*I, and *Pst*I-*Sca*I DNA fragments, which make up the 2.1-kb *Hin*dIII-*Sca*I DNA fragment of pYB232 (Fig. 1), were further subcloned into pBluescriptII KS(+) or SK(+) and used to create a series of nested deletions for sequencing. Appropriate-sized deletion derivatives were subjected to DNA sequence analysis. An open reading frame corresponding to the $tfdA_{RASC}$ gene contains 891 bases of coding sequence (45) aligned with the *tfdA* gene of *A. eutrophus* JMP134 (Fig. 2). The initiation codons differ for the two genes, with $tfdA_{RASC}$ encoding the traditional ATG while *tfdA* begins with GTG. Both genes terminate with a TAG stop codon; however, $tfdA_{RASC}$ is larger than *tfdA* by 30 nucleotides. Additional evidence that the open reading frame encodes the $tfdA_{RASC}$ gene is that it contained a *Pst*I site that, when cleaved, resulted in loss of dioxygenase activity (described above); also, the amino-terminal sequence was identical to that of purified $TfdA_{RASC}$ (described below). In all, there were 185 differences in the aligned nucleotide sequences (excluding the additional 30 bases of $tfdA_{RASC}$). Thus, the overall DNA sequence similarity for the two genes was 78.5%, which was close to that expected from Southern hybridization experiments. Analysis of the sequence showed that 110 of the 185 nucleotide differences occur in the third (or wobble) position of the codon or in another position that preserves the identity of the amino acid. This suggests that structural and functional requirements of the enzyme constrain the degree of sequence difference allowed. The degree of identity in the amino acid sequence was higher than that in the DNA sequence. BLAST searches (1) of existing gene and protein databases using both DNA and amino acid sequences of *tfdA* and $tfdA_{RASC}$ were performed, but no evidence of another highly related gene or gene product was found.

In contrast to the coding regions, the promoter regions of $tfdA_{RASC}$ and *tfdA* are not highly conserved. For example, the putative −10 and −35 regions involved in RNA polymerase binding possess only three identical positions in each region (Fig. 2). The weak homology to the consensus sequences for these regions in RASC is consistent with the observation that the cloned $tfdA_{RASC}$ gene is poorly expressed in *E. coli* unless it is under the control of a heterologous promoter. In contrast, these regions in *tfdA* from *A. eutrophus* JMP134 more nearly match the sequences for RNA polymerase-binding sites in *E. coli* (45).

The translated amino acid sequences of $TfdA_{RASC}$ and TfdA were aligned and compared (Fig. 3). The $TfdA_{RASC}$ enzyme is composed of 297 amino acids, while TfdA contains 287. As expected from the above DNA sequence comparison, all of the additional protein residues are at the carboxyl terminus. The data indicate 80.5% identity of amino acids for the first 287 residues. Of the 56 differences observed when the sequences of $TfdA_{RASC}$ and TfdA were compared, 42 represented conservative substitutions (as determined by analysis with Genetyx-Mac [Software Development Co. Ltd., Tokyo, Japan]). Allowing conservative substitutions, the two enzymes have 95.5% similarity for the first 287 residues. The positions of histidine residues, shown to be important for enzymatic function (16), are also highly conserved, with all nine histidine positions of TfdA being matched in $TfdA_{RASC}$ (Fig. 3). In

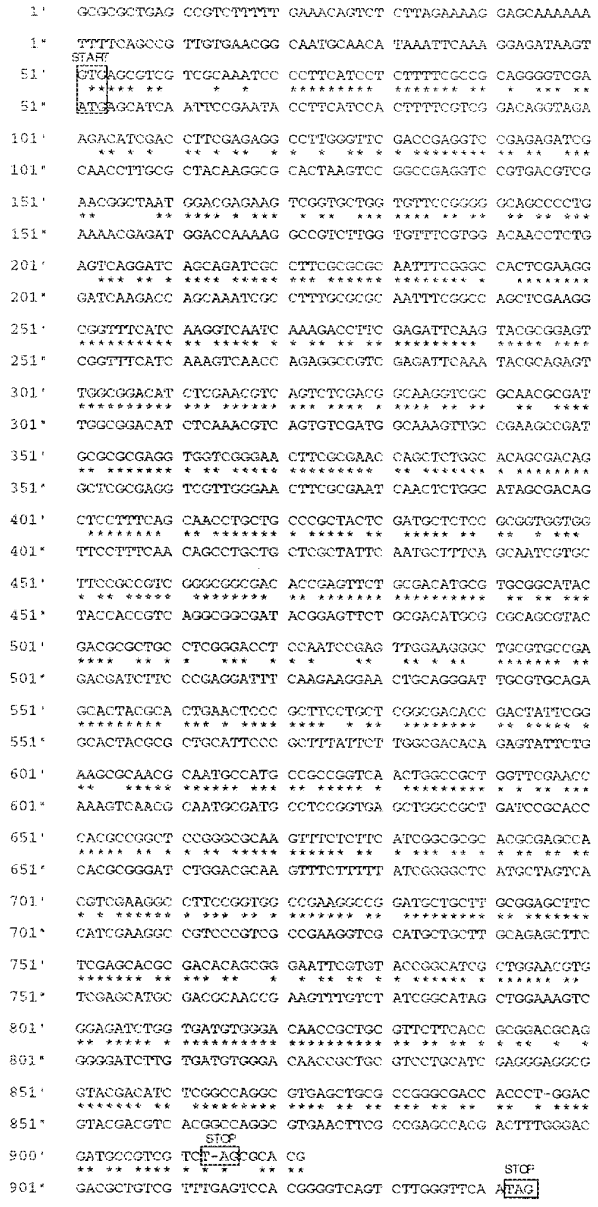


FIG. 2. Comparison of nucleotide sequences of the 2,4-D/α-ketoglutarate dioxygenase gene of *Burkholderia* sp. strain RASC ($tfdA_{RASC}$; bottom row) with that of *A. eutrophus* JMP134 (*tfdA*; top row). Identical nucleotides are indicated by asterisks. The initiation and termination codons and the putative RNA polymerase-binding sites are boxed.

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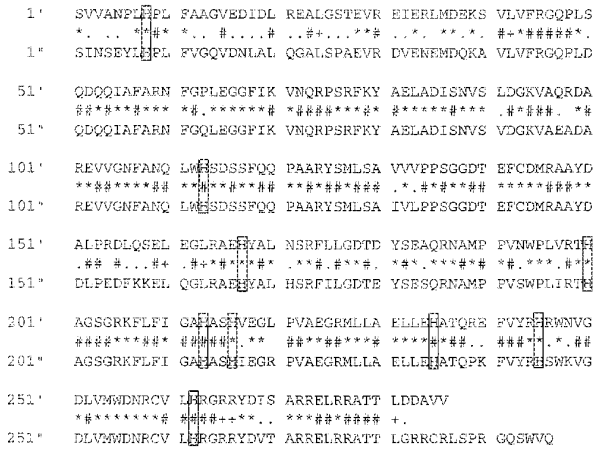


FIG. 3. Comparison of the amino acid sequences of 2,4-D/α-ketoglutarate dioxygenase from *Burkholderia* sp. strain RASC (bottom row) and *A. eutrophus* JMP134 (top row). Identical amino acids encoded by the identical codon are indicated by asterisks; degenerate codons differing at the wobble position or the first nucleotide are indicated by # or +, respectively. Amino acids which are related by conservative replacement are indicated by •. Histidine residues are boxed.

addition, $TfdA_{RASC}$ contains a 10th histidine at position 172, in contrast to TfdA, which contains an asparagine residue at that position.

Examination of Fig. 3 shows that the distribution of nonidentical residues in these enzymes was apparently not random. For example, 18 of 56 mismatches were found in the amino-terminal 41 residues of the enzyme. An additional pocket of mismatches (6 of 11 residues) exists between positions 151 and 162 of the enzyme. Finally, a string of five consecutive mismatches plus the additional 10 residues unique to $TfdA_{RASC}$ were found at the COOH terminus. Histidines were generally present in conserved regions, except for two that were present in the C terminus of the protein.

Purification and characterization of $TfdA_{RASC}$. $TfdA_{RASC}$ was purified, and typical results are summarized in Table 1. The dioxygenase activity of $TfdA_{RASC}$ appeared to be more labile than that of TfdA of *A. eutrophus* JMP134 (16) but was stabilized by the inclusion of glycerol and dithiothreitol in all buffers during the purification. Despite the lability of the enzyme, it was purified 117-fold to apparent homogeneity to give a specific activity of 10.5 U/mg of protein. This value compares well with that (16.9 U/mg) found for recombinant *A. eutrophus* TfdA isolated from *E. coli* (16).

Preliminary data indicate that cell extracts of strain RASC degraded 2,4-D by a ferrous ion- and α-ketoglutarate-dependent mechanism similar to that previously characterized in *A. eutrophus* JMP134 (15, 16). No 2,4-D/α-ketoglutarate dioxygenase activity was detected in cell extracts of strain RASC grown in the absence of 2,4-D. Moreover, maximal dioxygenase activity was found in cell extracts that were harvested during the exponential growth phase before 2,4-D was exhausted. As found for TfdA (16), the $TfdA_{RASC}$ enzyme was capable of using several phenoxyacetate compounds as substrates, including phenoxyacetate without chlorine substituents and the chlorinated compound 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The enzyme had greatest affinity for 2,4-D (K_m, approximately 2 μM), whereas the K_m values for phenoxyacetate and 2,4,5-T were approximately 310 and 42 μM, respectively. The two enzymes behaved similarly in all measured parameters, including cosubstrates, cofactor requirements, activity, preferred substrate, and substrate range.

TABLE 1. Purification of $TfdA_{RASC}$ from *Burkholderia* sp. strain RASC

Purification step	Total amt of protein (mg)	Activity (U)	Recovery (%)	Sp Act[a]	Fold purification
Cell extract	1,710	1,730	100	0.09	1
$(NH_4)_2SO_4$	1,020	2,400	139	0.21	2.3
DEAE-Sepharose	90	900	52	0.9	10
MonoQ	10	680	39	6.8	75.6
Superose 12	6.0	630	36	10.3	117

[a] Specific activity is expressed as units of activity per milligram of protein, where 1 U is the amount of enzyme that forms 1 μmol of 2,4-DCP per min.

The purified protein was composed of a single type of subunit with an apparent M_r of 34,000. This value is slightly larger than that (M_r = 32,190) reported for the *A. eutrophus*-derived enzyme (16) and is in good agreement with the value (M_r = 33,310) calculated from the sequence of the $tfdA_{RASC}$ gene. Antibodies generated against the *A. eutrophus* enzyme cross-reacted with the RASC-derived enzyme after denaturing gel electrophoresis (data not shown), which is consistent with the sequence similarity of the two proteins. In addition, the amino-terminal sequences of the two polypeptides (S-I-N-S-E-Y-G/L-H-P-L-F-V-G-Q-V for $TfdA_{RASC}$ and S-V-V-A-N-P-L-H-P-L-F-A-A-G-V for TfdA) revealed identity in 6 or 7 of 15 positions, with several additional conservative substitutions. In both cases, the N-terminal methionines were processed away.

Evolutionary considerations. The high degree of DNA sequence homology between *tfdA* and $tfdA_{RASC}$ and the similarity of the biochemical mechanisms suggest that the genes (proteins) are evolutionarily related to each other. Moreover, their unique features suggest that the proteins form a new class of α-ketoglutarate-dependent dioxygenases. The two genes differ from one another in that *tfdA* is carried on a broad-host-range plasmid whereas $tfdA_{RASC}$ is chromosomally encoded. Matheson et al. (36) have also recently shown that *tfdA* is chromosomally encoded in a bacterial strain that was independently isolated from an agricultural soil in mid-Michigan. Thus, it appears that the gene family is rather mobile and has been widely disseminated among phylogenetically diverse species.

The evolutionary origins of *tfdA* and $tfdA_{RASC}$ are unknown. However, since widespread exposure to 2,4-D has been limited to the last 50 years, it is conceivable that TfdA evolved from an ancestral protein that has activity with a naturally occurring analog of 2,4-D. The identities of the ancestral gene and the naturally occurring 2,4-D analog are unknown. We postulate that the substrate specificity of the ancestral gene has been altered through selection of variants with the ability to cleave the acetyl moiety from 2,4-D and that the gene has been recruited for 2,4-D catabolism.

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tfdA-Like Genes in 2,4-Dichlorophenoxyacetic Acid-Degrading Bacteria Belonging to the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* Cluster in α-*Proteobacteria*

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The 2,4-dichlorophenoxyacetate (2,4-D)/α-ketoglutarate dioxygenase gene (*tfdA*) homolog designated *tfdA*α was cloned and characterized from 2,4-D-degrading bacterial strain RD5-C2. This Japanese upland soil isolate belongs to the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* cluster in the α subdivision of the class *Proteobacteria* on the basis of its 16S ribosomal DNA sequence. Sequence analysis showed 56 to 60% identity of *tfdA*α to representative *tfdA* genes. A MalE-TfdAα fusion protein expressed in *Escherichia coli* exhibited about 10 times greater activity for phenoxyacetate than 2,4-D in an α-ketoglutarate- and Fe(II)-dependent reaction. The deduced amino acid sequence of TfdAα revealed a conserved His-X-Asp-X_{146}-His-X_{14}-Arg motif characteristic of the active site of group II α-ketoglutarate-dependent dioxygenases. The *tfdA*α genes were also detected in 2,4-D-degrading α-*Proteobacteria* previously isolated from pristine environments in Hawaii and in Saskatchewan, Canada (Y. Kamagata, R. R. Fulthorpe, K. Tamura, H. Takami, L. J. Forney, and J. M. Tiedje, Appl. Environ. Microbiol. 63:2266–2272, 1997). These findings indicate that the *tfdA* genes in β- and γ-*Proteobacteria* and the *tfdA*α genes in α-*Proteobacteria* arose by divergent evolution from a common ancestor.

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has been used as a model compound to study evolution of bacterial catabolic genes for anthropogenic chemicals (10, 11, 16, 20, 21, 26, 35, 36, 37). A number of 2,4-D-degrading bacterial strains have been isolated and found to be distributed over many different phylogenetic groups (3, 5, 10, 12, 16, 20, 21, 25, 26, 35, 37). Most of the strains have been classified into two groups on the basis of the genetic and enzymatic properties of their 2,4-D-catabolizing traits. One group is composed of various genera in the β and γ subdivisions of the class *Proteobacteria*. They have 2,4-D-catabolizing genes that are homologous to *tfd* genes found in *Ralstonia eutropha* JMP134, a well-studied 2,4-D degrader (5, 26). The other group is composed of strains in the α subdivision of the class *Proteobacteria*. Their 2,4-D-catabolizing genes have shown less than 60% homology by hybridization to the *tfd* genes in the β- and γ-*Proteobacteria* (20, 21, 26, 37).

The *tfdA* gene encodes an α-ketoglutarate (α-KG)-dependent 2,4-D dioxygenase that converts 2,4-D into 2,4-dichlorophenol and glyoxylate while oxidizing α-KG to CO_2 and succinate as an initial step of 2,4-D mineralization (8, 9, 31, 32). The *tfdA* sequences fall into three distinct classes for members of the β- and γ-*Proteobacteria* (26). The class I *tfdA* genes, including that of *R. eutropha* JMP134, are usually located on broad-host-range, self-transmissible plasmids (5, 19, 36). The class II *tfdA* gene, which is only found in the chromosome of *Burkholderia* sp. strain RASC, is 76% identical to the class I *tfdA* genes. The class III *tfdA* genes are 77 and 80% identical to the class I and class II *tfdA* genes, respectively. A *tfdA* phylogenetic tree with three distinct branches has been constructed, and interspecies gene transfer of the *tfdA* genes among the β- and γ-*Proteobacteria* was suggested by comparing the phylogenetic trees of the *tfdA* and 16S ribosomal (rDNA) genes (26).

An alignment of the *tfdA* genes was used to design PCR primer sets for amplifying *tfdA*-like genes from other sources (31, 37). In contrast to the 2,4-D-degrading β- and γ-*Proteobacteria*, none of 2,4-D-degraders belonging to the α-*Proteobacteria* has been found to yield *tfdA*-like PCR products (21, 26, 37). No hybridization was observed with *tfdA* even under low-stringency conditions, and α-KG-dependent 2,4-D dioxygenase activity was not detected. Therefore, the 2,4-D genes in this particular phylogenetic group were considered to be functionally and evolutionally different from the *tfdA* genes in the former phylogenetic groups (21, 26, 37).

In order to understand the origin and evolutionary relationship of the 2,4-D catabolic genes, it is important to characterize the 2,4-D genes in the α-*Proteobacteria*. Recently we isolated the 2,4-D-degrading bacterial strain RD5-C2 from Japanese upland soil (18). RD5-C2 is a member of the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* cluster (14, 29) in the α-*Proteobacteria*, like the 2,4-D degraders previously isolated from the pristine environments in Hawaii and in Saskatchewan, Canada, and Chile (21). In this paper we report that RD5-C2 and other degraders isolated from the pristine environments possess *tfdA*-like genes that encode a protein belonging to the α-KG-dependent dioxygenase family (8, 9, 31, 32). Significantly, we show that the recombinant RD5-C2 protein exhibits

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greater activity for nonchlorinated phenoxyacetate over 2,4-D in contrast to the more uniform activities observed for the canonical TfdA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. RD5-C2 and the other 2,4-D-degrading strains isolated from the pristine environments (21), HW13, HWK12, and BTH, were cultured on 2,4-D-containing basal medium (18) or HM medium (27). *Escherichia coli* HB101 and *E. coli* DH5α were used for the cloning and expression experiments, respectively. Luria-Bertani (LB) medium was used to grow *E. coli*. The *tfdA*-like gene from RD5-C2 (hereafter referred to as *tfdA*α) was cloned into pUC118 and then subcloned into pUC119. The plasmid vector pMAL-cRI (New England BioLabs) was used to construct the MalE-TfdAα fusion protein. The *tfdA*-like gene was fused to the *malE* gene, which encodes maltose-binding protein, and was expressed by using the *tac* promoter and translation initiation signals of maltose-binding protein.

Cloning and sequencing of *tfdA*α. To obtain whole DNA from the isolates, the cultures were grown on HM medium, washed with 1% NaCl, suspended in distilled water, frozen at −20°C, thawed at room temperature, and treated with proteinase K (10 μl of a 1-mg/ml concentration per 40 μl of sample) (Takara) in 40 mM Tris buffer solution (50 μl) containing 1% Tween 20, 0.5% Nonidet P-40 (Sigma), and 1 mM EDTA (pH 8.0). The mixture was incubated at 60°C for 20 min to digest proteins and then at 100°C for 5 min to inactivate the enzyme. After centrifugation (8,060 × *g*, 10 min) the supernatant was collected for gene amplification by PCR (15). A partial *tfdA*α fragment was amplified by using modified primers designed for the conserved regions of the *tfdA* genes from *R. eutropha* JMP134 and *Burkholderia* sp. strain RASC (3, 37): 5′-AC(C/G)GAGT TCTG(C/T)GA(C/T) ATG-3′ and 5′-(A/G)ACGCAGCG(G/A)TT(G/A)TCCC A-3′. The amplified *tfdA*α gene fragments were directly ligated into pT7Blue T-Vector (Novagen) and were transformed into *E. coli* HB101-competent cells according to the supplier's instructions. The inserted regions were amplified by PCR by using M13 primers M4 and RV (Takara) and were directly sequenced with an ABI 377 DNA sequencing system (Perkin-Elmer Japan) with the same primers.

The amplified *tfdA*α gene fragment was labeled with a DIG DNA Labeling Kit (Roche Molecular Biochemicals) and was shown to hybridize with a single ca. 4.0-kbp fragment of whole DNA *Sac*I digest (data not shown). DNA fragments of around 4.0 kbp were extracted from the gel, ligated into pUC118 to produce pTFD1-1, and transformed into *E. coli* DH5α-competent cells to create a genomic library. The DNA fragments were then screened by colony hybridization with the *tfdA*α gene fragment as a probe.

Plasmid DNA was purified from five positive clones, and the *Sac*I fragment was further digested with several restriction enzymes. The *Sal*I fragment was subcloned into pUC119, and the DNA sequence of the inserted fragment was determined as described previously. On the basis of the gene sequence, the following new primer set was constructed to amplify *tfdA*α from HW13, HWK12, and BTH: 5′-AC(C/G)GAGTTC(G/T)(C/G)CGACATGCG-3′ and 5′-GCGGT TGTCCCACATCAC-3′. The *tfdA*α genes were amplified, and the full sequences for HW13 and HWK12 and the partial sequence for BTH were determined by using a PCR in vitro cloning kit (Takara). All procedures described here were conducted according to the supplier's instructions.

Analysis of *tfdA*α sequence. The *tfdA*α sequences were compared with other available sequences by using the FASTA (28) and BLAST (2) algorithms. Sequences were aligned manually with the CLUSTALW program (34), and the sites where nucleotides were not resolved for all sequences were excluded from the alignments. Phylogenetic analysis was performed with three types of algorithms. CLUSTALW version 1.8 (34) was used as the software package for the neighbor-joining (NJ) analysis (30), and the bootstrap analysis based on 1,000 replicates was used to place confidence estimates on the tree. A maximum-likelihood (ML) analysis was carried out with the program package MOLPHY version 2.3b3 (1). An ML distance matrix was calculated by using NucML, and the NJ topology was reconstructed by using NJdist in MOLPHY as the starting tree for the ML method. An ML tree was obtained by using NucML with an R (local rearrangement search) option based on the HKY model (13). Local bootstrap probabilities were estimated by the resampling of estimated log-likelihood method (13, 24). A maximum-parsimony tree reconstruction was performed by using PAUP* software version 4.0b10 (33). A heuristic search was used with a random stepwise addition sequence of 100 replicates, tree-bisection-reconnection branch swapping, and the multrees option. A further analysis was run with 100 bootstrap replicates, each consisting of 30 additional random replicates. The Kishino-Hasegawa test (23) was carried out to survey the best topology having the highest log-likelihood value among the most parsimonious trees. A consensus tree was obtained by using the strict method.

Construction of *malE-tfdA*α fusion plasmid. From pTFD1-1, plasmid pNK2 was constructed by subcloning the appropriate *Sal*I fragment into pUC119. *tfdA*α of RD5-C2 was amplified by PCR by using the primers 5′-GCTCTAGAATGA CGGTCCTGATCCGGCAG-3′ and 5′-CCCAAGCTTTGTTATTGTGCCCGC TACTCC-3′ to give *Xba*I and *Hin*dIII ends, respectively, and was inserted into the *Xba*I and *Hin*dIII cloning sites of the pMAL-cRI vector. The plasmid was transformed into *E. coli* DH5α-competent cells according to the supplier's instructions.

Preparation of cell extracts and enzyme assay. Unless stated otherwise, the experiments were done at 4°C. *E. coli* DH5α (pUC119-*tfdA*α) was grown overnight at 30°C in LB medium supplemented with 100 μg of ampicillin/ml. *E. coli* DH5α (pMAL-cRI-*tfdA*α) was grown to mid-log phase at 30°C in LB medium supplemented with 2 g of glucose/liter and 100 μg of ampicillin/ml, followed by induction with isopropyl-[β]-D-thiogalactopyranoside at 0.3 mM for 2 h. Cells were harvested by centrifugation (6,000 × *g*, 15 min) and suspended in a solution containing 10 mM Tris, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM leupeptin (pH 7.6). The suspended cells were passed through a French press at 120 MPa, and cell extracts were obtained by centrifugation (100,000 × *g*, 45 min). The cell extracts were examined by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were assayed for TfdA enzyme activity. Gels were stained with Coomassie brilliant blue, and phosphorylase b (M_r, 97,400), bovine serum albumin (M_r, 66,200), ovalbumin (M_r, 45,000), carbonic anhydrase (M_r, 31,000), soybean trypsin inhibitor (M_r, 21,500), and lysozyme (M_r, 14,400) (Bio-Rad) were used as standards. The MalE-TfdAα fusion protein was further purified by using an amylose resin column (New England BioLabs) according to the supplier's instructions. The fusion protein eluate was dialyzed against 10 mM Tris–0.1 mM EDTA (pH 7.4) prior to the enzyme assay. Protein concentration was determined by using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

The enzyme activity was measured by the modified 4-aminoantipyrine method (8, 22). The typical assay mixture contained 1 mM α-KG, 50 μM ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$, and 1 mM 2,4-D in 10 mM imidazole buffer (pH 6.75). The enzyme reaction was conducted at 30°C and was quenched by the addition of

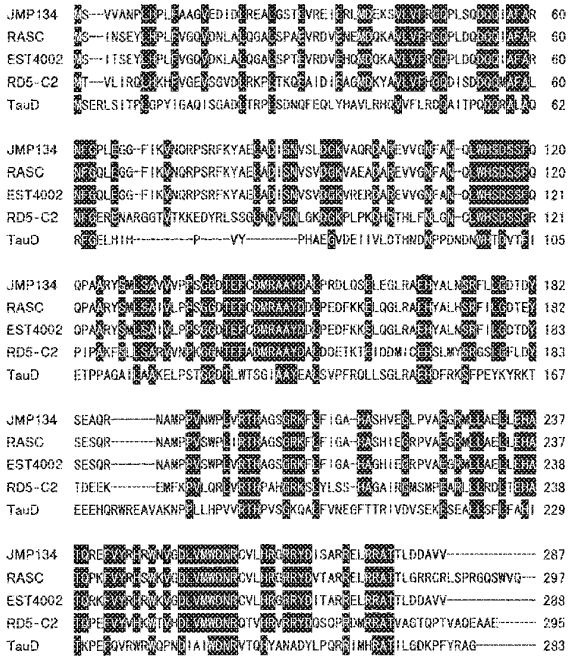


FIG. 1. Multiple alignment of the deduced amino acid sequences of RD5-C2 TfdAα and other α-KG-dependent dioxygenases. JMP134, RASC, and EST4002 are representative strains having class I, II, and III *tfdA* genes, respectively. TauD is taurine/α-KG dioxygenase of *E. coli*. Conserved residues are boxed.

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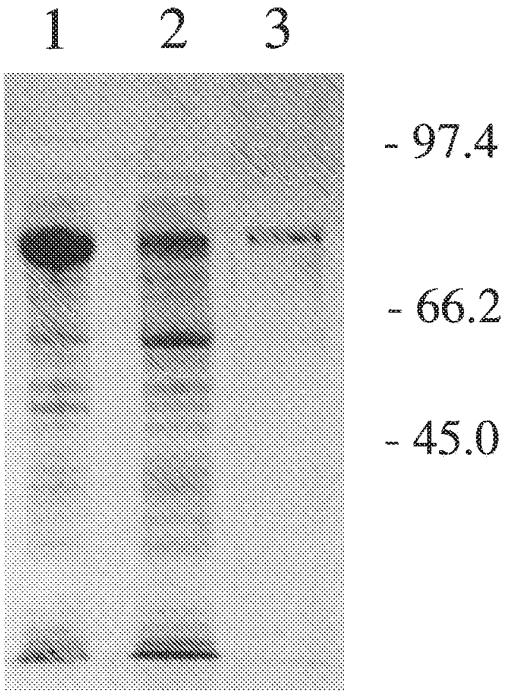


FIG. 2. Purification of MalE-TfdAα fusion protein from RD5-C2 (pMAL-cRI-tfdAα). Lane 1, whole-cell disruptant of RD5-C2 (pMAL-cRI-tfdAα); lane 2, supernatant of the disrupted cells; lane 3, purified MalE-TfdAα fusion protein eluted from an amylose resin column.

EDTA to give a final concentration of 5 mM. The concentration of released phenol derivative was measured as follows. One hundred microliters of a pH 10 buffer solution, 10 μl of 2% 4-aminoantipyrine, and 10 μl of 8% potassium ferricyanide were added to 1 ml of the reaction mixture. The absorbance at 510 nm was measured after 20 min. The assay was simultaneously conducted with purified *R. eutropha* JMP134 TfdA (9) for reference.

For the other phenoxyacetate derivatives, the corresponding phenol compounds were used as the standard in the assay. For the other α-ketoacids, phenoxyacetate was used as the substrate because this exhibited the highest activity among the phenoxyacetate derivatives used.

Nucleotide sequence accession number. The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank database, and the accession numbers for *tfdA*α of RD5-C2, HWK12, HW13, and BTH are AB074490, AB074491, AB074492, and AB074493, respectively.

RESULTS

***tfdAα* gene and TfdAα amino acid sequences of RD5-C2.** The *tfdA*α sequence was found to most closely match that of *tfdA* of *R. eutropha* JMP134 and showed 56 to 60% identity to representative *tfdA* genes of JMP134, RASC, and *Achromobacter xylosoxidans* EST4002 (E. Vedler, V. Koiv, and A. Heinaru, Analysis of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pEST4011 of *Achromobacter xylosoxidans* subsp. *denitrificans* strain EST4002; direct submission to GenBank [accession no. U32188], 1995), which were reported to have the class I, II, and III *tfdA* genes, respectively (26). The deduced amino acid sequence of RD5-C2 TfdAα was aligned with those of representative TfdA proteins and taurine/α-KG dioxygenase (TauD) from *E. coli*, which all belong to group II of the α-KG-dependent dioxygenase family (7, 8, 9, 17, 32) (Fig. 1). The data indicated 80 to 93% identity of amino acids among the three classes of TfdA, whereas TfdAα showed lower identity (45 to 48%) to all classes of TfdA, as expected from the gene sequence analysis. TfdAα also showed lower identity to TauD (27%) than the other TfdA proteins (28%). Despite the divergence of TfdAα compared to the other sequences, all of these proteins possessed the two His and one Asp considered to be iron-binding ligands and an Arg suspected of forming a salt bridge with the C-5 carboxylate of α-KG (17). The four predicted active-site residues in TfdAα are His115, Asp117, His264, and Arg279.

TABLE 1. Specific activity of the MalE-TfdAα fusion protein[a]

Substrate	Sp act	
	MalE-TfdAα (pmol/min · μg of protein)	TfdA (nmol/min · μg of protein)
2,4-D	2.6	7.9
4-Chlorophenoxyacetate	6.5	8.6
Phenoxyacetate	21	8.7

[a] Experiments were performed at 30°C in 10 mM imidazole buffer (pH 6.75) containing 1 mM substrate, 1 mM α-KG, 50 μM ferrous iron, and 50 μM ascorbate.

TfdA activity of the MalE-TfdAα fusion protein. When *tfdA*α was overexpressed in *E. coli* by using a pUC119 derivative (pNK2), TfdAα protein was produced, as shown by a predominant band exhibiting an M_r of 36,000 following denaturing gel electrophoresis. However, most of the recombinant wild-type protein was insoluble and possessed no activity even when prepared from cells grown at 30 or 25°C (9) or when examined with cells cultured in 1 M sorbitol and 2.5 mM glycyl betaine (4). In contrast, the MalE-TfdAα fusion protein (M_r, 76,000) exhibited increased solubility, allowing its partial purification by amylose resin chromatography (Fig. 2). As shown in Table 1, the specific activity of purified MalE-TfdAα protein was greatly reduced in comparison to that of *R. eutropha* TfdA. Significantly, however, the MalE-TfdAα protein exhibited higher activity for phenoxyacetate derivatives that were less chlorinated than 2,4-D. In contrast, TfdA showed similar activity towards these phenoxyacetates, as reported previously (8). Besides the difference in specific activities between the substrates, MalE-TfdAα showed the same traits as TfdA (8) in terms of dependency on α-KG and ferrous iron (Table 2) and

TABLE 2. Requirement of α-KG and ferrous iron for activity of the MalE-TfdAα fusion protein

Conditions	Sp act (pmol/min · μg of protein)
Control[a]	25.1
−α-KG[b]	3.8
−Ascorbate[b]	21.3
−Ferrous iron[b]	3.7
−Enzyme[b]	2.9
−Phenoxyacetate[b]	2.1

[a] Experiments were performed at 30°C in 10 mM imidazole buffer (pH 6.75) containing 1 mM phenoxyacetate, 1 mM α-KG, 50 μM ferrous iron, and 50 μM ascorbate.

[b] Each component was removed from the reaction mixture.

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TABLE 3. α-Ketoacid substrate specificity of the MalE-TfdAα fusion protein

Substrate[a]	Sp act (pmol/min · μg of protein)
α-KG	27.5
α-Ketoadipate	6.3
α-Ketovalerate	3.8
α-Ketobutyrate	2.5
α-Ketoisovalerate	1.3
α-Ketocaproate	0.6

[a] Experiments were performed at 30°C in 10 mM imidazole buffer (pH 6.75) containing 1 mM phenoxyacetate, 1 mM α-ketoacid, 50 μM ferrous iron, and 50 μM ascorbate.

in exhibiting the highest activity for α-KG among the α-ketoacids (Table 3).

Phylogenetic analysis of *tfdA*α genes from strain RD5-C2 and other 2,4-D-degrading α-*Proteobacteria*. Three 2,4-D-degrading strains belonging to α-*Proteobacteria*, previously isolated from pristine environments (21), were tested for the presence of a *tfdA*α-type gene by PCR amplification. The resulting *tfdA*α genes were sequenced and, along with RD5-C2 *tfdA*α, were subjected to phylogenetic analysis (Fig. 3). Since only four full-length open reading frames for *tfdA* are available in the DDBJ/EMBL/GenBank database, partial sequences (307 bp) of *tfdA* and *tfdA*α were used for the analysis. The *tfdA*α genes of α-*Proteobacteria* comprise a distinct cluster compared to the three reported clusters associated with the representative *tfdA* genes. Gene sequence identity among the *tfdA*α representatives was 81 to 99%. Based on the full-length sequence comparisons, a similar phylogenetic tree was obtained. In addition, all three algorithms presented similar topologies in both the full-length and partial sequences (data not shown).

DISCUSSION

Previous studies of α-proteobacterial 2,4-D degraders indicated that the genes responsible for 2,4-D degradation were different from the canonical *tfd* genes and that these bacteria lack a TfdA-like enzyme, suggesting a different origin of the biodegradative pathway compared to the β- and γ-*Proteobacteria* (21, 26, 37). Our studies refute this interpretation; rather, strain RD5-C2 and other 2,4-D degraders within the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* cluster of α-*Proteobacteria* do harbor a *tfdA*-like gene (*tdfA*α), and the RD5-C2 TfdAα protein exhibits TfdA-like activity. This is the first unequivocal

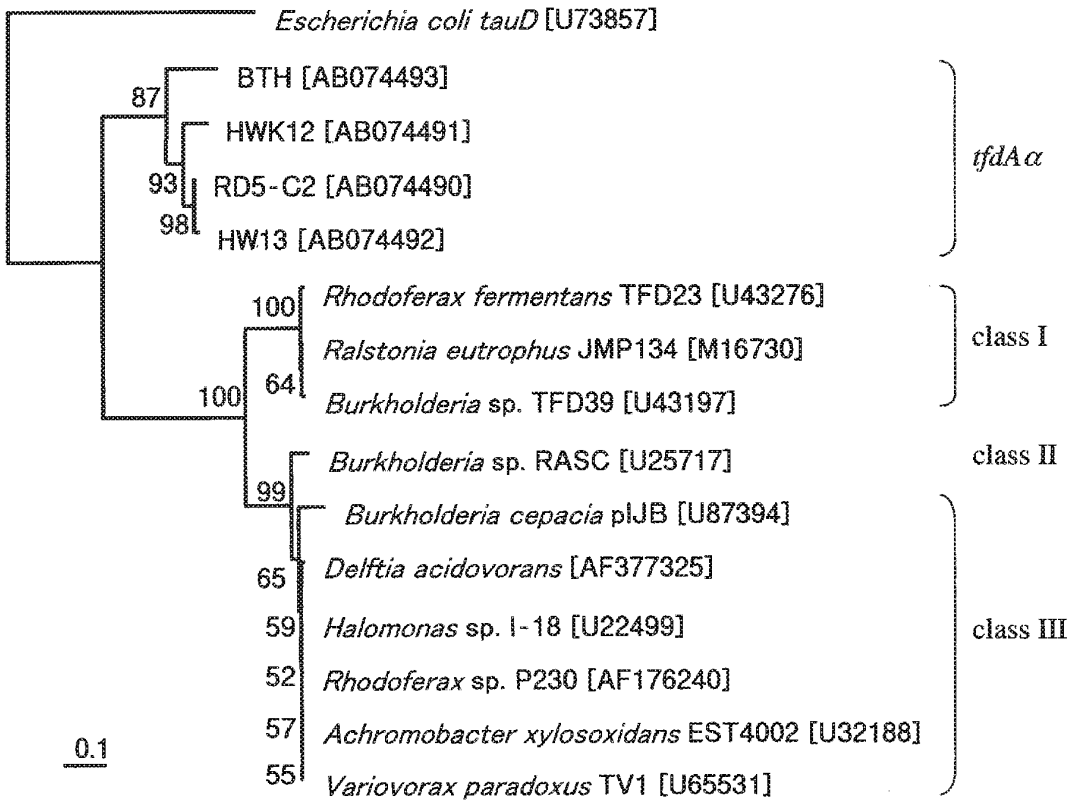


FIG. 3. Phylogenetic position of *tfdA*α among other genes of the α-KG-dependent dioxygenase family. The *tfdA* genes of JMP134, TFD39, and TFD23 are found in class I, the *tfdA* gene of RASC is in class II, and the other previously reported *tfdA* genes are class III representatives. *E. coli tauD* encodes taurine/α-KG dioxygenase. Designations in parentheses are DDBJ/EMBL/GenBank accession numbers. The phylogenetic tree was constructed on the basis of common partial sequences (307 bp) by using the NJ method. Bootstrap values above 50% are shown at nodes. The scale bar indicates substitutions per site.

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evidence that *tfdA*-type genes and TfdA-like enzymes exist among α-, β-, and γ-proteobacterial 2,4-D degraders. Because the FASTA homology search with the DDBJ/EMBL/GenBank database indicated no *tfdA*α-like genes among microorganisms other than those presented in Fig. 3, it is difficult to envisage the original function of these genes. The low sequence identity (56 to 60%) of *tfdA*α genes with representative *tfdA* genes explains why they did not hybridize in prior studies, even under low-stringency conditions (21, 26, 37).

All α-*Proteobacteria* strains used in this study were isolated from pristine environments and Japanese arable soil that had had no history of chemical treatment, suggesting that *tfdA*α genes are distributed among soil strains independent of 2,4-D exposure. These *tfdA*α genes are likely to have some original function(s), and some strains with the appropriate *tfdA*α genes incidentally degrade 2,4-D.

On the basis of the 30% amino acid identity between TauD from *E. coli* and TfdA from *R. eutropha* JMP134, Eichhorn et al. (7) suggested that the two dioxygenases might have arisen by convergent evolution rather than from a common ancestor. In contrast, the *tfdA* and *tfdA*α genes are likely to have arisen by divergent evolution from a common ancestor, because they exhibit higher similarities and are separately distributed in β- and γ-*Proteobacteria* and α-*Proteobacteria*, respectively. Horizontal gene transfer is a well-known phenomenon, and interspecies gene transfer of *tfdA* genes was demonstrated by phylogenetic comparison of *tfdA* and 16S rDNA genes (26). On the other hand, phylogenetic trees of *tfdA*α and 16S rDNA genes are congruent (18), indicating evolution of the *tfdA*α genes without horizontal gene transfer occurring. On the basis of the findings that microorganisms isolated from pristine environments harbor *tfdA*α and that TfdAα has higher activity for nonchlorinated phenoxyacetate than 2,4-D, the *tfdA*α genes could be the origin of the *tfdA* genes. Horizontal transfer by transposons and/or plasmids and further adaptations to the anthropogenic environments would give rise to the well-studied *tfdA* representatives. Plasmid pJP4, carrying *tfdA*, was shown to be transferred to strains of α-, β- and γ-*Proteobacteria*; however, the 2,4-D-degrading phenotype could not be expressed in α-*Proteobacteria* such as *Rhizobium* sp. and *Agrobacterium tumefaciens* (5). Distribution of *tfdA*-like genes among non-2,4-D-degrading soil bacteria in many different phylogenetic groups was reported, and nucleotide sequence analyses of four isolates showed the highest homology with canonical *tfdA* (16). Sequence analysis of the *tfdA* genes from the other strains could reveal the evolutionary relationship between the *tfdA* and *tfdA*α genes.

TfdAα was experimentally found to be an α-KG-dependent dioxygenase, as previously observed for TfdA. The amino acid sequence of TfdAα clearly belongs to group II of the α-KG-dependent dioxygenase family, since it contains multiple conserved residues that are thought to function in catalysis (8, 17). The substrate for ancestral TfdA is unknown, but naturally occurring aryl-ether compounds released during degradation of lignin are a reasonable possibility (16). In addition, it is possible that the TfdA ancestor functioned in the metabolism of a cinnamic acid (6).

In the present study we showed that 2,4-D-degrading α-*Proteobacteria* possess *tfdA*α genes that arose from a common ancestor to the canonical *tfdA* genes, and it is considered that ancestral *tfdA*α genes may serve as the origin of the *tfdA* genes on the basis of the following reasons. First, the 2,4-D-degrading α-*Proteobacteria* were isolated from pristine environments, widely distributed in Hawaii, and in Saskatchewan, Canada, and Chile and Japan, that had not been exposed to 2,4-D. Second, TfdAα is an α-KG- and Fe(II)-dependent dioxygenase that exhibits higher specific activity for nonchlorinated substrates. Thus, the chemistry of the TfdAα enzymes could be retained while modifying the substrate binding site to allow enhanced reactivity toward highly chlorinated 2,4-D to give rise to the TfdA activities. Further studies are needed to clarify the evolutionary relationships between *tfdA*α and the canonical *tfdA* genes and to understand the adaptation process of bacteria to chlorinated compounds.

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The Completely Sequenced Plasmid pEST4011 Contains a Novel IncP1 Backbone and a Catabolic Transposon Harboring *tfd* Genes for 2,4-Dichlorophenoxyacetic Acid Degradation

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The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacterium *Achromobacter xylosoxidans* subsp. *denitrificans* strain EST4002 contains plasmid pEST4011. This plasmid ensures its host a stable 2,4-D$^+$ phenotype. We determined the complete 76,958-bp nucleotide sequence of pEST4011. This plasmid is a deletion and duplication derivative of pD2M4, the 95-kb highly unstable laboratory ancestor of pEST4011, and was self-generated during different laboratory manipulations performed to increase the stability of the 2,4-D$^+$ phenotype of the original strain, strain D2M4(pD2M4). The 47,935-bp catabolic region of pEST4011 forms a transposon-like structure with identical copies of the hybrid insertion element IS*1071*::IS*1471* at the two ends. The catabolic regions of pEST4011 and pJP4, the best-studied 2,4-D-degradative plasmid, both contain homologous, *tfd*-like genes for complete 2,4-D degradation, but they have little sequence similarity other than that. The backbone genes of pEST4011 are most similar to the corresponding genes of broad-host-range self-transmissible IncP1 plasmids. The backbones of the other three IncP1 catabolic plasmids that have been sequenced (the 2,4-D-degradative plasmid pJP4, the haloacetate-catabolic plasmid pUO1, and the atrazine-catabolic plasmid pADP-1) are nearly identical to the backbone of R751, the archetype plasmid of the IncP1 β subgroup. We show that despite the overall similarity in plasmid organization, the pEST4011 backbone is sufficiently different (51 to 86% amino acid sequence identity between individual backbone genes) from the backbones of members of the three IncP1 subgroups (the α, β, and γ subgroups) that it belongs to a new IncP1subgroup, the δ subgroup. This conclusion was also supported by a phylogenetic analysis of the *trfA2*, *korA*, and *traG* gene products of different IncP1 plasmids.

Microbial degradation of 2,4-dichlorophenoxyacetic acid (2,4-D), a xenobiotic herbicide used worldwide for almost 60 years, is a well-studied process. Various soil bacteria can use 2,4-D as a carbon and energy source. Therefore, this compound has become a model for studying the evolution and distribution of genes for the degradation of chloroaromatic compounds. A number of bacterial strains belonging to different phylogenetic groups able to mineralize this compound have been found to possess genetically and enzymatically different 2,4-D-catabolic pathways (9, 10, 16–18). The best-studied 2,4-D degradation genes (located in a chromosome or a plasmid) are *tfd*-like (pJP4-like). The very recently sequenced 87,688-bp plasmid pJP4 (48) from *Wautersia eutropha* JMP134 (formerly *Ralstonia eutropha*) was originally isolated in Australia (8), and its *tfd* genes and the corresponding enzymes responsible for converting 2,4-D to 3-oxoadipate are well characterized (22, 23, 25, 26, 35, 58). Besides pJP4, there are only two cases in which the DNA regions containing *tfd* genes for the whole 2,4-D degradation pathway have been sequenced, a chromosomal transposon-like structure (about 30 kb) from *Delftia acidovorans* P4a (15) and Tn*5530* (41 kb) located in plasmid pIJB1 from *Burkholderia cepacia* 2a (36, 56). The *tfd* genes of these two elements are homologous to the pJP4 *tfd* genes but are organized in a different way.

Bacterial catabolic genes are often encoded by mobile genetic elements, including transposons and conjugative plasmids. Genes that encode degradation of man-made compounds are often found in IncP1 plasmids which are divided into three subgroups, the α, β, and γ subgroups (1). These plasmids are the most promiscuous self-transmissible plasmids that have been characterized to date (46) and thus could be responsible for efficient dissemination of genes encoding degradation of recently introduced xenobiotic compounds. The (almost) complete nucleotide sequences of four IncP1 catabolic plasmids, pUO1 (39), pADP-1 (30), pJP4 (48), and pTSA (47) coding for haloacetate, atrazine, 2,4-D, and *p*-toluenesulfonate degradation, respectively, have been determined, and they have all been shown to be members of the IncP1 β subgroup. Although only the catabolic region of the 2,4-D-degradative plasmid pIJB1 has been sequenced, it has been shown that this plasmid belongs to the same subgroup (36).

Nearly 20 years ago, several 2,4-D-metabolizing bacterial strains were isolated from different soil samples from Estonian agricultural enterprises. They all contained 2,4-D-degradative plasmids that were the same size (2; V. Kõiv, unpublished data). One strain, D2M4, containing plasmid pD2M4 (about 95 kb), was selected for further investigation as it showed the best growth characteristics on 2,4-D as a sole source of carbon and energy. However, the 2,4-D$^+$ phenotype of this strain was very unstable. Different laboratory manipulations were performed in order to obtain a more stable phenotype. As a result, strain EST4002 containing plasmid pEST4011 (approximately 70 kb, as estimated by restriction analysis) was isolated. This

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strain was determined to be a strain of *Achromobacter xylosoxidans* subsp. *denitrificans*. According to hybridization experiments, the *tfd*-like genes for 2,4-D degradation were all located in pEST4011. 2,4-D$^-$ strain EST4003 (obtained when EST4002 was grown in Luria-Bertani medium) contained plasmid pEST4012 (about 30 kb), from which the whole catabolic region had been deleted (28, 53).

In this paper we report the complete nucleotide sequence of a 2,4-D degradative plasmid, the 76,958-bp pEST4011 plasmid, and we describe and analyze all open reading frames (ORFs) and other features detected in pEST4011. The restriction patterns of pEST4011 and its ancestor, pD2M4, which is unstable in the laboratory, are compared. On the basis of multiple alignments of all available *trfA2*, *korA*, and *traG* gene products of different IncP1 plasmids, we show that the backbone of pEST4011 is quite different from that of other IncP1 plasmids. Consequently, we suggest that pEST4011 belongs to a new IncP1subgroup, the δ subgroup.

MATERIALS AND METHODS

Isolation of pEST4011 DNA. *A. xylosoxidans* subsp. *denitrificans* EST4002 cells harboring pEST4011 were maintained on minimal salts agar plates containing 5 mM 2,4-D. Plasmid pEST4011 was isolated from EST4002 cells that were grown overnight at 30°C in minimal salts medium supplemented with 5 mM 2,4-D. The culture obtained was inoculated into minimal salts medium supplemented with 20 mM sodium citrate and 0.2% Casamino Acids and grown overnight at 30°C. Plasmid DNA was isolated by using a method for isolation of large bacterial plasmids described by Hansen and Olsen (13), followed by CsCl buoyant density ultracentrifugation (38).

Construction of pEST4011 library. For construction of a pEST4011 library, partial digestion with Bsp143I was used to produce overlapping fragments that were approximately 1 kb long. These fragments were cloned into the BamHI site of the vector plasmid pBluescript II (SK) (Stratagene) and transferred into *Escherichia coli* DH5α cells. This and all other cloning procedures in this study were performed by using standard methods (38).

DNA sequencing. Templates for DNA sequencing were prepared by PCR amplification of inserted DNA from randomly selected insert-containing clones with the vector-specific primers T3 and T7. The PCR products obtained were directly sequenced by using the same primers (subsequent primer walking was used if necessary), a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech), and an ABI Prism 377 DNA sequencer (Applied Biosystems).

pEST4011 sequence assembly. All sequences were edited and analyzed and the complete sequence of pEST4011 was assembled by using the BioEdit sequence alignment editor, version 5.0.9 (12). Vector sequences were removed, and sequences were assembled into contigs manually according to alignment results with the most similar complete sequences obtained from the GenBank, including those of Birmingham IncP-alpha plasmids (R18, R68, RK2, RP1, RP4) (accession number L27758), *Enterobacter aerogenes* plasmid R751 (NC_001735), and *B. cepacia* 2a plasmid pIJB1 (AF029344). In order to close gaps, custom primers were designed by using the ends of each contig. These primers were used for PCR amplification of DNA fragments containing the necessary sequences; purified pEST4011 DNA was used as a template. Again, the PCR products obtained were directly sequenced by using the same primers. Finally, assembly of the pEST4011 nucleotide sequence was verified by PCR analysis by producing overlapping products covering the whole plasmid and by restriction analysis with BamHI, ClaI, EcoRI, HindIII, KpnI, MfeI, MluI, NdeI, StuI, and XmnI.

Resequencing the identical regions of pEST4011. In order to separately resequence the identical copies of IS*1071*::IS*1471* and the 6,991-bp duplications, two strategies were used. First, pEST4011 regions were cloned into the vector plasmid pBluescript II (SK) and sequenced by using vector-specific and custom primers. Second, two overlapping PCR products were generated by using one primer annealing outside the duplicated area and another primer annealing inside the duplicated area, and they were sequenced by using the same primers and subsequent primer walking.

Analysis of pEST4011 ORFs. The presence of ORFs in pEST4011 was analyzed by using the National Center for Biotechnology Information ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf.html). All predicted ORFs that were at least 150 bp long were analyzed further with the BLASTN and BLASTP programs (http://www.ncbi.nlm.nih.gov/BLAST), and only the ORFs with reasonable homology to some other known sequence were selected. Translation start codon positions were determined manually on the basis of the presence of a potential Shine-Dalgarno sequence upstream of a start codon.

Phenotypic analysis with Biolog GN2 microplates. One day before inoculation of Biolog GN2 plates (Biolog Inc., Hayward, Calif.), strain EST4002 harboring plasmid pEST4011 and strain EST4003 harboring plasmid pEST4012 were streaked on BUGM (Biolog Inc.) agar plates. The wells of the Biolog GN2 plates were inoculated with 150 μl of a bacterial suspension adjusted to an optical density at 580 nm of 0.150 for both strains. The plates were incubated at 30°C for 72 h. Development of color was automatically recorded by using a microplate reader with a 620-nm-wavelength filter.

Multiple alignment and phylogenetic tree construction. The amino acid sequences of different IncP1 *trfA2*, *korA*, and *traG* gene products were aligned, and bootstrapped neighbor-joining trees were derived by using the program CLUSTALX, version 1.8 (43), with default parameters. The PHYLIP 3.6 software package (http://evolution.genetics.washington.edu/phylip.html) programs SEQBOOT, PROTDIST, NEIGHBOR, CONSENSE, and PROML were used to calculate protein distances and to derive the corresponding phylogenetic trees.

Nucleotide sequence accession number. The complete nucleotide sequence of pEST4011 from *A. xylosoxidans* subsp. *denitrificans* strain EST4002 has been deposited in the GenBank database under accession number AY540995.

RESULTS AND DISCUSSION

Nucleotide sequence and organization of pEST4011. The 2,4-D-degradative plasmid pEST4011 is circular and 76,958 bp long and has an overall G+C content of 62.03%. Thus, pEST4011 is 7 kb larger than we previously calculated on the basis of restriction analysis. A circular physical map of pEST4011 is shown in Fig. 1. The first nucleotide of the HindIII restriction site between the *upf54.4* and *upf54.8* genes corresponds to position 1. The recognition site positions of 10 different restriction endonucleases are also shown in Fig. 1. Interestingly, 101 of 115 of these sites are located in the catabolic transposon of pEST4011. These restriction enzymes together with the PCR analysis were used to verify the assembly of pEST4011 (data not shown). Plasmid pEST4011 consists of the 29-kb IncP1 backbone loaded with the 48-kb catabolic transposon containing, among other genes, the *tfd*-like genes for complete 2,4-D degradation, bordered by identical IS*1071*:: IS*1471* hybrid insertion elements. The nucleotide sequence of this catabolic transposon is 99% identical to the sequence of Tn*5530* (41 kb) of pIJB1, except for duplication of a 6,991-bp region in the case of pEST4011. As a result, the pEST4011 region from position 27334 to position 34324 is identical to the adjacent region from position 34325 to position 41315, with only one mismatch. In addition, the pEST4011 region from position 20101 to position 31555 is 99% identical to the sequenced region of *Variovorax paradoxus* TV1 2,4-D-degradative plasmid pTV1 (49, 50). The IncP1 backbone of pEST4011 consists of the standard elements (Fig. 1), including the origin for the theta mode of replication (*oriV*) regulated by iterons; the region necessary for autonomous replication initiation, copy number control, and stable maintenance in the host cell; and two regions involved in plasmid conjugation, Tra1 (*tra* genes) with an origin of transfer (*oriT*) and Tra2 (*trb* genes). However, seven *trb* genes (*trbE* to *trbL*) essential for mating pair formation during conjugation are not present in pEST4011.

By using the National Center for Biotechnology Information ORF Finder program and BLAST similarity searches, 68 ORFs were identified in the pEST4011 sequence (Fig. 1; Table 1); 58 of these ORFs were named after the closest relative

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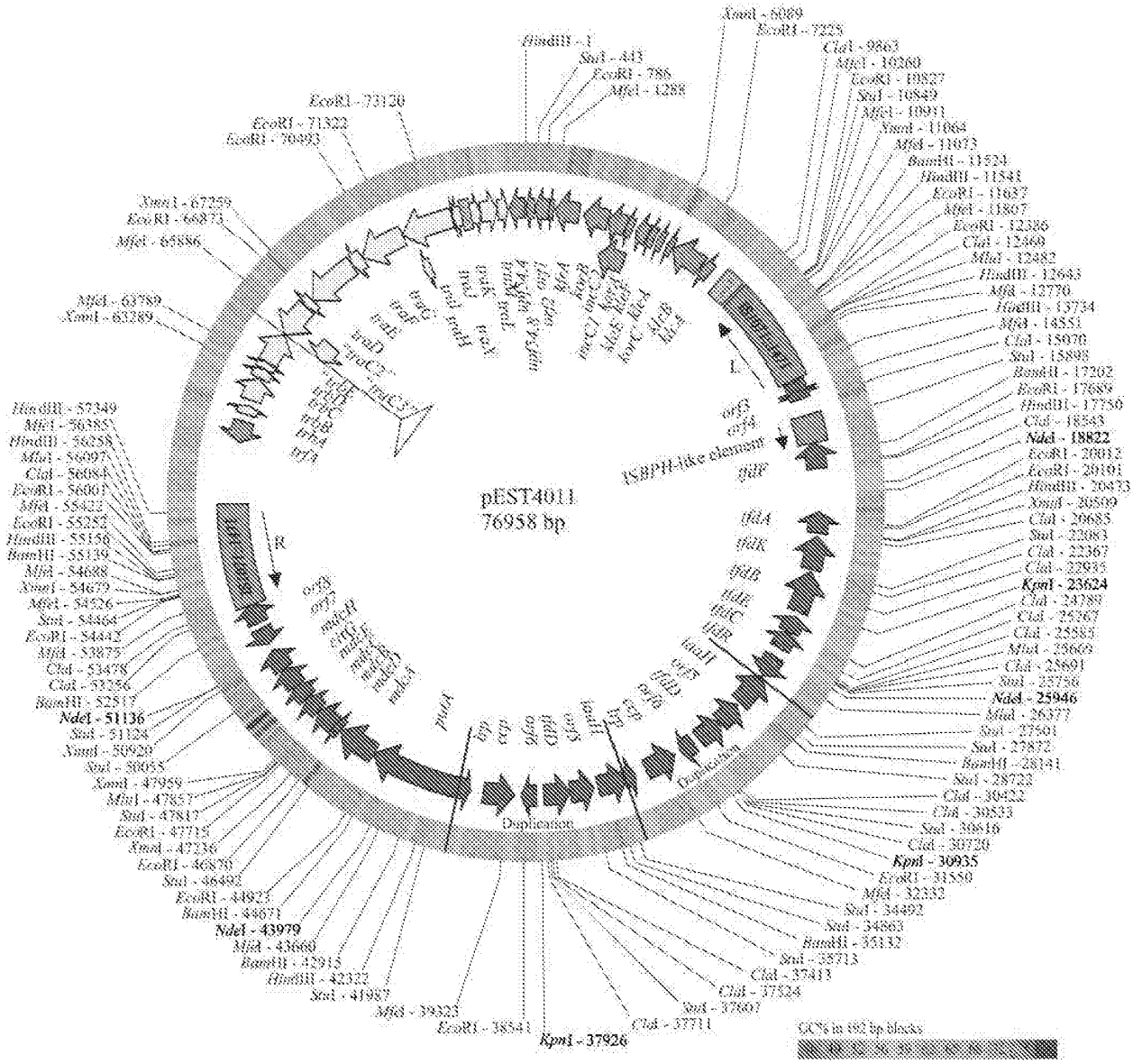


FIG. 1. Circular physical map of 76,958-bp plasmid pEST4011. The identified ORFs, the insertion elements, and the vegetative (*oriV*) and transfer (*oriT*) origins are shown inside the circle. The blue genes are necessary for plasmid autonomous replication initiation, copy number control, and stable maintenance in the host cell; the yellow genes are involved in plasmid conjugation; all genes in the pEST4011 catabolic transposon are purple. The 7-kb duplicated regions are indicated; the site from which the essential transfer genes *trbE* to *trbL* are missing is indicated by a line with a triangle at the end. R indicates the righthand copy of IS*1071*::IS*1471*, and L indicates the lefthand copy of IS*1071*::IS*1471*. The recognition site positions of 10 restriction endonucleases, which were used to verify the assembly of pEST4011, are indicated outside the circle. The colored circle indicates the G+C contents in 192-bp blocks starting from the position 1 (the first nucleotide in the corresponding HindIII recognition site). The products of the genes in quotation marks are truncated and thus probably not functional.

found in the GenBank database. Twenty-two ORFs are orientated in the same direction, whereas 46 are orientated in the opposite direction. Similarly, 34 ORFs are located both in the backbone and in the catabolic transposon of pEST4011 (9 of these ORFs are the *tfd* genes for 2,4-D catabolism). Six ORFs in the catabolic region (including one *tfd* gene) have identical copies due to duplication of a 7-kb DNA segment. Five ORFs code for putative transposases, and two of them are identical *tnp1471* genes of IS*1071*::IS*1471*. Ten ORFs encode putative transcriptional regulators. Four ORFs start with GTG, and the rest start with an ATG translation start codon. The translation stop codons are TGA (42 ORFs), TAA (15 ORFs), and TAG (12 ORFs). A ribosome-binding site with low similarity (two to four matches) to the Shine-Dalgarno consensus sequence (5′-TAAGGAGGT-3′) precedes 24 ORFs, while for 44 ORFs there are five to seven matches with the consensus sequence.

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TABLE 1. Locations and predicted functions of pEST4011 coding sequences

pEST4011 coding sequence	pEST4011 coordinates (5′ to 3′)	Function of closest relative (protein)[a]	Source	No. of amino acids in protein		% Identity/ % similarity	GenBank accession no.
				pEST4011 (alignment region)	Homolog (alignment region)		
upf54.8	350–6	Unknown (Upf54.8)	*Enterobacter aerogenes* plasmid R751	114 (1–114)	115 (1–115)	63/81	NC_001735
orf1	975–367	Cell filamentation protein (XF 1657)	*Xylella fastidiosa* 9a5c	202 (1–202)	203 (1–203)	82/93	AE003992
orf2	1160–972	Unknown (XF1656)	*Xylella fastidiosa* 9a5c	62 (1–62)	62 (1–62)	81/97	AE003992
kfrA	2241–1225	Regulation of *kfrA* transcription (KfrA)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	338 (1–338)	308 (1–308)	54/72	L27758
korB	3509–2436	Global transcriptional repressor (K or B)	*Enterobacter aerogenes* plasmid R751	357 (1–357)	349 (1–349)	73/87	NC_001735
incC1	4597–3506	Plasmid partitioning (IncC1)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	363 (1–363)	364 (1–364)	68/81	L27580
IncC2	4279–3506	Plasmid partitioning (IncC2)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	257 (1–257)	259 (1–259)	74/87	L27758
korA	4584–4276	Global transcriptional repressor (K or A)	*Enterobacter aerogenes* plasmid R751	102 (1–102)	100 (1–100)	73/90	NC_001735
kleF	5268–4747	Plasmid maintenance (KleF)	*Enterobacter aerogenes* plasmid R751	173 (1–173)	176 (1–176)	63/82	NC_001735
kleE	5593–5270	Plasmid maintenance (KleE)	*Enterobacter aerogenes* plasmid R751	107 (1–107)	109 (1–109)	61/79	NC_001735
kleA	5967–5710	Plasmid maintenance (KleA)	*Enterobacter aerogenes* plasmid R751	85 (1–85)	78 (1–78)	55/71	NC_001735
korC	6415–6158	Global transcriptional repressor (KorC)	*Enterobacter aerogenes* plasmid R751	85 (1–85)	85 (1–85)	68/84	NC_001735
klcB	7836–6430	KorA repression modulation (KlcB)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	468 (1–468)	461 (227–234) (1–461)[b]	53/72	L27758
klcA	8318–7884	Possible antirestriction system (KlcA)	*Enterobacter aerogenes* plasmid R751	144 (1–144)	142 (1–142)	75/87	NC_001735
tnp1471	13099–12062	Transposase (Tnp1471)	*Burkholderia cepacia* 2a plasmid pIJB1	345 (1–345)	345 (1–345)	100/100	AF029344
orf3	14087–14875	Unknown (CdoX3)	*Comamonas* sp. strain JS765	262 (143–262)	305 (138–256)	39/59	AF190463
orf4	14844–14479	Transcriptional regulator (COG0583)	*Ralstonia metallidurans*	121 (1–84)	305 (1–84)	90/98	NZ_AAA101000558
tnpBPH	15612–16595	Transposase (TnpBPH)	*Achromobacter georgiapolitanum* KKS102	327 (1–327)	321 (1–321)	65/78	AB047327
tfdF	17839–16766	Maleylacetate reductase (ClpF)	*Delfavibacter lusatiensis*	357 (1–357)	353 (1–353)	52/76	AJ536297
tfdA	20529–19666	2,4-D/alpha-ketoglutarate dioxygenase (TfdA)	*Delftia acidovorans* P4a	287 (1–287)	287 (1–287)	98/100	AY078159
tfdK	22069–20678	2,4-D transport protein (TfdK)	*Delftia acidovorans* P4a	463 (1–463)	463 (1–463)	99/99	AY078159
tfdB	23925–22168	2,4-Dichlorophenol hydroxylase (TfdB)	*Delftia acidovorans* P4a	585 (1–585)	586 (1–586)	99/99	AY078159
tfdE	24847–24140	Dienelactone hydrolase (TfdE)	*Delftia acidovorans* P4a	235 (1–235)	235 (1–235)	99/100	AY078159
tfdC	25628–24867	Chlorocatechol 1,2-dioxygenase (TfdCII)	*Ralstonia eutropha* JMP134 plasmid pJP4	253 (1–253)	253 (1–253)	89/97	U16782
tfdR	26946–26960	LysR-type transcriptional regulator (TfdR)	*Ralstonia eutropha* JMP134 plasmid pJP4	304 (1–304)	295 (1–295)	88/93	U16782
iaaH	28435–27014	Indole acetamide hydrolase (Tms2)	*Bradyrhizobium japonicum* USDA110	473 (1–473)	471 (1–471)	47/70	NC_004463
orf5	29621–28608	Unknown (ORF1)	*Delftia acidovorans* P4a	337 (1–337)	336 (1–336)	73/91	AY078159
tfdD	30735–29623	Chloromuconate cycloisomerase (TfdD)	*Delftia acidovorans* P4a	370 (1–370)	370 (1–370)	84/95	AY078159
orf6	31042–31671	Putative transposase (ORF4)	*Burkholderia cepacia* R34	209 (1–209)	371 (163–371)	94/99	AF169302
ccp	33282–31951	Chloride channel protein (EriC)	*Burkholderia fungorum*	443 (1–443)	459 (1–459)	41/65	ZP_00030265
lrp	34302–33793	Leucine-responsive regulatory protein (Lrp)	*Bradyrhizobium japonicum* USDA110	169 (1–169)	153 (1–153)	57/73	NC_004463
iaaH	35426–34191	Indole acetamide hydrolase (Tms2)	*Bradyrhizobium japonicum* USDA110	411 (1–411)	471 (1–423)	42/66	NC_004463
orf5	36612–35599	Unknown (ORF1)	*Delftia acidovorans* P4a	337 (1–337)	336 (1–336)	73/91	AY078159
tfdD	37726–36614	Chloromuconate cycloisomerase (TfdD)	*Delftia acidovorans* P4a	370 (1–370)	370 (1–370)	84/95	AY078159
orf6	38033–38662	Putative transposase (ORF4)	*Burkholderia cepacia* R34	209 (1–209)	371 (163–371)	94/99	AF169302
ccp	40273–38942	Chloride channel protein (EriC)	*Burkholderia fungorum*	443 (1–443)	459 (1–459)	41/65	ZP_00030265
lrp	41293–40784	Leucine-responsive regulatory protein (Lrp)	*Bradyrhizobium japonicum* USDA110	169 (1–169)	153 (1–153)	57/73	NC_004463
putA	41332–45132	Proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase bifunctional protein (COG4230)	*Pseudomonas syringae* pv. syringae B728a	1,266 (1–1266)	1,317 (1–1317)	60/78	ZP_00125171
mdcA	45142–46824	Malonate decarboxylase alpha subunit (MdcA)	*Xanthomonas axonopodis* pv. citri str. 306	560 (1–560)	548 (1–548)	79/90	NC_003919
mdcD	46834–47160	Malonate decarboxylase delta subunit (MdcD)	*Xanthomonas axonopodis* pv. citri str. 306	108 (1–108)	105 (1–105)	49/66	NC_003919
mdcB	47157–48050	Malonate decarboxylase beta subunit (MdcB)	*Xanthomonas axonopodis* pv. citri str. 306	297 (1–297)	306 (1–306)	55/71	NC_003919
mdcC	48061–48762	Malonate decarboxylase gamma subunit (MdcC)	*Xanthomonas axonopodis* pv. citri str. 306	233 (1–233)	234 (1–234)	49/69	NC_003919
mdcE	48771–49463	Holo-ACP synthetase (MdcE)[c]	*Xanthomonas axonopodis* pv. citri str. 306	230 (1–230)	213 (1–213)	31/53	NC_003919
citG	49460–50332	2′-(5′′-Triphosphoribosyl)-3′-dephospho-coenzyme A synthetase (CitG)	*Xanthomonas axonopodis* pv. citri str. 306	290 (1–290)	289 (1–289)	47/62	NC_003919
mdcH	50329–51237	Malonyl coenzyme A-ACP transacylase (MdcH)	*Xanthomonas axonopodis* pv. citri str. 306	302 (1–302)	306 (1–306)	45/63	NC_003919
orf7	52274–51534	Transcriptional regulator (COG1309)	*Burkholderia fungorum*	246 (1–246)	242 (1–242)	33/62	ZP_00033878
orf8	52526–53341	Putative exported protein, conserved in bacteria	*Bordetella pertussis* Tohama I	271 (1–263)	323 (1–265)	47/75	NP_879486
trp1471	56714–55677	Transposase (Tnp1471)	*Burkholderia capacia* 2a plasmid pIJB1	345 (1–345)	345 (1–345)	100/100	AF029344
trfA	61110–60241	Initiation of vegetative plasmid replication (TrfA2)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	289 (1–289)	285 (1–285)	77/88	L27758
trbA	61431–61808	Transcriptional repressor (TrbA)	*Enterobacter aerogenes* plasmid R751	125 (1–125)	120 (1–120)	63/80	NC_001735
trbB	62060–63022	Inner membrane ATPase (TrbB)	*Enterobacter aerogenes* plasmid R751	320 (1–320)	320 (1–320)	81/89	NC_001735
trbC	63034–63462	Involved in mating pair formation during conjugation (TrbC)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	142 (1–142)	145 (1–145)	66/84	L27758
trbD	63465–63776	Involved in mating pair formation during conjugation (TrbD)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	103 (1–103)	103 (1–103)	86/96	L27758

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trbE	63773–65146	Involved in mating pair formation during conjugation (TrbE)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	457 (1–457)	852 (1–457)	84/95	L27758	
traC2	66617–65130	Primase (TraC2)	*Enterobacter aerogenes* plasmid R751	495 (1–495)	1,448 (1–495)	83/92	NC_001735	
traC3	65942–65130	Primase (TraC3)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	270 (1–270)	1,061 (1–270)	85/93	L27758	
traD	66998–66621	Unknown (TraD)	*Enterobacter aerogenes* plasmid R751	125 (1–125)	129 (1–129)	62/79	NC_001735	
traE	69195–67003	Possible helicase (TraE)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	730 (1–730)	737 (1–737)	78/90	L27758	
traF	69740–69204	Involved in mating pair formation during conjugation (TrbE)	*Enterobacter aerogenes* plasmid R751	178 (1–178)	178 (1–178)	82/92	NC_001735	
traG	71650–69737	DNA transport during conjugation (TraG)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	637 (1–637)	635 (1–635)	83/95	L27758	
traI	73839–71647	DNA relaxase (TraI)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	730 (1–730)	732 (1–732)	59/83	L27758	
traH	72272–71946	Relaxosome stabilization (TraH)	Birmingham IncP-alpha plasmid (RP4, RK2, etc.)	108 (1–108)	119 (1–119)	61/75	L27758	
traX	73877–73836	Translation coupling (TraX)	*Enterobacter aerogenes* plasmid R751	13 (1–13)	13 (1–13)	54/85	NC_001735	
traJ	74263–73874	*oriT* recognizing protein (TraJ)	*Enterobacter aerogenes* plasmid R751	129 (1–129)	130 (1–130)	55/70	NC_001735	
traK	74655–75053	*oriT* binding protein (TraK)	*Enterobacter aerogenes* plasmid R751	132 (1–132)	132 (1–132)	57/75	NC_001735	
traL	75053–75778	Essential transfer protein (TraL)	*Enterobacter aerogenes* plasmid R751	241 (1–241)	241 (1–241)	83/94	NC_001735	
traM	75778–76215	Essential transfer protein (TraM)	*Enterobacter aerogenes* plasmid R751	145 (1–145)	146 (1–146)	56/82	NC_001735	
upf54.4	76933–76304	Unknown (Upf54.4)	*Enterobacter aerogenes* plasmid R751	209 (1–209)	217 (1–217)	51/72	NC_001735	

[a] As the whole catabolic transposon of pEST4011 (positions 9700 to 57634) is practically identical to Tn*5530* of *Burkholderia cepacia* 2a plasmid pJJ81 (accession number AF029344), the second-closest relatives are indicated for the pEST4011 coding sequences.

[b] The sequence of the Birmingham IncP-alpha plasmid *klcB* gene is interrupted by Tn*1*, which was removed by us before alignment.

[c] ACP, acyl carrier protein.

Genes involved in 2,4-D degradation. In *A. xylosoxidans* subsp. *denitrificans* EST4002, all *tfd* genes necessary for 2,4-D degradation (the pathway is shown in Fig. 2) are located on plasmid pEST4011 (Table 1; Fig. 1). Figure 3 shows the portion of the pEST4011 catabolic region containing the *tfd* genes, which are organized as follows. There is a cluster of genes comprising *tfdC* (which codes for 3,5-dichlorocatechol 1,2-dioxygenase) (21), *tfdE* (which codes for 2-chlorodienelactone hydrolase), *tfdB* (which codes for 2,4-dichlorophenol hydroxylase), *tfdK* (which codes for 2,4-D transporter), and *tfdA* (which codes for 2,4-D/α-ketoglutarate dioxygenase). *tfdR*, which encodes a LysR-type transcriptional activator (54), is located immediately upstream and is transcribed divergently from this cluster; almost 2 kb downstream of this cluster lies a gene for (2-chloro)maleylacetate reductase (*tfdF*); finally, 2,4-dichloro-*cis*,*cis*-muconate cycloisomerase is encoded by two identical copies of *tfdD* which are located 2.6 and 9.6 kb upstream of *tfdR*.

The *A. xylosoxidans* subsp. *denitrificans* EST4002 *tfdE*, *tfdB*, *tfdK*, and *tfdA* genes are almost identical to the same genes of the *D. acidovorans* P4a chromosomal transposon for 2,4-D degradation and are also organized in the same order (Table 1; Fig. 3). The *tfdC* and *tfdR* genes are most similar to the corresponding genes of *W. eutropha* JMP134 plasmid pJP4. The pEST4011 *tfdD* gene and *orf5*, which are immediately downstream of *tfdD*, are most similar to *D. acidovorans* P4a *tfdD* and *orf1*. The function of the product encoded by *orf1* is unknown, but this ORF has been found in many operons determining *ortho* cleavage pathways of chlorocatechols. The strongly conserved amino acid sequences encoded by these ORFs suggest that the products might play an essential role in the function of these operons (31). The chloromaleylacetate reductase encoded by pEST4011 *tfdF* is quite different from all other known chloromaleylacetate reductases; only 52% of its amino acids are identical to the amino acids of the closest relatives, the α-proteobacterial ClpF involved in chlorophenol degradation by *Defluvibacter lusatiensis* and *Bradyrhizobium japonicum* USDA 110 MacA (52% amino acid identity and 77% amino acid similarity).

All *tfd* gene products except TfdD and TfdF are very similar to pJP4 *tfd* gene products, including TfdA (84% amino acid identity and 94% amino acid similarity), $TfdB_{II}$ (91% amino acid identity and 97% amino acid similarity), $TfdC_{II}$ (89% amino acid identity and 97% amino acid similarity), $TfdE_{II}$ (79% amino acid identity and 93% amino acid similarity), and TfdK (74% amino acid identity and 90% amino acid similarity) (Fig. 3). The ancestors of the pEST4011 *tfdRCEBKA* gene cluster and the pJP4 $tfdRD_{II}C_{II}E_{II}F_{II}B_{II}K$ gene cluster probably had a common origin. In the case of pEST4011, the regions containing the *tfdD* and *tfdF* genes have been deleted. In the chromosomal transposon of *D. acidovorans* P4a only a portion of the *tfdRCEBKA* cluster is present in pEST4011 (Fig. 3). As *tfdF* and *tfdD* are not pJP4-like genes and are separated from the *tfdRCEBKA* gene cluster, they were probably individually recruited in the precursor of the pEST4011 catabolic transposon to replace the genes lost from the putative ancestor the *tfdRDCEFBK(A)* cluster during evolution. Vallaeys et al. have also shown that diverse gene cassettes were independently recruited during assembly of 2,4-D-catabolic pathways having different origins (51).

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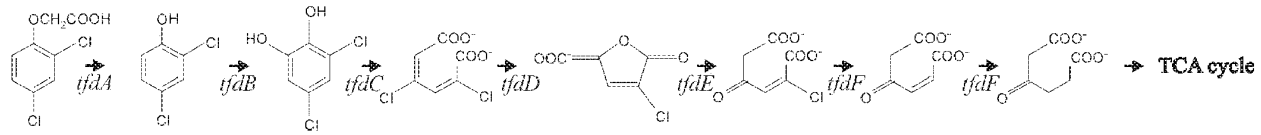


FIG. 2. 2,4-D degradation pathway encoded by *tfd* genes. TCA, tricarboxylic acid.

At a position 1.2 kb upstream of *tfdD* there is a gene (*ccp*) whose predicted product is most similar to *Burkholderia fungorum* EriC (41% amino acid identity and 65% amino acid similarity) and *Xanthomonas axonopodis* pv. citri str.306 (accession number NC_00125171) YadQ (37% amino acid identity and 65% amino acid similarity), which are the chloride-channeling proteins (37). As chloride ions are liberated during 2,4-D dissimilation, this protein may have a role in removal of these ions from cells.

The proposed functions and the levels of amino acid identity and similarity of other predicted gene products encoded by the pEST4011 catabolic transposon are shown in Table 1. In this transposon only 11 of 34 ORFs (nine *tfd* genes plus two copies of *ccp* coding for a chloride-channeling protein) are necessary for 2,4-D degradation. In addition to these ORFs, the presence of two copies of *orf5* could be bound to the same function (31). Five ORFs code for (putative) transposases, and four ORFs are translated into (putative) transcriptional regulators; the functions of two ORFs are unknown. The remaining 10 ORFs potentially code for different catabolic functions, including indole acetamide hydrolase (two copies of *iaaH*), proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase bifunctional protein (*putA*), and malonate decarboxylase (*mdc* operon of seven genes). As shown in Table 1, the closest relatives of these catabolic genes are found in bacteria known to be plant pathogens (*X. axonopodis* and *Pseudomonas syringae*) and in a nitrogen-fixing symbiontic bacterium (*B. japonicum*). We analyzed strains EST4002 (containing pEST4011) and EST4003 (containing the deletant plasmid pEST4012) by a microtiter plate-bound substrate utilization assay (Biolog Inc.). The scatter plot of substrate utilization activities for the two strains studied showed a high correlation (R^2 = 97.8%, $P < 0.0001$), which means that all 95 carbon substrates from Biolog GN2 microplate wells were utilized by these strains at approximately the same rate (Fig. 4).

Indole acetamide hydrolase (IaaH) performs the second reaction in the two-step pathway for synthesis of indole acetic acid (a plant hormone auxin) from tryptophan, which is found in associated plant-growth-promoting rhizobacteria (41). Thus, even if the *iaaH* gene codes for a functional enzyme, plasmid pEST4011 itself does not enable its host to synthesize auxin. The PutA bifunctional protein converts L-proline to glutamate for use as a carbon and nitrogen source; in addition, it acts as a repressor of its own expression in response to the proline supply (27). The Biolog analysis showed that strains EST4002 and EST4003 both used L-proline at physiological concentrations as a growth substrate (Fig. 4). Thus, this ability must rely on chromosomal genes.

Decarboxylation of malonate to acetate and CO_2 is a key reaction as it initiates decomposition of this compound in both

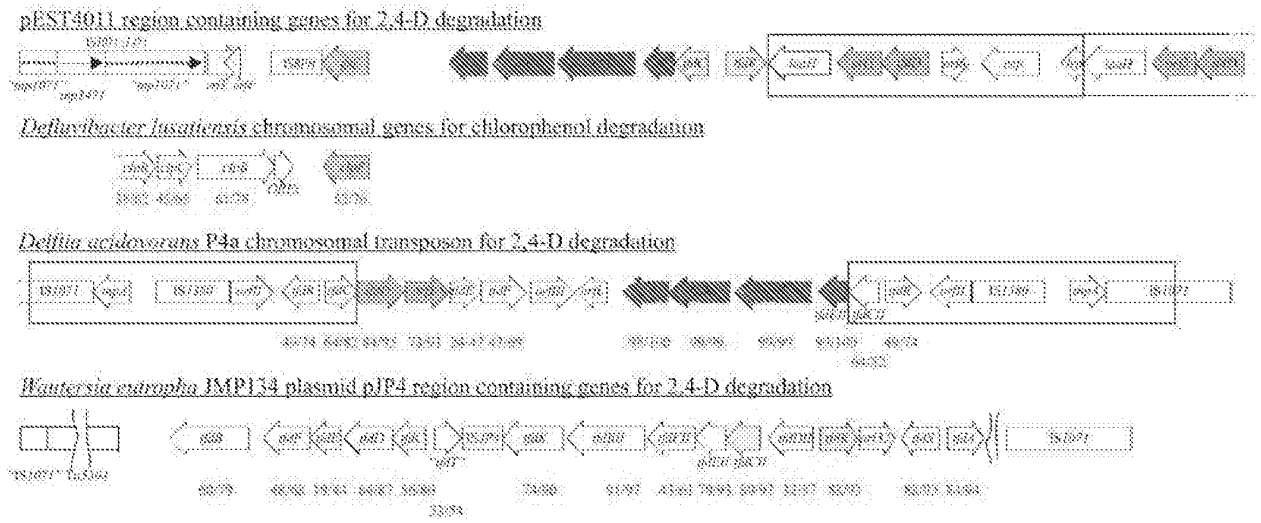


FIG. 3. Comparison of the arrangement of the *tfd* genes in plasmid pEST4011 and the homologous genes in *D. lusatiensis* (accession number AJ536297), *D. acidovorans* P4a (AY078159), and *W. eutropha* JMP134 plasmid pJP4 (AY365053). A pEST4011 *tfd* gene and its most similar counterpart are indicated by the same color. The red numbers below the genes indicate percentage of amino acid identity/percentage of amino acid similarity compared with the corresponding pEST4011 *tfd* analogue. The boxes around the genes indicate duplicated regions.

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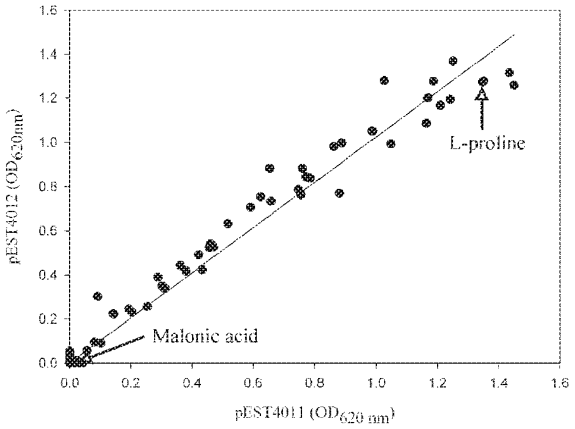


FIG. 4. Scatter plot of substrate utilization activities for strain EST4002 containing plasmid pEST4011 and strain EST4003 containing deletant plasmid pEST4012, with a regression line. All 95 carbon substrates from Biolog GN2 microplate wells are represented by dots, and the locations of L-proline and malonic acid are indicated by arrows. OD_{620nm}, optical density at 620 nm.

aerobic and anaerobic bacteria (7, 20). The predominant portion of malonate in the environment originates from industrial production; therefore, bacterial degradation of this compound is now of great interest. In the case of *B. cepacia* both strain 2a (which contains pIJB1) and mutant strain 2a-1 (which contains plasmid pIJB2, in which the whole catabolic region has been deleted) (56) were able to grow on malonate as a sole source of carbon and energy. Xia et al. (56) concluded that the ability to grow on malonate must reside on the chromosome, although the plasmid-borne analogues of *mdc* genes could also be active. According to the substrate utilization assay, strains EST4002 and EST4003 do not use malonic acid as a growth substrate (Fig. 4). Therefore, as the nucleotide sequences of pEST4011 and pIJB1 *mdc* operons are 100% identical, this gene cluster alone is not adequate for malonate dissimilation in either host. Consequently, it seems that the only detectable catabolic property of EST4002 provided by plasmid pEST4011 is the ability to degrade 2,4-D. However, it is possible that in other hosts this plasmid contributes to additional properties (for example, auxin biosynthesis or malonate dissimilation).

The (putative) regulatory proteins encoded by pEST4011 (for example, Lrp) may alter the expression pattern of both plasmid and chromosomal genes. Genome analyses have revealed that members of the Lrp family of transcriptional regulators are widely distributed among prokaryotes. The archetype leucine-responsive regulatory protein from *E. coli* is a global regulator involved in modulating a variety of metabolic functions, including catabolism and anabolism of amino acids, as well as pilus synthesis (4). Lrp has also been shown to be a positive modulator of conjugal transfer of F-like plasmids (5, 40). However, the metabolic patterns of strains EST4002 and EST4003 with the carbon substrates available on Biolog GN2 microplates are not significantly different (Fig. 4).

Insertion elements in pEST4011. The 48-kb catabolic transposon of pEST4011 is bordered by identical (two mismatches) copies of the hybrid insertion element IS*1071*::IS*1471* (positions 9700 to 14019 and 53315 to 57634) (Fig. 3). This element consists of the 1.1-kb IS*1471* element (which belongs to the IS*630* family described by Mahillon and Chandler [29]) inserted into the 3.2-kb class II transposable element IS*1071* (57). While IS*1071* flanks a variety of catabolic genes and operons (6), IS*1471* has been detected only in the hybrid IS*1071*::IS*1471* insertion elements present in pEST4011 and pIJB1. In the case of pIJB1, both copies of IS*1071*::IS*1471* flanking Tn*5530* have been sequenced only partially, but Poh et al. (36) have suggested that these copies are not identical.

At a position 1.5 kb upstream of the lefthand copy of IS*1071*::IS*1471* there is another insertion element in pEST4011 (Fig. 1), an IS*BPH*-like structure whose transposase gene is most similar to the *Achromobacter georgiopolitanum* KKS102 transposase gene *tnpBPH* of insertion element IS*BPH* (Table 1). In *A. georgiopolitanum* KKS102, IS*BPH* is located between *bphS* and *bphE*, the genes involved in biphenyl degradation (32).

Comparison of the pEST4011 backbone with other IncP1 backbones. A 29-kb region of pEST4011 (positions 1 to 9699 and 57635 to 76958) contains genes for plasmid replication initiation, stable maintenance in the host cell, and the conjugation machinery for plasmid transfer into new hosts (Fig. 1). The overall structure of the pEST4011 backbone is similar to that of the archetype plasmids belonging to the IncP1 α subgroup (RK2, etc.) (33) and IncP1 β subgroup (R751) (44) (Table 2 briefly describes all plasmids discussed in this paper). The predicted gene products of the backbones of the IncP1 plasmids (completely) sequenced by May 2004, including pUO1 (39), pADP-1 (30), pJP4 (48), pTSA (47), and pB4 (42), are 65 to 100% identical to those of R751. However, the corresponding gene products of the pEST4011 backbone show only 51 to 86% identity to either R751 or RK2 backbone gene products. The proposed functions and levels of amino acid identity and similarity of all predicted pEST4011 backbone gene products are shown in Table 1.

A comparison of the six IncP1 plasmids mentioned above (not including pJP4) (1, 39) showed that various resistance and catabolic determinants are inserted mostly into two regions in the plasmid backbones, between *oriV* and the *trfA* gene and between the Tra1 and Tra2 regions. When we compared plasmids pEST4011 and R751, the archetype plasmid of the IncP1 β subgroup, we found that pEST4011 contains only one catabolic transposon inserted between the core of *oriV* and the *trfA* gene (between iterons 2 and 3) (Fig. 5).

In the pEST4011 backbone two ORFs were found between the *upf54.8* and *kfrA* genes, namely, *orf1* and *orf2*, whose predicted amino acid sequences are most similar to the sequences of the *Xylella fastidiosa* 9a5c cell filamentation protein and to an unknown protein, respectively (Table 1). These two ORFs are not present in any of the plasmids mentioned above. In addition, two small genes, *kleB* and *kleG*, which are devoted to stable inheritance, as well as two genes having unknown functions, *kluA* and *kluB*, located in the R751 backbone were not identified in pEST4011.

In R751, RK2, pJP4, and pB4 there are two forms of the replication initiation protein TrfA encoded by the *trfA1* and *trfA2* genes, the larger TrfA-44 protein (382 to 407 amino acids) and the smaller TrfA-33 protein (284 to 289 amino acids). These proteins are translated from the same reading frame, and the translational start sites are 97 to 122 amino acids apart. It has

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TABLE 2. Plasmids discussed in this paper

Plasmid	Description[a]	GenBank accession no.	Reference(s)
RK2, RP4, etc.	IncP-1α, 60,099 bp, Amp^r Tet^r, isolated from enteric bacteria in Birmingham, UK	L27758	33
R751	IncP-1β, 53,425 bp, Tmp^r, isolated from *Enterobacter aerogenes* in UK	NC_001735	44
pJP4	IncP-1β, 87,688 bp, Hg^r, contains *tfd* genes for 2,4-D and 3-CBA degradation, isolated from *Rolstonia eutropha* JMP134 in Australia	AY365053	8
pIJB1	IncP-1β, about 102 kb, Hg^r, contains *tfd* genes for 2,4-D degradation located on Tn*5530*, isolated from *Burkholderia cepacia* 2a in UK	AF029344	36, 56
pUO1	IncP-1β, 67,066 bp, Hg^r, contains haloacetate-catabolic genes, isolated from *Delftia acidovorans* B in Osaka, Japan	NC_005088	39
pADP-1	IncP-1β, 108,845 bp, Hg^r, contains atrazine-catabolic genes, isolated from *Pseudomonas* sp. strain ADP in Minnesota	NC_004956	30
pB4	IncP-1β, 79,370 bp, Sm^r Em^r Amx^r, isolated from microbial community of activated sludge from wastewater treatment plant in Germany by biparental mating	NC_003430	42
pTSA	IncP-1β, about 72 kb, Hg^r, contains *p*-toluenesulfonate-catabolic genes, isolated from *Comamonas testosteroni* T-2 in Switzerland	AF303942, AF305549, AF311437, AF311820, AH010657, AY227144, AY227147, U73743	47
pEMT3	IncP-1, initially contained *tfd* genes for 2,4-D degradation, but the degradative capacity has disappeared, isolated from microbial community of agricultural soil in Michigan by biparental mating	AJ414161, AJ414162	11, 45
pQKH54	IncP-1γ, about 80 kb, Hg^r, isolated from epilithic microbial community of the River Taff in South Wales, UK, by triparental mating		14
pTV1	Unknown Inc group, about 200 kb, Hg^r, contains *tfd* genes for 2,4-D degradation, isolated from *Variovorax paradoxus* TV1 in Dijon, France	AB028643	49, 50

[a] Abbreviations: Amp, ampicillin; Tet, tetracycline; Tmp, trimethoprim; Hg, mercury; Sm, streptomycin; Em, erythromycin; Amx, amoxicillin; UK, United Kingdom; 3-CBA, 3-chlorobenzoate.

been shown that the smaller protein is sufficient for plasmid replication in many hosts (59). In pUO1, pTSA, and pEMT3 only the gene coding for TrfA-44 (406 to 411 amino acids) has been annotated, while in pADP-1 and pEST4011 only TrfA-33 proteins are encoded (303 and 289 amino acids, respectively). In pADP-1, the region corresponding to the N-terminal part of TrfA-44 is missing, and in pEST4011 the deletion also encompasses the *ssb* gene, which codes for a single-stranded DNA-binding protein involved in DNA replication, recombination, and repair and is present in all of the other plasmids mentioned above just upstream of *trfA*. In pEST4011 an additional 2,360 bp of DNA (positions 57635 to 59995) is inserted between the catabolic transposon and iteron 2 (positions 60146 to 60166) of *oriV*. The nucleotide sequence of this region is not similar to any sequence in the GenBank database.

In pEST4011, a large amount of DNA coding for the C-terminal half of TrbE and genes *trbF* to *trbN*, as well as the region coding for most of the C-terminal region of TraC2 and TraC3 and all of the *traC4* gene, which are present in R751, RK2, pJP4, pADP-1, pUO1, and pB4, are missing. In the latter plasmids, this region may also contain additional genes, but the important fact is that it includes seven *trb* genes (*trbE* to *trbL*) essential for mating pair formation during conjugation (1).

As in other IncP1 plasmids, five transcriptional regulators are encoded in the pEST4011 backbone. KorA, KorB, and KorC are three global repressors that regulate transcription of the genes necessary for plasmid replication, stable inheritance, and conjugation; TrbA is a transcriptional repressor of conjugation genes; and KfrA has been shown to regulate only its own transcription (1). Consensus sequences for the binding of these regulators have been determined for KorA, KorB, KorC, and TrbA (3, 33, 44). Most of these binding sites are also present in pEST4011; the only exception is one TrbA binding site present in the RK2 *traJ/traK* promoter-operator region (Table 3). Additionally, one KorB binding site upstream of *traJ* and one KorC binding site upstream of *klcA*, which are not present in R751 or RK2, were found in pEST4011. As shown in Table 3, the binding sites of KorA and KorB are well con-

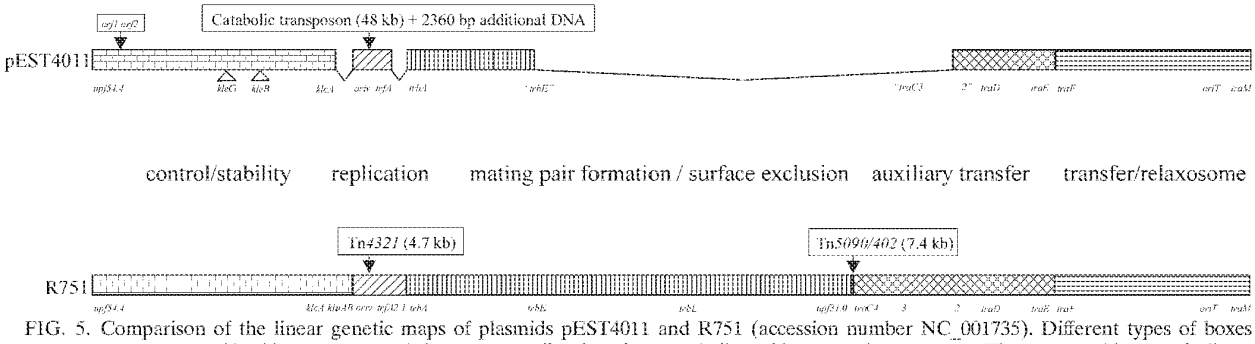


FIG. 5. Comparison of the linear genetic maps of plasmids pEST4011 and R751 (accession number NC_001735). Different types of boxes represent common sets of backbone genes, and the corresponding functions are indicated between the two maps. The arrows with boxes indicate insertions not found in the two plasmids; the pEST4011 sites from which the *kleG* and *kleB* genes are missing (compared with R751) are indicated by triangles. Some of the pEST4011 and R751 backbone gene positions are also indicated for orientation.

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