TABLE 3. DNA binding proteins encoded by the pEST4011 backbone

DNA-binding protein	Binding site coordinates in pEST4011	Binding site location in pEST4011	Binding site sequence in pEST4011 (5′ to 3′)[a]	Binding site sequence in R751 (5′ to 3′)[a]	Binding site sequence in RK2 (5′ to 3′)[a]
KorA	2314–2323	*kfrAp*	**TTTAGCTAAA**	**TTTAGCTGAA**	**TTTAGCTGAA**
	4659–4668	*korAp*	**TTTAGCTAAA**	**TTTAGCTAAA**	**TTTAGCTAAA**
	6054–6063	*kleAp*	**TTTAACGAAA**	**TTTAGCTAAA**	**TTTAGCTAAA**
	8401–8410	*klcAp*	**TTTAGCTAAA**	**TTTAGCTAAA**	**TTTAGCTAAA**
	61353–61362	*trfAp*	**TTTAGCTAAA**	**TTTAGCTAAA**	**TTTAGCTAAA**
KorB	2329–2341	*kfrAp*	**TTTAGCGGCTAAA**	**TTTAGCGGCTAAA**	**TTTAGCGGCTGAA**
	4690–4702	*korAp*	**TTTAGCCGCTAAA**	**TTTAGCGGCTAAA**	**TTTAGCCGCTAAA**
	6137–6149	*kleAp*	**TTTAGCCGCTAAA**	**TTTAGCCGCTAAA**	**TTTAGCCGCTAAA**
	8412–8424	*klcAp*	**TTTAGCCGCTAAA**	**TTTAGCCGCTAAA**	Not present
	61384–61396	*trfAp*	**TTTAGCGGCTAAA**	**TTTAGCGGCTAAA**	**TTTAGCGGCTAAA**
	61824–61836	*trbBp*	**TTTAGCGGCTAAA**	**TTTAGCCGCTAAA**	**TTTAGCGGCTAAA**
	69453–69465	*traF*	**TTTAGCGGCTAAA**	**TTTAGCGGCTAAA**	**TTTAGCCGCTAAA**
	73857–73869	*traX*	**TTTAGCGGCTAAA**	**TTTAGCCGCTAAA**	**TTTAGCGGCTAAA**
	74273–74285	Upstream of *traJ*	**TTTAGCGGCTAAA**	Not present	Not present
	76948–2	Upstream of *upf54.4*	**TTTAGCGGCTAAA**	**TTTAGCCGCTAAA**	**TTTAGCCGCTAAA**
KorC	6025–6040	*kleAp*	**TAGGGCAAAATGCCCTA**	**TAGGACAAAATGTCCTA**	**TAGGGCATTATGCCCTA**
	8373–8389	*klcAp*	**TAGGGCAAAATGCCCTA**	**TAGGACAAAATGTCCTA**	**TAGGGCATTATGCCCTA**
	8752–8768	Upstream of *klcA*	**TAGGGCAAAATGCCCTA**	Not present	Not present
TrbA	61253–61266	*trbAp*	**CTCGATGTATCGCT**	**ACAGCCAGGTCGAA**	**ACCGCTATATCGAA**
	62004–62017	trbBp	**GTTGGTATATCGTT**	**TTCGATATATCGTA**	**TTCGGTATATCGTT**
	71848–71861	*traGp*	**GTCAGTATAGCGGG**	**GGCAGTATATCGGG**	**ATCAGTATATCGTG**
	71867–71880	*traGp*	**TTCGGTATATCGAA**	**TTCGGTATATCGAA**	**TTCGGTATATCGAA**
	74493–74506	*traJp/traKp*	**ACCGATATAGCGAA**	**ACGGATATAGCGAA**	**ATGGATATACCGAA**
TraJ	74390–74404	*oriT*	**CGGTTAGCTAACTTC**	**CGCTTAGCTAACTTC**	**GGGTGGGCCTACTTC**
TraI	74409–74414	*oriT*	**ATCCTG**	**ATCCTG**	**ATCCTG**
TrfA	8790–8810	*oriV*	**TTTCTTTGACACTTGAGGGGC**	**GCTTCTTGACACTTGAGGGGC**	**TTTCATTGACACTTGAGGGGC**
	9285–9305c	*oriV*	**CACCCTAGACACTTGAGGGGC**	**TATCCATTGACACTTGAGGGGC**	**CACCTATTGACATTTGAGGGGC**
	9307–9327c	*oriV*	**AACTACAGACATTTGAGGGGC**	**GACTATTGACCCTTGAGGGGC**	**GAGTGATGACAGATGAGGGGC**
	9329–9349c	*oriV*	**CTTCGTTGACACTTGTGGGGC**	**GATCCTTGACACTTGACGGGC**	**GATCCTTGACACTTGAGGGGC**
	9352–9372c	*oriV*	**AACCCTAGACACTTGGGGGGC**	**GGGTGCTGACAGATGAGGGGC**	**GACTACTGACAGATGAGGGGC**
	9375–9395c	*oriV*	**TTCTATAGACACTTGAGGGGC**	**CCGTACTGACACTTGAGGGGC**	**CGGTATTGACACTTGAGGGGC**
	9501–9521c	*oriV*	**CACTGCAGACACTTGAGGGGC**	**CGATGCTGACAGATGAGGGGC**	**CGGCGTTGACAGATGAGGGGC**
	9536–9556c	*oriV*	**GTTCATTGACACTTGGGGGGC**	**GTTTCTTGACACGTTGGGGGGC**	**GGACGTTGACACTTGAGGGGC**
	9557–9577c	*oriV*	**TCACCGTGACACTTGGGGGGC**	**GGCTGGTGACAGTTGAGGGGC**	**CCTCACTGACAGATGAGGGGC**
	60146–60166c	*oriV*	**GCATGCAGACACTTGAGGGGC**	**ACTCTTTGACATTTGAGGGGC**	**TTTCATTGACACTTGAGGGGC**
	60176–60196c	*oriV*	**TTTCATTGACAGATGAGGGGC**	Not present	Not present

[a] The putative consensus operator sequence is indicated by boldface type.

served in the three plasmids, while the KorC binding sites of pEST4011 look like hybrids of the corresponding R751 and RK2 sites. Interestingly, the TrbA operator sequences are more heterogeneous and contain more mismatches with the proposed consensus binding site (WWCGATATATCGWW) (3). In the case of RK2, the individual TrbA operators contain one to three mismatches, while the corresponding sequences of pEST4011 and R751 have up to six mismatches. Only one TrbA binding site shown in Table 3, the site in the promoter region of the R751 *trbB* gene, is an exact match with the consensus sequence. Bingle and Zatyka (3) assumed that such suboptimal operators play an important role in the balanced regulation of plasmid transfer. Finally, the TraJ binding sites of pEST4011 and R751 are identical. This is in agreement with the fact that the amino acid sequence of the pEST4011 TraJ protein is more similar to the corresponding sequence of R751 than to the corresponding sequence of RK2, although the level of identity is only 55%.

The pEST4011 origin of replication (*oriV*) consists of the same components as the origins of replication described for other IncP1 plasmids (1, 24, 33): 10 binding sites (called iterons) for the TrfA protein (which initiates replication at *oriV*), including iterons 2 to 11 plus one additional iteron (iteron 1) not present in R751 or RK2 (Table 3; Fig. 6A); four binding sites for the host protein DnaA; three palindromic sequences which are also proposed to bind TrfA; an A-T-rich region; and a G-C-rich region. Table 3 shows that the conserved TGACA motif of the TrfA iterons of R751 and RK2 is much more frequently AGACA in pEST4011. Also, the second part of the pEST4011 iterons is more frequently CTTGAG than it is in R751 and RK2. In RK2 this sequence quite strictly alternates between CTTGAG and GATGAG.

The structure of the origin of transfer (*oriT*) is also similar to that of other IncP1 plasmids (1, 33). It consists of a relaxation region containing TraJ and TraI binding sites (Table 3; Fig. 6B) and of the *nic* site GC (positions 74414 and 74415 in pEST4011), in which one strand of DNA is nicked during conjugation (34).

Multiple alignments of *trfA2*, *korA*, and *traG* gene products of IncP1 plasmids. As described above, the backbone gene products of pEST4011 are homologous but still quite different from the corresponding products of both RK2 and R751, the

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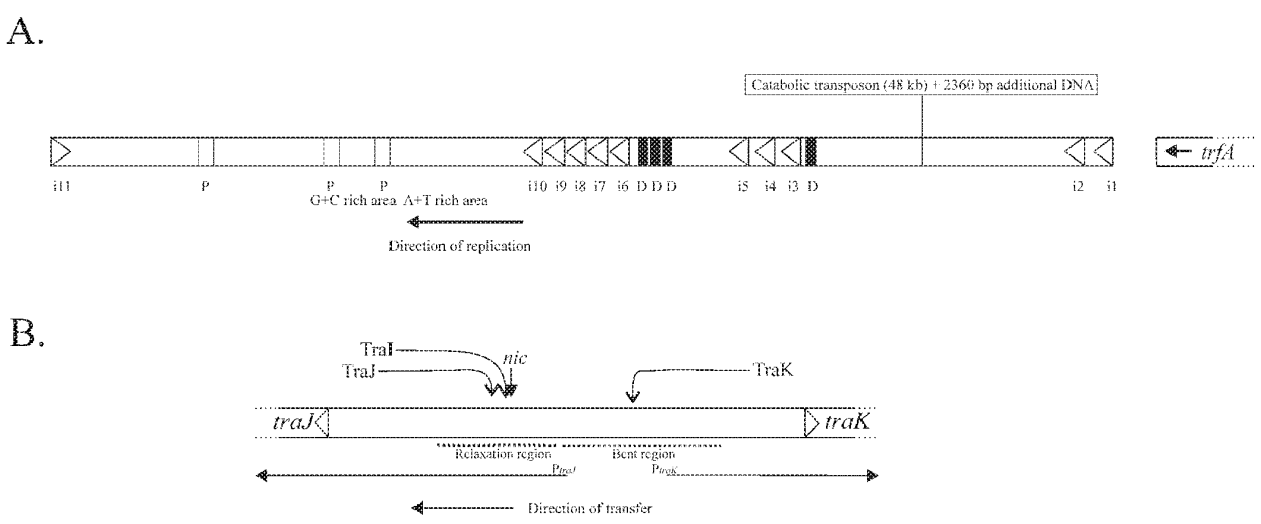


FIG. 6. (A) *oriV* region of pEST4011. The binding sites for replication initiation protein TrfA (iterons i1 to i11) together with the directions are indicated by triangles. The gray boxes represent palindromic sequences (P); the black boxes are DnaA binding sites (D). The G-C- and A-T-rich regions, the direction of replication, the site of additional DNA insertion, and the *trfA* gene (with the direction of transcription) are also indicated. (B) *oriT* region of pEST4011. The arrows indicate the binding sites for the TraI, TraJ, and TraK proteins and the *nic* site. The gray box represents the region that is highly conserved in different IncP1 plasmids. The translation start codons together with the directions are indicated for the *traJ* and *traK* genes. The relaxation region and the bent region are indicated by dotted lines; the directions of transcription from the promoters of *traJ* (P_{traJ}) and *traK* (P_{traK}) and the putative direction of DNA transfer during conjugation are indicated by horizontal arrows.

archetype plasmids of the IncP1 α and IncP1 β subgroups, respectively. In order to examine to which IncP1 subgroup pEST4011 belongs, we constructed multiple alignments of the available full-length amino acid sequences of the short form of TrfA (TrfA-33), KorA, and TraG of IncP1 plasmids (Table 2). In cases in which the coding sequences for TrfA-33 were not annotated, we generated the necessary sequences from TrfA-44 by deleting the N-terminal 122 amino acids in the case of pUO1 and pTSA and 123 amino acids in the case of pEMT3 (based on similarities with RK2 and R751). The bootstrapped neighbor-joining trees derived from these alignments (obtained by using the program CLUSTALX) (Fig. 7) showed that pEST4011 TrfA, KorA, and TraG are distinct from all other corresponding sequences and that they occupy individual branches of each tree. Similar results were obtained with the different PHYLIP programs used (data not shown). As C. M. Thomas and coworkers have assigned plasmid pQKH54 (14) to the γ subgroup (personal communication) and the amino acid sequence of TrfA of pQKH54 appeared to be different from the pEST4011 TrfA sequence (unpublished data), we placed pEST4011 in a new IncP1 subgroup, the δ subgroup. The other four IncP1 catabolic plasmids sequenced, pJP4, pADP-1, pUO1, and pTSA, together with pIJB1, possess the well-conserved IncP1 subgroup β backbone. The TrfA2 phylogenetic tree (Fig. 7A) shows that the 2,4-D-degradative plasmid pEMT3 is also different from all other IncP1 plasmids and could also form a separate subgroup. Unfortunately, the incompatibility group of the 2,4-D-degradative plasmid pTV1 is unknown (T. Vallaeys, personal communication).

Comparison of pEST4011 and its laboratory ancestor, pD2M4. The original strain D2M4, containing plasmid pD2M4 (about 95 kb), had a very unstable 2,4-D^+ phenotype; 99% of the cells lost this phenotype when they were grown in Luria-Bertani medium, and all of the 2,4-D^- clones studied contained no plasmid. Subsequently, a series of selections were performed (V. Kõiv, unpublished data); in each step D2M4 cells were cultivated in nonselective medium, and this was followed by selection on medium containing 2,4-D as a sole source of carbon and energy. The resulting 2,4-D^+ clone with the best growth on 2,4-D was selected for the next step. Finally, a strain with improved stability of the 2,4-D^+ phenotype was obtained; about 50% of the cells lost the 2,4-D^+ phenotype when they were grown in nonselective medium. The selected strain was designated EST4002, and it contained a smaller plasmid, pEST4002 (estimated size, 78 kb). In order to increase the stability of the plasmid and to overcome the difficulties with DNA extraction, pEST4002 was transferred by conjugation into a plasmid-free recipient strain, *Pseudomonas putida* PaW340 (28). The resulting 2,4-D^+ transconjugants contained plasmid pEST4011 (70 kb, as determined by restriction analysis). After maintenance of strain EST4002 on 2,4-D minimal medium agar plates for years, the strain acquired a very stable 2,4-D^+ phenotype; only 1 to 2% of the cells lost the phenotype when they were grown in Luria-Bertani medium. According to a restriction analysis this strain contained exactly the same pEST4011 plasmid as the 2,4-D^+ transconjugants of PaW340 (53).

Due to problems arising from instability of the 2,4-D^+ phenotype and the difficulties with DNA extraction, we were not able to maintain either the original strain D2M4 containing pD2M4 or plasmid pEST4002. As Mäe et al. have shown (28), plasmid pEST4002 had one additional 7.8-kb EcoRI restriction fragment compared with pEST4011. However, the exact location and size of the deletion were not determined. During the present study we compared the EcoRI and HindIII restriction patterns of pD2M4 (Kõiv, unpublished) and

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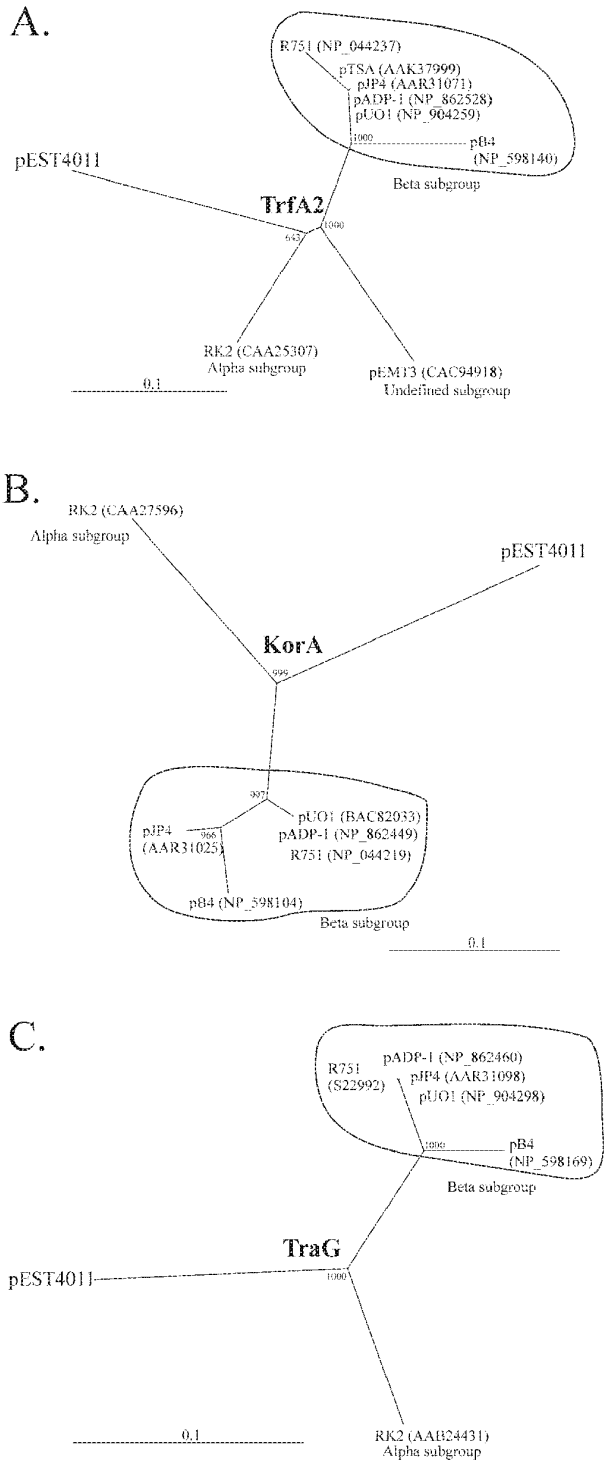


FIG. 7. Bootstrapped neighbor-joining trees derived from multiple alignments of *trfA2* (A), *korA* (B), and *traG* (C) gene products of different IncP1 plasmids, constructed with the CLUSTALX program. The GenBank accession numbers of the proteins are indicated in parentheses. The numbers at the nodes of the trees represent the bootstrap values (1,000 replicates) for each node.

pEST4011 (Fig. 8A and B). We found that pEST4011 had lost the approximately 23.5- and 20.5-kb HindIII restriction fragments and the approximately 26-, 9.5-, and 4.6-kb EcoRI fragments compared with pD2M4. In addition, the 19,609-bp HindIII fragment and the 6,991- and 10,872-bp EcoRI fragments of pEST4011 are not present in pD2M4. Instead of the 21,849-bp pEST4011 HindIII fragment, pD2M4 had a corresponding band at about 15 kb.

These results show that (i) pEST4011 lost approximately 25 to 29 kb of DNA from somewhere between position 57349 and position 66873 (i.e., between the righthand copy of IS*1071*:: IS*1471* and the *traD* gene) and (ii) pD2M4 did not contain the 6,991-bp duplication present in pEST4011 containing genes *lrp* to *iaaH* (Fig. 1 and 8C). Indeed, when pEST4011 was compared with R751, we noticed that in case of pEST4011 about 14 kb of backbone DNA containing (among other genes) a set of *trb* genes essential for conjugation (*trbE* to *trbL*) (Fig. 5) was missing. The remaining 11 to 15 kb could comprise additional DNA inserted between the Tra1 and Tra2 regions, as reported for all other IncP1 plasmids discussed in this study except pJP4 (1, 39, 48). Thus, on the one hand, this deletion somehow ensured its host a very stable 2,4-D$^+$ phenotype, and on the other hand, pEST4011 lost the ability to self-transmit.

The 6,991-bp duplication in the pEST4011 catabolic region is not present either in pD2M4 or in catabolic transposon Tn*5530* of pIJB1. At least two of six ORFs in this region can be directly related to 2,4-D degradation; namely, *tfdD* is responsible for the fourth step in the corresponding pathway (Fig. 2), and the *ccp* product may function as a channeling protein for the removal of chloride liberated during 2,4-D dissimilation from cells. The predicted amino acid sequence of the pEST4011 TfdD protein is far more similar to the sequence of TcbD of *Pseudomonas* sp. strain P51 plasmid pP51 (P27099) than to the sequence of pJP4 $TfdD_I$ or $TfdD_{II}$; the levels of amino acid identity are 83, 64, and 32%, respectively, and the levels of amino acid similarity are 96, 87, and 57%, respectively. The *tcb* genes of pP51 are responsible for degradation of 1,2,4-trichlorobenzene by its host strain (52), and the substrate for TcbD is 2,3,5-trichloro-*cis*,*cis*-muconate. The corresponding 2,4-D degradation intermediate and substrate for the pEST4011 and pJP4 *tfdD* gene products is 2,4-dichloro-*cis*,*cis*-muconate. Vollmer et al. (55) showed that although they could not measure enzyme kinetics with 2,3,5-trichloro-*cis*,*cis*-muconate as the substrate, 2,4-dichloro-*cis*,*cis*-muconate was the best substrate for pP51 TcbD and pJP4 $TfdD_I$. However, the catalytic efficiency of TcbD was somewhat lower than that of TfdD (k_{cat}/K_m, 53 and 120 μM^{-1} min^{-1}, respectively). Therefore, it is tempting to speculate that the 7-kb pEST4011 region was duplicated in order to increase the *tfdD* copy number and consequently the amount of the corresponding enzyme in a cell in order to degrade 2,4-D more efficiently.

Mercury resistance is a common heavy metal resistance in bacterial isolates from soil and water environments (19). As shown in Table 2, all catabolic IncP1 plasmids except the least-studied plasmid pEMT3 bear mercury resistance genes. Although plasmid pEST4011 does not carry any Hg^r determinants, it is probable that the precursor plasmid pD2M4 did. In pADP-1 and pUO1 the *mer* genes are located between the Tra1 and Tra2 regions; if this was the case for pD2M4 as well, the corresponding genes were lost during laboratory evolution.

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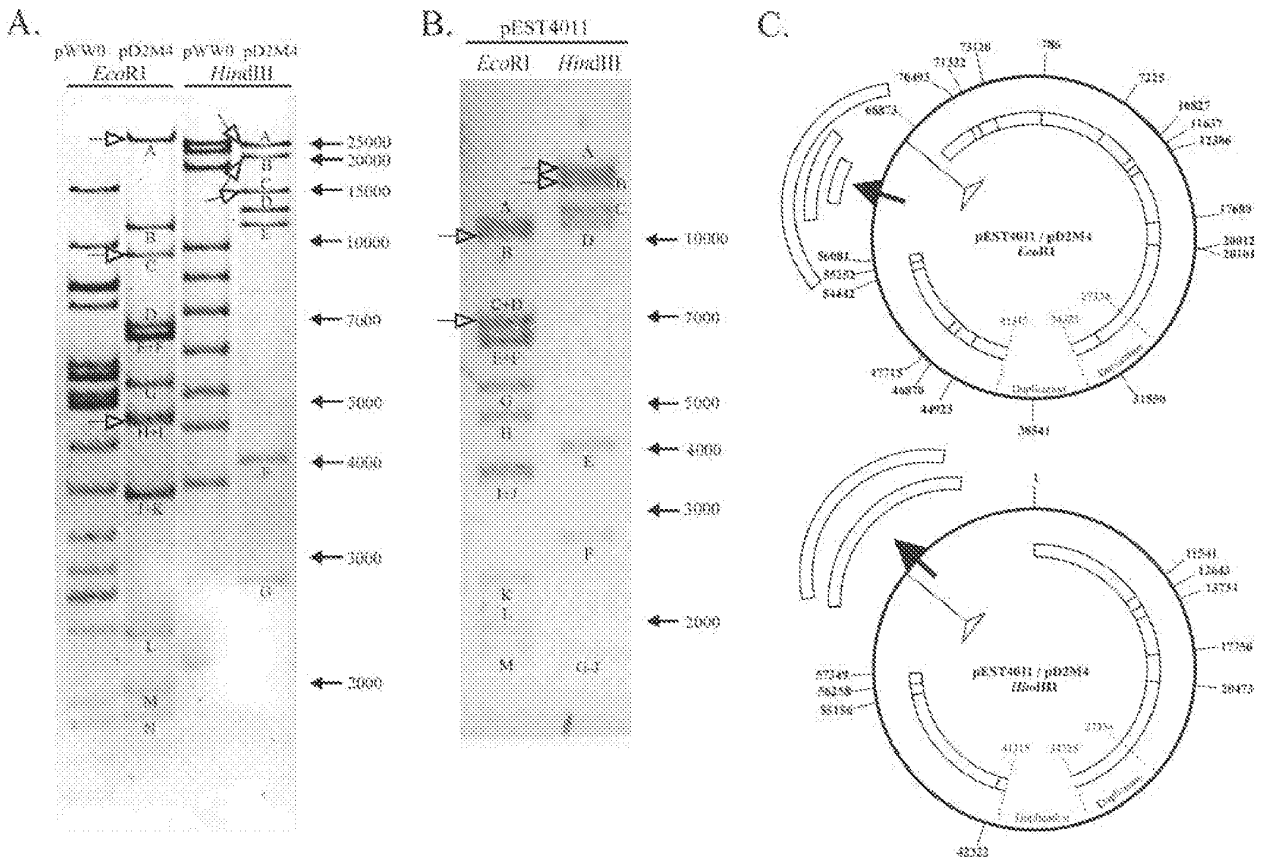


FIG. 8. (A) Restriction analysis of plasmid pD2M4 digested with EcoRI and HindIII. Plasmid pWW0 (accession number NC_003350) was digested with the same restriction endonucleases to provide size markers. The DNA fragments were separated in a 0.7% agarose gel. The numbers and arrows on the right indicate the approximate positions of the corresponding fragments (in base pairs). The arrows with the open arrowheads indicate the pD2M4 restriction fragments not present in pEST4011. (B) Restriction analysis of plasmid pEST4011 digested with EcoRI and HindIII. The DNA fragments were separated in a 0.8% agarose gel. The numbers and arrows on the right indicate the approximate positions of the corresponding fragments (in base pairs). The arrows with the open arrowheads indicate the pEST4011 restriction fragments not present in pD2M4. (C) Aligned circular restriction maps of plasmids pEST4011 and pD2M4. The outer circle with recognition positions of the corresponding restriction enzymes represents pEST4011. The inner discontinuous circle represents pD2M4, and the boxes indicate the corresponding digestion products. The restriction fragments of pD2M4 not present in pEST4011 are shown outside the circles, and the arrows indicate the sites in pD2M4 where these fragments are probably embedded in an unknown order. The line with the open triangle at the end indicates the site in pEST4011 where the essential transfer genes *trbE* to *trbL* are missing. The duplicated regions of pEST4011 are indicated by dotted lines.

However, mercury resistance has not been determined for either strain EST4002 or EST4003.

Our first conclusion is that it seems that despite similarities in overall organization, the backbones of IncP1 plasmids are far more heterogeneous than previously thought. Also, as shown in Table 2, very similar IncP1 backbones, although loaded with different additional functions, can be isolated from geographically distinct regions of the world. Second, diverse genes and gene cassettes are assembled into (2,4-D) catabolic pathways having different origins, but once a working set of necessary genes has been brought together (for example, in a composite catabolic transposon), these genes may spread horizontally as one unit and this unit may insert itself into different vehicles, like conjugative (broad-host-range) plasmids. Other catabolic and/or regulatory genes encoded by these composite transposons may be just entrapped passengers with no significance in one host, while they can probably confer an altered phenotype to another host in certain conditions. Finally, serious attention should be paid to the fact that the occurrence of laboratory evolution caused by continuous enrichment procedures during the isolation of natural catabolic plasmids may hinder studies and distort our understanding of evolution in nature.

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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

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Summary

Primers specific for the genes *tfdD*, *tfdE* and *tfdF*, derived from conserved amino acid sequence motifs of the corresponding homologous enzymes, and primers specific for the genes *tfdA* and *tfdB* as well as *tfdC* taken from the literature were applied in PCR reactions using the genomic DNA of *Delftia acidovorans* P4a as the template. PCR products were obtained with all primer pairs that were similar in size to those found with the genomic DNA of strains harbouring plasmid pJP4 as the carrier of *tfd* genes. The nucleotide sequences and the corresponding amino acid sequences of the PCR products obtained with Strain P4a were compared with the sequence databases. According to BLAST analyses, the partial sequences of *tfdA* and *tfdB* exhibited a 94–99% degree of identity with the homologous sequences of the 2,4-D-degrading strains *Achromobacter xylosoxidans* subsp. *denitrificans* EST4002 (pEST4011), *Burkholderia* sp. RASC, *Variovorax paradoxus* TV1 (pTV1) and *Burkholderia cepacia* 2a (pIJB1), whereas the partial sequences of the *tfdC*, *tfdD*, *tfdE* and *tfdF* genes revealed a 96–100% degree of identity with the homologous sequences of the chlorobenzene-utilizing strains *Ralstonia eutropha* NH9 (pENH91), *Pseudomonas chlororaphis* RW71 and *Pseudomonas* sp. P51 (pP51).

Introduction

In addition to contaminated soil and (ground)water, pollution problems in the chemical industry often extend to the facilities themselves. This was for example the case with a herbicide production plant, where building rubble examined after its demolition was found to be contaminated with a spectrum of chlorinated/methylated phenoxyalkanoates and phenols [1]. When this investigation began, making such material reusable by means of microbial decontamination had not been tried. A predominant characteristic of such concrete debris is the strongly alkaline conditions created in the

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aqueous surroundings and eluates. The initial question was whether this material harbours microorganisms that tolerate this inhospitable milieu, enabling them to establish and remain active in it. A microbial consortium was enriched from this material, which was able to completely mineralize the cocktail of contaminants under alkaline conditions. One of the strains isolated from such enrichment cultures was *Delftia* (formerly *Comamonas* [2]) *acidovorans* P4a capable of productively utilizing phenoxyacetate herbicides, i.e. 2,4-D and MCPA [3]. This strain showed alkalitolerant properties with a pH optimum of around 8.5 and activity up to pH 10. It proved stable even at pH values of up to pH 11.5, making *D. acidovorans* P4a suitable for application under *in situ* conditions [4].

One of the questions to be answered was how *D. acidovorans* P4a prefers such extreme milieu conditions. This knowledge is of fundamental interest, but it is finally this property which becomes effective in the bioremediation processes. Therefore, in the present study the molecular basis of herbicide degradation in Strain P4a was investigated by elucidating the genetic background of 2,4-D degradation. This is further considered a prerequisite for future tasks aiming at extending the degradative spectrum of the organism according to the spectrum of the pollutants found in the present case but also in other contaminated biotopes.

Materials and Methods

PCR Primers

Sequences of the primers specific for the genes *tfdA* (encoding 2,4-D/α-ketoglutarate dioxygenase) and *tfdB* (encoding dichlorophenol hydroxylase) were taken from VALLAEYS *et al.* [5]; those for *tfdC* (encoding dichlorocatechol 1,2-dioxygenase) were previously described by KLEINSTEUBER *et al.* [6]. For the construction of primers specific for the genes *tfdD*, *tfdE* and *tfdF* (encoding chloromuconate cycloisomerase, chlorodienelactone hydrolase and chloromaleylacetate reductase, respectively) the corresponding amino acid sequences of the homologous genes were aligned using the CLUSTAL W programme [7]. Degenerated primers were derived from conserved amino acid sequence motifs.

PCR

PCR was performed by using the genomic DNA from *Delftia acidovorans* P4a as the template. *Ralstonia eutropha* JMP134 (pJP4) and *Escherichia coli* HB101 (pJP4) were used as positive controls, the pJP4-free derivatives *R. eutropha* JMP222 and *E. coli* HB101 were used as negative controls. Template DNA was prepared from individual colonies grown on LB agar. The biomass was suspended in 50 µl of sterile distilled water, boiled for 10 min and chilled on ice before being sedimented by centrifugation at 23,000 g at 4 °C for 10 min. 2 µl of the supernatant was taken for a 50 µl PCR sample. PCR was carried out using the Multicycler PTC-200 (BIOZYM DIAGNOSTICS) or the Mastercycler 5330 (EPPENDORF). The DyNAzyme II Kit (BIOMETRA) was used with 10 pmol of each primer and 1 unit DNA polymerase being employed per 50 µl PCR sample. PCR was started by adding the template DNA; contamination control samples contained distilled water instead of the template DNA. The PCR was run according to the following protocol: initial denaturation for 3 min at 94 °C; 35 cycles of denaturation for 45 s at 94 °C, annealing for 30 s at 59 °C (*tfdA*), 57 °C (*tfdB*), 60 °C (*tfdC, tfdD, tfdE, tfdF*), extension for 2 min at 72 °C; final extension for 6 min at 72 °C. After the run, the samples were chilled at 4 °C. PCR products were separated by agarose gel electrophoresis using the 100-bp ladder (PROMEGA) as a molecular weight standard.

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After staining with ethidium bromide, the DNA from the appropriate bands was extracted and purified using the NucleoSpin Extract 2 in 1 Kit (MACHEREY-NAGEL).

DNA Sequencing

Nucleotide sequences of the PCR products were determined according to SANGER *et al.* [8]; both the forward and the reverse primers were applied. Cycle sequencing with fluorescence-labelled dideoxynucleotides was performed using the ABI Prism BigDye™ Terminator Sequencing Ready Reaction Kit (PE APPLIED BIOSYSTEMS). The samples contained 10 pmol of the primer, 30–90 ng template DNA and 4–8 µl of the ABI Prism reaction mixture in a total volume of 10–20 µl with sterile distilled water (HPLC grade). The sequencing reaction was performed in a Multicycler PTC-200 (BIOZYM DIAGNOSTICS) for 25 cycles using the following protocol: denaturation for 30 s at 96 °C; annealing and elongation for 4 min and 15 s at 60 °C. After the synthesis, the samples were chilled at 4 °C. The labelled DNA fragments were purified with Centri Sep columns (PRINCETON SEPARATIONS Inc.) according to the supplier's protocol and precipitated with ethanol. The dried DNA pellet was dissolved in 25 µl template suppression reagent (PE APPLIED BIOSYSTEMS). For the denaturation of DNA, the samples were treated for 2 min at 90 °C and immediately chilled on ice. Electrophoresis, excitation and collection of the fluorescence signals were performed with an ABI PRISM™ 310 Genetic Analyzer (PE APPLIED BIOSYSTEMS). Sequence data were analyzed with the Sequence Navigator Software (PE APPLIED BIOSYSTEMS). Comparison of sequences with DNA and protein sequences from sequence databases was performed with BLAST 2.0 ([9], http://www.ncbi.nlm.nih.gov/BLAST). The determined partial sequences of the genes *tfdA*, *tfdB*, *tfdC*, *tfdD*, *tfdE* and *tfdF* will appear in the GenBank as well as in the EMBL and DDBJ sequence databases under the accession Nos. AF377325, AF377326, AF077915, AF377327, AF377328 and AF377329, respectively.

Results

Development of PCR Primers Specific for the Genes tfdD, tfdE and tfdF

To develop primers aimed at detecting the *tfdD* gene encoding the 2,4-dichloromuconate cycloisomerase of *D. acidovorans* P4a, the amino acid sequences of TfdD from *Ralstonia eutropha* JMP134 (pJP4) [10], ClcB from *Pseudomonas putida* AC867 (pAC27) [11] and TcbD from *Pseudomonas* sp. P51 (pP51) [12] were aligned marking the conserved sequence motifs (Fig. 1). The degenerated nucleotide sequence of the forward primer was derived from the amino acid sequence motif AETIKII/AETIKVI as 5′-GC(G/T)-GA(A/G)-AC(C/A)-ATG-AAG-(AG)TC-ATC-3′. The reverse primer was derived from the sequence motif KLCNMGG with the nucleotide sequence 5′-(GC)CC-(GT)CC-CAT-GTT-GCA-(AC)AG-C-3′. The length of the expected *tfdD* fragment is comprised of 648 bp.

To develop the primers for the *tfdE* gene encoding the chlorodienelactone hydrolase of *D. acidovorans* P4a, the amino acid sequences of TfdE from *R. eutropha* JMP134 (pJP4) [13], ClcD from *P. putida* AC867 (pAC27) [11] and TcbE from *Pseudomonas* sp. P51 (pP51) [12] were used. According to the alignment, the conserved amino acid sequence motifs AQEIFG and GYYGVG were used (Fig. 2) to derive the specific primers 5′-GCG-CAG-GAG-ATT-TT(C/T)-GGG-3′ (forward) and 5′-CC(G/C)-ACG-CCG-TA(A/G)-TAG-CC-3′ (reverse). The DNA fragment of the *tfdE* gene should be 344 bp in size.

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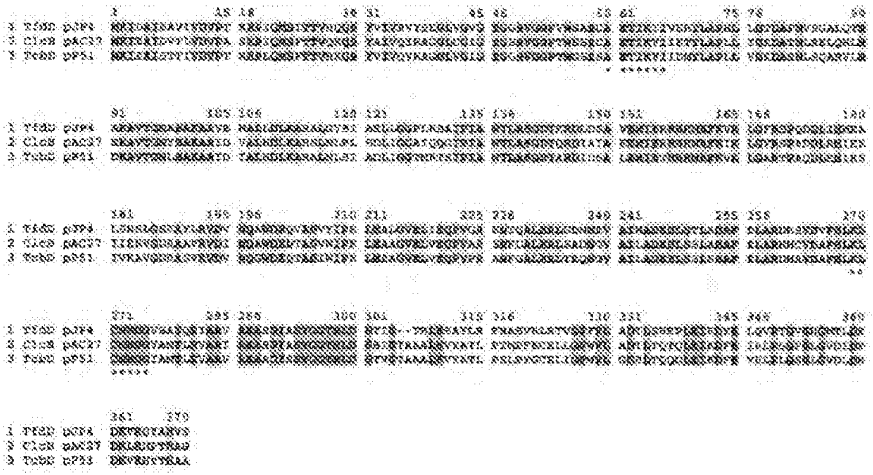


Fig. 1. Sequence alignment of TfdD (*Ralstonia eutropha* JMP134, pJP4) [10], ClcB (*Pseudomonas putida* AC867, pAC27) [11] and TcbD (*Pseudomonas* sp. P51, pP51) [12]
The conserved amino acid sequences are underlayed with grey, sequence motifs used for the construction of primers are indicated by asterisks.

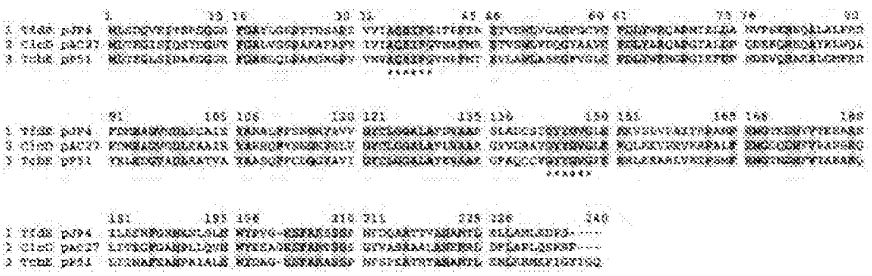


Fig. 2. Sequence alignment of TfdE (*Ralstonia eutropha* JMP134, pJP4) [13], ClcD (*Pseudomonas putida* AC867, pAC27) [11] and TcbE (*Pseudomonas* sp. P51, pP51) [12]
The conserved amino acid sequences are underlayed with grey, sequence motifs used for the construction of primers are indicated by asterisks.

To develop primers for the *tfdF* gene encoding the chloromaleylacetate reductase of *D. acidovorans* P4a, the amino acid sequences of TfdF from *R. eutropha* JMP134 (pJP4) [13], TcbF from *Pseudomonas* sp. P51 (pP51) [12] and TfDFII from *R. eutropha* JMP134 (pJP4) [14] were used for alignment; the conserved amino acid sequence

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motifs are indicated in Fig. 3. The forward primer with the sequence 5′-G(T/A)T-C(G/A)A-CCA-TCG-G(C/A)C-TCG-C-3′ was derived from the sequence motif GSTIGLA (all sequences but *tfdF*). The reverse primer was derived from LHHKLCH as 5′-GGC-A(G/C)A-GCT-TGT-G(A/G)T-G(T/C)A-G-3′. The size of the expected *tfdF* fragment amounts to 444 bp.

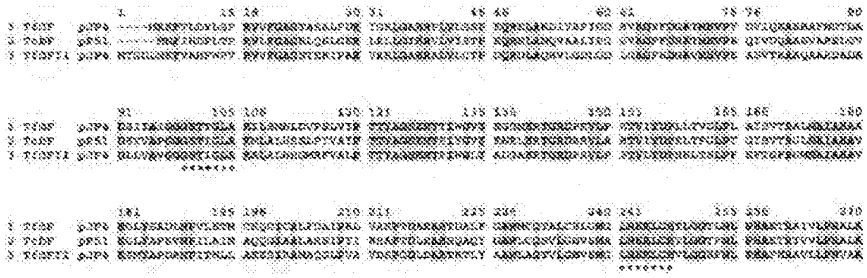


Fig. 3. Sequence alignment of TfdF (*Ralstonia eutropha* JMP134, pJP4) [13], TcbF (*Pseudomonas* sp. P51) [12] and TfdFII (*Ralstonia eutropha* JMP134, pJP4) [14]
The conserved amino acid sequences are underlayed with grey, sequence motifs used for the construction of primers are indicated by asterisks.

PCR Detection of the tfd Genes

The primers for *tfdA* and *tfdB* [5], *tfdC* [6] and the above-described primers for *tfdD*, *tfdE* and *tfdF* were applied for the detection of respective genes in *D. acidovorans* P4a. The PCR products were separated in agarose gels. Fig. 4 depicts the results obtained with the primers for *tfdA* and *tfdB*. It is apparent that Strain P4a harbours genes which result in amplification products corresponding in their molecular weight to those obtained with the genomic DNA from *R. eutropha* JMP134 (pJP4) as the positive control. Just as with the primers for *tfdC* and *tfdD* (Fig. 5) and for *tfdE* and *tfdF* (Fig. 6), PCR products of the appropriate sizes were obtained. Control reactions by applying genomic DNA from the negative *R. eutropha* JMP222 and *E. coli* HB101 control strains did not result in PCR products corresponding in size.

Sequence Analyses of the tfd Fragments

The sequence data of the individual PCR products were compared with nucleotide and amino acid sequences from the sequence databases by BLAST analysis. The results with the sequences showing the highest degree of similarity are summarized in Tab. 1. Accordingly, the *tfdC*, *tfdD*, *tfdE* and *tfdF* fragments of Strain P4a are highly similar to the sequences of the homologous genes from *R. eutropha* NH9 (pENH91) [15], *Pseudomonas chlororaphis* RW71 [16] and *Pseudomonas* sp. P51 (pP51) [12].

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Tab. 1. Comparison of the partial nucleotide and amino acid sequences of the *D. acidovorans* P4a *tfd* genes with database sequences
(Strains exhibiting the highest BLAST identity scores are listed).

Genes Strain P4a	Identity (nucleotide sequence)	Identity (amino acid sequence)	Identity with strain	Accession number[1)]
tfdA	99%	98%	*A. xylosoxidans* subsp. *denitrificans* EST4002 (pEST4011)	U32188
	94%	97%	*Burkholderia* sp. RASC	U25717
tfdB	99%	99%	*V. paradoxus* TV1 (pTV1)	AB028643
	99%	99%	*A. xylosoxidans* subsp. *denitrificans* EST4002 (pEST4011)	U32188
	96%	95%	*B. cepacia* 2a (pIJB1)	U88192
tfdC	100%	100%	*P. chlororaphis* RW71	AJ271325
	100%	100%	*R. eutropha* NH9 (pENH91)	AB019032
	98%	96%	*Pseudomonas* sp. P51 (pP51)	M57629
tfdD	100%	100%	*R. eutropha* NH9 (pENH91)	AB019032
	99%		*P. chlororaphis* RW71	AJ271325
tfdE	100%	100%	*Pseudomonas* sp. P51 (pP51)	M57629
	100 %	100 %	*R. eutropha* NH9 (pENH91)	AB019032
	100%	100%	*P. chlororaphis* RW71	AJ271325
tfdF	100%	100%	*Pseudomonas* sp. P51 (pP51)	M57629
	100%	100%	*R. eutropha* NH9 (pENH91)	AB019032
	99%	99%	*P. chlororaphis* RW71	AJ271325

[1)] The accession numbers refer to the GenBank, the EMBL as well as the DDJB sequence databases.

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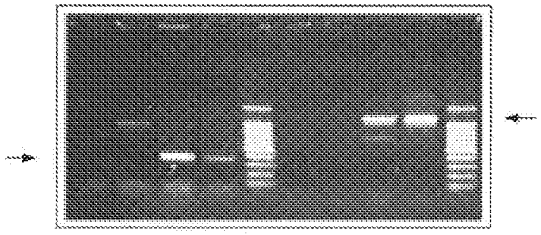


Fig. 4. PCR products with primers specific for the genes *tfdA* (Lanes 1–4) and *tfdB* (Lanes 6–9), separated in a 1.5% agarose gel
PCR products with Strain P4a (Lanes 4 and 9) corresponding in size to those obtained with *R. eutropha* JMP134 (pJP4) (Lanes 3 and 8) are indicated by arrows. Negative controls using *R. eutropha* JMP222 (Lanes 1 and 6) and *E. coli* HB101 (Lanes 2 and 7) are included. A 100-bp ladder was used as a molecular weight standard (Lanes 5 and 10).

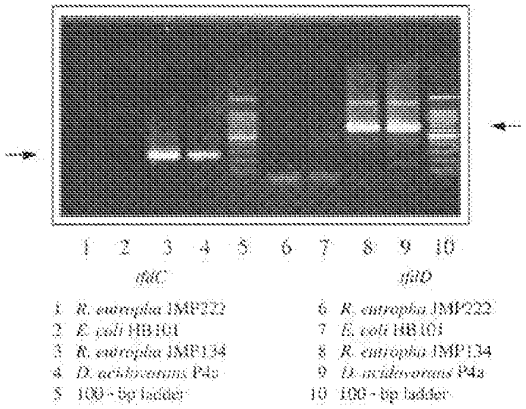


Fig. 5. PCR products with primers specific for the genes *tfdC* (Lanes 1–4) and *tfdD* (Lanes 6–9), separated in a 1.5% agarose gel
PCR products with Strain P4a (Lanes 4 and 9) corresponding in size to those obtained with *R. eutropha* JMP134 (pJP4) (Lanes 3 and 8) are indicated by arrows. Negative controls using *R. eutropha* JMP222 (Lanes 1 and 6) and *E. coli* HB101 (Lanes 2 and 7) are included. A 100-bp ladder was used as a molecular weight standard (Lanes 5 and 10).

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In contrast, in the case of *tfdA* and *tfdB* the greatest similarity was found with the homologous genes of strains *Achromobacter xylosoxidans* subsp. *denitrificans* EST4002 (pEST4011) [17], *Variovorax paradoxus* TV1 (pTV1) [18], *Burkholderia sp.* RASC (*tfdA*) [19] and *Burkholderia cepacia* 2a (pIJB1) (*tfdB*) [20]. With regards to *R. eutropha* JMP134 (pJP4), the similarity of the respective sequences with Strain P4a proved lower: Identity degrees of 87%, 66%, 72% and 76% were found on the basis of the amino acid sequences for *tfdA*, *tfdB*, *tfdC* and *tfdD*, respectively.

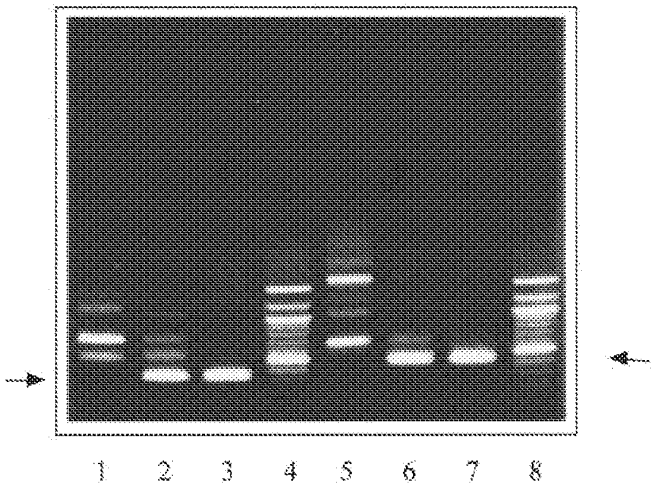


Fig. 6. PCR products with primers specific for the genes *tfdE* (Lanes 1–3) and *tfdF* (Lanes 5–7), separated in a 0.8% agarose gel
PCR products with Strain P4a (Lanes 3 and 7) corresponding in size to those obtained with *E. coli* HB101 (pJP4) (Lanes 2 and 6) are indicated by arrows. Negative controls using *E. coli* HB101 (Lanes 1 and 5) are included. A 100-bp ladder was used as a molecular weight standard (Lanes 4 and 8).

Discussion

Based on conserved amino acid sequence motifs of homologous enzymes from other bacterial strains, PCR primers specific for the genes *tfdD*, *tfdE* and *tfdF* were constructed. The application of these primers and of other primers specific for the genes *tfdA* and *tfdB* [5] as well as *tfdC* [6] resulted in the detection of all the genes of the 2,4-D degradation pathway in *D. acidovorans* P4a.

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Sequence analyses of the PCR products and comparison with known sequences revealed that the genes *tfdC, tfdD, tfdE and tfdF* of *D. acidovorans* P4a are highly similar to the homologous genes of strains *Pseudomonas chlororaphis* RW71 [16], *R. eutropha* NH9 (pENH91) [15] and *Pseudomonas sp.* P51 (pP51) [12]. *P. chlororaphis* RW71 and *Pseudomonas sp.* P51 (pP51) are characterized by their ability to utilize trichloro- and tetrachlorobenzene, whereas *R. eutropha* NH9 (pENH91) is known to degrade 3-chlorobenzoate. The genes encoding chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, chlorodienelactone hydrolase and chloromaleylacetate reductase are organized within one operon in these strains [21, 22, 23]. This might apply to the genes in Strain P4a due to the joint relationship of the individual gene sequences to those found in the strains mentioned above. Due to the high sequence similarities, a common origin of the chlorocatechol operons in these strains is likely.

In contrast, the PCR products obtained with the primers for *tfdA* and *tfdB* exhibit high similarity to sequences found with the 2,4-D-degrading strains *Achromobacter xylosoxidans* subsp. *denitrificans* EST4002 (pEST4011) [17], *Burkholderia* sp. RASC [19], *Variovorax paradoxus* TV1 (pTV1) [18] and *Burkholderia cepacia* 2a (pIJB1) [20]. These data indicate that the *tfdA/tfdB* genes and the *tfdCDEF* operon in Strain P4a have a different origin.

The species *D. acidovorans* ought to be suitable for biotechnological application under the milieu conditions found in aqueous solutions of building rubble. To tolerate alkaline growth conditions and to prefer organic acids as sources of carbon and energy for growth [2, 24] favours *D. acidovorans* for such a biotope. (Possibly and probably, alkalitolerant or alkaliphilic bacteria at all prefer acids for growth.) The disposition of this species for alkaline growth conditions is supported by the example of Strain MC1 also belonging to *D. acidovorans* [25]. It was isolated from the same surroundings as Strain P4a and is able to completely degrade phenoxyacetate and phenoxypropionate herbicides.

Expectedly, the results with Strain P4a suggest that the capability of degrading 2,4-D in *D. acidovorans* might have been acquired from horizontal gene transfer. The completion of the degradative pathway by recruiting the desired genes/operons from different origins tallies with the modular organization described for the 2,4-D degradative pathways [26, 27]. Subsequent investigations will help completely elucidate the genetic organization and regulation of the 2,4-D degradation pathway in *D. acidovorans* P4a.

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Root Nodule *Bradyrhizobium* spp. Harbor *tfdA*α and *cadA*, Homologous with Genes Encoding 2,4-Dichlorophenoxyacetic Acid-Degrading Proteins

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The distribution of *tfdA*α and *cadA*, genes encoding 2,4-dichlorophenoxyacetate (2,4-D)-degrading proteins which are characteristic of the 2,4-D-degrading *Bradyrhizobium* sp. isolated from pristine environments, was examined by PCR and Southern hybridization in several *Bradyrhizobium* strains including type strains of *Bradyrhizobium japonicum* USDA110 and *Bradyrhizobium elkanii* USDA94, in phylogenetically closely related *Agromonas oligotrophica* and *Rhodopseudomonas palustris*, and in 2,4-D-degrading *Sphingomonas* strains. All strains showed positive signals for *tfdA*α, and its phylogenetic tree was congruent with that of 16S rRNA genes in α-*Proteobacteria*, indicating evolution of *tfdA*α without horizontal gene transfer. The nucleotide sequence identities between *tfdA*α and canonical *tfdA* in β- and γ-*Proteobacteria* were 46 to 57%, and the deduced amino acid sequence of TfdAα revealed conserved residues characteristic of the active site of α-ketoglutarate-dependent dioxygenases. On the other hand, *cadA* showed limited distribution in 2,4-D-degrading *Bradyrhizobium* sp. and *Sphingomonas* sp. and some strains of non-2,4-D-degrading *B. elkanii*. The *cadA* genes were phylogenetically separated between 2,4-D-degrading and nondegrading strains, and the cadA genes of 2,4-D degrading strains were further separated between *Bradyrhizobium* sp. and *Sphingomonas* sp., indicating the incongruency of *cadA* with 16S rRNA genes. The nucleotide sequence identities between *cadA* and *tfdA* of 2,4,5-trichlorophenoxyacetate-degrading *Burkholderia cepacia* AC1100 were 46 to 53%. Although all root nodule *Bradyrhizobium* strains were unable to degrade 2,4-D, three strains carrying *cadA* homologs degraded 4-chlorophenoxyacetate with the accumulation of 4-chlorophenol as an intermediate, suggesting the involvement of *cadA* homologs in the cleavage of the aryl ether linkage. Based on codon usage patterns and GC content, it was suggested that the *cadA* genes of 2,4-D-degrading and nondegrading *Bradyrhizobium* spp. have different origins and that the genes would be obtained in the former through horizontal gene transfer.

2,4-dichlorophenoxyacetic acid (2,4-D) has been used as a herbicide since the 1940s, and a number of its degrading bacteria have been isolated worldwide from a variety of environments and extensively examined on a molecular basis (1, 2, 4, 17, 20, 24, 27, 34, 38, 39, 41). The most extensive studies have been done with *Ralstonia eutrophus* JMP134, which carries a 2,4-D-degrading gene cluster on the transmissible plasmid pJP4 (4, 24, 32). The genes and enzymes involved in the 2,4-D degradation pathway have been characterized in several isolates, and comparative studies of their phylogenetic positions, nucleotide and amino acid sequences, and gene organization and rearrangement have allowed us to understand adaptive and evolutionary processes for degrading this xenobiotic compound (2, 11, 24, 28, 41, 42, 43, 45).

2,4-D-degrading bacteria are considered to be categorized into three groups on evolutionary and physiological bases (20). The first group consists of copiotrophic bacteria belonging to the β and γ subdivisions of *Proteobacteria*, which include genera such as *Achromobacter*, *Burkholderia*, *Delftia*, *Halomonas*, *Pseudomonas*, *Ralstonia*, *Rhodoferax*, and *Variovorax* (2, 11, 28, 43). These bacteria have been isolated from agricultural and industrial soils and sediments and have *tfd* genes in common (2, 11, 28, 43). 2,4-D is transformed into 2,4-Dichlorophenol (2,4-DCP) by an α-ketoglutarate (α-KG)-dependent 2,4-D dioxygenase encoded by *tfdA* (9, 10), and 2,4-DCP is subsequently hydroxylated to 3,5-dichlorocatechol by 2,4-DCP hydroxylase encoded by *tfdB* (8, 26, 34). 3,5-Dichlorocatechol is further degraded through a modified *ortho*-cleavage pathway encoded by *tfdCDEF* (24, 34). Nucleotide sequence similarities of *tfdA*, *tfdB*, and *tfdC* in this group are more than 76, 34, and 55%, respectively (28, 43), and the *tfd* genes are usually present on a transmissible plasmid (2, 4, 28). 2,4-D degraders in this group are supposed to obtain the 2,4-D-catabolizing ability by independent *tfd* gene recruitment through horizontal gene transfer (11, 18, 19, 32, 42, 44). The incongruency of the phylogeny between the *tfd* genes and 16S rRNA genes supports this suggestion (28, 43).

The second group consists of α-*Proteobacteria* which are closely related to a *Bradyrhizobium* sp. based on their 16S rRNA gene sequences (16, 20). They are oligotrophic and have been isolated at first from pristine environments in Hawaii, Canada, and Chili (20) and subsequently also from arable soil in Japan (16). Although 2,4-D-degrading genes in this group

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TABLE 1. Strains used in this study

Strain	*tfdA*α[a]	*cadA*[a]	16S[b]	2,4-D[c]	4-CPA[c]	PAA[c]	Relevant characteristic(s)	Reference
Bradyrhizobium spp.								
RD5-C2	+	+	e	+	+	+	2,4-D degrader; Japanese isolate	16
HW13	+	+	e	+	+	+	2,4-D degrader; Hawaiian isolate	20
HWK12	+	+	e	+	+	+	2,4-D degrader; Hawaiian isolate	20
BTH	+	+	e	+	+	+	2,4-D degrader; Canadian isolate	20
USDA 110	+	−	j	−	−	−	*B. japonicum* type strain	30
USDA 94	+	+	e	−	+	+	*B. elkanii* type strain	30
jwc91-2	+	+	e	−	+	−	U.S. isolate from *Amphicarpaea bracteata*	33
th-b2	+	+	e	−	−	−	U.S. isolate from *Amphicarpaea bracteata*	33
DesT1	+	+	e	−	+	−	U.S. isolate from *Desmodium glutinosum*	33
DesB1	+	+	e	−	−	−	U.S. isolate from *Desmodium glutinosum*	33
ApB16	+	−	j	−	−	−	U.S. isolate from *Apios americana*	33
BDV5111	+	−	e	−	−	−	Australian isolate from native shrubby legumes	25
BDV5329	+	−	j	−	−	−	Australian isolate from native shrubby legumes	25
BDV5419	+	−	e	−	−	−	Australian isolate from *Hardenbergia violacea*	25
BDV5680	+	−	j	−	−	−	Australian isolate from *Bossiaea ensata*	25
Sphingomonas sp.								
B6-5	+	−		−	−	−	Originally a 2,4-D degrader and then lost the ability; Canadian isolate	11
B6-10	+	+		+	−	−	2,4-D degrader; Canadian isolate	11
TFD26	+	+		+	+	+	2,4-D degrader; U.S. isolate	41
TFD44	+	+		+	+	+	2,4-D degrader; U.S. isolate	11
A. oligotrophica JCM1494	(+)	−		−	−	−		36
R. palustris JCM2524	(+)	−		−	+	−		36

[a] +, PCR amplified and sequenced; (+), detected by Southern hybridization; −, not detected.
[b] e, 16S rRNA sequence belongs to the cluster of *B. elkanii*; j, 16S rRNA sequence belongs to the cluster of *B. japonicum*.
[c] +, degrading ability; −, no degrading ability.

had not been characterized until recently, remarkable findings have been made. *CadAB* genes, which have a nucleotide sequence similar to that of *tftAB* encoding 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) oxygenase of *Burkholderia cepacia* AC1100 (3), were found in *Bradyrhizobium* sp. strain HW13 isolated from Hawaiian soil (23). In addition, a *tfdA*-like gene (*tfdA*α) was found in strain RD5-C2 isolated in Japan (16, 17). The *CadAB* genes were transformed into *Sinorhizobium meliloti*, and the transformant acquired the degrading activities for 2,4-D and its related compounds. The gene homologs were also found in other Hawaiian and Canadian isolates by Southern hybridization (23). The *tfdA*α gene showed approximately 60% identity to canonical *tfdA*, and the gene was expressed in *Escherichia coli* as a MalE-TfdAα fusion showing α-KG-dependent 2,4-D dioxygenase activity. The protein exhibited about 10 times greater activity for phenoxyacetate (PAA) than 2,4-D. *tfdA*α was also found in the other 2,4-D-degrading strains isolated from pristine environments (17).

Members of the third group belong to a species of the genus *Sphingomonas*, which consists of copiotrophic α-*Proteobacteria*. They have been isolated from agricultural and industrial samples in the same way as the first group (28, 39, 43). Of the genes involved in 2,4-D degradation in this group, *tfdB* and *tfdC* show similarity to those of the first group, but the initial step of 2,4-D oxidation is not likely performed by α-KG-dependent 2,4-D dioxygenase but by another enzyme system (11, 28, 39, 43). Recently, the presence of *cadA* gene homologs was suggested by Southern hybridization that used a *cadA* gene fragment as a probe of the 2,4-D-degrading *Bradyrhizobium* strain HW13 (23).

The 2,4-D-degrading *Bradyrhizobium* strains have conspicuous characteristics, because they were isolated from pristine environments with no exposure to 2,4-D, and they have two different systems for degrading 2,4-D. Therefore, it is supposed that they could provide useful information on the origin and evolutionary relationship of the 2,4-D catabolic genes. As both *tfdA*α and *cadAB* are carried independently of the presence of 2,4-D in this group of 2,4-D degraders, the enzyme systems should maintain their original functions in addition to degrading 2,4-D, and it is expected that the phylogenetically related *Bradyrhizobium* spp. also have *tfdA*α and *cadAB*.

In this paper, we report that *tfdA*α genes are present in all *Bradyrhizobium* strains tested, which include type strains of *Bradyrhizobium japonicum* USDA110 and *Bradyrhizobium elkanii* USDA94 and those isolated from nodules of American and Australian native legumes. None of the strains showed 2,4-D-degrading activity; however, some strains showed activity toward less chlorinated phenoxyacetate derivatives. In addition, *tfdA*-like genes were found in 2,4-D-degrading *Sphingomonas* strains. On the other hand, *cadA* was found only in the *Bradyrhizobium* and *Sphingomonas* strains, which can degrade the phenoxy compounds. We also suggest that *cadA* was acquired via lateral gene transfer in *Bradyrhizobium* sp. and *Sphingomonas* sp. based on their GC content and codon usage frequencies.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used are listed in Table 1. *Bradyrhizobium* strains, *Agromonas oligotrophica* JCM1494, and *Rhodopseudomonas palustris* JCM2524 were grown at 25°C on modified HM medium (29). *Sphingomonas* strains were grown at 25°C on peptone-yeast extract-tryptone-glucose medium (20).

DNA preparation and analysis of *tfdA*α and *cadA*. Total DNA samples were prepared as described previously (14, 17). A partial *tfdA*α fragment was amplified by using primers designed for the conserved regions of the *tfdA*α genes of

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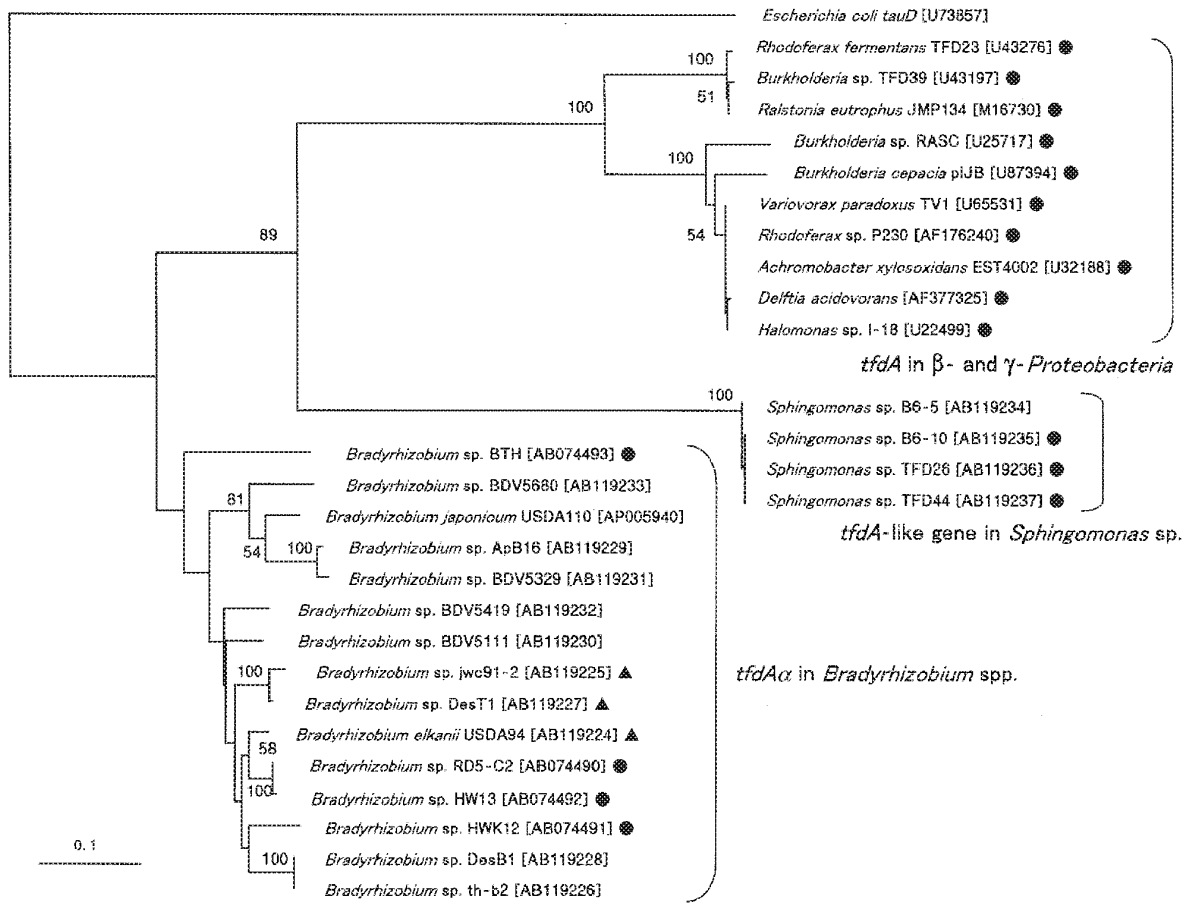


FIG. 1. Phylogenetic position of *tfdA*α in *Bradyrhizobium* spp. and the *tfdA*-like gene of *Sphingomonas* sp. beside canonical *tfdA* in β- and γ-*Proteobacteria*. *E. coli tauD* encodes taurine/α-KG dioxygenase. 2,4-D-degraders (●) and 4-CPA- and/or PAA-degraders (▲) are indicated. Designations in brackets are DDBJ, EMBL, and GenBank database accession numbers. The phylogenetic tree was constructed based on 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.

Bradyrhizobium 2,4-D-degrading strains (17). A partial *cadA* fragment was amplified with primers designed from the *cadA* sequence of strain HW13 (23): 5′-AAGCTGCA(A/G)TTTGA(A/G)AA(C/T)GG-3′ and 5′-(A/C)GGATTGA AGAAATCCTG(A/G)TA-3′. The amplified gene fragments were sequenced directly or after cloning into a pT7Blue T-Vector (Novagen) with an ABI 377 DNA sequencing system (Perkin-Elmer Japan) by using the same primers or the M13 primers M4 and RV (Takara). DNA sequences around the PCR-amplified fragments were determined by using a PCR in vitro cloning kit (Takara). To examine the distribution of *tfdA*α and *cadA* gene homologs among the bacterial strains used, the amplified *tfdA*α and *cadA* gene fragments of strain RD5-C2 were labeled by a DIG DNA labeling kit (Roche Molecular Biochemicals) and used as probes for Southern hybridization analysis. The DNA samples were digested by SalI and blotted onto Hybond N nylon membranes (Amersham) and then hybridized with each labeled probe under low-stringency conditions as described previously (23). All procedures described here were conducted according to the suppliers' instructions.

The *tfdA*α and *cadA* sequences were aligned by using the CLUSTAL W (version 1.8) program (40), and the sites where nucleotides were not resolved for all sequences were excluded from the alignments. Phylogenetic analysis was performed with the CLUSTAL W program using the neighbor-joining (NJ) method (37), and bootstrap analysis based on 1,000 replicates was used to place confidence estimates on the trees.

Degrading ability for 2,4-D and its derivatives. Cells grown on the designated medium were aseptically harvested by centrifugation (6,000 × *g* for 10 min) and resuspended in the same medium containing 2,4-D, 4-chlorophenoxyacetic acid (4-CPA), or PAA (each at a concentration of 10 mg/liter) to give a final turbidity of 0.25 at 600 nm. Cell suspensions were incubated at 25°C for 1 week, and the amount of remaining substrate was measured with a high-performance liquid chromatograph (LC-10AT; Shimadzu, Kyoto, Japan), equipped with a SUMIPAX ODS-A212 column (interior diameter, 6 mm; length, 150 mm) (Sumika Chemical Analysis Service, Osaka, Japan). The mobile phase was a mixture of acetonitrile–0.1% acetic acid (60/40) with a flow rate of 1.0 ml/min. 2,4-D, 4-CPA, and PAA (and their corresponding phenol derivatives) were detected by a Shimadzu SP10-10A UV-visible light detector at 284, 279, and 269 nm, respectively, and identified based on their retention times. Chemicals used were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Comparison of codon usage frequency and GC content. Codon usage patterns and the GC content of the *tfdA*α and *cadA* genes of the 2,4-D-degrading and non-2,4-D-degrading *Bradyrhizobium* strains were compared with those of the nodulation genes (*nod*), NifA-regulated genes, and housekeeping genes of *Bradyrhizobium* spp. (31, 35, and www.kazusa.or.jp/codon) to elucidate the difference in origin of *tfdA*α and *cadA* in this genus. Nineteen genes of *B. japonicum* (35) and the *dnaK* and *gyrB* genes of *B. elkanii* were used as the housekeeping genes of *Bradyrhizobium* spp. Patterns of synonymous codon frequency except for stop codons and codons for methionine and tryptophan were grouped by cluster analysis (Ward linkage using squared Euclidean distance) by using the software SPSS for Windows (version 11.0.1 J, SPSS Inc., Chicago, Ill.). The comparison was also made for *tfdA*-like and *cadA* genes of the *Sphingomonas* strains. The genes *bglI*, *ces10*, and *ugpG* were used as the housekeeping genes of *Sphingomonas* spp.

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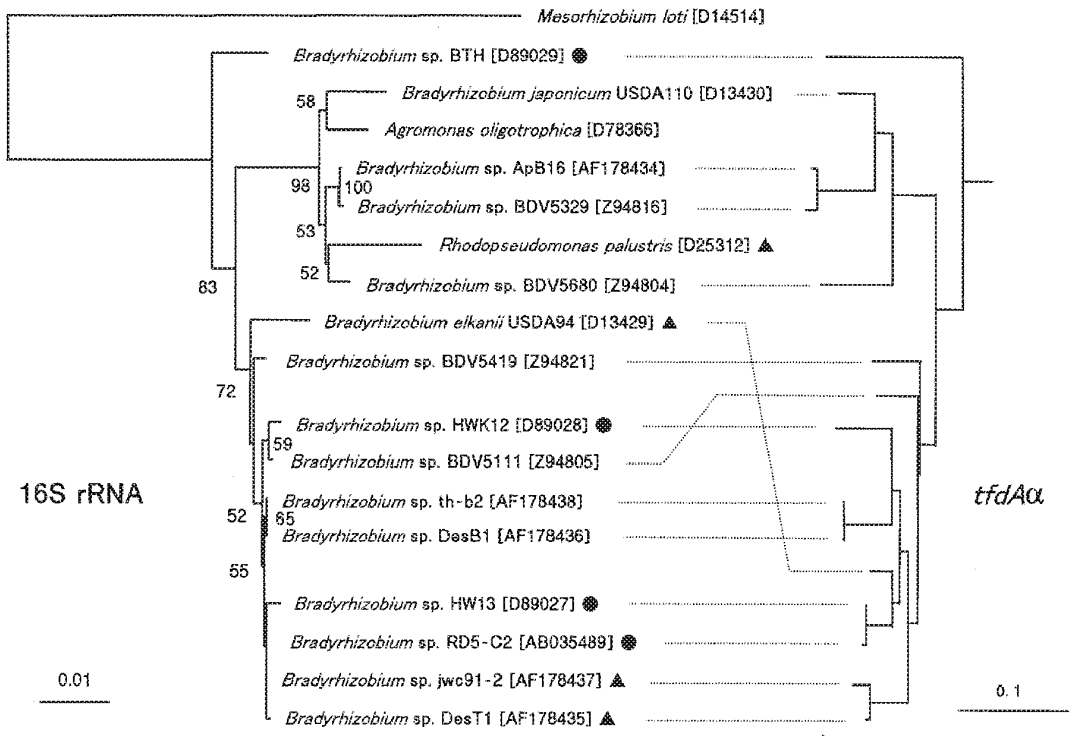


FIG. 2. Phylogenetic relationship of *Bradyrhizobium* strains based on 16S rRNA and *tfdAα* sequences. 2,4-D-degraders (●) and 4-CPA- and/or PAA-degraders (▲) are indicated. Designations in brackets are DDBJ, EMBL, and GenBank database accession numbers. The phylogenetic tree was constructed based on common partial sequences (1300 bp) by using the NJ method. Bootstrap values above 50% are shown at nodes. The scale bar indicates substitutions per site.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the DDBJ, EMBL, and GenBank databases under the accession numbers AB119224 to AB119237 for *tfdAα* and AB119238 to AB119248 for *cadA*.

RESULTS

Phylogenetic analysis of *tfdAα* and *cadA* genes in *Bradyrhizobium* strains and *Sphingomonas* 2,4-D degraders. *tfdAα* gene fragments were amplified by PCR from all the *Bradyrhizobium* and *Sphingomonas* strains used in this experiment (Table 1) but not from *A. oligotrophica* JCM1494 or *R. palustris* JCM2524. Based on the nucleotide sequences, a phylogenetic tree of *tfdAα* was constructed together with canonical *tfdA* gene sequences from β- and γ-*Proteobacteria* (Fig. 1). *E. coli tauD*, encoding taurine/α-KG dioxygenase (6), was used as an outgroup. The *tfdAα* and *tfdA* genes were clearly separated, and the *tfdA*-like genes of *Sphingomonas* sp. were positioned beside the *tfdA* cluster. The nucleotide sequence identities between *tfdAα* and *tfdA* were 51 to 57%, and those of *Sphingomonas* sp. with *tfdAα* and *tfdA* were 48 to 51% and 46 to 49%, respectively.

To examine the evolutionary characteristics of *tfdAα* in *Bradyrhizobium* spp., phylogenetic trees of *tfdAα* and the 16S rRNA genes were compared. As shown in Fig. 2, *Bradyrhizobium* strains were separated into two groups based on the 16S rRNA genes, resulting in clusters including *B. japonicum* or *B. elkanii*. The strains belonging to the cluster of *B. japonicum* in 16S rRNA genes clustered also in *tfdAα*; the same was true for the strains belonging to the cluster of *B. elkanii*, indicating congruency in the phylogenetic trees between *tfdAα* and the 16S rRNA genes. The nucleotide sequence identities of *tfdAα* in *B. japonicum* and *B. elkanii* were 81 to 87%.

In contrast to the pattern of distribution of *tfdAα*, *cadA* was amplified by PCR only in the 2,4-D-degrading *Bradyrhizobium* and *Sphingomonas* strains and in the non-2,4-D-degrading *Bradyrhizobium* strains USDA94, jwc91-2, th-b2, DesT1, and DesB1. The phylogenetic tree of *cadA* is shown in Fig. 3 with *tftA* of 2,4,5-T-degrading *Burkholderia cepacia* AC1100 as an outgroup. The nucleotide sequence identities of *cadA* and *tftA* were 46 to 53%. In the α-*Proteobacteria*, *cadA* genes were phylogenetically separated between 2,4-D-degrading and non-degrading strains, and the separation between the 2,4-D-degrading *Bradyrhizobium* sp. and *Sphingomonas* sp. was apparent with 74 to 78% of the nucleotide sequence identities. As a result, congruency of *cadA* with 16S rRNA genes was not observed between *Bradyrhizobium* sp. and *Sphingomonas* sp.

Southern hybridization. Positive signals by a *tfdAα* probe of strain RD5-C2 were detected in all the *Bradyrhizobium* strains and phylogenetically related strains of *A. oligotrophica* JCM1494 and *R. palustris* JCM2524 in which no *tfdAα* fragment was amplified by PCR, suggesting the inefficiency of the primers used (Fig. 4a). No *tfdAα* homologs were detected in the 2,4-D-degrading *Sphingomonas* strains even under low-stringency conditions; however, positive signals were observed

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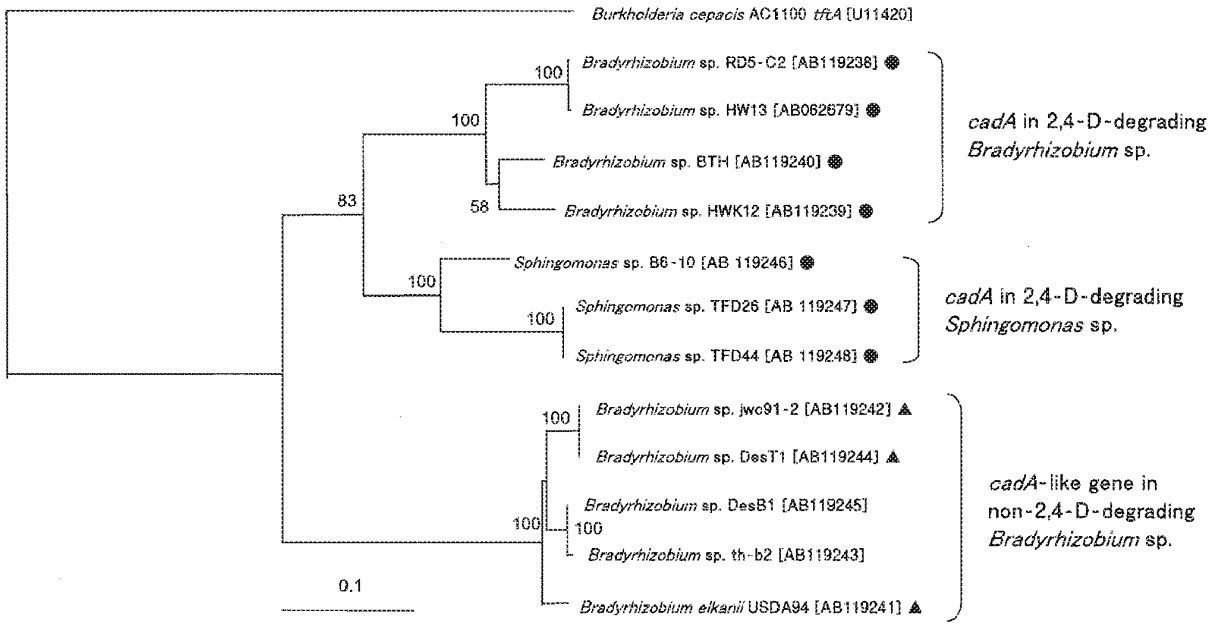


FIG. 3. Phylogenetic tree of *cadA* from 2,4-D-degrading α-*Proteobacteria*. *Burkholderia cepacia* AC1100 *tftA* encodes 2,4,5-T oxygenase. 2,4-D-degraders (●) and 4-CPA- and/or PAA-degraders (▲) are indicated. Designations in brackets are DDBJ, EMBL, and GenBank database accession numbers. The phylogenetic tree of *cadA* was constructed on the basis of common partial sequences (375 bp) by using the NJ method. Bootstrap values above 50% are shown at nodes. The scale bar indicates substitutions per site.

when a PCR-amplified *tfdA*-like gene fragment of B6-5 was used as a probe (Fig. 4b). When *cadA* was used to probe RD5-C2, it hybridized with DNA of the 2,4-D-degrading *Bradyrhizobium* and *Sphingomonas* strains, and weak signals were also observed in *Bradyrhizobium* strains USDA94, jwc91-2, th-b2, DesT1, and DesB1 (Fig. 4c). Stronger signals were detected in the *Bradyrhizobium* strains when a *cadA*-like probe of USDA94 was used (Fig. 4d).

Degradation of 2,4-D and its related compounds. The abilities of the strains used in the present study to degrade 2,4-D and its related compounds are indicated in Table 1 and in Fig. 1 to 3. The 2,4-D-degrading *Bradyrhizobium* and *Sphingomonas* strains degraded 4-CPA and PAA equally well except for *Sphingomonas* sp. strain B6-10, which did not degrade 4-CPA and PAA. Strains BTH, TFD26, and TFD44 did not degrade phenol, which accumulated during the degradation of PAA. None of the other strains degraded 2,4-D; however, strains USDA94, jwc91-2, DesT1, and *R. palustris* JCM2524 degraded 4-CPA. They did not degrade 4-chlorophenol beyond what accumulated during the degradation of 4-CPA. PAA was not degraded by any strains other than the 2,4-D degraders and USDA94. The degrading abilities were repeatedly observed and were not lost during successive cultivation under nonselective conditions on 2,4-D.

Codon usage frequency and GC content. The genes of the *Bradyrhizobium* spp. examined were separated into two groups based on their GC content as summarized in Table 2. Distinctions made between the two groups on the basis of the GC content and codon usage patterns were in good agreement (data not shown). The first group, which had a lower total GC content (55.7 to 59.4%), consisted of *cadA* of 2,4-D-degrading *Bradyrhizobium* sp., *nod*, and the NifA-regulated genes of *Bradyrhizobium* spp. (31, 35, and www.kazusa.or.jp/codon). The second group was made up of the *cadA* gene of non-2,4-D-degrading *Bradyrhizobium* sp., the *tfdA*α gene of both 2,4-D-degrading and nondegrading *Bradyrhizobium* spp., and the housekeeping genes of *Bradyrhizobium* spp. (35) and showed a higher total GC content (63.9 to 65.7%). The difference between the two groups was distinctive when the GC bias at the third codon position was considered.

In *Sphingomonas* sp., *tfdA*-like genes were similar to the housekeeping genes on the basis of a higher GC content (64.8 and 68.2%, respectively) and distinguished from *cadA* with a lower GC content (55.9%).

DISCUSSION

A number of 2,4-D degraders that belong to a variety of genera have been isolated worldwide, and it has been revealed that there are at least two catabolic systems for 2,4-D degradation. 2,4-D degraders in β- and γ-*Proteobacteria* use α-KG-dependent 2,4-D dioxygenase encoded by *tfdA* (11, 28, 44), whereas 2,4-D degrading α-*Proteobacteria* use the enzyme that is similar to 2,4,5-T oxygenase, encoded by *cadAB* (23). Besides these, *tfdA*α genes have been found in 2,4-D-degrading *Bradyrhizobium* strains in α-*Proteobacteria* that were isolated from pristine environments (17). However, the evolutionary origin of the genes still remains unknown because there are very few similar genes and amino acid sequences in databases. As *cadAB* and *tfdA*α genes have been found in microorganisms isolated from pristine environments where lava was deposited 4,800 years ago (20), we speculate that ancestral *cadAB* and

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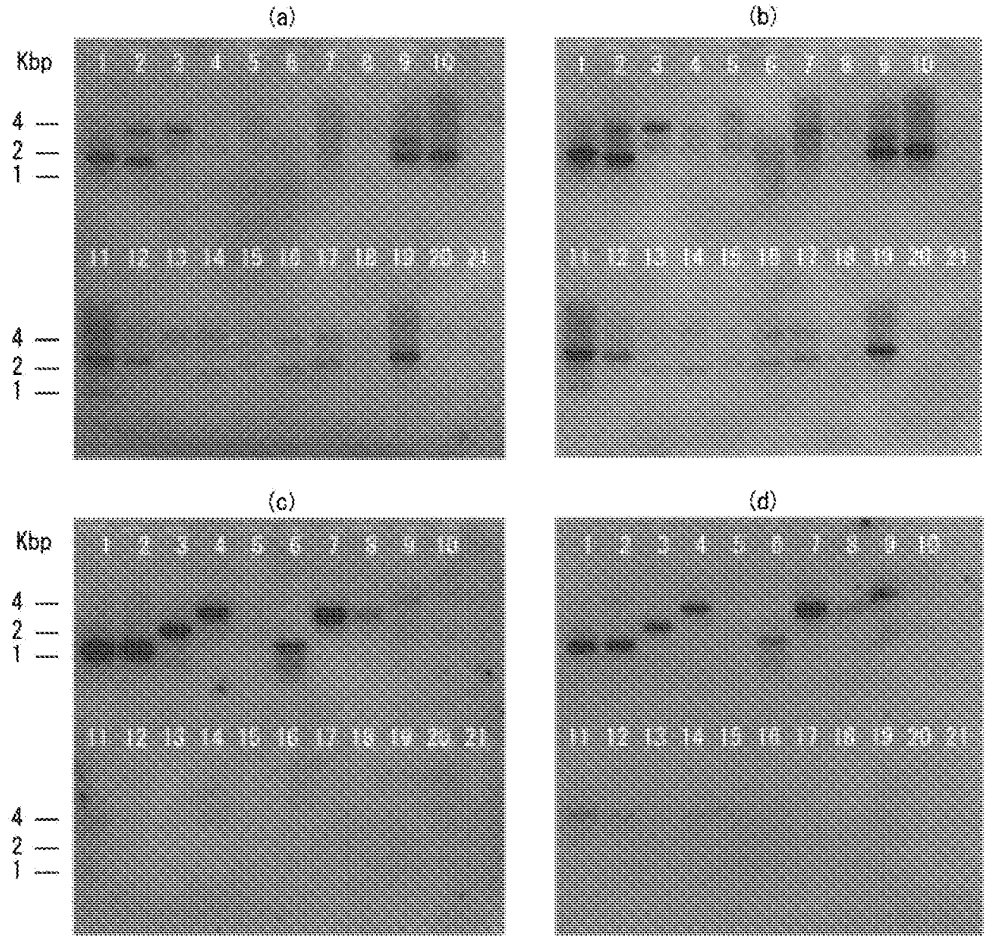


FIG. 4. Southern hybridization by the *tfdAα* (a) and *cadA* (c) probes from 2,4-D-degrading strain RD5-C2 in *Bradyrhizobium* sp., by the *tfdA*-like gene (b) from *Sphingomonas* sp. strain B6-5, and by the *cadA*-like gene (d) from *Bradyrhizobium* sp. strain USDA94. Results for the following strains are shown: 2,4-D-degrading strains RD5-C2 (lane 1), HW13 (lane 2), HWK12 (lane 3), and BTH (lane 4) in *Bradyrhizobium* sp.; 2,4-D-degrading strains B6-5 (2,4-D-degrading ability lost) (lane 5), B6-10 (lane 6), TFD26 (lane 7), and TFD44 (lane 8) in *Sphingomonas* sp.; *B. elkanii* USDA94 (lane 9); *B. japonicum* USDA110 (lane 10); *Bradyrhizobium* sp. strains jwc91-2 (lane 11), DesT1 (lane 12), DesB1 (lane 13), th-b2 (lane 14), ApB16 (lane 15), BDV5111 (lane 16), BDV5329 (lane 17), BDV5419 (lane 18), and BDV5680 (lane 19); *Agromonas oligotrophica* JCM1494 (lane 20); and *Rhodopseudomonas palustris* JCM2524 (lane 21).

tfdAα genes in this group of bacteria may serve as the origin of the *tftAB* and *tfdA* genes, respectively.

In this study, we revealed that all of the *Bradyrhizobium* strains including type strains of *B. japonicum* USDA110 and *B. elkanii* USDA94 have the gene *tfdAα*. The gene seems to be ubiquitous among *Bradyrhizobium* spp. independent of their 2,4-D-degrading ability. Therefore, TfdAα should have an original substrate or function, which should have no relation to xenobiotic compounds. Naturally occurring aryl ethers and cinnamic acid are proposed as candidate substrates (5), as well as naturally produced organohalogen compounds (13). Very recently, the complete genomic sequence of *B. japonicum* USDA110 has been determined (22). Gene arrangements around *tfdAα* were almost identical between strains RD5-C2 and USDA110 (our unpublished observations), and there are no related *tfd* genes (*tfdB* and *tfdCDEF*) that have been found in the 2,4-D-degrading plasmid pJP4. As genes for D-β-hydroxybutyrate dehydrogenase and oxidoreductase are present just upstream of *tfdAα*, the function of TfdAα might be related to fatty acid metabolism. As shown in Fig. 5, alignment of the deduced amino acid sequence of *B. japonicum* USDA110 TfdAα with the other α-KG-dependent 2,4-D dioxygenase indicates that the predicted active-site residues are well conserved among them as follows: two His residues and one Asp residue, which are suspected of being iron-binding ligands; a Thr and an Arg residue, suspected of forming a salt bridge with the C-5 carboxylate of α-KG; and a His and an Asp residue, suspected of forming a salt bridge with the 2,4-D carboxylate (7, 15). Therefore, it is supposed that TfdAα in *Bradyrhizobium* spp. would belong to the α-KG-dependent dioxygenase family (15).

The phylogenetic trees of the *tfdAα* gene and the corresponding 16S rRNA genes were almost congruent, indicating the evolution of the genes without horizontal gene transfer in

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TABLE 2. GC content of *tfdA*α and *cadA* genes of *Bradyrhizobium* spp. and *Sphingomonas* spp.

Gene	No. of genes	GC content (mol%)			
		Total	1st codon position	2nd codon position	3rd codon position
*tfdA*α of 2,4-D-degrading *Bradyrhizobium* sp.	4	64.4	65.3	39.2	89.0
*tfdA*α of non-2,4-D-degrading *Bradyrhizobium* spp	11	63.0	64.9	37.3	86.9
cadA of 2,4-D-degrading *Bradyrhizobium* sp.	4	55.7	59.1	43.8	64.5
cadA of non-2,4-D-degrading *Bradyrhizobium* sp.	5	65.7	64.1	48.4	84.6
nod genes of *Bradyrhizobium japonicum*[a]	12	57.8	Na[b]	Na	64.4
NifA-regulated genes of *B. japonicum*[a]	14	57.7	Na	Na	69.4
Housekeeping genes of *B. japonicum*[a]	19	64.9	Na	Na	86.4
nod genes of *B. elkanii*[c]	18	59.4	63.2	45.6	69.4
Housekeeping genes of *B. elkanii*[d]	2	64.3	66.3	38.3	88.1
tfdA-like genes of 2,4-D-degrading *Sphingomonas* sp.	4	64.8	67.3	45.2	81.7
cadA genes of 2,4-D-degrading *Sphingomonas* sp.	3	55.9	59.1	41.4	67.3
Housekeeping genes of *Sphingomonas* spp.[e]	3	68.2	71.9	47.6	85.3

[a] Ramseier and Gottfert (35).
[b] Not available.
[c] Codon usage tabulated from GenBank (www.kazusa.or.jp/codon).
[d] *dnaK* and *gyrB* (GenBank accession no. AY328393 and AB070585, respectively).
[e] *bglI*, *ces10*, and *ugpG* (GenBank accession no. AF305688, AF305841, and AF461178, respectively).

Bradyrhizobium spp. As *tfdA*α was also detected in phylogenetically closely related *A. oligotrophica* and *R. palustris*, the distribution of *tfdA*α seems not to be limited within *Bradyrhizobium* spp. However, no genes similar to *tfdA*α could be detected in *Rhizobium loti* and *Sinorhizobium meliloti*, both of which are symbiotic nitrogen-fixing bacteria belonging to α-*Proteobacteria* (12, 21).

The 2,4-D-degrading *Sphingomonas* sp. has been believed to have no *tfdA* gene homologs based on the negative results of PCR amplification and hybridization experiments (11, 28, 43, 44). In this study, however, *tfdA*-like gene fragments were amplified by PCR and detected by Southern hybridization in *Sphingomonas* strains. The phylogenetically distinct position of *Sphingomonas tfdA*-like genes suggests that the evolution occurred without recent horizontal gene transfer.

In addition to the previous evidence that 2,4-D-degrading *Bradyrhizobium* and *Sphingomonas* strains have *cadA* homologs (23), the genes were also detected in the non-2,4-D-degrading *Bradyrhizobium* strains (Table 1 and Fig. 3). In contrast to *tfdA*α, however, the distribution was limited to strains USDA94, jwc91-2, th-b2, DesT1, and DesB1, which belong to the group of *B. elkanii*, based on the 16S rRNA gene sequences, and have been isolated in the United States (33). A worldwide survey of the geographical distribution of *cadA* in 2,4-D-degrading *Bradyrhizobium* spp. and non-2,4-D-degrading *Bradyrhizobium* spp. could provide information on the endemicity of the gene.

The phylogenetic tree of *cadA* suggests the separate evolution of the gene in 2,4-D-degraders versus non-2,4-D-degraders (Fig. 3). The former cluster was made up of two distinct clusters of *Bradyrhizobium* sp. and *Sphingomonas* sp., suggesting the independent evolution in the genera from a common ancestral gene. The incongruency of *cadA* with the 16S rRNA genes would be an indication of the horizontal transfer of *cadA* between the genera, and the distinct separation of the tree between 2,4-D-degrading *Bradyrhizobium* sp. and *Sphingomonas* sp. suggests that they could be separated at an early stage of evolution.

The apparent differences in the codon usage patterns and the GC content of *cadA* between 2,4-D-degrading and non-2,4-D-degrading *Bradyrhizobium* spp. (Table 2) also suggests a different origin of *cadA* in 2,4-D-degraders versus nondegraders. Ramseier and Gottfert reported that *B. japonicum* nodulation and the NifA-regulated genes have different codon usage and GC content from the housekeeping genes in *B. japonicum* and suggested that nodulation and the NifA-regulated genes were acquired relatively late in evolution via lateral gene transfer from other microorganisms (35). The presence of the DNA region of lower GC content (680 kb), in which the symbiotic island region (410 kb) was included, was demonstrated in the complete genomic sequence of *B. japonicum* USDA110 (22). The codon usage patterns and the GC content of *cadA* of non-2,4-D-degrading *Bradyrhizobium* sp. were similar to those of the housekeeping genes, whereas for *cadA* of 2,4-D-degrading *Bradyrhizobium* sp., they were similar to those of the symbiotic genes, suggesting the possibility that the *cadA* gene of 2,4-D-degrading *Bradyrhizobium* sp. was obtained through horizontal gene transfer. The same assumption could be made for *cadA* of *Sphingomonas* sp., in which the GC content was distinctively distinguished from that of the housekeeping genes.

Among the non-2,4-D-degrading *Bradyrhizobium* strains having *cadA* homologs, USDA94, jwc91-2, and DesT1 degraded 4-CPA. They degrade 4-CPA with the accumulation of 4-chlorophenol as an intermediate metabolite, suggesting that the *cadA* homologs are involved in the cleavage of the aryl ether linkage. The *cadA* gene with 2,4-D-degrading ability might have emerged from *cadA* homologs, which had been distributed among the limited group of *Bradyrhizobium* spp. in the form of naturally occurring aryl ether compounds as substrates. As 2,4-D-degrading *Bradyrhizobium* strains were isolated from pristine environments, *cadA* should have evolved from natural compounds and the degradation of 2,4-D would be coincidental. The inability to degrade 4-chlorophenol also suggests the unrelated presence of the gene with the 2,4-D-degrading pathway. The limited distribution of *cadA* homologs in *Bradyrhizobium* spp. indicates that the genes are not essential for their proliferation.

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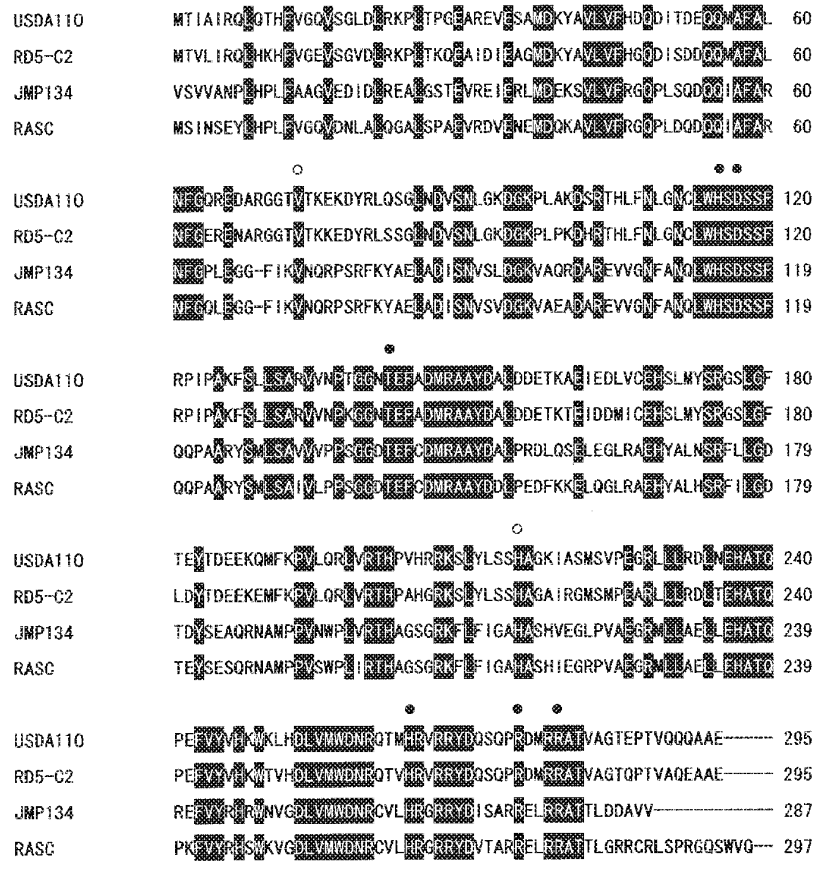


FIG. 5. Multiple alignment of the deduced amino acid sequences of TfdAα of *Bradyrhizobium* strains RD5-C2 and USDA110 and TfdA of *Ralstonia eutropha* JMP134 and *Burkholderia* sp. strain RASC. Conserved residues are boxed. The predicted iron- and α-KG-binding ligands are marked with filled circles. The predicted 2,4-D-binding ligands in TfdA are marked with open circles.

It has been revealed that two systems for 2,4-D degradation, TfdA and CadAB, have evolved in *Bradyrhizobium* spp. and *Sphingomonas* sp. in α-*Proteobacteria*. Although the α-KG-dependent 2,4-D dioxygenase activity was demonstrated in TfdAα (17), a good agreement between the presence of *cadA* and the ability to degrade phenoxy compounds suggests that the CadAB system might have a greater responsibility for the degradation. The distinct separation of *cadA* between *Bradyrhizobium* 2,4-D degraders and nondegraders in phylogeny and GC content suggests their evolution from different origins. A comparison of their genetic organization around *cadA* would provide useful information on the evolution of 2,4-D degraders.

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Complete Genomic Sequence of Nitrogen-fixing Symbiotic Bacterium *Bradyrhizobium japonicum* USDA110 (Supplement)

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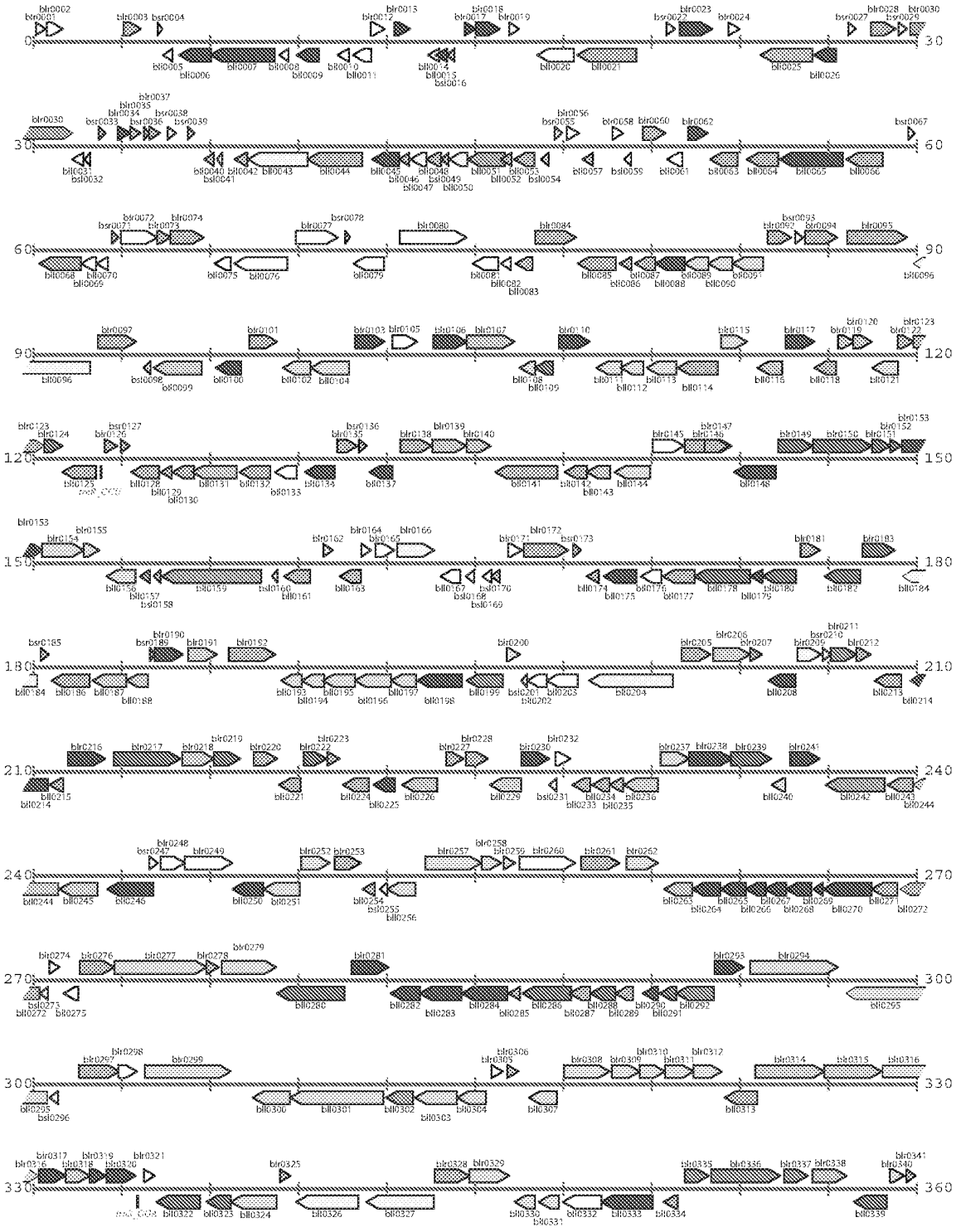
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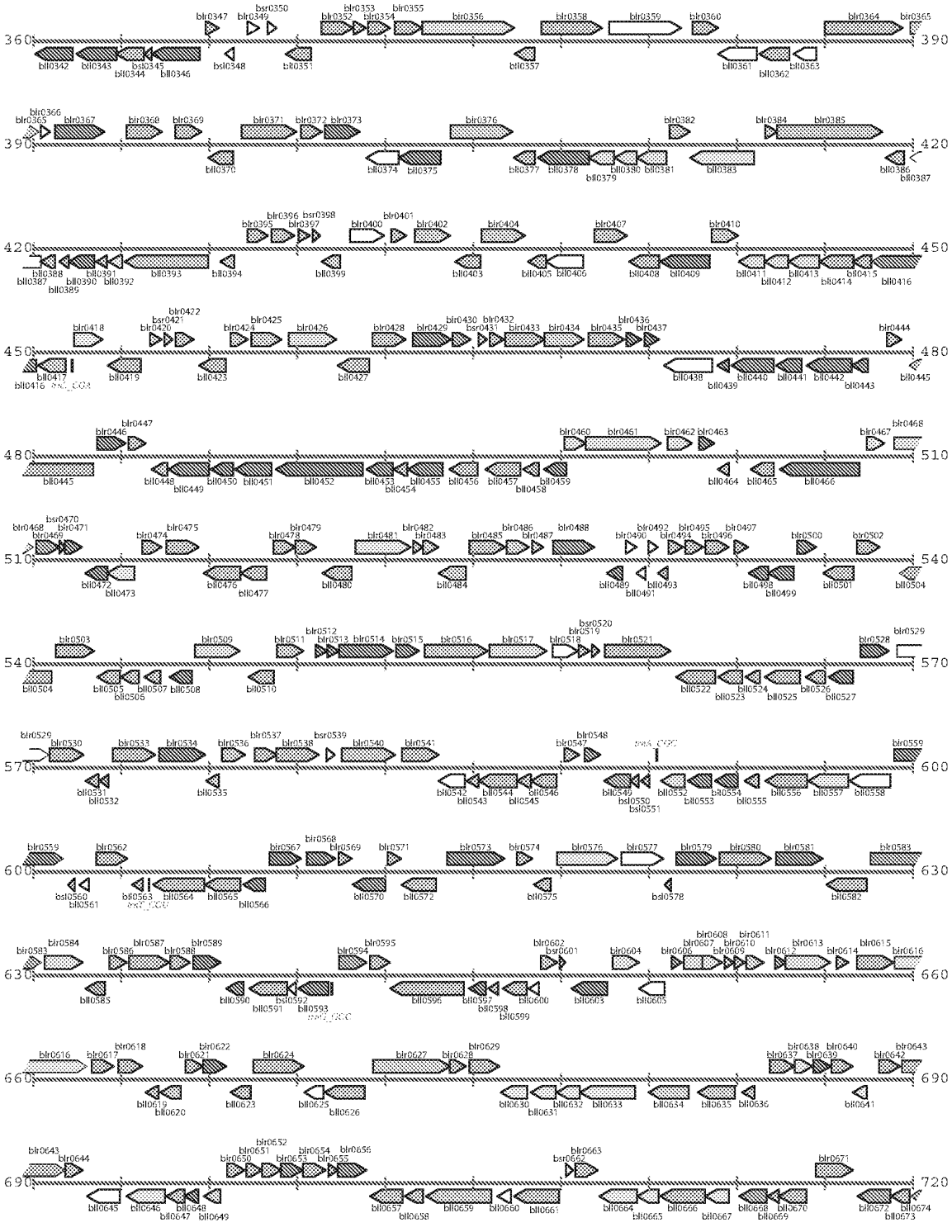
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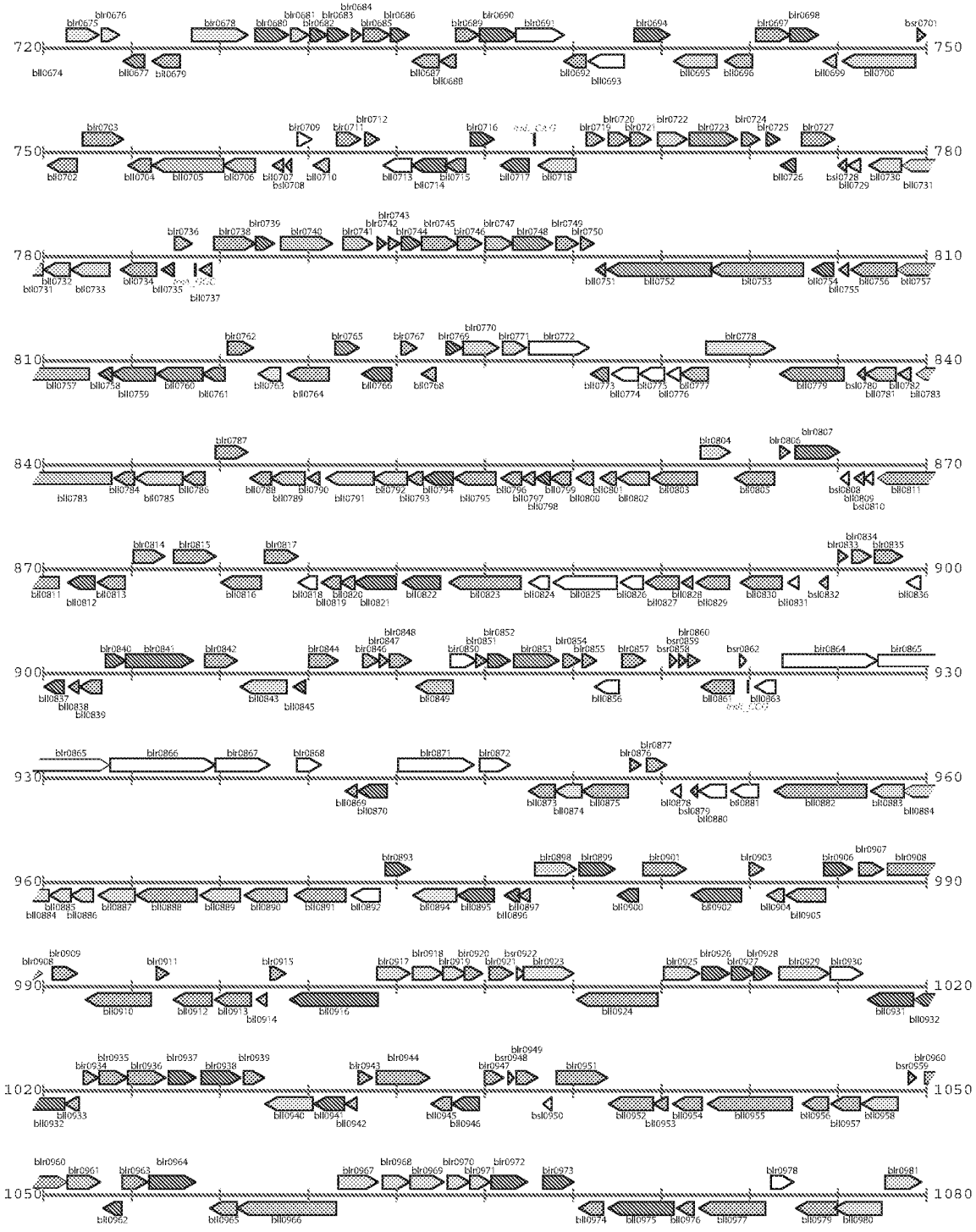
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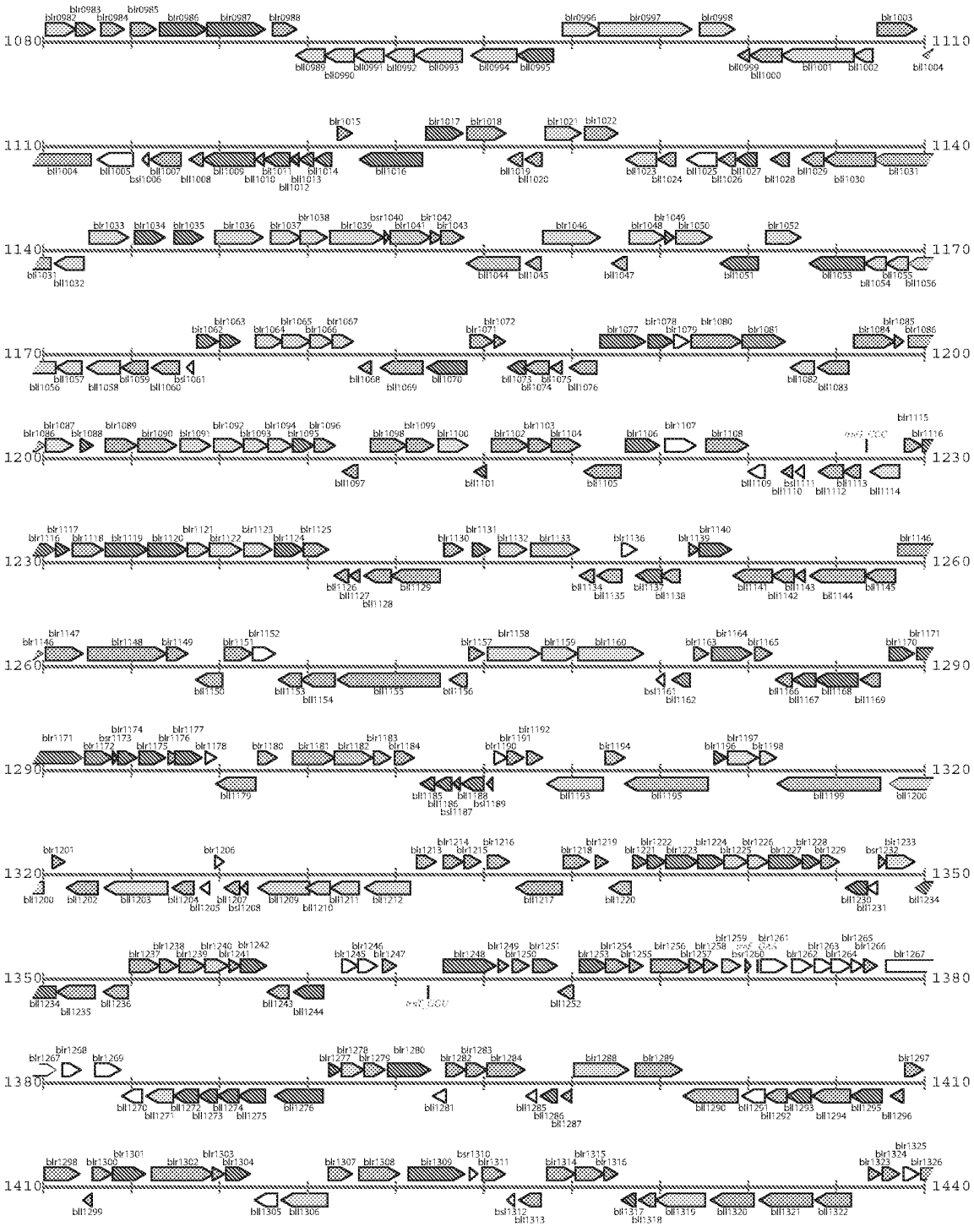
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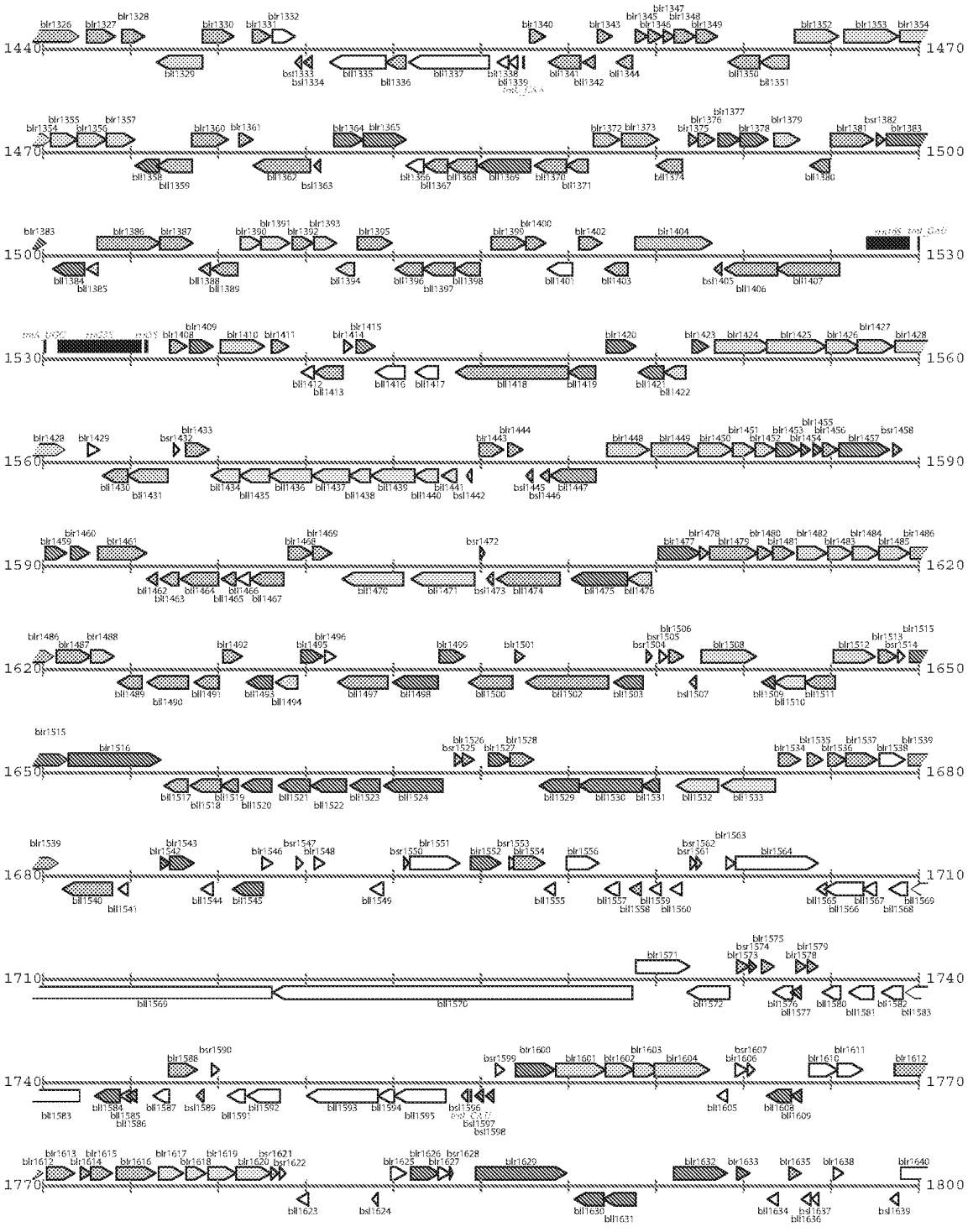
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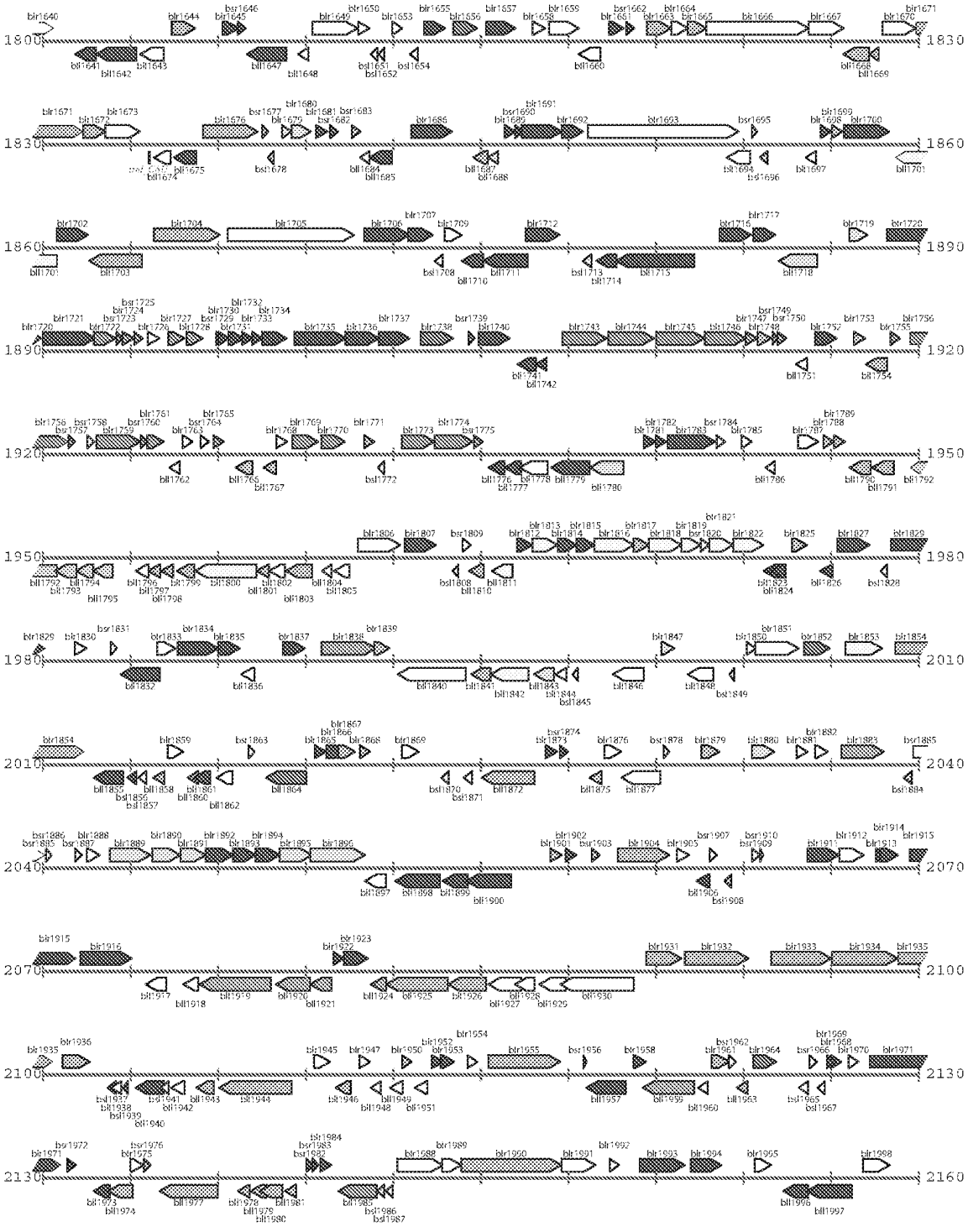
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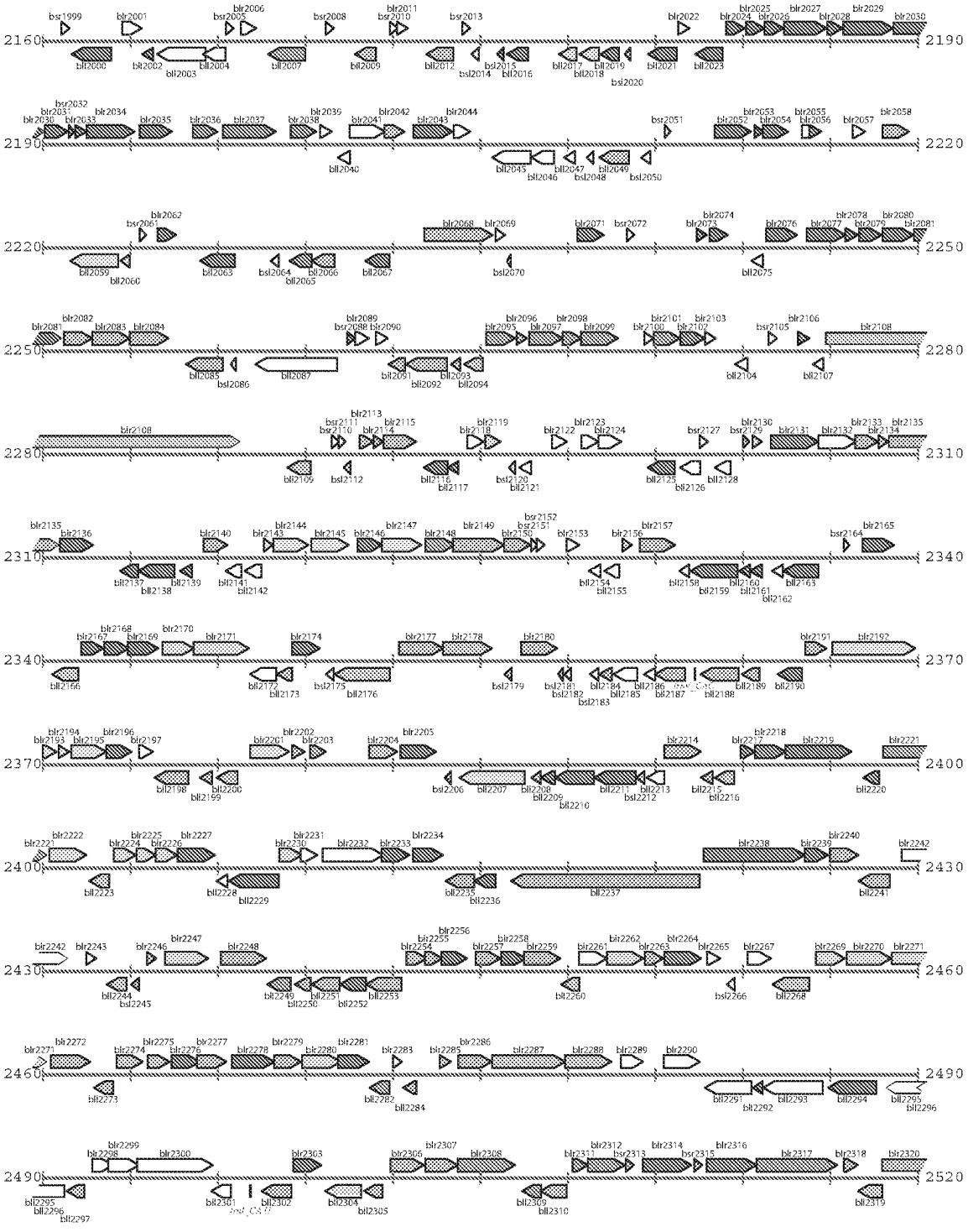
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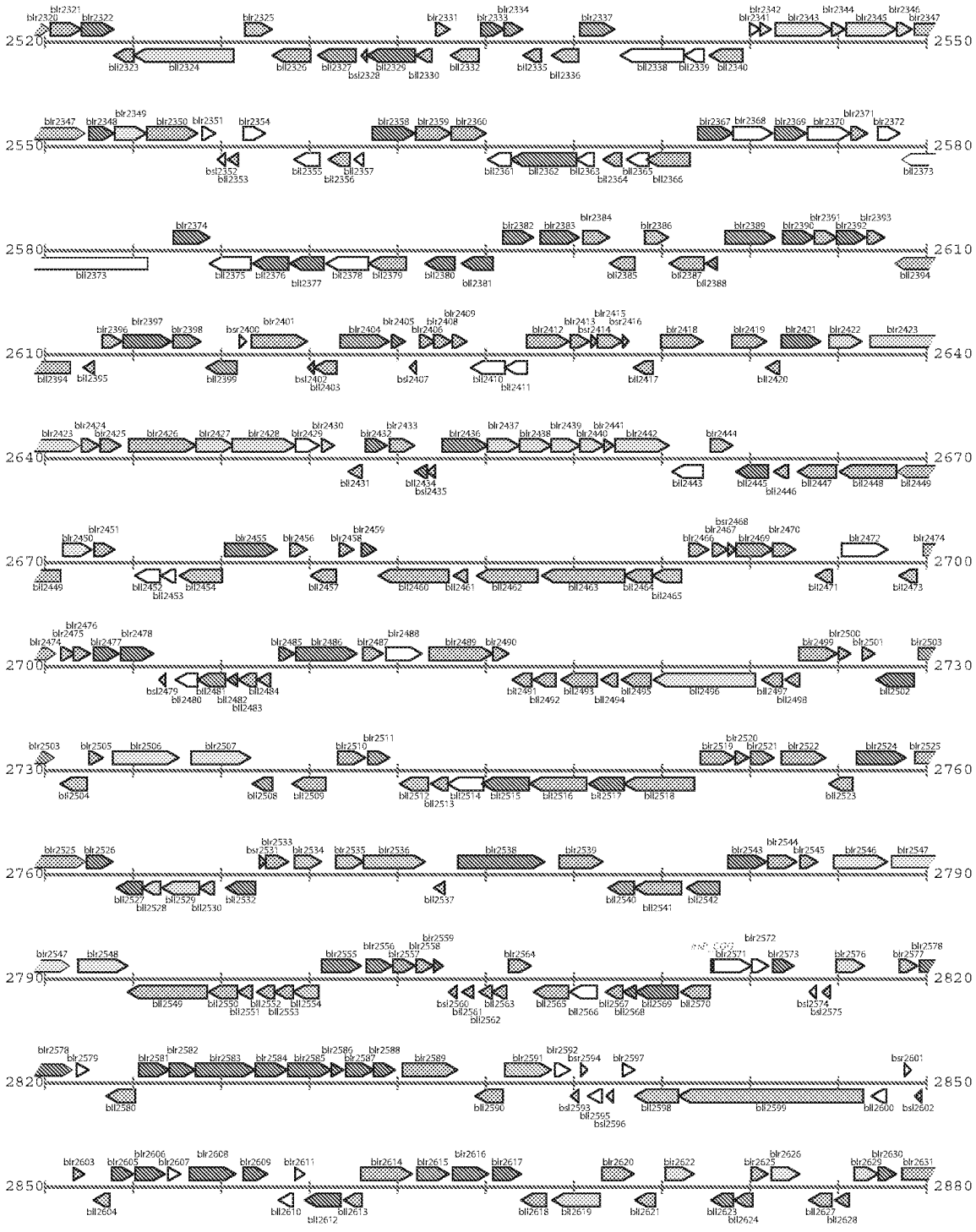
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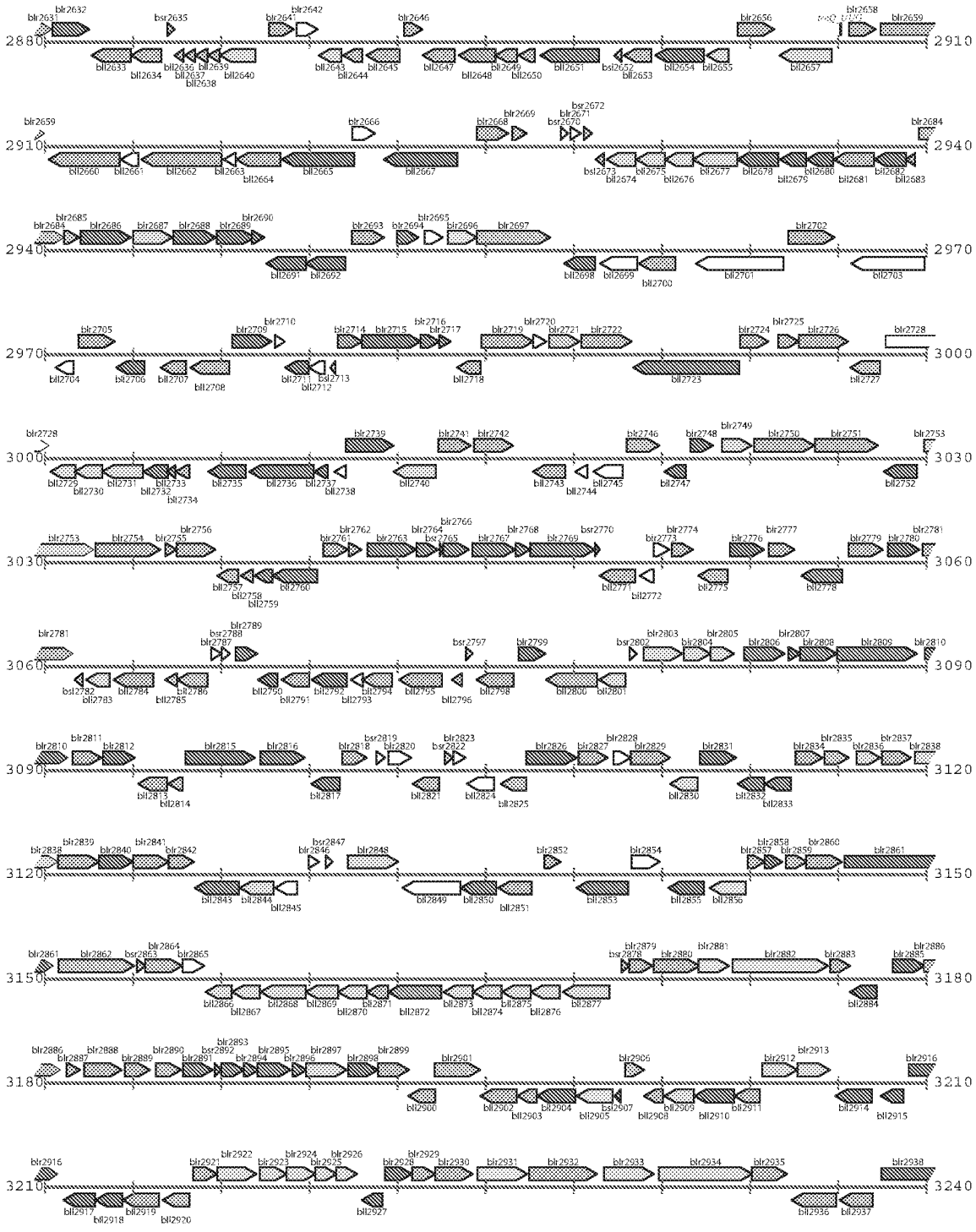
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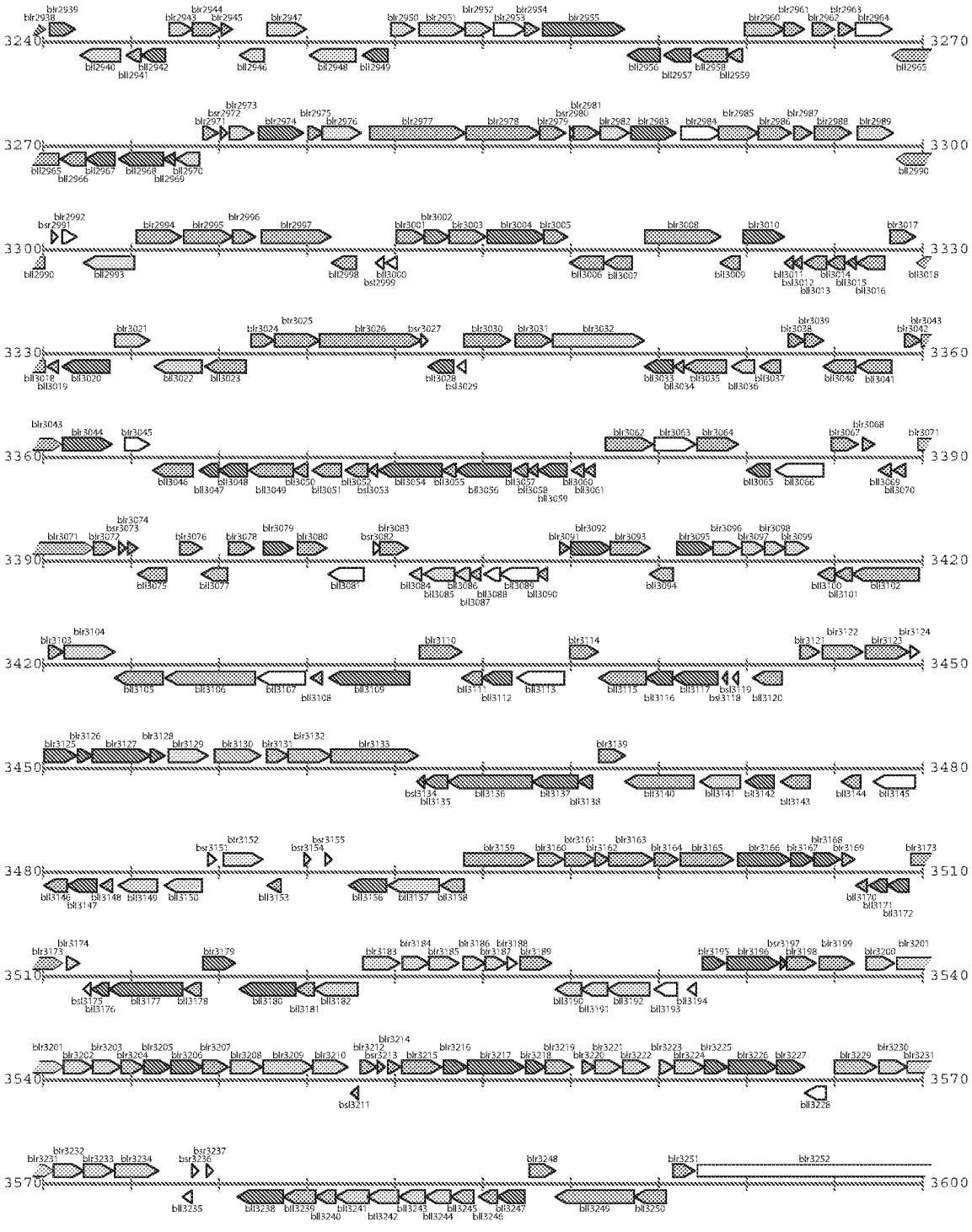
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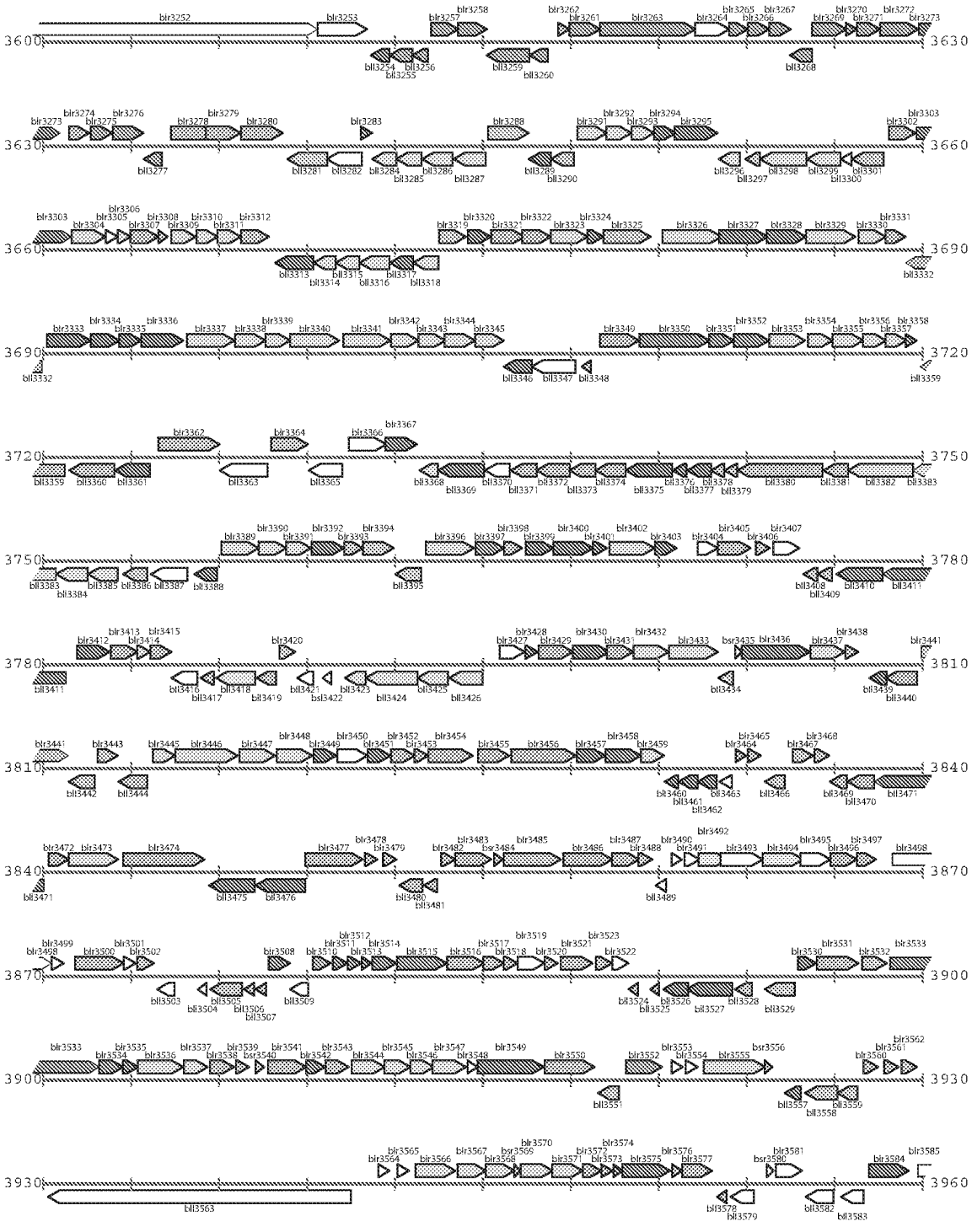
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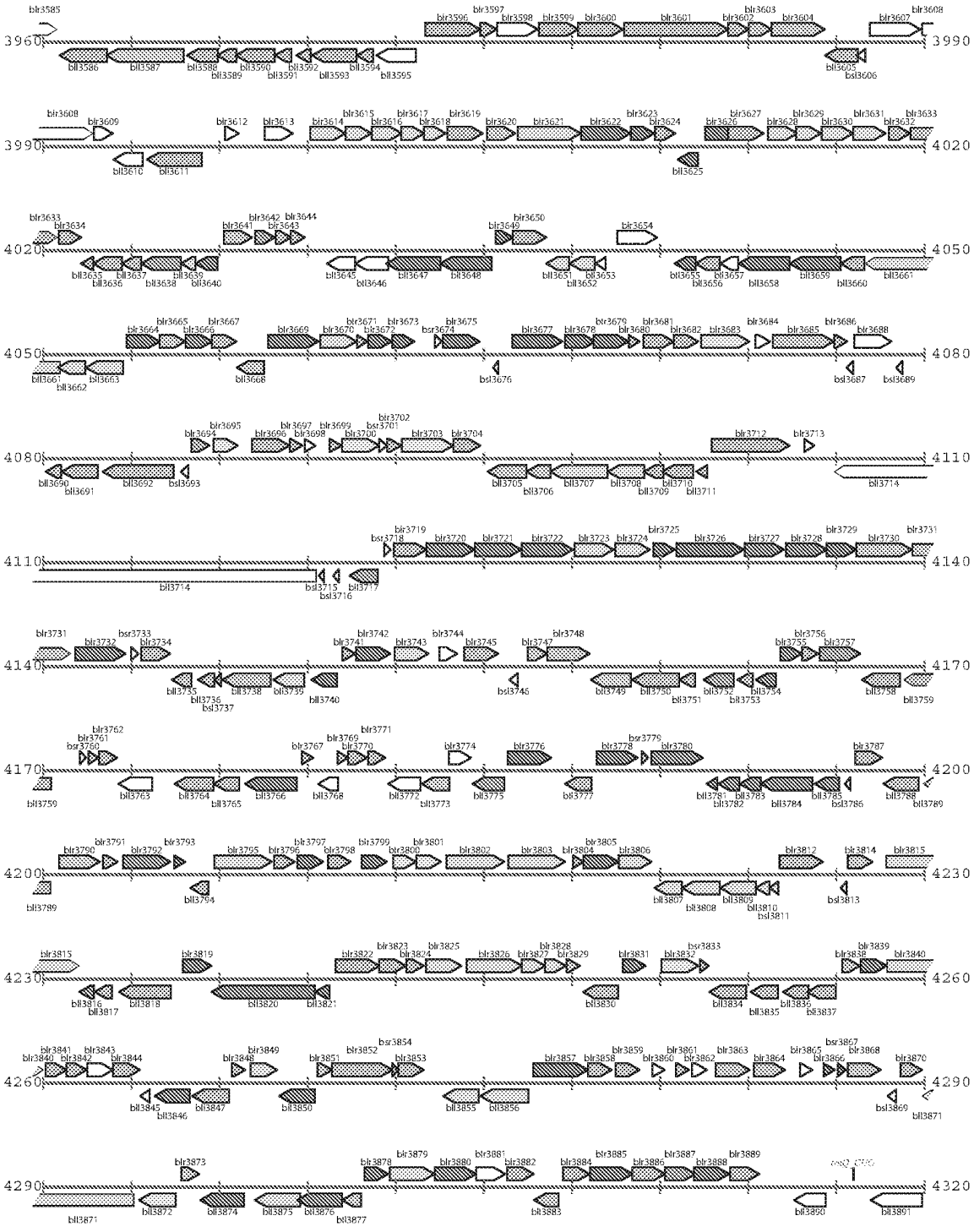
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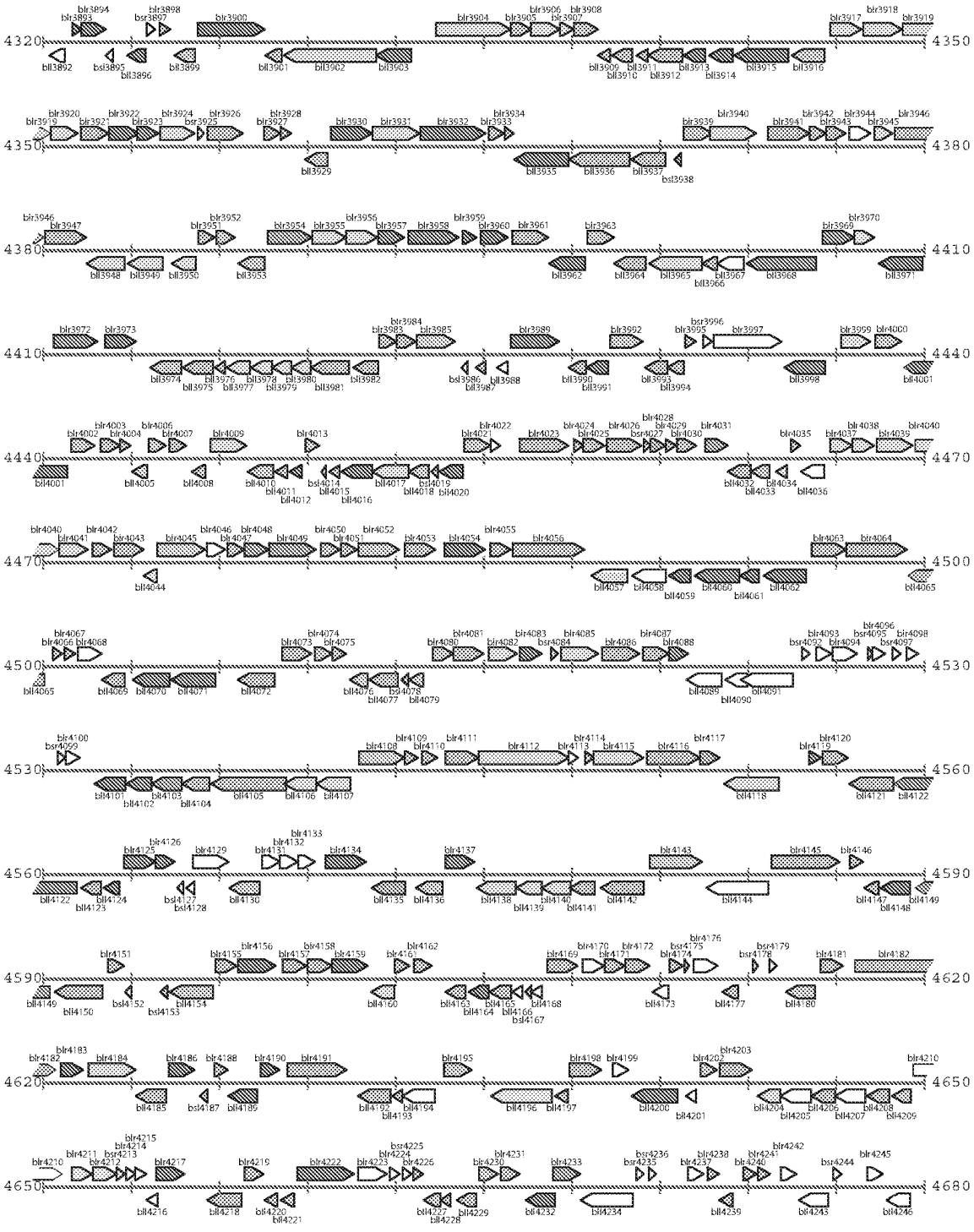
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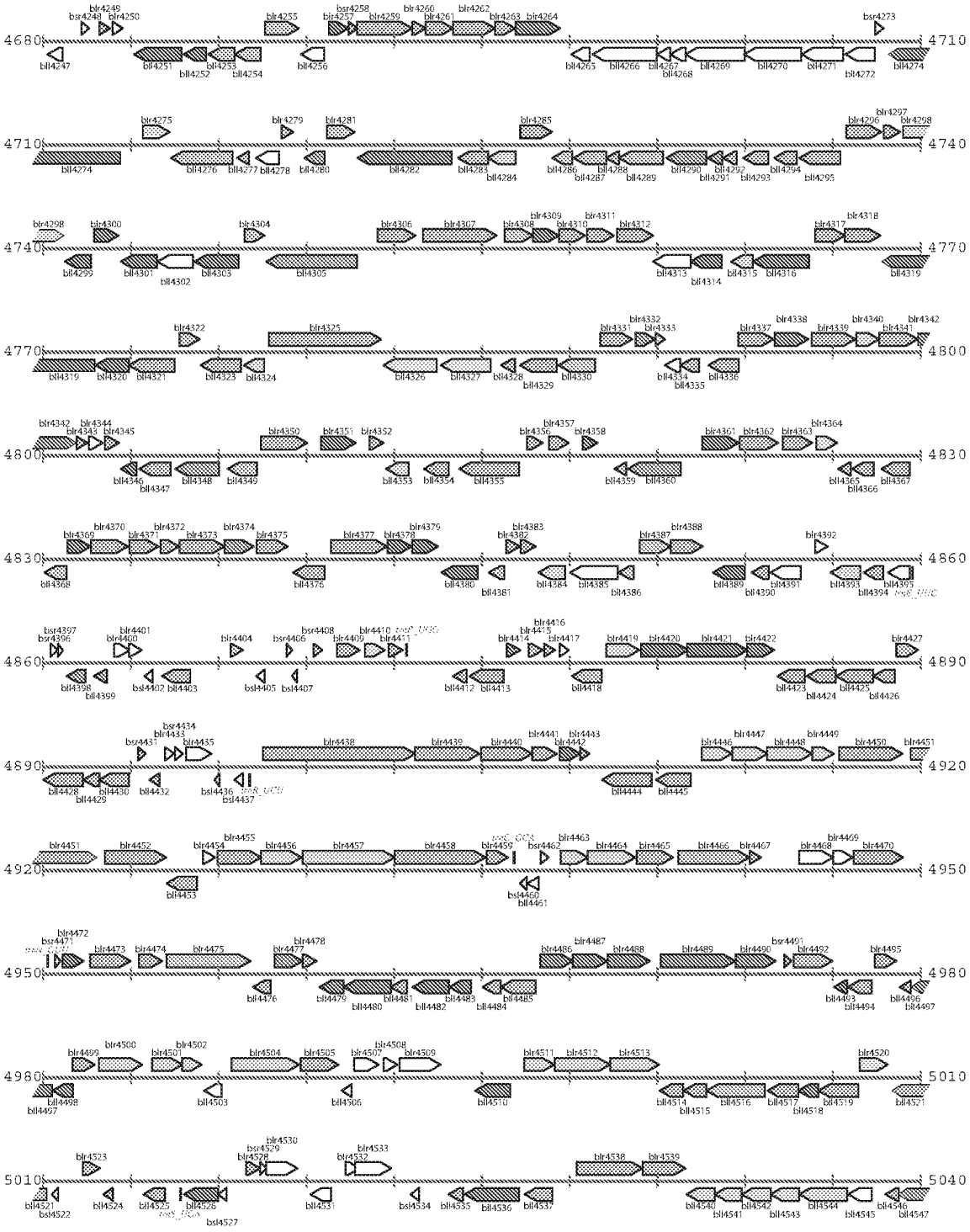
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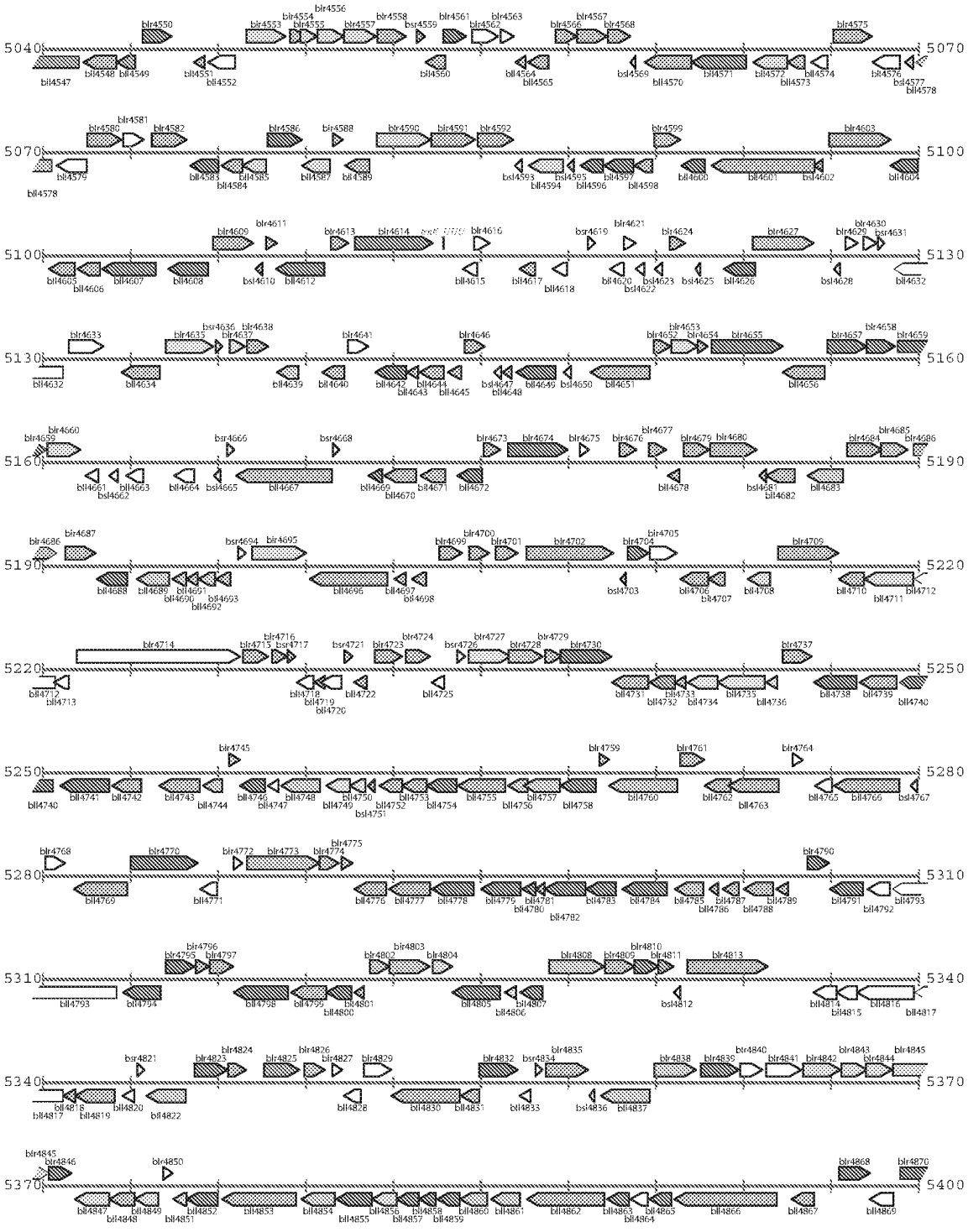
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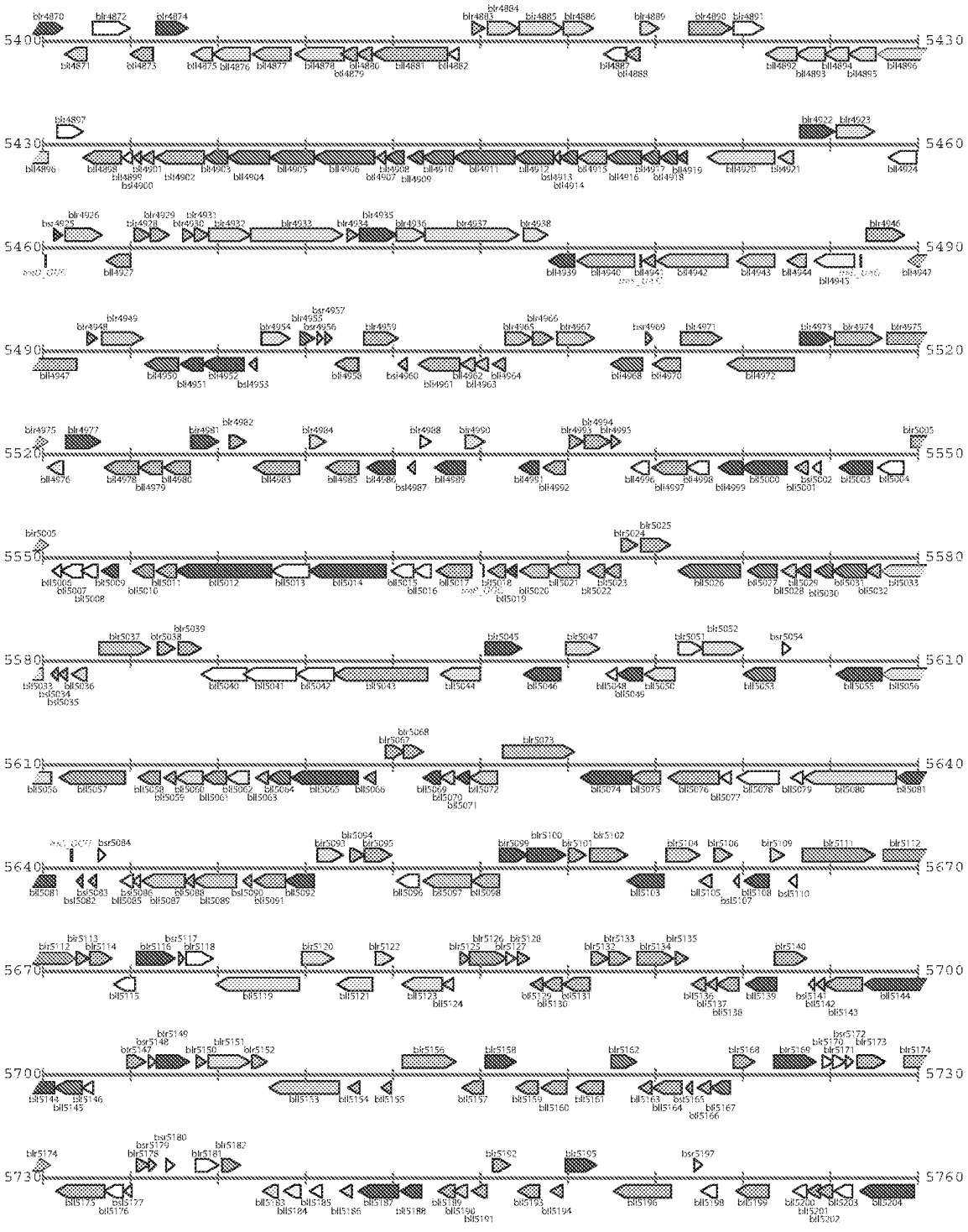
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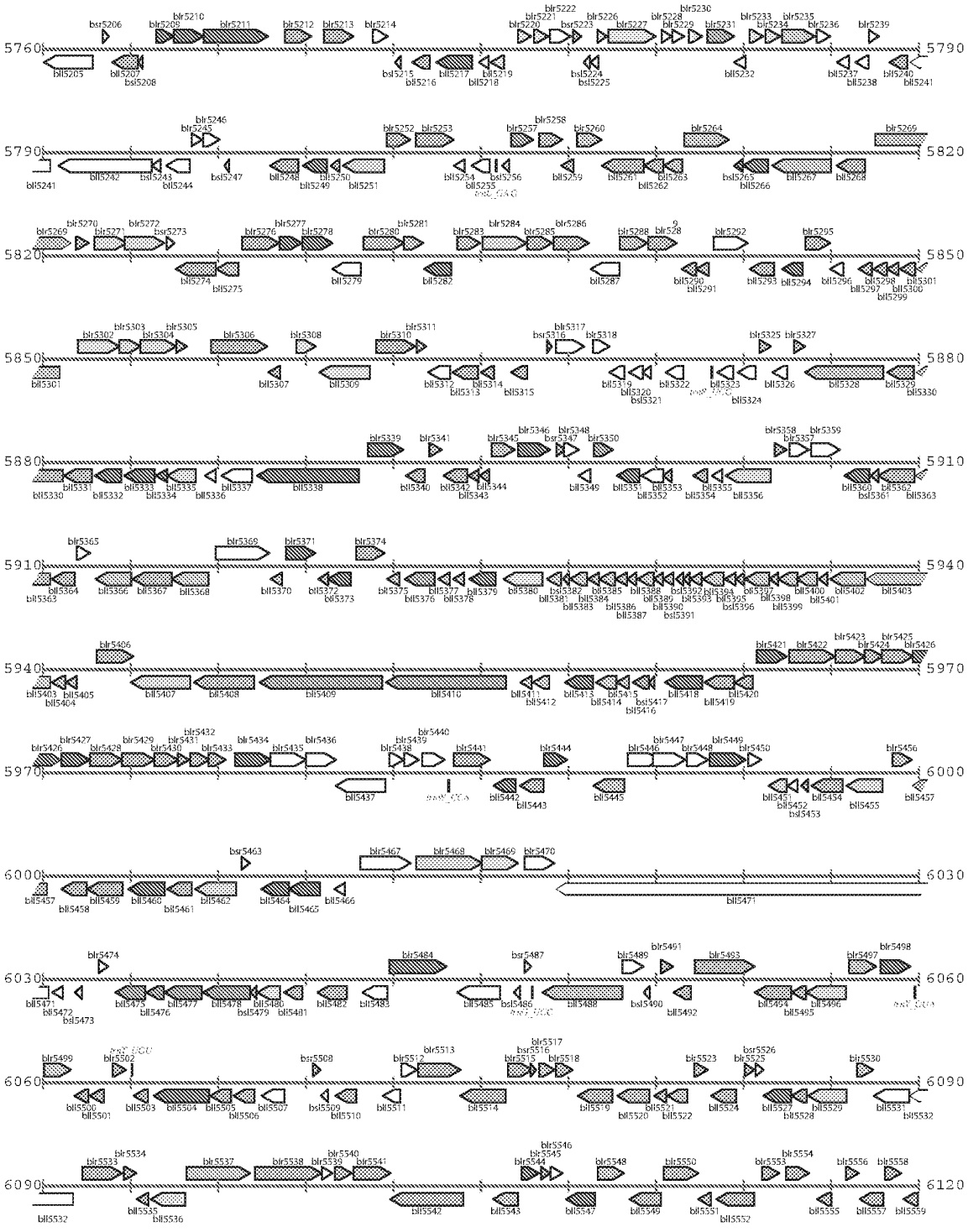
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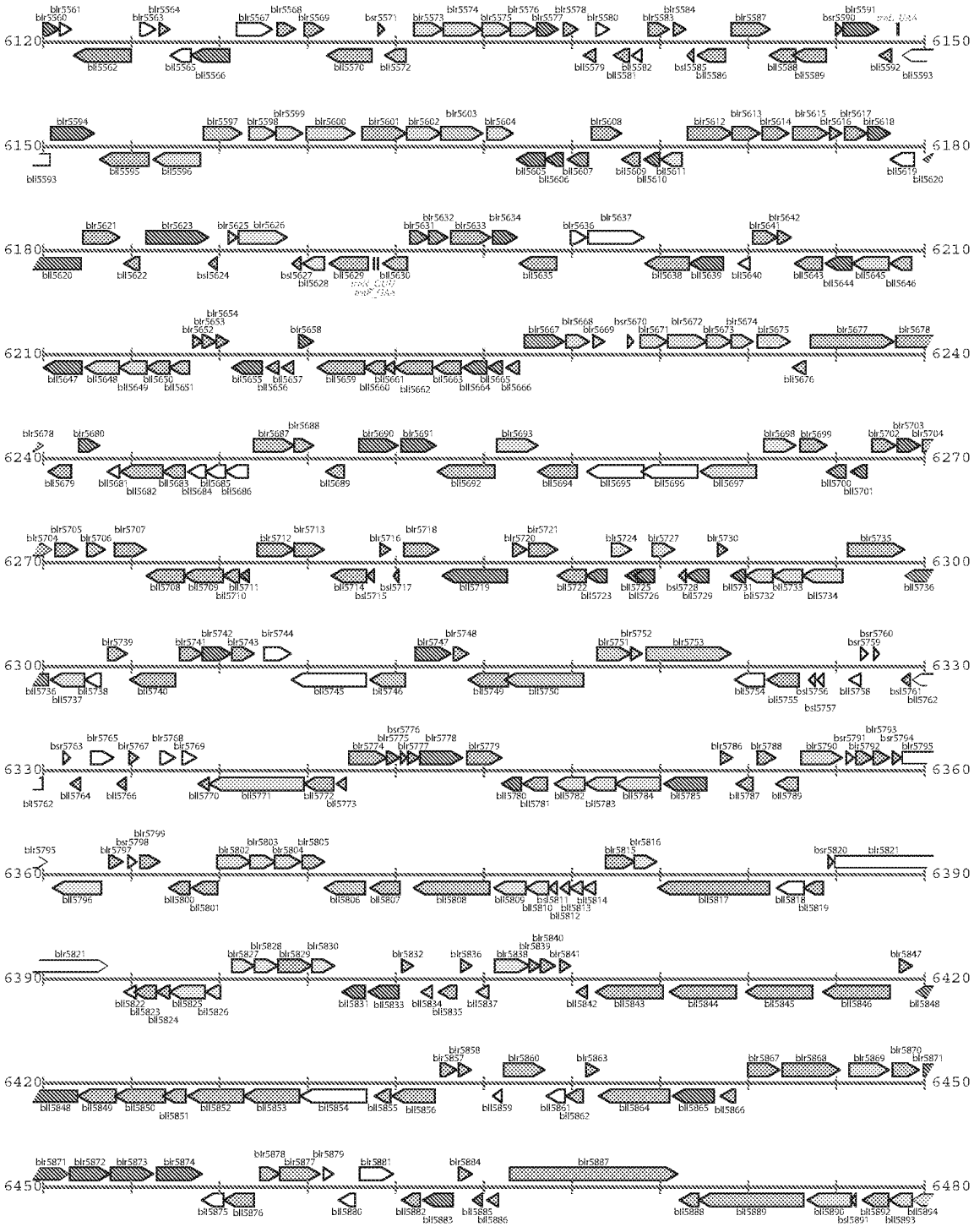
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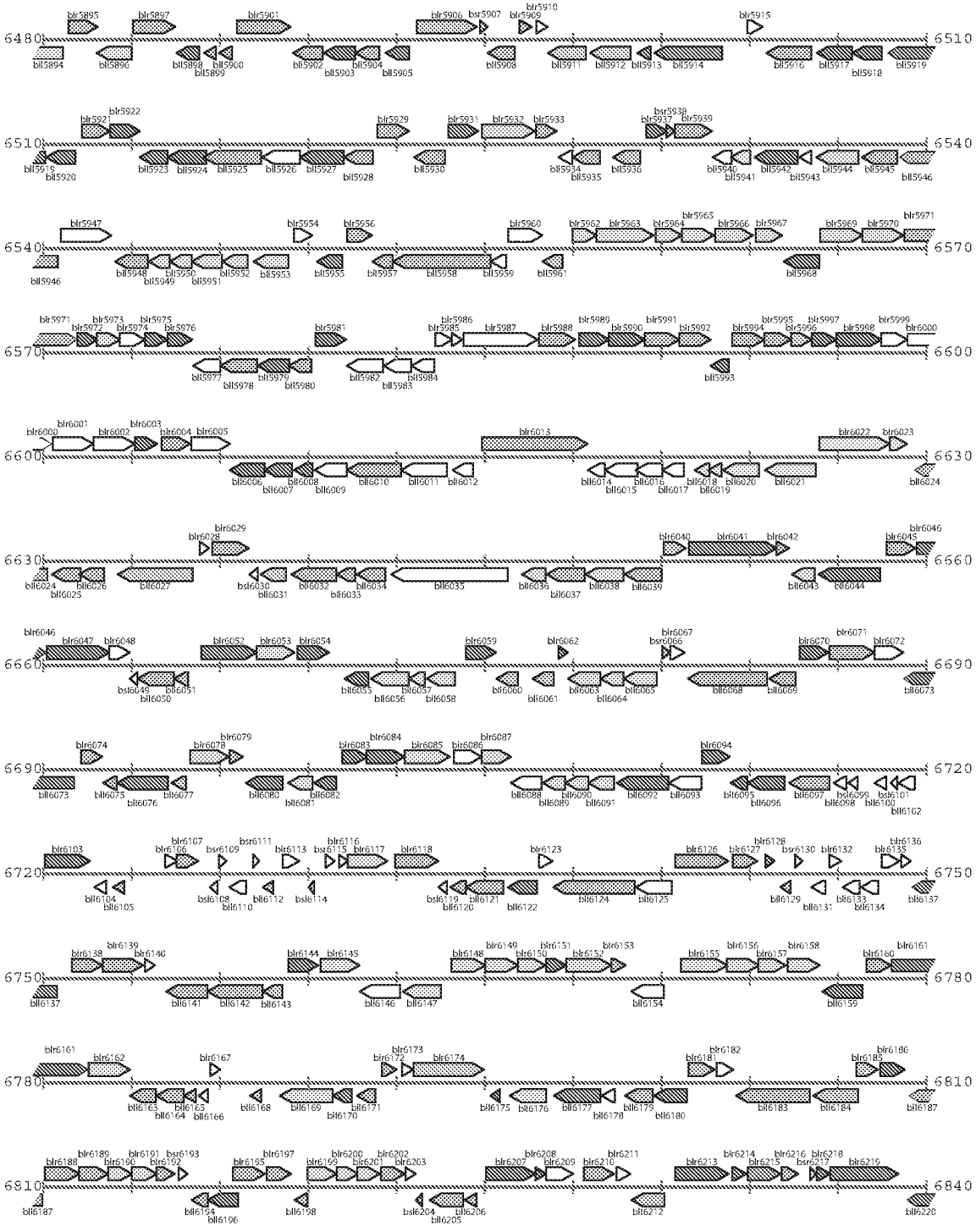
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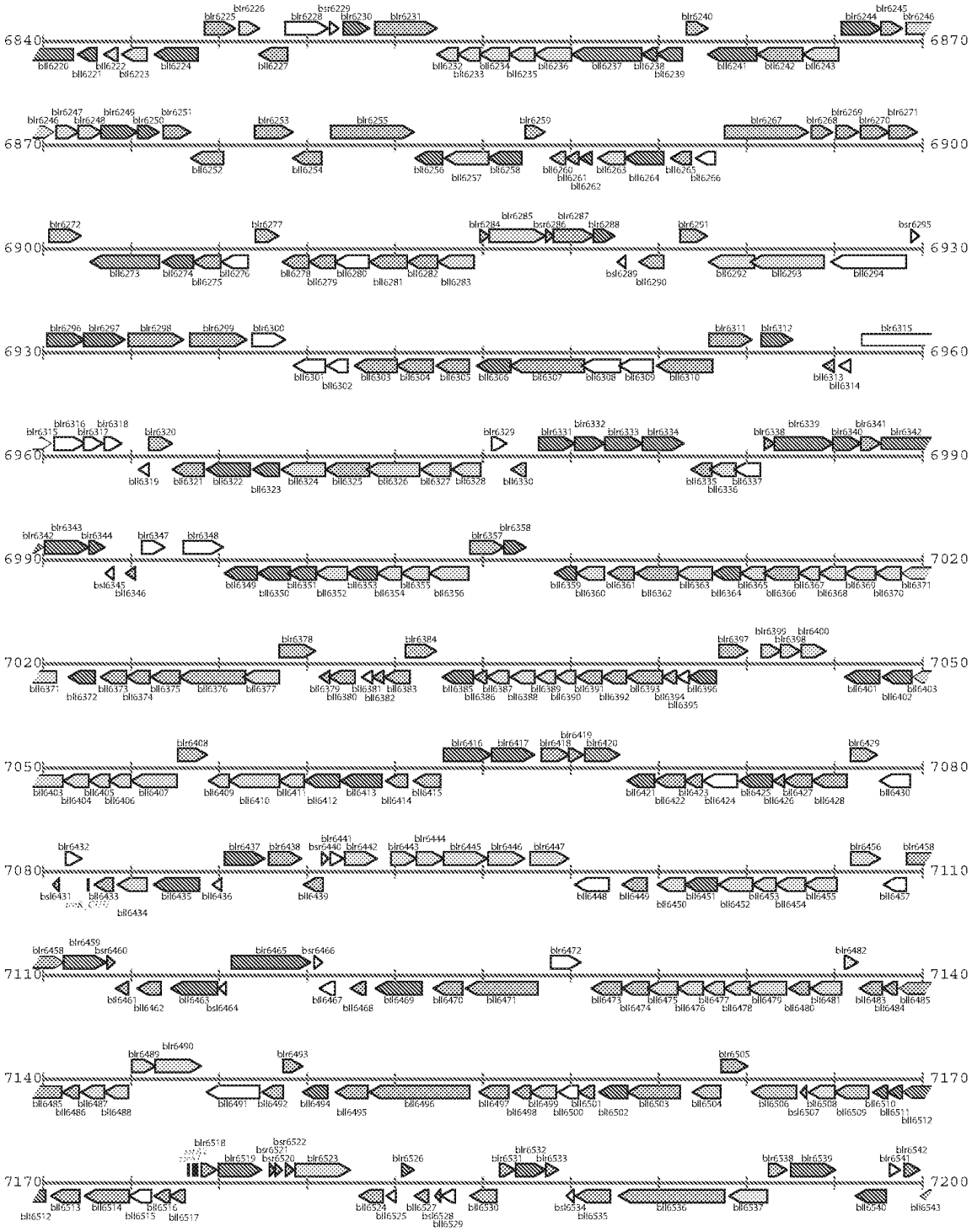
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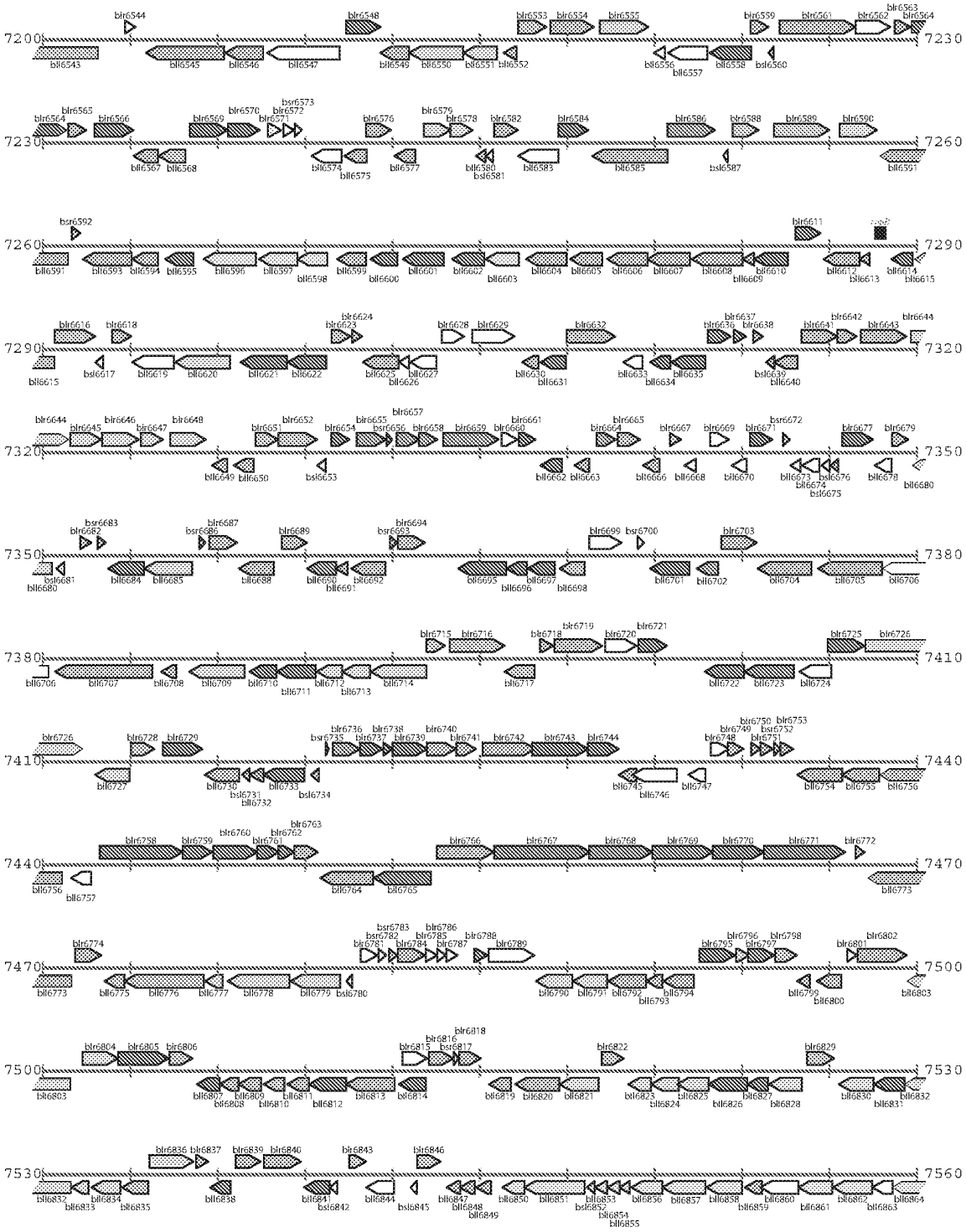
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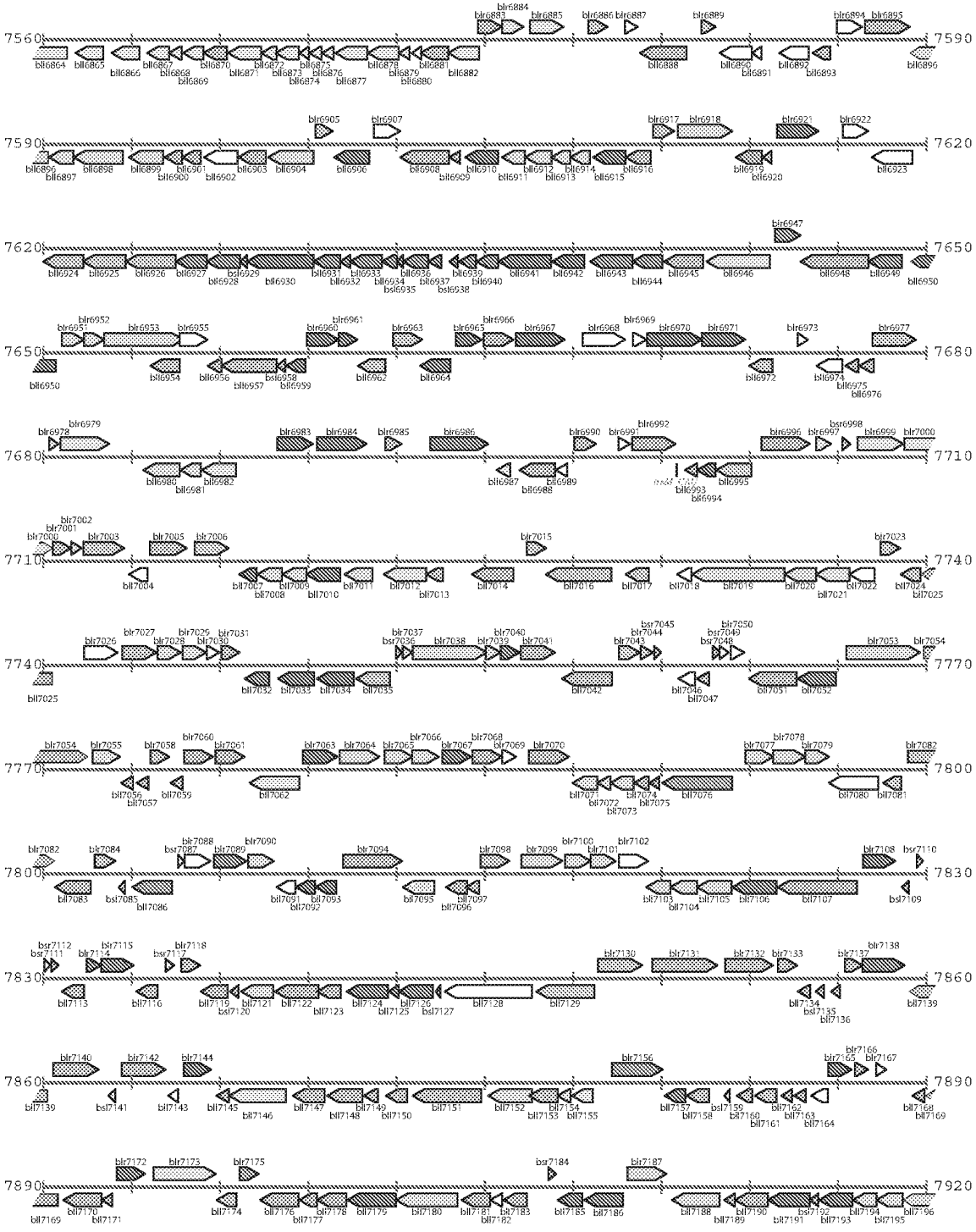
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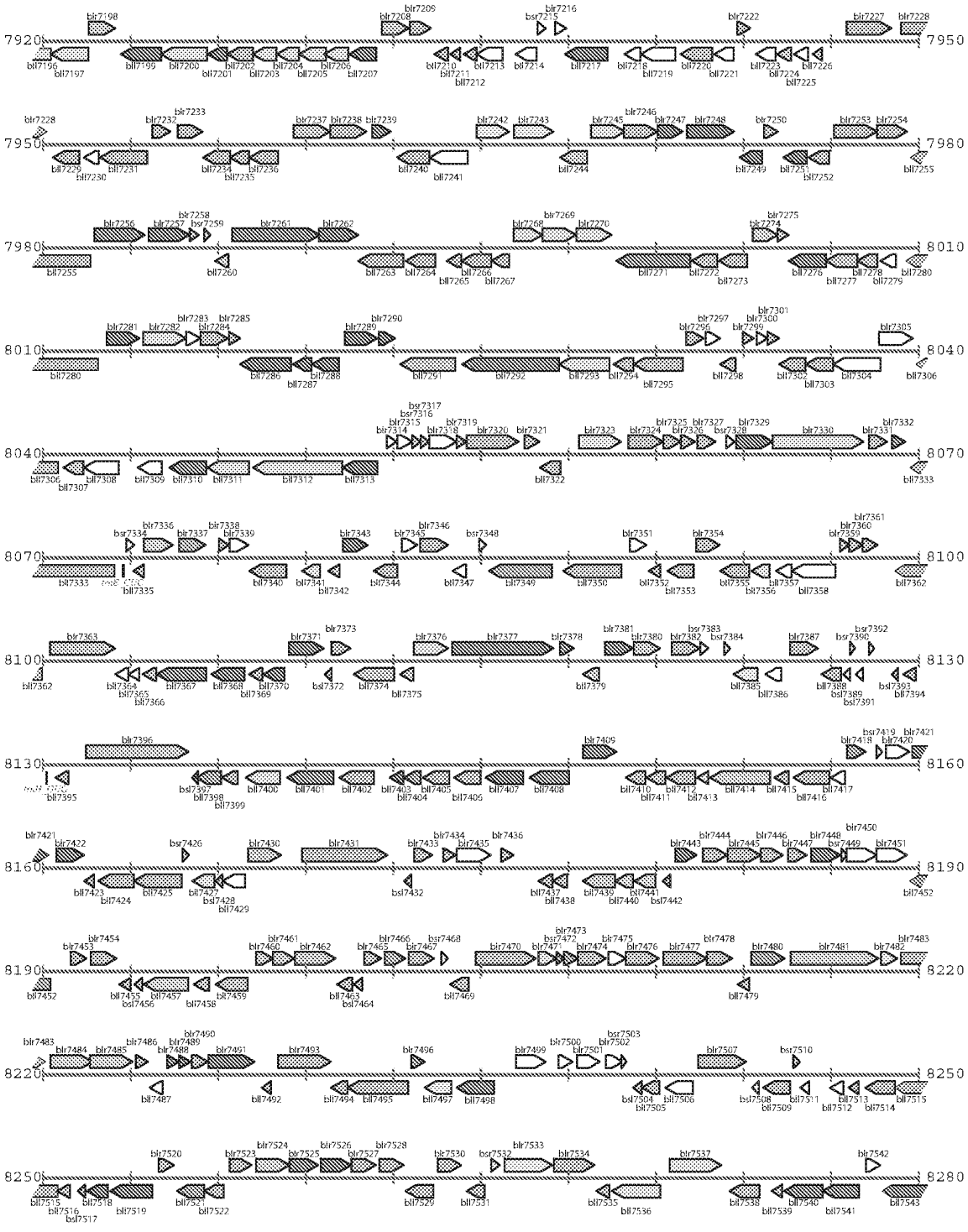
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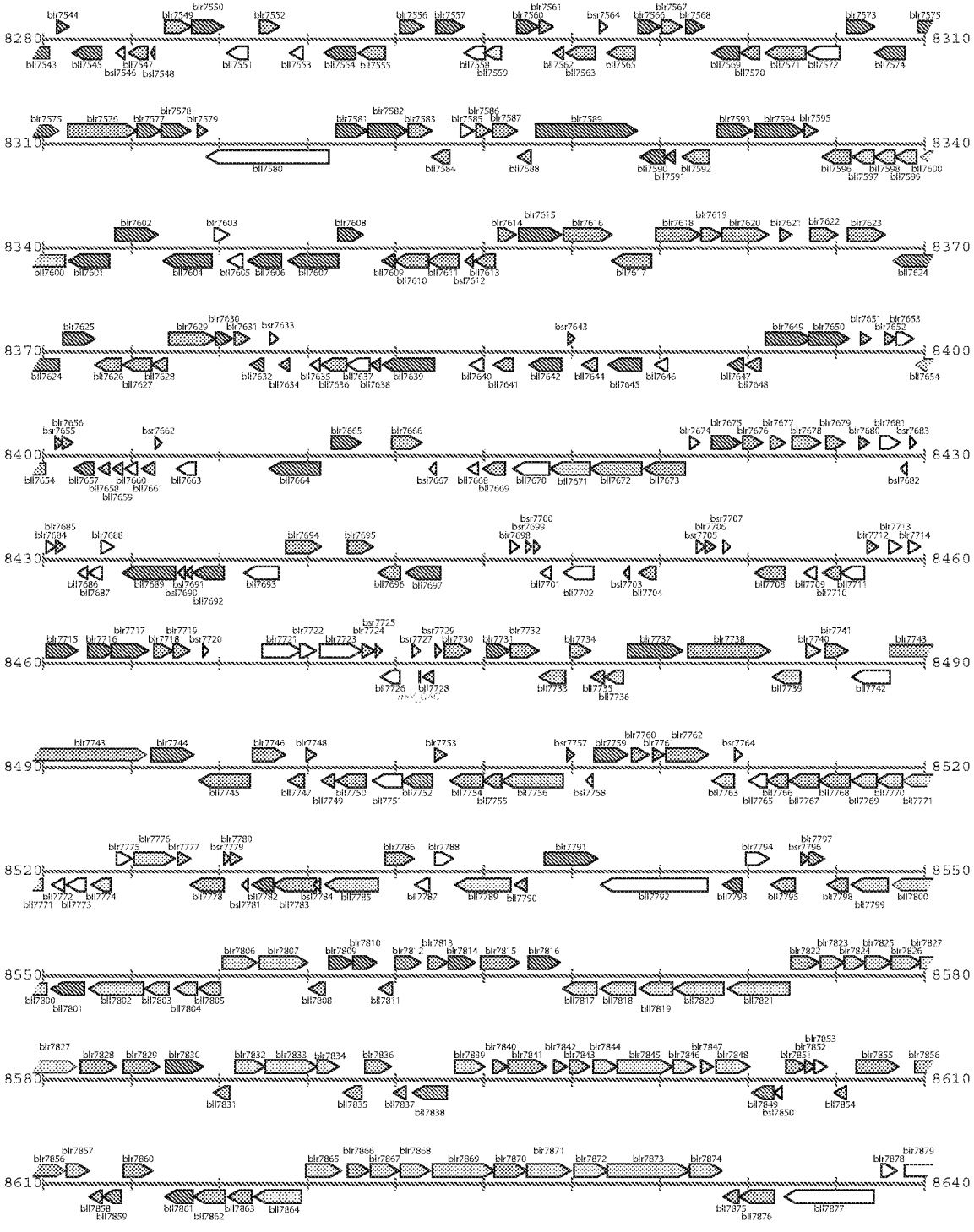
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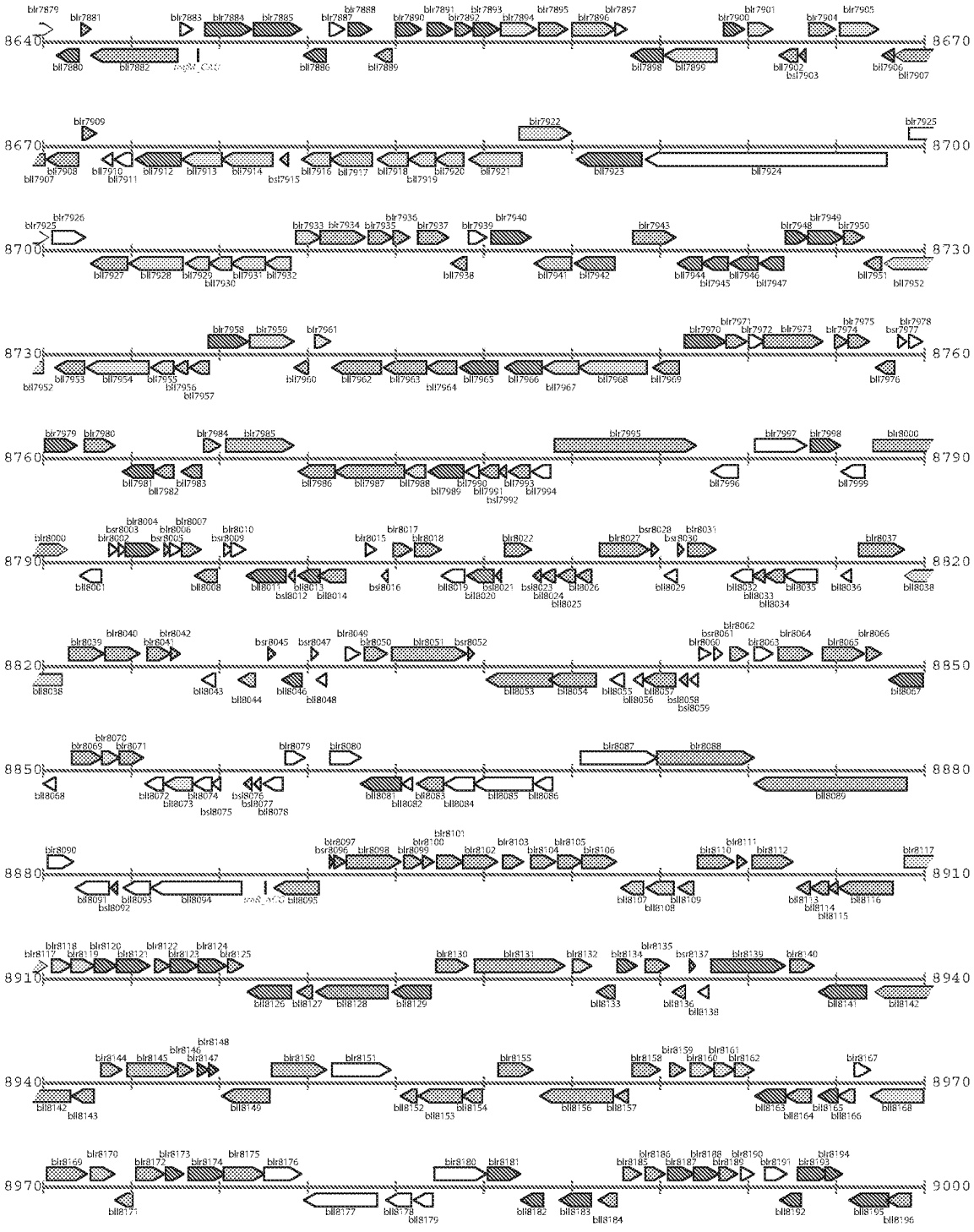
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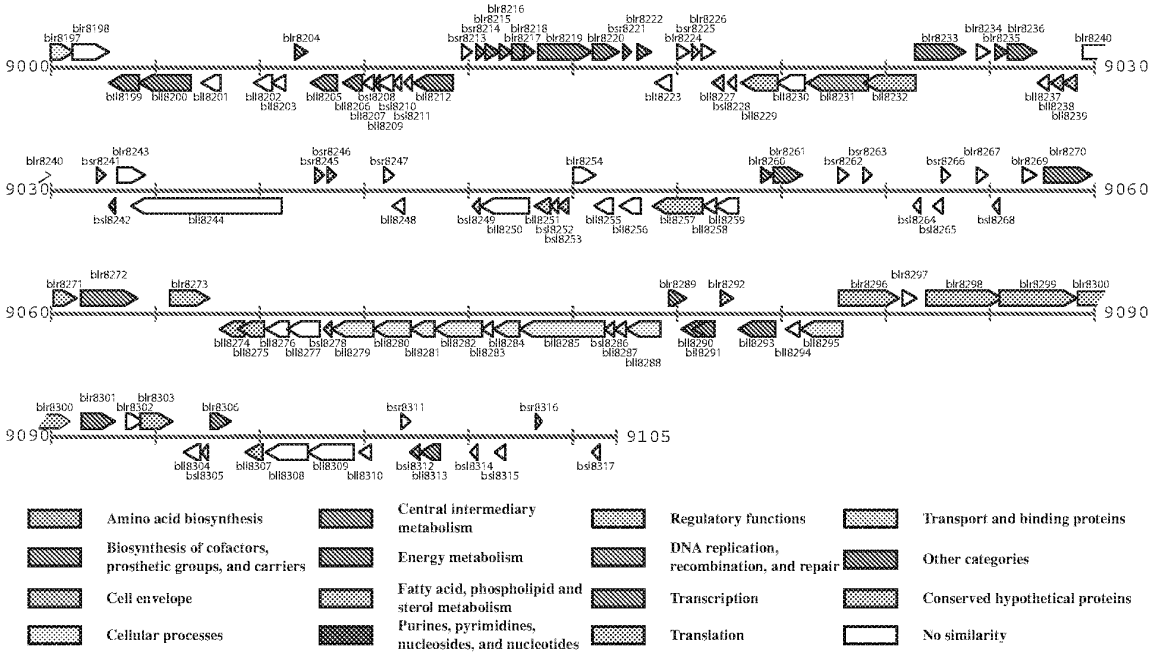


Figure 1. The gene map of the genome of *Bradyrhizobium japonicum* USDA110. The circular chromosome (9,105,828 bp) was opened at the *Pac* I recognition site, and is represented by a linear map starting from this junction (map position 0/100). Green bars show the scale in 3-kb increments with numerals in kb. On both sides of the scale, the potential protein-encoding genes assigned on the basis of computer prediction and the similarity search are shown as boxes with arrowheads indicating the reading direction. The detailed assignment procedures are described in the main article in this issue (pp. 189–pp. 197). The potential genes whose function could be evaluated by similarity search were classified into 14 functional categories, and are represented by different color codes. The RNA-encoding genes are represented by black bars and boxes. The sequences as well as the gene information shown in this paper are available in the Web database, RhizoBase, at http://www.kazusa.or.jp/rhizobase/. The sequence data analyzed in this study have been registered in DDBJ/GenBank/EMBL under accession number BA000040.

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Aminoacyl Stem	D domain stem	D domain loop	D domain stem	Anticodon domain stem	Anticodon domain loop	Anticodon domain stem	Variable region	TψC domain stem	TψC domain loop	TψC domain stem	Aminoacyl stem
trnR-CCU (122357-122281)											
GGTCCCG	TA GCTC	AGCCGGATA	GAGC G	GCGGT	TTCCTAA	ACCGT	AGGTC	GGATG	TTCGAGT	CATCC	CGGGATC G
trnS-GGA (333607-333518)											
GGAGAGA	TG GCC	GAGTGGCTTAA	GGC G	CACGG	TTGGAAA	GCGTG	TGTSCGGGAAACCGTACC	GTGGG	TTCGAAT	CCCAC	TCTCTCC G
trnG-CCA (451390-451361)											
GGAGAGG	TG GCA	GAGTGGTTGAA	TGC A	CCGCA	CTCGAAA	TGCGG	CATAGGTGCAAGCCTATC	GGGGG	TTCGAAT	CCCTC	CCTCTCC G
trnA-CGC (591251-591326)											
GGGGCCA	TA GCTC	AGCTGGGA	GAGC G	CGTCG	TTCGCAA	TGACG	AGGTC	GGCGG	TTCGATC	CGGGC	TGGCTCC A
trnI-CAU (604005-603930)											
GCCGCAA	TA GCTC	AGTCGGTA	GAGC A	CGTCA	TTCGTAA	TGACG	GGGTC	GGGGG	TTCGAAT	CCCTC	TTGCGGC A
trnG-GCC (640199-640125)											
GCGGGTG	TA GCTC	AGTGGTA	GAGC A	CGACC	TTGCCAA	GGTCG	GGGTC	GAGGG	TTCGAGC	CCCTT	CGCCCGC T
trnL-CAG (766675-766761)											
GCCCAGG	TG GTG	GAATTGGTAGA	CGC G	CTGGC	TTCAGGT	GCCAG	TGGCTTAACGGCCGT	GAAGG	TTCGAGT	CCTTT	CCTGGGC A
trnA-GGC (785224-785149)											
GGGGCCA	TA GCTC	AGCTGGGA	GAGC G	CTTGC	ATGGCAT	GCAAG	AGGTC	GGCGG	TTCGATC	CCGCC	TGGCTCC A
trnR-CCG (924007-923931)											
GCACCCG	TA GCTC	AGCTGGATA	GAGC G	TTGCC	CTCCGAA	GGCAA	AGGTC	ACACG	TTCGAAT	CGTGT	CGGGTGC G
trnG-CUC (1228026-1228099)											
GCGGGCG	TA GCTC	AATGGTA	GAAC G	GCAGC	TTCCCAA	GCTGC	ATAC	GAGGG	TTCGATT	CCCTT	CGCCCGC T
trnI-GAU (1363162-1363088)											
GCTGCCG	TA GCTC	AGTGGTA	GAGC A	CTCCA	TTGGTAA	TGGAG	AGGTC	GACAG	TTCAATC	CTGTC	TGGCAGC A
trnF-GAA (1374308-1374383)											
GCCCAGG	TA GCTC	AGTTGGTA	GAGC A	TGCGA	CTGAAAA	TCGCA	GTGTC	GGTGG	TTCGATT	CGGCC	CCTGGGC A
trnL-CAA (1456534-1456450)											
GCCCCTG	TA GCG	GAACTGGTAGA	CGC G	CTCGA	CTCAAAA	TCGAG	TTCCGCAAGGAGT	GCTGG	TTCGATT	CCGGC	CAGGGGC A
trnT-GAU (1529942-1530018)											
GGGCTTG	TA GCTC	AGTTGGTTA	GAGC G	CGCGC	TTGATAA	GCGTG	AGGTC	GGAAG	TTCAAGT	CTTCC	CAGGCCC A
trnA-UGC (1530052-1530127)											
GGGGCCA	TA GCTC	AGCTGGGA	GAGC G	CGTGC	TTTGCAA	GCAAG	AGGTC	GTCGG	TTCGATC	CCGTC	TGGCTCC A
trnI-CAU (1754724-1754649)											
GGGCCTG	TA GCTC	AATGGTTA	GAGC C	GGCCG	CTCATAA	CGGTC	TGGTT	GCAGG	TTCGAGT	CCTGC	CGGGCCC A
trnI-CAU (1833703-1833628)											
GGGCCTG	TA GCTC	AATGGTTA	GAGC C	GGCCG	CTCATAA	CGGTC	TGGTT	GCAGG	TTCGAGT	CCTGC	CGGGCCC A
trnV-CAC (2362416-2362342)											
GGGCGCA	TA GCTC	AGCGGGA	GAGC G	TTCTC	TTCACAC	GGGAG	AGGTC	CAAGG	TTCGATC	CCTTG	TGCGCCC A
trnI-CAU (2497176-2497101)											
GGGCCTG	TA GCTC	AATGGTTA	GAGC C	GGCCG	CTCATAA	CGGTC	TGGTT	GCAGG	TTCGAGT	CCTGC	CGGGCCC A
trnP-CGG (2812727-2812803)											
CGGAGTG	TG GCTC	AGCCGGTA	GAGC A	CTGCG	TTCGGGA	CGCAG	GGGTC	GCAGG	TTCGAAT	CCTGC	CACTCCG A
trnQ-UUG (2907070-2907144)											
TGGGGCG	TA GCC	AAGCGGTAA	GGC A	GCGGA	TTTTGAT	TCCGC	CATTC	GGAGG	TTCGATC	CCTCC	CGCCCCA G
trnQ-CUG (4317579-4317652)											
TGGGGAA	TG CTCT	AACTGCA	GCAC A	ACAGA	CTCTGAC	TCTGT	TTGT	CTTGG	TTCGAAT	CCAGG	TTCCCCA G
trnE-UUC (4859741-4859667)											
GCTCCCC	TC GTCT	ATCGTTA	GGAC G	CCACC	CTTTCAC	GGTGG	AGAG	AGCGG	TTCGATT	CCGCT	AGGGAGC G
trnP-UGG (4872412-4872488)											
CGGGGTA	TA GCGC	AGCCTGGTA	GCGC G	GCAGT	TTTGGGT	ACTGC	AGGTC	GTTGG	TTCGAAT	CCAGC	TGCCCCG A
trnR-UCU (4897125-4897049)											
GGTCCCA	TA GCTC	AACTGGATA	GAGT A	GCGGA	TTTCTAC	TCCGC	GGGTT	GCAGG	TTCGAGT	CCTGC	TGGGATC G
trnC-GCA (4936093-4936166)											
GGCCACG	TG GCG	GAGTGGTTA	CGC GC	CGGT	CTGCAAA	ACCG	TTTAC	TCGGG	TTCGAGT	CCGGA	CGTGGCC T
trnN-GUU (4950144-4950218)											
TCCTCGG	TA GCTC	AGCGGTA	GAGC A	TCCGA	CTGTTAA	TCGGA	TGGTC	GCTGG	TTCGAAT	CCAGC	CCGGGGA G
trnS-UGA (5014774-5014665)											
GGAAGGG	TG GCC	GAGTGGTTTAA	GGC A	CCGGT	CTTGAAA	ACCGG	CGTGCCTGCAAGGGTACC	GTGGG	TTCGAAT	CCCAC	CCCTTCC G
trnX-UUU (5113708-5113783)											
GAGCGCG	TA GCTC	AGCCGGTA	GAGC A	CGTGA	CTTTTAA	TCACG	GGGTC	CTGGG	TTCGAGC	CCCAG	CGCGCTC A
trnD-GUC (5460155-5460079)											
GCGGGAG	TA GCTC	AGTTGGTTA	GAGC G	CCGGC	CTGTCAC	GCCGG	AGGTC	GCGGG	TTCGAGC	CCGGT	CTCTCGC G
trnV-UAC (5480560-5480484)											
GGGCGGT	TA GCTC	AGCTGGTTA	GAGC A	TCTCG	TTTACAC	CGAGA	GGGTC	CGCGG	TTCGAAT	CCGYG	ACCGCCC A
trnL-UAG (5488101-5488017)											
GCGGGCG	TG GCG	GAACTGGTAGA	CGC G	CTGGA	TTTAGGT	TCCAG	TGACGAAAGTTGT	GGGGG	TTCGAGT	CCCTC	CGCCCGC A
trnP-GGG (5565170-5565093)											
CGGAGCG	TG GCGC	AGCCCGGTTA	GCGC A	CTAGT	CTGGGAG	ACTAG	GGGTC	GGAGG	TTCAAAT	CCTCT	CGCTCCG A
trnS-GCU (5640958-5641050)											
GGAGAGG	TG GCC	GAGTGCCTGAA	GGC A	ACGCT	TTGCTAA	AGCGT	CATACGGTCTCAAGCTGTATC	GAGGG	TTCGAAT	CCCTC	CCTCTCC G
trnI-CAG (5805593-5805509)											
GCGCTCG	TG GCG	GAACTGGTAGA	CGC G	CTGCC	TTGAGGT	GGCAG	TGAGTAAAATCGT	GGGGG	TTCGAGT	CCCTC	CGAGCGC A
trnR-UCG (5872973-5872897)											
GCGCTCG	TA GCTC	AGCTGGATA	GAGC A	TCGGA	TTTCGAT	TCGGA	GGGTC	GGAGG	TTCGAAT	CCTTC	CGAGCGC G
trnW-CCA (5983963-5983888)											
AGGAGTG	TA GCTC	AATTGGTA	GAGC A	CCGGT	CTCCAAA	ACCGG	GGGTC	GCAGG	TTCGAGC	CCTGC	CACTCCT G
trnG-UCC (6046825-6046752)											
GCGGGTG	TA GCTC	AATGGTA	GAGC AG	CAGC	CTTCCAA	GCTG	AATAC	GAGGG	TTCGATT	CCCTT	CACCCGC T
trnT-GUA (6059945-6059860)											
GGGGGAG	TG TCCC	GAGTGGCAAA	GGG A	GCTGA	CTGTAAA	TCAGC	CGCCTCATGGCTTC	GCAGG	TTCGAGT	CCTGC	CTCCCCC A
trnT-UGU (6063033-6063107)											
GCCGGCT	TA GCTC	AGCGGTA	GAGC AG	CGGT	TTTGTAA	ACCG	AAGGTC	GGGGG	TTCAATC	CCCTC	AGCCGGC A
trnL-UAA (6149083-6149171)											
GCGGGCA	TG GCG	GAATTGGTAGA	CGC A	AGGGA	CTTAAAA	TCCCT	CGGTGCGCAAGCGCTGT	GCCGG	TTCGACC	CCGGG	TGCCCGC A
trnN-GUU (6191320-6191247)											
CAAGGGA	TA GCTC	AGTTGGTTA	GAGC A	GATGA	CTGTTAA	TCATC	TTGTC	GCGGG	TTCGATT	CCGGC	TCCATCG C
trnF-GAA (6191390-6191318)											
GCAGGACG	TA GCTC	ATGGA	GAGC G	TCGCG	CTGAACG	CCCGA	AGGC	ATCGG	TTCGATT	CCGGT	CTCTGGC A
trnS-CUU (7081599-7081524)											
GGGCGCA	TA GCTC	AGTTGGTA	GAGC A	GCTGA	CTCTTAA	TCAGC	GGGTC	CCAGG	TTCGAGC	CCTGG	TGCGCCC A
trnH-GAU (7701576-7701500)											
GGCGGGG	TA GCTC	AGCTGGTTA	GAGC A	CGGGA	ATCATAA	TCCTG	GGGTC	GGGGG	TTCGAGT	CCCTC	TCCCGCT A
trnM-CAU (8072813-8072739)											
GCTCCCT	TC GTCT	AGCGGTTA	GGAC G	CGGCC	CTCTCAA	GGCTG	AAAC	AGGGG	TTCGATT	CCCGT	AGGGAGC G
trnA-GUG (8130188-8130112)											
GCAGTTG	TA GCTC	AGTTGGTTA	GAGC G	CCTGT	CTGTGGA	ACAGG	AGGTC	GGTGG	TTCGAGC	CCACC	CAACTGT A
trnV-GAC (8472878-8472804)											
GGGCACG	TA GCTC	AGCGGGA	GAGC A	CTACC	TTGACAT	GGTAG	GGGTC	ACAGG	TTCGATC	CCTGT	CGTGCCC A
trnEM-CAU (8645335-8645259)											
CGCGGGG	TG GAGC	AGCCCGGTA	GCTC G	TCAGG	CTCATAA	CCTGA	AGGTC	ATAGG	TTCAAAT	CCTAT	CCCCGCA A
trnR-ACG (8887625-8887549)											
GCGCCCG	TA GCTC	AGCTGGATA	GAGC A	TCAGA	CTACGAA	TCTGA	GGGTC	GGACG	TTCGAAT	CGTTC	CGGGCGC G

Figure 2. Structure of the tRNA genes in the genome of *B. japonicum*. The nucleotide sequences, nucleotide positions and the structural domains for the 50 tRNA genes in the genome are tabulated.

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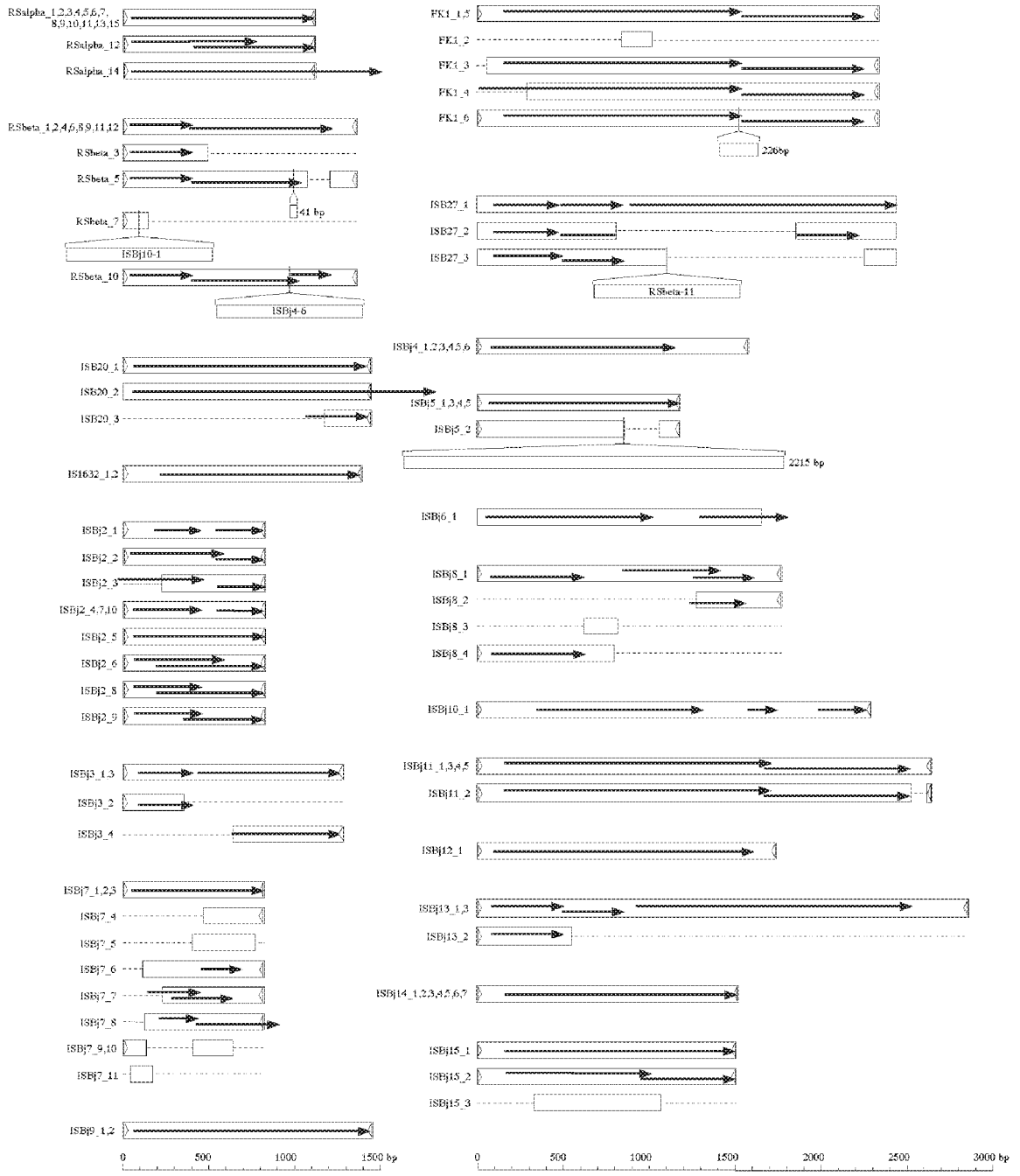


Figure 3. Structure of insertion sequences. The structures of insertion sequences found in the *B. japonicum* genome are depicted as open boxes. Horizontal broken lines show the deleted regions. Open triangles at the ends of the open boxes represent the terminal inverted repeats. Horizontal arrows indicate the positions of genes for putative transposases. The positions of insertions are indicated as vertical bars with open boxes.

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Table 1. Codon usage frequency for the *B. japonicum* genome. The frequency of the usage of each codon in the chromosome is shown as permillage of overall counts. Numerals in parentheses indicate the actual number of the codons.

UUU	F	6.5	(17263)	UCU	S	2.0	(5317)	UAU	Y	10.2	(27071)	UGU	C	1.3	(3537)
UUC	F	30.7	(81106)	UCC	S	12.8	(33935)	UAC	Y	11.8	(31127)	UGC	C	7.8	(20715)
UUA	L	0.7	(1875)	UCA	S	2.8	(7526)	UAA	*	0.5	(1340)	UGA	*	1.8	(4730)
UUG	L	8.5	(22439)	UCG	S	21.4	(56669)	UAG	*	0.9	(2247)	UGG	W	13.1	(34723)
CUU	L	7.8	(20633)	CCU	P	3.7	(9855)	CAU	H	9.2	(24305)	CGU	R	6.6	(17570)
CUC	L	34.5	(91273)	CCC	P	14.1	(37353)	CAC	H	11.4	(30119)	CGC	R	39.8	(105051)
CUA	L	1.6	(4240)	CCA	P	3.4	(8930)	CAA	Q	5.7	(15151)	CGA	R	4.2	(11080)
CUG	L	45.2	(119368)	CCG	P	31.2	(82485)	CAG	Q	26.1	(68871)	CGG	R	15.2	(40248)
AUU	I	6.3	(16538)	ACU	T	2.5	(6558)	AAU	N	8.6	(22608)	AGU	S	2.2	(5824)
AUC	I	44.8	(118461)	ACC	T	28.0	(73936)	AAC	N	18.9	(50070)	AGC	S	16.6	(43933)
AUA	I	1.4	(3660)	ACA	T	3.2	(8533)	AAA	K	5.9	(15570)	AGA	R	1.7	(4427)
AUG	M	23.8	(62797)	ACG	T	20.4	(53809)	AAG	K	30.2	(79816)	AGG	R	4.3	(11479)
GUU	V	6.1	(16052)	GCU	A	7.6	(20128)	GAU	D	18.3	(48252)	GGU	G	8.3	(22013)
GUC	V	37.3	(98480)	GCC	A	52.3	(138210)	GAC	D	36.2	(95729)	GGC	G	58.2	(153914)
GUA	V	2.0	(5388)	GCA	A	10.8	(28623)	GAA	E	15.6	(41303)	GGA	G	7.1	(18733)
GUG	V	29.3	(77305)	GCG	A	53.0	(139975)	GAG	E	36.8	(97131)	GGG	G	9.6	(25256)

Table 2. Codon-anticodon recognition pattern for the *B. japonicum* genome. Corresponding amino acids and tRNA species are given as a one-letter abbreviation and italic letters. Numerals in parentheses indicate the number of the genes on the chromosome.

UUU F	*tmF-GAA*(2)	UCU S	*tmS-GGA*	UAU Y	*tmY-GUA*	UGU C	*tmC-GCA*
UUC F		UCC S		UAC Y		UGC C	
UUA L	*tmL-UAA*	UCA S	*tmS-UGA*	UAA -		UGA -	
UUG L	*tmL-CAA*	UCG S	*tmS-CGA*	UAG -		UGG W	*tmW-CCA*
CUU L	*tmL-GAG*	CCU P	*tmP-GGG*	CAU H	*tmH-GUG*	CGU R	*tmR-ACG*
CUC L		CCC P		CAC H		CGC R	
CUA L	*tmL-UAG*	CCA P	*tmP-UGG*	CAA Q	*tmQ-UUG*	CGA R	*tmR-UCG*
CUG L	*tmL-CAG*	CCG P	*tmP-CGG*	CAG Q	*tmQ-CUG*	CGG R	*tmR-CCG*
AUU I	*tmI-GAU*	ACU T	*tmT-GGU*	AAU N	*tmN-GUU*(2)	AGU S	*tmS-GCU*
AUC I		ACC T		AAC N		AGC S	
AUA I	*tmI-CAU* (3)	ACA T	*tmT-UGU*	AAA K	*tmK-UUU*	AGA R	*tmR-UCU*
AUG M	*tmM-CAU*	ACG T	*tmT-CGU*	AAG K	*tmK-CUU*	AGG R	*tmR-CCU*
	tmfM-CAU						
GUU V	*tmV-GAC*	GCU A	*tmA-GGC*	GAU D	*tmD-GUC*	GGU G	*tmG-GCC*
GUC V		GCC A		GAC D		GGC G	
GUA V	*tmV-UAC*	GCA A	*tmA-UGC*	GAA E	*tmE-UUC*	GGA G	*tmG-UCC*
GUG V	*tmV-CAC*	GCG A	*tmA-CGC*	GAG E	*tmE-CUC*	GGG G	*tmG-CCC*

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Table 3. List of insertion sequences.

name	length (bp)	direction	position
RSα1	1116	>>	1860456 to 1861571
RSα2	1116	>>	1883172 to 1884287
RSα3	1116	>>	1904907 to 1906022
RSα4	1116	>>	1962375 to 1963490
RSα5	1116	>>	1979002 to 1980117
RSα6	1116	>>	2066180 to 2067295
RSα7	1116	>>	2244775 to 2245890
RSα8	1116	>>	2338099 to 2339214
RSα9	1116	<<	4666443 to 4667558
RSα10	1116	<<	5141398 to 5142513
RSα11	1116	>>	5397253 to 5398368
RSα12	1117	<<	6289836 to 6290952
RSα13	1116	>>	8285049 to 8286164
RSα14	1114	>>	9024824 to 9025937
RSα15	1116	<<	9079784 to 9080899
RSβ1	1364	>>	14651 to 16014
RSβ2	1364	<<	1764678 to 1766041
RSβ3	495	>>	1845813 to 1846307
RSβ4	1364	>>	1897147 to 1898510
RSβ5	1232	<<	1906041 to 1906198 ···join··· 1906200 to 1906281 ···join··· 1906323 to 1907314
RSβ6	1364	>>	2079936 to 2081299
RSβ7	148	>>	2102124 to 2102213 ···join··· 2104519 to 2104576
RSβ8	1364	<<	2292940 to 2294303
RSβ9	1364	<<	8330204 to 8331567
RSβ10	1363	>>	9012887 to 9013858 ···join··· 9016459 to 9016849
RSβ11	1364	>>	9027142 to 9028505
RSβ12	1364	>>	9050401 to 9051764
FK1_1	2343	<<	1801035 to 1803377
FK1_2	175	>>	1814969 to 1815143
FK1_3	2284	>>	1846314 to 1848597
FK1_4	2051	<<	1874302 to 1876352
FK1_5	2342	>>	1984494 to 1986835
FK1_6	2366	>>	9013859 to 9015407 ···join··· 9015634 to 9016450
IS1632_1	1395	<<	1937403 to 1938797
IS1632_2	1395	>>	1876354 to 1877748
ISB20_1	1447	<<	1806977 to 1808423
ISB20_2	1437	>>	2071254 to 2072690
ISB20_3	273	>>	9007146 to 9007418
ISB27_1	2440	>>	1856563 to 1859002
ISB27_2	1384	>>	2019241 to 2020624
ISB27_3	1286	<<	9026963 to 9027141 ···join··· 9028506 to 9029612
ISBj2_1	832	<<	1754822 to 1755653
ISBj2_2	830	>>	1806143 to 1806972
ISBj2_3	617	>>	1819675 to 1820291
ISBj2_4	832	>>	1839309 to 1840140
ISBj2_5	830	>>	1884292 to 1885121
ISBj2_6	831	<<	1974704 to 1975534
ISBj2_7	832	>>	2027184 to 2028015
ISBj2_8	828	>>	2068499 to 2069326
ISBj2_9	831	>>	2113280 to 2114110
ISBj2_10	832	>>	4286526 to 4287357
ISBj3_1	1284	>>	1683969 to 1685252
ISBj3_2	356	<<	1837736 to 1838091
ISBj3_3	1284	>>	4320931 to 4322214
ISBj3_4	645	<<	4322817 to 4323461
ISBj4_1	1585	<<	5122910 to 5124494
ISBj4_2	1584	>>	5747864 to 5749447
ISBj4_3	1587	>>	6668584 to 6670170
ISBj4_4	1585	<<	8289146 to 8290730
ISBj4_5	1585	>>	8460055 to 8461639
ISBj4_6	1586	<<	8987162 to 8988747
ISBj5_1	1183	>>	21909 to 23091
ISBj5_2	978	>>	1975539 to 1976396 ···join··· 1978610 to 1978729
ISBj5_3	1183	>>	2702540 to 2703722
ISBj5_4	1183	>>	8293173 to 8294355
ISBj5_5	1183	>>	8985099 to 8986281
ISBj6_1	1656	>>	9090881 to 9092536
ISBj7_1	813	>>	1812994 to 1813807
ISBj7_2	823	<<	1834493 to 1835316
ISBj7_3	815	<<	1841201 to 1842016
ISBj7_4	365	>>	1863302 to 1863667
ISBj7_5	507	>>	2057308 to 2057815
ISBj7_6	707	<<	2063237 to 2063944
ISBj7_7	592	>>	2126971 to 2127563
ISBj7_8	707	<<	9009467 to 9010174
ISBj7_9	371	>>	1854166 to 1854304 ···join··· 1854304 to 1854537
ISBj7_10	371	<<	9031638 to 9031871 ···join··· 9031871 to 9032009
ISBj7_11	130	<<	2061993 to 2062123
ISBj8_1	1776	<<	2014819 to 2016594
ISBj8_2	496	>>	2329929 to 2330424
ISBj8_3	195	>>	9006817 to 9007011
ISBj8_4	806	<<	9004162 to 9004967
ISBj9_1	1454	>>	9058516 to 9059969
ISBj9_2	1454	<<	5740825 to 5742278
ISBj10_1	2298	<<	2102214 to 2104511
ISBj11_1	2649	>>	1870866 to 1873514
ISBj11_2	2555	<<	2051930 to 2051956 ···join··· 2053700 to 2056228
ISBj11_3	2649	<<	2155260 to 2157908
ISBj11_4	2649	<<	3891046 to 3893694
ISBj11_5	2649	<<	5543048 to 5545696
ISBj12_1	1743	<<	2051957 to 2053699
ISBj13_1	2865	>>	1940519 to 1943383
ISBj13_2	555	<<	2314664 to 2315218
ISBj13_3	2865	<<	2331891 to 2334755
ISBj14_1	1524	<<	2118632 to 2120155
ISBj14_2	1524	<<	2161005 to 2162528
ISBj14_3	1524	>>	2202550 to 2204073
ISBj14_4	1524	>>	3958041 to 3959564
ISBj14_5	1524	<<	5146231 to 5147754
ISBj14_6	1524	<<	7370865 to 7372388
ISBj14_7	1524	>>	8715111 to 8716634
ISBj15_1	1509	<<	1982676 to 1984184
ISBj15_2	1507	<<	2131756 to 2133262
ISBj15_3	738	<<	8995098 to 8995835

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Table 4. Structural features of insertion sequences.

name	size (bp)	inverted repeat (bp)	direct repeat (bp)	family	number of full-length copies	number of partial copies
RSα	1116	5	4[a]	IS630 family	15	0
RSβ	1364	13	3	IS3 family	9	3
FK1	2342	48	6 or 8	IS21 family	3	3
IS1632	1395	23	4 or 9	IS256 family	2	0
ISB20	1447	15	4[a]	IS4 family	1	2
ISB27	2440	0	8	IS66 family	1	2
ISBj2	832	4	4[a]	IS3 family	9	1
ISBj3	1284	26	3	IS3 family	2	2
ISBj4	1585	25	2[b]	IS110 family	6	0
ISBj5	1183	3	4[a]	IS630 family	4	1
ISBj6	1656	0	2	unknown	1	0
ISBj7	823	16	0	IS6 family	3	8
ISBj8	1776	14	4[a]	IS1380 family	1	3
ISBj9	1454	15	4[a]	IS4 family	2	0
ISBj10	2298	13	7	IS21 family	1	0
ISBj11	2649	25	6 or 7	IS21 family	4	1
ISBj12	1743	10	0	unknown	1	0
ISBj13	2865	28	8	IS66 family	2	1
ISBj14	1524	16	6	IS5 family	7	0
ISBj15	1509	16	6	IS5 family	2	1

[a]These recognized "CTAG" as the target sequence, except for RSα9, RSα12, ISBj9-1. [b]All of couple of bases was "TC."

Table 5. List of DNA elements inserted in tRNA genes.

name	length (bp)	GC contents	position	duplication (bp)	tRNA gene
trnR1 element	30529	56.5%	924008 to 954536	40[a]	*trnR*-CCG[c]
trnF1 element	9063	54.7%	1374384 to 1383446	17	*trnF*-GAA
trnI1 element	51727	59.5%	1702922 to 1754648	49	*trnI*-CAU copy3[c]
trnI2 element	23912	59.4%	2473189 to 2497100	49	*trnI*-CAU copy1[c]
trnQ1 element	6144	59.5%	4317653 to 4323796	17	*trnQ*-CUG
trnS1 element	8796	60.3%	5014775 to 5023570	32	*trnS*-UGA
trnK1 element	48532	60.2%	5113784 to 5162315	45	*trnK*-UUU
trnP1 element	17798	57.1%	5547295 to 5565092	45	*trnP*-GGG
trnR2 element	4214	59.5%	5868683 to 5872896	48	*trnR*-UCG
trnK2 element	34462	61.1%	7047062 to 7081523	45[b]	*trnK*-CUU
trnK3 element	52391	62.5%	6994626 to 7047016	45	*trnK*-CUU
trnK4 element	31318	62.1%	6963263 to 6994580	45[b]	*trnK*-CUU
trnM1 element	81959	62.4%	7619541 to 7701499	16	*trnM*-CAU
trnR3 element	96530	62.6%	8791019 to 8887548	51	*trnR*-ACG

[a]The duplication stretched more 43 bp upstream of gene. [b]The patterns contained 1 base of mismatch either duplication. [c]The elements occurred to duplicate at 5′-end of tRNA genes.

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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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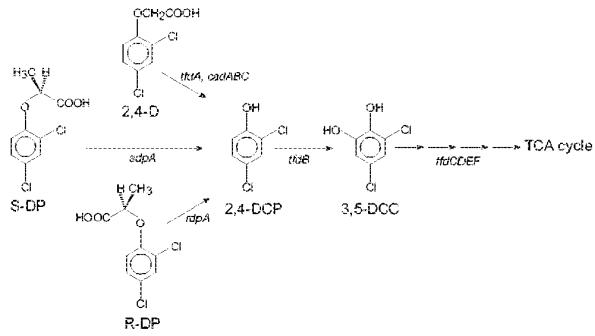


FIG. 1. Modified *ortho*-pathway for the degradation of chlorinated phenoxyalkanoate herbicides. 2,4-D, 2,4-dichlorophenoxyacetate; *R*-DP and *S*-DP, *R*- and *S*-enantiomers of 2(2,4-dichlorophenoxy)propionate, respectively; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; TCA, tricarboxylate.

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 etc aaA TGC ATG CTG CAC TGT CCC CC-3′; lowercase letters denote a linker sequence) and rdp_rev (5′-atg gta ggt etc age get 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 ATC AC-3′	
sdp_fT[c]	5′-ATG CAR ACI ACI CTG CAR ATT 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*91* 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.

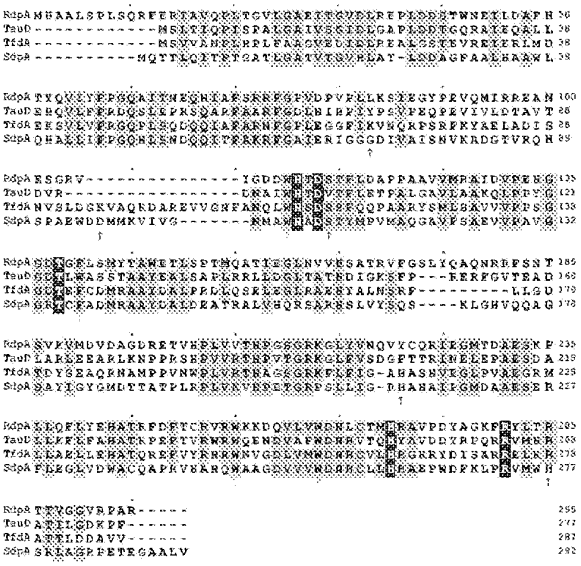


FIG. 2. Sequence alignment of RdpA and SdpA with other group II αKG-dependent dioxygenases. TfdA, *R. eutropha* 2,4-D/αKG dioxygenase (accession no. A27082); TauD, *P. aeruginosa* taurine/αKG dioxygenase (accession no. NP_252624). Conserved Fe(II)- and αKG-binding residues are shaded in black; other 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%.

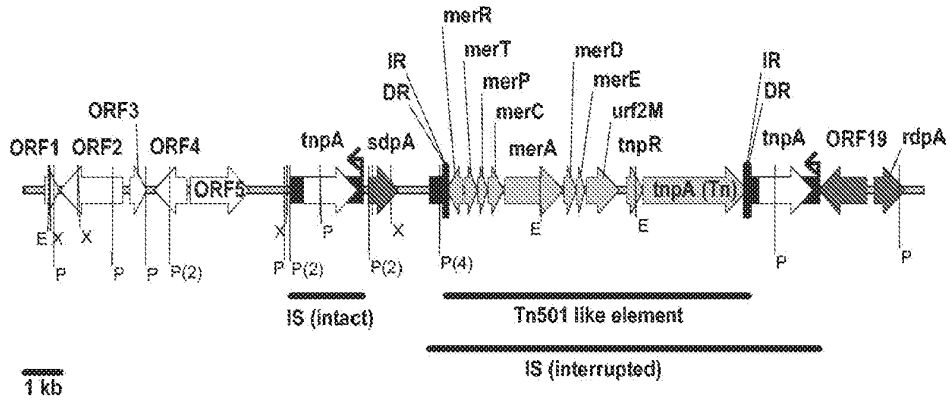
The amino acid sequences of both dioxygenases were analyzed on the basis of the alignment shown in Fig. 2. It shows that an internal sequence tentatively aligned to positions 257 to 287 of TfdA by Westendorf et al. (57), comprises positions 85 to 131 of RdpA.

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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A



B

IS*91* family: AnnnATAGGAAnTTnAAnnnn

putative IS*91*
element strain MC1: ccagATAGGAAaTTcAAccgc

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 *sdpA*-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 $HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R/K$, 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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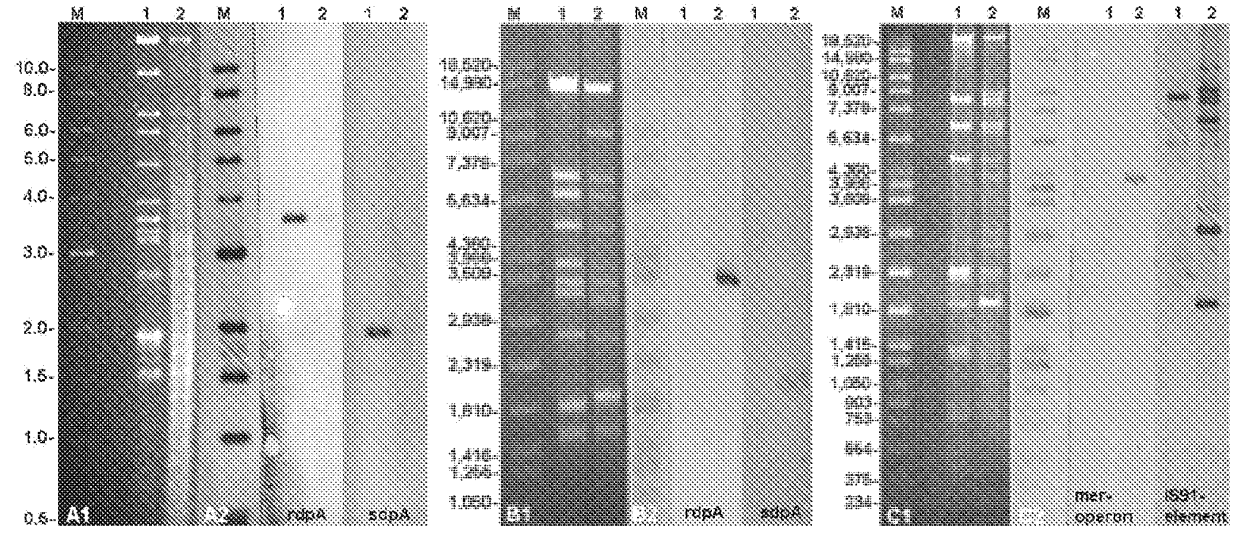


FIG. 4. Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of plasmid pMC1 with pMC1-DP$^-$, the plasmid of mutant MC1-DP$^-$. (A) Localization of *rdpA* and *sdpA* within the genome of strain MC1. Plasmid DNA (lane 1) and genomic DNA (lane 2) was digested with 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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Genetic Analysis of Phenoxyalkanoic Acid Degradation in *Sphingomonas herbicidovorans* MH

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Phenoxyalkanoic acid degradation is well studied in *Beta-* and *Gammaproteobacteria*, but the genetic background has not been elucidated so far in *Alphaproteobacteria*. We report the isolation of several genes involved in dichlor- and mecoprop degradation from the alphaproteobacterium *Sphingomonas herbicidovorans* MH and propose that the degradation proceeds analogously to that previously reported for 2,4-dichlorophenoxyacetic acid (2,4-D). Two genes for α-ketoglutarate-dependent dioxygenases, $sdpA_{MH}$ and $rdpA_{MH}$, were found, both of which were adjacent to sequences with potential insertion elements. Furthermore, a gene for a dichlorophenol hydroxylase (*tfdB*), a putative regulatory gene (*cadR*), two genes for dichlorocatechol 1,2-dioxygenases ($dccA_{I/II}$), two for dienelactone hydrolases ($dccD_{I/II}$), part of a gene for maleylacetate reductase (*dccE*), and one gene for a potential phenoxyalkanoic acid permease were isolated. In contrast to other 2,4-D degraders, the *sdp*, *rdp*, and *dcc* genes were scattered over the genome and their expression was not tightly regulated. No coherent pattern was derived on the possible origin of the *sdp*, *rdp*, and *dcc* pathway genes. $rdpA_{MH}$ was 99% identical to $rdpA_{MC1}$, an (*R*)-dichlorprop/α-ketoglutarate dioxygenase from *Delftia acidovorans* MC1, which is evidence for a recent gene exchange between *Alpha-* and *Betaproteobacteria*. Conversely, $DccA_I$ and $DccA_{II}$ did not group within the known chlorocatechol 1,2-dioxygenases, but formed a separate branch in clustering analysis. This suggests a different reservoir and reduced transfer for the genes of the modified *ortho*-cleavage pathway in *Alphaproteobacteria* compared with the ones in *Beta-* and *Gammaproteobacteria*.

Phenoxyalkanoic acid herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), chiral mecoprop [(*R,S*)-2-(4-chloro-2-methyl-phenoxypropanoic acid)], and chiral dichlorprop [(*R,S*)-2-(2,4-dichlorophenoxypropanoic acid)] (Fig. 1) are widely used against broadleaved weeds in agriculture, lawn pastures, and industries. They were introduced in large amounts into the environment in the 1940s and 1950s (1, 10, 57). 2,4-D, mecoprop, and dichlorprop are synthetic compounds and have only been applied for some decades. However, microorganisms able to use them as the sole carbon and energy source have been isolated from different environments (21, 36, 52, 53, 60). 2,4-D-degrading strains from the *Beta-* and *Gammaproteobacteria* groups mostly harbor similar genes for 2,4-D degradation (17, 24, 34, 50, 51), which supports that frequent gene exchange has occurred and hence their adaptation and wide distribution (17).

In many cases, the first step in the degradation of phenoxyalkanoic acids is the enzymatic conversion of the acids to the corresponding phenols, then via the catechols, and through the modified *ortho*-cleavage pathway (21, 43). The best-studied degradation pathway is that of 2,4-D in the soil bacterium *Ralstonia eutropha* JMP134(pJP4) (Fig. 1). Both the genetic and the enzymatic details of the pathway in this bacterium have been established. *R. eutropha* JMP134(pJP4) degrades 2,4-D by an α-ketoglutarate-dependent dioxygenase that cleaves the ether bond to produce 2,4-dichlorophenol (15, 16, 45). 2,4-Dichlorophenol in turn is hydroxylated to 3,5-dichlorocatechol by a phenol-hydroxylase ($TfdB_{I/II}$) (13, 29, 40). 3,5-Dichlorocatechol undergoes *ortho*-ring fission to 2,4-dichloro-*cis,cis*-muconate, a reaction catalyzed by chlorocatechol 1,2-dioxygenase ($TfdC_{I/II}$) (29, 39–41). 2,4-Dichloro-*cis,cis*-muconate is then converted to *cis*-2-chlorodienelactone and further to 2-chloromaleylacetate by chloromuconate cycloisomerase ($TfdD_{I/II}$) and dienelactone hydrolase activity ($TfdE_{I/II}$), respectively (27, 40). Finally, chloromaleylacetate is reduced to 3-oxoadipate via maleylacetate by maleylacetate reductase ($TfdF_{I/II}$) (40, 41). Tett et al. (48) showed that (*R*)-mecoprop degradation in *Alcaligenes denitrificans* proceeds analogously to 2,4-D degradation in *R. eutropha* JMP134(pJP4). Similarly, phenol hydroxylase, chlorocatechol 1,2-dioxygenase, and chloromuconate cycloisomerase activities are induced in *Delftia acidovorans* MC1 upon exposure to dichlorprop. This suggests that dichlorprop is also metabolized through a modified *ortho*-cleavage pathway (6).

Sphingomonas herbicidovorans MH is a versatile phenoxyalkanoic acid degrader and grows on mecoprop and dichlorprop as the sole carbon and energy sources (22, 26, 60). Although strain MH metabolizes both enantiomers, it preferentially uses the (*S*) enantiomer. Nickel et al. (38) showed that *S. herbicidovorans* MH harbors two distinct α-ketoglutarate-dependent dioxygenase activities that are involved in the enantioselective degradation of mecoprop and dichlorprop to achiral phenols. However, nothing is known about the genetic background of the mecoprop and dichlorprop degradation pathway in strain MH. *S. herbicidovorans* MH belongs to the *Alphapro-*

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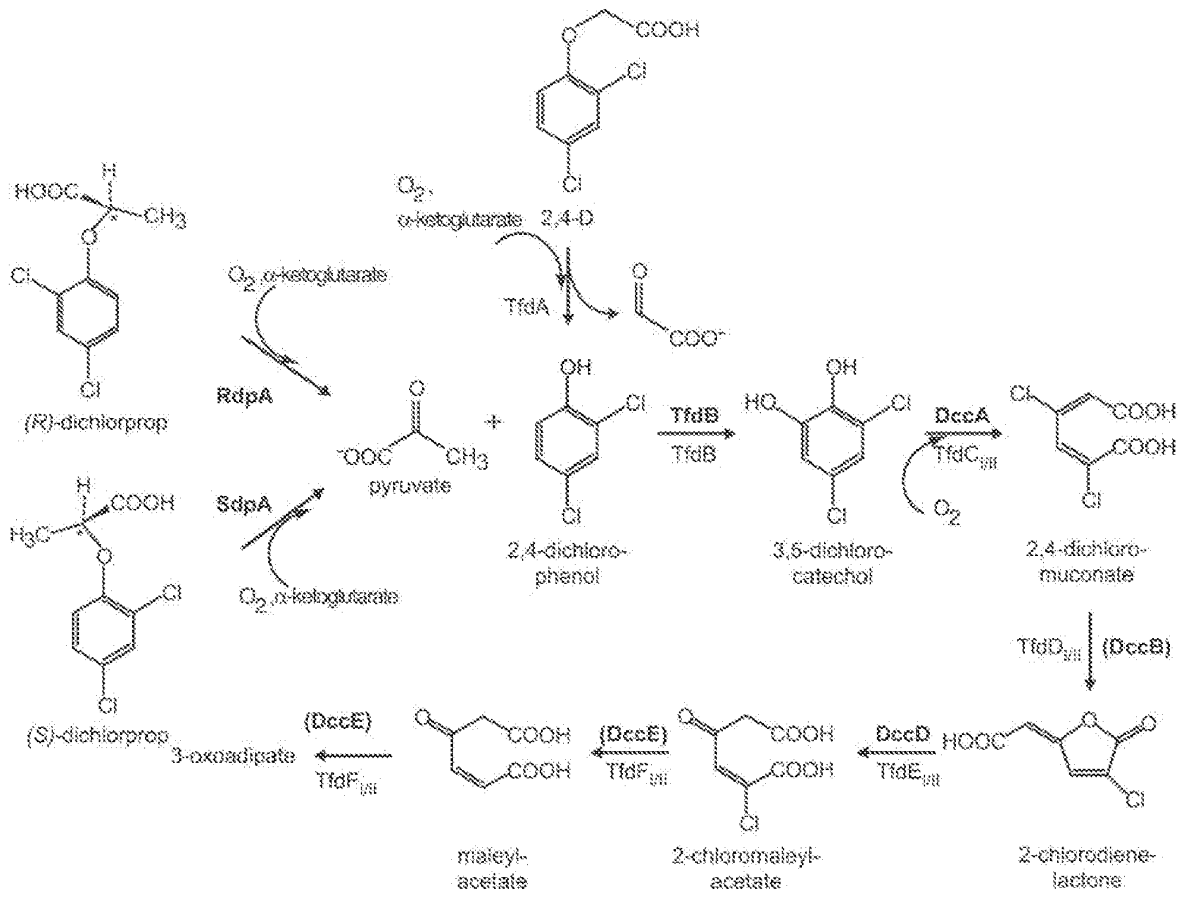


FIG. 1. Pathway proposed for dichlorprop degradation in *S. herbicidovorans* MH and for 2,4-D degradation in *R. eutropha* JMP134(pJP4) (30, 39–41). The enzymes of the pathway in *S. herbicidovorans* MH are printed in boldface. The enzymes of the genes, which have not yet or partially been isolated from *S. herbicidovorans* MH are displayed within parentheses.

teobacteria, whereas other well-studied phenoxyalkanoic acid degraders such as *R. eutropha* JMP134(pJP4) and *D. acidovorans* MC1 belong to the *Betaproteobacteria* and the *Gammaproteobacteria*. Total DNA from phenoxyalkanoic acid-degrading *Alphaproteobacteria* did not hybridize to *tfdA* gene probes encoding α-ketoglutarate-dependent 2,4-D dioxygenase (17, 24, 34, 50, 51). PCR experiments aimed at amplifying DNA fragments from *Sphingomonas* strains with *tfdA* primers also failed (44, 50). Therefore, the hypothesis was formulated that *Alphaproteobacteria* might harbor different dioxygenases catalyzing the initial step of phenoxyalkanoic acid degradation. Only recently, the first *tfdA* homologous gene, *tfdAα*, was isolated from a 2,4-D-degrading bacterium belonging to the *Bradyrhizobium-Agromyces-Nitrobacter-Afipia* (BANA) cluster of *Alphaproteobacteria* (23). On the other hand, fragments similar to phenol hydroxylase (e.g., *tfdB* like) and chlorocatechol 1,2-dioxygenase genes (e.g., *tfdC* like) have also been amplified from *Sphingomonas* strains with degenerate primers in the PCR (31, 50). Whereas the *tfdB*-like gene fragments from *Sphingomonas* strains were more than 60% similar to *tfdB* genes from *Beta-* and *Gammaproteobacteria*, *tfdC*-like gene fragments showed little similarity to other known chlorocatechol 1,2-dioxygenase genes and formed a rather coherent group among themselves (50). Hence, *Alphaproteobacteria* might not only harbor different dioxygenases but also different chlorocatechol degradation genes, and it was thus proposed that gene flow might be less common between *Alpha-* and *Beta-* or *Gammaproteobacteria* than among *Beta-* and *Gammaproteobacteria* themselves (17, 18).

In order to test this hypothesis and to elucidate the phenoxyalkanoic acid degradation pathway, we isolated and characterized several genes from *S. herbicidovorans* MH by PCR amplification with degenerated primers, DNA-DNA hybridization, cosmid library construction, and DNA sequencing. We propose that these genes are involved in phenoxyalkanoic acid degradation in strain MH and are equivalent to those known from the 2,4-D degradation pathway of *R. eutropha* JMP134(pJP4). The expression of the characterized genes was analyzed in hybridization experiments with mRNA isolated from *S. herbicidovorans* MH cultures that were induced with mecoprop or 2,4-D. The two chlorocatechol 1,2-dioxygenases were expressed in *Escherichia coli* and characterized in terms of their substrate specificities. Finally, the phylogenetic relationships of the *S. herbicidovorans* MH chlorocatechol pathway genes were compared to those of other phenoxyalkanoic acid degradation pathways.

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TABLE 1. Plasmids constructed in this work

Plasmid	Relevant characteristics (reference)	Source
pMec1	0.3-kb PCR-fragment obtained with CCDb and CCDe primers cloned into pGEM-T Easy; contains part of *dccA*	Total DNA
pMec2	1.5-kb SalI-fragment in pUC28 (5), contains part of the chlorocatechol degradation cluster II	Cosmid 9H11
pMec4	2.3-kb SalI-fragment in pUC28, contains part of the chlorocatechol degradation cluster I	11D11
pMec5	0.3-kb PCR-fragment obtained with RDPin_f and RDPin_r primers; contains part of *rdpA*	Total DNA
pMec7	$dccA_I$ in pGEM-T Easy	pMec4
pMec9	$dccA_I$ in pRSET6α (NdeI-BamHI cloned) (46)	pMec7
pMec10	2.8-kb NcoI-fragment in pUC28; contains *rdpA* and a transposase	10A12
pMec12	4.5-kb BamHI-fragment in pUC28; contains part of the chlorocatechol degradation cluster II	9H11
pMec14	0.7-kb PCR-fragment in pGEM-T Easy, contains *rdpA*	pMec10
pMec16	5-kb KpnI-fragment in pUC28; contains *sdpA*	10A12
pMec18	0.7-kb PCR-fragment in pGEM-T Easy, contains *sdpA*	pMec16
pMec20	$dccA_{II}$ in pGEM-T Easy	pMec12
pMec21	$dccA_{II}$ in pET8c (46)	pMec20
pMec23	1.1-kb PCR-fragment obtained with primers tfdBup and tfdBlow cloned into pGEM-T Easy, contains part of *tfdB*	total DNA
pMec25	5.5-kb SalI-fragment in pUC18Not; contains *tfdB*	11D11

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. herbicidovorans* MH was grown in baffled Erlenmeyer flasks shaking at 30°C either in complex medium (59) or in mineral medium supplemented with the appropriate carbon and energy source. The mineral medium was prepared as described in Nickel et al. (38) and was modified by reducing the amount of added peptone to 10 mg/liter and by adding 10-mg/liter yeast extract. *E. coli* strains were grown at either 30 or 37°C in Luria-Bertani (LB) medium (42) with the appropriate antibiotic. Ampicillin and kanamycin were added to final concentrations of 50 μg/ml, and chloramphenicol was added to 25 μg/ml. If necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Biosynth AG, Staad, Switzerland) was added to a final concentration of 50 μg/ml.

Standard molecular techniques. Cloning and digestions were done according to established procedures (4, 42). Restriction enzymes and other DNA-modifying enzymes were purchased from Promega (Wallisellen, Switzerland) and Fermentas (Nunningen, Switzerland). Plasmids and cosmids were isolated by the boiling Miniprep method, the alkaline lysis method according to Sambrook et al. (42), or with the E.Z.N.A. plasmid Miniprep kit II (Peqlab Biotechnologies GmbH, Baden-Dättwil, Switzerland) as suggested by the manufacturer. Gel extraction was carried out with the MinElute gel extraction kit (QIAGEN AG, Basel, Switzerland) according to the protocol of the supplier.

Construction of a cosmid library of *S. herbicidovorans* MH. The SuperCos1 cosmid vector kit (Stratagene, Amsterdam, The Netherlands) was used to construct a cosmid library. *S. herbicidovorans* MH was grown in 500 ml of mineral medium containing 200-mg/liter (*R,S*)-mecoprop until a turbidity of 0.15 (measured at 546 nm) was reached. The culture was harvested by centrifugation for 30 min at 6,000 × *g*. The pellet was resuspended in 15 ml of lysis buffer (10 mM NaCl, 20 mM Tris-HCl, pH 8, 1 mM EDTA, 100-μg/ml proteinase K, and 0.5% [wt/vol] sodium dodecyl sulfate) and incubated overnight at 50°C. The DNA was isolated by phenol-chloroform extraction (42), precipitated with ethanol, and dissolved in 4 ml of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) and stored at 4°C. Partial digestion of the DNA was done with Sau3AI using different enzyme concentrations for 15 min at 37°C. The digestion was stopped by adding EDTA to a final concentration of 1 mM. The preparation of the host strain *E. coli* XL-1 BlueMR, the packaging, and the titer determination were carried out according to the protocol of the supplier (Stratagene). Finally, about a thousand clones were picked and grown overnight in microtiter plates in LB medium supplemented with kanamycin at 30°C. Glycerol was added to a final concentration of 15%, and the cosmid library was stored at −80°C.

Screening for genes involved in phenoxyalkanoic acid degradation by PCR. To isolate *rdpA*, we amplified a fragment with the PCR primers RDPin_fG and RDPin_r (55) from chromosomal DNA of *S. herbicidovorans* MH. Oligonucleotides for PCR were obtained from Microsynth GmbH (Balgach, Switzerland). The following conditions were applied: initial denaturation for 5 min at 93.5°C; denaturing for 30 s at 93.5°C; annealing for 30 s at 52, 49, 46, 43, 41, and 39°C; extension for 45 s at 72°C (5 cycles at each annealing temperature, 10 cycles at 39°C) followed by a final extension for 4 min at 72°C. A chlorophenol hydroxylase gene fragment (*tfdB*) was amplified with the tfdBup and tfdBlow primers from total DNA of *S. herbicidovorans* MH as described by Vallaeys et al. (50).

To screen for a chlorocatechol 1,2-dioxygenase gene, the degenerate PCR primers CCDb and CCDe described by Leander et al. (31) were used. The PCR protocol was as follows: initial denaturation for 6 min at 93.5°C, 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 48°C, and extension for 1 min at 72°C followed by one extension cycle for 6 min at 72°C. PCR fragments were cloned in pGEM-T Easy (Promega) and sequenced to check the insert (Table 1).

Hybridization of the cosmid library and construction of plasmids harboring genes involved in phenoxyalkanoic acid degradation. For colony blotting of the cosmid library, the colonies were grown overnight on LB agar plates at 37°C. Colony material was transferred to Hybond-XL membrane (Amersham Biosciences, Dübendorf, Switzerland) by direct contact. The colony material on the membrane was lysed twice with a solution of 0.5 M NaOH for 2 min and washed twice with a solution of 1 M Tris-HCl (pH 8). The DNA was fixed on the membrane by UV light (254 nm, 120 mJ/cm^2) in a Stratalinker model 1800 (Stratagene).

The cosmid library of *S. herbicidovorans* MH was hybridized with the inserts of pMec1 (*dccA* fragment) and pMec5 (*rdpA* fragment). The fragments were radioactively labeled with [α-^{32}P]dATP using the Random Primed DNA labeling kit according to the supplier's protocol (Roche Applied Science, Rotkreuz, Switzerland). Positively reacting cosmids were isolated, digested, and checked by Southern hybridization. Additionally, all positive-reacting cosmids were hybridized in the same way with the insert of pMec23 to screen for the *tfdB* gene. Suitable fragments were then subcloned in pUC vectors (5), yielding the plasmids listed in Table 1.

Transposon insertion and sequencing. The EZ::TN<KAN-2> Tnp transposome kit (Epicentre Technologies, Madison, Wis.) was used for the insertion of a kanamycin resistance marker and for sequencing priming sites into the plasmids with cloned *S. herbicidovorans* MH DNA. The insertion reaction was carried out according to the protocol of the supplier. DNA sequencing was performed on double-stranded DNA templates with the Thermo Sequenase primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences) and M13 primers (5) or KAN-2 FP-1/RP-1 primers (Epicentre Technologies). Sequencing reactions were analyzed on an automated DNA sequencer model 4200 IR^2 (LI-COR, Inc., Lincoln, Nebr.). Primers for sequencing were labeled at the 5′ end with IRD-700 or IRD-800 and were purchased from MWG Biotech AG (Ebersberg, Germany). Sequencing data were analyzed with DNASTAR software (DNASTAR, Inc., Madison, Wis.) and compared to the databases by BLAST searches (www.ncbi.nlm.nih.gov/BLAST) (47).

Construction of expression plasmids pMec9 and pMec21, expression of DccA$_{I/II}$ in *E. coli*, and preparation of cell extract. The $dccA_I$ gene was reamplified from a cosmid fragment by PCR with the primer pair CCD2/CCD3 (5′-GAGAGGTCATATGAGCAATCGT-3′, 5′-AACGCGAGGATCCGCAGACCAT-3′), thereby introducing an NdeI restriction site and a BamHI restriction site (underlined). The $dccA_{II}$ gene was amplified by PCR with the forward primer ClcA2_for (5′-AGGTTCCATGGGCAATCG-3′) to introduce an NcoI site and the reverse primer ClcA2_rev (5′-AGGATCCGTGCCTGTCAG-3′) to create a BamHI site. The fragments were first cloned into pGEM-T Easy and verified by

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sequencing. They were then digested with the respective enzymes and cloned in the same restriction sites in pRSET6a and pET8c, respectively (46) (Table 1).

To express the proteins, a preculture of *E. coli* BL21(DE3)(pLysS) harboring pMec9 or pMec21 was grown overnight at 37°C. A volume of 1% (vol/vol) of the preculture was transferred to fresh medium, and the cells were grown at 30°C until a turbidity of 0.4 to 0.6 (measured at 546 nm) was reached, after which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.05 mM. The culture was incubated for another 3 h at 30°C, and the cells were harvested by centrifugation. A pellet of approximately 1 g (wet weight) was resuspended in 2.5 ml of a solution of 40 mM Tris-HCl, pH 7.4, containing 0.3 mM EDTA. The cells were broken by one passage through a French Pressure cell at 1 MPa. The lysate was centrifuged at 16,000 × *g* for 30 min at 4°C, and the supernatant was recovered and stored at −20°C until used as cell extract in enzyme activity measurements.

Enzyme assay. Enzymatic activity of chlorocatechol 1,2-dioxygenase with all substrates was measured spectrophotometrically at 260 nm except for protocatechuate, which was monitored at 290 nm. To 485 μl of buffer (containing 40 mM Tris-HCl, 0.3 mM EDTA, pH 7.4), 10 μl of cell extract of *E. coli* BL21(DE3)(pLysS) harboring pMec9 or pMec21 containing between 15 and 30 μg of total protein was added. The reaction was started by adding the substrate from a 20 mM stock in methanol to a final concentration of 0.4 mM. To calculate activity, extinction coefficients were taken from Broderick and O'Halloran (7) and Dorn and Knackmuss (11).

Induction experiment. *S. herbicidovorans* MH was grown on mineral medium with 20 mM pyruvate to a turbidity of 0.4 to 0.6 (measured at 546 nm). The culture was induced by the addition of (*R*)-, (*S*)-mecoprop, or 2,4-D dissolved in dimethyl sulfoxide (DMSO; 10 g/liter) to a final concentration of 100 mg/liter and incubated for another 30 min at 30°C. A culture to which only DSMO was added was used as a negative control. To stop induction and prevent RNA degradation, 1 volume of culture was added to 2 volumes of RNA protector immediately after sampling (QIAGEN AG). The mixture was incubated at room temperature for 5 min, centrifuged at 13,000 × *g* for 1 min, and the supernatant was decanted. The pellets were stored at −20°C.

RNA extraction. RNA was extracted with the RNeasy Mini kit from QIAGEN AG according to the supplier's protocol. The RNA, dissolved in RNase-free water, was then stored by adding 2 volumes of ethanol and 0.1 volume of 5 M sodium acetate (pH 5.2) at −20°C. RNA concentrations were determined spectrophotometrically at 260 nm.

In vitro synthesis of antisense mRNA. The pGEM-T Easy plasmids pMec14, -18, and -20, harboring *rdpA*, *sdpA*, and $dccA_H$, respectively, were linearized by restriction enzyme digestion in order to allow insert-specific transcription from either the T7 or the SP6 promoter. In vitro transcription reactions were carried out with biotin-16-UTP and T7 or SP6 RNA polymerase with the Riboprobe in vitro transcription systems (Promega) according to the protocol of the supplier. The size and yield of all antisense mRNA probes were checked on 1% agarose gels.

Dot blot hybridization of mRNA. Total RNAs were washed with 70% ethanol and dissolved in 50 μl of RNase-free water. Equal volumes of RNA dilutions (0.5, 0.1, and 0.05 μg per 50 μl) were blotted onto positively charged nylon membranes (QIAGEN GmbH) in a dot blot manifold (Invitrogen Life Technologies, Basel, Switzerland) containing a 96-well 3-mm gasket. The samples were washed with an equal volume of 1× SSC (0.15 M NaCl, 15 mM sodium citrate) directly after their application. On each blot, a series of DNA standards were included (not shown). The standards contained 100, 50, 10, 1, or 0.1 ng of DNA/50 μl of plasmid pMec14, -18, or -20.

Phylogenetic analysis. Amino acid sequences were aligned with the software tool CLUSTALX (49). Phylogenetic analysis was done with the maximum-likelihood method by using the software Tree-Puzzle (http://www.tree-puzzle.de).

Chemicals. (*R,S*)-, (*R*)-mecoprop, and 2,4-D were purchased at Riedel-de Haën (Buchs, Switzerland; PESTANAL, 99% purity). (*S*)-Mecoprop had been synthesized previously by Zipper (58). Catechol, 4-chloro-, 3,5-dichloro-, 4,5-dichloro-, tetrachloro-, 3-methyl-, and 4-nitrocatechol were purchased from Sigma-Aldrich (Buchs, Switzerland; 97 to 99% purity). 3-Chlorocatechol was bought at Promochem GmbH (Wesel, Germany; 99% purity). All other chemicals were either obtained from Fluka (Buchs, Switzerland) or from Merck AG (Dietikon, Switzerland).

Nucleotide sequence accession number. Nucleotide sequences determined in this study were deposited in the EMBL database under accession no. AJ628859 to AJ628863.

RESULTS

Sequence determination of *sdpA*, *rdpA*, and *tfdB*. Degradation of phenoxyalkanoic acid herbicides usually begins with an ether cleavage to the corresponding phenol by α-ketoglutarate-dependent dioxygenases (15, 16, 45, 55, 56). By using a primer set previously developed for isolating a gene fragment encoding a (*R*)-dichlorprop dioxygenase from *D. acidovorans* MC1 (55), a 0.3-kb fragment was amplified from total DNA of *S. herbicidovorans* MH. This PCR-amplified fragment was cloned (pMec5) and sequenced to confirm its identity to the dichlorprop dioxygenase gene. It was subsequently used to screen a cosmid library of *S. herbicidovorans* MH. Several cosmids were positively hybridizing with the pMec5 insert, and a 2.8-kb NcoI-fragment was identified from cosmid 10A12 to contain the region of identity to pMec5. This fragment was subcloned (pMec10), sequenced, and analyzed in detail (Table 2). The sequence revealed two open reading frames (ORFs), one of which was almost identical to the (*R*)-dichlorprop dioxygenase gene *rdpA* from *D. acidovorans* MC1 ($rdpA_{MC1}$; Table 2 and Fig. 2A). At the nucleotide level, the 888-bp *rdpA* ORF from *S. herbicidovorans* MH contained one neutral mutation compared to $rdpA_{MC1}$, whereas the predicted $RdpA_{MH}$ protein had a 100% identical amino acid sequence to $RdpA_{MC1}$. RdpA belonged to the class of α-ketoglutarate-dependent dioxygenases and showed a 35% identical amino acid sequence to TauD from *Pseudomonas aeruginosa* and percentages of identities below 33% to other members of this enzyme class (Table 3). Upstream of $rdpA_{MH}$, a 1,200-bp region was found which was similar to an insertion element from *Ralstonia* sp. strain JS705 (Table 2). The transposase ORF comprised 1,149 nucleotides.

The cosmid 10A12 was subsequently hybridized under low-stringency conditions with a 0.7-kb EcoRI fragment of pMec10 containing the $rdpA_{MH}$ gene, which revealed a weak signal in addition to the *rdpA*-containing fragments itself (not shown). The signal could be located to a 5-kb KpnI fragment, which was cloned (pMec16) and partially sequenced. An ORF of 864 nucleotides was identified whose predicted amino acid sequence showed significant similarity to the group of α-ketoglutarate-dependent dioxygenases and highest similarity to (*S*)-dichlorprop/α-ketoglutarate dioxygenase from *D. acidovorans* MC1 (Table 2). For this reason, this ORF was designated $sdpA_{MH}$. The predicted amino acid sequence for $SdpA_{MH}$ was 63% identical to SdpA from *D. acidovorans* MC1 ($SdpA_{MC1}$) and 37% to TfdA from *R. eutropha* JMP134(pJP4) (Table 3). Upstream of $sdpA_{MH}$, a similar insertion element was identified as the one in the region upstream of $rdpA_{MH}$. However, the complete ORF of this element was not present on the insert of pMec16.

With the primer pair tfdBup and tfdBlow, a 1.1-kb PCR-fragment was amplified from total DNA of *S. herbicidovorans* MH, cloned (pMec23), and sequenced. The insert was then used to hybridize cosmids 10A12, 9H11, and 11D11, which harbor other genes of phenoxyalkanoic acid degradation. A 5.5-kb SalI fragment from 11D11 was subsequently cloned (pMec25) and partially sequenced. The sequence revealed two complete ORFs and one incomplete ORF, whose deduced amino acid sequences showed similarity to known proteins in the databank (Table 2). The deduced amino acid sequence of the incomplete ORF at the left end of the insert was similar to CadR from *Bradyrhizobium* sp. strain HW13 and was therefore tentatively designated *cadR* (25). The ORF downstream showed low similarity (<26%) to the C-terminal region of

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TABLE 2. Genes and corresponding products isolated from *S. herbicidovorans* MH

Plasmid	ORF[a]	Position (nt)[b]	Probable function or product	Homologous protein[c]	Source	Reference accession no.	% Amino acid identity[d]	No. of amino acids[e]
pMec10	*tnpA*	226–374	Transposase	TnpA	*Ralstonia* sp. strain JS705	CAD60927	87 (352/403)	403/414
	rdpA	1733–2620	(*R*)-Dichlorprop/α-ketoglutarate dioxygenase	RdpA	*D. acidovorans* MC1	AAM90962	100 (295/295)	295/295
pMec16	(*tnpA*)	(1–447)	Transposase (C terminus)	Transposase	*Ralstonia* sp. JS705	CAD60927	79 (117/148)	-/414
	sdpA	750–1613	(*S*)-Dichlorprop/α-ketoglutarate dioxygenase	SdpA	*D. acidovorans* MC1	AAM90963	58 (171/290)	287/292
pMec25	(*cadR*)	(1–972)	Transcriptional regulator	CadR	*Bradyrhizobium* sp. HW13	BAB78520	65 (181/276)	-/336
	ORF2	1062–2627[f]	Outer membrane receptor protein	COG1629	*Novosphingobium aromaticivorans*	ZP_00096178	26 (129/488)	521/743
	tfdB	2654–4453	Dichlorophenol hydroxylase	TfdB	*D. acidovorans* MC1	AAM76774	57 (334/585)	600/586
pMec4 (cluster I)	(*dccE*)	(1–247)	(Partial) maleylacetate reductase	TfdF	*Burkholderia cepacia*	AAK81685	53 (42/78)	-/357
	$dccA_I$	514–1350	Chlorocatechol 1,2-dioxygenase	TfdC	*R. eutropha* JMP134(pJP4)	P05403	53 (134/255)	278/255
	$dccD_I$	1293–2015	Dienelactone hydrolase	$TfdE_{II}$	*R. eutropha* JMP134(pJP4)	P94136	47 (106/222)	240/235
pMec2 + 12 (cluster II)	*tfdK*	595–1968	Putative transport protein	TfdK	*R. eutropha* JMP134(pJP4)	AAC44725	36 (159/440)	457/463
	(*orfB*)	2494–2865[g]	ISCc3 transposase *orfB* (C terminus)	ISCc3 transposase OrfB	*Caulobacter crescentus* CB15	NP_419441	65 (81/123)	-/326
	$dccA_{II}$	3320–4087	Chlorocatechol 1,2-dioxygenase	TfdC	*R. eutropha* JMP134(pJP4)	BAA74530	51 (125/242)	256/251
	($dccD_{II}$)	4100–(4705)	(Interrupted dienelactone hydrolase)	$TfdE_{II}$	*R. eutropha* JMP134(pJP4)	P94136	50 (88/173)	-/235
	(*tnpA*)	(5161–4677)	Transposase (C terminus)	TnpA of IS*6100*	*Salmonella enterica* subsp.	AAG03007	100 (156/156)	-/264

[a] Incomplete ORFs are set in parentheses.
[b] The position is given respectively to the nucleotides of the plasmid insert.
[c] Given is the most similar protein identified by BLASTX.
[d] The values in parentheses refer to the number of identical amino acids over the aligned region.
[e] The number of amino acids of the protein in *S. herbicidovorans* MH is given first, and the number of the reference protein is given second.
[f] The exact start position of the ORF could not be determined. Other possible start codons are at nucleotides 969, 1185, and 1290.
[g] The nucleotide region represents the part that is similar to the C terminus of outer membrane proteins, since no start codon was found upstream of position 2494, and at nucleotide 2389, a stop codon is encoded.

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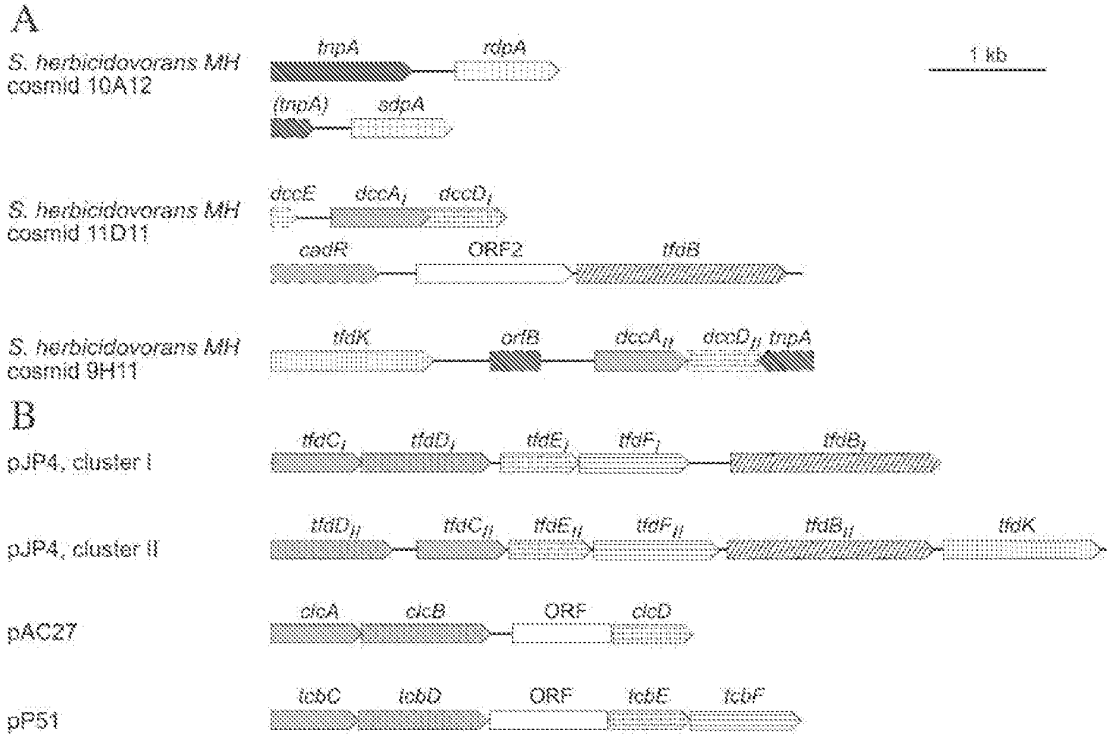


FIG. 2. (A) Configuration of phenoxyalkanoic acid degradation genes in *S. herbicidovorans* MH; (B) comparison of the gene clusters for the modified *ortho*-cleavage degradation pathway in different bacteria. pJP4, *R. eutropha* JMP134 (M35097, U16782); pAC27, *P. putida* (M16964); pP51, *Pseudomonas* sp. (M57629). The arrows indicate the localization, size, and direction of the transcription of the genes. Dashed lines around boxes represent incompletely sequenced ORFs. Similar patterns of hatching and shading between genes indicate DNA homology or amino acid similarity of the translated gene products. The following enzymes are shown (the genes that code for them are in parentheses): α-ketoglutarate-dependent dioxygenases (*rdpA* and *sdpA*), 2,4-D transport protein (*tfdK*), (chloro)phenol hydroxylases (*tfdB*, $tfdB_I$, and $tfdB_{II}$), chlorocatechol 1,2-dioxygenases ($dccA_I$, $dccA_{II}$, $tfdC_I$, $tfdC_{II}$, *clcA*, and *tcbC*), chloromuconate cycloisomerases ($tfdD_I$, $tfdD_{II}$, *clcB*, and *tcbD*), dienelactone hydrolases ($dccD_I$, $dccD_{II}$, $tfdE_I$, $tfdE_{II}$, *clcD*, and *tcbE*), maleylacetate reductases (*dccE*, $tfdF_I$, $tfdF_{II}$, and *tcbF*), and transposases (*tnpA* and *orfB*).

outer membrane receptor proteins. The ORF starting at nucleotide 2654 and comprising 1,800 bp showed similarity to a dichlorophenol hydroxylase gene and was designated *tfdB* (Table 2).

Sequence determination and organization of two chlorocatechol degradation gene clusters. To determine whether genes for chlorocatechol degradation through an *ortho*-cleavage pathway were present in the genome of strain MH, a PCR was carried out with the conserved primer set CCDb and CCDe (31). Amplified fragments were cloned and sequenced to determine the nature of the insert. One clone (pMec1) was found to contain a part of a putative chlorocatechol 1,2-dioxygenase

TABLE 3. Identity of RdpA and SdpA to other α-ketoglutarate-dependent dioxygenases (determined by BlastP two sequences)

Protein	Strain	No. of amino acids	Accession no.	Substrate	% Identity (no. with identity/total)	
					RdpA	SdpA
RdpA	*S. herbicidovorans* MH	295	AJ628859	(*R*)-Dichlorprop		30 (79/262)
	Delftia acidovorans MC1	295	AAM90962	(*R*)-Dichlorprop		
SdpA	*S. herbicidovorans* MH	287	AJ628860	(*S*)-Dichlorprop	30 (79/262)	
TauD	*E. coli* K-12	283	AAC73471	Taurine	33 (94/281)	29 (82/280)
TauD	*Pseudomonas aeruginosa*	277	NP 252624	Taurine	35 (99/282)	32 (91/277)
AtsK	*Pseudomonas putida*	301	AAD31784	Alkyl sulfate esters	31 (93/293)	27 (77/277)
TfdA	*Burkholderia cepacia* (pIJB)	288	AAB47567	2,4-D	28 (82/290)	35 (90/252)
TfdA	*Burkholderia* sp. strain RASC	297	AAB17363	2,4-D	26 (79/293)	37 (107/284)
TfdA	*Ralstonia eutropha* JMP134(pJP4)	287	AAA21983	2,4-D	30 (88/288)	37 (107/287)
RdpA	*Delftia acidovorans* P4a	287	AAM76772	2,4-D	28 (81/288)	36 (105/286)
SdpA	*Delftia acidovorans* MC1	292	AAM90963	(*S*)-Dichlorprop	27 (75/268)	63 (183/290)
TfdAα	Strain RD-5-C2 (member of BANA cluster)	295	BAB92964	2,4-D	28 (83/287)	36 (106/288)
TfdA	*Achromobacter xylosoxidans* subsp. *denitrificans*	287	AAF59421	2,4-D	27 (80/288)	37 (106/286)

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gene (Table 1). The cosmid library was then hybridized with the pMec1 insert to retrieve the complete gene for chlorocatechol 1,2-dioxygenase. Interestingly, two different fragments of cosmids 9H11 and 11D11 were hybridizing, which were both subcloned and sequenced (pMec2 and pMec4). The insert of pMec4 revealed three ORFs, the deduced amino acid of the sequence of one of which had significant similarity to chlorocatechol 1,2-dioxygenases (Table 2). This ORF was tentatively designated $tcbA_I$, for dichlorocatechol 1,2-dioxygenase. The exact start of $tcbA_I$ could not be determined completely, since several alternative possible start codons occurred in sequential position (i.e., at positions 265, 367, 400, 472, and 514). However, on the basis of the position of a putative Shine-Dalgarno sequence (AGGAGA at position 500), we propose the $tcbA_I$ gene start at position 514. Interestingly, flanking the $tcbA_I$ gene were ORFs putatively encoding a dienelactone hydrolase (designated $tcbD_I$) (Fig. 2A; Table 2) and a maleylacetate reductase. The maleylacetate reductase ORF was not completely recovered on the insert of pMec4.

The left end of the insert of pMec2 contained a part of a chlorocatechol 1,2-dioxygenase gene. To retrieve the complete ORF, a 4.5-kb BamHI fragment from cosmid 9H11, which partially overlapped the insert of pMec2, was cloned (pMec12) and sequenced. The whole ORF encoding the putative chlorocatechol 1,2-dioxygenase was obtained and tentatively designated $tcbA_{II}$, since it was not identical to $tcbA_I$. Flanking the $tcbA_{II}$ on the inserts of pMec2 and pMec12 were ORFs for another dienelactone hydrolase and a putative transport facilitator protein (Table 2; Fig. 2A). However, the second possible ORF for dienelactone hydrolase ($tcbD_{II}$) was interrupted by a part of an insertion element identical to IS*6100*. The ORF upstream of $tcbA_{II}$ most likely codes for a transporter protein similar to the TfdK 2,4-D transporter. Between $tcbA_{II}$ and *tfdK*, the sequence revealed part of another insertion element (IS*Cc3*). The sequence information thus indicated that *S. herbicidovorans* MH harbored at least two complete genes for a chlorocatechol 1,2-dioxygenase, which were both located upstream of a (partial) dienelactone hydrolase gene. Evidence was also found for a chloromaleylacetate reductase upstream of $tcbA_I$. Except for a gene for chloromuconate cycloisomerase, this would provide an almost complete modified *ortho*-cleavage pathway to strain MH.

Expression of $TcbA_I$ and $TcbA_{II}$ in *E. coli* BL21(DE) (pLysS). The $tcbA_I$ and $tcbA_{II}$ ORFs were expressed in *E. coli* BL21(DE3)(pLysS) harboring pMec9 and pMec21. In both cell extracts, bands of the expected sizes of 30.9 and 27.9 kDa, respectively, were seen on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (not shown), which verified the translation of both genes in *E. coli*. Incubation of the cell extracts with 4-methylcatechol clearly showed the appearance of 4-methylmuconate with a maximal absorption at 255 nm (not shown). The substrate range for both enzymes was determined in cell extracts from *E. coli* BL21(DE3)(pLysS) by incubation with different substituted catechols (Table 4). $TcbA_I$ and $TcbA_{II}$ exhibited essentially the same substrate range (Table 4). Both enzymes converted chloro- and methyl-substituted catechols; the highest activity was detected with 4-methylcatechol, whereas 3,5-dichlorocatechol was preferred to monosubstituted catechols and catechol. 4,5-Dichlorocatechol was also converted, although at very low rates. No activity was measured with tetrachlorocatechol, *p*-nitrocatechol, protocatechuate, and hydroquinone.

TABLE 4. $TcbA_I$ and $TcbA_{II}$ activities with different substrates measured in cell extract of *E. coli* BL21(DE3)(pLysS) harboring pMec9 or pMec21

Substrate	% Activity ± standard deviation[a]	
	$TcbA_I$	$TcbA_{II}$
Catechol	23 ± 2	19 ± 3
3-Chlorocatechol	41 ± 2	34 ± 2
4-Chlorocatechol	25 ± 1.6	22 ± 0.7
3-Methylcatechol	62 ± 3	47 ± 3
4-Methylcatechol	100 (1.48–2.59)	100 (1.12–2.15)
3,5-Dichlorocatechol	56 ± 4	49 ± 3
4,5-Dichlorocatechol	0.7 ± 0.3	0.6 ± 0.3
Tetrachlorocatechol	<0.01	<0.01
p-Nitrocatechol	<0.01	<0.01
Protocatechuate	<0.01	<0.01
Hydroquinone	<0.01	<0.01

[a] Percent enzyme activities ± standard deviation compared to the value obtained with 4-methylcatechol. The data represent means of three independent cell extracts, of which each was measured three times with all substrates, except for tetrachloro- and *p*-nitrocatechol, protocatechuate, and hydroquinone, which represent means of two independent cell extracts. The values in parentheses represent the range of specific activity in the cell extracts (in units per milligram of protein).

Expression of *rdpA*, *sdpA*, $tcbA_I$, and $tcbA_{II}$. To investigate whether *rdpA*, *sdpA*, and $tcbA_{I/II}$ were involved in phenoxyalkanoic acid degradation in *S. herbicidovorans* MH, we grew the strain in batch cultures with pyruvate and pulsed the culture with (*R*)-, (*S*)-mecoprop, or 2,4-D. The formation of specific mRNAs was then determined by dot blot hybridizations (Fig. 3). mRNAs for *sdpA* and *rdpA* were always present, irrespective of the addition of mecoprop and 2,4-D, but mRNA for $tcbA_{I/II}$ appeared specifically after exposure to both mecoprop and 2,4-D (Fig. 3). It has to be noted, however, that the hybridization experiments cannot distinguish between $tcbA_I$ and $tcbA_{II}$ because of their high similarity. These results strongly suggest that mecoprop and 2,4-D induce a modified *ortho*-cleavage pathway involving $tcbA_I$ and/or $tcbA_{II}$.

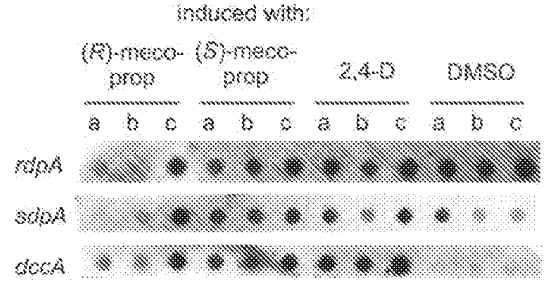


FIG. 3. Dot blot hybridization of total RNA isolated from batch cultures of *S. herbicidovorans* MH after induction with (*R*)-, (*S*)-mecoprop, or 2,4-D. Antisense mRNA probes used for hybridization are indicated on the left. (*R*)-, (*S*)-mecoprop and 2,4-D on the top show the induction substrate; DMSO represents the uninduced state. a, b, and c represent individual replicates.

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DISCUSSION

Phenoxyalkanoic acid degradation in *S. herbicidovorans* MH. Here we studied the genetic background of phenoxyalkanoic acid degradation in *S. herbicidovorans* strain MH. Several candidate genes were isolated, which showed similarity at the amino acid level to other proteins involved in phenoxyalkanoic acid and chlorocatechol degradation. Strain MH harbors two genes encoding two α-ketoglutarate-dependent dioxygenases (named $RdpA_{MH}$ and $SdpA_{MH}$), which were different from the archetypal TfdA enzyme (Table 3) but very similar to two dioxygenases from *D. acidovorans* MC1. Enzymes from *D. acidovorans* MC1 catalyze the enantioselective reaction of dichlorprop to dichlorophenol (55). $RdpA_{MH}$ and $SdpA_{MH}$ have been expressed in *E. coli* and indeed catalyze the enantioselective conversion of mecoprop and dichlorprop (T. A. Müller, unpublished data). This strongly suggests that $RdpA_{MH}$ and $SdpA_{MH}$ catalyze the first step in the degradation pathway of mecoprop and dichlorprop in strain MH (Fig. 1). Both genes were constitutively expressed and were not specifically induced upon exposure to mecoprop or 2,4-D. Previously, however, it was observed that the (*R*)-specific enzyme is only present when strain MH is grown on (*R*)-mecoprop, (*R*)-dichlorprop, or their racemates, whereas (*S*) activity is always present except when the strain is grown on the (*R*)-enantiomer (38). Therefore, it might be that expression of $rdpA_{MH}$ and $sdpA_{MH}$ is further regulated on the translational or posttranslational level.

S. herbicidovorans MH was shown to harbor two distinct chlorocatechol 1,2-dioxygenases genes, designated $dccA_I$ and $dccA_{II}$. Both genes were induced when strain MH was exposed to mecoprop or 2,4-D. When expressed in *E. coli*, $DccA_I$ and $DccA_{II}$ showed activity toward different substituted catechols but preferred 3,5-dichlorocatechol to monosubstituted catechols. Both enzymes showed essentially the same substrate specificities. From these results, we conclude that $DccA_I$, $DccA_{II}$, or both are involved in dichlorprop, mecoprop, and 2,4-D degradation in *S. herbicidovorans* MH. However, we do not know whether the two dichlorocatechol 1,2-dioxygenases have different roles.

We hypothesize that the *tfdB*-like gene is involved in mecoprop and dichlorprop degradation in strain MH, although this was not specifically tested here. The finding of other genes from the modified *ortho*-cleavage pathway (i.e., dienelactone hydrolase and maleylacetate reductase) is further evidence to propose that 3,5-dichlorocatechol and 3-methyl-5-chlorocatechol are processed through a metabolic pathway similar to the one for 2,4-D degradation in *R. eutropha* JMP134(pJP4) (Fig. 1). Currently, no information is available concerning the location of a chloromuconate cycloisomerase gene, the product of which would be needed to complete the pathway.

Genetic organization of phenoxyalkanoic degradation. The genes for phenoxyalkanoic acid degradation in *S. herbicidovorans* MH were not organized in one or two operons but scattered over the genome. The $rdpA_{MH}$ and $sdpA_{MH}$ gene were located on the same cosmid but separated by at least 10 kb. The genes for chlorophenol and chlorocatechol degradation were organized in three clusters, which were located on different cosmids. Such a gene organization is in contrast to that of the *tfd* genes for 2,4-D degradation in *R. eutropha* JMP134(pJP4), which are present in one compact region of 22 kb (29). A dispersed gene organization seems to be common for *Sphingomonas* strains. For instance, the *pcp* genes coding for pentachlorophenol degradation in *Sphingobium chlorophenolicum* ATCC 39723 are organized on noncontiguous parts of the DNA (8). A scattered gene organization was also found for the dioxin-degradative genes in *Sphingomonas* sp. strain RW1 (2). The *lin* genes for lindane degradation in *Sphingomonas paucimobilis* are present in five different operons and unlinked clusters (28). This contrasting gene organization in sphingomonads may have consequences for regulation of pathway expression. Expression of $rdpA_{MH}$ and $sdpA_{MH}$ in strain MH was constitutive, whereas the $dccA_{I/II}$ genes were inducibly expressed. This scheme is similar to the one for expression of the lindane pathway in *S. paucimobilis*, where the first three genes are constitutively expressed and only the genes for chlorohydroquinone conversion are inducible (35). In contrast, the 2,4-D pathway in *R. eutropha* JMP134(pJP4) is very tightly regulated (32, 33). It might be that the organization observed in *S. herbicidovorans* is not optimized yet and that regulatory networks controlling *rdpA* and *sdpA* expression will evolve when the strain remains being exposed to phenoxyalkanoic acid-containing environments. On the other hand, the absence of inducible expression might just be a different strategy found in sphingomonads compared to pseudomonads, which nonetheless results in functional catabolic pathways. When considering that inducible catabolic pathways often seem to need a threshold concentration before induction takes place, it may even be an advantageous strategy to continuously produce the first enzymes necessary for the pathway.

Potentially two clusters might be involved in chlorocatechol degradation in strain MH. Both clusters clearly have a different gene order than the typical operons for chlorocatechol such as the *clcABD* genes from *P. putida* (pAC27) (14), the *tfdCDEF* genes in *R. eutropha* JMP134(pJP4) (9, 19, 20, 40) and the *tcbCDEF* genes in *Pseudomonas* sp. strain P51 (54) (Fig. 2B). It was noteworthy that a gene for a chloromuconate cycloisomerase was not present within the regions sequenced from strain MH. Furthermore, cluster II was flanked by sequences with clear homology to (parts of) IS elements that even interrupted $dccD_{II}$; this points to (recent) genetic rearrangements.

Evolutionary aspects of the phenoxyalkanoic acid degradation pathway in *S. herbicidovorans* MH. It has been proposed that phenoxyalkanoic acid degradation genes are highly mobile and thus are easily transferred among bacteria (17, 24, 34, 50, 51). On the other hand, gene flow is assumed to be less frequent between *Alphaproteobacteria* and *Beta-* and *Gammaproteobacteria* than among *Beta-* and *Gammaproteobacteria* (17). The unique combination and organization of the phenoxyalkanoic acid degradation genes in *S. herbicidovorans* MH indicate that a recent gene exchange between *Alpha-* and *Betaproteobacteria* must have taken place. This can be concluded from the finding that $RdpA_{MH}$ is 100% identical to $RdpA_{MC1}$ from *D. acidovorans* MC1, a member of the *Betaproteobacteria*, and also clearly contradicts the hypothesis that *tfdA*-like genes of *Alphaproteobacteria* and *Beta-* and *Gammaproteobacteria* have arisen by divergent evolution (23). The transposase genes located next to $rdpA_{MH}$ and $sdpA_{MH}$ also support the hypothesis of gene mobilization. The transposase gene located nearby $rdpA_{MH}$ is very similar to a presumably promiscuous IS element which was implicated in transferring the toluene dioxy-

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genase from *Ralstonia* sp. strain JS745 to *Ralstonia* sp. strain JS705 (37).

In contrast to the α-ketoglutarate-dependent dioxygenase genes, chlorocatechol 1,2-dioxygenases from *Beta-* and *Gammaproteobacteria* present in the database are less related to $DccA_{I/II}$ than they are among themselves. In a phylogenetic analysis, strain MH's chlorocatechol 1,2-dioxygenases clustered together with chlorocatechol 1,2-dioxygenases from gram-positive bacteria forming a separate branch (not shown), which is in agreement with the results obtained by Eulberg et al. (12). Six different groups of chlorocatechol 1,2-dioxygenase could be defined: (i) ClpC, (ii) *Sphingomonas* homologs, (iii) TfdC_pJP4 homologs, (iv) the TcbC-TetC group, (v) $TfdC_{II}$_pJP4 homologs, and (vi) the ClcA homologs. Subgroups i and ii build new branches, whereas the classification of subgroups iii to vi agrees with the one described by Armengaud et al. (3). The two novel groups comprise sequences from *Alphaproteobacteria* and support our hypothesis that *Sphingomonas* strains have not exchanged their chlorocatechol 1,2-dioxygenase genes recently with those from *Beta-* and *Gammaproteobacteria*. The phenoxyalkanoic acid degradation pathway of strain MH has been demonstrated to be unique in its composition, genetic organization, and regulation, and it will be important to further study its characteristics in order to broaden our understanding of pathways different from the canonical 2,4-D degradation pathways in *Beta-* and *Gammaproteobacteria*.

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Physiological and genetic characteristics of two bacterial strains utilizing phenoxypropionate and phenoxyacetate herbicides

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Abstract

Two strains, *Rhodoferax* sp. P230 and *Delftia (Comamonas) acidovorans* MC1, have previously been shown to carry activities for the degradation of the two enantiomers of (*RS*)-2-(2,4-dichlorophenoxy-)propionate (dichlorprop) and (*RS*)-2-(4-chloro-2-methylphenoxy-)propionate (mecoprop) and, in addition, are capable of degrading phenoxyacetate derivatives 2,4-dichlorophenoxyacetate (2,4-D) and 4-chloro-2-methylphenoxyacetate (MCPA). Metabolism of the herbicides is initiated by α-ketoglutarate-dependent dioxygenases for both enantiomers of the phenoxypropionate herbicides and for 2,4-D. These activities were constitutively expressed for both enantiomers of dichlorprop in strain MC1 and for the *R*-enantiomer in strain P230. Enzyme activities for the complete degradation of phenoxyacetate and phenoxypropionate herbicides were induced during incubation on either of these herbicides. Strain MC1 has about threefold higher activities for the degradation of dichlorprop and for growth on this substrate (μ_{max} = 0.15 h^{-1}) than strain P230; the maximum growth rate on 2,4-D amounts to 0.045 h^{-1} with strain MC1. Dichlorprop is utilized faster than mecoprop and the *R*-enantiomers are cleaved with higher rates than the *S*-enantiomers. The degradation of the chlorophenolic intermediates seems to proceed via the modified *ortho* cleavage pathway as indicated by activities of the respective enzymes. The enzymatic results were supported by genetic investigations by which the presence of the genes *tfdB* (encoding a dichlorophenol hydroxylase), *tfdC* (encoding a chlorocatechol 1,2-dioxygenase) and *tfdD* (encoding a chloromuconate cycloisomerase) could be demonstrated in both strains by PCR after application of respective primers. The presence of the *tfdA* gene (encoding a 2,4-D/α-ketoglutarate dioxygenase) was only shown for strain P230 but was lacking in strain MC1. Sequence analysis of the *tfd* gene fragments revealed high homology to the degradative genes of other proteobacterial strains degrading chloroaromatic compounds. Strain MC1 carries a plasmid of about 120 kb which apparently harbors herbicide degradative genes as concluded from deletion mutants which have lost 2,4-D[phenoxalkanoate]/α-ketoglutarate dioxygenase activities for cleavage of the *R*- and *S*-enantiomer, and of 2,4-D. For strain P230, no plasmid could be demonstrated; the activity was stably conserved in this strain during growth under nonselective conditions.

Keywords: phenoxyalkanoate herbicides – enantiospecific degradation – 2,4-D[phenoxalkanoate]/α-ketoglutarate dioxygenase – *tfd* genes – *Delftia (Comamonas) acidovorans* MC1 – *Rhodoferax* sp. P230

Introduction

Microbial degradation of phenoxyalkanoic acid herbicides is well known and has most exemplarily been described for *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134 from genetic, biochemical and regulatory points of view (Don and Pemberton 1981, 1985; Streber *et al.* 1987; Perkins and Lurquin 1988; Pieper *et al.* 1988; Kaphammer *et al.* 1990; Perkins *et al.* 1990; Fukumori and Hausinger 1993; Matrubutham and Harker 1994; You and Ghosal 1995; Leveau and Van der Meer 1996; Filer and Harker 1997; Leveau *et al.* 1998). This strain degrades phenoxyacetate derivatives (2,4-D, MCPA) but does not productively utilize phenoxypropionate and phenoxybutyrate derivatives. Such high substrate specificity is remarkable in view of the close structural similarities and is a feature in phenoxyalkanoic acid utilization by various strains (Sinton *et al.* 1986) including isolates obtained

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from the herbicide production site considered in our case, e.g. *Delftia acidovorans* P4a which only degrades phenoxyacetates (Hoffmann *et al.* 1996) or strains of *Rhodococcus* and *Aureobacterium* which only attack phenoxybutyrates (Mertingk *et al.* 1998); none of these strains did utilize phenoxypropionate derivatives. Activity for the degradation of phenoxypropionates was often described to be associated with bacterial consortia (Kilpi 1980; Pfarl *et al.* 1990; Kelly *et al.* 1991). Only few examples of pure cultures are known to completely degrade compounds like 2-(2,4-dichlorophenoxy-)propionic acid (2,4-DP; dichlorprop) and 2-(4-chloro-2-methylphenoxy-)propionic acid (MCPP; mecoprop), e.g. strain MH (Horvath *et al.* 1990) meanwhile identified as *Sphingomonas herbicidovorans* (Nickel *et al.* 1997). Essential to remark is the fact that the above mentioned herbicides are racemic compounds. Thus two different enzymes with distinct specificity of cleaving the *R*- and *S*-enantiomers have to be taken into account (Armstrong *et al.* 1993; Zipper *et al.* 1996). This has been confirmed by separating two enzymes after growing *Delftia acidovorans* MC1 on (*RS*)-2,4-DP (Müller and Babel 1999). Such specificity is confirmed also by the fact that e.g. *Alcaligenes denitrificans* is able to utilize only the *R*-configuration of mecoprop (Tett *et al.* 1994).

In our attempt to detoxify herbicide-contaminated building rubble we have screened for strains exhibiting phenoxypropionate degrading activity. Two strains have previously been isolated from this material, i.e. *Rhodoferax* sp. strain P230 (Ehrig *et al.* 1997) and *D. acidovorans* strain MC1 (Müller *et al.* 1999) which completely degrade (*RS*)-2,4-DP and (*RS*)-MCPP. Besides these compounds both strains utilize phenoxyacetate derivatives in addition to certain chlorophenols. Strains with such a profile in substrate utilization should be favorable candidates for bioremediation purposes as sites of the chemical industry are often polluted by a mixture of contaminants. To apply such strains as biocatalysts in biotechnological processes detailed knowledge on the biochemical and physiological properties and their genetic basis in general and with special reference to the specific performances are required to exploit the degradative potential both under qualitative and quantitative aspects. Essential physiological and genetic features were investigated in these two strains.

Materials and methods

Strains and cultivation. Strain P230 was isolated from contaminated building rubble (Ehrig *et al.* 1997); strain MC1 was obtained from the biofilm established on the matrix of a fixed-bed reactor which was run for several months under aerobic conditions for degrading a herbicide mixture obtained from eluates of contaminated building rubble (Müller *et al.* 1999).

Both strains were grown on a mineral salts solution containing (in mg/l): NH_4Cl, 760; KH_2PO_4, 340; K_2HPO_4, 485; $CaCl_2 \cdot 6H_2O$, 27; $MgSO_4 \cdot 7H_2O$, 71.2; $FeSO_4 \cdot 7H_2O$, 4.98; $CuSO_4 \cdot 5H_2O$, 0.785; $MnSO_4 \cdot 4H_2O$, 0.81; $ZnSO_4 \cdot 7H_2O$, 0.44; $Na_2MoO_4 \cdot 2H_2O$, 0.25. At batch conditions the respective herbicides were supplied as a source of carbon and energy at a concentration of 0.2 g/l; at continuous cultivation the herbicides were supplied at a concentration of 2 g/l. In the case of strain P230 0.5 g/l of yeast extract in addition to thiamin (5 mg/l) and biotin (2 mg/l) were fed. Strain MC1 was grown prototrophically on the respective herbicides. Growth under nonselective conditions was performed either on LB medium or PYE medium (peptone 3.0 g/l, yeast extract 3.0 g/l and fructose 1.8 g/l) if not indicated otherwise. Batch cultivation was performed in 100-, and 500-ml shaking flasks on a rotary shaker with 200 rpm at 30 °C. Fed-batch and continuous cultivation of the strains were performed in a 1-l laboratory fermenter under aerobic conditions, at 30 °C and pH value of 8.5. The dilution rates applied ranged from 0.05 to 0.12 h^{-1} with strain MC1 and from 0.02 to 0.04 h^{-1} with strain P230. The maximum growth rates were obtained from transient-state cultivation technique as described elsewhere (Müller *et al.* 1995).

Induction studies. Strain P230 was grown under nonselective conditions on fructose (2 g/l) and yeast extract (0.5 g/l). In the case of strain MC1 colonies were taken from agar plates cultivated under selective conditions on (*RS*)-2,4-DP, inoculated into PYE medium and grown as an overnight culture. Cells from both strains were harvested, washed and transferred into mineral salts solution and incubated aerobically at 30 °C in the presence of 100–200 mg/l of herbicide in the absence and presence of streptomycin (50 mg/l) to prevent the expression of degradative enzymes as desired.

Enzyme activities. 2,4-D(phenoxyalkanoate)/α-ketoglutarate dioxygenase was determined according to Fukumori and Hausinger (1993) measuring the liberation of the phenolic intermediate (2,4-dichlorophenol; DCP) in a colorimetric assay after reaction with 4-aminoantipyrin. 2,4-Dichlorophenol hydroxylase was measured according to Beadle and Smith (1982) on basis of the disappearance of NADPH. Catechol 1,2-dioxygenase activity was determined by the method of Nakazawa and Nakazawa (1970) determining the formation of the *cis,cis*-muconic acid derivative by using 3,5-dichlorocatechol (Promochem, Wesel, Germany) as the substrate. *Cis,cis*-muconate cycloisomerase was assayed according to Schmidt and Knackmuss (1980)

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applying 3,5-dichlorocatechol and the partially purified type II catechol 1,2-dioxygenase (kindly provided by M. Schlömann, University of Stuttgart) in order to generate the *cis,cis*-2,4-dichloromuconic acid derivative in a precultivation phase; the activity was calculated by using an extinction coefficient of 9,700 l/mole cm (Pieper 1986). The enzyme activities were expressed as nmole/min mg protein (mU/mg).

Amplification of tfd gene fragments and DNA sequence analysis. 2,4-D degradative genes were detected by using degenerated PCR primers derived from conserved sequence motifs of the genes *tfdA* and *tfdB* (Vallaeys *et al.* 1996), *tfdC* (Kleinsteuber *et al.* 1998) and *tfdD* (Schäfer *et al.* 1998). The following oligonucleotide pairs were used for PCR amplification of the respective *tfd* gene fragments (expected fragment length according to the *tfdA* and *tfdB* genes and the *tfdCDEF* operon of *Ralstonia eutropha* JMP134): tfdA-1f [5'-ACG GAG TTC TGY GAY ATG-3'] and tfdA-2r [5'-AAC GCA GCG RTT RTC CCA-3'] amplifying a 363-bp *tfdA* fragment; tfdB-1f [5'-CGC AYA TCA CCA AYC ARC-3'] and tfdB-2r [5'-ATA GCG CTG RTT CAT YTC-3'] amplifying a 1.1-kb *tfdB* fragment; tfdC-1f [5'-TGG CAY TCS ACG CCY GAT-3'] and tfdC-2r [5'-TTC RAA GTA GTA TTG CGT GGT-3'] amplifying a 261-bp *tfdC* fragment; tfdD-1f [5'-GCK GAR ACM ATC AAG RTC ATC-3'] and tfdD-2r [5'-SCC KCC CAT GTT GCA MAG C-3'] amplifying a 648-bp *tfdD* fragment. The following primer combinations were used to test the gene cluster structure of the *tfdC* and *tfdD* genes: tfdC-1f and tfdD-2r amplifying a 1.2-kb fragment; tfdC-1f and tfdD-1r ([5'-GAT GAY CTT GAT KGT YTC MGC-3'], reverse complementary to tfdD-1f) amplifying a 599-bp fragment; tfdC-2f ([5'-ACC ACG CAA TAC TAC TTY GAA-3'], reverse complementary to tfdC-2r) and tfdD-1r amplifying a 359-bp fragment; tfdC-2f and tfdD-2r amplifying a 986-bp fragment. Oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany). 25 pmol of each primer were added to a 50-µl PCR sample. Total genomic DNA used as template was prepared from single colonies grown on LB agar as described elsewhere (Kleinsteuber *et al.* 1998) or from liquid cultures continuously grown on the herbicides as sole carbon source using the NucleoSpin C+T kit (Macherey-Nagel, Düren, Germany). PCR was performed on a multicycler PCT-200 (Biozym) using the DyNAzyme II PCR kit (Biometra, Göttingen, Germany). All ingredients were aliquoted from a mastermix. For each run, a PCR with only water instead of the template DNA was performed as a contamination control. *Ralstonia eutropha* JMP134 was used as a positive control, its pJP4-free derivative JMP222 as a negative control. PCR conditions for the amplification of *tfdA*-like and *tfdB*-like genes were according to Vallaeys *et al.* (1996), for the *tfdC*, *tfdD* and *tfdCD* fragments the PCR protocol described by Kleinsteuber *et al.* (1998) was applied. After electrophoresis in 1.5% agarose and ethidium bromide staining the PCR products were purified using the NucleoSpin Extract 2 in 1 kit (Macherey-Nagel, Düren, Germany), and the concentration of the purified DNA was determined by measuring the absorbance at 260 nm.

Cycle sequencing of the PCR products was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Weiterstadt, Germany) with 30–100 ng of template DNA, 10 pmol of the respective primer and 4 µl premix in a total volume of 20 µl. After 25 cycles of 30 s at 96 °C and 4:15 min at 60 °C the extension products were purified by Centri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA). 4 µl of the purified sample were mixed with 20 µl template suppression reagent (TSR, PE Applied Biosystems), denaturated at 95 °C for 2 min and chilled on ice. Electrophoresis, data collection and analysis were carried out on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). Each PCR product was sequenced with the forward and the reverse primers, respectively. The forward and the reverse sequences were analyzed by the Sequence Navigator software, contigs were assembled by the Autoassembler software (PE Applied Biosystems). The BLASTN and BLASTX programs (version 2.0.9.; Altschul *et al.* 1997) were used to search for similar sequences in the nucleotide and protein sequence databases. The partial sequences of the putative *tfd* genes will appear in the GenBank, EMBL and DDBJ nucleotide sequence databases under the accession nos. AF176240, AF176241, AF176242, AF176243 and AF176244.

Plasmid isolation and Southern hybridization. Plasmid DNA was prepared from 20–100-ml overnight cultures grown in LB medium using the Nucleobond AX100 kit (Macherey-Nagel, Düren, Germany) according to the manufacturers' protocol for the isolation of low-copy plasmids. With strain P230, additionally the plasmid isolation method according to Kado and Liu (1981) and a method described by Müller *et al.* (1998) were applied. Furthermore, the NucleoSpin kit (Macherey-Nagel) and the Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany) were used.

Plasmid DNA was separated electrophoretically in 0.8% agarose, stained with ethidium bromide and blotted onto a nylon membrane (Boehringer Mannheim) by standard procedures according to Sambrook *et al.* (1989). Purified PCR products were digoxygenin-labeled using the DIG DNA Labeling kit (Boehringer Mannheim). For hybridization and im-

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munodetection the DIG Easy Hyb Wash and Block Buffer Set and the DIG Nucleic Acid Detection kit (Boehringer Mannheim) were used. Hybridization with the *tfdB, tfdC* and *tfdD* probes was performed at 55 °C.

Analytical methods. Biomass concentration was determined by measurement of optical density at 700 nm (OD700) and by dry mass determination. Phenoxyalkanoic acids were determined by HPLC as described by Oh and Tuovinen (1990). The enantiomers of the phenoxypropionates were analyzed by HPLC according to the method given by Zipper *et al.* (1996).

Chemicals. The *R*- and *S*-enantiomers of MCPP were obtained from the racemic mixture following the procedure of Matell (1953). The *R*-and *S*-enantiomers of 2,4-DP were synthesized as practiced by Zipper (1998). We are grateful to Dr. H.-P. E. Kohler (EAWAG Dübendorf, Switzerland) for providing some (*S*)-2,4-DP in advance of our own preparation.

Results

Induction phenomena

Growth of strain P230 and strain MC1 under nonselective conditions revealed etherolytic activity to various phenoxyalkanoates resulting in the liberation and excretion into the medium of chlorinated phenols. Incubation of strain P230 in the presence of streptomycin showed degradation of (*RS*)-2,4-DP which amounted up to 50% of the racemic substrate added. Application of the individual enantiomers indicated exclusive degradation of (*R*)-2,4-DP, the rate of which amounted to 10 ± 2 nmol/min · mg dry weight under these conditions. As with 2,4-D, the *S*-enantiomer was not attacked to a significant extent by these cells (Fig. 1A). Induction of etherolytic activity with respect to (*S*)-2,4-DP should have occurred within about 6–8 h during incubation on (*RS*)-2,4-DP in the absence of streptomycin as follows from the patterns of substrate utilization in Figs. 1A and 1B. At the same time the degradation of 2,4-D was initiated. After being induced the degradation of phenoxyacetate (2,4-D) and phenoxypropionate [(S)-2,4-DP] herbicides proceeded simultaneously in this strain which was also the case for both enantiomers of dichlorprop and mecoprop (not shown). In addition, disappearance of DCP from the culture broth indicated the induction of the whole degradative pathway.

In the case of strain MC1 etherolytic activities with respect to the degradation of both enantiomers of (*RS*)-2,4-DP were constitutively expressed. Whereas the *R*-enantiomer was completely cleaved under these conditions the degradation of the *S*-enantiomer and consequently that of the racemic mixture ceased at a certain level by cultures exposed to streptomycin (Fig. 2A). 2,4-D was, if at all, attacked to a very low degree only. These patterns, i.e. limitations in the extent of the cleavage reaction, are referred to a shortage in the replenishment of α-ketoglutarate at incomplete phenoxyalkanoate degradation (Müller and Babel 2000; see Discussion). Cells grown on (*RS*)-2,4-DP were also capable of completely degrading 2,4-D. Vice versa, cells induced for the degradation of 2,4-D did utilize (*RS*)-2,4-DP. The latter was not suppressed by

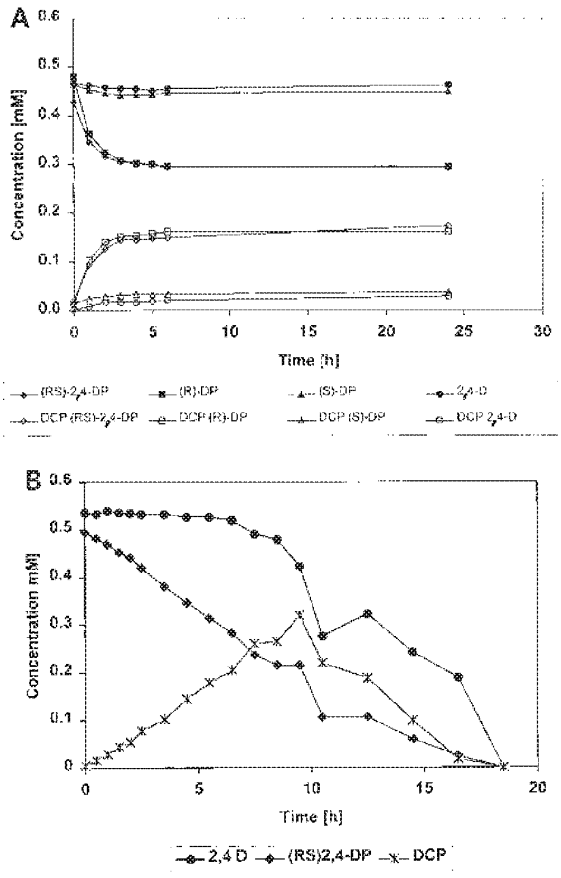


Fig. 1. Degradation pattern of phenoxyalkanoates by *Rhodoferax* sp. P230. The strain was precultivated on 2 g/l fructose and 0.5 g/l yeast extract. Cells from the logarithmic growth phase were collected, washed and resuspended under sterile conditions in mineral medium and incubated with the various herbicides in the absence and presence, respectively, of streptomycin. A, presence of streptomycin: consumption of the individual substrates and formation of DCP, respectively, under non-inductive conditions. B, absence of streptomycin: induction and degradation pattern during simultaneous presence of (*RS*)-2,4-DP and 2,4-D.

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incubating 2,4-D-induced cells in the presence of streptomycin. Induction of the whole degradative pathway occurred within about 3–5 h as monitored by the disappearance of DCP from the medium and reached maximum rates after about 10 h (not shown). Induced cells showed sequential degradation of the phenoxypropionate [(*RS*)-2,4-D] and phenoxyacetate (2,4-D) herbicides under batch conditions (Fig. 2B).

The degradative properties were not stably conserved by strain MC1: growth on complex medium resulted in a drastic loss of phenoxy herbicide-utilizing activity which amounted up to 90% in an overnight culture as indicated by either measuring etherolytic cleavage activities in comparison to cultures grown on (*RS*)-2,4-DP or by staining colonies grown on complex medium for the liberation of dichlorophenol after spraying and incubating for one hour with (*RS*)-2,4-DP. Moreover, these activities were completely lost and the degradation of the herbicides could not be restored after only two to three passages on complex medium. For this reason specific activities were not indicated with cultures of strain MC1 after nonselective growth. In contrast to strain MC1, cultivation of strain P230 did not lead to a remarkable loss of the degradative properties even after many generations of growth under nonselective conditions.

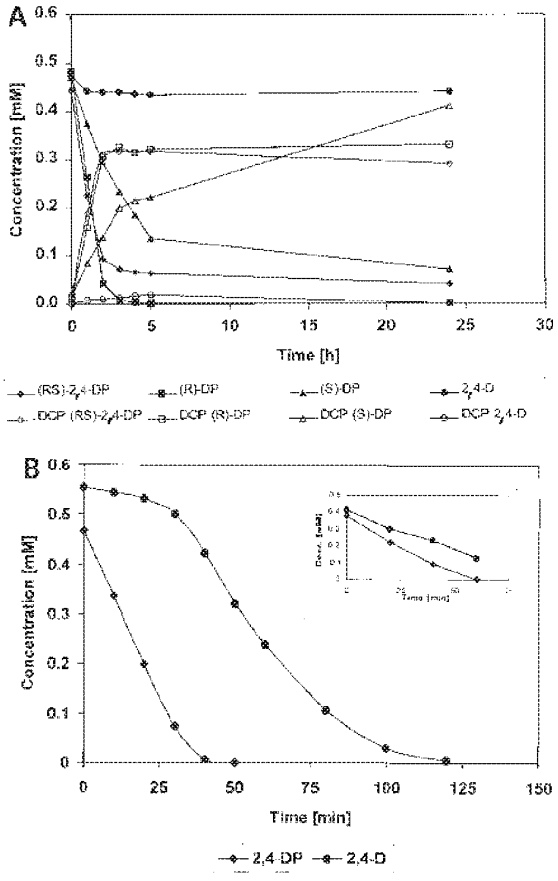


Fig. 2. Degradation pattern of phenoxyalkanoates by *Delftia acidovorans* MC1. The strain was precultivated on PYE medium. Cells from an overnight culture were collected, washed and resuspended under sterile conditions in mineral medium and incubated with the various herbicides in the absence and presence, respectively, of streptomycin. A, presence of streptomycin: consumption of the individual substrates and formation of DCP, respectively, under noninductive conditions. B, absence of streptomycin: induction and degradation pattern during simultaneous presence of (*RS*)-2,4-DP and 2,4-D (in the inset the degradation characteristics by applying single substrates only are shown).

Substrate conversion properties

Strain MC1 is able to grow prototrophically on various phenoxyalkanoates. The maximum growth rates obtained on dichlorinated phenoxyacetate and phenoxypropionate herbicides are shown in Table 1 indicating a rate about threefold as high for (*RS*)-2,4-DP compared to 2,4-D. This property is reflected by the degradation rates of various phenoxyalkanoates (Fig. 1). In addition, these figures make evident, that the dichlorinated derivatives of the phenoxypropionates are degraded with higher rates than the chloromethyl-substituted compounds; the *R*-enantiomers are equally or faster degraded than the *S*-enantiomers. During the degradation experiments some increase in the degradation rates were observed the values of which are shown in brackets. This might result from further induction of cleavage activity but can likely be referred to metabolic reasons in correlation to the regeneration of α-ketoglutarate (Müller and Babel 2000).

In contrast to strain MC1, strain P230 was more difficult to grow on the herbicides and required supplements. Steady state conditions at continuous cultivation on 2 g/l of (*RS*)-2,4-DP were obtained in the presence of 0.5 g/l of yeast extract fed in addition to vitamins (thiamin 5 mg/l, biotin 2 mg/l). The maximum rate at which this strain could grow on (*RS*)-2,4-DP was around 0.04 h^{-1} under these conditions (Table 1). With 2,4-D no stable herbicide consumption (steady state) could be reached in continuous cultivation even in the presence of yeast extract. 2,4-D accumulated even at low dilution rates (around 0.02 h^{-1}) although this strain in general was able to completely degrade this compound under batch conditions. Again the growth properties are reflected by the rates of degradation of the various compounds (Fig. 1). The consumption rate of 2,4-D was only around 10% of the value found with dichlorprop (Table 1). Phenoxybutyrate derivatives

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Table 1. Kinetic parameters of herbicide utilization

Parameter	Strain MC1 Growth on				Strain P230 Growth on	
	(*RS*)-2,4-DP		2,4-D		(*RS*)-2,4-DP	2,4-D
Maximum growth rate (h^{-1})	0.15		0.045		0.037	no steady state
Degradation rate (nmole/min mg dry mass)						
(*RS*)-2,4-DP	31.65		19.95	(29.75)[1)]	10.0	3.1
(*R*)-2,4-DP	21.15	(27.5)	29.5		16.3	n.d.
(*S*)-2,4-DP	4.8	(6.85)	8.45	(14.85)	3.45	n.d.
(*RS*)-MCPP	12.5	(26.65)	9.85	(18.2)	n.d.	n.d.
(*R*)-MCPP	17.9		11.15		5.6	n.d.
(*S*)-MCPP	4.1	(8.65)	5.75	(12.95)	5.85	n.d.
2,4-D	7.6	(n.d.)	9.0	(11.9)	1.4	0.8

Strain MC1 was continuously cultivated on (*RS*)-2,4-DP and 2,4-D at rates of 0.05 h^{-1} and 0.03 h^{-1} respectively. Strain P230 was cultivated on (*RS*)-2,4-DP and 2,4-D under batch conditions.

[1)] Figures correspond to initial and final (within the time of measurement, i.e. up to 6 h) rates; no figure in brackets means constancy of the degradation rate over the assay time.

n.d., not determined

Table 2. Assay conditions for TfdA activity (mU/mg).

Substrate	Assay	Strain MC1	Strain P230
(*R*)-2,4-DP	Complete	124	23.7
	– Ascorbic acid	62	7.0
	– αKG	7	0.2
	– Fe^{2+}	6	<0.1
(*S*)-2,4-DP	Complete	59	1.0
	– Ascorbic acid	2	0.6
	– αKG	1	0.7
	– Fe^{2+}	1	<0.1
2,4-D	Complete	32	n.d.
	– Ascorbic acid	19	n.d.
	– αKG	3	n.d.
	– Fe^{2+}	0	n.d.

Strain MC1 was continuously grown on (*RS*)-2,4-DP at a dilution rate of 0.05 h^{-1}. Strain P230 was derived from batch cultures induced and grown over three passages on (*RS*)-2,4-DP.

n.d., not determined

were not utilized to a significant extent by either of the strains (not shown, cf. (Ehrig *et al.* 1997; Müller *et al.* 1999)).

Enzyme activities

The initial steps of (*RS*)-2,4-DP and 2,4-D degradation, i.e. the etherolytic cleavage, proved to be catalyzed by α-ketoglutarate- and Fe^{2+}-dependent dioxygenases. Omission of either of both components led to an almost complete loss of enzyme activity in the two strains (Table 2). Highest activity with strain MC1 was obtained with (*R*)-2,4-DP; with (*S*)-2,4-DP and 2,4-D, respectively, only about 50% and 25% of this value were found. Remarkably, after omission of ascorbic acid about 50% of the activity remained with (*R*)-2,4-DP and 2,4-D whereas it was almost completely lost with (*S*)-2,4-DP. A similar picture was obtained with (*R*)-2,4-DP in the case of strain P230; the activity with regard to the *S*-enantiomer was low even under conditions of the complete assay (cf. Table 1). This might result from instabilities of the enzyme after cell disintegration as the racemic mixture was utilized by induced whole cells without complications (Fig. 1B). In general, the cleavage activity of strain P230 was significantly lower in comparison to strain MC1. This corresponds in its proportion to the degradation rates of the substrates with whole cells (Table 1). Further metabolism of the chlorophenolic intermediates seems to proceed via the modified *ortho* cleavage pathway in both strains. Enzyme activities representative of this metabolic route were found as shown in Table 3. Again higher rates were measured with strain MC1 compared to strain P230.

Genetic background

PCR primers specific for the 2,4-D degradative genes *tfdA* (encoding a 2,4-D/α-ketoglutarate dioxygenase), *tfdB* (encoding a dichlorophenol hydroxylase), *tfdC* (encoding a chlorocatechol 1,2-dioxygenase) and *tfdD* (encoding a chloromuconate cycloisomerase) were applied to elucidate the genetic background of phenoxy herbicide utilization in the present strains. With the *tfdA*-specific primers, a specific PCR product was only

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Table 3. Enzyme activities for the utilization of phenoxy herbicides.

Enzyme	Substrate	Activity (mU/mg)	
		Strain MC1	Strain P230
2,4-D(PA)/α-KG dioxygenase[1)]	(*RS*)-2,4-DP	73	21
	2,4-D	11	6.9[2)]
Phenol hydroxylase	2,4-dichlorophenol	145	18
Catechol 1,2 dioxygenase	3,5-dichlorocatechol	385	71
cis,cis-Muconic acid cycloisomerase	2,4-dichloro-*cis,cis*-muconate	130	12

Enzyme activities were measured after chemostatic growth on (*RS*)-2,4-DP at D = 0.05 h^{-1} (MC1) and D = 0.02 h^{-1} (P230), respectively.

[1)] Designation of the etherolytic cleavage activities as 2,4-D(phenoxyalkanoate)/α-ketoglutarate dioxygenase instead of 2,4-D/α-ketoglutarate-dioxygenase (TfdA) in correspondence to the broad substrate spectrum utilized.

[2)] After fed-batch growth on 2,4-D in the presence of equimolar concentration of fructose; the feeding cycles were adjusted to the consumption characteristic of 2,4-D.

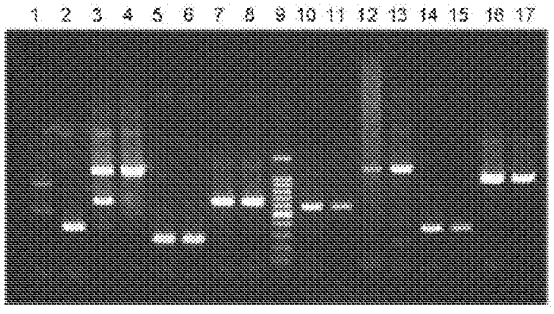


Fig. 3. Agarose gel electrophoresis of the PCR products amplified from the *tfd*-specific primers. Lanes: 1. Strain MC1, primers tfdA-1f and tfdA-2r; 2. Strain P230, primers tfdA-1f and tfdA-2r; 3. Strain MC1, primers tfdB-1f and tfdB-2r; 4. Strain P230, primers tfdB-1f and tfdB-2r; 5. Strain MC1, primers tfdC-1f and tfdC-2r; 6. Strain P230, primers tfdC-1f and tfdC-2r; 7. Strain MC1, primers tfdD-1f and tfdD-2r; 8. Strain P230, primers tfdD-1f and tfdD-2r; 9. 100 bp ladder (Promega); 10. Strain MC1, primers tfdC-1f and tfdD-1r; 11. Strain P230, primers tfdC-1f and tfdD-1r; 12. Strain MC1, primers tfdC-1f and tfdD-2r; 13. Strain P230, primers tfdC-1f and tfdD-2r; 14. Strain MC1, primers tfdC-2f and tfdD-1r; 15. Strain P230, primers tfdC-2f and tfdD-1r; 16. Strain MC1, primers tfdC-2f and tfdD-2r; 17. Strain P230, primers tfdC-2f and tfdD-2r

detected with strain P230, whereas with strain MC1 the amplification of a *tfdA* fragment failed. As the side-chain cleaving activity for 2,4-D and 2,4-DP seemed to be unstable in strain MC1 under nonselective conditions, the PCR was performed with template DNA isolated from liquid cultures continuously grown on the herbicides as sole carbon source. However, this approach confirmed the results from PCR experiments with template DNA extracted from LB-grown colonies. Using the *tfdB*-, *tfdC*- and *tfdD*-specific primer pairs, amplification products of the appropriate sizes were obtained with both strains, reproducibly also under nonselective growth conditions. Furthermore, the application of the various *tfdC* and *tfdD* primer combinations resulted in the amplification of *tfdCD* fragments of the appropriate sizes, indicating that the *tfdC* and *tfdD* genes are organized in a gene cluster in both strains (Fig. 3).

Sequencing of the PCR products revealed high homology to the degradative genes of other chloroaromatics degrading proteobacterial strains. As calculated by BLAST search in the sequence databases, the partial *tfdA* nucleotide sequence of strain P230 (acc. no. AF176240) with a length of 327 bp is identical to the 318-bp partial *tfdA* nucleotide sequence of *Variovorax paradoxus* TV1 (acc. no. U65531), the most similar complete sequence was the *tfdA* gene from *Burkholderia* sp. RASC (acc. no. U25717) with 95% amino acid sequence identity. The 1122-bp partial *tfdB* sequences of strains P230 (acc. no. AF176241) and MC1 (acc. no. AF176242) are identical to each other and 99% identical (1118 nucleotides and 373 of 374 amino acids, respectively) to the *tfdB* gene of the *Achromobacter xylosoxidans* plasmid pEST4011 (acc. no. U32188). Sequencing of the 261-bp *tfdC* fragment of strain P230 failed, as sequence data were ambiguous, indicating that more than one sequence was amplified from this strain by the tfdC-1f and tfdC-2r primers. The 936-bp partial *tfdCD* sequence of strain P230 (acc. no. AF176243) assembled from the sequences of the PCR products amplified from primer pairs tfdC-2f and tfdD-2r, tfdC-2f and tfdD1r, and tfdD-1f and tfdD-2r, respectively, shows 99% identity (935 nucleotides) to the homologous region of the chlorocatechol degradative operon *cbnABCD* of *Ralstonia eutropha* plasmid pENH91 (acc. no. AB019032), while the deduced amino acid sequences were 100% identical to the *cbnA* and *cbnB* gene products. The 1156-bp partial *tfdCD* sequence of strain MC1 (acc. no. AF176244) assembled from the sequences of the PCR products amplified from the various tfdC and tfdD primer combinations is identical to the homologous region of the *cbnABCD* operon of *R. eutropha* plasmid pENH91. Remarkably, the 231-bp partial *tfdC* sequence (amplified from primers tfdC-1f and tfdC-2r) of strain MC1 published previous-

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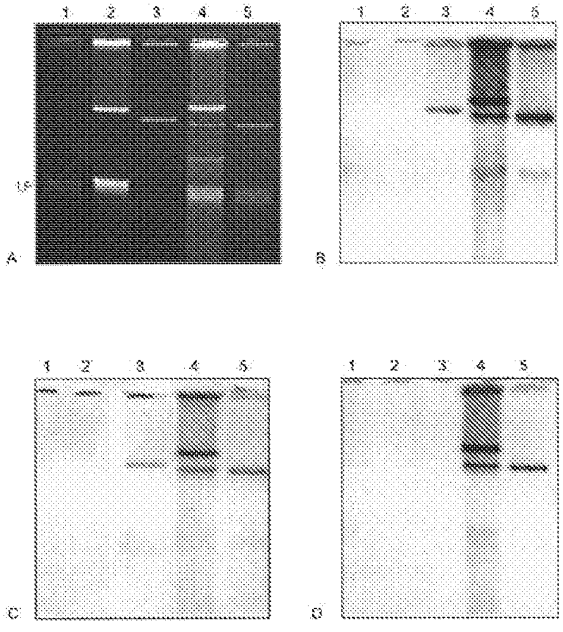


Fig. 4. Agarose gel electrophoresis of plasmid DNA (A) and Southern hybridization with the *tfdB* (B), *tfdC* (C), and *tfdD* (D) probes from strain MC1. Plasmids of known molecular weight were applied as size markers. Lanes: 1. Strain P230; 2. pWWO (117 kb) from *Pseudomonas putida* mt-2; 3. pJP4 (80 kb) from *Ralstonia eutropha* JMP134; 4. Strain MC1 (wild type); 5. Strain MC1-Mut (degradation-defective mutant).
LF: linear fragments

ly (Kleinsteuber *et al.* 1998; acc. no. AF077917) differs from this contig formed by the other *tfdCD* sequences, as was confirmed by repeated PCR amplification and sequencing of the PCR product with primers tfdC-1f and tfdC-2r. Strain MC1 obviously possesses two different *tfdC*-like genes, and with the primer pair tfdC-1f and tfdC-2r this previously sequenced *tfdC* is preferably amplified, whereas the other primer combinations prefer the *tfdCD* gene cluster sequences. According to the genetic organization of the *tfd* genes in *R. eutropha* JMP134 plasmid pJP4, we rename the previously described *tfdC*-like gene of strain MC1 (acc. no. AF077917) *tfdCII*. This partially sequenced gene exhibits 94% amino acid sequence identity to *tfdCII* from pJP4 and only 59% to *tfdC* of pJP4.

As xenobiotic-degradative genes are often located on plasmids, both herbicide-degrading strains were screened for the presence of plasmids. No plasmid was detected in strain P230 despite the application of various extraction methods, indicating that the herbicide degradative trait is encoded chromosomally or on a very large plasmid that could not be detected by the isolation procedures. With strain MC1, two plasmid bands of about 120 kb and 80 kb were detected (Fig. 4A). Whereas the 80-kb plasmid band could be obtained reproducibly, the 120-kb plasmid band was often lost. Remarkably, the lack of the 120-kb band correlated with the loss of the phenoxy herbicide-degrading activity. Mutants spontaneously emerging under nonselective conditions and defective in 2,4-D and 2,4-DP cleaving activity exhibited various smaller plasmids, while the 120-kb band was always absent. The-80 kb mutant plasmid band was also found in wild-type plasmid preparations from LB cultures grown overnight which had been inoculated from selective medium, indicating that a deletion of about 40 kb frequently occurs in liquid complex medium. Mutant strains carrying only the 80-kb plasmid or sometimes smaller plasmids were also obtained from repeated passages on LB agar plates. Southern hybridization with the *tfdB*-, *tfdC*- and *tfdD*-specific PCR products as probes revealed plasmidal localization of the *tfdB*, *tfdC* and *tfdD* genes (Figs. 4B–4D). Hybridization signals were detected not only with the wild-type 120-kb plasmid but also with the 80-kb mutant plasmid, which is consistent with the finding that the 2,4-D-/2,4-DP-defective mutants are still able to mineralize 2,4-dichlorophenol.

Discussion

Rhodoferax sp. P230 and *Delftia acidovorans* MC1 are characterized by their ability to completely utilize phenoxyacetate and enantiomeric phenoxypropionate herbicides. For both strains phenoxypropionates [(*RS*)-2,4-DP] are the preferred substrates in comparison to phenoxyacetates (2,4-D) as derived e.g. from growth rates. Despite of these similarities general differences emerged between the two strains. *Rhodoferax* sp. P230 in general was difficult to cultivate under heterotrophic conditions. This applied to either conventional substrates (fructose, acetate, gluconate) but the more to herbicides; growth required supplements such as biotin and thiamine but even more yeast extract to stabilize growth under continuous conditions. With (*RS*)-2,4-DP, continuous growth could be established under these conditions at dilution rates below 0.04 h^{-1}; with 2,4-D, in contrast, steady state conditions could never be reached. This might be caused by too low a substrate consumption rate with 2,4-D as followed from enzyme activities (Table 3), i.e. too low an energy flow rate, to enable both maintenance and growth (cf. (Müller and Babel 1996)). By contrast, *D. acidovorans* MC1 grew well under prototrophic conditions on phenoxypropionate [(RS)-2,4-DP; μ_{max} = 0.15 h^{-1}]

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and phenoxyacetate (2,4-D, μ_{max} = 0.05 h^{-1}) herbicides as the substrates.

The enantiomeric substrates were completely utilized by both strains. Taking into account that *R*- and *S*-enantiomers have to be considered to be individual substrates (Armstrong *et al.* 1993, Zipper 1998) two distinct enzymes were surmised for the initial step of cleaving the substrate. Their presence follows from the constitutive and inductive patterns in the various strains. Accordingly, *R*-cleaving activity was constitutively established in strain P230 comparably to the enzyme responsible for the cleavage of the *S*-enantiomer in the case of *S. herbicidovorans* MH (Nickel *et al.* 1997). Corresponding cleavage activities for the other enantiomers were induced in the presence of the racemic substrate. Two distinct enzymes have also been confirmed with strain MC1 in which activities for cleavage of both enantiomers were constitutively expressed. Here, two separate enzymes with different specificity with respect to the two enantiomers could be demonstrated by chromatographic separation (Müller and Babel 1999). In addition, the *S*-specific enzyme activity seems to be responsible for the cleavage of 2,4-D too, as followed from the chromatographic separation of the proteins: the enzyme responsible for the etherolytic cleavage of 2,4-D co-eluted with that for (*S*)-2,4-DP both in ion exchange and hydrophobic interaction chromatography (Müller and Babel 1999). Such dual substrate specificity has been assumed to be the case also with the enzyme of *S. herbicidovorans* MH (Nickel *et al.* 1997).

Despite of this fact, 2,4-D was not found to disappear to a significant extent under non-induced conditions in either of our strains. Enzymatic investigations have shown that in both strains the degradation of the two enantiomers of (*RS*)-2,4-DP and of 2,4-D is initiated by an α-ketoglutarate-dependent dioxygenase. Consequently, the availability of α-ketoglutarate in this two-substrate reaction is a necessary pre-condition to maintain the etherolytic cleavage of phenoxyalkanoates. Its replenishment is restricted in the case of incomplete degradation by liberating DCP as a dead-end product which holds the more with phenoxyacetates than with phenoxypropionates (Müller and Babel 2000).

Further degradation seems to follow the modified *ortho* cleavage pathway, as confirmed by enzymatic and genetic investigations. Application of specific PCR primers and DNA sequence analyses revealed the presence of *tfdB*-, *tfdC*- and *tfdD*-like genes in both strains with high similarity to the homologous genes of other chloroaromatic-degrading proteobacterial strains. Sequence data suggest a mosaic-like structure, being composed of DNA fragments of different bacterial origins, a well-known phenomenon described for other 2,4-D degrading strains (Fulthorpe *et al.* 1995; Vallaeys *et al.* 1996, 1999). According to the phylogenetic trees of the *tfdB*-like and *tfdC*-like gene families as described elsewhere (Vallaeys *et al.* 1999), the *tfdB* genes in both our strains are from the *A. xylosoxidans* plasmid pEST4011-like type; by contrast, the *tfdC* and *tfdD* genes apparently arranged in one gene cluster belong to the *Pseudomonas* sp. P51-like group of chlorocatechol catabolic operons. Furthermore, strain MC1 additionally harbors a *tfdCII* gene of the *A. xylosoxidans* pEST4011-like type. Taking into account the sequence ambiguities of the *tfdC*-specific PCR product of strain P230, this might also be possible in this case. Regarding the congruent pattern of the *tfdB, tfdC* and *tfdD* organization in both strains, the common ancestry of these herbicide-degrading strains isolated from the same site of contaminated building rubble is likely. This also holds for *Delftia acidovorans* P4a isolated from the same habitat (Hoffmann *et al.* 1996), which has nearly identical *tfdB* and *tfdC* genes as strains P230 and MC1 and a nearly identical *tfdA* gene as strain P230 (D. Hoffmann, personal communication).

Despite these common genetic characteristics of strains P230 and MC1, there is a fundamental difference in the genetic encoding of the initial enzymatic step in herbicide degradation. With strain P230, a *tfdA*-like gene with high similarity to the homologous genes of other 2,4-D cleaving strains was detected. According to the classification of *tfdA-like* genes (McGowan *et al.* 1998), it is a class III type *tfdA* gene. Class III *tfdA* genes have also been found in randomly isolated and phylogenetically diverse bacteria not degrading 2,4-D, suggesting that *tfdA*-like genes are maintained by these bacteria for another function not related to herbicide degradation (Hogan *et al.* 1997). In contrast, no *tfdA*-like gene could be detected in strain MC1 even under selective conditions, despite 2,4-D/α-ketoglutarate dioxygenase activity being measured. Consequently, this activity must be encoded by a non-*tfdA*-like gene. Whether this gene also encodes one or both of the 2,4-DP cleaving activities remains unknown. However, the spontaneous loss of 2,4-D cleaving activity occurring under nonselective conditions seemed to be accompanied by the loss of 2,4-DP cleaving activities and correlated with the lack of the 120-kb plasmid band, suggesting at least a linkage between the respective genes. Further experiments are required to clarify whether the herbicide-cleaving activities of strain MC1 are actually encoded on the deleted 40-kb plasmid fragment. If the genes encoding the 2,4-DP cleaving dioxygenases are actually localized on this plasmid fragment this will explain the instability of the plasmid and of the degradation property under non-selective conditions, since maintenance of plasmids with constitutively expressed catabolic genes under non-selective

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conditions represents a physiological burden to the cell (Sayler *et al.* 1990).

Although the nucleotide sequences of various 2,4-D/α-ketoglutarate dioxygenase genes (*tfdA*) are known, there are not yet any data concerning the genetic encoding of the initial cleaving step in the degradation of the phenoxypropionic acid herbicides. Further genetic studies on strain MC1 will provide insights into the molecular reasons for the substrate spectra/substrate specificity of phenoxy herbicide-cleaving enzymes, and may yield more information on the evolutionary origin of phenoxy herbicide-degradative genes and on the mechanisms of recruiting existing genes by microorganisms to establish novel degradative pathways.

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Sir:

In response to the non-final Office Action mailed March 10, 2020, Applicant submitted a response on September 9, 2020 with an accompanying supporting Declaration under 37 CFR 1.132. Upon further review of those documents Applicant realized that citation 12 listed below the Tables presented in both Appendix II and III contained a transposition of two numbers. Accordingly, the relevant citation should have been "WO2005/107437" and not "WO2005/104737".

Respectfully submitted,

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(54) Title: NOVEL HERBICIDE RESISTANCE GENES

(57) Abstract: 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.

NOVEL HERBICIDE RESISTANCE GENES

Cross-Reference to Related Application

[0001] This application claims priority to U.S. provisional application Serial No. 60/567,052, filed April 30, 2004.

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 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 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-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 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 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.

[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.

[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*.

[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).

[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.

[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.

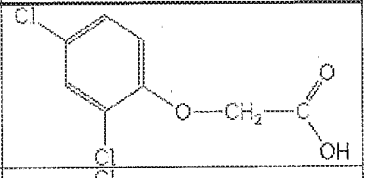
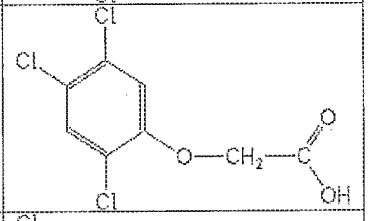
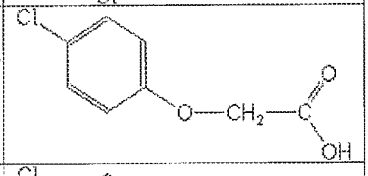
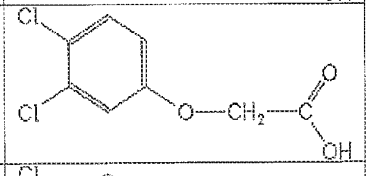
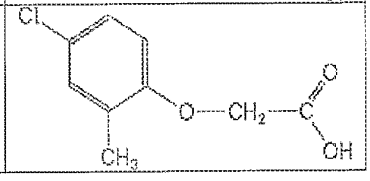
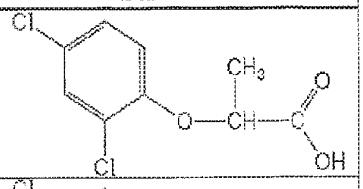
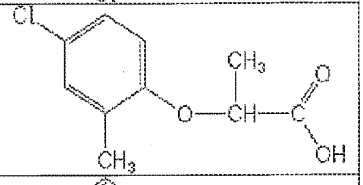
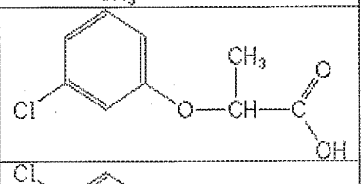
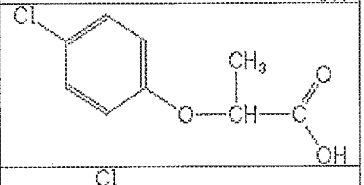
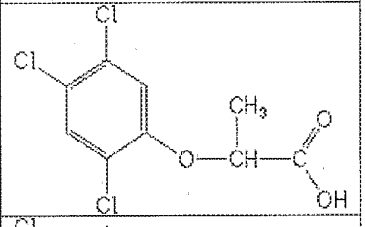
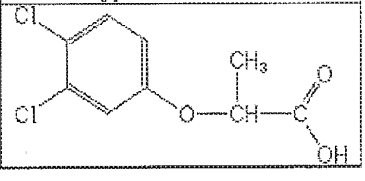
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	
MCPA	94-74-6	25 – 4000	125 - 1550	

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
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 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.

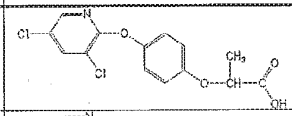
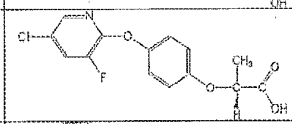
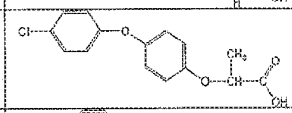
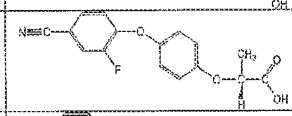
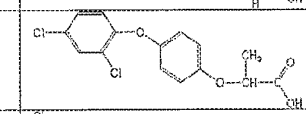
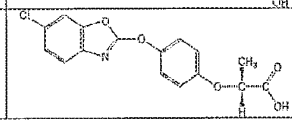
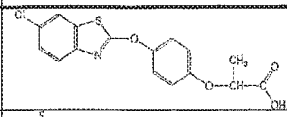
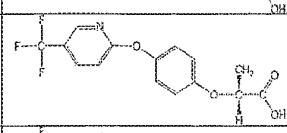
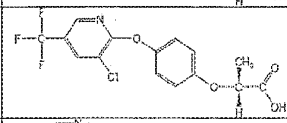
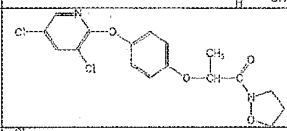
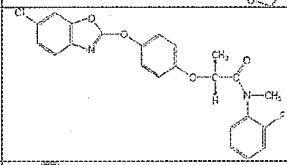
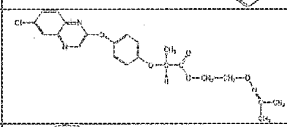
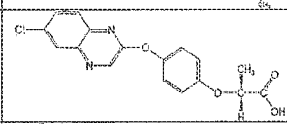
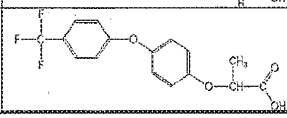
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	
Fenoxaprop	66441-23-4	10-2000	20-200	

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
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 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 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, 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% 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 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 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 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.

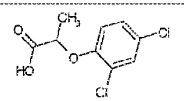
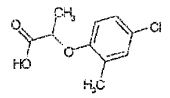
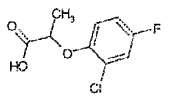
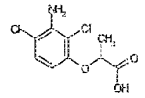
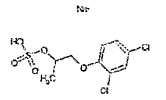
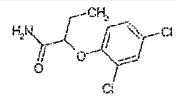
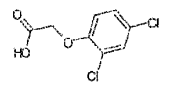
[00105] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed (auxins) and grass weeds (AOPPs). This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can 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.

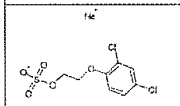
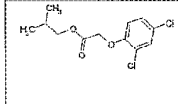
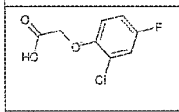
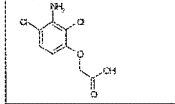
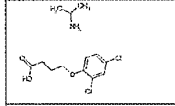
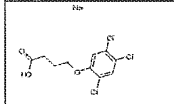
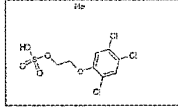
[00106] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable 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**).

[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.

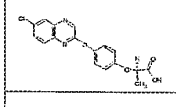
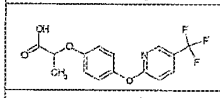
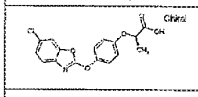
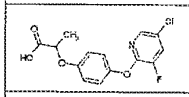
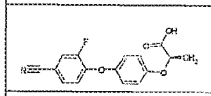
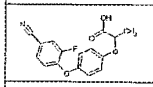
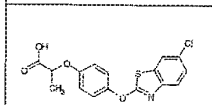
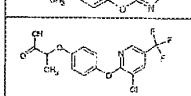
Table 3: Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

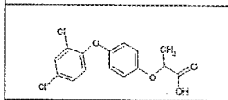
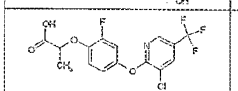
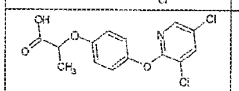
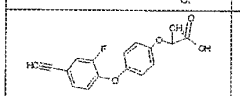
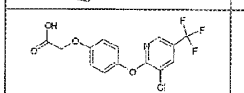
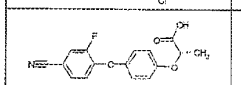
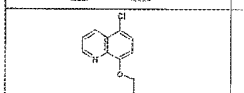
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383

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

[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)
	18706	(R)-quizalofop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17
	11044492	(R)-fenoxaprop	0.408	0.597
	34697	(R,S)-clodinofop	0.295	1.98
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiaprop	0.199	1.055
	66905	haloxyfop	0.172	1.63

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	460511	(R,S)-diclofop	0.155	1.663
	25646		0.144	1.69
	70222	(R,S)-chlorazifop	0.128	1.584
	199608	cyhalofop	0.114	1.26
	43865	haloxyfop-oxyacetate	0.004	0.053
	7466	(S)-cyhalofop	0.003	0.017
	204558	Cloquintocet	0	0.001

[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

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),

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

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.*).

[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.

[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:

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

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,

"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

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,

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.

[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

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.

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.

[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.

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.

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.

[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

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

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

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

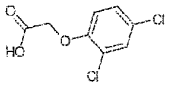
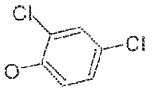
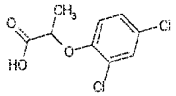
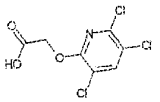
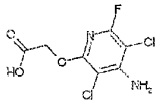
[00187] *Arabidopsis* seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 µL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @ 250 rpm). Bleach solution was decanted inside a sterile hood and rinsed three times with sterile water.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

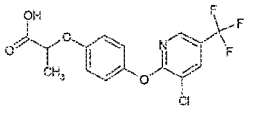
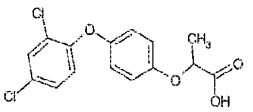
Table 6. *Arabidopsis* plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

	GR_{50} (nM)				
Chemical tested	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.

	GR_{50} (nM)				
Chemical tested	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.

[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

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

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

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).

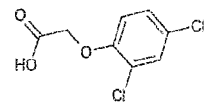
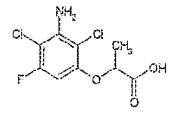
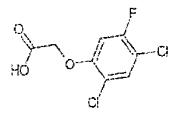
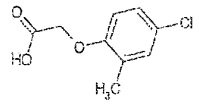
[00209] **Table 4** shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly

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*.

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.

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 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 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.

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

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.

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,

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).

[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

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).

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

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.

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

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

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.

[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

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).

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

[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.

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
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) 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	90.7	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

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.

[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

[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

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.

[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

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).

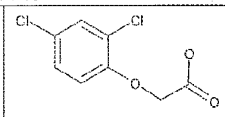
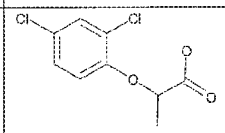
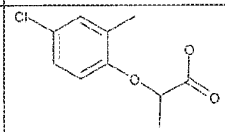
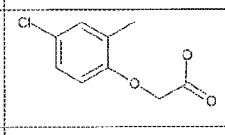
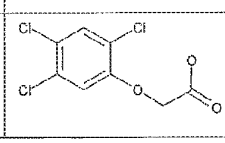
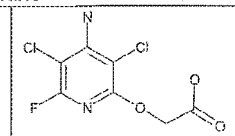
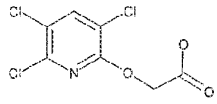
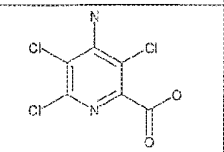
Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Phenoxy auxins							
2,4-D		0.2	10	< 0.01	0.04	50	> 4
dichlorprop		2	5	0.01	1.5	2.5	150
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T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

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.

[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

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

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

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.

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

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

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

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

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.

[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.

[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.

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.

[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

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

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.

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

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.

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 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)

[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).

[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

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.

[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

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

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 actetosyringone, 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

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					

[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

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.

[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

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.

[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**).

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

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**).

[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.

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.

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

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

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

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.

[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%

[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.

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.

[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.

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

[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
1	2	pos	***
2	1	pos	***
3	1	pos	***
4	2	pos	***
5	1	pos	***
6	1	pos	neg
7	1	pos	****
8	1	pos	*****
9	2	pos	*
10	1	pos	*
11	1	pos	*****
12	1	pos	*

Line	Copy #	PTU	Western
43	1	pos	*****
44	low DNA	pos	*****
45	1	pos	*****
46	2	pos	*****
47	1	pos	***
48	1	pos	***
49	3	faint pos	neg
50	1	pos	****
51	4	pos	neg
52	2	pos	****
53	1	pos	***
54	1	pos	****

Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western
13	1	pos	*
14	1	pos	**
15	1	pos	****
16	1	pos	*****
17	1	pos	*
18	1	pos	***
19	1	pos	**
20	1	pos	*****
21	2	pos	*****
22	1	pos	*****
23	1	pos	*****
24	4	pos	* or neg
25	1	pos	****
26	1	pos	****
27	low DNA	pos	*****
28	low DNA	pos	**
29	low DNA	pos	*****
30	17	pos	*
31	low DNA	pos	*****
32	low DNA	pos	*****
33	low DNA	pos	****
34	low DNA	faint pos	*****
35	low DNA	pos	****
36	low DNA	pos	neg
37	low DNA	pos	****
38	low DNA	neg	*****
39	1	pos	****
40	low DNA	pos	*
41	low DNA	pos	*
42	low DNA	pos	**
standard	AAD1	5ug/ml	*****
standard	AAD1	0.5ug/ml	**

Line	Copy #	PTU	Western
55	1	pos	neg
56	5	pos	*
57	2	faint pos	neg
58	8	pos	****
59	2	pos	****
60	5	pos	*****
61	1	pos	**
62	1	pos	**
63	1	pos	***
64	1	pos	***
65	3	pos	****
66	5	pos	*
67	6	pos	*
68	Low DNA	pos	neg
69	Low DNA	pos	****
70	low DNA	pos	**
71	low DNA	faint pos	**
72	low DNA	pos	****
73	low DNA	neg	***
74	low DNA	faint pos	***
75	Low DNA	pos	***
76	Low DNA	pos	neg
77	low DNA	faint pos	****
78	low DNA	pos	****
79	1	pos	**
80	low DNA	pos	***
81	low DNA	pos	***
82	low DNA	pos	***
83	low DNA	neg	****
84	low DNA	pos	***
85	low DNA	pos	**
86	low DNA	pos	*

[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.

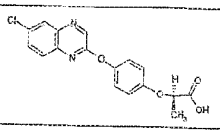
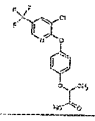
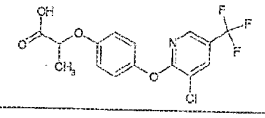
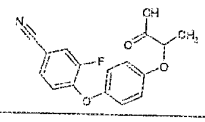
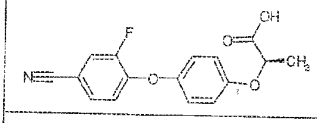
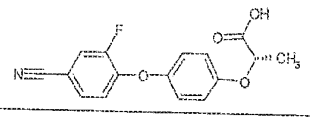
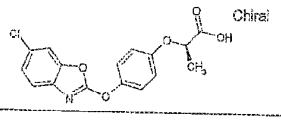
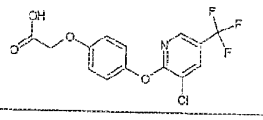
[00439] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (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
	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 florometer (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

Claim 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.

Claim 2.

The cell of claim 1 wherein said plant cell is selected from the group consisting of dicotyledonous cells and monocotyledonous cells.

Claim 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.

Claim 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.

Claim 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.

Claim 6.

The plant of claim 5 wherein said herbicide is a phenoxy auxinic herbicide.

Claim 7.

The plant of claim 5 wherein said herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

Claim 8.

The plant of claim 5 wherein said herbicide is an aryloxyphenoxypropionate.

Claim 9.

The plant of claim 5 wherein said herbicide is selected from the group consisting of fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, metamifop, cyhalofop, and clodinofop.

Claim 10.

The plant of claim 5 wherein expression of said polynucleotide renders said plant resistant to both a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

Claim 11.

The plant of claim 5 wherein said plant further comprises a second herbicide resistance gene.

Claim 12.

The plant of claim 11 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).

Claim 13.

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 of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

Claim 14.

The method of claim 13 wherein said herbicide is an R-enantiomer of a chiral phenoxy auxin.

Claim 15.

The method of claim 14 wherein said herbicide is selected from the group consisting of R-dichlorprop and R-mecoprop.

Claim 16.

The method of claim 13 wherein said herbicide is an achiral phenoxy auxin.

Claim 17.

The method of claim 16 wherein said herbicide is selected from the group consisting of 2,4-D and MCPA.

Claim 18.

The method of claim 13 wherein said first herbicide is an aryloxyphenoxypropionate and said plant is a monocot.

Claim 19.

The method of claim 18 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, turf grass, oats, sorghum, and pasture grasses.

Claim 20.

The method of claim 13 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

Claim 21.

The method of claim 20 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

Claim 22.

The method of claim 13 wherein said method comprises applying a second herbicide.

Claim 23.

The method of claim 22 wherein said first herbicide and said second herbicide are applied sequentially.

Claim 24.

The method of claim 22 wherein said first herbicide and said second herbicide are applied concurrently.

Claim 25.

The method of claim 22 wherein said first herbicide is a phenoxy auxin and second herbicide is an aryloxyphenoxypropionate.

Claim 26.

The method of claim 13 wherein said plant is resistant to glyphosate.

Claim 27.

The method of claim 14 wherein said R-enantiomer is a component of a racemic mixture.

Claim 28.

The method of claim 22 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

Claim 29.

The method of claim 28 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.

Claim 30.

The method of claim 28 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.

Claim 31.

The method of claim 30 wherein said second herbicide is an acetolactate synthase inhibitor selected from the group consisting of imidazolinones, sulfonyl ureas, and triazolopyrimidine herbicides.

Claim 32.

The method of claim 31 wherein said second herbicide is an imidazolinone selected from the group consisting of imazamox, imazethapyr, imazaquin, and imazapic.

Claim 33.

The method of claim 28 wherein said first herbicide is a phenoxy auxin and said second herbicide is selected from the group consisting of glyphosate and glufosinate.

Claim 34.

The method of claim 33 wherein said phenoxy auxin is 2,4-D and said second herbicide is glyphosate.

Claim 35.

The method of claim 28 wherein said first herbicide is an aryloxyphenoxypropionate and said second herbicide is glyphosate.

Claim 36.

The method of claim 35 wherein said first herbicide is selected from the group consisting of quizalofop, haloxyfop, and cyhalofop.

Claim 37.

The method of claim 31 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.

Claim 38.

The method of claim 28 wherein said method further comprises applying a third herbicide.

Claim 39.

The method of claim 38 wherein said herbicides are 2,4-D; quizalofop; and glufosinate.

Claim 40.

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.

Claim 41.

The method of claim 40 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.

Claim 42.

The method of claim 40 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.

Claim 43.

The method of claim 42 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate

Claim 44.

A polynucleotide optimized for expression in a plant wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

Claim 45.

The polynucleotide of claim 44 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

Claim 46.

The polynucleotide of claim 44 wherein said polynucleotide comprises SEQ ID NO:5.

Claim 47.

An isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell.

Claim 48.

The polynucleotide of claim 47 wherein said promoter is a plant promoter.

Claim 49.

The polynucleotide of claim 47 wherein said promoter is a cassava vein mosaic virus promoter.

Claim 50.

A method of selecting for a transformed plant cell wherein said method comprises subject a plurality of plant cells to transformation with a polynucleotide of claim 44 or 47 and 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, wherein said herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxyalkanoate.

Claim 51.

The method of claim 50 wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

Claim 52.

A polynucleotide of claim 47 or 50 wherein said polynucleotide is used as a selectable marker in the method of claim 50.

Claim 53.

A method of detecting whether a plant comprises a polynucleotide of claim 47 or 50 wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

Claim 54.

The method of claim 53 wherein said method comprises assaying said sample for the presence of a protein encoded by said polynucleotide.

Claim 55.

The method of 53 wherein said method comprises using a PCR primer or probe to detect for the presence of said polynucleotide.

Claim 56.

The method of 54 wherein said method comprises using an antibody to detect for the presence of said protein.

Claim 57.

A seed comprising a plant cell of claim 1.

Claim 58.

A plant grown from the seed of claim 57.

Claim 59.

A part, progeny, or asexual propagate of the plant of claim 5.

Claim 60.

The method of claim 13 wherein said method is used to treat or prevent herbicide-resistant weeds.

Claim 61.

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*.

Claim 62.

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.

Claim 63.

A method of conferring 2,4-D herbicide resistance to a crop wherein said method comprises introducing into at least one plant cell of said crop a nucleic-acid-encoding enzyme having R-specific aryloxyalkanoate dioxygenase activity relative to a 2,4-D herbicide.

Claim 64.

A method of controlling glyphosate-resistant weeds in a field of glyphosate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 47, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

Claim 65.

The method of claim 64 wherein said herbicide is a phenoxy auxin.

Claim 66.

The method of claim 64 wherein said herbicide is an aryloxy phenoxypropionate.

Claim 67.

The method of claim 64 wherein said plants are dicots.

Claim 68.

The method of claim 64 wherein said plants are monocots.

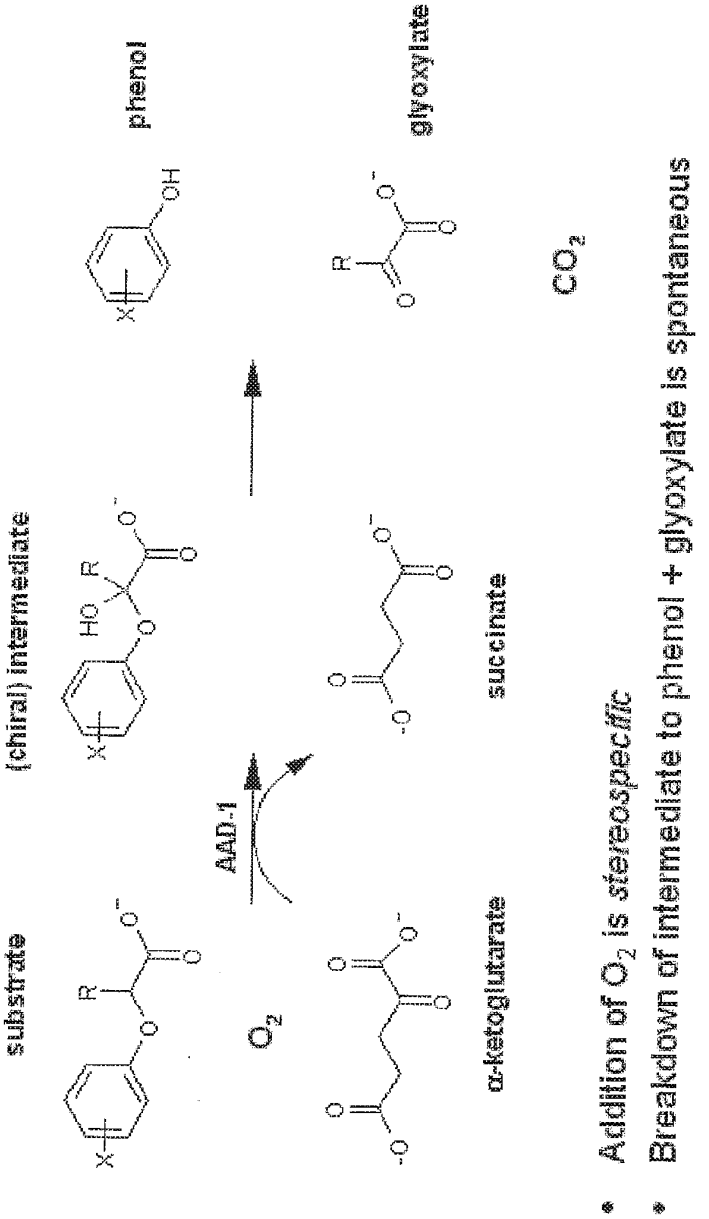

substrate
(chiral) intermediate
phenol
glyoxylate
AAD-1
O_2
α-ketoglutarate
succinate
CO_2
* Addition of O_2 is *stereospecific*
* Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

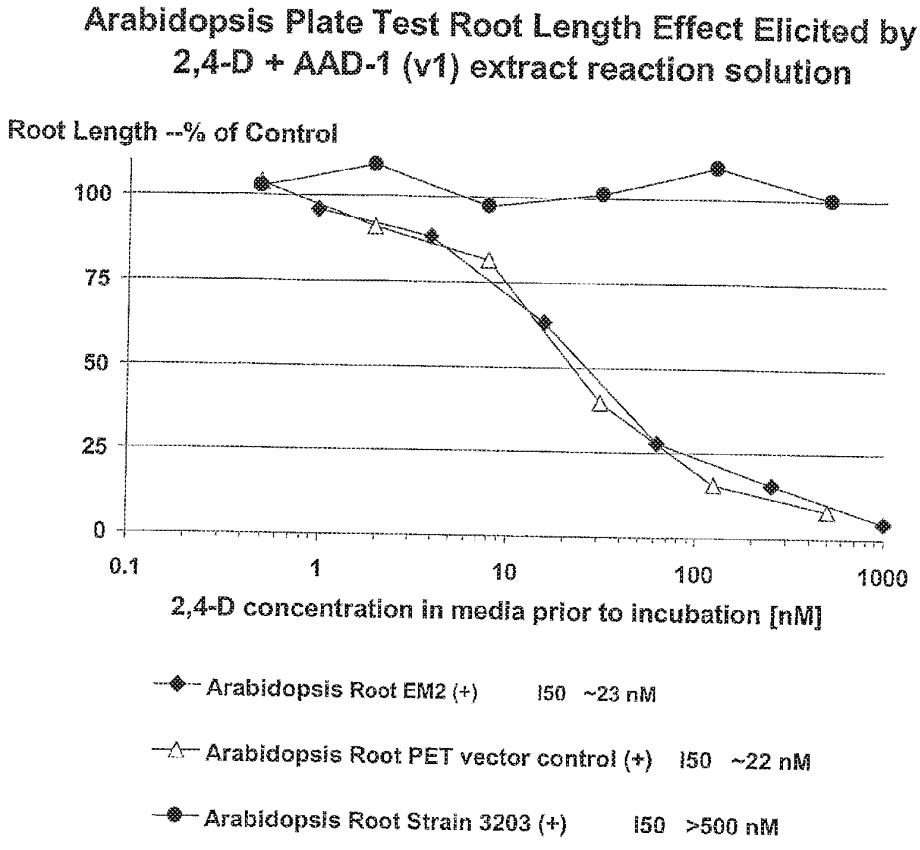

Arabidopsis Plate Test Root Length Effect Elicited by
2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

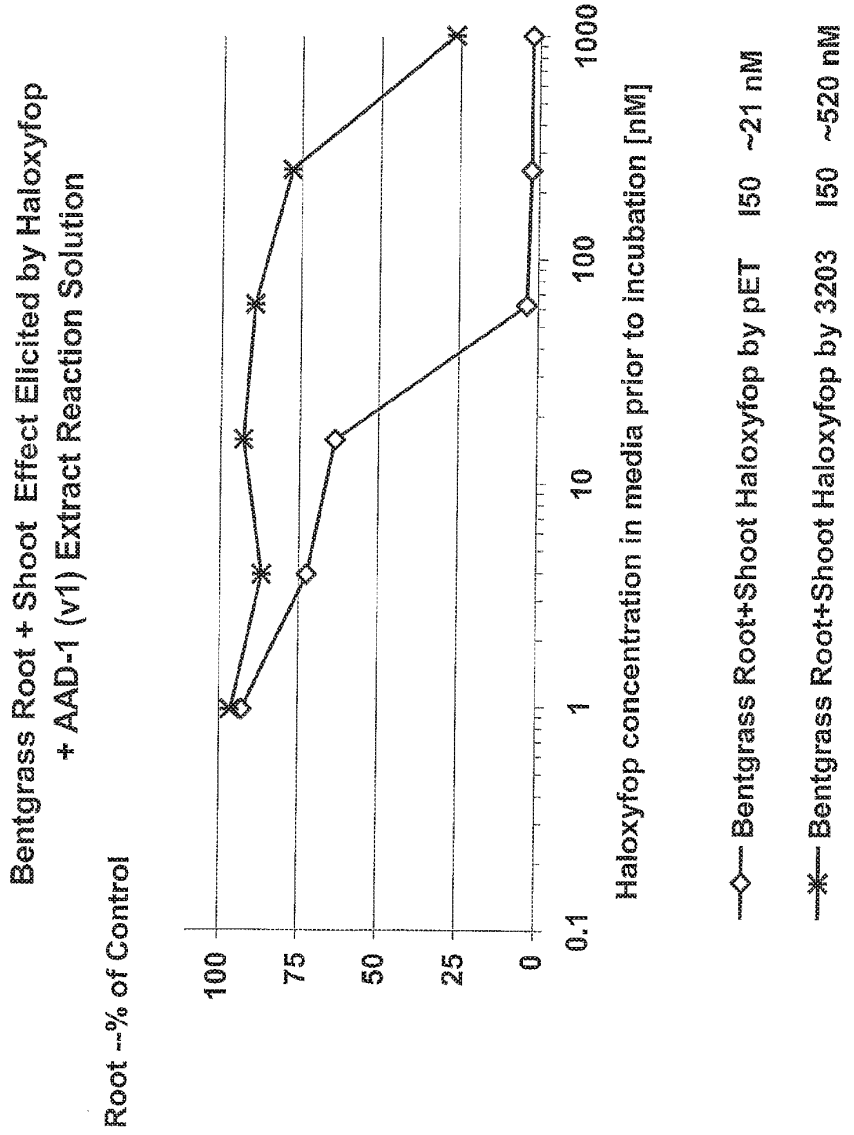

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

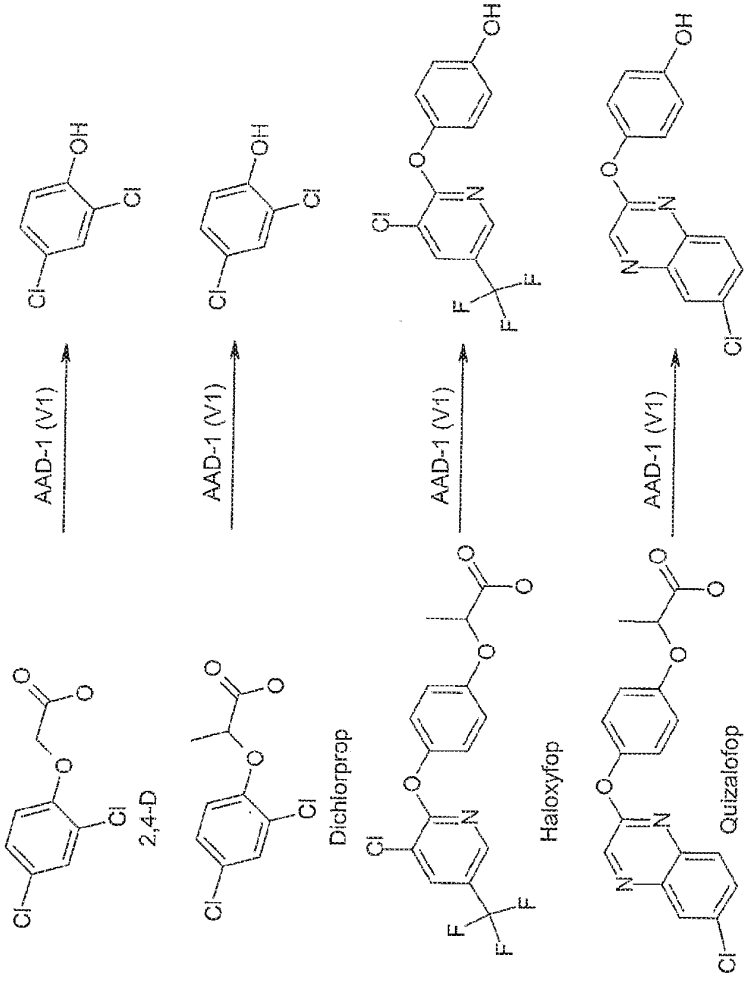

AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
2,4-D
Dichlorprop
Haloxyfop
Quizalofop

Fig. 4

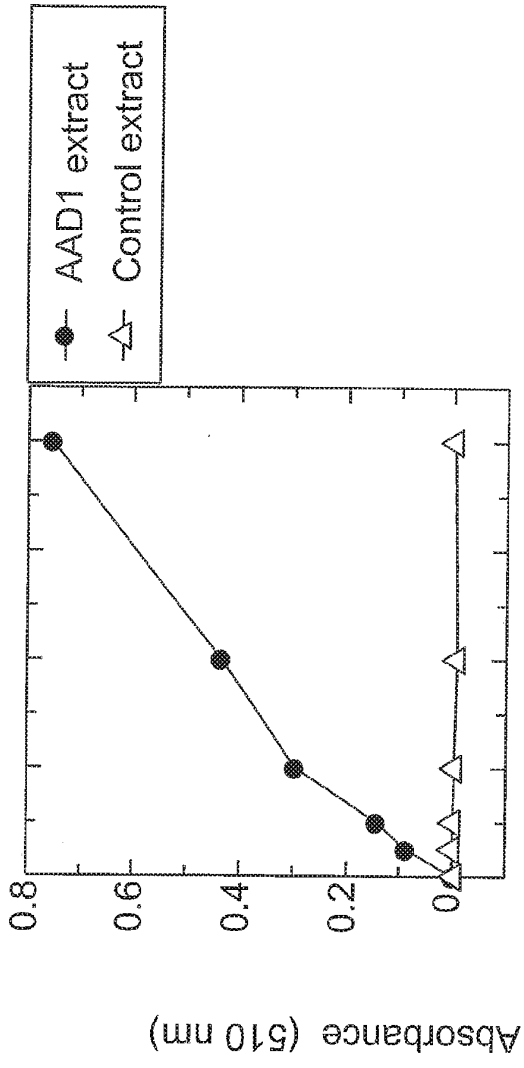

AAD1 extract
Control extract
Absorbance (510 nm)
0.8
0.6
0.4
0.2
0
0
2
4
6
8
µl Extract

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

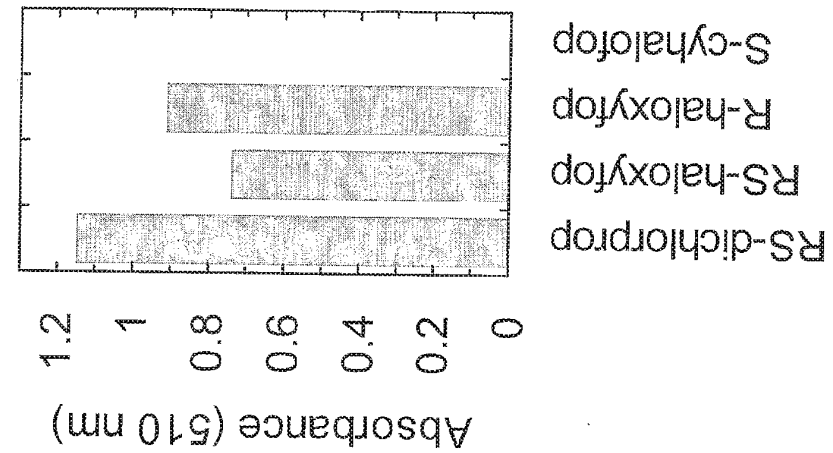

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
RS-dichlorprop
RS-haloxyfop
R-haloxyfop
S-cyhalofop

Fig. 6B

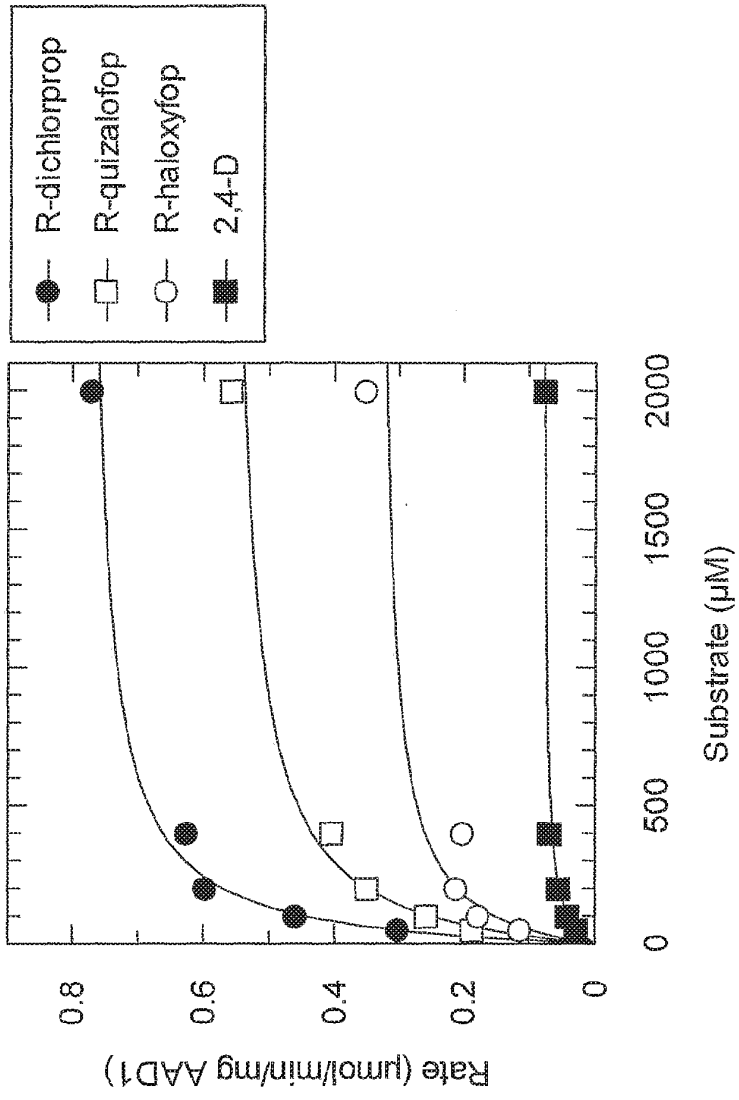

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (µmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (µM)

Fig. 7

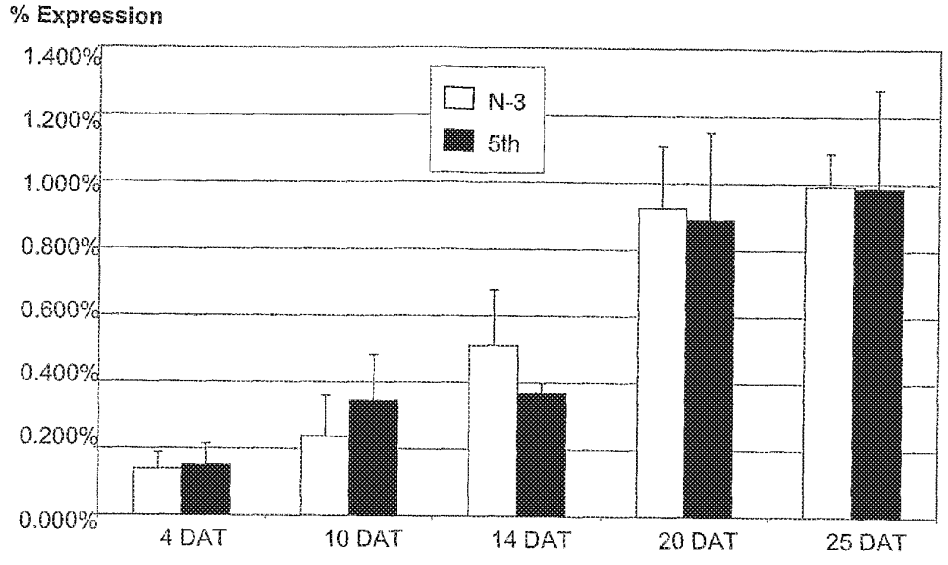

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

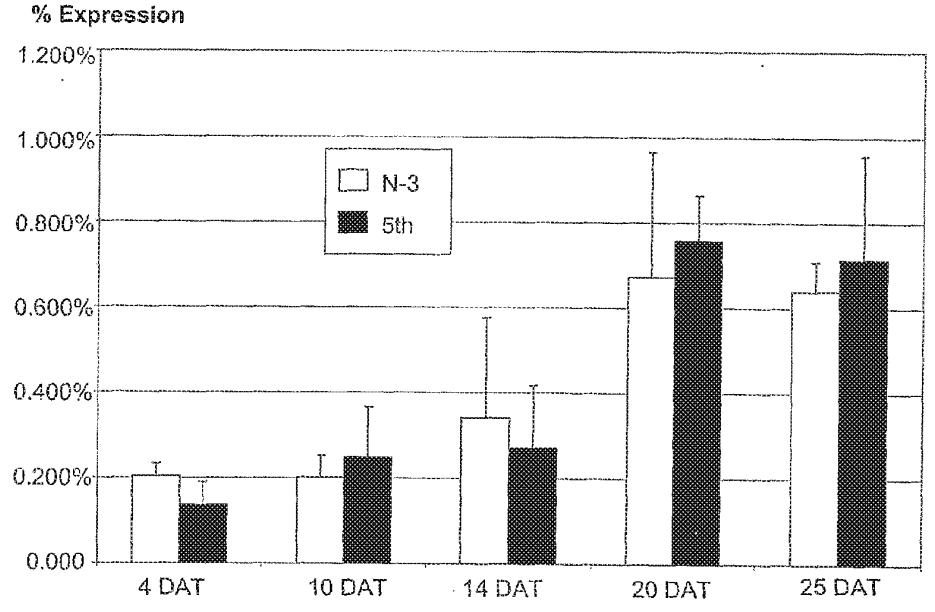

% Expression
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

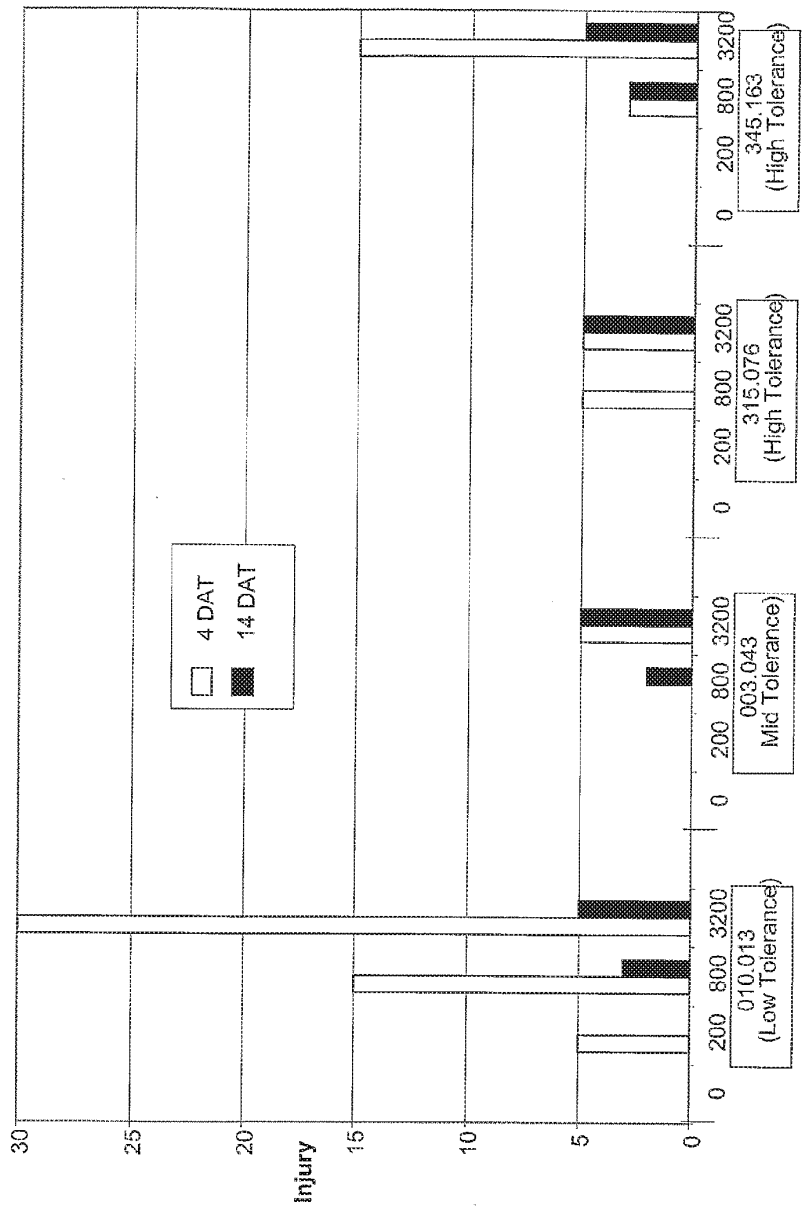

4 DAT
14 DAT
Injury
0
5
10
15
20
25
30
0
200
800
3200
010.013
(Low Tolerance)
003.043
Mid Tolerance)
315.076
(High Tolerance)
345.163
(High Tolerance)

Fig. 9A

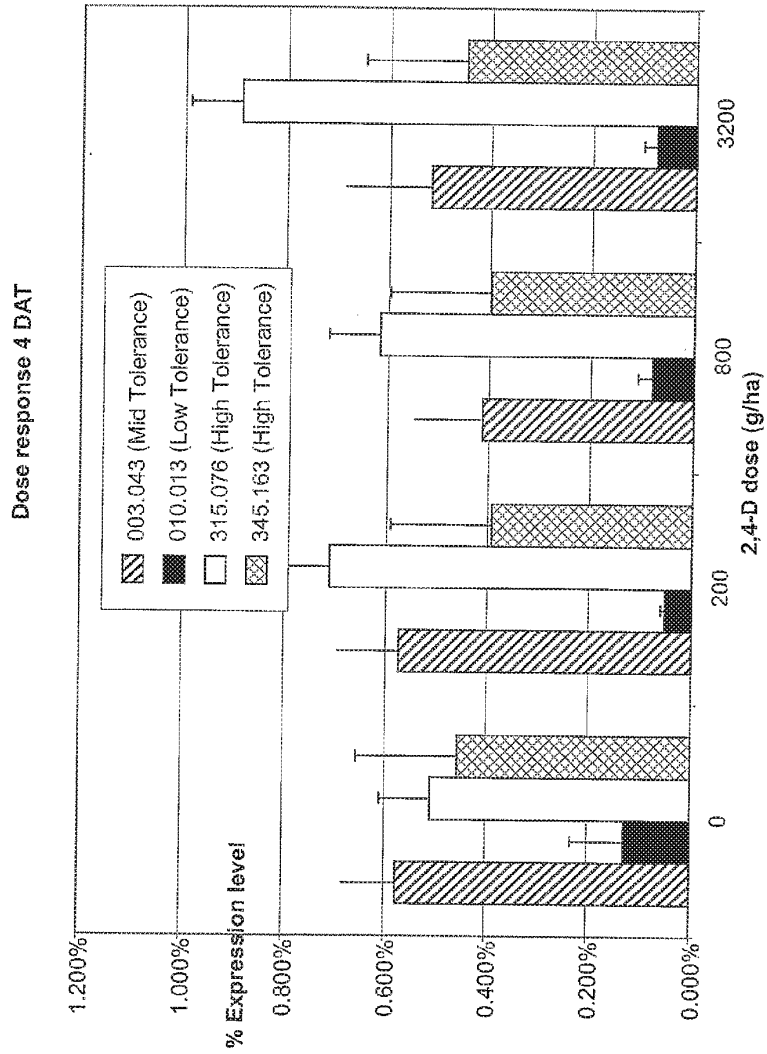

Dose response 4 DAT
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

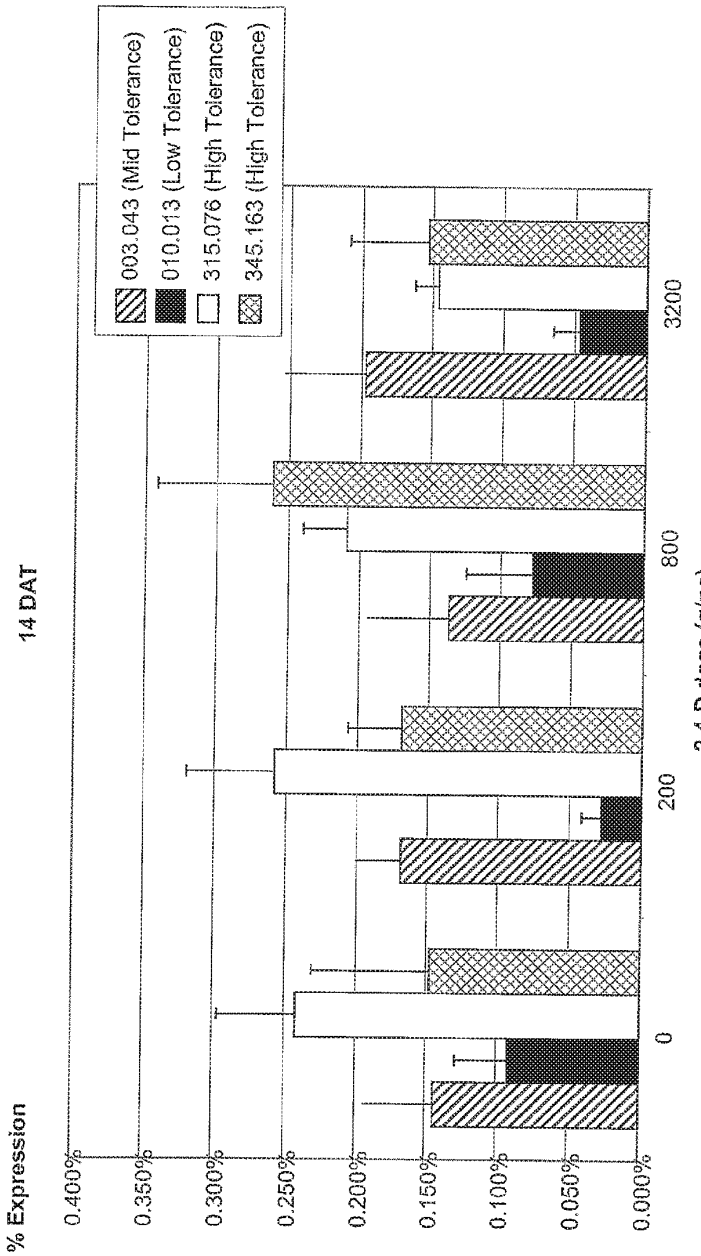

003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
14 DAT
% Expression
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

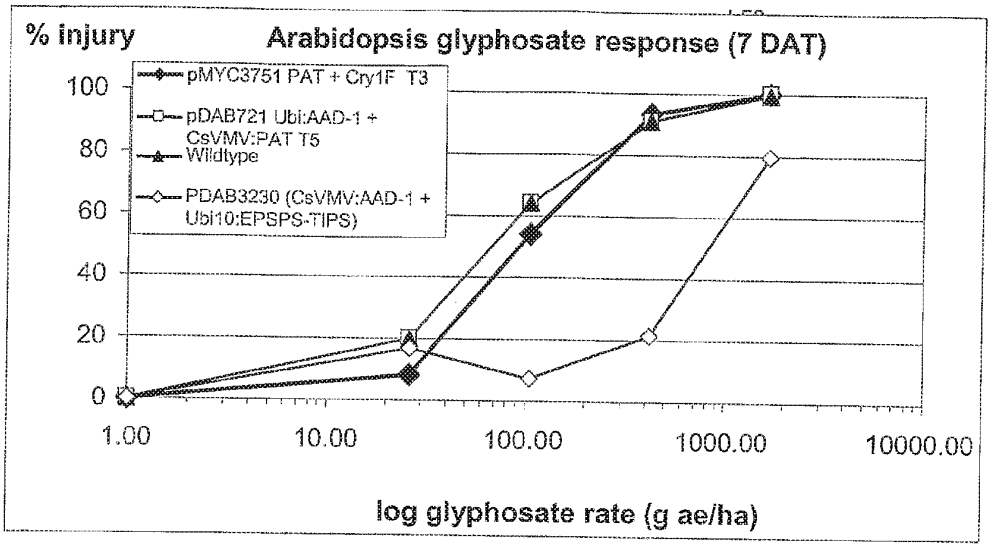

% injury
Arabidopsis glyphosate response (7 DAT)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)

Fig. 10

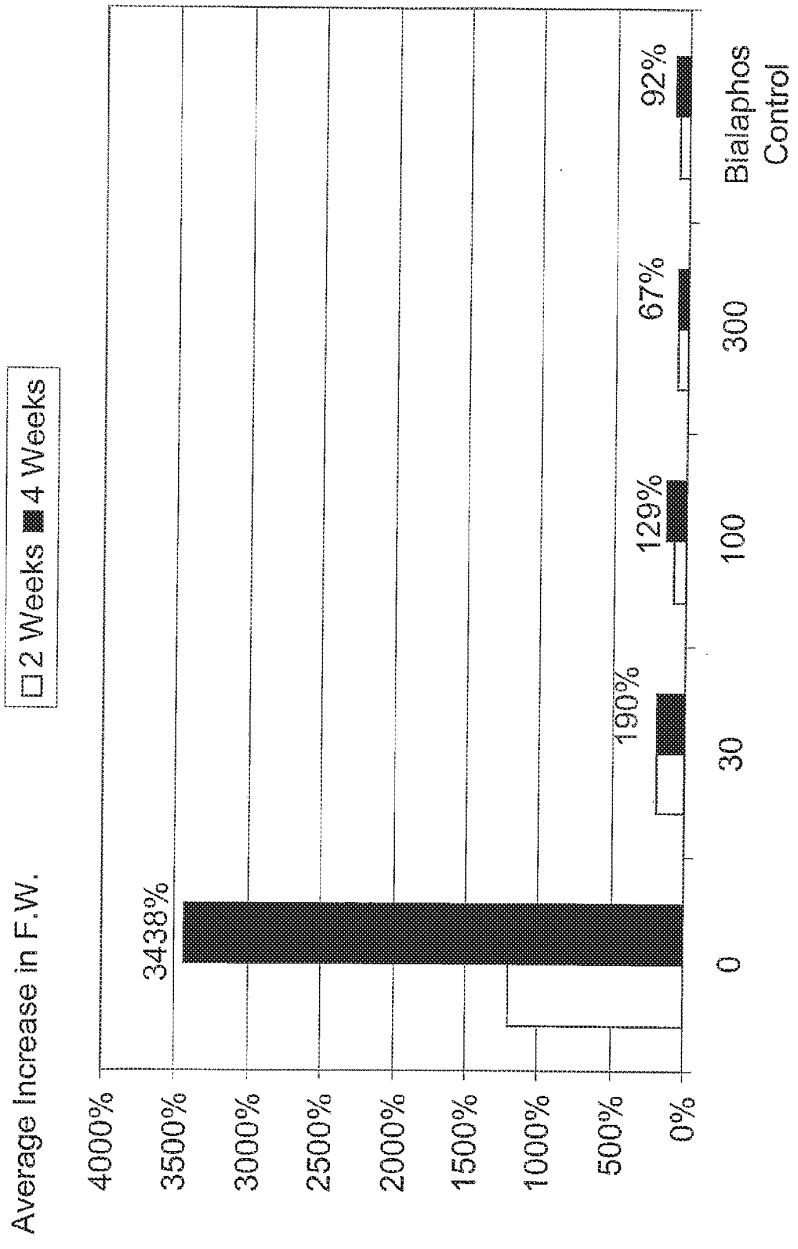

Average Increase in F.W.
2 Weeks
4 Weeks
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

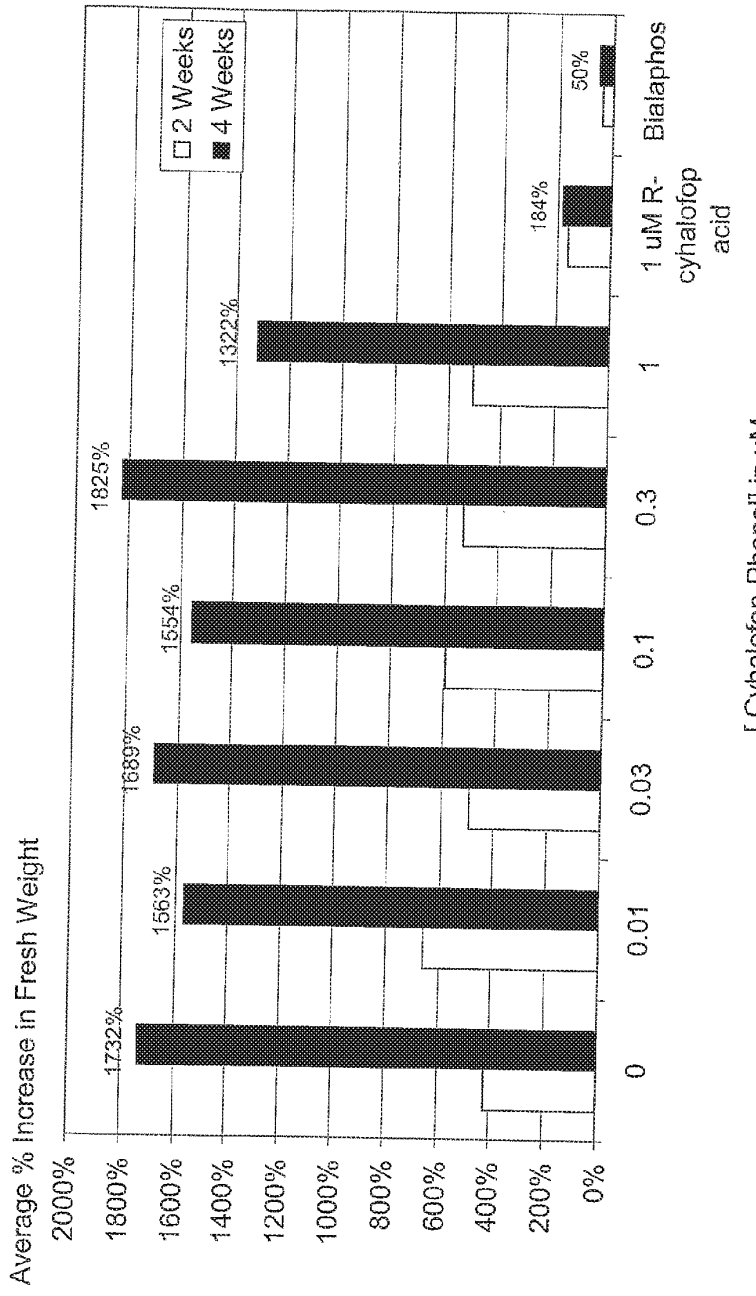

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
2 Weeks
4 Weeks
1732%
1825%
1563%
1689%
1554%
1322%
184%
50%
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

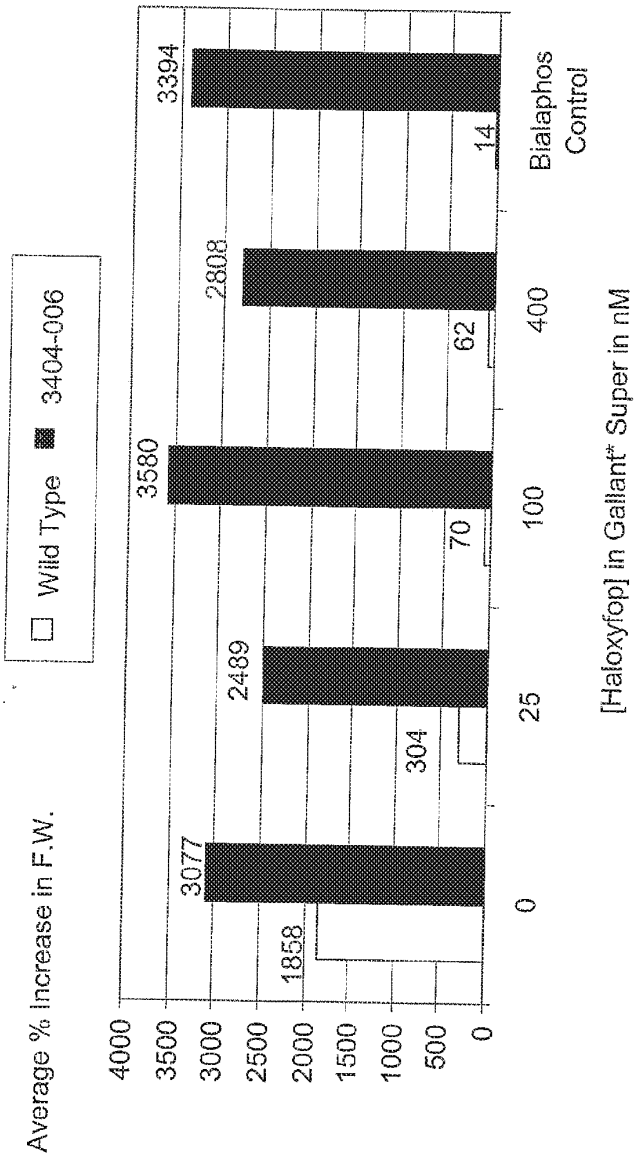

Average % Increase in F.W.
Wild Type
3404-006
4000
3500
3000
2500
2000
1500
1000
500
0
3077
1858
2489
304
3580
70
2808
62
3394
14
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant® Super in nM

Fig. 13

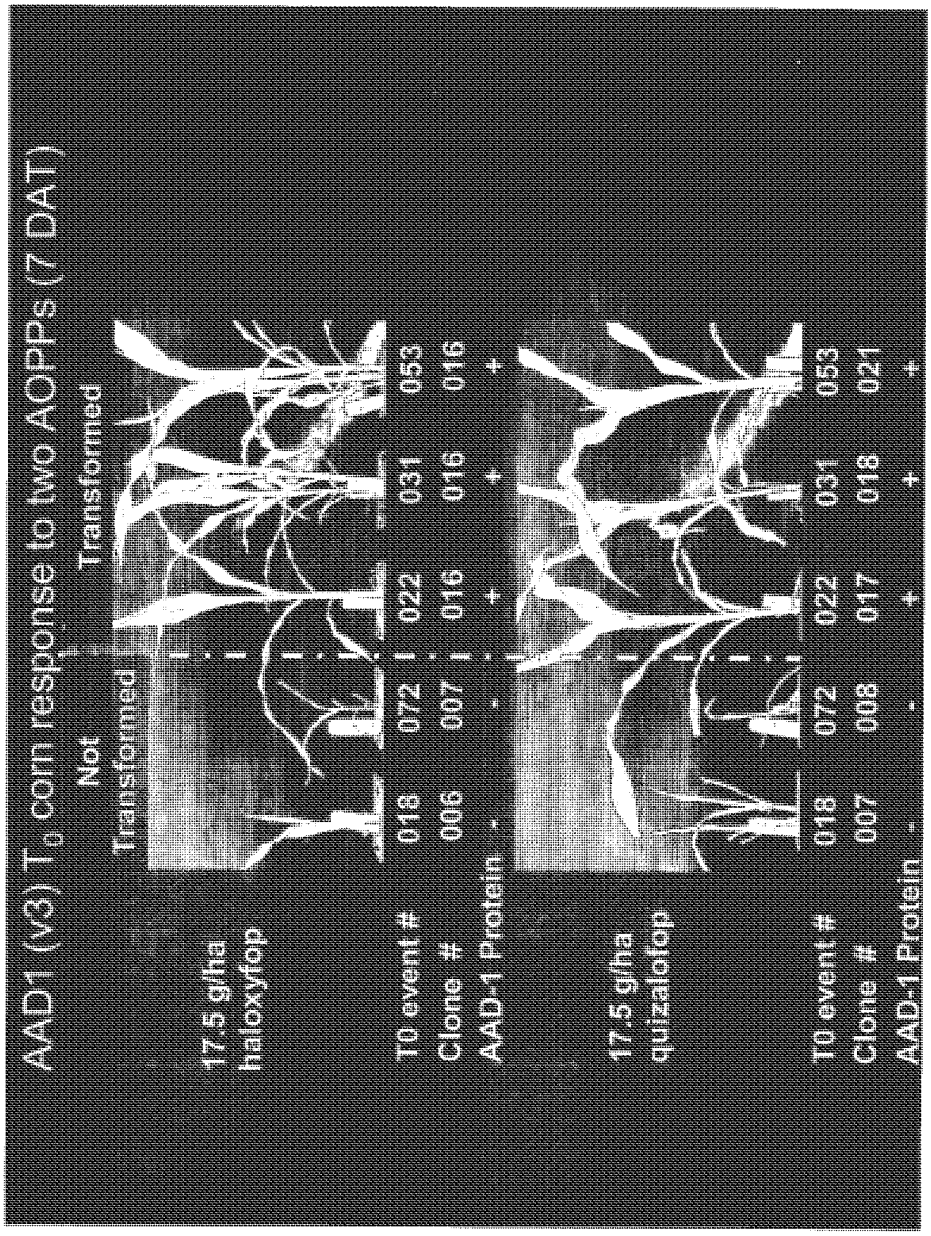

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

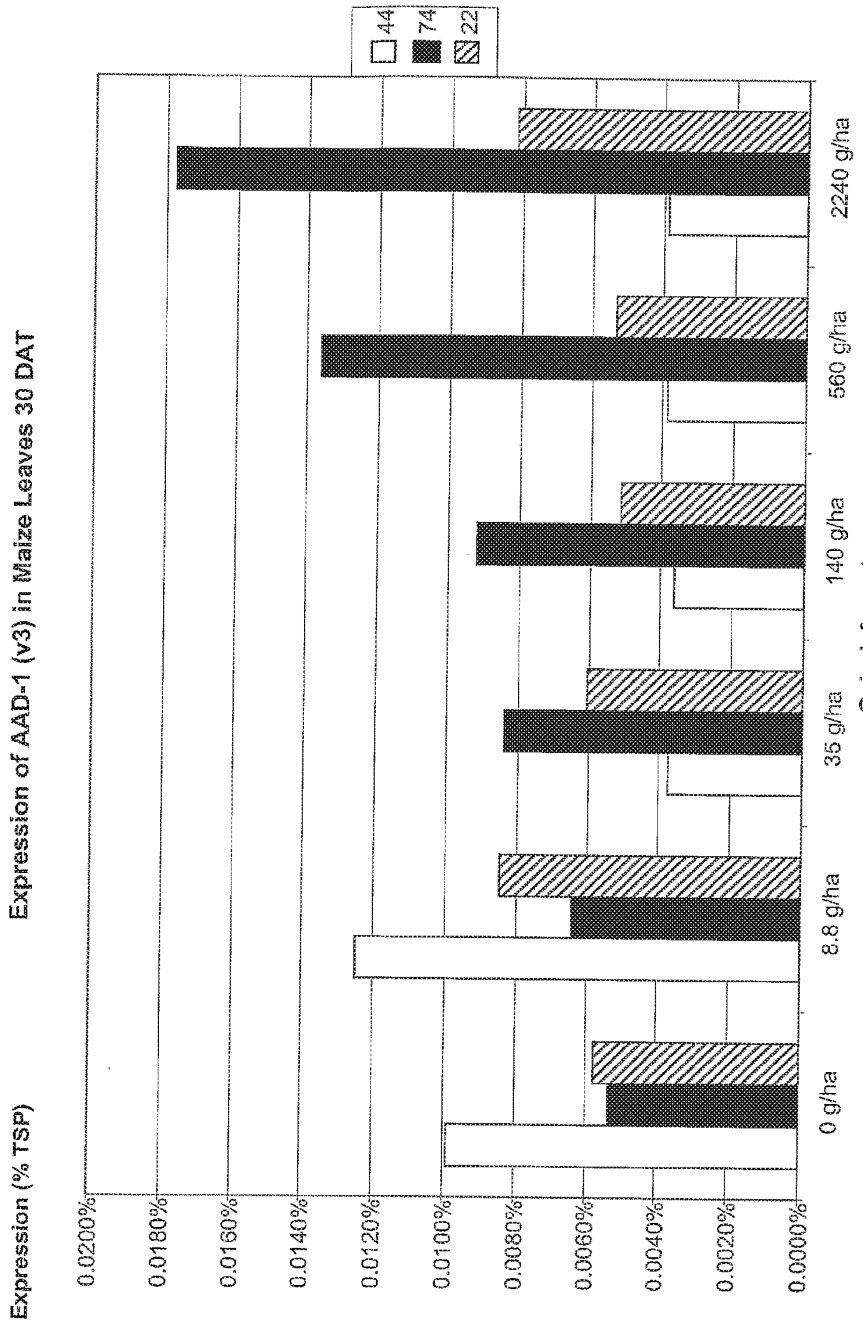

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
44
74
22
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate

Fig. 15

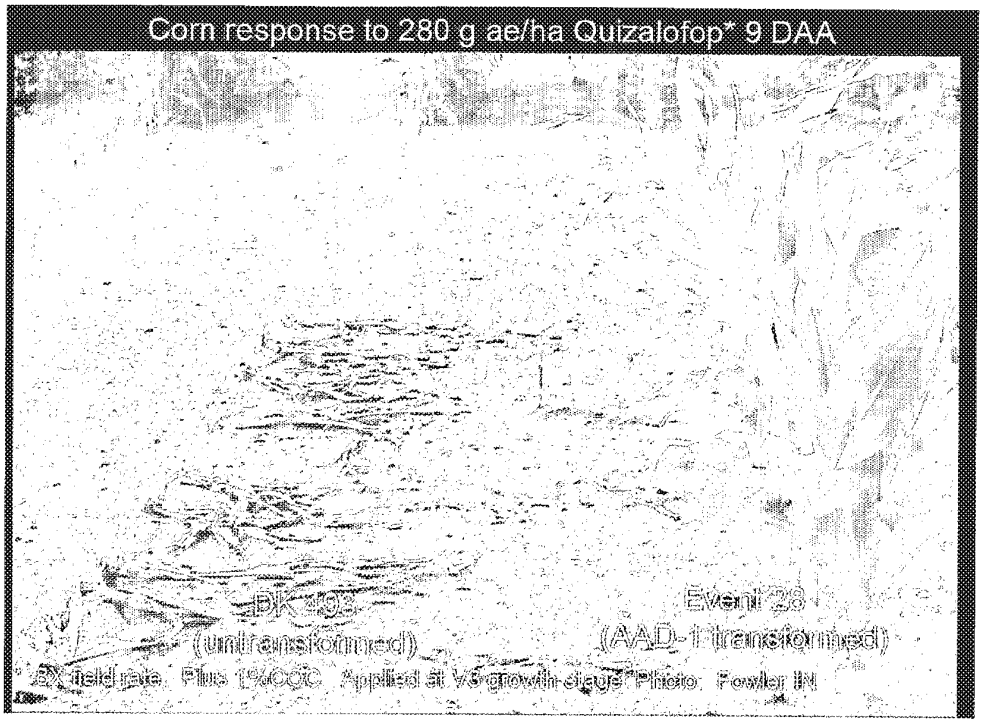

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 493
(untransformed)
Event 28
(AAD-1 transformed)
2X field rate. Plus 1%COC. Applied at V6 growth stage *Photo: Fowler IN

Fig. 16

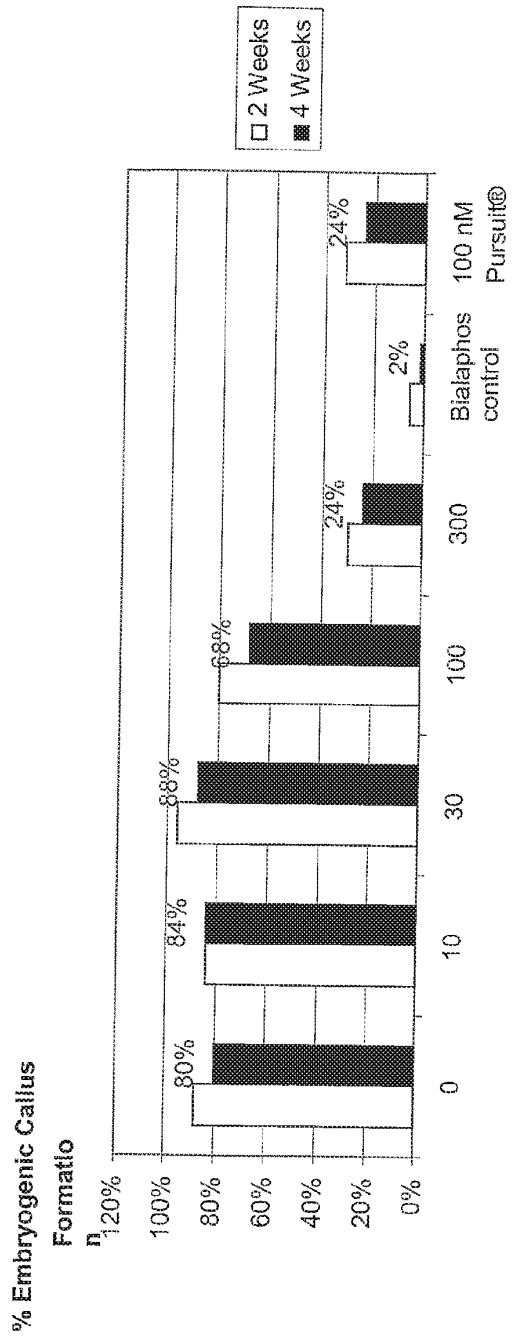
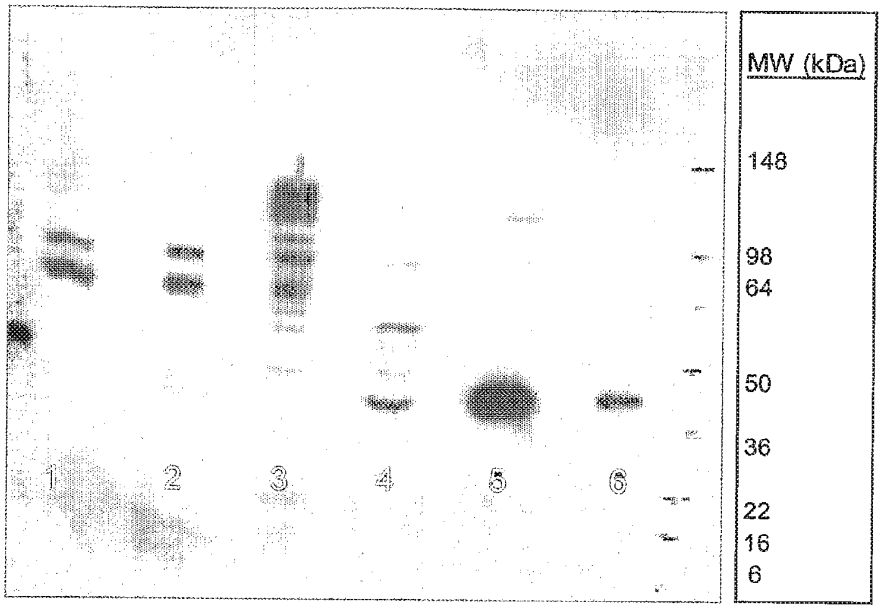

% Embryogenic Callus Formatio n
120%
100%
80%
60%
40%
20%
0%
80%
84%
88%
68%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
[R-cyhalofop ACID] in nM
2 Weeks
4 Weeks

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

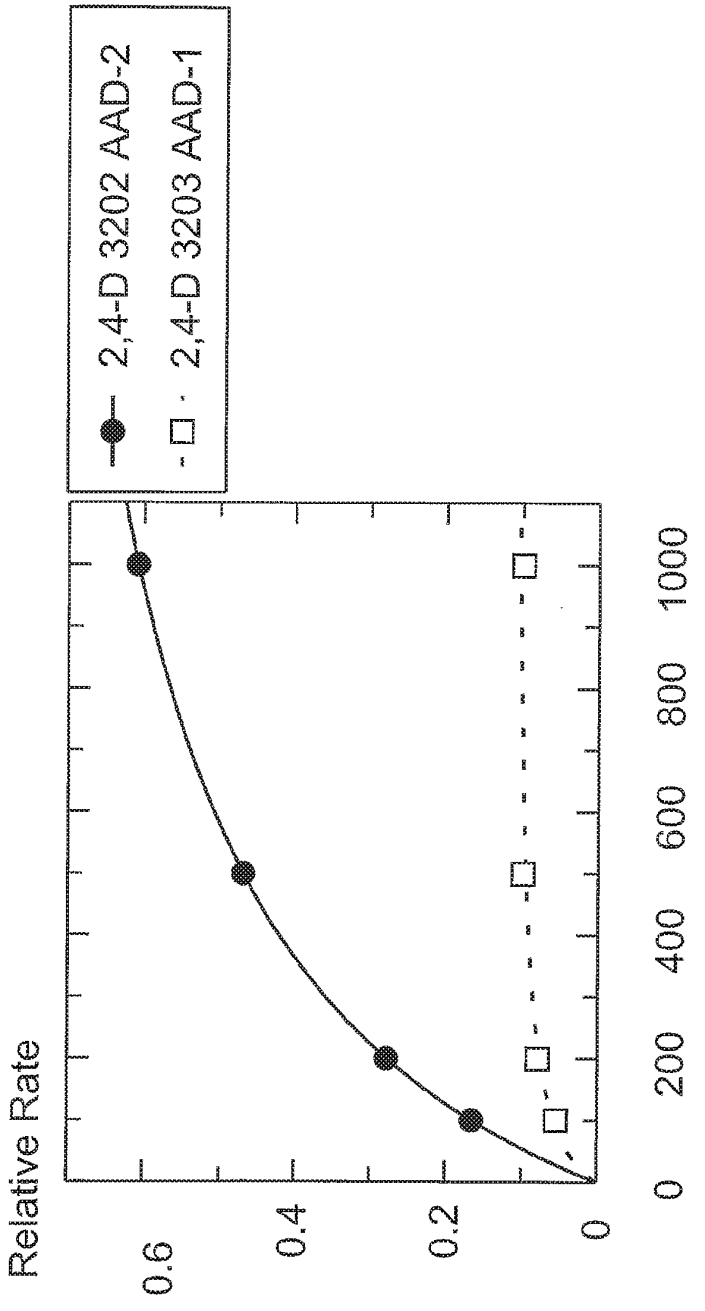

2,4-D 3202 AAD-2
2,4-D 3203 AAD-1
Relative Rate
0
0.2
0.4
0.6
0
200
400
600
800
1000
[2,4-D], µM

Fig. 19

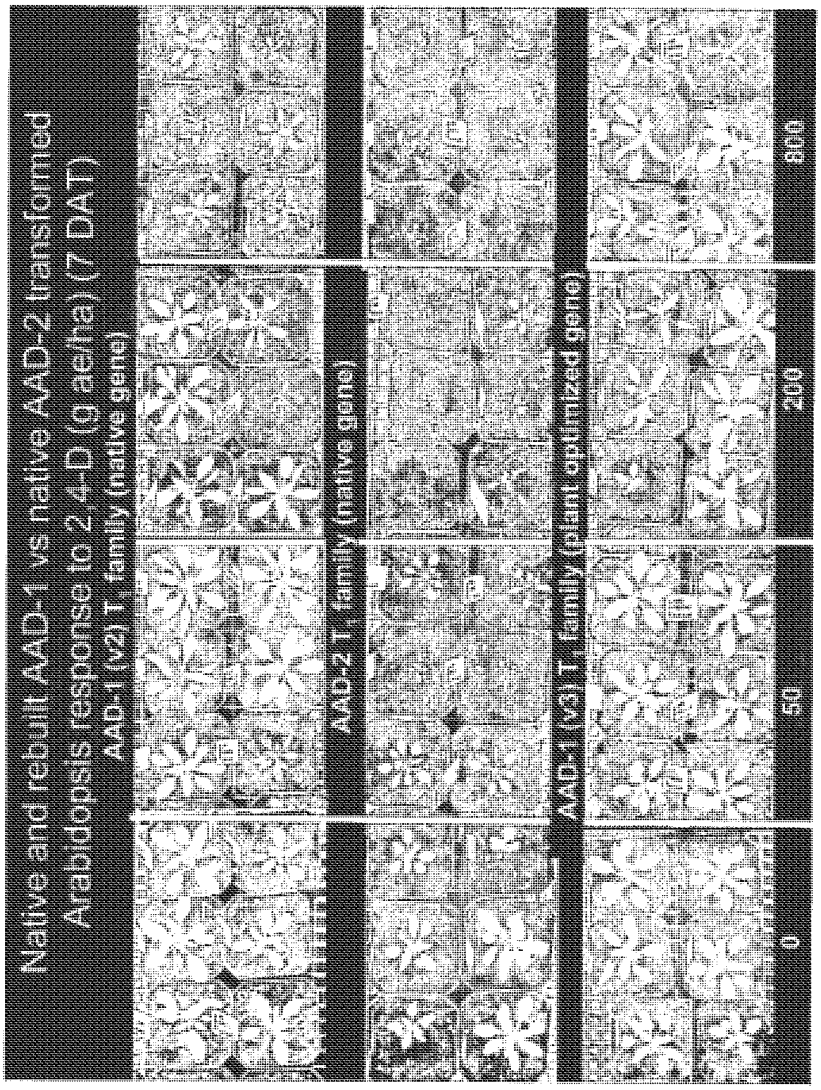

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (ester) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

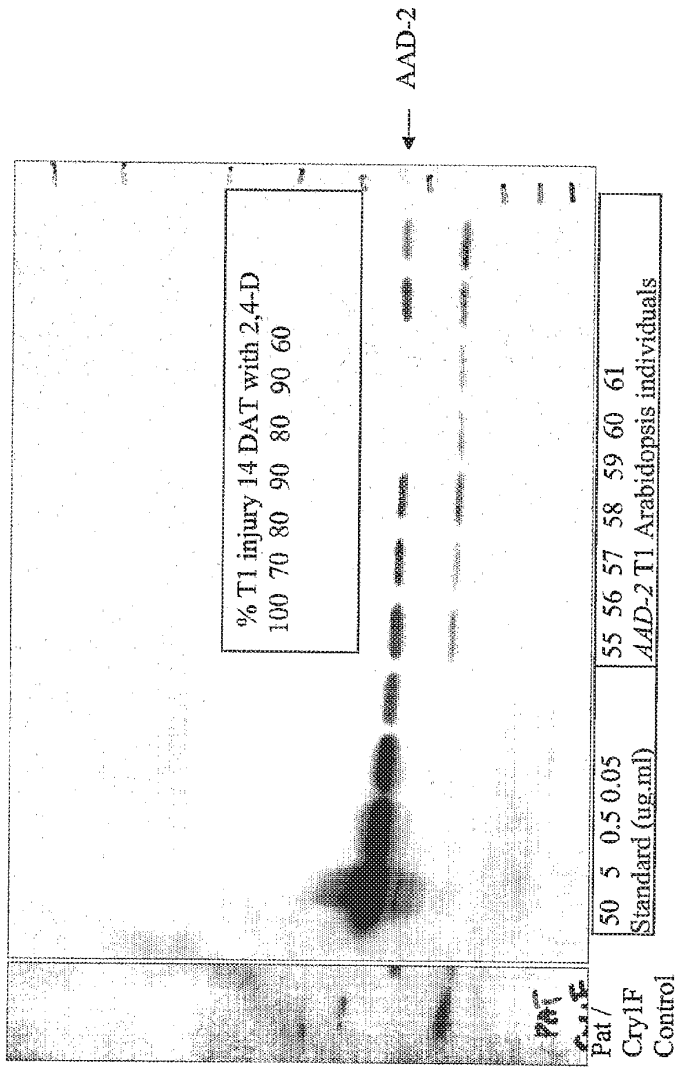

AAD-2
% T1 injury 14 DAT with 2,4-D
100 70 80 90 80 90 60
50 5 0.5 0.5 0.05
Standard (ug.ml)
55 56 57 58 59 60 61
AAD-2 T1 Arabidopsis individuals
Pat / Cry1F Control

Fig. 21

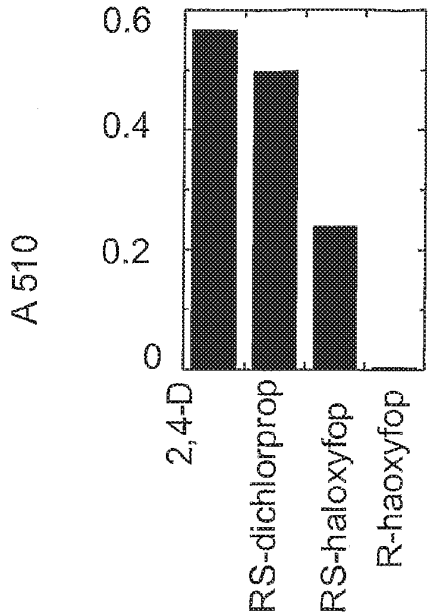


Fig. 22

SEQUENCE LISTING

<110> Dow AgroSciences LLC

<120> Novel Herbicide Resistance Genes

<130> DAS-115XC1

<150> US 60/567,052
<151> 2004-04-30

<160> 32

<170> PatentIn version 3.2

<210> 1
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer used to amplify the rdpA/AAD-1 (v1) gene

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<210> 2
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer used to amplify the rdpA/AAD-1 (v1) gene

<400> 2
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<210> 3
<211> 915
<212> DNA
<213> Sphingobium herbicidovorans

<220>
<221> misc_feature
<222> (1)..(18)
<223> Primer linker

<220>
<221> misc_feature
<222> (907)..(915)
<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

ggccccgtcg atcccgtgcc cctgctcaag agcatcgaag ggtatccaga ggtgcagatg 300

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

gactacgcgg gcaagttccg ctacctgacg cgcaccacgg tcggtggcgt gcgcccggcg 900

cgctagtaac tcgag 915

<210> 4
<211> 897
<212> DNA
<213> Artificial Sequence

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<223> AAD-1 (v2) primary sequence

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<222> (1)..(2)
<223> primer linker

<220>
<221> misc_feature
<222> (891)..(897)
<223> primer linker

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tgaccggcgt cctgggcgcc gagatcaccg gcgtcgacct gcgcgagccg ctcgacgaca 120

gcacctggaa cgaaatcctc gacgcgttcc acacttacca ggtcatctat tttcccggcc 180

aggcgatcac caacgaacag cacatcgcct tcagccggcg cttcggcccc gtcgatcccg 240

tgcccctgct caagagcatc gaagggtatc cagaggtgca gatgatccgc cgcgaagcca 300

acgaaagcgg gcgtgtgatc ggtgatgact ggcacaccga cagcaccttc ctggacgcac 360

cgccggccgc cgtggtgatg cgcgcgatcg acgtgcccga gcatggcggc gacaccggtt 420

ttctgagcat gtacaccgcg tgggagacgc tgtcgcccac catgcaggcc accatcgaag 480

ggttgaacgt agtgcacagc gccacgcgtg tgttcggctc gctctaccag gcccagaacc 540

ggcgcttcag caacaccagc gtcaaggtga tggacgtcga cgcgggcgac cgtgaaaccg 600

tgcacccccc ggtggtgacc catccgggca gcggctgcaa gggcctgtac gtgaaccagg 660

tctattgcca gcgcatcgag ggcatgaccg atgccgaaag caaaccgctg ctgcagttcc 720

tgtacgagca tgcgacacgg ttcgatttca cctgccgcgt gcgctggaag aaggaccagg 780

tcctggtctg ggacaacctg tgcacgatgc accgggccgt acccgactac gcgggcaagt 840

tccgctacct gacgcgcacc acggtcggtg gcgtgcgccc ggcgcgctag tgagctc 897

<210> 5
<211> 919
<212> DNA
<213> Artificial Sequence

<220>
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<222> (1)..(2)
<223> primer linker

<220>
<221> misc_feature
<222> (6)..(8)
<223> additional alanine codon (GCT)

<220>
<221> misc_feature
<222> (894)..(919)
<223> primer linker

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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

cacctccagc tgctgttgtg atgagggcca tagatgttcc tgagcatggc ggagacactg 420

ggttcctttc aatgtacaca gcttgggaga ccttgtctcc aaccatgcaa gccaccatcg 480

aagggctcaa cgttgtgcac tctgccacac gtgtgttcgg ttcctctac caagcacaga 540

accgtcgctt cagcaacacc tcagtcaagg tgatggatgt tgatgctggt gacagagaga 600

cagtccatcc cttggttgtg actcatcctg gctctggaag gaaaggcctt tatgtgaatc 660

aagtctactg tcagagaatt gagggcatga cagatgcaga atcaaagcca ttgcttcagt 720

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

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<210> 7
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> 3'saci primer

<400> 7
gagctcacta gcgcgccggg cgcacgccac cga 33

<210> 8
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> BstEII/ Del NotI 5' primer

<400> 8
tggtggtgac ccatccgggc agcggctgca agggcc 36

<210> 9
<211> 295
<212> PRT
<213> Sphingobium herbicidovorans

<400> 9

Met His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
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
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255
```

```
Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295
```

<210> 10
<211> 295
<212> PRT
<213> Artificial Sequence

<220>
<223> AAD-1 v2 Translation

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> Different from v1

<220>
<221> MISC_FEATURE
<222> (212)..(212)
<223> Different from v1

<400> 10

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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
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phé Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Cys Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295
```

<210> 11
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<212> PRT
<213> Artificial Sequence

<220>
<223> AAD-1 v3 Translation

<220>
<221> MISC_FEATURE
<222> (2)..(3)
<223> Different from V1

<400> 11

Met Ala His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile
1 5 10 15

Ala Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val
20 25 30

Asp Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp
35 40 45

Ala Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr
50 55 60

Asn Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro
65 70 75 80

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
115 120 125

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

COR-AAD1-00001043

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
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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 cgggctgctg ttcgcgatc 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
```

COR-AAD1-00001045

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'(speI)

<400> 14
actagtaaca aagaaggaga tataccatga cgat 34

<210> 15
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> br jap 3' (xhoI)

<400> 15
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<210> 16
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 forward primer

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<210> 17
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 reverse primer

<400> 17

caggaaacag ctatgac 17

<210> 18
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> NcoI of Brady

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<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

COR-AAD1-00001047

```
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

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Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
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Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
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Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
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COR-AAD1-00001048

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Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
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Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
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Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
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Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
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Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
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His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
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Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
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Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
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Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
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Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
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Phe Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val
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Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
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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
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Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
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Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
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Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
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Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
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Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
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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
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gctggaattg caatgagaag cctcactgct gcagtagttg cagctggtgg gaatgcaagt 600

tatgtccttg atggtgtccc cagaatgagg gaaaggccca tcggtgacct tgtggctggc 660

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tttggagttt ctgttgaaca ttctggcaac tgggatcgtt tccttgtaca tggaggtcag 960

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tctgagaaca gtgtaactgt gtctggacct cccagagact tcagtggcag aaaggttctc 1200

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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
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Gly Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe
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195 200 205

Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
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Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
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PTO/SB/06 (09-11)
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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	09/09/2020	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
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	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 ($)	ADDITIONAL FEE ($)
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	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".	/ANGELA D JOHNSON/
*** 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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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Environmental Behavior of the Chiral Herbicide Haloxyfop. 1. Rapid and Preferential Interconversion of the Enantiomers in Soil

Thomas Poiger,* Markus D. Müller, Hans-Rudolf Buser, and Ignaz J. Buerge

Agroscope, Institute for Plant Production Sciences, Schloss 1, CH-8820 Wädenswil, Switzerland

Supporting Information

ABSTRACT: Haloxyfop-methyl is a chiral herbicide that was first introduced as racemate and later replaced by "haloxyfop-P-methyl", mainly consisting of the *R*-enantiomer, which carries the herbicidal activity. We studied the ester cleavage of haloxyfop-methyl and further degradation and chiral inversion of the acid enantiomers in three different soils using enantioselective gas chromatography−mass spectrometry. Our results confirm the rapid ester hydrolysis of haloxyfop-methyl with half-lives of a few hours and indicate that hydrolysis is weakly enantioselective. Further degradation of haloxyfop was slower with half-lives of several days. In all three soils, *S*-haloxyfop was rapidly converted to *R*-haloxyfop. In sterile soil, no degradation and no inversion were observed, indicating that both processes are biologically mediated. In soil where 50% of the water had been replaced by deuterium oxide, significant H−D exchange in haloxyfop was observed, pointing to a reaction mechanism involving abstraction of the proton at the chiral center of the molecule.

KEYWORDS: *haloxyfop, soil, enantioselective degradation, chiral inversion, enantioselective GC-MS*

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INTRODUCTION

The aryloxyphenoxy-propionate herbicides (FOPs) control graminaceous weeds in broad-leaved crops such as soybean or sugar beet and are applied postemergent at rates of typically 100−400 g active ingredient/ha. They can be formulated alone or in combination with other herbicides or a safener to enhance selectivity also in cereals.[1] At present, 10 compounds belong to the FOPs, which share a common mode of action by inhibiting acetyl coenzyme A carboxylase (ACCase) and hence the synthesis of fatty acids in sensitive plants.[2] The compounds are applied as esters, which are rapidly hydrolyzed in soil and plants to their herbicidally active acid metabolites.

All FOPs possess an asymmetrically substituted carbon atom in the propionic acid moiety (Figure 1) and are therefore chiral.[3] Initially, the FOPs were developed and introduced in the 1970s and 1980s as racemates.[4] Later, it was recognized that the herbicidal activity is essentially associated with the *R*-enantiomers, and some of the racemic compounds were replaced by the enantioenriched "P" compounds, which offered higher specific activity and lower use rates ("chiral switch"). The suffix "P", as in haloxyfop-P, relates to the fact that all *R*-enantiomers of FOPs show dextro-(+)-rotation of the plane of polarized light.

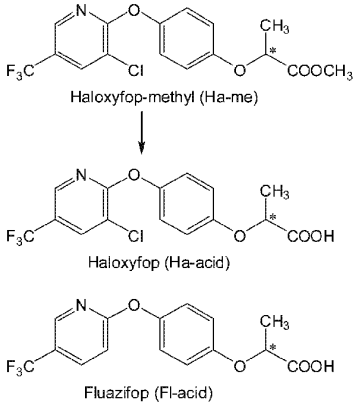


Figure 1. Structures of haloxyfop-methyl, the major herbicidally active soil metabolite haloxyfop, and fluazifop, used as internal standard. The asterisk indicates the asymmetrically substituted carbon.

The stereochemistry of substituted propionic acids has been the subject of a series of papers not least because aryl propionic acids (APAs), such as ibuprofen or naproxen, are well-known nonsteroidal anti-inflammatory drugs.[5,6] These drugs, when administered as racemates, are rapidly and almost completely converted to the *S*-enantiomers, which carry the desired therapeutic activity (note that the *S*-configuration in APAs topologically corresponds to the *R*-configuration in FOPs). It is now generally accepted that the chiral inversion of APAs occurs through formation of a (diastereomeric) coenzyme A-thioester with subsequent epimerization of the activated ester by an epimerase.[5,6]

Whereas the mechanism and the rates for the interconversion of the APA enantiomers in mammals are well described and understood, comparatively less is known for the herbicidal FOPs. Plant uptake is mainly through leaves but, after ester cleavage in soil, to a lesser extent also through roots. The enantiomer composition of residual haloxyfop in weeds and crops is unknown and, consequently, the current residue definitions do not state stereochemistry (e.g., "sum of haloxyfop, including haloxyfop-P, its esters, and its conjugates

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Table 1. Characterization of Soils Used for Incubation Experiments

site name	altitude (m asl)	land use	soil type	sand (%)	silt (%)	clay (%)	pH ($CaCl_2$)	organic carbon (%)	soil moisture[a] (% w/w) (% of MWHC)[b]
Realp	2120	alpine pasture	clay	12.2	39.0	48.8	4.0	18.1	63–64
									59–60
Neualp	955	fertilized pasture	sandy loam	59.5	20.8	19.7	5.5	4.2	31–32
									39–40
Dübendorf	440	arable land	loam	40.5	32.3	27.2	7.2	1.9	19–20
									37–39

[a]Soil moisture varied slightly from experiment to experiment due to different water volumes used for spiking. [b]Maximum water-holding capacity.

expressed as haloxyfop"),[7] and residue enforcement methods (e.g., the German official multiresidue method)[8] do not address enantiomer composition. Moreover, the fate of individual stereoisomers in soil is not well understood. Although the rapid enantiomerization of several FOPs, for example, fluazifop, diclofop, and fenoxaprop, in soil was previously described,[3,9−11] a deeper insight into the mechanism and actual conversion rates are still missing.

In this paper, we describe an enantioselective analytical method used to isolate milligram quantities of single enantiomers of haloxyfop (both acid and methyl ester) based on HPLC as well as a method for the enantioselctive analysis of these compounds in soil extracts based on GC-MS. The latter was also used to analyze the compounds in plant extracts as reported in our companion paper.[12] Our results confirm the rapid hydrolysis of haloxyfop-methyl (Ha-me) to haloxyfop (Ha-acid) in soil. We then examine the rapid and preferential interconversion and further degradation of Ha-acid in soil as determined by incubating the racemic compound and the pure *R*- and *S*-enantiomers. Three different soils are used to determine if soil properties have any influence on the enantioselectivity of transformation. Control experiments in sterile soil are performed to determine whether the conversion is biologically mediated. A hypothesis on the mechanism of conversion in soil is postulated on the basis of H−D exchange experiments and mass spectrometric analysis. Mathematic modeling is applied to derive kinetic parameters for *R*/*S* interconversion and degradation in soil.

MATERIALS AND METHODS

Reference Materials. Racemic haloxyfop-methyl and haloxyfop-P-methyl (*rac*-Ha-me and *R*-Ha-me; 99.2 and 99.1%, respectively) were obtained from Dow AgroSciences, Hitchin, UK, and *rac*-haloxyfop (*rac*-Ha-acid, 99.8%) was from Riedel de Haen, Seelze, Germany. Pure *R*- and *S*-enantiomers of Ha-me and Ha-acid were isolated from the racemates by enantioselective HPLC (see below). *rac*-Fluazifop (*rac*-Fl-acid, 94.5%, from Dr. Ehrenstorfer, Augsburg, Germany) and *rac*-fluazifop-methyl (*rac*-Fl-me), prepared by methylation of *rac*-Fl-acid using diazomethane (see below), were used as internal standards. For CAS names of the various compounds used, see Supporting Information Table S2.

Semipreparative Isolation of *R*- and *S*-Enantiomers of Ha-me and Ha-acid. Pure *R*- and *S*-enantiomers of Ha-me were prepared from the racemic compound in milligram quantities by enantioselective HPLC according to a method published by CIPAC.[13] The enantiomers were separated on a silica gel column coated with cellulose tricinnamate (Chiralcel OK, 25 cm × 4 mm, 10 μm particle size; Daicel Chemical Industries, Osaka, Japan) at ambient temperature using heptane/isopropanol/methanol (85:5:10) as eluent at a flow rate of 1 mL/min and UV detection at 280 nm. *R*- and *S*-enantiomers of Ha-me eluted baseline separated after 6.7 and 8.8 min, respectively, with peak widths of 1.0 and 1.5 min (chromatogram, see Supporting Information Figure S5).

Pure enantiomers of Ha-acid were prepared using the same column and heptane/isopropanol/acetic acid (95:5:0.1) as eluent at a flow rate of 1 mL/min and UV detection at 280 nm. *R*- and *S*-Ha-acid eluted baseline separated after 13.5 and 20.4 min, respectively (peak widths of 4 and 6 min; chromatogram see Supporting Information Figure S6). A total of 10 injections of *rac*-Ha-me and *rac*-Ha-acid (0.5 mg in 100 μL), respectively, were made, and fractions were collected at the exit of the UV detector while the UV trace was observed. The pooled fractions contained approximately 2 mg of each enantiomer with >99% enantiomeric purity as determined by enantioselective GC-MS after methylation with diazomethane when appropriate (see below). Isolated enantiomers were stored in mobile phase until used for incubation experiments. Aliquots were then evaporated to dryness and redissolved in spiking solution (water or water/methanol, see below).

Incubation of Ha-me and Ha-acid in Soil. Soil samples were collected at sampling locations in close proximity to those of the Swiss soil monitoring network (NABO, www.bafu.admin.ch/boden). The soils were selected to cover a wide range of properties, in particular pH and organic carbon (Table 1). Standard equipment was used for sampling soil from the top 10 cm. The soils were kept in the dark at ≈4 °C for no more than 6 months prior to use.

Portions of 2 mm sieved, field-moist soil (100 g) were spread on glass dishes, and the test substance was applied using a small spray bottle (1−2 mL of an aqueous solution containing 100 μg per enantiomer of Ha-acid). For experiments with Ha-me (only with soil Neualp), the spike solution contained 30% methanol as cosolvent to increase the solubility (note the solubility of Ha-me in water is 9.1 mg/L[7]). The resulting spike levels were 1 μg enantiomer/g moist soil and thus high enough to allow for reliable quantification down to 1% of the initial concentration. This spike level is somewhat higher than the concentrations expected from typical application rates in the field of up to 160 g/ha (0.21 μg/g assuming uniform incorporation in the top 5 cm soil layer and a bulk density of 1.5 g/cm^3) but is not expected to affect soil microorganisms. Separate experiments were carried out with racemic compounds and with pure *R*- and *S*-enantiomers. The soils were then thoroughly mixed, transferred to Erlenmeyer flasks, closed with air-permeable cellulose plugs, and incubated at 20 °C in the dark for up to 4 days. The moisture of the soils (24−174 g water/100 g dry soil, corresponding to 37−60% of the maximum water-holding capacity) was regularly checked by weighing and kept constant by the addition of distilled water. At appropriate time intervals, aliquots of 10 g of soil were removed for extraction and analysis.

Sterile Control Experiments. Abiotic transformation of Ha-acid was studied using a γ-irradiated soil (soil Dübendorf, Table 1) as sterile control. Portions of 200 g of soil in closed screw-cap glass jars were exposed for a few hours to γ-irradiation (dosimeter controlled total dose of 30 kGy) from a commercial ^{60}Co source (Leoni Studer-Hard, Däniken, Switzerland). The glass jars were kept tightly closed after irradiation until the start of the incubation experiments to ensure sterile conditions. Spiking was done in the same way as for the nonsterile soils except that the soil was kept in the glass jars during spiking. Nevertheless, sterile conditions were broken upon spiking. As the incubation experiments lasted for only a few days, this was not expected to allow for restoration of any substantial biological activity in the soil.

H−D Exchange Experiments. Three 100 g portions of 2 mm sieved, field-moist soil (Neualp, Table 1) were spread on glass dishes

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and allowed to dry to approximately 50% of their initial water content. The evaporated water was replaced by dropwise addition of deuterium oxide (Fluka, Buchs, Switzerland) to achieve an approximate 1:1 ratio of H_2O/D_2O. As D_2O can be toxic to soil microorganisms, a larger proportion was avoided. The soils were then spiked in the same way as the other soils (separate experiments with *rac*-Ha-acid and with pure *R*- and *S*-enantiomers). Soil moisture was maintained by the addition of a 1:1 mixture of distilled water and deuterium oxide.

Extraction and Derivatization. Soil samples from experiments with Ha-acid (10 g) were collected from the incubation vessels in 20 mL glass scintillation vials. Immediately after weighing, 10 mL of methanol and 100 μL of a methanolic solution of *rac*-Fl-acid (0.1 μg/μL) were added and the vials closed and vigorously shaken by hand for approximately 1 min and then stored at −20 °C until further processed (typically within 1 day). The vials were centrifuged at a relative centrifugal force (RCF) of 720 for 5 min (rotor with a radius of 16 cm, 2000 rpm), and the clear supernatants were removed into 30 mL glass vials, diluted with 10 mL of distilled water, and acidified to pH ≈1 by the addition of 4 drops of 5 M sulfuric acid. The extracts were then partitioned twice with 5 mL of dichloromethane, and the combined organic extracts were allowed to evaporate to dryness overnight in a fume hood. The extracts were then redissolved in a few drops of methanol and methylated by the addition of approximately 300 μL of an ethereal diazomethane solution until the yellow color of the diazomethane persisted (see ref 14 and cautionary note therein). The reaction mixtures were allowed to stand for 15 min and then carefully evaporated to dryness in a stream of air, and the dry residues were dissolved in 5 mL of hexane. An aliquot of 1 μL was used for GC-MS analysis. Absolute recoveries over the extraction and derivatization procedure, determined in soil Neualp, were 71% for both Fl-acid and Ha-acid, respectively (spike level, 1 μg/g moist soil). This indicates that both compounds show very similar behavior and that Fl-acid is thus an appropriate internal standard for Ha-acid. The method precision, expressed as relative standard deviation of multiple extractions, on average was 3.2% (range, from 0.3 to 5.9%).

In exploratory experiments with Ha-me, the same extraction procedure was used. In contrast to the experiments with Ha-acid, prior to the extraction the soil samples were fortified with 10 μg of *rac*-Fl-acid in 100 μL of methanol and 10 μg of *rac*-Fl-me in 100 μL of ethyl acetate as internal standards. The samples were derivatized with diazoethane (for a description of the preparation, see ref 12) rather than diazomethane, to allow for the simultaneous determination of parent Ha-me as well as Ha-acid formed during incubation. An aliquot of 1 μL was used for GC-MS analysis. Absolute recoveries over the extraction and derivatization procedure were 68 and 71% for Fl-me and Ha-me, respectively (spike level, 1 μg/g moist soil).

Several control experiments were conducted to ensure that no artifacts were produced during extraction and derivatization. Selected samples were spiked only with Fl-acid and then derivatized with diazomethane or diazoethane to confirm that no (<1%) ethyl ester was formed upon methylation and vice versa. Samples spiked only with Fl-me were derivatized with diazoethane to confirm that no transesterification occurred (<1% Fl-et present in ethylated soil extract dissolved in ethyl acetate). Finally, soils freshly spiked with single isomers of Ha-me and Ha-acid were processed to confirm that no hydrolysis and no enantiomerization occurred during sample preparation.

Enantioselective GC-MS Analysis. A Quattro Micro GC-MS/MS system (Waters Corp., Milford, MA, USA) connected to a HP6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) fitted with a PAL autosampler (CTC Analytics, Zwingen, Switzerland) was used in the electron ionization (EI, 70 eV, 180 °C) mode. Full-scan EI mass spectra (m/z 35−435, 0.5 s/scan, nominal mass resolution) were recorded for analyte detection and identification. Analysis of the methyl and ethyl esters was carried out in the selected ion monitoring mode for optimum sensitivity (cycle time = 0.5 s) using the m/z values listed in Table 2.

A custom-made 20 m GC column coated with OV 1701 containing 15% (w/w) permethyl-β-cyclodextrin with a film thickness of 0.1 μm (BGB Analytics, Boeckten, Switzerland) was used for separation. The GC conditions were as follows: split/splitless injection (240 °C, 48 s splitless time); temperature program, 50 °C for 2 min, increase to 120 °C at a rate of 25 °C/min and to 220 °C (upper temperature limit for this type of columns) at 2 °C/min. Helium was used as carrier gas at a flow rate of 2 mL/min. The column showed excellent stability, low bleed, and good and constant, nearly baseline, resolution of Ha-me, Ha-et, Fl-me, and Fl-et enantiomers (Table 2).

Table 2. GC-MS Masses Used for Determination of Target Compounds, Retention Times of the Enantiomers, and Chromatographic Resolution Achieved with the Permethyl-β-Cyclodextrin Column

target compound	m/z (all M^+)	retention times (min)	resolution
fluazifop-methyl (Fl-me)	341.1	38.5/38.7	1.4
fluazifop-ethyl (Fl-et)	355.1	39.9/40.1	1.3
haloxyfop-methyl (Ha-me)	375.1	41.3/41.5	1.3
haloxyfop-D_1-methyl	376.1	41.3/41.5	1.3
haloxyfop-ethyl (Ha-et)	389.1	42.7/42.9	1.2

Kinetic Evaluation of Experimental Data. All kinetic analyses of experiments were performed using the modeling software Aquasim, version 2.1b.[15] Degradation and isomerization were implemented as first-order processes based on the conceptual model given in Figure 2,

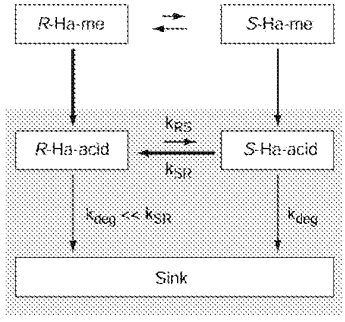


Figure 2. Reaction scheme and definitions of reaction rate constants used in the model calculations. The shaded area highlights the portion that was covered by model calculations.

and the corresponding rate constants were determined by fitting the modeled curves to measured data. The rate equations used for curve fitting were

$$d[S]/dt = k_{RS} \times [R] - (k_{SR} + k_{deg}) \times [S]$$

$$d[R]/dt = k_{SR} \times [S] - (k_{RS} + k_{deg}) \times [R]$$

Initial enantiomer concentrations were not fixed, but were fitted as additional parameters, with the following exceptions: the initial concentration of *S*-Ha-acid in the experiment with enantiopure *R*-Ha-acid was set to zero (and vice versa), and equal initial concentrations of *R*- and *S*-Ha-acid were assumed in the experiments with *rac*-Ha-acid.

Fitting was done in several steps. First, all rate constants, including separate degradation rate constants for each enantiomer, were fitted individually for each experiment. This approach did not yield satisfying solutions for the degradation rate constants because isomerization (particularly $S \rightarrow R$) is substantially faster than degradation. It is thus not possible to determine whether the *S*- or the *R*-enantiomer is more rapidly degraded because a small change in the isomerization rate constants would compensate for that difference. Therefore, a more robust fitting approach was used assuming that both enantiomers are degraded with an equal rate constant, k_{deg}. Isomerization rate constants k_{RS} and k_{SR}, respectively, were fitted for each soil simultaneously with data from the experiments with *rac*-Ha-acid and the pure *R*- and *S*-

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enantiomers, using the enantiomer fractions[16] ([R]/([R] + [S]) and [S]/([R] + [S]), respectively) rather than individual concentrations as fit targets. The fitted values for k_{RS} and k_{SR} were then fixed, and k_{deg} values were determined for each individual experiment by fitting of the total concentrations (sum of *R*- and *S*-enantiomers).

RESULTS AND DISCUSSION

Analytical Considerations. For the sensitive and accurate enantioselective determination of Ha-me and Ha-acid in soil, chiral GC with mass spectrometric detection was used. Ha-acid and Fl-acid (internal standard) were converted to the respective methyl esters using diazomethane. In the initial experiments with Ha-me, selected samples were derivatized with diazo*ethane* to allow for simultaneous detection of Ha-acid (as ethyl ester) and Ha-me. Experiments with sterile soil showed that the extraction and derivatization procedures did not change the enantiomer ratios of Ha-acid.

Several chiral GC columns were evaluated for resolution of haloxyfop and fluazifop enantiomers. Whereas a dimethyl-*tert*-butyldimethylsilyl-β-cyclodextrin column had shown excellent enantiomer resolution of various phenoxypropionic acid herbicides,[17] this selector was found to provide insufficient chromatographic resolution of haloxyfop and fluazifop enantiomers. Eventually, a column coated with OV 1701 containing 15% (w/w) permethyl-β-cyclodextrin provided best resolution and allowed elution of the target analytes below 200 °C and, thus, well below the temperature limit of the column of 220 °C (Figure 3).

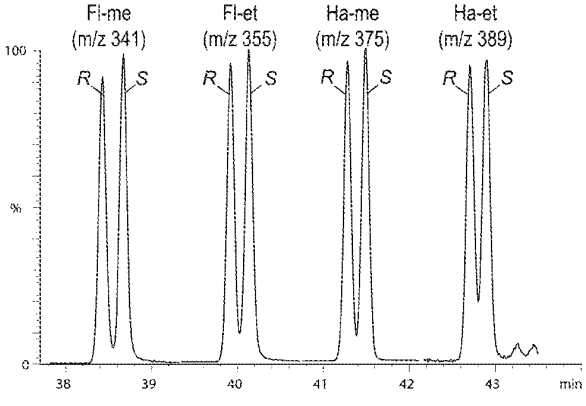


Figure 3. EI SIM chromatograms of racemic reference compounds of the methyl and ethyl esters of fluazifop (internal standard) and haloxyfop, analyzed on the enantioselective permethyl-β-cyclodextrin column.

The same enantiomer elution order (*R* prior to *S*) was observed for Ha-me, Ha-et, and Fl-me on this chiral selector. It is interesting to note that the same elution order was observed for ibuprofen, when considering its reverted absolute configuration at the α-carbon atom (*S* prior to *R*).[18] This indicates a common principle toward chiral recognition of the propionic acid moiety by this cyclodextrin derivative.

Whereas the enantiomer composition of Ha-acid was expected to change during incubation, the racemic fluazifop, serving as an internal standard, theoretically should always show enantiomer ratios of 1:1. The actual ratios for *rac*-Fl-acid (analyzed as methyl and ethyl esters) on a new GC column were indeed very close to 1:1, but with aging of the column, they were shifted toward a small excess in the later eluted *S*-enantiomer (Figure 3), which is commonly observed in enantioselective GC. In this particular case, the shift of apparent peak area ratios was small (approximately 0.9:1.1), and it was not due to peak tailing. Using the observed apparent ratio of the internal standard it was possible to apply a correction factor to the experimental data and thus to obtain accurate enantiomer ratios for haloxyfop.

Exploratory Soil Incubations of Ha-me. In initial experiments, *rac*-Ha-me or its pure enantiomers were incubated for 0, 2, and 4 h in soil Neualp (Table 1). In Figure 4 we show

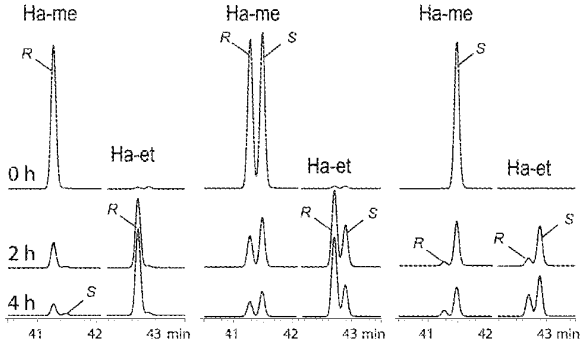


Figure 4. EI SIM chromatograms (*m*/*z* 375 + 389) of extracts of soil Neualp spiked with *R*- (left), *rac*- (middle), and *S*-Ha-me (right) immediately after spiking (top) and 2 h (middle) and 4 h later (bottom). Signals are normalized to the peaks for Fl-me used as internal standard. Note the rapid disappearance of Ha-me and the concurrent formation of Ha-acid (analyzed as ethyl ester).

chromatograms from these incubations. In all three experiments, Ha-me rapidly disappeared with half-lives of <2 h, and approximately 75% of the applied Ha-me was transformed to Ha-acid. The chromatograms from the incubation with *rac*-Ha-me indicate that degradation is enantioselective with faster dissipation of the *R*-enantiomer (residual composition, *R* < *S*). Degradation of the enantiopure compounds was associated with formation of small amounts of the antipode, indicating that enantiomerization occurred, although at a much slower rate compared to degradation.

This initial set of experiments confirmed that rapid degradation of Ha-me was leading to Ha-acid, consistent with previous studies.[7,19] Indeed, the 0 h ("quasi-zero") samples already showed the presence of some small amounts of free acid (analyzed as ethyl esters, Figure 4), whereas standards of Ha-me contained no detectable amounts of free acid. Apparently, the short time (a few minutes) required for preparation (spiking, mixing of soil, sampling) was sufficient to form free acid by hydrolysis of the methyl ester. In the 2 h sample, then, the presence of acid was significant, thus clearly indicating rapid hydrolysis of the methyl ester to the free acid. In a review,[20] the properties of microbial carboxy esterases were described and enantioselective cleavage of, for example, substituted phenoxypropionic acids was reported. Apparently, these esterases are widespread in soil microbiology, which could explain the fast ester cleavage of Ha-me.

Closer inspection of the siguals for Ha-acid (analyzed as its ethyl ester) in Figure 4, from incubation of *rac*- and particularly from *S*-Ha-me, indicates that the proportion of *R*-Ha-acid formed is larger than would be expected from the stereochemical composition of the precursor. Furthermore, the proportion of the *R*-enantiomer increases from the 2 h to the

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4 h sample. This indicates that Ha-acid enantiomers formed in soil are rapidly interconverted, with preference for the *R*-enantiomer. This interconversion appears to be faster than the actual degradation of Ha-acid, as its impact on the enantiomer composition is clearly observed. As Ha-acid is the active metabolite of Ha-me and is present in soil much longer than Ha-me, its enantiomer composition determines the residual herbicidal activity. Therefore, we investigated the degradation and enantiomerization of Ha-acid in soil in more detail.

Incubation of *rac*-, *R*-, and *S*-Ha-acid in Three Different Soils. Separate incubation experiments were conducted with racemic and enantiopure Ha-acid to investigate possible enantioselective degradation as well as conversion of one enantiomer to the other. From the experiment with Ha-me it was expected that changes in enantiomeric composition are fast. Therefore, short initial sampling intervals of 4 h were chosen, except for soil Dübendorf (Table 1), for which 1 day intervals were used.

In all three soils, the enantiomer composition of spiked Ha-acid changed rapidly as indicated by the chromatograms shown in Figure 5. In fact, the enantiomer ratio after 8 h of incubation

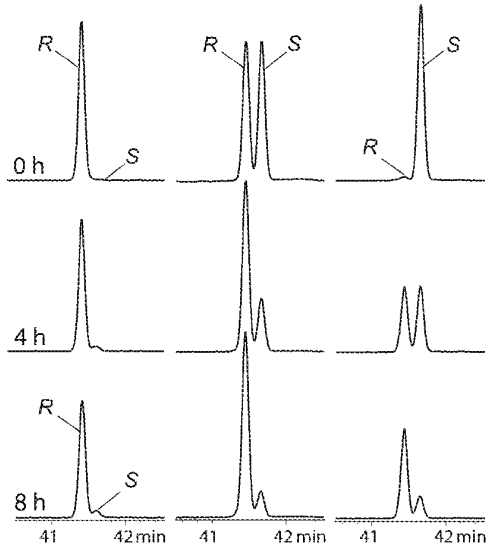


Figure 5. EI SIM chromatograms (m/z 375) of extracts of soil Neualp spiked with *R*- (left), *rac*- (middle), and *S*-Ha-acid (right) immediately after spiking (top) and 4 h (middle) and 8 h later (bottom), analyzed after methylation. Signals are normalized to the peaks for fluazifop used as internal standard. Note the rapid disappearance of *S*-Ha-acid and the concurrent formation of the *R*-enantiomer.

was almost independent of the initial enantiomer composition. The change in composition was faster than the overall dissipation of Ha-acid in soil. It involved the formation of *R*- from *S*-Ha-acid and, to a lesser degree, also the reverse reaction. The enantiomer composition eventually approached a steady state, where the concentration of the *R*-enantiomer was about 10 times that of the *S*-enantiomer. The composition in the three soils, expressed as enantiomer fraction (here defined as concentration of *S*-enantiomer divided by total concentration), is shown in Figure 6. From the curves it is suggested that the final enantiomer composition is independent of the initial composition and very similar in all three soils, whereas the rate at which this composition was achieved differed slightly (curve fitting, see below).

In the sterile soil, no degradation and no change in enantiomer composition were observed over the first 4 days of incubation, indicating that degradation as well as enantiomerization in soil are microbially mediated processes. After prolonged incubation, some conversion was observed, probably due to regrowth of microorganisms as sterile conditions were broken with spiking and sampling.

Kinetic Modeling of Degradation and Enantiomerization of Ha-acid in Soils. The experimental data in the three nonsterile soils were fitted using the conceptual model shown in Figure 2. In total, three rate constants were fitted to describe the conversion of *R*- to *S*-Ha-acid and vice versa, as well as overall degradation. The actual degradation of Ha-acid was found to be much slower than enantiomerization. Consequently, the degradation rate constants could not be fitted individually for each enantiomer and were thus assumed to be equal for *R*- and *S*-Ha-acid. This does not necessarily mean that the enantiomers were degraded equally quickly, but rather that any differences in degradation rates would be compensated by the rapid enantiomerization.

As an example, the fitting of degradation and enantiomerization of Ha-acid in soil Neualp is shown in Figure 7, where the fitted curves describe the observed behavior (formation and dissipation of enantiomers as well as overall degradation) correctly. The kinetic parameters thus derived for this and the two other soils are shown in Table 3. Overall, degradation was faster in soil Neualp (half-lives = ln $2/k_{deg}$, 2.3−4.0 days) than in the other two soils (6.0−9.3 days). Degradation in the experiments with *rac*-Ha-acid was consistently slower than in experiments with the pure enantiomers. This may be due to the fact that *rac*-Ha-acid was spiked with a slightly lower water volume, and the soil was thus a little less moist (Table 1).

The rate constants for conversion of *R*- to *S*-Ha-acid (k_{RS}) were only somewhat higher than the degradation rate constants in the same soils, whereas the rate constants for the reverse reaction were substantially (≈20 times) higher.

Due to rapid enantiomerization, a steady-state enantiomeric composition was achieved within 1−2 days of incubation (Figure 6). From the enantiomerization rate constans (k_{RS} and k_{SR}) equilibrium constants (K_{enant}) for the enantiomerization of Ha-acid can be calculated as

$$K_{enant} = k_{RS}/k_{SR}$$

The value of K_{enant} corresponds to the enantiomer ratio S/R at steady state. As shown in Table 3, the values for K_{enant} ranged from 0.061 to 0.082 and were thus very similar in all three soils.

This is in contrast to the situation when a chiral compound is degraded enantioselectively, but with no apparent enantiomerization, such as with the chiral fungicide metalaxyl.[21] In that case, the enantiomer ratio (ER) changes with incubation time according to the equation

$$\ln(ER) = \ln(ER_0) - \Delta k \times t$$

where Δk is the difference of the degradation rate constants of the two enantiomers and ER_0 is the enantiomer ratio at time zero. This equation indicates that the enantiomer ratio will not reach a steady state at any given time.

In a recent paper on diclofop-methyl,[10] a herbicide that is structurally closely related to Ha-me, the authors used the above equation to evaluate their experimental data. However, they did not take into consideration the rapid enantiomeriza-

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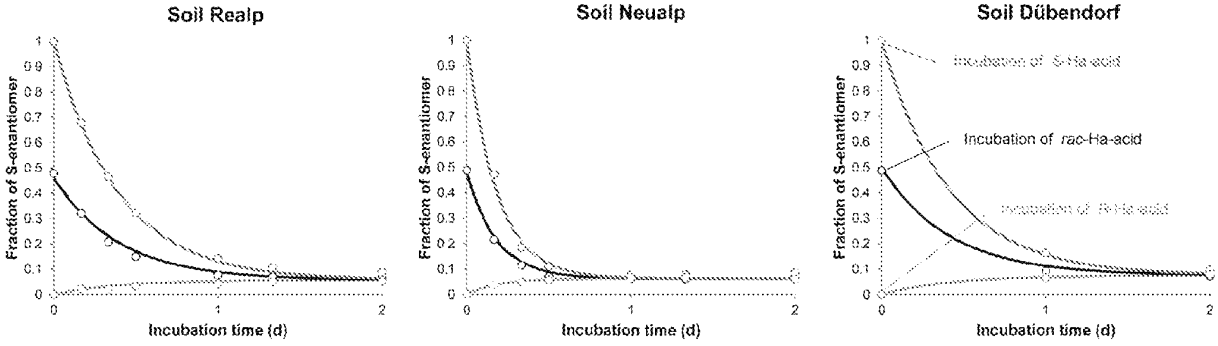


Figure 6. Change of enantiomeric composition of Ha-acid in soils, expressed as enantiomer fraction (concentration of *S*-enantiomer divided by total concentration) during incubation of racemic and enantiopure Ha-acid. Lines indicate the fitted curves. Note that the final composition is independent of the initially applied substance.

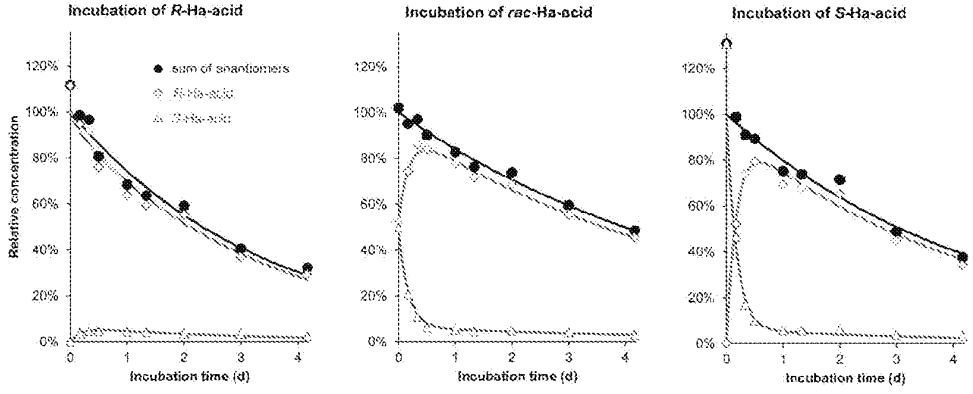


Figure 7. Concentrations of *R*-, *S*-, and total Ha-acid in soil Neualp after spiking of *R*- (left), *rac*- (middle), and *S*-Ha-acid (right). Curves were fitted according to the conceptual model shown in Figure 2.

Table 3. Degradation and Enantiomerization Rate Constants (± Standard Deviation Calculated by the Software) of Ha-acid in Three Soils Determined in Experiments with *rac*-Ha-acid and the Pure *R*- and *S*-Enantiomers Using the Kinetic Scheme Shown in Figure 2[a]

soil	spiked with	k_{deg} (day^{-1})	k_{RS} (day^{-1})	k_{SR} (day^{-1})	k_{RS}/k_{SR}
Dübendorf	*R*-Ha-acid	0.103 ± 0.012	0.184 ± 0.015	2.25 ± 0.11	0.082 ± 0.008
	rac-Ha-acid	0.080 ± 0.012			
	S-Ha-acid	0.115 ± 0.013			
Neualp	*R*-Ha-acid	0.298 ± 0.029	0.353 ± 0.086	5.21 ± 0.46	0.068 ± 0.018
	rac-Ha-acid	0.173 ± 0.017			
	S-Ha-acid	0.225 ± 0.020			
Realp	*R*-Ha-acid	0.107 ± 0.002	0.147 ± 0.073	2.40 ± 0.26	0.061 ± 0.031
	rac-Ha-acid	0.074 ± 0.022			
	S-Ha-acid	0.113 ± 0.028			

[a]Note that k_{deg} values were fitted for each experiment individually, whereas k_{RS} and k_{SR} were fitted simultaneously for all experiments with a particular soil.

tion of diclofop acid. To test the applicability of our approach for evaluation of Ha-acid to other, similar cases, we used the data for diclofop acid from ref 10 and applied the same conceptual model as for Ha-acid to derive the kinetic parameters. The resulting plots and kinetic parameters are shown in the Supporting Information (Figures S1–S4; Table S1). Indeed, acceptable fits for diclofop acid were obtained in this way from those previously published data.

Comparison of Haloxyfop Behavior in Soil with That of Other Phenoxy Acids. Isomerization of chiral pesticides in the environment was described for numerous compounds from different chemical families.[22] Rapid conversion of enantiomers with clear preference for the *R*-enantiomer was also observed for other FOPs, for example, for fluazifop,[9] diclofop,[3,10] and fenoxaprop.[3] It is also consistent with field experiments with Ha-me, where it was shown that the efficacy of the substance against weeds depended strongly on the enantiomeric

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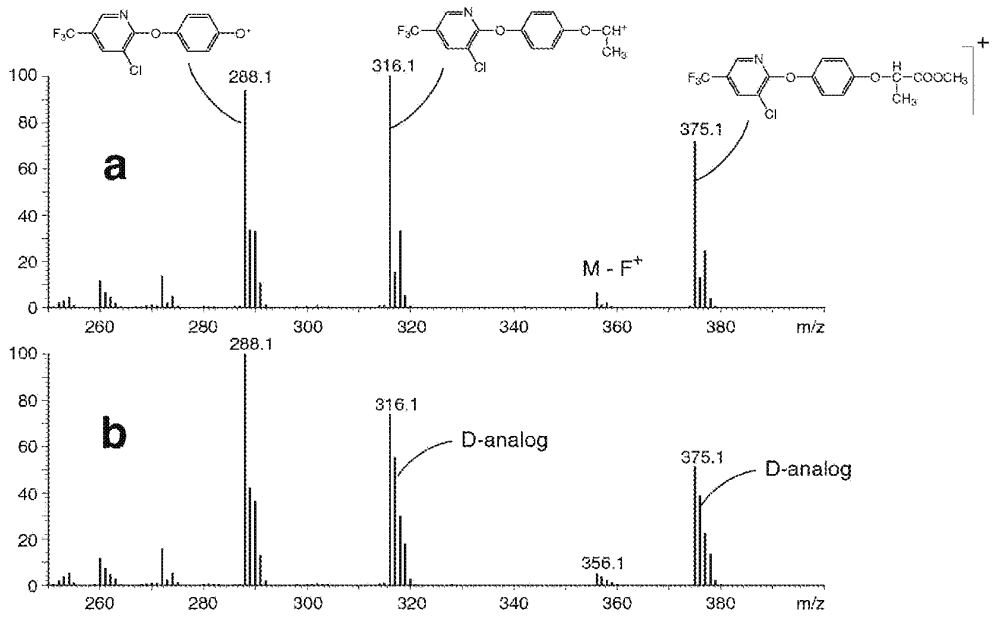


Figure 8. Partial mass spectra of (a) Ha-me (reference compound) and of (b) Ha-acid after 4 h of incubation in soil in the presence of D_2O and subsequent methylation. Note the incorporation of a single deuterium into Ha-acid as indicated by the changed isotope profiles of the molecular and the major ions at m/z 375 and 316, respectively (panel b).

composition when applied postemergent (thus applied to the leaves), whereas it was practically independent of the enantiomeric composition when applied pre-emergent (and is thus taken up from soil).[11] In the latter study, residues extracted from soil 7 days after application of Ha-me consisted mainly of *R*-Ha-acid.

Conversion of enantiomers was also observed for phenoxypropionic acid herbicides such as mecoprop and dichlorprop.[17,23] However, in contrast to our results with Ha-acid, degradation appeared to be faster than enantiomerization.[17] The enantioselectivity of degradation/enantiomerization of mecoprop and dichlorprop varied over a wide range and apparently changed with soil pH values (see ref 24 and references therein), whereas no pH dependence with respect to the preferential formation of the *R*-enantiomer was observed with Ha-acid in the three soils studied here.

Mechanistic Considerations from H−D Exchange Experiments. Full-scan MS clearly indicated the incorporation of deuterium when Ha-acid was incubated in soil where water was partially replaced with D_2O (H/D ratio of about 1:1). The EI mass spectra of Ha-me (Figure 8), Ha-et, and Fl-me are very similar in that all three show intense molecular ions and agree in their major fragmentation pathways. Ha-me (M^+ = 375) and Ha-et (M^+ = 389) yield the same major fragment ions at m/z 316 (loss of $COOCH_3$ and $COOC_2H_5$, respectively) and m/z 288 (loss of $CHCH_3COOCH_3$ and $CHCH_3COOC_2H_5$, respectively). No significant differences were observed in the spectra of the enantiomers.

As shown in Figure 8, the mass spectrum of Ha-acid after incubation in D_2O-containing soil and analyzed as methyl ester clearly shows changed isotopic patterns of M^+ (note increased signal of m/z 376) and for the main fragment ion at m/z 316 (increased signal of m/z 317) due to the incorporation of a single D into the molecule. The exact location of D was deduced from the fact that the isotopic pattern of fragment ion m/z 288 (loss of the complete propionic acid side chain) was still the same as with the native compound. This locates the D label at one of two carbons (C-2 or C-3) of the propionic acid moiety, most likely at C-2 (the methin-H), in the same sense as was observed with the propionic acid herbicide mecoprop.[23] The amount of D incorporation, estimated from the signal intensities at m/z 375 and 376 and when corrected for ^{13}C contributions, was about 35%.

The H−D exchange presumably follows a scheme such as outlined in Figure 9 via a planar transitional enolate structure

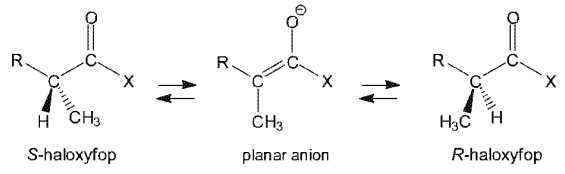


Figure 9. Proposed mechanism for the conversion of the enantiomers of Ha-me and Ha-acid in soil. "X" depicts a methoxy, a hydroxy, or a possible intermediate coenzyme A ester, analogous to that proposed for aryl propionic acid isomerization in mammalian metabolism.[6]

(back/front addition of H/D). However, the exchanged proton located next to the carboxylic acid moiety is only weakly acidic. Consequently, this reaction requires enzymatic activity such as in biologically active soils. For APAs it was shown that epimerization proceeds via formation of a coenzyme A thioester.[6] The enzyme that catalyzes epimerization of the coenzyme A esters of APAs in rats (2-arylpropionyl-coenzyme A epimerase) was isolated from rat liver[25] and was subsequently shown to be closely related, if not identical, to the 2-methylacyl-coenzyme A racemases present in peroxisomes and mitochondria,[26] which are involved in the metabolism of branched-chain fatty acids.[27] Although the epimerase is not enantioselective,

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the net formation of an excess of one enantiomer results from enantioselective formation of the coenzyme A thioester.[6]

It is interesting to note that in the WHO JMPR evaluation of haloxyfop[28] the reviewer concluded that in mammals Ha-me undergoes rapid ester cleavage in vivo, followed by an equally rapid and almost complete conversion of *S*- to *R*-Ha-acid. There seems to be a striking similarity in the findings from the studies in rodents, dogs, and monkeys with our finding of the rapid transformations of Ha-me and Ha-acid in biologically active soils. This may indicate that the same biochemical processes are involved in soil as in mammalian conversion of haloxyfop enantiomers, and these may even be very closely related to those involved in the conversion of APA enantiomers.

In contrast to these similarities in behavior of Ha-acid enantiomers in mammalian and soil metabolism, no conversion of Ha-acid enantiomers and no enantioselectivity in degradation of Ha-me or Ha-acid were found in plants.[12] Consequently, the residues in plants most likely reflect the stereoisomer composition of the compound that was applied (uptake by leaves) or that was formed in soil (root uptake).

ASSOCIATED CONTENT

Supporting Information

Figures S1–S6 and Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Complete Genomic Sequence of Nitrogen-fixing Symbiotic Bacterium *Bradyrhizobium japonicum* USDA110

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Abstract

The complete nucleotide sequence of the genome of a symbiotic bacterium *Bradyrhizobium japonicum* USDA110 was determined. The genome of *B. japonicum* was a single circular chromosome 9,105,828 bp in length with an average GC content of 64.1%. No plasmid was detected. The chromosome comprises 8317 potential protein-coding genes, one set of rRNA genes and 50 tRNA genes. Fifty-two percent of the potential protein genes showed sequence similarity to genes of known function and 30% to hypothetical genes. The remaining 18% had no apparent similarity to reported genes. Thirty-four percent of the *B. japonicum* genes showed significant sequence similarity to those of both *Mesorhizobium loti* and *Sinorhizobium meliloti*, while 23% were unique to this species. A presumptive symbiosis island 681 kb in length, which includes a 410-kb symbiotic region previously reported by Göttfert et al., was identified. Six hundred fifty-five putative protein-coding genes were assigned in this region, and the functions of 301 genes, including those related to symbiotic nitrogen fixation and DNA transmission, were deduced. A total of 167 genes for transposases/104 copies of insertion sequences were identified in the genome. It was remarkable that 100 out of 167 transposase genes are located in the presumptive symbiotic island. DNA segments of 4 to 97 kb inserted into tRNA genes were found at 14 locations in the genome, which generates partial duplication of the target tRNA genes. These observations suggest plasticity of the *B. japonicum* genome, which is probably due to complex genome rearrangements such as horizontal transfer and insertion of various DNA elements, and to homologous recombination.

Key words: *Bradyrhizobium japonicum* USDA110; genome sequencing; symbiosis; nodulation; nitrogen fixation

1. Introduction

Advances in DNA manipulation and sequencing technology have drastically changed the strategies for studying genetic systems of various living organisms, especially bacteria. Sequencing of the entire genomes followed by investigation of gene function using sequence information has proven to be effective; therefore, the complete genome structures of nearly 100 bacteria have been determined to date and genome sequencing of several hundred other species is in progress. Complete genomic sequences provide not only the information needed to perform functional analysis of the genes but also new insights into gene function, gene evolution, and genome evolution by comparing the gene components and gene organization in the genomes among species. This approach is called "comparative genomics."

Symbiotic nitrogen fixation is a vital component of the global nitrogen cycles and agricultural practices. The establishment of rhizobia-legume symbiosis requires the creation of developmental and functional programs. To understand the mechanisms which underlie this symbiosis, the physiology, ecology and genetics of rhizobia have been studied extensively. In addition, the recent introduction of genomic approaches has accelerated the accumulation of information on gene and genome structures as well as gene function. The complete structure of a broad host-range symbiotic plasmid (pNGR234a) of *Rhizobium* sp. NGR234 was reported in 1997.[1] Since

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then, the nucleotide sequences of the entire genomes of two rhizobia, *Mesorhizobium loti* and *Sinorihzobium meliloti* have been determined.[2,3] Accordingly, many comprehensive functional analyses of genes based on the genome sequences have been conducted using a microarray technology. These analyses include identification of genes that are up-regulated or down-regulated during the process of symbiotic nitrogen fixation.

Among rhizobia, *Bradyrhizobium japonicum* is the most agriculturally important species because it has the ability to form root nodules on soybeans (*Glycine max*). *B. japonicum* strain USDA110, which was originally isolated from soybean nodule in Florida, USA in 1957, has been widely used for the purpose of molecular genetics, physiology, and ecology, because of its superior characteristics regarding symbiotic nitrogen fixation.[4,5] The genome structure of *B. japonicum* USDA110 is similar to that of *M. loti* MAFF303099[2] in that many of the genes for symbiotic nitrogen fixation are clustered on the chromosome,[6,7] and a 410-kb DNA region identified as a symbiotic region is rich in these genes. A physical map of the genome and an ordered BAC (Bacterial Artificial Chromosome) library which covers most of the genome were reported.[8] To better understand the whole genetic system carried by this organism including the genes required for symbiotic nitrogen fixation, we determined the nucleotide sequence of the entire genome of *B. japonicum* USDA110, which comprises a single circular chromosome. In this paper, we describe the characteristic features of the genome of *B. japonicum* and compare them with two previously sequenced rhizobial strains, *M. loti* and *S. meliloti*.

2. Materials and Methods

2.1. Bacterial strain and genomic libraries

Bradyrhizobium japonicum USDA110 was provided by Dr. Michael J. Sadowsky at University of Minnesota.

For DNA sequencing, five random libraries with four types of cloning vectors were constructed from the total cellular DNA of *B. japonicum* to minimize cloning bias: BRE and BRB with inserts of approximately 1.0 kb (element clones) and 2.7 kb (bridge clones), respectively, were both cloned into M13mp18; BRP with approximately 9 kb (plasmid clones) was cloned into pUC18; BRC containing 25 kb inserts (cosmid clones) was cloned in cosmid vector pKS800;[9] and BRL bearing inserts of approximately 50 kb was cloned in BAC vector pBeloBACII.

2.2. DNA sequencing

The nucleotide sequence of the entire genome of *B. japonicum* USDA110 was determined by the whole-genome shotgun strategy combined with the "bridging shotgun" method.[10]

One strand of the element clones and both strands of the clones from the other four libraries were sequenced using the Dye-terminator Cycle Sequencing kit with DNA sequencers type 377XL (Applied Biosystems, USA), and ET-Terminator kit with a RISA-384 DNA sequencer (Shimazu Corporation, Japan). The accumulated sequence files were assembled using the Phrap program (Philip Green, University of Washington, Seattle, USA). The end-sequence data from the bridge, plasmid, cosmid, and BAC clones facilitated the gap-closure process as well as accurate reconstruction of the entire genome. The final gaps in the sequences were filled by the primer walking method. A lower threshold of acceptability for the generation of consensus sequences was set at a Phred score of 20 for each base. The integrity of the reconstructed genome sequence was assessed by walking through the entire genome with the end sequences of the cosmid or BAC clones.

2.3. Gene assignment and annotation

Coding regions were assigned by a combination of computer prediction and similarity search as described previously. Briefly, prediction of protein-coding regions was carried out with the Glimmer 2.02 program.[11] Prior to prediction, the matrix was generated for the *B. japonicum* genome by training with a dataset of 1593 open reading frames (ORFs) that showed a high degree of sequence similarity to genes assigned in the genomes of *M. loti*, *S. meliloti*, and *Agrobacterium tumefaciens* at the amino acid level. All of the predicted protein-encoding regions equal to or longer than 90 bp were translated into amino acid sequences, which were then subjected to similarity search against the non-redundant protein database (nr-database) with the BLASTP program.[12] In parallel, the entire genomic sequence was compared with those in the nr-protein database using the BLASTX program to identify genes that had escaped prediction and/or those smaller than 90 bp, especially in the predicted intergenic regions. For predicted genes that did not show sequence similarity to known genes, only those equal to or longer than 150 bp were considered as candidates.

Functions of the predicted genes were assigned on the basis of the sequence similarity of their deduced products to those of genes of known function. For genes that encode proteins of 100 amino acid residues or more, a BLAST score of 10^{-20} was considered significant. A higher E-value was considered significant for genes encoding smaller proteins.

Genes for structural RNAs were assigned by similarity search against the in-house structural RNA database that had been generated based on the data in GenBank (rel. 124.0). tRNA-encoding regions were predicted by use of the tRNA scan-SE 1.21 program[13] in combination with the similarity search.

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Comparison of the structures of the gene components among *B. japonicum*, *M. loti*, and *S. meloloti* was performed by taking two factors, the BLAST2 bit score and the ratio of alignment length, into consideration. A lower threshold of acceptability was set at one-fourth of the bit score reported by self-comparison of the translated amino acid sequences by the BLASTP program. Only amino acid sequences whose alignments extended over at least 0.6 times the length of the query sequence were considered similar. With lower stringency, two protein-encoding genes were considered similar if the BLAST2 score was lesser than 10^{-4}.

The GC skew analysis was performed as described by Lobry.[14]

3. Results and Discussion

3.1. Sequence determination of the entire genome

The entire genome of *B. japonicum* was sequenced according to the modified whole genome shotgun method as described in Materials and Methods. Briefly, a total of 101,181 random sequence files corresponding to approximately 6 genome-equivalents were assembled to generate draft sequences. Finishing was then carried out by visually editing the above sequences, followed by gap closing, and additional sequencing to obtain the sequence data having a Phred score of 20 or higher. The integrity of the final genome sequence was assessed by comparing the insert length of each cosmid and BAC clone with the computed distance between the end sequences of the clones. The genome of *B. japonicum* USDA110 thus deduced was a circular chromosome of 9,105,828 bp, and the average GC content was 64.1%. No plasmid was detected in the course of genome sequencing. The nucleotide position was numbered from one of the recognition sites of the restriction enzyme *Pac* I (Fig. 1 and Fig. 1 in the Supplement section) according to the physical map of the genome by Kündig et al,[6] although inconsistency was found between the published physical map and the genome sequence determined in this study with respect to another *Pac* I restriction site, possibly due to genome rearrangements.

3.2. Sequence features of the genome

The second inner circle of Fig. 1 shows the average GC percent of every 10 kb for the entire genome. Uneven distribution of GC contents was observed in several locations of the genome including a putative symbiotic region (section 3.5.2) and putative insertion elements in tRNA genes (section 3.5.4).

A GC skew analysis was performed to locate a probable origin and terminator of DNA replication.[14] As a result, shift of GC skew was observed in two regions of the genome at coordinates 700 kb and 4890 kb, as shown in Fig. 1 (the innermost circle). In *Caulobacter*

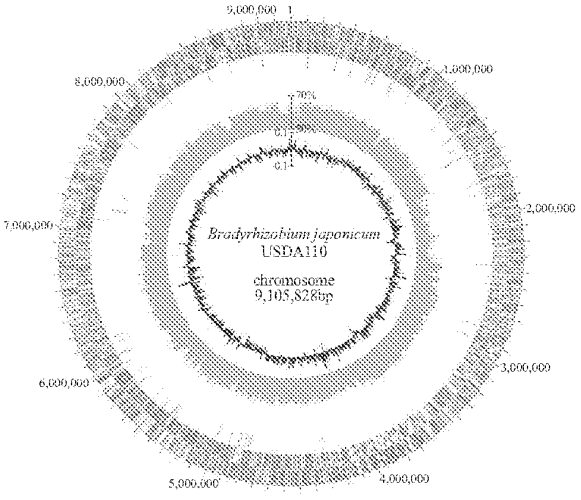


Figure 1. Circular representation of the chromosome of *Bradyrhizobium japonicum* USDA110. The scale indicates the location in bp starting from the *Pac* I recognition site. The bars in the first and second outermost circles show the positions of the putative protein-encoding genes in the clockwise and counterclockwise directions, respectively. Genes whose functions could be deduced by sequence similarity to genes of known function are depicted in green, and those whose function could not be deduced are in red. The bars in the third circle indicate the positions of predicted tRNA genes and those in the fourth circle the positions of genes for structural RNAs including rRNAs and small RNAs. The innermost and the second circles with scales show the GC skew and the average GC percent, respectively, calculated using a window-size of 10 kb.

crecentus, a member of alpha-proteobacteria, the origin of DNA replication has experimentally been located between two genes, *CC0001* and *CC3763* (*hemE*), where the shift of GC skew was detected.[15,16] In *A. tumefaciens* and *S. meliloti*, the same region is anticipated to be the origin because a shift of GC skew was observed in the region spanning the *CC0001* homologue and *hemE* genes despite of no experimental evidences.[17,18] A cluster of genes containing *parA*, *parB*, *gidA*, *gidB*, *thdF*, *rho*, *aroE*, *dnaQ*, and *CC3761* homologue was conserved near this region in the above three species. Although no clear shift of GC skew was detected in *M. loti*, it is assumed that the same region functions as the replication origin, because the gene cluster containing the above gene set is conserved.[2] In *B. japonicum*, a cluster of *parA*, *parB*, *gidA*, *gidB*, *thdF*, *rho*, *CC0001* homologue, *aroE* and *dnaQ* genes (*bll0630–bll0641*) but not *hemE* (*bll2399*), was found at coordinates 675–689 kb. These observations strongly suggest that this region contains an origin of replication.

Another shift of GC skew was detected at the diagonal position of the putative replication origin in the genome of *B. japonicum* (coordinates 4900 kb) as well

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Table 1. Features of the assigned protein-encoding genes and their functional classification.

	Number of genes	%
Amino acid biosynthesis	173	2.1
Biosynthesis of cofactors, prosthetic groups, and carriers	179	2.2
Cell envelope	109	1.3
Cellular processes	302	3.6
Central intermediary metabolism	198	2.4
Energy metabolism	303	3.6
Fatty acid, phospholipid and sterol metabolism	212	2.5
Purines, pyrimidines, nucleosides, and nucleotides	86	1.0
Regulatory functions	567	6.8
DNA replication, recombination, and repair	96	1.2
Transcription	49	0.6
Translation	203	2.4
Transport and binding proteins	752	9.0
Other categories	1119	13.5
Subtotal of genes similar to genes of known function	**4348**	**52.3**
Similar to hypothetical protein	2506	30.1
Subtotal of genes similar to registered genes	**6854**	**82.4**
No similarity	1463	17.6
Total	**8317**	**100.0**

as those of *C. crecentus*, *A. tumefaciens* and *S. meliloti*, and it is likely that DNA replication terminates in this region. In *Escherichia coli*, a 28-mer sequence [-GGTGCGCATAATGTATATTATGTTAAAT-], designated as *dif*, is present in this region, which is required to convert a dimer chromosome to monomers after aberrant DNA duplication.[19] A similar sequence was found in the *B. japonicum* genome near the position where the shift of GC skew was detected at the coordinates 4,996,159–4,996,186, while the similar sequences were also observed in *M. loti*, *A. tumefaciens*, and *S. meliloti*.

3.3. Assignment of protein- and RNA-encoding genes

The potential protein-encoding regions were assigned using a combination of computer prediction by the Glimmer program and similarity search. Glimmer predicted a total of 13,619 potential protein-encoding genes in the genome after training with a dataset of sequences of highly probable protein-encoding genes in the genomes of three bacteria: *M. loti*, *S. meliloti*, and *A. tumefaciens*. By taking the sequence similarity to known genes and the relative positions into account to avoid overlaps as described in Materials and Methods, the total number of potential protein-encoding genes finally assigned to the genome was 8317 (Table 1 and Fig. 1 in the Supplement section). The average gene density was one gene in every 1095 bp. The putative protein-encoding genes thus assigned to the genome starting with either an ATG, GTG, TTG, or ATT codon are denoted by a serial number with three letters representing the species name (b), whether the ORF was longer than or shorter than 100 codons (l or s), and the transcription direction on the circular map (r or l) (Fig. 1). The codon usage frequency of the whole gene components in the genome is tabulated in Table 1 in the Supplement section.

One copy of an rRNA gene cluster was identified on the genome in the order of 16S-*trnI*-*trnA*-23S-5S at the coordinates of 1,528,226–1,533,622 (Fig. 1 in the Supplement section).

A total of 50 tRNA genes including those in the rRNA gene cluster, which represent 46 tRNA species, were assigned on the genome by sequence similarity to known bacterial tRNA genes and computer prediction using the tRNA scan-SE program (Fig. 1 and Table 1, and Table 2, Fig. 1, Fig. 2 in the Supplement section). There were three copies of *trnI*-CAU genes of completely identical sequences. Two genes, *trnN*-GUU (coordinates 6,191,247–6,191,320) and *trnF*-GAA (coordinates 6,191,318–6,191,390), were located in the same direction with a 2-bp overlap. Most of the tRNA genes were dispersed on the genome and were likely to be transcribed as single units except those in the rRNA gene cluster and *trnN*-*trnF* genes described above.

As to small RNA-encoding genes, potential genes showing sequence similarity to RNase P subunit B (*rnpB*) and tmRNAs (*ssrA*) were assigned to the genome. tmRNA is known to be involved in the degradation of aberrant proteins in the cells.[20] In *B. japonicum*, *ssrA* codes for two RNA components, tmRNA-acceptor RNA and tmRNA-coding RNA, the latter of which has been reported as unknown RNA (*sra*) involved in the development of symbiotic root nodules.[21]

3.4. Functional assignment of protein-encoding genes

Translated amino acid sequences of 8317 potential protein-encoding genes in the genome were compared with the sequences in the nr-protein databases with the BLASTP program as described in Materials and Methods. According to the results, 4348 (52%) were similar to genes of known function, 2506 (30%) showed similarity to hypothetical genes, and the remaining 1463 (18%) showed no significant similarity to any registered genes (Table 1 and Fig. 1).

The potential protein-encoding genes whose function could be anticipated were classified into 14 categories, each with different biological roles, according to the principle of Riley.[22] The numbers of genes in each category are summarized in Table 1, and the name of each gene is listed in RhizoBase at http://www.kazusa.or.jp/rhizobase/. The location,

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length and direction of these genes are indicated on the gene map in the Supplement section (Fig. 1), with color codes corresponding to functional categories.

3.5. *Characteristic features of the predicted genes and the genome*

3.5.1. *Comparison of gene components among three rhizobia*

To date, annotation information of the whole gene components in two rhizobia, *M. loti* and *S. meliloti*, have been published, and a total of 7281 and 6205 potential protein-encoding genes have been assigned to the respective genomes. Genes of *B. japonicum* were compared with those of *M. loti* and *S. meliloti* under the criteria described in Materials and Methods. Comparison with higher stringency indicated that 2798 *B. japonicum* genes, comprising 34% of the 8317 potential protein-encoding genes, have matched genes in both the *M. loti* and *S. meliloti* genomes, 632 of which were genes of unknown function. Seven hundred seventy-five (9%) and 584 (7%) *B. japonicum* genes were commonly found in either the *M. loti* or *S. meliloti* genome, respectively.[2,3] The remaining 4160 genes (50%) were unique to *B. japonicum*. Two hundred thirty-three of these were genes of known function, including those for NolY (*bll2016*), NoeD (*blr1633*), NodY (*blr2024*), nodulation protein (*blr2062*), NoeE (*blr2073* and *blr2074*), NolZ (*bsl2015*), and NolM (*bsr2032*). With lower stringency, where most members of a given gene family can be grouped into a cluster, 5382 *B. japonicum* genes (65%) showed significant sequence similarity to genes in both *M. loti* and *S. meliloti*. Five hundred eighty-six (7%) and 420 (5%) *B. japonicum* genes were conserved in either the *M. loti* or *S. meliloti* genome, respectively, and 1929 genes (23%) were only found in *B. japonicum*. Both results indicate that significant portions of the gene components in the genomes are unique to the species.

3.5.2. *Symbiosis island*

The symbiosis island was first identified in *M. loti* ICMP3153 as a transmittable DNA segment of approximately 500 kb in length containing a cluster of symbiotic genes.[23] It was shown that this region was inserted into a phe-tRNA gene in the genome with a 17-bp duplication of the 3′ terminal portion of the gene. A 611-kb DNA region with lower GC contents flanked by a complete and a partial phe-tRNA genes with a 17-bp duplication was also reported as a putative symbiosis island in *M. loti* MAFF303099.[2] In *B. japonicum*, on the other hand, a 410-kb DNA segment containing clusters of genes for symbiotic nitrogen fixation has been assigned as a symbiotic region.[7] Sequence analysis of the entire *B. japonicum* genome revealed the presence of a 680-kb DNA region of lower GC content (59.4%, see Fig. 1) at the coordinates 1681–2362 kb, in which the 410-kb symbiotic DNA region described above (coordinates 1,878,486–2,289,058) was completely included. A val-tRNA gene, instead of the phe-tRNA gene in *M. loti*, was found at one end of this region at the coordinates 2,362,342–2,362,416, but no trace of terminal duplication was detected in the other end of this region. However, a 45-bp segment of the 3′ terminal portion of the val-tRNA gene was found at the coordinates 7,939,490–7,939,534, 3528-kb apart from the intact val-tRNA gene, and a putative integrase gene (*bll7217*) was found next to this tRNA gene segment, as in *M. loti*. Considering the fact that the GC content of a region (coordinates 7934–7940 kb) adjacent to this segment were lower than the average of the entire genome (59.2% vs. 64.1%), it can be postulated that an ancient larger symbiosis island was split into two, the 680-kb DNA region (a putative symbiosis island A at the coordinates 1681–2362 kb) and the 6-kb DNA region (a putative symbiosis island B at the coordinates 7934–7940 kb), by a large-scale genome rearrangement.

A total of 655 potential protein-encoding genes were tentatively assigned to putative symbiosis islands A and B. Three hundred one out of 655 genes (46%) showed sequence similarity to genes of known function. These included two clusters of genes *nifDKENX*-[8 genes]-*nifS*-[2 genes]-*nifB*-[9 genes]-*nifH*-[3 genes]-*fixBCX* (*blr1743–bsr1775* at the coordinates 1,907,825–1,935,084) and *nolZY*-[2 genes]-*nolA*-[1 gene]-*nodD2*-[1 gene]-*nodD1YABCSUIJ-nolMNO-nodZ-fixR-nifA-fixA* (*bsl2015–blr2038* at the coordinates 2,175,594–2,199,393) as previously reported by Kündig et al.[6] In addition, *trbLFGI* (*blr1617-blr1618-blr1619-blr1620*) were found at the coordinates 1,773,989–1,777,834, while *traG* was disrupted, which may impair the self-transmissibility of the symbiosis island(s) in the USDA110 strain. Four genes, *nodM* (*blr1632*), *nolK* (*bll1630*), *noeL* (*bll1631*), and *noeD* (*blr1633*), formed a gene cluster at the coordinates 1,788,234–1,794,230. Three gene clusters for unknown ABC transporters, *blr1601* (substrate-binding protein)-*blr1602* (permease)-*blr1603* (permease)-*blr1604* (ATP-binding protein), *blr1679* (permease)-*blr1680* (permease) and *blr2170* (substrate-binding protein)-*blr2171* (permease), and genes for sensor/regulator hybrid of a two-component signal transduction system, *bll2176* (sensor/regulator hybrid) and *blr2178* (sensor/regulator hybrid), were also found in the putative symbiosis islands. It is remarkable that 100 out of 167 genes for transposases were located in presumptive symbiosis island A. This accounts for 60% of the transposase genes in the genome and 15% of the putative protein-encoding genes in symbiosis island A.

3.5.3. *Insertion sequences*

A total of 167 genes for putative transposases were assigned to the genome of *B. japonicum*. Of these, 133 were identified as components of insertion sequences (ISs), and

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the remaining 34 were not due to structural rearrangements. With respect to ISs, 104 copies were classified into 20 groups, each comprising 1 to 15 members on the basis of the type and number of transposases. Structural features of each IS group are summarized in Tables 3 and 4 and Fig. 3 in the Supplement section.

Most of the members exhibited typical characteristics of ISs, such as terminal inverted repeats of 3 to 48 bp and direct repeats at the insertion sites. It has been reported that insertion of RSα occurs at a target sequence 5′-CTAG-3′ in *B. japonicum*.[24] Analysis of the entire genome sequence in this study revealed that 7 groups including RSα seemed to have preferable sequences for insertion, and it is notable that 6 of them, which comprise 26 IS members (34% of the ISs in the genome), targeted 5′-CTAG-3′. The tetramer sequence 5′-CTAG-3′ may play a significant role in the transposition of ISs and IS-mediated genome rearrangement in *B. japonicum*.

It is remarkable that 63 out of 104 ISs (60%) were located in presumptive symbiosis island A. ISs were also frequently found in a 206-kb DNA region at the coordinates 8975-0-75 kb. Of 224 putative protein-encoding genes assigned to this region, 89 (40%) did not show any sequence similarity to genes of known function, and 36 of the remaining 135 genes of known function code for transposases. This region had lower GC content (60.2%) than the rest of the genome (Fig. 1), and a gene for probable site-specific integrase/recombinase (*blr8174*) was identified near the border. These characteristic features may indicate that this region is a mobile genetic element,[25] though terminal duplication was not detected.

3.5.4. Insertion of DNA elements in tRNA genes

Duplication of portions of tRNA genes was found at 14 locations in the genome. They were separated from the respective intact genes by DNA segments of 4 to 97 kb; therefore, it is likely that these are traces of insertion of DNA elements into tRNA genes.[25] Table 5 in the Supplement section lists the species of tRNA genes, their positions, the length of duplications and DNA regions that separate the duplicated tRNA gene segments. The size of the presumptive DNA elements is diverse among the insertions, and no apparent sequence similarity was detected among them except that many of them contained putative genes for integrases. The GC contents of these DNA elements were lower than the average GC content of the genome. These features, common characteristics of mobile genetic elements, strongly suggest that insertion of the DNA elements into tRNA genes took place, though the origin of the element as well as the ability of the extant elements to transpose remain to be studied.

In and near the putative symbiosis island A, 3 copies of ile-tRNA genes (Fig. 2 in the Supplement section) and 2 copies of 49-bp 3′ terminal portions of the genes (coordinates 1,702,873–1,702,921 and 2,473,140–2,473,188, respectively) were found. Two pairs of 49-bp terminal portions were assigned as the *trnI1* and *trnI2* elements as shown in Table 5 in the Supplement section. There was another copy of the 49-bp 3′ terminal portion of the ile-tRNA gene at the coordinates 7,676,010–7,676,058, in the *trnM1* element, and the origin of this segment is unknown. This feature may indicate that complex genome rearrangements took place before and after insertion of the symbiosis island.

3.5.5. Genes related to symbiotic nitrogen fixation

Genes related to various steps of the symbiotic nitrogen fixation process have been identified in the genome of *B. japonicum*, as listed in the gene category list of RhizoBase at http://www.kazusa.or.jp/rhizobase/. Remarkable features noted for the *B. japonicum* genome so far are itemized below.

1. Genes showing sequence similarity to that of *virA* (*blr2697*) and *virG* (*blr2694*) were identified. Homologues of *virD* and *virE* were not detected.

2. There was a large gene cluster (*blr2358–bll2381*) at the coordinates 2,561,178–2,595,273 containing *exoP* (succinoglycan biosynthesis transport protein: *bll2362*), *exoZ* (probable exopolysaccharide production protein: *blr2369*),[26] 6 genes similar to those for glycosyl transferase, which is likely to be involved in exopolysaccharide synthesis.

3. Two adjacent genes, *blr1993* and *blr1994*, located in the presumptive symbiosis island were assigned as putative polygalacturonase and pectinesterase, respectively. Outside of the symbiosis island, *bll7427*, *blr3367*, and *bll2241* showed significant sequence similarity to those for ligninase (*ligE*), cellulase, and endo-1,4-beta-xylase, respectively. These genes may take part in the degradation of plant cell walls during the infection process.[27]

4. The sequence of the putative gene product of *blr0241* is 80% identical with that of ACC deaminase of *M. loti* (*mlr5932*). This gene is likely to be involved in the reduction of ethylene concentration during the nodule formation process.[28]

5. *nolK/noeL/nodM/noeD* (*bll1630-bll1631-blr1632-blr1633*) formed a gene cluster in the presumptive symbiosis island at the coordinates 1,788,234–1,794,230. A homologue of *nodQ* (*bll1475*) was also found but was 178 kb distant from *nolK* outside of the symbiosis island.

6. Of the four copies of *dnaJ* homologues (*bll0184*, *blr0680*, *blr2626*, *blr4653*), *blr0680* showed the highest degree of sequence similarity to that of *dnaJ* in *Rhizobium leguminosarum*, which was reported to be

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involved in the regulation of nodule formation and nitrogen fixation.[29]

7. *blr1755* showed significant sequence similarity to *iscN* of *R. etli*, which plays a critical role in nitrogen fixation.[30] It formed a cluster with the *fixU* and *nifS* homologues (*bsr1757* and *blr1756*, respectively), as in *R. etli*.

8. Two adjacent genes, *nfeD* (*bll4952*) and a homologue of a gene for stomatin-like protein (*bll4951*), both of which are assumed to be involved in nodulation competitiveness,[31,32] were identified at the coordinates 5,494,753–5,496,917.

9. The putative products of *fixK2/fixLJ/fixNOPQ/fixGHIS* genes (*bll2757-bll2759-bll2760-blr2763-blr2764-bsr2765-blr2766-blr2767-blr2768-blr2769-bsr2770*) are the oxygen-sensing cascade and high-affinity terminal oxidase that are required for microaerobic respiration and nitrogen fixation in nodules.[33] This gene cluster was located at a position 674 kb from main symbiosis island A, similar to its location on the *M. loti* chromosome.[2] This system may have originated from ancient non-symbiotic bradyrhizobia.

3.5.6. Other features

In addition to the various characteristics of genes in the *B. japonicum* genome described above, other remarkable features are itemized below.

1. All the genes for the key carbon fixation enzymes seem to be present. These include genes related to the Calvin-Benson-Bassham (CBB) cycle (*cbbR-cbbFPTALSX*) which formed a cluster (*bll2580–blr2587*) at the coordinates 2,822,117–2,831,210, a PEP carboxylase gene (*blr2955*), and a carbonic anhydrase gene (*bll2065*). *bll1137*, *bll4865*, and *bll4863* also showed sequence similarity to the gene for carbonic anhydrase. A gene showing sequence similarity to that for ribulose-phosphate 3-epimerase (*blr2588*) was located downstream of *blr2587*, as in *S. meliloti*,[34] suggesting that this gene may be a part of the gene cluster for the CBB cycle. These fixation systems of carbon dioxide are probably required for chemoautotrophic growth of *B. japonicum* USDA110 in the presence of hydrogen gas as an energy source.[4]

2. Ni-Mo hydrogenase mediates uptake of dihydrogen via nitrogenase, and increases N_2 fixation efficiency. Two sets of a hydrogenase gene cluster were found, one inside and another outside of the symbiotic region (coordinates 1,888,916–1,902,575 and 7,624,531–7,649,197, respectively). Interestingly, a complete set of *hupNCUVSLCDFGHIJK*, *hypABFCD'E*, and *hoxXA* were located outside of the symbiosis island, and were highly homologous to the uptake hydrogenase genes of *B. japonicum* strain USDA122DES.[35] On the other hand, some variant genes for uptake hydrogenase (*hupSLC*, *hypAFDE*) were found inside the symbiosis region.[7]

3. It was shown that *B. japonicum* had a NapAB-type periplasmic dissimilatory nitrate reductase, as does *S. meliloti*.[36] *napEDABC* formed a cluster, while *norCB*, *nirK*, and *nosZ* were dispersed throughout the genome.[37] The putative products of these genes are likely to be involved in denitrification from nitrate to N_2.

4. Homologues of *trbBCDEJLFGI* (*bll8288-bll8287-bsl8286-bll8285-bll8284-bll8282-bll8281-bll8280-bll8279*), *traG* (*bll0043*), and *traF* (*bll0047*), which were essential for conjugative transfer of Ti-plasmid in *A. tumefaciens*,[38] were assigned to the putative mobile genetic element at the coordinates 8975-0-75 kb (see section 3.5.3). Taking into consideration the fact that the homologues are found on either the symbiosis island or pMLb in *M. loti*, these genes may play a role in the transfer of this genetic element. Inconsistency between the previously reported physical map of the genome and the sequence determined in this study was attributed to this region, suggesting that this element may be self-transmissibile.[6]

5. A significant number of genes for flagella formation and mobility, including those for chemotaxis, *cheAWY* (*blr2192-blr2193-blr2194*), were found in *B. japonicum*.[39]

6. A homologue of a *tfdA*-like gene (ketoglutarate-dependent 2,4-dichlorophenoxyacetate dioxygenase), which is involved in the degradation of 2,4-D (2,4-dichlorophenol) and phenoxyacetate, was found (*bll1493*).[40]

7. *B. japonicum* USDA110 simultaneously possesses type III and IV secretion systems. Gene homologues of *ctpACDFGHI*, *pilA* for the type IV secretion system (*bsl7141-bll1440-bll1439-bll1437-bll1436-bll1435-bll1434*, and *bsl6587*) were found mainly outside of symbiosis islands, whereas those for *rhcC1 C2 JNQRSTUV* for the type III secretion system (*bll1811-bll1842-blr1813-blr1816-blr1818-blr1819-bsr1820-blr1821-blr1822-bll1800*) were located on symbiosis island A. Interestingly, genes for the type III and type IV secretion systems exist within symbiosis islands of *M. loti* strains MAFF303099 and R7A, respectively.[41] These secretion systems may be involved in fine turning of host-specific nodulation.

The sequences, as well as the gene information shown in this paper, are available in the Web database,

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RhizoBase, at http://www.kazusa.or.jp/rhizobase/. The sequence data analyzed in this study appears in DDBJ/GenBank/EMBL under accession number BA000040.

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Isolation and characterisation of new Gram-negative and Gram-positive atrazine degrading bacteria from different French soils

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Abstract

The capacity of 12 soils to degrade atrazine was studied in laboratory incubations using radiolabelled atrazine. Eight soils showed enhanced degradation of this compound. Twenty-five bacterial strains able to degrade atrazine were isolated by an enrichment method from 10 of these soils. These soils were chosen for their wide range of physico-chemical characteristics. Their history of treatment with atrazine was also variable. The genetic diversity of atrazine degraders was determined by amplified ribosomal restriction analysis (ARDRA) of the 16S rDNA gene with three restriction endonucleases. The 25 bacterial strains were grouped into five ARDRA types. By sequencing and aligning the 16S rDNA genes, the isolates were shown to belong to the Gram-negative species *Chelatobacter heintzii*, *Aminobacter aminovorans*, *Stenotrophomonas maltophilia* and to the Gram-positive genus *Arthrobacter crystallopoietes*. These species were not described previously as being capable of atrazine degradation. Most Gram-negative bacteria could mineralise ^{14}C ring labelled atrazine and carried the *atz*A, *atz*B, *atz*C and *trz*D genes. Gram-positive strains could convert atrazine to cyanuric acid and carried only the *atz*B and *atz*C genes. In this study, we describe the atrazine degradation capacities and corresponding genes in bacterial species that were not known as atrazine degraders. We report for the first time the occurrence of the *trz*D gene in these atrazine-mineralising bacteria and we demonstrate the potential use of colony hybridisation to isolate bacteria involved in atrazine degradation. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Atrazine-degrading bacterium; Diversity; *atz*A; *atz*B; *atz*C; *trz*D

1. Introduction

For 30 years, the herbicide atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine), which inhibits electron transport in photosynthesis, has been widely used in many parts of the world to control a variety of broad-leaf weeds in cropped and non-cropped soils. Despite its low water solubility, atrazine has a high mobility in soil which results in the contamination of soil [1,2], surface water [3], ground water [4], rain water [5] and of tile-drain water [6]. Frequently, atrazine contamination levels in water exceed the maximum pesticide contaminant level for drinking water, which is 0.1 μg l^{-1} in Europe and 3 μg l^{-1} in the USA. Atrazine is considered a relatively persistent chemical in the environment with a half-life ranging from a few weeks to months and with low ring mineralisation [7]. The dissipation of atrazine in soils can be due to chemical or to biological processes. The dechlorination of atrazine to hydroxyatrazine can be catalysed in soil [8], but atrazine is mainly degraded biologically. Fungi [9,10] and bacteria [11–14] can degrade atrazine via *N*-dealkylation or dehalogenation reactions. The ability of fungi to degrade atrazine and other xenobiotics could be due to the action of enzymes involved in the lignin degrading system and, particularly, the extracellular peroxidases. In the case of *s*-triazine degrading bacteria, a cytochrome P-450 like monooxygenase could be involved in the *N*-dealkylation of atrazine [14,15].

It has been known for a few years that certain soils are capable of rapidly degrading the *s*-triazine ring (enhanced degradation). This rapid dissipation has been linked to repeated treatments with atrazine [16–18] especially in soils with a high pH (from neutral to alkaline) [19].

Recently, mixed communities of microorganisms [20],

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consortia [21] or pure strains of *Pseudomonas* sp. ADP [22] and *Pseudaminobacter* sp. [23] have been shown to be capable of complete mineralisation of atrazine to carbon dioxide and ammonia at high rates. The ability of microorganisms to use atrazine as a sole nitrogen source [22] and/or carbon source [23,24] was clearly demonstrated.

The atrazine catabolic genes *atz*ABC have been characterised from *Pseudomonas* sp. strain ADP [25–28]: the genes *atz*A, -B, -C which encode three hydrolases transforming atrazine into cyanuric acid are highly conserved [23,29] and are located on a large plasmid, pADP1 [30], which is self transmissible to Gram-negative bacteria and widespread. However, Topp et al. [31] have isolated a Nocardioides sp. which degrades atrazine to hydroxyatrazine and finally to *N*-ethylammelide. The enzyme responsible for the first step of atrazine degradation is a novel chlorohydrolase. Recently, a gene responsible for the cleavage of cyanuric acid (*trz*D) was described in *Pseudomonas* NRRLB-12227 [32]. This bacterium was isolated as a melamine degrader. The occurrence of such genes encoding the cleavage of cyanuric acid has never been reported in atrazine degraders.

The aim of this work was to analyse the diversity of atrazine degrading microorganisms isolated from French soils. These soils were chosen for their wide range of physico-chemical characteristics and for their different histories of treatment with atrazine. Atrazine degrading bacteria were isolated from each soil using an enrichment technique. Then, the strains were characterised on a taxonomic basis by amplified ribosomal restriction analysis (ARDRA) and sequencing of the 16S rDNA gene. The capacity of pure strains to degrade atrazine was tested and the occurrence of known degrading genes (*atz*A, *atz*B, *atz*C and *trz*D) was determined for each strain.

2. Materials and methods

2.1. Sampling sites and kinetics of atrazine degradation in soils

The bacteria described in this paper were isolated from 12 cultivated and grassland soils from different areas in France. Soil samples were collected in the 0–15-cm top horizon layer. The main soil properties, crop and herbicides histories are reported in Table 1. Before use, the soil was partially air dried and sieved to 2–3 mm. Atrazine degradation in soils was measured in laboratory incubations. For this purpose, the moisture content of the soils was adjusted to 100% of the soil water holding capacity, taking into account the volume of added atrazine solutions. Chemical grade non-labelled atrazine (chemical purity 99%, Novartis) was used to prepare solutions of herbicide after mixing with the appropriate ^{14}C ethyl chain labelled atrazine (specific activity 1776 MBq mmol^{-1}, purity >98%, Isotopchim) or ^{14}C ring uniformly labelled atrazine (specific activity 910.2 MBq mmol^{-1}, purity >98%, Sigma). 10 g of soil (on a dry weight basis) was incubated in a closed 1-l jar at 20°C in the dark. Three replicates were prepared for each soil. The final atrazine concentration was 0.5 mg kg^{-1} (the equivalent of 1.5 kg ha^{-1} on the basis of 3000 tonnes of soil per hectare) and each soil sample received 1667 Bq. The [^{14}C]CO_2 evolved was trapped in 5 ml of 0.2 N NaOH and periodically measured using a liquid scintillation analyser (1900TR-Tri-Carb, Packard, France).

2.2. Enrichment, isolation

Selective enrichment cultures were used to obtain mixed bacterial populations that were capable of atrazine degradation. 10 g of soil (on a dry weight basis) was incubated in 90 ml of atrazine medium (AM) containing (per liter of deionised water): 1.6 g K_2HPO_4; 0.4 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.02 g $CaCl_2$; 30 mg atrazine; 10 ml of a sodium citrate stock solution; 1 ml of a salt stock solution; 1 ml of a vitamin stock solution; 1 ml of a $FeSO_4 \cdot 6H_2O$ stock solution. The sodium citrate stock solution contained 100 g l^{-1} sodium citrate. The salt stock solution contained 2 g l^{-1} boric acid; 1.8 g l^{-1} $MnSO_4 \cdot H_2O$; 0.2 g l^{-1} $ZnSO_4$; 0.1 g l^{-1} $CuSO_4$; 0.25 g l^{-1} Na_2MoO_4. The vitamin stock solution contained 100 mg l^{-1} of thiamine–HCl and 40 mg l^{-1} of biotin. The $FeSO_4 \cdot 6H_2O$ stock solution contained 5 g l^{-1} $FeSO_4 \cdot 6H_2O$. The $FeSO_4 \cdot 6H_2O$ and vitamin stock solutions were filter sterilised, kept at 4°C and supplemented with sodium citrate and cycloheximide (50 mg l^{-1}) after autoclaving. Cultures were incubated aerobically on an orbital shaker (150 rpm) at 28°C in the dark. All enrichment cultures were subcultured five times on the same medium at 2-week intervals during 2.5 months. Suspensions/dilutions of each enrichment were plated on a solidified medium consisting of AM supplemented with 15 g agar (Biokar Diagnostics, France) per liter. Atrazine was added to the agar medium as a 2 ml l^{-1} methanolic solution to a final concentration of 500 mg l^{-1} that formed a chalky suspension [22]. The plates were incubated at 28°C. Colonies that developed cleared zones in the atrazine medium agar were purified and routinely maintained on this medium. Cleared zones around colonies could be due to atrazine being dissolved and/or transformed and/or mineralised. Strains were kept frozen at −80°C with 12.5% glycerol.

The capacity of strains to degrade atrazine was tested in pure culture. Three repeats of each culture were done for each isolate. A fresh cell suspension (OD 0.5 at 620 nm) was incubated aerobically at 20°C with shaking (120 rpm) in a sterile jar (250 ml) containing a flask with 10 ml of AM (described previously) supplemented with ^{14}C ethyl chain labelled atrazine or ^{14}C ring uniformly labelled atrazine described previously. Each culture contained a total radioactivity of 1667 Bq. Atrazine degradation was ana-

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lysed by reverse phase HPLC using a Microsorb-MV C_{18} column (length 25 cm, internal diameter 4.6 mm, Varian) and a system LC Star (Varian). The isocratic HPLC mobile phase contained methanol–water (75/25, v/v). The flow rate was adjusted to 1 ml min^{-1} and chromatography was conducted at 30°C. Labelled metabolites were analysed using a radioactivity detector (Flo-One, βeta Radiomatic, Camberra Company). The CO_2 evolved was trapped in 5 ml of 0.2 N NaOH and $[^{14}C]CO_2$ was periodically measured by liquid scintillation counting.

2.3. Amplification of 16S rDNA gene and restriction analysis (PCR RFLP)

Bacterial DNA was extracted using the Non-organic DNA Extraction Kit (Intergen Company, USA) according to the manufacturer's instructions modified by adding a lysozyme treatment (final concentration 1 mg ml^{-1}). 16S rRNA genes were amplified with universal primers 27f (5′-AGAGTTTGATC[C/A]TGGCTCAG-3′) and 1492r (5′-TACGG[A/T/C]TACCTTGTTACGACTT-3′) [33]. PCR reactions were done in a total reaction volume of 50 μl containing: 5 μl of *Taq* polymerase buffer 10× (Appligene-Oncor, France), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 1.25 U of *Taq* DNA polymerase (Appligene-Oncor, France) and 25 ng of DNA template. DNA amplification was carried out using an Omnigene thermocycler Hybaid (Ceralabo). The following programme was used: denaturation for 4 min at 95°C; then 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final extension step consisting of 72°C for 15 min and 30°C for 3 min. Amplification products were analysed by electrophoresis on a 1% agarose (Sigma) gel in 1×TBE (Interchim) and stained with ethidium bromide (200 μl l^{-1}) in order to visualise DNA bands. Amplification products were digested independently overnight at 37°C with three restriction endonucleases: *Alu*I, *Hha*I and *Hae*III (Appligene-Oncor, France). PCR RFLP profiles were performed by electrophoresis on 3% gel Small Fragment Agarose (Appligene-Oncor, France) in 1×TBE (Interchim). Staining of the gel was done with ethidium bromide and pictures were recorded with the imaging system Bioprint (Vilber Lourmat, France). 16S rDNA PCR products were sequenced by Genome Express (France) to precisely identify the genus and the species of the different strains. Primers used for sequencing were: the 27f and 1492r primers, which were used to amplify the 16S rDNA and two additional internal primers, 341f (5′-CCTACGGGAGGCAGCAG-3′) and 926r (5′-CCGTCAATTCMTTTRAGTTT-3′). The sequencing reaction was performed by PCR amplification in a final volume of 20 μl using 100 ng of PCR products, 5 pmol of primer and 9.5 μl of DyeTerminators premix according to the Applied Biosystems protocol. After heating to 94°C for 2 min the reaction cycles were as follows: 25 cycles of 30 s at 94°C, 30 s at 55°C, and 4 min at 60°C (9600 thermal cycler, Perkin Elmer). Removal of excess DyeTerminators was performed using Quick Spin columns (Roche Biochemical). The samples were dried in a vacuum centrifuge and dissolved in 4 μl of deionised formamide EDTA pH 8.0 (5/1). The samples were then loaded onto an Applied Biosystems 373A sequencer and run for 12 h on a 4.5% denaturing acrylamide gel. New partial sequences were aligned together with published sequences from GenBank using ClustalW.

The percentage similarity between 16S rDNA of the strains from this study and the nearest neighbour found in GenBank was estimated over an average of 1300 bp for the submitted sequences, for other sequences similarity was estimated over an average of 900 bp.

2.4. Amplification of atzA, atzB, atzC and trzD

For all pure strains, we attempted to amplify the genes *atz*A, *atz*B, *atz*C with primers described by de Souza et al. [29]. From the gene sequence (accession number AF086815) described by Karns [32], we designed primers for the amplification of the *trz*D gene: the forward primer was *trz*D F1 (5′-CCTCGCGTTCAAGGTCTACT-3′), position 919–938 on the sequence, and the reverse primer was *trz*D R1 (5′-TCGAAGCGATAACTGCATTG-3′), position 1753–1772 on the sequence. The primers were synthesised by Oligo Express, France. PCR reactions were done under the same conditions as the 16S rDNA amplification. Cycles used were as follows: 1 cycle at 95°C for 4 min; 5 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 2 min; 1 cycle at 94°C for 1 min, 59, 58, 57, 56°C for 1 min, 72°C for 2 min; 26 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; 1 cycle at 72°C for 3 min. The PCR products were sequenced by Genome Express (France), using the same method as for 16S rDNA.

2.5. Probe labelling and Southern hybridisation

Probes were produced by PCR using the primers described previously. The probes for the *atz*A, *atz*B and *atz*C genes were prepared using genomic DNA extracted from *Pseudomonas* sp. ADP and for the *trz*D gene using genomic DNA extracted from the newly isolated strain Cit1. The PCR reaction conditions were as described above, except that the dNTP concentrations were as follows: dATP, dCTP and dGTP 200 mmol l^{-1} each; dTTP 26 mmol l^{-1}; Dig 11-dUTP 13 mmol l^{-1} (Roche Biochemical).

Total genomic DNA of each strain was digested with the restriction endonuclease *Eco*RI (Appligene-Oncor, France) electrophoresed on 0.9% agarose (Sigma) gel in TAE buffer (Polylabo, France). The Dig labelled DNA molecular mass marker III (Roche Biochemical) was also loaded on each gel. DNA fragments were then transferred to a Biodyne Plus membrane (Gelman Sciences) using a Vacu Gene apparatus (Pharmacia). The membranes were

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washed in 5×SSC and air-dried. DNA was cross-linked to the membrane by UV irradiation for 3 min. Hybridisation with *atz*A, *atz*B, *atz*C and *trz*D probes was carried out under two stringency conditions: posthybridisation washes were performed twice in 2×SSC and 0.1% SDS for 5 min at room temperature and twice in 0.1%×SSC and 0.1% SDS solution for 15 min at 68°C (high stringency) or twice in 2×SSC and 0.1% SDS for 5 min at room temperature and twice in 2×SSC and 0.1% SDS for 15 min at 68°C (low stringency). The hybridised probe was detected using the Dig Luminescent Detection Kit (Roche Biochemical) and CDP Star as a chemiluminescent substrate according to the manufacturer's conditions.

2.6. Colony hybridisation

Trials were made to attempt direct isolation from soil of bacteria carrying the degrading genes. The colony hybridisation method of Grunstein and Hogness [34] was used on filter replicates from AM plates inoculated with diluted enrichment cultures and incubated in the same conditions as previously described. Filters were then hybridised using a mixture of *atz*A, *atz*B, *atz*C and *trz*D probes described above. Positive colonies were isolated from the original plates and characterised as described for the other isolates.

3. Results and discussion

3.1. Atrazine degradation kinetics in the 12 French soils studied

The first objective of this study was to analyse the diversity of atrazine degrading species and the polymorphism of the atrazine degrading genes in different soils. For this purpose, 12 soils were chosen with a wide range of physico-chemical characteristics (Table 1) and were characterised for their ability to degrade atrazine. Representative kinetics of ^{14}C ethyl chain labelled atrazine degradation are given for the 12 soils in Fig. 1. Based on their ability to mineralise atrazine, the soils were placed in three groups. Three soils (Cîteaux, Côte St André and Epoisses) were placed in group A. In these soils, atrazine was mineralised very rapidly: up to 52%, 61% and 52% of the initially applied atrazine, respectively, over an incubation period of 131 days with no lag phase (Fig. 1A). These three soils showed enhanced degradation of atrazine.

Five soils (Baccon, Chigy, Dijon, Le Rheu and Loustalet) were placed in group B. In these soils more than 50% of the initially applied atrazine was degraded over an incubation period of 131 days with variable lag phases (Fig. 1B). These five soils presented an intermediate adaptation to atrazine degradation.

Four soils (Plumelec, Salinis, La Bouzule 1 and La Bouzule 2) were placed in group C. In these soils, only 38%, 31%, 25% and 19% of the initially applied atrazine, respectively, was degraded over an incubation period of 131 days (Fig. 1C). These soils presented no adaptation to atrazine degradation. This may be due to soil pH values lower than 6.0 and/or absence of atrazine treatments for the last 3 years (Table 1). Some authors have reported that enhanced degradation of atrazine occurs mainly in soils with a pH above 6.5 [19] and/or with repeated atrazine applications [16–18]. So microbial adaptation could be favoured by repeated atrazine treatments, which act as a selective pressure for atrazine degraders in the bacterial community [17].

Our results are in agreement with observations described previously [18,19], except for the results of the soils of Le Rheu and Loustalet. In these other studies, the kinetics of atrazine degradation in soils was determined using ^{14}C ring labelled atrazine, in the present work we used

Table 1
Main physical and chemical characteristics of the different soils and crop history

Soil	Particle size (%)			C (g kg^{-1})	N (g kg^{-1})	C/N (ratio)	pH (H_2O)	CEC (cmol$^+$ kg^{-1})	Cultures	Treatment	Microbial biomass (mg C kg^{-1} soil)
	Clay	Silt	Sand								
La Bouzule (1), Nancy	24.2	48	8.5	14.0	–	–	6.2	14.2	maize, rotation	alachlor, bentazon, etc.	213
La Bouzule (2), Nancy	51.7	35.7	3.9	13.0	–	–	7.3	22.1	maize, rotation	alachlor, bentazon, etc.	449
Baccon	37.1	57.1	5.7	15.6	1.7	9.2	7.2	23.4	maize, rotation	atrazine/fenoxaprop, etc.	320
Chigy	41.2	51.0	5.8	64.5	6.5	9.9	7.8	38.0	maize, continuous	atrazine, alachlor, etc.	480
Cîteaux	17.8	56.9	25.3	9.9	1.4	9.8	7.9	11.0	maize, rotation	atrazine	309
Côte Saint André	18.2	38.7	43.0	13.8	1.3	10.6	7.0	9.1	maize, continuous	atrazine	231
Dijon	33.4	50.5	16.1	13.9	1.4	10.2	7.5	22.2	grassland	atrazine, ametryn, etc.	390
Epoisses	46.1	47.2	6.7	20.4	2.2	9.2	6.7	33.5	maize, continuous	atrazine	375
Le Rheu	16.6	70.7	12.6	11.4	1.2	9.2	6.2	7.3	maize, continuous	atrazine	204
Loustalet	19.2	65.7	15.0	17.7	1.9	9.2	6.5	9.7	maize, continuous	atrazine	235
Plumelec	16.1	48.2	35.6	29.4	2.6	11.4	5.7	13.0	maize, continuous	atrazine	209
Salinis	25.3	57.0	17.6	51.1	3.4	14.9	5.5	15.3	maize, continuous	atrazine	137

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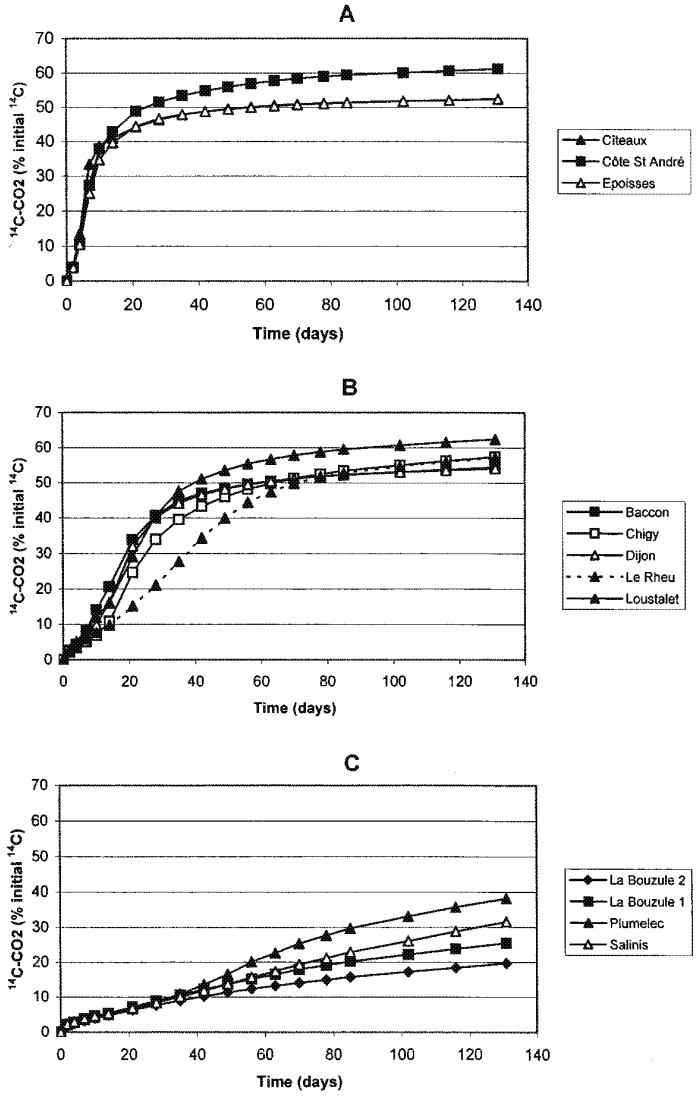


Fig. 1. Kinetics of degradation of ^{14}C ethyl chain labelled atrazine in different soils. A: Soils showing enhanced atrazine degradation. B: Soils showing intermediate adaptation to atrazine degradation. C: Soils not showing enhanced atrazine degradation.

^{14}C ethyl chain labelled atrazine. Identical patterns were obtained for kinetics of ^{14}C mineralisation from both the side chain and the ring with all of the soils we tested. The soils of Le Rheu and Loustalet were considered not adapted to the degradation of atrazine and had pH values below 6.5 in previous studies [19]. Our study showed that these soils (Fig. 1B) had intermediate pH values (6.2 and 6.5 respectively) with degraded atrazine to 57 and 62% of the initially applied atrazine respectively after 131 days of incubation. In our case, the pH value does not seem to be a meaningful parameter in the understanding of the capacity of the soils to degrade atrazine. Finally, Tessier [35] showed spatial variability of the pH and of the atrazine degradation in the same plot. This observation could also explain the difference between our results and the results of Houot et al. [19].

3.2. Isolation of atrazine degrading microorganisms

To isolate atrazine degrading bacterial strains, we used an enrichment and isolation method used in other laboratories [20,22,23,36,37]. In order to obtain the largest possible collection of degrading species, we attempted to isolate strains from all soils, even those with a slow mineralisation of atrazine.

Twenty-five atrazine degrading bacterial strains were isolated from 10 geographically different soils (Table 2). The additional strain, Ep 2-1c, was isolated using colony hybridisation. We were able to isolate atrazine degrading bacteria from three of the four soils that degrade atrazine slowly. No atrazine degrading microorganisms were isolated from the La Bouzule 1 soil. Although atrazine was mineralised in this soil, the density of atrazine degrading

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Table 2
Ribosomal diversity (16S rDNA), presence of *atz*A, *atz*B, *atz*C and *trz*D genes, atrazine degrading capacity and 16S rDNA gene sequencing of atrazine degrading soil bacteria

Source	Strain	ARDRA type	Nearest neighbours and accession number	Similarity (%)	Atrazine degradation	Genes	Southern hybridisation
La Bouzule 2	Pel 1-4	1	*Chelatobacter heintzii* AJ011762	97	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
La Bouzule 2	Pel 3-4	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
La Bouzule 2	Pel A	1	*Chelatobacter heintzii* AJ011762	99	dechlorination	*atz*A	*atz*A
Baccon	Bacc 6	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Baccon	Bacc 2-3	1	*Chelatobacter heintzii* AJ011762	100	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Baccon	Bacc 3-4	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Cîteaux	Cit 1[a]	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Cîteaux	Cit A	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Cîteaux	Cit 2[b]	2	*Arthrobacter crystallopoietes* X80738	99	cyanuric acid production	*atz*B, *atz*C	*atz*B, *atz*C and a weak signal with *atz*A probe
Côte Saint André	Côte 1-4	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Dijon	Dij 1	2	*Arthrobacter crystallopoietes* X80738	99	cyanuric acid production	*atz*B, *atz*C	*atz*B, *atz*C and a weak signal with *atz*A probe
Epoisses	Ep1	2	*Arthrobacter crystallopoietes* X80738	99	Cyanuric acid production	*atz*B, *atz*C	*atz*B, *atz*C and a weak signal with *atz*A probe
Epoisses	Ep 2	3	ND	–	cyanuric acid production	*atz*B, *atz*C	–
Epoisses	Ep 2-1c[c]	4	*Aminobacter aminovorans* AJ011759	100	cyanuric acid degradation	*atz*C *trz*D	*atz*C, a*trz*D
Le Rheu	LR 1-3b	1	*Chelatobacter heintzii* AJ011762	97	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Le Rheu	LR 3-3	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Le Rheu	LR A	1	*Chelatobacter heintzii* AJ011762	94	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Loustalet	Lous 2-3	1	*Chelatobacter heintzii* AJ011762	98	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Loustalet	Lous 3	1	*Chelatobacter heintzii* AJ011762	99	dechlorination	*atz*A	*atz*A
Loustalet	Lous 3-4	5	*Stenotrophomonas maltophilia* AF012541	98	dechlorination	*atz*A	*atz*A
Loustalet	Lous 5	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Plumelec	Plum 1	1	*Chelatobacter heintzii* AJ011762	99	dechlorination	*atz*A	*atz*A
Salinis	Sal 1	1	*Chelatobacter heintzii* AJ011762	100	dechlorination	*atz*A	*atz*A
Salinis	Sal 2	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Salinis	Sal 1-3	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D
Salinis	Sal B	1	*Chelatobacter heintzii* AJ011762	99	ring cleavage	*atz*A, *atz*B, *atz*C and *trz*D	*atz*A, *atz*B, *atz*C and *trz*D

ND: not identified.
Similarity (%): percent similarity between 16S rDNA of the strains from this study and the nearest neighbour found in GenBank (similarity was estimated over an average of 1300 bp for the submitted sequences Cit 1, Cit 2 and Ep 2-1c; for other sequences similarity was estimated over an average of 900 bp).
[a]16Sr DNA sequence submitted to GenBank (accession number AF256055).
[b]16Sr DNA sequence submitted to GenBank (accession number AF329834).
[c]16Sr DNA sequence submitted to GenBank (accession number AF329835).

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microorganisms was probably very low. In all soils with enhanced atrazine degradation, we were able to isolate atrazine degrading strains except for the soil of Chigy.

3.3. Diversity of strains: taxonomic characterisation by ARDRA and 16S rDNA sequencing

3.3.1. ARDRA analysis

All atrazine degrading strains were characterised by ARDRA with the three restriction endonucleases (*Alu*I, *Hha*I and *Hae*III). ARDRA analysis of the 16S rDNA genes allowed discrimination between five different ARDRA types. Among them, ribotypes 3, 4 and 5 were represented by single isolates, the other two ribotypes included several isolates. Two of the five ribotypes belonged to Gram-positive bacteria (Table 2).

Ribotype 2 only included isolates from Dijon, Epoisses and Cîteaux soils and ribotypes 3, 4 and 5 were only detected from Epoisses and Loustalet soils. In contrast, ribotype 1 was detected in nine out of the 10 soils studied. This suggests that certain ribotypes may be soil specific whereas others are ubiquitous.

3.3.2. Sequencing of the 16S rDNA gene: identification of new atrazine degrading strains belonging mainly to the Chelatobacter and Arthrobacter genera

The 16S rDNA genes of all the strains belonging to ARDRA types 1 and 2 were sequenced. The 16S rDNA genes of the two strains Ep 2-1c and Lous 3-4 were also sequenced. The 16S rDNA gene of strain Ep 2, which belongs to ARDRA type 3, was not sequenced since we were not able to repeatedly extract its genomic DNA.

Among the 26 strains isolated, 22 were Gram-negative. The sequences of the 16S rDNA (1340 bp) from these Gram-negative strains were aligned with GenBank database sequences. Results and percentages of similarity are reported in Table 2. The strains of ribotype 1 were classified as Bacteria, phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Rhizobiaceae and were identified as *Chelatobacter heintzii* (nearest neighbour accession number: AJ011762). Strain Ep 2-1c was classified as Bacteria, phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Phyllobacteriaceae and was identified as *Aminobacter aminovorans* (nearest neighbour accession number: AJ011759). Strain Lous 3-4 (ribotype 5) was classified as Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Xanthomonadales, family Xanthomonadaceae and was identified as the iron oxidising lithotrophic bacterium *Stenotrophomonas maltophilia* (nearest neighbour accession number: AF012541). The three other strains isolated in this study were closely related to Gram-positive bacteria with a high G+C content and the analysis of the sequences showed that these strains were classified as Bacteria, phylum Actinobacteria, class Actinobacteria, subclass Actinobacteridae, order Actinomycetales, suborder Micrococcineae, family Micrococcaceae and were identified as *Arthrobacter crystallopoietes* (nearest neighbour accession number: X80738). The 16S rDNA sequences of strains Cit 1, Cit 2 and Ep 2-1c were deposited in GenBank under the accession numbers AF256055, AF329834 and AF32835, respectively. Among the 26 isolated strains from 10 geographically different soils, 20 strains belonged to the genus *Chelatobacter* and three strains to the genus *Arthrobacter*.

This is the first report of strains belonging to the *C. heintzii*, *A. aminovorans*, *S. maltophilia* and *A. crystallopoietes* species that are capable of mineralising or degrading atrazine. These bacteria are known to be ubiquitous in natural environments and seem to be particularly adapted to degrade xenobiotics like atrazine. Different strains of *C. heintzii*, *A. aminovorans* and *S. maltophilia*, capable of degrading carbofuran, have been isolated [38]. A strain of *C. heintzii* ATCC 29600 [39] contains a gene encoding a monooxygenase, which degrades nitrilotriacetate. In other studies, strains of *Arthrobacter* sp. were described as being heavy metal resistant [40] or as capable of degrading phenanthrene or carbaryl [41,42]. Another example of the metabolic versatility of *Arthrobacter* species is that we have found that a bacterial suspension of strain Ep 1 can degrade 50% of initially applied ^{14}C labelled carbofuran in 15 days and 35% of initially applied ^{14}C labelled methylamine in 30 days (data not shown). However, with this strain, we were not able to amplify a methylcarbamate degrading gene (*mcd*) responsible for the hydrolysis of carbofuran to carbofuran phenol [43]. Desaint et al. [38] reported that the *mcd* gene was detected in only 45% of carbofuran degrading bacteria described in their study.

A high number of atrazine degrading strains belonging to the *Chelatobacter* genus were isolated from a high number of geographically different soils. However, it should be remembered that the enrichment procedure might select for soil microorganisms adapted to the complex medium supplemented with atrazine that we used. Thus the actual enrichment might introduce a bias in the representativeness of the isolates compared to soil population. Desaint et al. [38] used a different isolation procedure, and reported a wider diversity of carbofuran degrading bacteria than we have. Colony hybridisation using probes for degradation genes as presented in this study might be a promising alternative to the enrichment procedure for describing atrazine degrading populations.

We have demonstrated that some strains assigned to the species *C. heintzii* are able to mineralise atrazine. Our strains of *Chelatobacter*, although taxonomically related to the strains of *Pseudaminobacter* described by Topp et al. [23], constitute a new group of atrazine degrading bacteria, which has not been described before (Fig. 2A). In the same way, strains assigned to the species *A. crystallopoietes* (order Actinomycetales) described in our study and distantly related to *Nocardioides* described by Topp et al. [31] constitute a new group of atrazine degraders (Fig.

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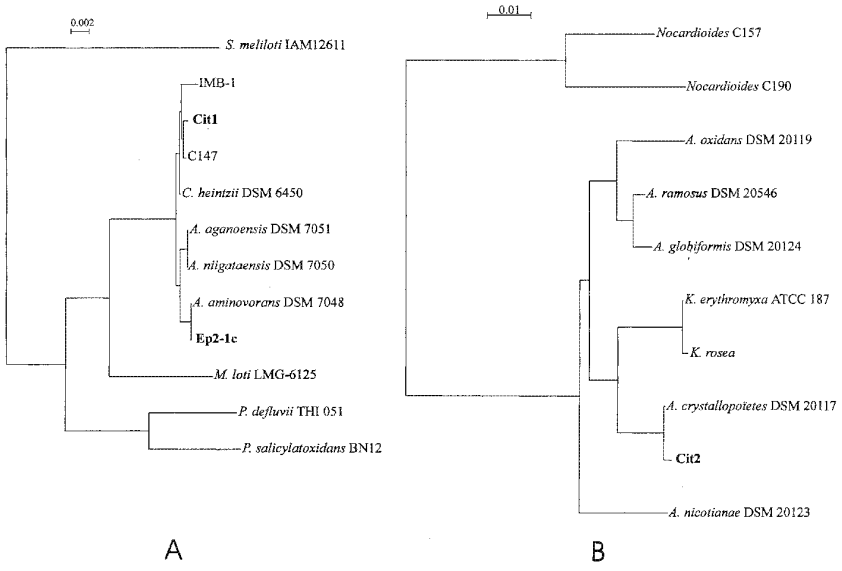


Fig. 2. Phylogenetic trees based on the 16S rDNA sequence alignments. Sequences were aligned using ClustalW, distances were calculated using the Kimura 2 parameter method. The trees were built using the neighbour joining method. A: Tree showing the estimated relationship between the Gram-negative strains Cit 1 and Ep 2-1c, IMB-1, C147, *Sinorhizobium meliloti*, *Mesorhizobium loti*, *C. heintzii*, species of the genus *Aminobacter* and species of the genus *Pseudaminobacter*. Accession numbers of these related sequences are as follows: strain Cit 1, AF256055; strain Ep 2-1c, AF329835; methylotrophic proteobacterium IMB-1, AF034798; *Pseudaminobacter* C147, AF246220; *S. meliloti* IAM12611, D12783; *M. loti*, LMG-6125, X67229; *C. heintzii* DSM 6450, AJ011762; *A. aganoensis* DSM 7051, AJ011760; *A. niigataensis* DSM 7050, AJ011761; *A. aminovorans* DSM 7048, AJ011759; *P. defluvii* THI051, D32248; *P. salicylatoxidans* BN12, AF072542. The bar indicates 0.002 substitution per nucleotide position. B: Tree showing the estimated relationship between the Gram-positive strain Cit 2 and species of the genus *Arthrobacter*, species of the genus *Kocuria* and species of the genus *Nocardioides*. Accession numbers of these related sequences are as follows: Cit 2, AF329834; *A. oxidans* DSM 20119, X83408; *A. ramosus* DSM 20546, X80742; *A. globiformis* DSM 20124, X80736; *A. crystallopoietes* DSM 20117, X80738; *A. nicotianae* DSM 20123, X80739; *K. erythromyxa* ATCC 187, Y11330; *K. rosea*, X87756; *Nocardioides* C157, AF253509 and *Nocardioides* C190, AF253510. The bar indicates 0.01 or 0.02 substitution per nucleotide position.

2B). Our work enlarges the array of bacterial genera and species able to degrade or mineralise atrazine.

3.4. Capacity of the isolates to degrade atrazine and occurrence of *atzA*, *atzB*, *atzC* and *trzD* genes

3.4.1. Gram-negative bacteria

Kinetics of atrazine ring or side chain mineralisation are presented in Fig. 3A,B. Only one curve is shown for each type of kinetics. Among the 20 strains of *C. heintzii*, 16 mineralised ^{14}C ring labelled atrazine to carbon dioxide at 60–90% of the initially applied atrazine over an incubation period of 15 days. For all these 16 *C. heintzii* strains, the *atz*A, *atz*B and *atz*C genes were amplified using primers described by de Souza et al. [29]. The expected 0.5-kb *atz*A or *atz*B PCR products and the expected 0.6-kb *atz*C PCR product were obtained. The occurrence of the *atz*ABC genes was confirmed by hybridisation of total genomic DNA using the *atz*A, *atz*B and *atz*C probes produced by PCR from genomic DNA of *Pseudomonas* sp. ADP. The corresponding PCR products were then sequenced for strains Cit 1 and Cit A and were found to be homologous at respectively 99%, 98% and 100% with the *atz*A, *atz*B and *atz*C genes of *Pseudomonas* sp. ADP. The *atz*A, *atz*B and *atz*C genes of strain Cit 1 were deposited in GenBank under the accession numbers AF364900, AF364901 and AF364902, respectively. PCR RFLP analysis of *atz*ABC genes using *Alu*I showed that *atz*ABC genes of other Gram-negative isolates were not different from those of Cit 1 and Cit A. These sequence homologies are in agreement with the conclusions of de Souza et al. [29] that the atrazine catabolism genes *atz*ABC are widely distributed and highly conserved in other atrazine degrading Gram-negative bacteria belonging to the genera *Agrobacterium*, *Alcaligenes* and *Ralstonia*. Topp et al. [23] came to the same conclusions with *Pseudaminobacter* sp. strains.

From all of the 16 *C. heintzii* strains, the *trz*D gene described by Karns [32], which is responsible for cyanuric acid cleavage, was amplified and the expected 0.8-kb PCR product was obtained. The *trz*D PCR product was sequenced for strain Cit 1 and Cit A and found to be 100% homologous with the *trz*D gene of *Pseudomonas* sp. strain NRRLB-12227. The *trz*D sequence of strain Cit 1 was deposited in GenBank under the accession number AF364903. The homology of the *trz*D gene among the *C. heintzii* strains was confirmed by hybridisation of the total genomic DNA using a *trz*D probe produced by PCR from the genomic DNA of strain Cit 1. This is the first report of the presence of a *trz*D-like gene in atrazine mineralising bacteria. Karns [32] cloned and sequenced this

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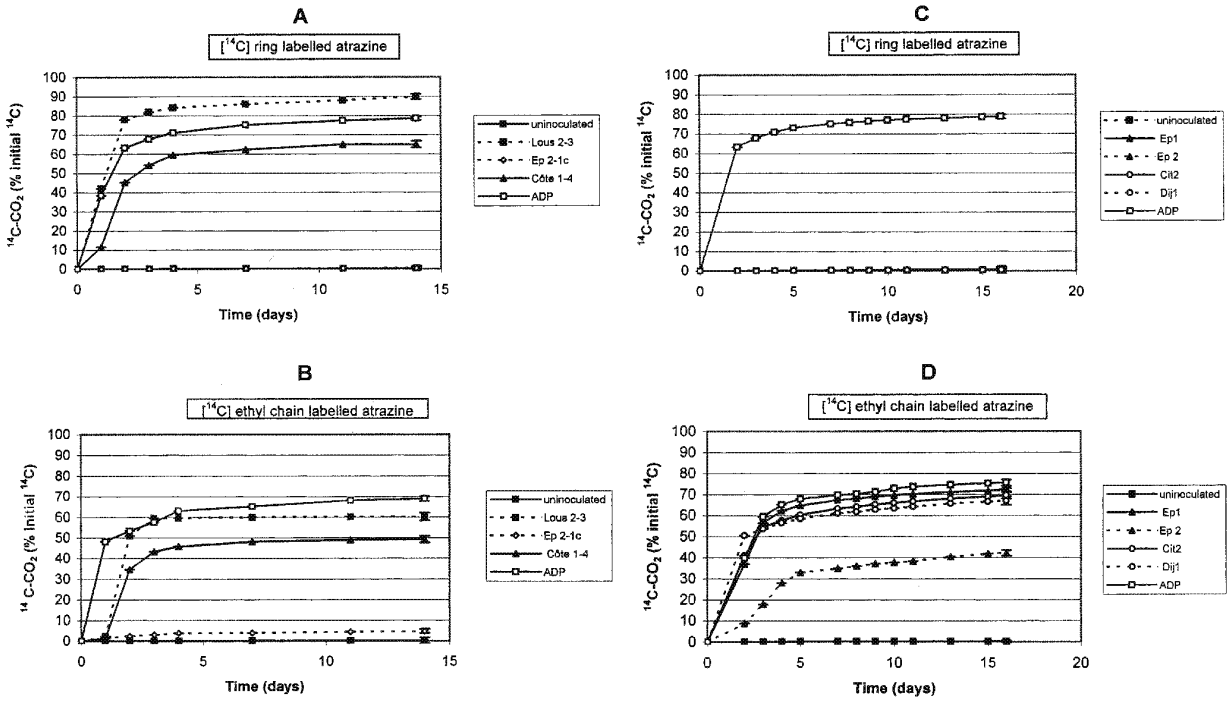


Fig. 3. Degradation kinetics of ^{14}C ring labelled atrazine (A, C) or ^{14}C ethyl chain labelled atrazine (B, D) for representative Gram-negative strains of *C. heintzii* (A, B) or Gram-positive strains of *A. crystallopoietes* (C, D) in pure culture.

gene, which encodes a cyanuric acid amidohydrolase, from *Pseudomonas* sp. strain NRRLB-12227 which did not mineralise atrazine but did mineralise melamine. The presence of the gene *trz*D in atrazine mineralising strains and in other *s*-triazine degrading strains shows that this gene is also widespread. Moreover, genomic DNA of *Pseudomonas* sp. ADP slightly hybridised with the *trz*D probe under non-stringent conditions. This result suggests that the *Pseudomonas* sp. ADP gene responsible for the cleavage of cyanuric acid has a weak sequence homology with the *trz*D gene of *Pseudomonas* NRRLB-12227 (data not shown). The presence of the *trz*D gene in our atrazine degrading strains confirms that they were able to convert atrazine to CO_2 and NH_4^+ by using a combination of the two catabolic pathways described by de Souza et al. [29] and Karns [32].

Four other strains of *C. heintzii* isolated from four different soils did not mineralise ^{14}C ring labelled atrazine. Degradation kinetics of ^{14}C ring labelled -atrazine or ^{14}C ethyl chain labelled atrazine did not show any $^{14}CO_2$ production (Fig. 3A,B). HPLC analyses showed the accumulation in the culture medium of a compound which co-elutes with a hydroxyatrazine standard (data not shown). Moreover, a cleared zone with a crystalline precipitate was observed on the surface of the AM isolation plates, which could be hydroxyatrazine. Combining PCR amplifications and Southern hybridisation results shows that only the *atz*A gene was present and 99% homologous to the *Pseudomonas* sp. ADP *atz*A gene. The *atz*B, *atz*C and *trz*D genes were not detected in these strains. Topp et al. [23] reported that four strains of *Pseudaminobacter* sp. carry only the *atz*A gene, the genes *atz*B and *atz*C were absent and that two other strains of *Pseudaminobacter* sp. carry only the *atz*A and *atz*C genes.

3.4.2. Gram-positive bacteria

Of the Gram-positive strains isolated in this study, none mineralised ^{14}C ring labelled atrazine, but the three strains Cit 2, Ep 1 and Dij 1 isolated from three different soils were able to cleave and mineralise ^{14}C ethyl chain labelled atrazine to 70% of the initially applied atrazine (Fig. 3C,D). HPLC analyses of the culture medium showed accumulation of an intermediate compound which was radiolabelled only when ^{14}C ring labelled and not chain labelled atrazine was used. This compound co-elutes with standard cyanuric acid (data not shown).

Amplification of *atz*A, *atz*B, *atz*C and *trzD* genes using the primers described previously was attempted. We obtained the expected 0.5-kb *atz*B and 0.6-kb *atz*C PCR products for the three strains Cit 2, Ep 1, and Dij 1. The *atz*B and *atz*C genes were sequenced for the two strains Cit 2 and Dij 1 and found to be respectively 97% and 100% homologous with the *atz*B and *atz*C genes of *Pseudomonas* sp. ADP. The *atz*B and *atz*C genes of strain Cit 2 were deposited in GenBank under the accession numbers AF364904 and AF364905, respectively. PCR RFLP analysis of *atz*BC genes using *Alu*I showed that *atz*BC genes of other Gram-positive isolates were not dif-

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ferent from those of Cit 2 and Dij 1. The occurrence of the *atz*B and *atz*C genes was confirmed by hybridisation of total genomic DNA using the *atz*B and *atz*C probes described previously. However, genomic DNA of strains Cit 2, Ep 1 and Dij 1 slightly hybridised under low stringency conditions with the *atz*A probe, suggesting that the gene of these three strains responsible for the atrazine dechlorination presents a weak sequence homology with the *atz*A gene of *Pseudomonas* sp. ADP (data not shown). In the same hybridisation conditions, we confirmed that these strains lacked the *trz*D gene. Strain Ep 2 degraded atrazine slowly (45% of the initially applied atrazine) with accumulation of cyanuric acid. For this strain, there was no amplification of the genes *atz*A and *trz*D, only the *atz*B and *atz*C genes were amplified.

The isolated strains Ep 1, Ep 2, Cit 2 and Dij 1 degraded atrazine to cyanuric acid but carry only the *atz*B and *atz*C genes, which are responsible for the degradation of hydroxyatrazine to *N*-isopropylammelide and to cyanuric acid, respectively. The *atz*B and *atz*C genes were functional in these strains, because Ep 1, Ep 2, Cit 2 and Dij 1 degraded hydroxyatrazine (data not shown). These strains probably hydrolysed atrazine to hydroxyatrazine by means of a novel chlorohydrolase. Recently Topp et al. [31] described a Gram-positive strain of *Nocardioides* sp., which converted atrazine through hydroxyatrazine to the end product *N*-ethylammelide by means of a novel chlorohydrolase thus demonstrating the possibility of diversity in atrazine chlorohydrolases and in the corresponding genes.

To confirm the hypothesis of atrazine degradation to cyanuric acid by strain Ep 1, a cell suspension of strain Ep 1 (*atz*B and *atz*C) was mixed with a cell suspension of strain Ep 2-1C (*atz*C and *trz*D) which degrades cyanuric acid, in a culture medium (AM) containing ^{14}C ring uniformly labelled atrazine. Kinetics of atrazine mineralisation of strains Ep 1 and Ep 2-1c is given in Fig. 4. The mixed culture mineralised ^{14}C ring uniformly labelled atrazine (50% of the initially applied atrazine). It appears that strain Ep 1 converted atrazine to cyanuric acid, which was then mineralised by strain Ep 2-1c carrying the *trz*D gene.

The same results were observed with the strains Ep 2, Cit 2 and Dij 1.

In this study, we have reported the occurrence of the

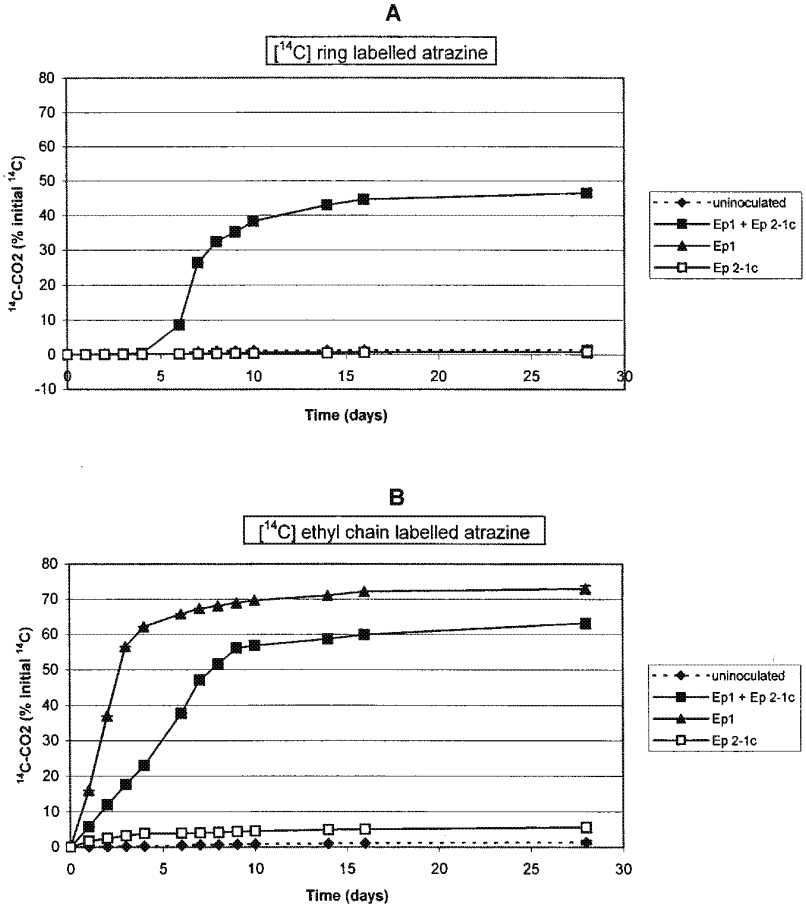


Fig. 4. Degradation kinetics of ^{14}C ring labelled atrazine (A) or ^{14}C ethyl chain labelled atrazine (B) for strain Ep 1 (*atz*B and *atz*C), strain Ep 2-1c (*atz*C and *trz*D) in pure culture and strains Ep 1 and Ep 2-1c in mixed culture.

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Table 3
Distribution of isolates among the soils grouped according to their atrazine degrading capability

Soil type (number of soil in each group)	Atrazine degradation (%)	Taxonomic position of the strains isolated from the soils (number of strains)	Phenotype of the strains
No adaptation (4)	below 38%	*Chelatobacter* (5)	mineralisation
		Chelatobacter (3)	dechlorination
Intermediate adaptation (5)	50–61%, variable lag phase	*Chelatobacter* (8)	mineralisation
		Chelatobacter (1)	dechlorination
		Stenotrophomonas (1)	dechlorination
		Arthrobacter (1)	cyanuric acid production
Enhanced degradation (3)	50–62%, no lag phase	*Arthrobacter* (2)	cyanuric acid production
		Chelatobacter (3)	mineralisation
		Unidentified Gram-positive bacteria Ep 2	cyanuric acid production

*atz*ABC and *trz*D genes, which are highly conserved in different strains isolated from geographically different soils. This observation is in agreement with previously published work [23,29,37]. We suggest that the different associations of atrazine degrading genes in our isolates might be the result of horizontal gene transfer. Several studies of other catabolic degrading genes involved in 2,4-D degradation [44] or in naphthalene degradation [45] suggested that horizontal gene transfer could play a major role in gene flow in the environment.

3.5. *Distribution of isolates among soils as related to their atrazine degrading capacity*

The 26 atrazine degrading strains isolated from 10 soils could be assigned to only three genera of Gram-negative strains and two genera of Gram-positive strains. From Table 3, we can observe that in non-adapted soils only strains belonging to the *Chelatobacter* genus occurred. In contrast, Gram-positive atrazine degrading bacteria occurred only in soils showing intermediate or enhanced degradation capacity. Gram-positive atrazine degrading bacteria are not able to mineralise the atrazine ring, thus suggesting that in soils adapted to atrazine degradation, complete mineralisation of this compound could be done by bacterial consortia. The diversity observed in non-adapted soils seems to be lower than in other soils. Repeated application of atrazine seems to increase the diversity (potentially by gene transfer) of atrazine degrading bacteria that were isolated. There is no apparent relationship between the diversity of isolated atrazine degrading bacteria and the physical or chemical properties of the soils. The use of colony hybridisation in order to isolate degrading bacteria without an enrichment procedure would be of great interest to further investigate the diversity of these bacteria.

Our results indicate that among all isolated strains, some mineralised atrazine rapidly (60–90% of the initially applied atrazine in 15 days) and that these strains carry the *atz*ABC and *trz*D genes. We report for the first time the occurrence of the *trz*D gene in atrazine mineralising bacteria. Moreover, Gram-positive isolates appear to degrade atrazine to cyanuric acid via a novel chlorohydrolase and via the *atz*B and *atz*C genes. The different soil isolates mineralising ring or side chain labelled atrazine carry different combinations of genes. It may be interesting to determine the location of atrazine degrading genes in our strains to explain this variability. The use of the atrazine mineralising strains or consortia for the depollution of contaminated sites will be considered in future work.

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Stereospecific degradation of the phenoxypropionate herbicide dichlorprop

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Abstract

2,4-Dichlorophenoxyacetic acid (2,4-D)/α-ketoglutarate (α-KG) dioxygenase, TfdA, from *Ralstonia eutropha* JMP134, was purified from recombinant cells and shown by gas chromatographic and colorimetric methods to degrade only the *S* enantiomer of dichlorprop, a phenoxypropionate herbicide. Similarly, cell extracts of *Burkholderia cepacia* RASC, containing a biochemically and genetically related α-KG-dependent dioxygenase, also were shown to oxidize (*S*)-dichlorprop using chiral HPLC and colorimetric methods. In contrast, cell extracts of a mecoprop-degrading strain of *Alcaligenes denitrificans* were shown to catabolize (*R*)-dichlorprop. Although the *A. denitrificans* activity exhibited stereospecificity opposite to that of the JMP134 and RASC strains, its cofactor requirements were found to be characteristic of an α-KG-dependent dioxygenase. A PCR amplification product from the DNA of this strain was shown to encode an amino acid sequence that was 95% and 86% identical to the corresponding region of TfdA in RASC and JMP134, respectively. Thus, closely related herbicide-degrading gene products appear to be capable of exhibiting opposite stereochemical degradative capabilities. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Stereospecificity; Herbicide; Dichlorprop; α-Ketoglutarate; TfdA

1. Introduction

Ring-substituted phenoxyacetates and phenoxypropionates are widely used as herbicides to control the growth of broadleaf weeds and woody plants [1]. While phenoxyacetates such as 2,4-dichlorophenoxyacetic acid (2,4-D) lack a chiral carbon atom, phenoxypropionates can exhibit either of two enantiomeric forms because of the methyl group attached to the C-2 carbon atom. Notably, only the *R* enantiomers are active towards plants [2]. A current trend in agriculture is increasing use of only the active enantiomer of chiral herbicides in order to reduce environmental loads of organopesticides [2]. For example, about 75% of dichlorprop (2-(2,4-dichlorophenoxy)propionate) and mecoprop (2-(2-methyl-4-chlorophenoxy)propionate) are sold in their active enantiomeric forms in the European Union, with the expectation that

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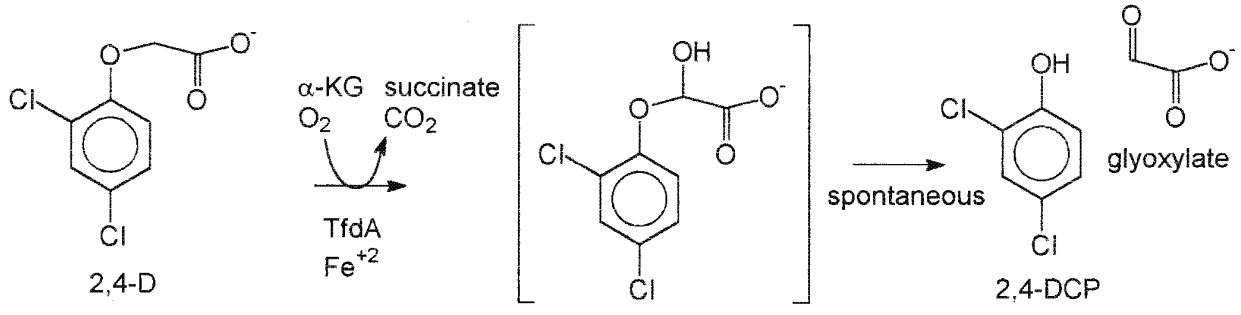
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within 5 years only the *R* isomer will be commercially available (R. Akers, personal communication).

Phenoxyacid herbicides are rapidly degraded in soil by bacteria and fungi [1]. One of the best characterized herbicide-degrading organisms is *Ralstonia eutropha* (formerly *Alcaligenes eutrophus* [3]) strain JMP134, capable of using 2,4-D as its sole carbon and energy source. The first step in 2,4-D catabolism is its conversion to 2,4-dichlorophenol (2,4-DCP), catalyzed by the enzyme 2,4-D/α-ketoglutarate (α-KG) dioxygenase [4] or TfdA [5]. This ferrous ion-dependent enzyme couples the oxidative decarboxylation of α-KG to hydroxylation of the substrate at the C-2 carbon to yield a postulated hemiacetal intermediate which spontaneously decomposes to form 2,4-DCP and glyoxylate [4] (Scheme 1). The chlorinated phenol is subsequently metabolized by a series of well-studied enzymes that eventually generate an intermediate of the tricarboxylic acid cycle [6]. It is important to emphasize, however, that not all 2,4-D-degrading microorganisms use this pathway. The biochemistry of phenoxypropionate degradation is much less well understood than that of 2,4-D. Here too, however, side chain removal to release the corresponding phenol has been shown to be an essential step in catabolism of these compounds (e.g., Refs. [7–14]).

Studies to characterize the stereospecificity of enzymes that degrade phenoxypropionate herbicides can have important biotechnological ramifications. For example, a synthetic process could be devised in which a particular herbicide is initially synthesized as the racemic mixture, the *S* enantiomer is degraded by a stereoselective enzyme, and the remaining *R* isomer is purified away from the other reaction products which are recycled. As an alternate illustration, the genes encoding an enzyme specific for the *R* isomer could be used to develop genetically engineered crops that are resistant to the herbicide of interest. Genetic engineering has already been used for crop protection from (achiral) phenoxyacetate herbicides: cotton has shown enhanced tolerance towards 2,4-D when *tfdA* was transformed directly into the plant [15] or into root-associated microbes [16].

Here, we demonstrate that purified *R. eutropha* TfdA specifically degrades the *S* enantiomer of dichlorprop and characterize the kinetics of this process. In addition, we establish that *Burkholderia cepacia* strain RASC, a 2,4-D-degrader containing a TfdA with 80% sequence identity to that from JMP134, has the same stereopreference for the *S* enantiomer. By contrast, we show that cell extracts of a strain of *Alcaligenes denitrificans*, intact cells of which are known to utilize the *R* enantiomer of mecoprop [14], specifically degrade the *R* enantiomer of dichlorprop in an α-KG-dependent process. Although the latter activity exhibits opposite stereospecificity from that found in the 2,4-D-degrading strains, we provide evidence that a *tfdA*-like gene is present in *A. denitrificans*, suggesting that all of these enzymes are closely related to each other.



Scheme 1. Reaction catalyzed by TfdA with 2,4-D.

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2. Experimental

2.1. Chemicals

(+)-(*R*)-Dichlorprop was a kind gift from BASF. Racemic and (+)-(*R*)-mecoprop were generously donated by A.H. Marks. Racemic dichlorprop and 2,4-D were purchased from Aldrich.

2.2. Bacterial strains

R. eutropha JMP134 [17] and *B. cepacia* RASC [18] were obtained from the Research on Microbial Evolution laboratory at Michigan State University. A strain of *A. denitrificans* isolated for its ability to degrade mecoprop [14] was obtained from Hilary Lappin-Scott (University of Exeter).

2.3. Activity assay with colorimetric detection of derivatized phenols

The standard assay mix contained 1 mM α-KG, 50 μM each of Fe(II) and ascorbate, 1 mM phenoxyacid substrate in 10 mM imidazole buffer (pH 6.75), and enzyme. In studies of cell extracts, the concentrations of selected reagents were varied in some experiments as noted in the text. The iron and ascorbate were either supplied together as iron(II) ascorbate salt or separately as $(NH_4)_2Fe(SO_4)_2$ and ascorbic acid. Reactions were carried out at 30°C and terminated by addition of EDTA to a final concentration of 5 mM. The phenolic product was detected at 510 nm following addition of 4-aminoantipyrene, pH 10 borate buffer, and potassium ferricyanide, as described [19]. Measured extinction coefficients for the 4-aminoantipyrene adducts were 15,700 M^{-1} cm^{-1} for 2,4-DCP and 11,300 M^{-1} cm^{-1} for 4-chloro-2-methylphenol.

2.4. Preparation of cell extracts and purified enzyme

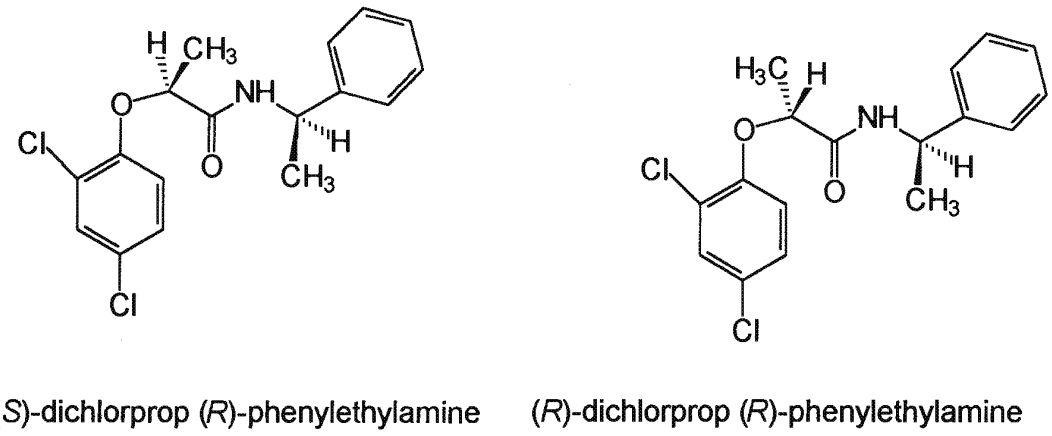
TfdA was purified from *Escherichia coli* DH5α cells carrying the cloned *R. eutropha* JMP134 *tfdA* gene on plasmid pUS311 by sequential chromatography on DEAE Sepharose and MonoQ (Pharmacia) columns [4,20].

2,4-D and mecoprop degraders were grown aerobically at 30°C in MMO mineral salts medium [21] with 50 ppm yeast extract and the relevant herbicide at initial concentrations between 1 and 5 mM. Cells were harvested when 60–90% of the herbicide was consumed as measured by disappearance of the chromophore at 230 nm. Cell extracts were prepared by resuspending the bacteria in Tris buffer (pH 7.7) with 1 mM EDTA followed by disruption in a French pressure cell and ultracentrifugation (100,000 × *g*, 30 min) or centrifugation in a microcentrifuge (16,000 × *g*, 5 min). Protein concentrations were determined by using the BioRad protein assay with bovine serum albumin as the standard.

2.5. Formation of diastereomers and gas chromatography (GC)

The levels of (*R*)- and (*S*)-dichlorprop in samples were assessed by using a modification of a published GC method [22]. Aqueous reaction mixtures (1 ml) were acidified with 25 μl of HCl (conc.) and extracted with 1 ml methylene chloride. The organic phase (0.5 ml) was dried under a gentle stream of air, 1 drop of thionyl chloride was added, and the sample was incubated 10 min over a steam bath. Following removal of the thionyl chloride under vacuum, the sample was dissolved in 0.1 ml chloroform with 1 drop of (*R*)-phenylethylamine added, allowed to react 25 min at room temperature to form diastereomers (Scheme 2), dried, and transferred to 0.5 ml of ethyl acetate. An aliquot (1 μl) of the sample was injected onto a DB-5 column (polymethylsiloxane with 5% phenyl groups; J&W Scientific), with a temperature program set to increase linearly from 200 to 270°C in 7 min, and the analytes were monitored by electron capture detection (rather than the less sensitive flame ionization detection originally used [22]). Peaks were identified by

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(*S*)-dichlorprop (*R*)-phenylethylamine (*R*)-dichlorprop (*R*)-phenylethylamine

Scheme 2. Formation of diastereomers of dichlorprop and (*R*)-phenylethylamine.

comparison with the retention times of the diastereomeric amides of authentic (*R*)-dichlorprop and (*RS*)-dichlorprop.

2.6. High pressure liquid chromatography (HPLC)

Dichlorprop was reacted with cell extracts or purified enzyme as described for the activity assay, and the reactions were stopped with 5 mM EDTA. Aliquots were injected onto a Nucleodex α-PM column (4.0 mm i.d. × 200 mm length; Machery-Nagel, Germany). The eluent was 30% phosphate buffer (50 mM, pH 3) and 70% methanol at a flow rate of 0.7 ml min^{-1}. Species eluting from the column were monitored with UV detection at 230 nm.

2.7. DNA hybridization, amplification and sequencing

Total genomic DNA was isolated from *A. denitrificans* [23] and digested with *Bam*HI. Following separation on a 0.8% agarose gel, the DNA was transferred [23] to a Hybond N membrane (Amersham). Probes for $tfdA_{RASC}$ (a 1-kb *Pst*I fragment [18]) and for $tfdA_{JMP134}$ (an 801-bp *Sty*I fragment [24]) were labeled with digoxigenin according to the Genius™ System instructions (Boehringer-Mannheim Biochemicals). The membrane was blotted with the probes at high stringency (68°C, 50% formamide) or low stringency (68°C, no formamide) using the protocol from the same manual.

A. denitrificans DNA was used as template DNA for PCR amplification with TVU and TVL primers which amplify a 360-bp internal fragment of both RASC and JMP134 *tfdA* [25]. The product was purified by using the GFX PCR DNA and gel band purification kit (Pharmacia Biotech) and was sequenced in both directions by using dye terminator chemistry at the MSU-DOE-PRL Plant Biochemistry DNA Sequencing Facility with TVU and TVL as primers. The nucleotide and amino acid sequences of the amplified product (not including the primer sequence) were aligned to those of JMP134 and RASC using protocols in the GCG suite of programs [26].

2.8. Data analysis

Data were fitted to straight lines, exponential decays, or square hyperbola using the program KaleidaGraph (Synergy software). As well, progress curves of 2,4-DCP production vs. time were fitted to Eq. (1) by non-linear regression using the same program.

$$A_t = A_f(1 - e^{-k(\text{inact})t}) \quad (1)$$

In this equation, A_t is the absorbance at time t, A_f is the final absorbance, and k_{inact} is the rate of inactivation.

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3. Results

3.1. TfdA stereospecificity

The stereospecificity of purified TfdA from *R. eutropha* JMP134 was investigated using two methods. The first approach used derivatization with (*R*)-phenylethylamine followed by GC analysis to separate and quantitate the two diastereomers. During the reaction of racemic dichlorprop with TfdA, the percentage of *S* relative to *R* enantiomer decreased over time, suggesting that this enzyme uses the *S* isomer (Fig. 1). The second procedure used to assess stereospecificity involved a colorimetric assay for 2,4-DCP release. 2,4-DCP was produced by TfdA acting on racemic dichlorprop but not on (*R*)-dichlorprop (Fig. 2), a result in agreement with the GC results for this enzyme. The enzyme lost activity over time due to oxidative inactivation events that are minimized by the presence of ascorbic acid [27]. Based on initial rates, (*S*)-dichlorprop utilization (0.81 μmol min^{-1} mg^{-1} protein) was 6% of that observed for 2,4-D decomposition (13 μmol min^{-1} mg^{-1} protein) under saturating substrate concentrations.

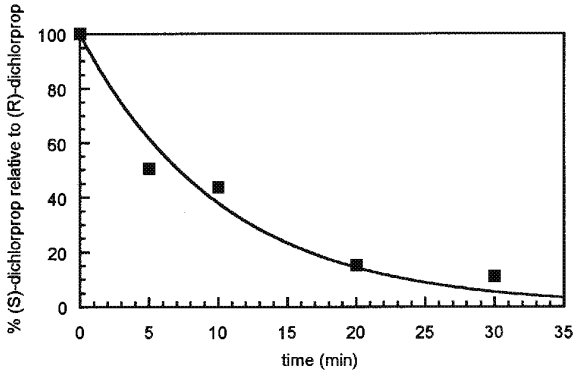


Fig. 1. Stereoselectivity of dichlorprop decomposition catalyzed by TfdA. TfdA from JMP134 (50 μg) was incubated with racemic dichlorprop (100 μM) and ascorbic acid, ferrous ion, and α-KG using standard concentrations for the indicated time periods. Following extraction, the remaining dichlorprop was converted to the (*R*)-phenylethylamine diastereomers and analyzed by GC/electron capture detection. Data were fitted to an exponential decay with a decay rate of 0.096 ± 0.011 min^{-1}.

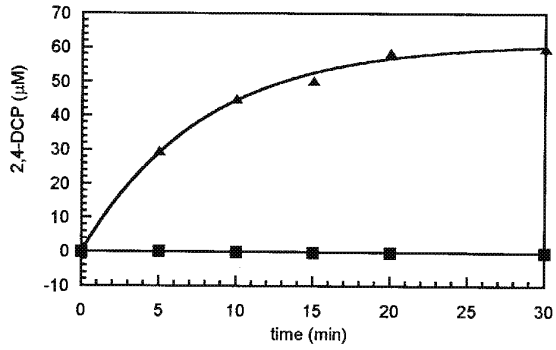


Fig. 2. 2,4-DCP production by TfdA acting on dichlorprop. TfdA from JMP134 (10 μg ml^{-1}) was incubated with a racemic mixture of dichlorprop (▲) at 2 mM or with (*R*)-dichlorprop at the same concentration (■). 2,4-DCP was measured colorimetrically. Data for the racemic dichlorprop were fitted to Eq. (1) ($k_{inact} = 0.131 \pm 0.004$ min^{-1}), while data for the (*R*)-dichlorprop were fitted by linear regression (slope of -0.02 μM min^{-1}).

3.2. Herbicide-degrading activities in cell extracts

Cell extracts were examined from JMP134 and two additional phenoxyacid herbicide degraders: *B. cepacia* RASC, a 2,4-D degrader with a TfdA that is 80% identical to that of *R. eutropha* JMP134 [18], and a strain of *A. deni-*

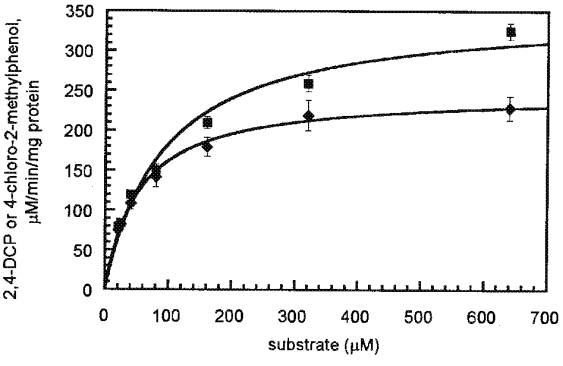


Fig. 3. Kinetic analysis of *A. denitrificans* cell extract activities towards (*R*)-mecoprop and (*R*)-dichlorprop. Cell extracts of *A. denitrificans* were examined for their abilities to decompose (*R*)-dichlorprop (■) and (*R*)-mecoprop (◆). Initial rates calculated from the phenolic product release (based on 6 min time points, triplicate samples) were fitted to the Michaelis–Menten equation to yield values of $K_m = 91 \pm 16$ μM and $V_{max} = 351 \pm 21$ μM min^{-1} mg^{-1} protein for (*R*)-dichlorprop and $K_m = 52 \pm 4$ μM with $V_{max} = 247 \pm 6$ μM min^{-1} mg^{-1} protein for (*R*)-mecoprop. Mecoprop velocity data were corrected for ~1% contamination with a phenolic compound presumed to be 4-chloro-2-methylphenol.

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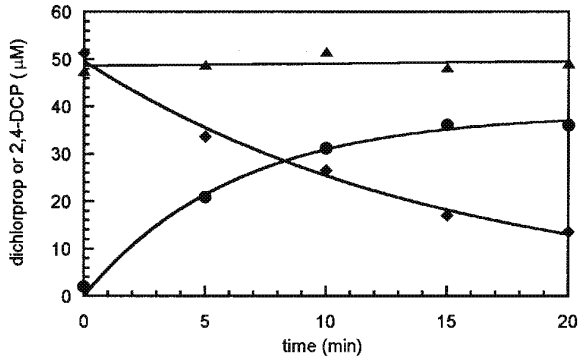


Fig. 4. Stereospecificity of dichlorprop-degrading activity of *A. denitrificans* cell extracts as measured by chiral HPLC. Concentrations of (*S*)-dichlorprop (▲), (*R*)-dichlorprop (◆), and 2,4-DCP (●) were measured at selected time points after incubation of *A. denitrificans* cell extracts (50 μl) with racemic dichlorprop (0.1 mM) and the standard reaction components. Dichlorprop was quantitated by using chiral HPLC and 2,4-DCP was quantitated colorimetrically. Data for the (*S*)-dichlorprop were fit to a straight line of slope 0.05 μM min^{-1}, for (*R*)-dichlorprop to exponential decay with an exponent of -0.067 min^{-1}; and for 2,4-DCP to Eq. (1) with an apparent inactivation rate of 0.16 min^{-1}.

trificans isolated for its ability to degrade (*R*)-mecoprop [14]. Cell extracts of JMP134 and RASC exhibited highest activity towards 2,4-D, but showed ~20% of this activity towards dichlorprop. We attribute the cell extract activity towards phenoxy herbicides in both these strains to TfdA, since Tn5 disruption of the *tfdA* gene in either strain will prevent the conversion of 2,4-D to 2,4-DCP by intact cells [5,18]. By contrast to the specificity of the abovementioned strains, *A. denitrificans* extracts were most active with phenoxypropionates including mecoprop, dichlorprop, 2-(2,4-dibromophenoxy)propionate, and 2-(4-chlorophenoxy)propionate, with only minor activity towards the phenoxyacetates 2,4-D and 2-chloro-4-methylphenoxyacetic acid (~13 fold less than the activity towards dichlorprop). Cell extracts of *A. denitrificans* demonstrated a slightly higher V_{max} towards the dichlorprop than towards mecoprop, the substrate on which it was isolated, but slightly less affinity (higher K_m) (Fig. 3). The *A. denitrificans* activity was dependent on α-KG and ferrous ion, and was rapidly lost (in 2 min) in the absence of ascorbic acid. These cofactor requirements are characteristic of α-KG-dependent dioxygenases.

3.3. Stereospecificity of activity in cell extracts

The stereospecificity of herbicide decomposition by cell extracts was measured using an HPLC column with a chiral stationary phase, which permitted direct separation without prior derivatization of the enantiomers of dichlorprop. In agreement with the results using purified enzyme, cell extracts of JMP134 degraded the *S* enantiomer. Similar results were obtained with

```
                150       160       170       180       190       200
JMP134          RAAYDALPRDLQSELEGLRAEHYALNSRFLLGDTDYSEAQRNAMPPVNWPLVRTHAGSGRK
                ||||| ||:|:::||:|||||||||||||||:||||||||:||||||||:|||||||||||||
A.denitrificans RAAYDDLPEDFKKELQGLRAEHYALNSRFILGDTDYSESQRNAMPPVSWPLVRTHAGSGRK
                |||||||||||||||||||||||||||||:||||||||:||||||||||||||||:|||||||||
RASC            RAAYDDLPEDFKKELQGLRAEHYALHSRFILGDTEYSESQRNAMPPVSWPLIRTHAGSGRK
                170       180       190       200       210       220

                   210       220       230       240       250
JMP134          FLFIGAHASHVEGLPVAEGRMLLAELLEHATQREFVYRHRWNVGDLVM
                ||||||||:|:|| ||||||||||||||||||||:|||||  |:|||||||
A.denitrificans FLFIGAHAGHIEGRPVAEGRMLLAELLEHATQRKFVYRHSWKVGDLVM
                ||||||||:|||||||||||||||||||||||| ||||||||||||||
RASC            FLFIGAHASHIEGRPVAEGRMLLAELLEHATQPKFVYRHSWKVGDLVM
                   230       240       250       260       270
```

Fig. 5. Comparison of an *A. denitrificans* TfdA-like sequence with the enzymes from RASC and JMP134. The deduced sequence encoded by the PCR amplification product arising from *A. denitrificans* DNA was compared to the indicated portions of the RASC and JMP134 TfdA sequences. Identical amino acids are indicated by a solid vertical bar and similar amino acids by a dashed bar.

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RASC extracts (data not shown). By contrast, *A. denitrificans* extracts degraded (*R*)-dichlorprop (Fig. 4) with concurrent 2,4-DCP production.

3.4. Molecular biological comparison of strains

The similarity in cofactor requirements among the three strains raised the question of whether the *A. denitrificans* activity was due to a gene related in sequence to those which encode the two TfdA enzymes in the other species. *A. denitrificans* genomic DNA hybridized at low stringency to a probe for $tfdA_{JMP134}$ and at high stringency to a segment of $tfdA_{RASC}$ (data not shown). Amplification of *A. denitrificans* DNA with primers specific for *tfdA* yielded a 360-bp DNA fragment with a sequence that was 94% identical to that from RASC and 78% to that from JMP134, in a 327-bp overlap. The translated sequence (109 amino acids long) had 95% identity with TfdA from RASC and 86% identity to that from JMP134 (Fig. 5).

4. Discussion

4.1. R. eutrophus JMP134 TfdA oxidizes the S isomer of phenoxypropionates

α-KG dependent dioxygenases typically exhibit stereoselectivity in the hydroxylations they catalyze (e.g., Refs. [28–31]); thus, it was not surprising to find that *R. eutrophus* JMP134 TfdA oxidizes a single enantiomer (the *S* isomer) of dichlorprop. Based on this result with phenoxypropionate, the enzyme likely catalyzes stereochemically selective oxidation at the *pro-R* position when acting on the prochiral 2,4-D.

4.2. Related gene products can degrade opposite stereoisomers

The above studies demonstrate that the herbicide-degrading activity of *A. denitrificans* exhibits opposite stereochemistry than that found in strains JMP134 or RASC; yet, each of these activities are α-KG-dependent and the enzymes are likely to be genetically related. The present work extends the conclusions derived from studies with a strain of *Sphingomonas herbicidovorans* that is capable of degrading both enantiomers of mecoprop [32]. In that strain, two α-KG-dependent dioxygenases appear to be present, each specific for a separate enantiomer of the substrate [33]. Based on our findings, it is probable that the two *S. herbicidovorans* enzymes are closely related in sequence. The ability for closely related enzymes to recognize opposite stereoisomers has precedent. For example, recent crystallographic characterization of two tropinone reductases demonstrate the presence of almost identical overall protein folding, but the closely related enzymes exhibit opposite substrate stereoselectivities due to differences in a few charged residues at the active site [34].

4.3. Biotechnological implications

The work described here may have biotechnological applications. An *S* isomer selective dichlorprop-degrading enzyme, such as TfdA, may be used to facilitate the preparation of the desired *R* isomer, while a gene encoding an *R* isomer selective enzyme, such as that in *A. denitrificans*, may be useful for development of herbicide-resistant transgenic plants. Also, in understanding the role of microorganisms in the environmental fate of phenoxypropionates, it is useful to have analytical techniques such as those used in this research capable of distinguishing the enantiomers.

Acknowledgements

We thank Peter Chapman for suggesting that dichlorprop could be used to probe the stereospecificity of TfdA, Paul Loconto of the Engineering Research Labs of MSU for assistance in setting up the GC method and suggesting the

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use of electron capture detection, and Hilary Lappin-Scott for providing us with the mecoprop-degrading strain of *A. denitrificans*. This work was supported by the National Science Foundation (MCB9603520), the National Science Foundation–Science and Technology Center Grant DEB9120006, and the Michigan State University Agricultural Experiment Station.

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Plant and Soil Enantioselective Biodegradation of Racemic Phenoxyalkanoic Herbicides

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ABSTRACT The biodegradation of the chiral phenoxyalkanoic herbicides 2-(2,4-dichlorophenoxy)propionic aid (2,4-DP) and 2-(4-chloro-2-methylphenoxy)propionic acid (MCPP) was investigated using enantioselective HPLC and chiroptical detection. Racemic mixtures of 2,4-DP and MCPP were applied to three species of turf grass, four species of broadleaf weeds, and soil. Preferential degradation of the S-(–) enantiomer of each herbicide was observed in most species of broadleaf weeds and soil, while the degradation in all species of grass occurred without enantioselectivity. The biodegradation in all systems appeared to follow pseudo first-order kinetics with the fastest degradation occurring in broadleaf weeds, followed by the grasses. The slowest degradation was observed in soil. The results of this work illustrate the need to characterize both enantiomers of chiral agrochemicals in order to have an accurate understanding of their distribution and fate in the environment. *Chirality 11:330–337, 1999.*
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KEY WORDS: Chirobiotic T; teicoplanin; enantioselective chromatography; column-switching; kinetics

The majority of chiral agrochemicals are applied to the environment as racemates (i.e., 50:50 mixtures of enantiomers). Enantiomers often have different biological and physiological properties; therefore, investigations that treat racemates as though they were single entities can produce inaccurate and misleading results.[1,2] In many cases, one enantiomer can produce a completely different biological response than its corresponding antipode.[3,4] Almost all environmental regulations in the United States treat racemates as though they are single molecules with the same biological and physiological properties.

Although herbicides and pesticides are often designed to be applied to and affect specific types of plants or animals, they often are widely dispersed throughout the ecosystem and they, and/or their degradation products, can be found in various food chains.[5–7] In order to understand the distribution, effect, and fate of chiral agrochemicals it is necessary to perform enantioselective biodegradation and toxicological studies.

The phenoxyalkanoic herbicides are widely used for the pre- and post-emergent control of broadleaf weeds in field grass, turf grass, and cereal crops.[8] First introduced at the end of World War II, about 55 million pounds of acid equivalent of these herbicides are now used annually in the United States alone.[9] These compounds mimic the action of plant growth hormones (auxins) in certain types of broadleaf weeds causing abnormal growth and development of the plant.[8] This unnatural development ultimately leads to the death of the plant.

Dichlorprop (2,4-DP) and mecoprop (MCPP) are two widely used, chiral phenoxyalkanoic herbicides (see Figure 1). Each herbicide exists as a pair of enantiomers with the R-(+) enantiomer of each being responsible for the majority of herbicidal or auxin activity and the S-(–) enantiomer of each possessing slight anti-auxin activity.[8,10] Despite this fact, both herbicides continue to be produced and applied as racemic mixtures. In cases of racemic pesticides and herbicides in which only one enantiomer produces the desired effect, 50% of that material is unnecessarily introduced into the environment. A considerable amount of information has been reported about the degradation of phenoxyalkanoic herbicides by various bacteria, soil microbes, and plants.[11–20] However, most of this research has treated racemic 2,4-DP and MCPP as single compounds, failing to acknowledge that both enantiomers may not be undergoing the same biological/biochemical processes.

To gain a more complete understanding of the fate of these herbicides in the environment, it is necessary to examine the fate of both enantiomers of 2,4-DP and MCPP in various environmental systems. In other work, Faller et al.[21,22] reported that the (–) enantiomer of α-1,2,3,4,5,6-hexachlorocyclohexane (α-HCH) was more prevalent off of the east British coast, whereas (+) α-HCH was more prevalent in the central North Sea. In another example, Garrison et al.[23] reported the preferential degradation of S-(–) 2,4-

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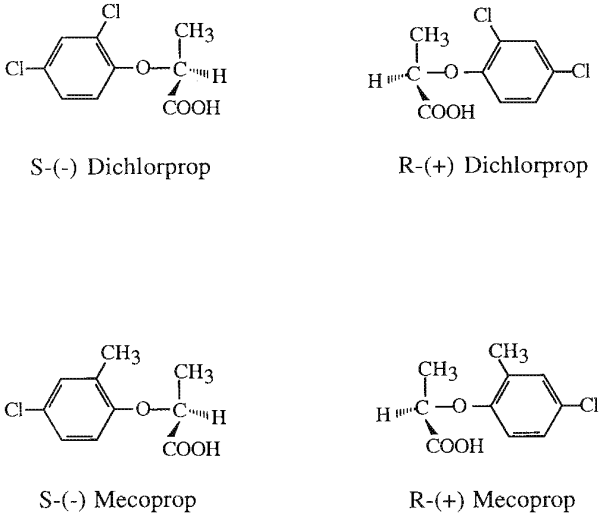


Fig. 1. Structures of the phenoxyalkanoic herbicides dichlorprop and mecoprop.

DP in soil, while Ludwig et al.[24] reported the preferential degradation of R-(+) 2,4-DP by marine microorganisms. Most recently the biodegradation of chlordane in fish was shown to be stereoselective.[25] The goal of this research is to investigate and compare the biodegradation of both enantiomers of 2,4-DP and MCPP in the specific environments in which they are used (e.g., various species of grass, broadleaf weeds, and soil). To our knowledge, no broad-scale study such as this has been reported.

MATERIALS AND METHODS

Plant Growth and Care

All grass and clover were grown indoors at approximately 23°C in circular containers 4 cm deep and 30 cm in diameter that contained commercial potting soil. Each species was grown in separate containers with a population density of approximately 3–5 plants per cm^2. Holes were placed in the container to allow for drainage. Seeds for rye grass (*Lolium perenne*), fescue (*Festuca arundinacea*), blue grass (*Poa pratensis*), and medium red clover (*Trifolium pratense*) were generously donated by Missouri Southern Seeds (Rolla, MO), whereas dandelions (*Taráxacum officinále*), broadleaf plantain (*Plantágo májor*), and narrowleaf plantain (*Plantágo lanceoláta*) were sprayed outdoors or transplanted and sprayed indoors. The indoor plants received approximately 150 mL of tap water daily and approximately 12 h of sunlight each day followed by 12 h of darkness. The grass and clover samples were sprayed at 4 months of age. They were approximately 7 cm in height. The dandelion leaves ranged from 7–11 cm in length and central seed-bearing stalks were 15–20 cm in height. The leaves of both species of plantain ranged from 3–6 cm in length and their central seed-bearing stalks were 5–7 cm in height. Neither the plants nor the soil had previous exposure to herbicides of any kind.

Herbicide Application and Extraction for Plant Samples

Methanol, acetonitrile, triethylamine, acetic acid, and methylene chloride were obtained from Fischer Scientific Co. (St. Louis, MO). 2,4-DP was obtained from Aldrich (Milwaukee, WI) and MCPP was obtained from AccuStandards Inc. (New Haven, CT). Both herbicides were greater than 95% pure. The R-(+) enantiomer of each herbicide was obtained from Reidel de Haen (Germany) and shipped via Aldrich. Five mg of racemic 2,4-DP or racemic MCPP was dissolved in 2.5mL of methanol and added to 60 mL of deionized water. Each 62.5 mL portion of solution was applied evenly to the 700 cm^2 plots of plants grown indoors or on the 1,400 cm^2 plots outdoors using a pneumatic sprayer. There was no rainfall during the course of the outdoor experiments. At given time intervals (see Results and Discussion) after application, approximately 15 g of plants (aerial and root portions) were individually collected from random locations in the soil and washed with tap water. The plants were weighed, homogenized in a glass grinder, and added to a 250 Erlenmeyer flask with 150 mL of methanol. The glass grinder was rinsed with methanol and the washings were added to the Erlenmeyer flask. This mix-

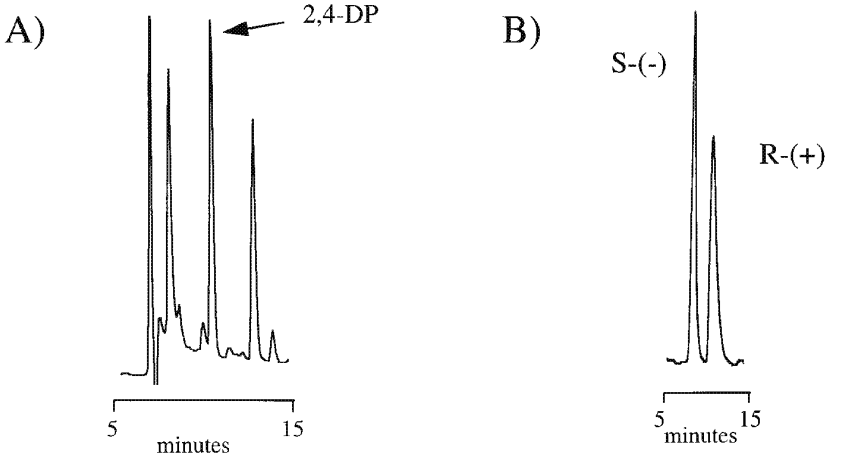


Fig. 2. Chromatograms showing A) the achiral (C_{18}) separation of a sample of 2,4-DP extracted from rye grass and B) the enantiomeric separation of 2,4-DP switched from chromatogram A (using a 6-port switching valve, 20 µl of sample taken) to the Chirobiotic T column for enantiomeric resolution. See Experimental Section for chromatographic conditions.

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ture was sonicated (Fischer model FS-28, 720 Watts at 43 kHz) for 2 h. After sonication, the solution was gravity filtered using Whatman's filter paper (Medium-qualitative #2) and transferred to a 500-mL separatory funnel. To this solution, an equal volume of 1% HCl was added to protonate the herbicide. The solution was then extracted with 75 mL (3 × 25 mL) of methylene chloride and collected in a beaker. The methylene chloride was allowed to evaporate just to dryness by passing a slow stream of nitrogen over the sample leaving a dark residue. This residue was dissolved in 1 or 2 mL of methanol and analyzed by HPLC. The recovery for this extraction procedure was 97% with an relative standard deviation (RSD) of 5% (n = 4). The entire growing/spraying experiment was performed in duplicate for each species of plants. Experiments were performed on broadleaf plantain both outdoors and indoors, yielding the same results under both conditions. Samples from individual days were analyzed in triplicate with an RSD of peak area of 0.3%.

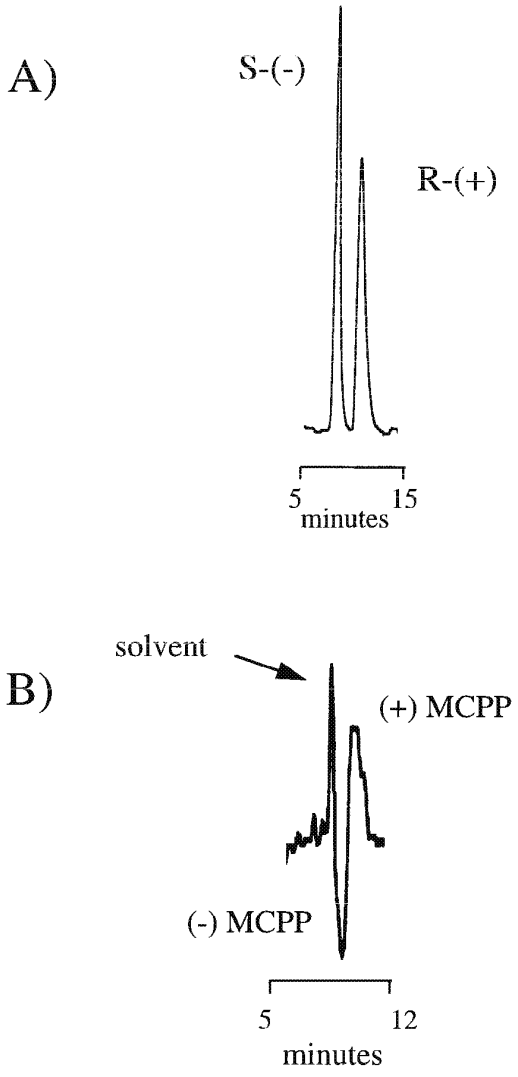


Fig. 3. Chromatograms obtained from A) an enantiomeric separation of mecoprop using a UV-vis detector and B) the same separation using a chiroptical detector. Note that the peak areas are equivalent in these chromatograms.

Herbicide Application and Extraction for Soil Samples

Approximately 500 g of moist topsoil was removed from the ground to a depth of 20 cm. The soil was classified as Union silt loam and consisted of 20% clay, 1.5% organic matter, and had a pH of 6.0. This soil was transferred to a beaker, spiked with 2 mg of 2,4-DP or MCPP, mixed thoroughly, and placed in a dark drawer. The soil was kept moist by adding 50 mL of tap water daily. At selected time intervals, portions from three locations within the bulk soil were removed and placed in a small beaker. The total mass of the soil sample was 35 g. To this beaker, 150 mL of methanol was added and the mixture was sonicated in an ultrasonic bath for 1 h. After sonication, the soil was allowed to settle under the influence of gravity. The methanol extract was decanted and filtered with filter paper by gravity. The filtered solution was evaporated just to dryness by passing a stream of nitrogen over the sample leaving a yellow-brown residue. To this residue, 1 to 2 mL of methanol was added and the dissolved sample was analyzed by HPLC. The entire experiment and analysis of the soil was performed in duplicate. The recovery from soil extraction was 93% with an RSD of 6% (n = 4). Samples from individual days were analyzed in triplicate with an RSD of peak area of 0.3%.

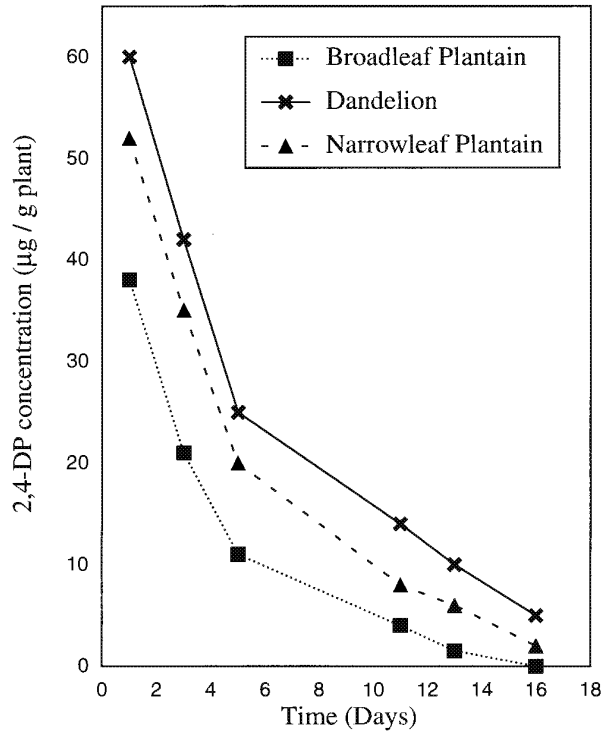


Fig. 4. Degradation of dichlorprop in three species of broadleaf annuals.

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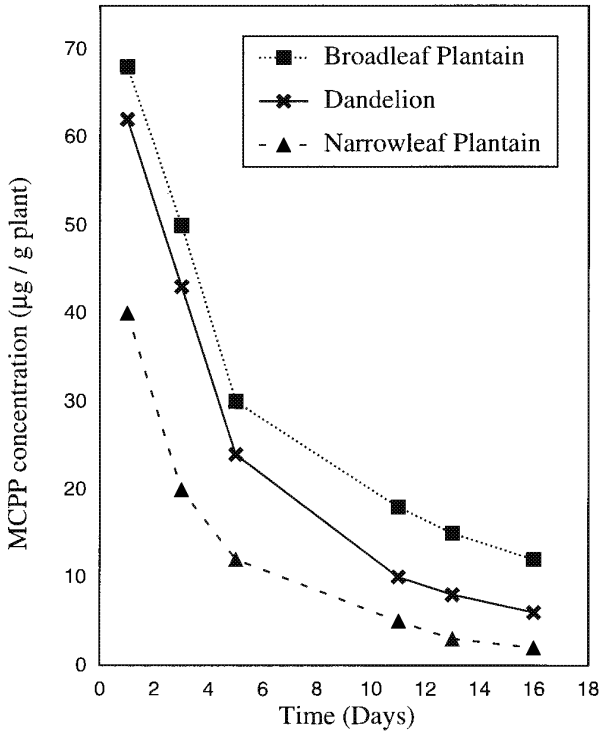


Fig. 5. Degradation of MCPP in three species of broadleaf annuals.

Chromatographic Conditions

HPLC analysis was performed using Shimadzu (Kyoto, Japan) LC-6A pumps with a Shimadzu SCL-6B system controller. Analysis of the plant extracts was performed using a column switching design. The 5 µm C_{18} column and the Chirobiotic T column (both were 25 cm by 4.6 cm i.d.) were obtained from Advanced Separation Technologies (Whippany, NJ). The mobile phase for the C_{18} separation was delivered at 1 mL/min and consisted of 60:40 methanol:1% triethylammonium acetate (pH = 4.1). The mobile phase for the Chirobiotic T column was also delivered at 1 mL/min and consisted of 5:95 methanol:1% triethylammonium acetate (pH = 4.1). Detection of the eluent from the C_{18} column was accomplished at 230 nm using a Shimadzu SPD-2AM detector. Detection of the eluent from the Chirobiotic T column was accomplished at 240 nm using a Shimadzu SPD-6A detector. The chiral detector was obtained from Product Development Resources (Palm Beach Gardens, FL). Figure 2 shows the chormatograms obtained from A) separation of the rye grass extract on an achiral C_{18} column, and B) the enantiomeric resolution of 2,4-DP, which was switched to the chiral Chirobiotic T column. The quantitation of the herbicides was accomplished using the chromatograms obtained from the C_{18} column, whereas the enantiomeric ratios of the herbicides were determined using the Chirobiotic T column.

RESULTS AND DISCUSSION

Assignment of the Peaks

To determine the elution order of each enantiomer, the racemic samples were spiked with standards of the R-(+) enantiomer. As a secondary means of confirming the optical rotation of each eluting peak, a chiral detector was placed in series with the UV detector. This detector operates on the principles of ellipsometry. A dextrorotatory (+) or a levorotatory (–) sign is assigned to a molecule on the basis of its ability to rotate light from the sodium D line (589.3 nm). Although this detector does not operate at the sodium D line, it does operate in the visible region near the sodium D line (675 nm). Investigations have shown that the ORD spectrum of a molecule can change dramatically at lower wavelengths, near absorption bands. However, the phase change (δ) experienced by a plane polarized wave as it interacts with an optically active sample changes slowly at wavelengths far from an absorption band. Thus, for compounds with no absorption bands in the visible region of the electromagnetic spectrum, the direction of rotation of plane polarized light at the sodium D line is usually the same as that for the chiral detector (under analogous conditions).[26] This behavior was confirmed for these herbicides as well. Figure 3 shows the enantioselective separations obtained using A) UV detection and B) a micropolarimetry detector placed in series. This confirms that the first

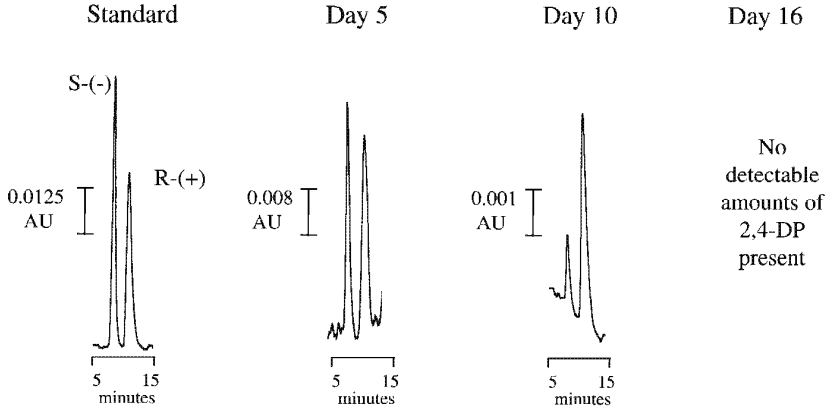


Fig. 6. Enantiomeric separations of dichlorprop extracted from broadleaf plantain. The enantiomeric ratios (%S:%R) from left to right are 50:50, 40:60, and 14:86.

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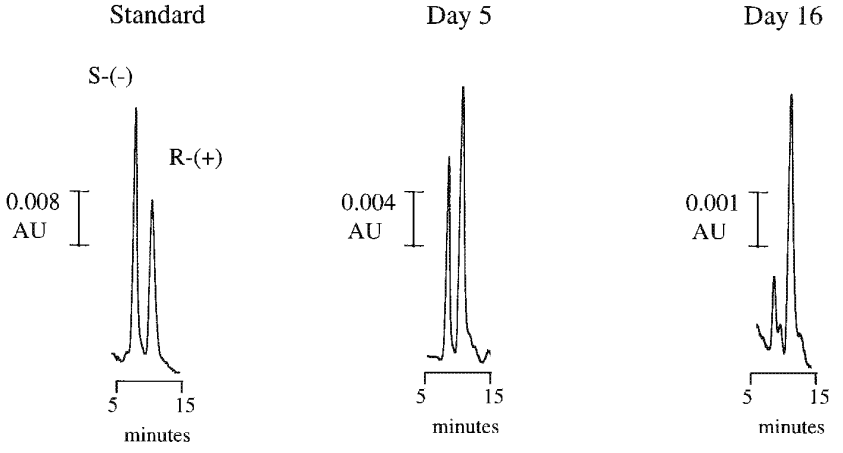


Fig. 7. Enantiomeric separations of mecoprop extracted from broadleaf plantain. The enantiomeric ratios (%S:%R) from left to right are 50:50, 36:64, and 12:88.

eluting peak is the S-(–) enantiomer of MCPP and 2,4-DP and the second eluting peak is the R-(+) enantiomer of MCPP and 2,4-DP.

Analysis of 2,4-DP and MCPP in Taráxacum officinále (Dandelion), Plantágo májor (Broadleaf Plantain), and Plantágo lanceoláta (Narrowleaf Plantain)

Figures 4 and 5 show the analysis of the total concentration of both herbicides extracted from dandelions, broadleaf plantain, and narrowleaf plantain at various time intervals. In both cases, the concentration decreased sharply the first week after application and then decreased at a slower rate. This trend is observed for all species of broadleaf weeds. During the course of the experiments, all species of broadleaf flora exhibited outward signs of stress (i.e., curling of the leaves, leaves turning brown or yellow); however, not all of the broadleaf flora were completely destroyed after 16 days. In most cases, complete herbicidal degradation was not observed by the 16th day.

In order for a foliage-applied, postemergent herbicide to be effective, certain conditions must be met. The target plants must be able to absorb the herbicide and the herbicide must be translocated to the sites of action.[8] Herbicide uptake by plants has been shown to be dependent on such environmental factors as air temperature, soil temperature, and relative humidity.[8] As indicated previously, it is known that the R-(+) enantiomer of each herbicide is thought to be responsible for the herbicidal activity. Figures 6 and 7 show that the extracted herbicides appear to be racemic on the first day after application. These figures also show that the S-(–) enantiomer of each herbicide is degraded faster in all three species of broadleaf weeds than the R-(+) enantiomer.

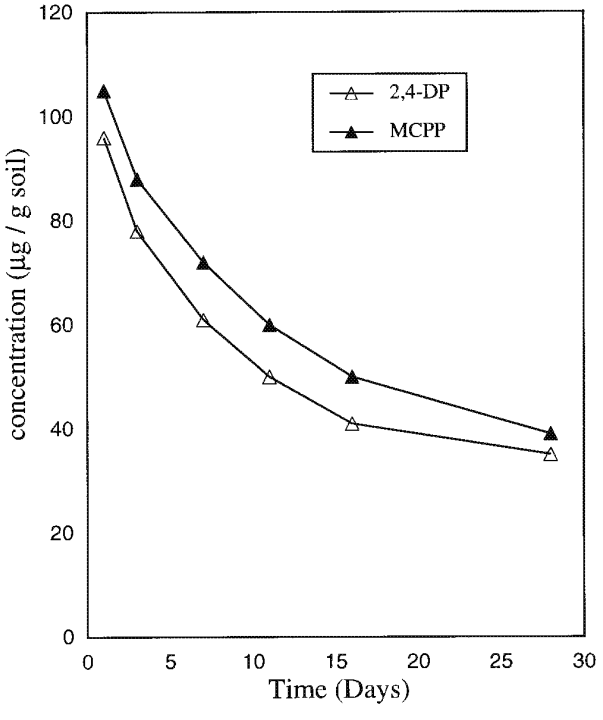


Fig. 8. Degradation of dichlorprop and mecoprop in soil.

Analysis of 2,4-DP and MCPP in Trifolium pratense (Medium Red Clover)

Both herbicides show a somewhat similar total degradation profile in clover as in broadleaf weeds. However, in contrast to broadleaf weeds, the clover does not exhibit selectivity for the enantiomers of these herbicides. All extracted samples contained racemic mixtures of the herbicides for all intervals during the course of 16 days. Also, by the 16th day, the concentrations of both herbicides were below the limits of detection for our instrumentation and all clover plants were completely destroyed. While this data appears to contradict that observed for the other species of broadleaf flora, it may be indicative of different mechanisms for degradation between the clover and other annuals and further illustrates that the fate of racemic compounds in different biological systems can vary tremendously.

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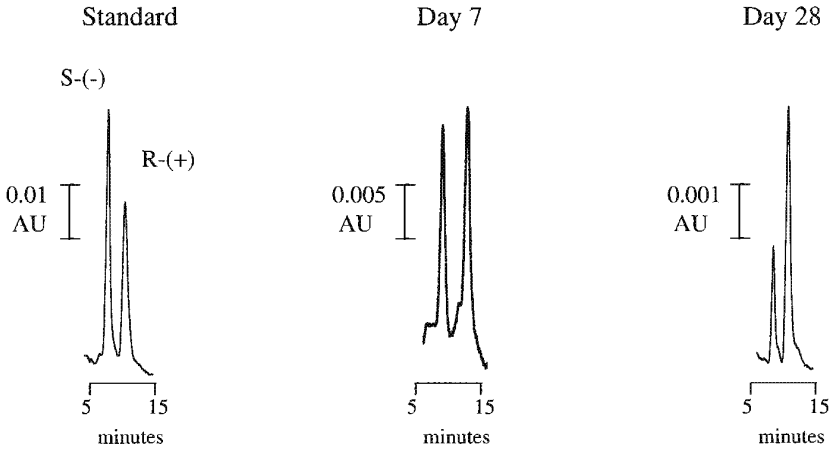


Fig. 9. Enantiomeric separations of dichlorprop extracted from soil. The enantiomeric ratios (%S:%R) from left to right are 50:50, 50:50, 37:63, and 17:83.

Analysis of 2,4-DP and MCPP in Lolium perenne (Perennial rye grass), Festuca arundinacea (Fescue), and Poa pratensis (Blue Grass)

The concentration profile of both herbicides decreases with time although at a rate much slower than in the broadleaf flora. None of the three species of grass displayed any symptoms of stress or injury during the course of the investigation. This is not surprising because these herbicides are known to be ineffective in controlling most species of grass. The process by which these herbicides were removed from the grasses was not enantioselective. This indicates that both enantiomers are degraded or removed at equal rates.

Analysis of 2,4-DP and MCPP in Soil

Figure 8 shows the analysis of the total concentration of both herbicides extracted from soil spiked with 2,4-DP and MCPP. The total herbicide concentration in the soil decreased over the course of 28 days. Figures 9 and 10 show that the degradation process was selective for the S-(–) enantiomer of both herbicides. As shown in Table 1, however, the degradation rate in soil was much slower than that observed for any of the plants. The disappearance of the S-(–) enantiomer of both herbicides was about 3 to 5 times slower in soil than in broadleaf flora. These observations agree with previous reports on the degradation of 2,4-DP in soil.[23,27] Conversely, earlier studies on the degradation of MCPP in soil and by soil bacteria indicated that the process was enantioselective for R-(+) MCPP.[16,28] However, a more recent report gave the opposite results by showing the enantioselective degradation of S-(–) MCPP in soil taken from a plot near Wädenswil, Switzerland.[28] The results showed that the rate for degradation of S-(–) MCPP was about 1.5 times faster than that of R-(+) MCPP. Clearly, there are conflicting results for the enantioselective biodegradation of MCPP in soil. It may be possible that these are because of variations in the microbial populations in different locations and soil types or because of other environmental differences.

Comparison of Degradation Rates

The biodegradation of 2,4-DP and MCPP appeared to follow pseudo first-order kinetics. The pseudo first-order degradation rates were calculated for the enantiomers of

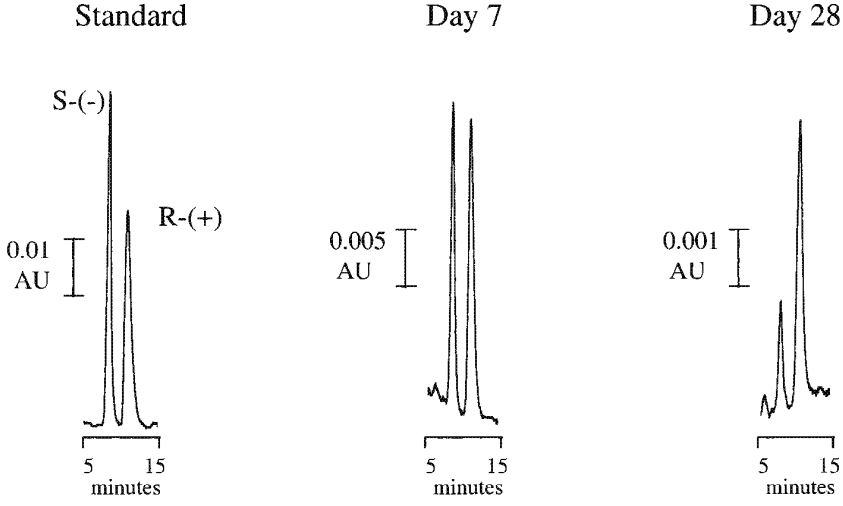


Fig 10. Enantiomeric separations of mecoprop extracted from soil. The enantiomeric ratios (%S:%R) from left to right are 50:50, 38:62, and 21:79.

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TABLE 1. Pseudo first-order rate constants for the biodegradation of 2,4-DP and MCPP*

Plant investigated	Rate constants ($\times 10^{-2}$ d^{-1})[a]			
	(R)-2,4-DP	(S)-2,4-DP	(R)-MCPP	(S)-MCPP
Taráxacum officinalé (dandelion)	12.1	25.1	19.8	29.7
Plantágo major (broadleaf plantain)	18.8	36.9	11.1	21.3
Plantágo lanceoláta (narrowleaf plantain)	17.2	34.2	9.3	22.6
Trifolium pratense (medium red clover)	20.5	20.5	19.3	19.3
Festuca arundinacea (fescue)	4.2	4.2	5.7	5.7
Lolium perenne (rye grass)	17.4	17.4	5.6	5.6
Poa pretensis (blue grass)	7.8	7.8	5.9	5.9
Soil	2.6	8.2	2.6	6.0

*The relative standard deviation for the rate constants was 1.5% for the plant samples and 1.8% for soil.

both herbicides in all studies from regression plots of $\ln(c/c_0) = -kt$. The results of these plots are given in Table 1. The rates ranged from the lowest value 2.6 d^{-1} for R-(+) MCPP in soil to the highest value of 36.9 d^{-1} for S-(-) 2,4-DP in broadleaf plantain. The fastest degradation rates were observed for both herbicides in broadleaf weeds, followed by the grasses, whereas the slowest rates were observed in soil. In the cases where the degradation was enantioselective (i.e., broadleaf weeds), the rates for the S-(-) enantiomers of both herbicides were approximately twice those of the R-(+) enantiomers. The S-(-) enantiomers of both herbicides were degraded twice as fast as the R-(+) enantiomers in soil with the R-(+) enantiomer of 2,4-DP being the most resistant to degradation in soil.

CONCLUSIONS

Three types of floral degradation patterns were observed for both 2,4-DP and MCPP: 1. preferential enantioselective degradation of the S-(-) enantiomer with the plants being killed (i.e., broadleaf weeds); 2. both enantiomers degraded quickly at equal rates and completely within 1.5 to 2 weeks with the plants being killed (i.e., medium red clover); and 3. both enantiomers degraded slowly at equal rates without harming the plants (i.e., all species of grass). In general, both herbicides were degraded more quickly and completely by plants than by soil microbes. Herbicides were still prevalent in soil samples after 28 days, whereas their concentrations in grass and broadleaf annuals were significantly lower or undetectable. In addition, the R-(+) enantiomer of both herbicides was more resistant to degradation in plants and especially in soil. The results of this work illustrate the need to characterize both enantiomers of chiral agrochemicals in various ecological systems in order to have an accurate understanding of their distribution and fate in the environment. As demonstrated, degradation patterns can differ greatly in different media and even between different species of plants.

Because many chiral pesticides and herbicides are racemic mixtures, there is an obvious way to reduce environmental pollution (i.e., pollution prevention) involving these compounds and/or their metabolites. By manufacturing and applying only the active enantiomers we can immediately reduce the amount of these biocides introduced into the environment by 50% (or more for certain diastereomeric compounds) without jeopardizing their beneficial effects. This switch from racemic mixtures to pure active enantiomers is already occurring in the pharmaceutical industry as a result of FDA regulatory policies[29] and other concerns. It may be logical to examine the possibility of doing an analogous switch for agrochemicals.

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Effect of tillage on microbial characteristics and herbicide degradation in a Sharkey clay soil

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Field and laboratory studies were conducted at Stoneville, MS, from 1996 to 1998 to determine the influence of subsoiling (SS) and conventional tillage (CT) of a Sharkey clay soil on microbial characteristics and herbicide degradation. Soil samples obtained from imazaquin-treated and nontreated plots from the soybean row and interrow position were analyzed. Because only the row position is actually disturbed by SS, a comparison of row and interrow position on the parameter was conducted. Imazaquin (preemergence, 140 g ai ha^{-1}) had no effect on microbial populations, microbial enzyme activity (fluorescein diacetate [FDA] hydrolysis and triphenyl-tetrazolium chloride [TTC] dehydrogenase), and organic carbon content. Estimates of microbial activity based on FDA hydrolysis and TTC dehydrogenase activity indicated greater activity under CT; however, microbial biomass and organic carbon were not affected by tillage or row position. A laboratory study assessed the degradation of carboxyl- and ring-labeled 2,4-D as influenced by tillage and row position. Soils from CT plots had an initially higher mineralization rate of ^{14}C carboxyl-labeled 2,4-D compared to soils from SS plots; however, no effect of tillage or row position was observed on the cumulative amount of $^{14}CO_2$ evolved 14 d after treatment (DAT) in 1996 and 18 DAT in 1998. In studies with ring-labeled 2,4-D, a higher $^{14}CO_2$ evolution was detected in soils obtained from SS plots, regardless of row position, whereas a greater amount of radioactivity was observed in the unextractable fraction from CT soils. Because differences in 2,4-D mineralization between tillage regimes were minimal, adoption of SS as a tillage practice for heavy clay soils in the Mississippi Delta may have a limited effect on microbial characteristics and biodegradation of soil-applied herbicides.

Nomenclature: 2,4-D; 2,4-DCP, 2,4-dichlorophenol; imazaquin; FDA, fluorescein diacetate; TTC, triphenyl-tetrazolium chloride; soybean, *Glycine max* (L.) Merr.

Key words: Enzymatic activity, microbial metabolism, subsoiling.

Tillage systems can have a major effect on the physical, chemical, and biological properties of soils. The effects of tillage systems, including conventional tillage (CT), reduced tillage, and no tillage, on soil properties have been extensively reviewed (Griffith et al. 1992; Locke and Bryson 1997). CT, mainly accomplished by a moldboard plow or disk, is characterized by a high degree of soil disturbance. Under CT, plant residues are completely incorporated into the upper soil layer, whereas under reduced-tillage and no-tillage systems, a cover of vegetation or plant residue is preserved.

Soils managed using reduced tillage generally have more surface plant residues, higher moisture content, and better structure and aggregation compared to soils managed under CT (Doran 1980; Griffith et al. 1992; Locke and Bryson 1997). Accumulation of plant residues on the soil surface fosters higher levels of organic matter in the surface soil, promoting better structure, fertility, water infiltration, and water-holding capacity (Kirchner et al. 1993; Wesley et al. 1994). Shading action of ground cover under reduced-tillage systems lowers soil surface temperatures, and fluctuations are not as dramatic compared to temperatures of soils managed under CT (Eghball et al. 1994). The increased plant residue present in soils under reduced tillage also provides a natural barrier against evaporation, promoting higher moisture levels and protecting soil surfaces from erosion.

Accelerated degradation of organic matter because of increased microbial oxidation has been observed in soils under CT, whereas soils managed under reduced tillage typically have higher amounts of organic carbon and mineralizable nitrogen (Doran 1980; Eghball et al. 1994). The presence of decaying plant material on the surfaces of soils under reduced tillage affects the quality, composition, and solubility of soil organic components and the degree of humification. Free sugars or complex polysaccharides associated with high levels of organic matter in reduced-tillage soils serve as labile substrates for microorganisms (Eghball et al. 1994). Reduced-tillage systems may create an optimal environment for microbial growth and activity and other desirable characteristics associated with soil quality (Wagner et al. 1995).

Elevated microbial and soil enzyme activity observed in soils subjected to reduced tillage can promote the biodegradation of compounds such as atrazine (Levanon et al. 1994), bentazon (Wagner et al. 1996), or fluometuron (Zablotowicz et al. 1994). Increased polymerization of organic substances also occurs under reduced tillage, which promotes formation of nonextractable humus–herbicide complexes (Foster and McKercher 1973; Greer and Shelton 1992; Locke and Bryson 1997; Stott et al. 1983). Soil microflora participate in the biotransformation of herbicides by several processes, including co-metabolism, metabolism, polymerization, and mineralization (Bollag and Liu 1990).

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Table 1. Sampling dates, environmental parameters, and assays performed for microbial characterization in 1996, 1997, and 1998.[a]

Year	Date	Sample timing	Temperature[b]	Rainfall[b]	Assay[c]					
					FDA	TTC	Microbial population	Microbial biomass/ organic carbon	2,4-D mineralization	2,4-D metabolite
1996	May 15	Initial characterization	20	28	*		*			
	May 31	2 WAP	27	34	*		*		*	
	June 26	5 WAP	28	6	*		*			
1997	April 21	Initial characterization	18	0	*	*				
	June 20	2 WAP	25	79	*	*		*		
	July 13	5 WAP	30	0.3	*	*				
1998	June 17	2 WAP	29	3	*			*	*	*

[a] Abbreviations: FDA, fluorescein diacetate hydrolysis activity; TTC, triphenyl-tetrazolium chloride dehydrogenase activity; WAP, wk after soybean planting.

[b] Average temperature [(max + min)/2] and rainfall reported during the week prior to sampling.

[c] An asterisk indicates an assay was performed.

Levanon et al. (1994) suggested that the activity of microbial enzymes in polymerization and the adsorption of herbicides to organic matter are the primary mechanisms reducing the movement of herbicides under reduced tillage. The accumulation of plant residues and organic matter in the upper layer of reduced-tillage soils can create additional herbicide adsorption sites (Locke and Bryson 1997). The degree of adsorption of a specific pesticide to soil colloid can determine its bioavailability to microorganisms (Anderson 1981; Greer and Shelton 1992; Locke and Bryson 1997). Adsorbed pesticides can be protected from microbial degradation, which can increase persistence in soil.

Subsoiling (SS), or deep tillage conducted in the fall, can increase yield of nonirrigated soybean (*Glycine max*) grown on heavy clay soils of the Mississippi Delta (Wesley and Smith 1991; Wesley et al. 1994). Using a subsoiler equipped with parabolic shanks creates only a small slot in the soil surface in the area of major root development. Thus, the degree of soil disturbance is lower under SS than CT. Although the depth of cultivation is increased, SS can leave more plant residues on the soil surface. Although SS has been shown to be beneficial in several soil types, limited research has been conducted to predict the effect of SS on microbial and enzymatic activity and the effect on herbicide dissipation. Therefore, the objectives of this study were to examine the effect of tillage systems, in concert with imazaquin applied preemergence, on soil microbial characteristics and to determine the effect of SS on 2,4-D dissipation in a Mississippi Delta Sharkey clay soil.

Materials and Methods

Site and Soil Description

Field studies were conducted at the Delta Research and Extension Center, Stoneville, MS, from 1996 to 1998. The soil is a Sharkey clay (very fine, smectitic, thermic Chromic Epiaquert) with 3% sand, 32% silt, 66% clay, 3.0% organic matter, and pH 6.4. Two tillage systems, SS and CT, were evaluated. Tillage treatments were initiated 3 yr before initiation of the study and were maintained in the same plots during the 3-yr study. SS was accomplished using a subsoiler unit equipped with two parabolic shanks spaced 1 m apart. All SS plots were tilled to a depth of 40 to 45 cm each fall, with six rows (subsoiler slot positions) per plot. Although SS plots were cultivated with a spike-tooth harrow to create a smooth seedbed, the soil was less disturbed compared to the CT treatment. CT, also performed each fall, was achieved by three passes with an off-set disk harrow, adjusted to operate 15 cm deep, followed by a single pass with a field cultivator 6 cm deep.

'DPL 3589' soybean was planted 3 cm deep at a rate of 33 seeds m^{-1} of row, spaced 1 m apart with six rows per plot. In subsoiled plots, soybean rows were planted exactly over subsoiler slot positions. Immediately after planting, imazaquin was applied at 140 g ai ha^{-1} to corresponding plots. Nontreated plots receiving no imazaquin were also included for comparison. Five soil samples were randomly taken from the soil surface (0 to 5 cm) of each plot using a shovel for initial microbial characterization before soybean planting and 2 and 5 wk after planting (WAP) as summarized in Table 1. To determine the effect of row position (subsoiler shank position) on microbial populations, enzyme activity, organic carbon, microbial biomass, and biodegradation of herbicides, samples taken 2 and 5 WAP were also obtained from the soybean row and interrow (between soybean rows) positions. Soil samples were composited, passed through a 3-mm sieve, and stored moist for approximately 2 wk in the dark at 5 C until analysis.

Microbial Populations

Estimates of soil microbial propagules were determined in soil samples from all sampling dates in 1996 (Table 1) by serial dilutions and spiral plating[1] on selective and semiselective growth media, as described previously (Reddy et al. 1995; Wagner et al. 1995). Total bacteria, gram-negative bacteria, and fluorescent pseudomonads were enumerated on 10% tryptic soy agar containing cycloheximide (100 g ml^{-1}), 10% tryptic soy agar with crystal violet (5 g ml^{-1}) and cycloheximide (100 g ml^{-1}), and S1 media (Gould et al. 1985), respectively. Total fungus populations were determined on Rose Bengal potato dextrose agar (Martin 1950). Colonies of fluorescent pseudomonads, gram-negative bacteria, total fungi, and total bacteria were counted after 2, 3, 5, and 7 d of incubation at 28 C, respectively. Enumeration was conducted using four replications from imazaquin-treated and nontreated plots. Propagule counts were expressed as $\log_{10}$ colony-forming units g^{-1} soil (oven-dry wt).

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Enzyme Activity

Fluorescein diacetate (FDA) hydrolytic activity was used as an estimate of soil microbial activity during the 3 yr of the study (Table 1) using a modification of the method described by Schnürer and Rosswall (1982). Two grams of soil (fresh wt) from each sampling date was placed into 50-ml polypropylene centrifuge tubes; 15 ml 0.1 M potassium phosphate buffer (pH 7.6) and 0.5 g FDA in 250 µl acetone were added and tubes were capped and vortexed. Samples were incubated for 2 h on a reciprocal shaker at room temperature (22 to 24 C). The assay was terminated by extraction with 15 ml acetone for 30 min on a reciprocal shaker and was clarified by centrifugation (10 min at 6000 × *g*). Fluorescein (product) concentration was determined spectrophotometrically at 490 nm (extinction coefficient of 80.3 mM cm^{-1}). Experiments were conducted using two replications containing substrate and one control without substrate to correct for background.

A second assay determined triphenyl-tetrazolium chloride (TTC) dehydrogenase activity (Casida 1977) to assess soil enzyme activity in the 1997 samples (Table 1). Four grams of soil (fresh wt) was incubated in 8 ml of 3% aqueous TTC solution for 24 h at 37 C. Assays were conducted with three replicates containing TTC and one control incubated with 8 ml of deionized water. The reaction was terminated by addition of 24 ml of methanol, and soil was extracted for 30 min on a reciprocal shaker. The reaction mixture was clarified by centrifugation (10 min at 6000 × *g*). The concentration of 2,3,4-triphenyl-tetrazolium formazan (product) was determined spectrophotometrically at 485 nm after correcting for coloration due to extractable soil components.

Soil Microbial Biomass and Organic Carbon Content

Microbial biomass was determined in soil samples obtained 2 WAP in 1997 and 1998, employing a chloroform fumigation–incubation method (Jenkinson and Powlson 1976). Soil samples were sterilized by 48 h of exposure to chloroform in a dessicator, after which the dessicator was vented under vacuum several times for complete removal of residual chloroform from the soil. Twenty grams of sterilized soil (oven-dried equivalent) was transferred into biometer flasks[2] and reinoculated with 2 ml of unsterilized soil suspension (1:200 dilution). Microbial biomass was determined by comparing respiration from two replicates of sterilized, reinoculated soil and two replicates of untreated soil for each sample. CO_2 evolved from decomposition of fumigated microflora or endogenous respiration was trapped in sidearms of the flasks containing 10 ml of NaOH (1 N) during 2 wk of incubation at 28 C. Evolved CO_2 was determined by titration with 0.5 M HCl after adding 5 ml of 2 N $BaCl_2$ and phenolphthalein indicator (1 mg ml^{-1} ethanol) to a 5-ml NaOH subsample. The microbial biomass carbon (g C kg^{-1} soil, oven-dry wt) was calculated as Biomass C = F_C/0.45, where F_C is (CO_2-C evolved by fumigated soil) − (CO_2-C evolved by unfumigated soil) (Jenkinson and Ladd 1981).

The effect of tillage systems, row position, and imazaquin application on soil organic carbon (g organic C kg^{-1} soil, oven-dry wt) was determined using a modified Mebius procedure (Nelson and Sommers 1982). Triplicate samples of soils (300 mg, air dried) obtained in 1997 and 1998 2 WAP were analyzed.

2,4-D Mineralization

The effect of tillage systems and row position on herbicide degradation was evaluated in the laboratory using soil biometer flasks (Bartha and Pramer 1965) with 2,4-D as a model substrate. Radiolabeled carboxyl- and ring-labeled 2,4-D was used to determine the effect of soil management systems on various microbial transformations, including side-chain decarboxylation, ring cleavage, or oxidative coupling reactions (polymerization). Soil samples collected from herbicide nontreated plots of both tillage systems and row positions 2 WAP in 1996 and 1998 were analyzed. Studies were initiated approximately 2 wk after sampling. Twenty-five grams of soil (oven-dry wt) were placed in biometer flasks and adjusted to 30% moisture content. Soils were treated with 1.0 ml of a methanol : water (1:4, v/v) solution containing ^{14}C uniformly labeled[3] (98% radiochemical purity) and technical-grade 2,4-D[4] (99% purity) to achieve a final concentration of 10 g g^{-1} soil. Total radioactivity added per flask was 171 and 217 Bq g^{-1} soil of carboxyl-labeled material and 373 and 319 Bq g^{-1} soil of ring-labeled material in 1996 and 1998, respectively. Each tillage and row position combination was replicated with four flasks. Biometer flask side arms contained 10 ml of NaOH (1 N) to trap evolved $^{14}CO_2$ and were replaced every second day. Soils treated with carboxyl-labeled 2,4-D were incubated for 14 and 18 d, whereas soils treated with ring-labeled material were incubated for 20 and 26 d at 28 C in 1996 and 1998, respectively. Trapped $^{14}CO_2$ was determined in 1996 from 250-L aliquots of NaOH mixed with 250 L of distilled water and added to 15 ml of scintillation cocktail[5]. In 1998, evolved $^{14}CO_2$ was determined from 1-ml aliquots of NaOH added to 15 ml of scintillation cocktail.[6] Radioactivity was determined by liquid scintillation counting[7] (LSC). After completion of the 1996 study, 5 g of soil from each flask were extracted twice with methanol (15 ml and 10 ml, respectively) to determine the extractable radioactivity using a modified method described by Zablotowicz et al. (1998). In 1998, soils (25 g) were extracted twice with 50 ml of methanol. Aliquots (1 ml) were added to 15 ml of scintillation cocktail[8] and radioactivity was determined by LSC. Unextractable radioactivity was determined from air-dried methanol-extracted soils by oxidation. Duplicate 300-mg subsamples were mixed with 200 mg of cellulose into cellulose thimbles and combusted with a biological materials oxidizer[9] at 482 C for 4 min. Released $^{14}CO_2$ was trapped in a combination of carbon absorbent[10] and scintillation cocktail[11] and was counted by LSC.

2,4-D Metabolite Determination

Formation of metabolites from ring-labeled 2,4-D in CT and SS plots in 1998 soil samples was determined using thin layer chromatography (TLC) as described by Zablotowicz et al. (1998). Twenty-five grams of soil (interrow position, oven-dry wt) were placed in 250-ml polypropylene bottles, treated with ^{14}C uniformly ring–labeled[5] and technical grade 2,4-D[6] in a methanol : water solution (1:4, v/v) to attain a concentration of 10 g g^{-1} soil with 319 Bq g^{-1} soil. Moisture content was adjusted to 30%. Samples were

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TABLE 2. Soil microbial populations in a Sharkey clay soil at 2 and 5 wk after soybean planting (WAP) as influenced by tillage systems and row position in 1996.

		Microbial population							
		2 WAP				5 WAP			
Tillage system	Row position	Total fungi	Total bacteria	Gram-negative bacteria	Fluorescent pseudomonads	Total fungi	Total bacteria	Gram-negative bacteria	Fluorescent pseudomonads
		$\log_{10}$ colony-forming units g^{-1} soil							
Conventional	Row	5.31	7.78	6.17	4.44	4.99	7.34	5.30	2.27
Conventional	Interrow	5.56	8.03	5.96	4.62	4.83	7.28	5.15	2.26
Subsoiling	Row	4.94	7.87	6.19	4.56	4.94	7.28	5.78	3.84
Subsoiling	Interrow	5.19	7.83	6.03	4.28	4.66	7.31	5.44	2.64
LSD (0.05)		0.37	0.23	NS	NS	0.13	NS	0.41	1.54

incubated in the dark at 28 C for 4 or 8 d and were extracted twice with methanol as described previously (Zablotowicz et al. 1994). Methanol extracts were reduced in a rotary evaporator to approximately 10 ml. The methanol concentrate was diluted with 100 ml of deionized water, acidified to pH 3.0 with HCl (1 N), and passed through a C-18 solid-phase extraction column.[12] Extracted 2,4-D and metabolites were eluted from the column with 3 ml methanol. Aliquots (40 µl) were spotted on 250-µm silica TLC plates[13] and developed 10 cm with benzene : acetic acid : acetone (25:25:1, v/v/v) solvent. The distribution of radioactivity in the developed TLC plates was analyzed with an imaging scanner.[14] The R_f values for 2,4-D and 2,4-dichlorophenol (2,4-DCP) were 0.16 and 0.40, respectively.

Statistical Analysis

The field experiment was conducted using a split-split plot design with four replications. Data obtained from microbial enumeration, FDA, TTC, and organic carbon were analyzed with tillage treatment as the main plot unit, herbicide treatment (imazaquin or no herbicide) as the subplot, and row position the sub-subplot. Data obtained from soil microbial biomass determination and 2,4-D mineralization were analyzed as a split plot design (only nontreated soils were assessed). All data were subjected to analysis of variance, and means were separated using Fisher's Protected LSD test. All statistical determinations were made at the 0.05 level of significance, unless otherwise noted. Because of year interactions, data from each year of the study were analyzed separately.

Results and Discussion

Effect of Imazaquin

Preemergent application of imazaquin at 140 g ai ha^{-1} did not affect microbial characteristics of a Sharkey clay soil. Although deleterious effects of certain herbicides on soil microbial populations have been reported in the literature (Biederbeck et al. 1987; Levanon et al. 1994; Moorman and Dowler 1991; Narain Rai 1992), no effect of imazaquin on microbial populations, soil enzyme activity, microbial carbon, or organic carbon was observed in this study (data not shown). Subsequently, data were averaged over imazaquin treatment and analyzed as a split plot design. Because of an interaction, the combination of tillage system and row position are discussed separately.

Microbial Populations

At initial characterization (before soybean planting), microbial populations did not differ between tillage management systems (data not shown). Soil samples collected from CT plots 2 WAP in 1996 contained higher counts of total fungi than that found in soils from SS plots for their respective row positions (Table 2). Total bacteria were greatest in soil from CT plots in the interrow position; however, all other microbial populations evaluated were not affected by tillage or row position. In contrast, higher numbers of gram-negative bacteria and fluorescent pseudomonads 5 WAP were found in soil from SS plots in the row position compared to both positions in soil from CT plots. The number of fluorescent pseudomonads was low 5 WAP, which may have been related to high temperatures and drier soil.

Microbial populations and their enzyme activities in soils are subject to seasonal fluctuations (Kaiser and Heinemeyer 1993; Kirchner et al. 1993). Warmer temperatures and adequate moisture typically found in the spring promote the growth of crops and other vegetation, providing additional substrate for proliferation of soil microbes. Water availability is a key environmental parameter influencing the quantity and diversity of microflora in soil (Anderson 1981, 1984). Increased rainfall and temperature and the presence of soybean roots may have promoted microbial growth at later sampling dates (Table 2). Although microbial populations increased from the initial sampling time to 2 WAP, numbers declined by 5 WAP (Table 2). In 1996, dry soil conditions in combination with higher average temperatures were present 5 WAP (Table 1). Surface soil temperatures increased during the season, resulting in periods of water deficit that typically reduce microbial populations. Thus, effects of tillage on microbial populations in a Sharkey clay soil are difficult to assess because of temporal variations.

Although significant differences in microbial populations were observed, the effects of tillage system or row position were not consistent. The effect of SS on microbial propagules observed in this study was much lower than effects reported in previous studies that compared no-tillage and CT systems in other Mississippi Delta soils (Reddy et al. 1995; Wagner et al. 1996; Zablotowicz et al. 1994). Soils used in these studies had lighter texture (e.g., Dundee silt loam [fine-silty, mixed, thermic Aeric Ochraqualf]), which may have been more responsive to tillage than Sharkey clay soils. Subtle changes in the soil microbial community ob-

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TABLE 3. Fluorescein diacetate (FDA) hydrolysis and triphenyl-tetrazolium chloride (TTC) dehydrogenase activity in a Sharkey clay soil as influenced by tillage system and row position.[a]

		FDA hydrolysis activity							TTC dehydrogenase activity		
		1996			1997			1998	1997		
Tillage	Position	Initial	2 WAP	5 WAP	Initial	2 WAP	5 WAP	2 WAP	Initial	2 WAP	5 WAP
		nmol g^{-1} soil h^{-1}									
Conventional	Row	264	261	265	155	193	126	299	2.5	1.1	1.6
Conventional	Interrow	----	276	271	----	217	108	301	----	1.2	1.0
Subsoiling	Row	198	155	249	103	131	111	166	1.2	0.6	1.3
Subsoiling	Interrow	----	178	229	----	163	76	215	----	0.8	1.0
LSD (0.05)		NS	46	NS	NS	28	34	71	1.1	0.4	NS
LSD (0.10)		48	----	----	41	----	----	----	----	----	----

[a] Abbreviation: WAP, weeks after soybean planting.

served in this study would be expected to have minimal effect on the potential for microbial herbicide metabolism.

Microbial Enzyme Activity

At initial sampling dates in 1996 and 1997, increased FDA activity (33 to 50%) was associated with soils from CT compared to SS plots; however, these differences were only statistically different at the 0.10 level of significance (Table 3). During all 3 yr of the study, CT soils (from both row and interrow positions) had higher FDA hydrolytic activity compared to soil from SS plots 2 WAP. In 1996 5 WAP, no differences in FDA hydrolysis attributable to tillage or row position were observed, although in 1997, soils collected from SS plots in the interrow position had lower FDA activity than other soil treatments. FDA hydrolysis is a simple, nonspecific, but sensitive technique to determine microbial activity in soils (Dick 1984; Reddy et al. 1995; Wagner et al. 1995). The substrate, FDA, is nonspecific for a wide spectrum of enzymes, including proteases, lipases, and esterases, and its hydrolysis has been highly correlated with the de-esterification of fenoxaprop-ethyl (Zablotowicz et al. 2000). In addition, a good correlation was found between FDA activity and soil respiration. However, FDA hydrolytic activity may represent enzymatic activity from living and dead cells as well as extracellular enzymes associated with clay minerals, organic matter, or fungal biomass (Schnürer and Rosswall 1982). FDA hydrolysis has also been suggested as a useful parameter to assess soil quality (Dick 1994).

TTC dehydrogenase activity is based on the ability of a soil to use TTC as an alternative electron acceptor and is used as an indicator for total microbial activity estimated only in living cells (Casida 1977). At initial characterization in 1997, TTC dehydrogenase activity was 108% greater in CT compared to SS soils (Table 3). Higher dehydrogenase activity was also observed in CT soils compared to SS soils collected 2 WAP when comparing respective row positions. However, no differences were observed 5 WAP. Results from the TTC assays confirmed results from the FDA hydrolysis assays, indicating greater biological activity in soils from CT compared to SS plots, regardless of row position. The magnitude of enzyme activity is dependent on several factors, such as microbial populations and composition, environmental conditions (temperature, moisture, pH), and carbon substrate availability (Dick 1984, 1994; Sinsabaugh 1994). Under CT, incorporation of plant residues into the surface soil provided more labile carbon substrates to support microbial activity and may be the major factor responsible for higher enzyme activities in CT soils compared to SS soils.

TABLE 4. Influence of tillage system and row position on radioactivity recovered from a Sharkey clay soil treated with 10 μg g^{-1} 2,4-D (^{14}C carboxyl-labeled) in 1996 and 1998.[a]

			Cumulative $^{14}CO_2$ recovery											
			Incubation time (DAT)											
Year	Tillage	Position	2	4	6	8	10	12	14	16	18	ME	UE	Recovery
			% of applied											
1996	Conventional	Row	22.5	41.6	57.7	69.5	74.4	76.4	77.7	----	----	1.0	15.0	93.7
	Conventional	Interrow	21.6	41.5	59.5	70.8	75.5	77.0	77.9	----	----	1.0	15.5	94.4
	Subsoiling	Row	19.0	38.1	51.6	66.9	73.6	75.9	77.1	----	----	1.0	14.9	92.9
	Subsoiling	Interrow	19.5	38.7	55.3	68.3	73.3	75.2	76.5	----	----	0.9	15.0	92.6
	LSD (0.05)		1.1	2.6	3.2	2.0	2.0	NS	NS	----	----	NS	NS	NS
1998	Conventional	Row	18.9	42.3	59.8	70.6	76.0	79.2	81.0	82.1	82.9	1.0	12.2	96.1
	Conventional	Interrow	18.4	36.0	51.8	64.0	70.1	74.6	77.3	79.0	80.3	1.6	13.3	95.1
	Subsoiling	Row	13.2	33.2	51.8	63.9	70.0	74.0	76.7	78.5	80.0	1.7	11.8	93.5
	Subsoiling	Interrow	15.7	38.1	56.6	68.0	72.8	76.3	78.5	79.9	81.0	1.3	11.9	94.1
	LSD (0.05)		1.7	8.2	NS	6.5	NS	NS	NS	NS	NS	0.5	NS	NS

[a] Abbreviations: DAT, days after treatment; ME, methanol-extractable fraction; UE, unextractable fraction.

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Soil Microbial Biomass and Organic Carbon

Tillage system and row position had no effect on either soil microbial biomass carbon or total soil organic carbon (data not shown). Only small differences in microbial populations attributed to tillage systems have been observed in this study; thus, alterations in soil microbial biomass would not have been expected. Crop rotation sequences and climate have been found to have greater influence on soil microbial biomass than tillage (Biederbeck et al. 1987; Eghball et al. 1994; Moorman and Dowler 1991). Cropping systems that produce more plant residues and do not greatly disturb the soil surface tend to have greater amounts of soil organic matter (Eghball et al. 1994). Subsoiling techniques cause minimal disturbance to the soil surface; therefore, an increase in soil organic matter due to reduced oxidation would only be expected over several years.

2,4-D Mineralization and Metabolite Determination

Although 2,4-D was chosen as a model substrate, differences in degradation of 2,4-D due to tillage system may be used as an indicator for biodegradation characteristics of other herbicides in a Sharkey clay soil. In 1996, a more rapid initial mineralization ($^{14}CO_2$ evolution) of carboxyl-labeled 2,4-D was detected in CT compared to SS (Table 4). Specifically, in the initial 8 d after treatment (DAT), cumulative $^{14}CO_2$ evolution was higher in CT compared to SS soils for their respective row positions. In soils collected in 1998, initially higher mineralization was observed 2 DAT when comparing CT and SS soils, regardless of row position. Mineralization detected in the row position 4 DAT was significantly higher in CT (Table 4). This initial, more rapid mineralization of carboxyl-labeled 2,4-D in CT compared to SS soils may reflect levels of increased microbial activity, as observed in the FDA hydrolytic and TTC dehydrogenase enzyme assays.

Although the highest initial rate of mineralization of carboxyl-labeled 2,4-D occurred in CT soils, the level of $^{14}CO_2$ evolution in soils from SS plots increased over time to a level equivalent to that of CT soils, indicating that microbial populations in SS soils adapted to degrade 2,4-D. Mineralization of the carboxyl-labeled 2,4-D side chain occurred without a lag period, indicating the presence of metabolizing microorganisms and enzyme systems to facilitate this transformation (Kunc and Rybarova 1983). In both years, about 78 and 83% of the applied carboxyl-labeled 2,4-D was mineralized to $^{14}CO_2$ after 14 and 18 d of incubation, respectively (Tables 4). Minimal radioactivity (about 1%) was recovered by methanol extraction at termination of studies, indicating almost complete metabolism of the acetic acid moiety of 2,4-D. Between 12 and 15% of initially applied radioactivity was associated with the unextractable fraction. A satisfactory level of mass balance (total recovery) of 93 to 96% was observed.

In 1996, a higher initial mineralization rate of ring-labeled 2,4-D occurred in soils from CT compared to SS plots in the interrow position (Table 5). However, more 2,4-D was mineralized to $^{14}CO_2$ in soils from SS compared to CT, regardless of row position at the termination of the study (18 DAT). In 1998, a higher mineralization rate was detected 2 DAT in soils obtained from CT compared to SS,

TABLE 5. Influence of tillage system and row position on radioactivity recovered from a Sharkey clay soil treated with 10 μg g^{-1} 2,4-D (^{14}C ring-labeled) in 1996 and 1998.[a]

Year	Tillage	Position	Cumulative $^{14}CO_2$ recovery											ME	UE	Recovery
			Incubation time (DAT)													
			2	4	6	8	10	12	14	16	18	20	22	24	26	
			% of applied													
1996	Conventional	Row	4.4	15.0	25.1	34.9	39.4	41.4	42.8	44.3	45.3	—	—	—	—	3.5	35.9	84.8
	Conventional	Interrow	5.0	16.5	29.6	38.4	41.7	43.1	44.2	45.4	46.3	—	—	—	—	3.2	34.0	82.5
	Subsoiling	Row	4.5	15.4	23.9	35.3	40.5	42.8	44.4	45.9	48.0	—	—	—	—	3.9	30.6	81.9
	Subsoiling	Interrow	4.3	14.9	25.0	36.3	41.6	43.7	45.2	46.7	47.7	—	—	—	—	3.5	30.1	81.3
	LSD (0.05)		0.5	NS	2.0	1.8	1.5	1.5	1.5	1.4	1.2	—	—	—	—	0.5	3.7	NS
1998	Conventional	Row	3.2	15.1	28.7	38.7	44.0	47.6	49.9	51.4	52.6	53.6	54.4	54.9	55.4	3.0	28.8	87.2
	Conventional	Interrow	3.0	12.6	25.3	35.6	41.7	45.9	48.8	50.6	52.1	53.3	54.3	54.9	55.4	3.1	29.0	87.5
	Subsoiling	Row	2.4	13.4	27.3	38.3	44.2	48.5	51.4	53.3	55.0	56.4	57.5	58.3	59.0	3.3	25.2	87.5
	Subsoiling	Interrow	2.6	13.8	27.8	38.7	44.4	48.3	51.0	52.9	54.5	55.7	56.7	57.4	58.0	3.1	25.6	86.7
	LSD (0.05)		0.2	1.8	2.6	2.4	2.0	1.8	1.6	1.4	1.2	1.1	1.0	0.8	0.8	0.2	2.0	NS

[a] Abbreviations: DAT, days after treatment; ME, methanol-extractable fraction; UE, extractable fraction.

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TABLE 6. Influence of tillage systems on metabolite formation in a Sharkey clay soil treated with 10 μg g^{-1} 2,4-D (^{14}C ring-labeled) in 1998.[a]

	Incubation time (DAT)							
	4				8			
Tillage system	ME	2,4-D	2,4-DCP	UE	ME	2,4-D	2,4-DCP	UE
	% of applied							
Conventional	34.7	21.1	12.9	37.7	12.3	6.9	5.2	34.1
Subsoiling	35.2	26.7	7.7	39.8	16.6	10.0	6.5	32.0
LSD (0.05)	NS	3.3	3.8	NS	NS	NS	NS	NS

[a] Abbreviations: DAT, days after treatment; 2,4-DCP, 2,4-dichlorophenol; ME, methanol-extractable; UE, unextractable.

regardless of row position (Table 5). However, at later sampling dates, cumulative $^{14}CO_2$ evolution increased in SS compared to CT soils regardless of row position.

Total cumulative mineralization of ring-labeled 2,4-D was approximately 45 to 59% of the applied radioactivity in 1996 and 1998, respectively (Table 5). Only approximately 4% of the initial radioactivity was recovered by methanol extraction, indicating nearly complete degradation of applied 2,4-D. A higher percentage of ^{14}C was recovered in the unextractable fraction in CT compared to SS soils, regardless of row position in both years of the study. The trend of greater mineralization of the 2,4-D ring in SS soils and greater incorporation of ring-labeled 2,4-D in the unextractable fraction in CT soils suggests altered pathways of 2,4-D metabolism due to tillage. Perhaps oxidative coupling of the 2,4-D ring moiety into soil humus was facilitated under CT, whereas more complete oxidation occurred in SS soils. Mass balance using ring-labeled 2,4-D ranged from 81 to 87%, with a higher efficiency in 1998 (Table 5). Although mineralization studies were conducted in soil biometer flasks to minimize loss of radioactivity, some loss of volatile compounds can be expected in this controlled environment (Bartha and. Pramer 1965). Possible loss of ^{14}C could have occurred via volatilization of 2,4-D itself and the metabolites, 2,4-DCP and 2,4-dichloroanisole (Smith 1985), and could have occurred during sampling of NaOH solutions or during soil extraction. However, volatility losses of 2,4-DCP and 2,4-dichloroanisole would only be detectable for the ring-labeled 2,4-D.

In 1998, an additional short-term incubation study assessed ^{14}C ring-labeled 2,4-D dissipation and metabolite formation in soil from CT and SS plots. Approximately 35 and 15% of the initial radioactivity was recovered by methanol extraction after 4 and 8 DAT, respectively (Table 6). Likewise, about 38 and 33% of the initial 2,4-D was unextractable 4 and 8 DAT, respectively. TLC analysis of methanol extracts indicated a rapid dissipation of 2,4-D with 21 and 27% of the initial 2,4-D recovered 4 DAT from CT and SS, respectively. Results also indicate that 2,4-DCP was the major metabolite formed and accumulated in a Sharkey clay (Table 6). A higher percentage of 2,4-DCP was initially accumulated in CT (Table 6), supporting results of a more rapid mineralization of the acetate side chain of 2,4-D in CT compared to SS soils. Up to 43% of the extractable radioactivity 8 DAT was recovered as 2,4-DCP.

Mineralization of carboxyl-labeled 2,4-D occurred more rapidly and to a greater extent compared to ring-labeled 2,4-D. Microbial metabolism of 2,4-D proceeds by initial cleavage of the ether-linked acetic acid side chain forming glycolic acid, which can be utilized as an energy source and readily metabolized to CO_2 (Foster and McKercher 1973; Kunc and Rybarova 1983; Stott et al. 1983). Mineralization of the ring structure of 2,4-D molecules requires ring cleavage and dechlorination. Transformation of the ring moiety of 2,4-D is mediated by a limited group of microorganisms possessing specific enzyme systems. The mineralization of ring-labeled 2,4-D exhibited a lag period up to 4 d in both soils, indicating growth or an adaptation period of 2,4-D–metabolizing bacteria. Typically, the acetic acid side chain is used for general metabolism by soil microorganisms. Thus, greater $^{14}CO_2$ evolution was observed in soils treated with carboxyl-labeled 2,4-D compared to ring-labeled 2,4-D. In contrast, 2,4-D and its metabolites can be subjected to enzyme-mediated oxidative coupling reactions and incorporated into relatively resistant humic acid polymers (Sarkar et al. 1988; Wilson and Cheng 1978; Zablotowicz et al. 1994). In general, incorporation of phenolic compounds into humic acid polymers or diffusion into the interior of humic acid particles reduces the concentration of free 2,4-D molecules in soil solutions, thereby stabilizing them against biodegradation. Incorporation of 2,4-D and its metabolites into soil as chemically bound compounds can resist solvent extractions (Sarkar et al. 1988; Stott et al. 1983; Wilson and Cheng 1978, Zablotowicz et al. 1994). Continuous incorporation of plant residues in CT may stimulate microorganisms that produce enzymes responsible for oxidative coupling of phenolic compounds and may explain the higher unextractable portion of ring-labeled herbicide in soil from CT plots.

Tillage systems evaluated in this study resulted in small and transient changes in microbial populations. Microbial activity measured by two distinct assays was consistently higher in CT compared to SS soils, which most likely enabled the initially more rapid mineralization rate of carboxyl-labeled 2,4-D in CT systems. CT operations completely incorporated crop residues into the upper soil layer (plow layer), thereby providing intimate contact between microorganisms and substrate. Although the soil microbial biomass and organic matter content were not affected by tillage systems, crop residues incorporated in CT soils may have provided readily available carbon and nitrogen sources to support increased microbial activity. Because SS had only minor effects on biodegradation of 2,4-D, minimal effects on microbial decomposition of other soil-applied herbicides would be expected. Previous work also indicated that SS does not affect the environmental fate of a common soil-

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applied herbicide used in soybean (Seifert 1999). Therefore, adoption of SS techniques in the Mississippi Delta would most likely have minimal effects on soil microbial properties and the biodegradation of soil-applied herbicides.

Sources of Materials

[1] Spiral System Instruments, 7830 Old Georgetown Road, Bethesda, MD 20814.

[2] Bellco Glass Inc., 340 Edrudo Road, Vineland, NJ 08360.

[3] 2,4-D (ring- and carboxyl-labeled), Sigma Chemical Co., 3050 Spruce Street, St. Louis, MO 63103.

[4] 2,4-D (technical grade), ChemService, P.O. Box 3108, West Chester, PA 19381.

[5] ScintiSafe Plus 50% scintillation cocktail, Fisher Scientific, 711 Forbes Avenue, Pittsburgh, PA 15219.

[6] Hi-Ionic-Fluor scintillation cocktail, Fisher Scientific, 711 Forbes Avenue, Pittsburgh, PA 15219.

[7] Model Packard TriCarb 4000 series, Packard Instruments Co., 800 Research Parkway, Meriden, CT 06450.

[8] EcoLume scintillation cocktail, ICN, 3300 Hyland Avenue, Costa Mesa, CA 92626.

[9] Packard TriCarb Oxidizer 306, Packard Instruments Co., 800 Research Parkway, Meridian, CT 06450.

[10] Carbo-Sorb E, Packard Instrument Co., 800 Research Parkway, Meriden, CT 06450.

[11] Permafluor E+, Packard Instrument Co., 800 Research Parkway, Meriden, CT 06450.

[12] J. T. Baker, 222 Red School Lane, Phillipsburg, NJ 08865.

[13] Fisher Scientific, 711 Forbes Avenue, Pittsburgh, PA 15219.

[14] Bioscan Imaging System 200, Bioscan, Inc., 4590 Macarthur Boulevard, Washington, DC 20007.

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Enhanced Mineralization of [U-^{14}C]2,4-Dichlorophenoxyacetic Acid in Soil from the Rhizosphere of *Trifolium pratense*

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Enhanced biodegradation in the rhizosphere has been reported for many organic xenobiotic compounds, although the mechanisms are not fully understood. The purpose of this study was to discover whether rhizosphere-enhanced biodegradation is due to selective enrichment of degraders through growth on compounds produced by rhizodeposition. We monitored the mineralization of [U-^{14}C]2,4-dichlorophenoxyacetic acid (2,4-D) in rhizosphere soil with no history of herbicide application collected over a period of 0 to 116 days after sowing of *Lolium perenne* and *Trifolium pratense*. The relationships between the mineralization kinetics, the number of 2,4-D degraders, and the diversity of genes encoding 2,4-D/α-ketoglutarate dioxygenase (*tfdA*) were investigated. The rhizosphere effect on [^{14}C]2,4-D mineralization (50 μg g^{-1}) was shown to be plant species and plant age specific. In comparison with nonplanted soil, there were significant ($P < 0.05$) reductions in the lag phase and enhancements of the maximum mineralization rate for 25- and 60-day *T. pratense* soil but not for 116-day *T. pratense* rhizosphere soil or for *L. perenne* rhizosphere soil of any age. Numbers of 2,4-D degraders in planted and nonplanted soil were low (most probable number, <100 g^{-1}) and were not related to plant species or age. Single-strand conformational polymorphism analysis showed that plant species had no impact on the diversity of α-*Proteobacteria tfdA*-like genes, although an impact of 2,4-D application was recorded. Our results indicate that enhanced mineralization in *T. pratense* rhizosphere soil is not due to enrichment of 2,4-D-degrading microorganisms by rhizodeposits. We suggest an alternative mechanism in which one or more components of the rhizodeposits induce the 2,4-D pathway.

The rhizosphere is the zone of soil directly influenced by the presence of plant roots. It receives inputs of an array of low (e.g., sugars, organic and amino acids, phenolics, and other secondary metabolites)- and high (e.g., cellulose, lignin, mucilage, proteins)-molecular-mass compounds as a result of rhizodeposition and is a zone of complex plant-microbe interactions (56). Seminal work by Hsu and Bartha (23) demonstrated that mineralization of organophosphate pesticides in the rhizosphere is increased relative to that in nonplanted soils. Since this study, numerous reports of rhizosphere-enhanced biodegradation have been published for several classes of organic pollutants, including polyaromatic (2, 35, 48) and aliphatic (5, 38, 48) hydrocarbons, chlorophenols (3, 13), chlorophenoxyacetic acids (3), chlorobenzoates (46), *s*-triazines (32), and surfactants (29).

One suggested reason for rhizosphere-enhanced biodegradation is the possible presence, as a result of rhizodeposition, of high densities of active and diverse heterotrophic microorganisms on root surfaces, a situation which will facilitate complex degradative pathways through activities of consortia and conjugative horizontal transfer of catabolic genes carried on mobile genetic elements (6). Another suggested reason for rhizosphere-enhanced xenobiotic biodegradation is that components of the rhizodeposits (exudates, root materials, or their decomposition products) may be structural analogs for the pollutant in question (6, 20). It follows that the presence of a pollutant analog in the rhizosphere may select for pollutant-degrading microorganisms capable of directly using the analog for growth, act as a cooxidized substrate during cometabolism, or serve as an effector molecule in the induction of xenobiotic catabolic pathways (43). Furthermore, some investigators (45, 47, 57) hold the view that the rhizodeposition of compounds stimulating xenobiotic biodegradation may not be merely a passive process that fortuitously results in enhanced biodegradation. On the contrary, it is suggested that the plant, on sensing the presence of a xenobiotic in its rhizosphere, actively alters the composition of its rhizodeposits to stimulate xenobiotic degradation and that this response is an extension of the plant's defenses against natural allelopathic chemicals. However, despite much speculation regarding the causes, most experimental studies are descriptive and solely report the phenomenon of rhizosphere-enhanced biodegradation. Thus, empirical studies are required in order to dissect the mechanisms of enhanced biodegradation in the rhizosphere. Only if we gain a better understanding of plant-soil-microorganism-pollutant interactions can we rationally exploit the system for effective rhizoremediation of contaminated land.

One xenobiotic for which rhizosphere-enhanced biodegradation has been reported is 2,4-dichlorophenoxyacetic acid (2,4-D) (3), a herbicide widely used for the control of broad-leaved weeds in both agriculture and domestic applications. Many 2,4-D-degrading bacteria have been isolated both from 2,4-D-exposed (9, 26) and from nonexposed (pristine) (24, 27) soils. Of these, the best studied is the β-proteobacterium *Ralstonia eutropha* JMP134(pJP4), originally isolated from 2,4-D-exposed soil (9). The initial step in 2,4-D breakdown, as described for *R. eutropha* JMP134(pJP4), is mediated by an

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TABLE 1. Chemical properties of Sourhope soil[a]

Soil	pH[b]	Total organic carbon (mg g^{-1})[c]	Freundlich parameters for adsorption of 2,4-dichlorophenol[d]		
			K_f	n	K_{OC}
Nonplanted	4.03 (0.007)	24.61 (0.80)	5.75 (1.02)	0.83 (0.006)	233.6
L. perenne rhizosphere	4.02 (0.003)	21.03 (0.51)	5.60 (1.02)	0.82 (0.007)	266.3
T. pratense rhizosphere	4.04 (0.008)	24.52 (0.71)	5.47 (1.01)	0.83 (0.005)	223.1

[a] Soil was sampled from the rhizospheres of *L. perenne* and *T. pratense* after 60 days of plant growth. Values are means (standard errors), $n = 4$.
[b] Determined in $CaCl_2$ after removal of root debris.
[c] Determined by wet oxidation after removal of root debris.
[d] The concentration dependence of 2,4-dichlorophenol adsorption to *L. perenne*, *T. pratense* (with incorporated root debris), and nonplanted soil was investigated after 1 h (25°C) over triplicate initial aqueous concentrations of 0.2, 2, 10, 50, and 250 μg of [^{14}C]2,4-dichlorophenol ml^{-1}. Data are expressed as the linear form of the Freundlich equation ($r^2 > 0.99$ in all cases), $\log S = \log K_f + n \log A$, where S is the adsorbed concentration (in micrograms per gram), A is the aqueous concentration (in micrograms per milliliter), $\log K_f$ is the intercept, and n is the elasticity of sorption. K_{oc} is calculated as K_f/percent total organic carbon × 100.

α-ketoglutarate-dependent dioxygenanse, encoded by the *tfdA* gene, which cleaves the acetate side chain to produce 2,4-dichlorophenol (15, 16, 49). *tfdA* shows only low homology to one other previously characterized gene (*tauD* of *Escherichia coli* [53]). Other 2,4-D-degrading isolates are distributed among the α, β, and γ subdivisions of the class *Proteobacteria* (24, 27, 34). Isolates in the β- and γ-*Proteobacteria* possess *tfdA*-like genes with more than 76% sequence similarity to the canonical *tfdA* genes in *R. eutropha* JMP134(pJP4) (34). Until recently it was thought that isolates belonging to the α-*Proteobacteria* did not possess *tfdA*-like genes (27, 28, 34), and Kitagawa et al. (28) have characterized a novel gene cluster, *cadRABKC*, that is responsible for 2,4-D degradation in *Bradyrhizobium* sp. strain HW13 but shows no similarity to *tfdA* genes. *cadA* homologs have been detected in other bradyrhizobia and in α-proteobacterial degraders belonging to the genus *Sphingomonas* (28). However, more recent research has reported that slow-growing 2,4-D-degrading bacteria isolated from pristine soils and belonging to the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* (BANA) cluster within the α-*Proteobacteria* possess *tfdA*-like genes with approximately 60% sequence identity to the canonical *tfdA* gene (25).

The high diversity uncovered in *tfdA*-like genes suggests that the selection for genotypes may have been operating before the first use of 2,4-D in the early 1940s, and several workers have postulated the existence of an ancestral *tfdA* gene encoding a degradative enzyme with the highest affinity for a naturally occurring analog of 2,4-D (11, 17, 22, 25). Indeed, the protein encoded by the *tfdA*-like gene in BANA cluster isolates from pristine soil has a higher affinity for nonchlorinated phenoxyacids than for 2,4-D (25). The facts that pristine soils can mineralize 2,4-D (17) and that 2,4-D-degrading bacteria can be isolated from them (24, 27) suggest that *tfdA*-like genes are maintained for a function other than 2,4-D degradation. Dunning Hotopp and Hausinger (11) tested a range of alternative substrates for canonical TfdA and concluded that the original substrate may have been a plant-derived compound, such as a cinnamic acid derivative. Given the diversity of compounds produced in the rhizosphere, it is reasonable to hypothesize that other natural substrates for TfdA may be found in the rhizodeposits and that this may explain rhizosphere-enhanced mineralization of 2,4-D. In this study we monitored 2,4-D mineralization kinetics in pristine soils collected from the rhizospheres of *Lolium perenne* and *Trifolium pratense* and related them to the number of 2,4-D degraders and the diversity of *tfdA*-like genes. We present evidence to suggest that enhanced mineralization in the *T. pratense* rhizosphere is not due to selection of 2,4-D-degrading microorganisms in the rhizosphere by rhizodeposit analogs of 2,4-D but may be due to induction of the 2,4-D pathway by one or more components of the rhizodeposits.

MATERIALS AND METHODS

Soil. The brown forest soil used in this study was collected from the Macaulay Land Use Research Institute experimental site at Rigg Foot (Sourhope Research Station, Cheviots, Scotland). The Rigg Foot site is upland grassland, dominated by *Agrostis capillaris*, and has not been exposed to 2,4-D. Detailed vegetation and soil data can be obtained from the Natural Environmental Research Council (United Kingdom) Soil Biodiversity website (http://mwnta.nmw.ac.uk/soilbio/baseline98A.htm). Soil samples were collected from a depth of 10 to 40 cm, sealed in sterile polyethylene bags, and transported to the laboratory, where they were sieved (mesh size, <2.8 mm) and stored at 4°C until use. Some properties of the soil are shown in Table 1.

Experimental design. Plants were grown in boiling tubes containing field-moist (0.36 g of H_2O g of dry $soil^{-1}$; 62.3% maximum holding capacity) soil (equivalent dry weight, 12 g). Replicate tubes were planted with 20 seeds of either *L. perenne* or *T. pratense* (Herbiseed, Twyford, England) per tube. As controls, additional tubes were left nonplanted but were otherwise treated identically to the planted tubes. Tubes were closed with polyurethane foam bungs and incubated at 20°C with a light-dark cycle of 16 h of light (4,200 lx) and 8 h of dark. The water content was gravimetrically adjusted to the initial field value with sterile distilled water every 2 days. Toward the end of the time course, plants began to show signs of nutrient stress. Therefore, at 87 and 106 days after planting, tubes for each planting treatment received a solution of NH_4NO_3 and KH_2PO_4 as part of the 2-day watering to give 35, 25, and 30 μg of N, P and K, respectively, g of $soil^{-1}$. Additional tubes received no fertilizer. Four replicate tubes per plant treatment were destructively sampled 0, 25, and 60 days after planting, and three replicates were sampled for each plant × fertilizer treatment on day 116: shoots were excised and discarded, and the root was chopped finely with a sterile scalpel and homogenized with the soil (i.e., the entire below-soil-surface contents of the tube were defined as the rhizosphere). Plants within replicate treatments grew equally well (coefficient of variation for dry weight of shoots, <25%). Subsamples were taken for determination of 2,4-D mineralization potential and microbiological and molecular analysis.

Soil biochemical and microbiological analysis. Soil dehydrogenase activity was determined by the iodonitrotetrazolium chloride (INT; Sigma-Aldrich Co. Ltd., Gillingham, Dorset, United Kingdom) method (51, 55). An aqueous 0.2% (wt/vol) solution of INT was added to the soil sample (dry weight, 0.5 g). Following incubation in the dark (25°C, 48 h), the reaction was terminated by addition of 10 ml of *N,N*-dimethyl formamide–ethanol (1:1, vol/vol), soil was removed by centrifugation, and the absorbance of the supernatant was determined at 464 nm. The amount of INT formezan (INTF) produced was calculated by reference to an INTF calibration curve.

Soil subsamples (dry weight, 0.5 g) were used as the basis for a 10-fold dilution series for the determination of culturable bacterial numbers and most probable numbers of 2,4-D degraders ($MPN_{2,4\text{-}D}$). For culturable numbers, dilutions (50 μl) were spread onto nutrient agar (Oxoid), and plates were incubated at 25°C

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and counted after 72 h. For $MPN_{2,4\text{-}D}$, ignition tubes (five replicates for each dilution) containing 1.8 ml of basal medium supplemented with L-[U-^{14}C]2,4-D (0.050 g liter^{-1}; 28.7 kBq liter^{-1}; Sigma-Aldrich Co., Ltd.) were inoculated with dilutions (200 μl). The basal medium (modified from reference 10) was composed of the following (measured in grams per liter unless otherwise stated): Na_2HPO_4 (2.78), KH_2PO_4 (1.0), $CaNO_3 \cdot 4H_2O$ (0.05), $(NH_4)_2SO_4$ (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), Casamino Acids (0.005), and trace element solution (1 ml per liter) (27). To obtain 10^{-1} most-probable-number (MPN) dilutions, soil (0.2 g [dry weight basis]) was weighed directly into ignition tubes. After incubation (9 weeks, 20°C), the remaining radioactivity was quantified, and tubes containing ≤60% of that for the noninoculated controls were scored positive. $MPN_{2,4\text{-}D}$ estimates were derived by reference to MPN tables (1). A dilution series constructed from 25-day harvest soil that had previously been exposed to 2,4-D (50 μg g^{-1}) for 36 days was also used to inoculate MPN tubes.

[U-^{14}C]2,4-D and [U-^{14}C]2,4-dichlorophenol fate. [^{14}C]2,4-D mineralization was determined for soil harvested after 0, 25, 60, or 116 days of plant growth. [^{14}C]2,4-dichlorophenol mineralization was determined for the 25-day harvest sample only. In addition, [^{14}C]2,4-D mineralization kinetics were also recorded for nonplanted soil exposed previously to 2,4-D (50 μg g^{-1}; 36 days). Soil subsamples (4 g [dry weight basis]) in EPA vials (40 ml; gas-tight tetrafluoroethylene-silicone septa; Sigma-Aldrich Co., Ltd.) were amended with [U-^{14}C]2,4-D or [U-^{14}C]2,4-dichlorophenol (50 μg g^{-1}; 250 Bq g^{-1}) in a sufficient volume of sterile distilled water to bring the soil moisture content to 70% of the maximum moisture-holding capacity. A test tube (internal diameter, 75 by 9 mm) containing NaOH (1 ml; 1 M) was placed on the surface of the soil to trap the $^{14}CO_2$ released. Vials were incubated at 25°C. On sampling days, the NaOH solution was taken for quantification of radioactivity and replaced with fresh NaOH solution. At the end of the mineralization experiment (36 days), soil subsamples (0.5 g [wet weight]) were extracted with water (1 ml) by vortexing (30 s), the soil was removed by centrifugation (16,100 × *g*, 5 min), 800 μl of the supernatant was decanted, and 200 μl was used for ^{14}C determination. The water-extracted soil pellet was further extracted with methanol (1 ml, 30 s), 1 ml of the supernatant was decanted, and radioactivity in a 400-μl aliquot was determined. The remaining aqueous and methanolic supernatants were stored at −20°C for high-performance liquid chromatography (HPLC) analysis. The recovery efficiency of this sequential extraction method was tested by using soil freshly spiked with 2,4-D and was found to be 99.7% ± 0.5%.

The extracted soil pellet was dried (105°C), and levels of nonextractable residues were determined by CrO_3 oxidation as described by Dalal (7) by using NaOH (2 ml; 4 M) to trap the $^{14}CO_2$ evolved. In all cases, radioactivity was quantified by using a Beckman LS6000TA Liquid Scintillation system programmed to count each sample for 5 min. UltimaGold (Packard Bioscience, Groningen, The Netherlands) was used as the scintillant in a 1:4 (for aqueous extract and 1 M NaOH trap samples) or 1:10 (for methanolic samples) sample-to-cocktail ratio. HionicFluor (Packard) was used as the scintillant for 4 M NaOH traps (sample-to-cocktail ratio, 1:12).

HPLC analysis of extracts was performed as described previously (44).

DNA template isolation. DNA was extracted from soil exactly according to the method of Griffiths et al. (19) except that bead beating was performed using white quartz sand (0.2 g) mixed with glass beads (0.3 g; 425 to 600 U) (both from Sigma-Aldrich Co. Ltd.) as the matrix and a mini-beadbeater (Biospec Products) set to beat for 30 s at 2,500 rpm.

PCR amplification and cloning. Two primer sets were used: those designed by Vallaeys et al. (52) by sequence alignment of the *tfdA* genes from *R. eutropha* JMP134(pJP4) and *Burkholderia* sp. strain RASC and those (*tfd*Aα1 [5′-CCGG CGTCGATCTGCGCAAG-3′] and *tfd*Aα2 [5′-GTTGACGACGCGCGCCGA CA-3′]) designed to amplify a 359-bp region of the *tfdA*-like gene (encoding 2,4-D α-ketoglutarate-dependent dioxygenase) recently characterized in α-*Proteobacteria* (25) by alignment of complete *tfdA*-like sequences from strains RDS-C2 (GenBank accession number AB074490), HWK12 (AB074491), and HW13 (AB074492). Reaction mixtures contained 1× PCR buffer, 2.5 mM $MgCl_2$, 1× Q solution, 1 U of HotStarTaq DNA polymerase (QIAGEN, Crawley, United Kingdom), deoxynucleoside triphosphate solution (200 μM [each] dATP, dCTP, dGTP, and dTTP), forward and reverse primers (0.4 μM each), 0.5 μl of template DNA, and sterile distilled water up to 25 μl. Reaction mixtures were incubated at 95°C for 15 min to activate the HotStarTaq prior to the start of the temperature program. For the *tfd*Aα primer set, the temperature program was 40 cycles of denaturation (1 min at 94°C), annealing (45 s at 67°C), extension (1 min at 72°C), and final extension (10 min at 72°C). For the Vallaeys et al. (52) primer set, we used a touchdown program: 23 cycles of denaturation (1 min at 94°C), annealing (1 min at 65°C, decreasing by 1°C cycle^{-1} to 42°C), and extension (1.5 min at 72°C), followed by 17 cycles of denaturation (1 min at 94°C), annealing (1 min at 42°C), extension (1.5 min at 72°C), and final extension (10 min at 72°C). The ability of *tfd*Aα1 and -2 to amplify *tfdA*-like gene fragments from soil was confirmed by sequencing. Putative partial *tfdA*-like fragments obtained by PCR amplification of DNA extracted from nonplanted soil before and after 2,4-D treatment (Fig. 5) were ligated into the pGEM-T Easy vector (Promega, Southampton, United Kingdom) and transformed into *E. coli* JM109 competent cells (Promega), according to the manufacturer's instructions. White colonies were checked for inserts by PCR using primers *tfd*Aα1 and -2. Plasmid DNA extracted (QIAprep Spin Miniprep kit; QIAGEN) from six unique (as determined by single-strand conformational polymorphism [SSCP]) randomly selected clones was used as the basis for sequencing (Comfort Read; MWG Biotech, Ebersberg, Germany) using M13 primers.

Analysis of cloned *tfdA*-like sequences. Cloned *tfdA*-like sequences were compared with selected reference *tfdA*-like sequences in the GenBank database. Partial cloned sequences and full reference sequences were aligned by using ClustalX (version 1.81), and reference sequences were trimmed so that only contiguous nucleotides were used for subsequent analysis. A phylogenetic tree was constructed by using the neighbor-joining method and Jukes-Cantor distances (Phylip, version 3.6a3). Bootstrap analysis with 100 replicates was used to place confidence estimates on the tree. *E. coli tauD* (encoding taurine/α-ketoglutarate dioxygenase [53]) was used as an outgroup.

SSCP analysis. The SSCP analysis protocol was based on the method described by Schwieger and Tebbe (41). Prior to electrophoretic analysis, aliquots of PCR products were mixed with denaturing loading buffer (95% formamide, 10 mM NaOH, 0.25% [wt/vol] bromophenol blue, 0.25% [wt/vol] xylene cyanol [41]) in a 1:5 sample-to-buffer ratio. Samples were denatured (95°C for 3 min) and then immediately cooled on ice water. Denatured samples (4 μl) were loaded onto a 0.6X SequaGel MD (National Diagnostics, Hull, United Kingdom) gel (40 by 33 cm with 0.4-mm spacers) and run at 600 V and 21 ± 2°C for 20 h by using a Hoefer SQ3 sequencing tank and an EPS601 (Amersham Pharmacia Biotech) power pack. The gel was silver stained according to the Promega Silver Sequence DNA sequencing system (39). The SSCP gels were composed of sample lanes with several conformer bands per lane. Band positions were assigned and scored either 1, for present, or 0, for absent. The resulting binary matrix was used as the basis for cluster analysis using the matching binary similarity coefficient and single linkage method (Intercooled Stata 7 for Windows; Stata Corporation, College Station, Tex.).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AY193866 to AY193870.

RESULTS

Culturable numbers and dehydrogenase activity. In soil harvested after only 25 days of plant growth, plant treatment had a significant effect ($P = 0.006$ by analysis of variance [ANOVA]) on culturable numbers (Fig. 1A). The effect was most pronounced in the *T. pratense* rhizosphere (CFU were 4.0×10^7, 1.6×10^7, and 5.8×10^6 g^{-1} for *T. pratense*, *L. perenne*, and nonplanted soils, respectively). The *T. pratense* rhizosphere also showed significantly increased dehydrogenase activity on day 25 ($P = 0.017$ by ANOVA) and day 60 ($P = 0.013$ by ANOVA) (Fig. 1B). Results for both culturable number and dehydrogenase activity followed the same treatment order (from highest to lowest, *T. pratense*, *L. perenne*, and nonplanted soils).

$MPN_{2,4\text{-}D}$. Before the pristine soil was exposed to 2,4-D, $MPN_{2,4\text{-}D}$ were low (<100 g^{-1}; below detectable limits in some replicates) for material harvested either from nonplanted soil or from *L. perenne*- or *T. pratense*-planted soil (Fig. 2A). In contrast to culturable numbers or dehydrogenase activity (Fig. 1), $MPN_{2,4\text{-}D}$ were not related to planting treatment ($P = 0.063$ by the Kruskal-Wallis test) or harvest day ($P = 0.33$ by the Kruskal-Wallis test) (Fig. 2A). However, soil sampled from 25-day-old rhizospheres or nonplanted controls and incubated in the presence of 2,4-D (50 μg g^{-1}, 36 days) had significantly ($P < 0.001$ by the Kruskal-Wallis test) higher $MPN_{2,4\text{-}D}$ (Fig. 2B) than non-2,4-D-exposed soil (Fig. 2A). In 2,4-D-exposed

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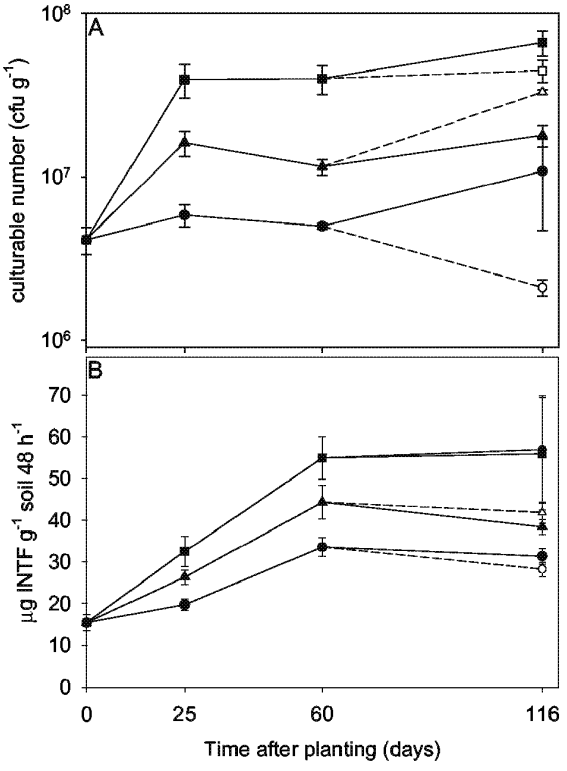


FIG. 1. Effects of plant species and plant age on the number of culturable bacteria (A) and dehydrogenase activity (B) in soil sampled from the rhizosphere of *L. perenne* (▲) or *T. pratense* (■) or in nonplanted controls (●). Soils were fertilized on days 87 and 106 with NH_4NO_3 and KH_2PO_4 to give 35 μg of N g^{-1}, 25 μg of P g^{-1}, and 30 μg of K g^{-1}. Open symbols, fertilized treatments. Error bars, standard errors.

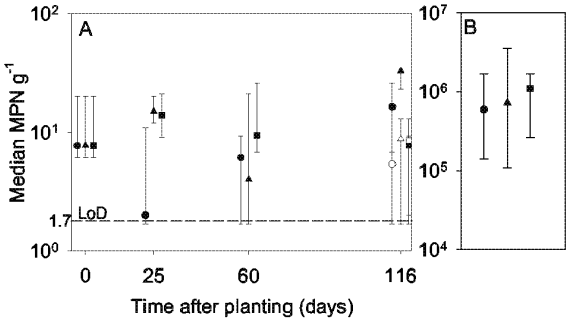


FIG. 2. (A) Effects of plant species and plant age on $MPN_{2,4\text{-}D}$ in pristine soil sampled from the rhizosphere of *L. perenne* (▲) or *T. pratense* (■) or from nonplanted controls (●). Soils were fertilized on days 87 and 106 with NH_4NO_3 and KH_2PO_4 to give 35 μg of N g^{-1}, 25 μg of P g^{-1}, and 30 μg of K g^{-1}. Open symbols, fertilized treatments. (B) $MPN_{2,4\text{-}D}$ in soil sampled from 25-day-old rhizospheres or nonplanted controls and incubated in the presence of 50 μg of 2,4-D g^{-1} for 36 days. Error bars, maximum and minimum values. LoD, limit of detection.

soil, median $MPN_{2,4\text{-}D}$ ranged from 6×10^5 g^{-1} (nonplanted) to 1×10^6 g^{-1} (*T. pratense*), although plant treatment did not have a significant effect ($P = 0.91$ by the Kruskal-Wallis test).

2,4-D and 2,4-dichlorophenol mineralization and 2,4-D ^{14}C mass balance. Cumulative $^{14}CO_2$ evolution from nonplanted, *L. perenne*-planted, or *T. pratense*-planted soil amended either with [U-^{14}C]2,4-D over the entire time course (0 to 116 days) (Fig. 3A to E) or with [U-^{14}C]2,4-dichlorophenol at the day-25 harvest (Fig. 3B) was determined. Mineralization of 2,4-D by nonplanted soil previously exposed to 2,4-D (50 μg g^{-1}, 36 days) is shown for comparison (Fig. 3A). To enable a quantitative comparison of mineralization kinetics, curves were fitted to the function $Y = a[1 + (t/t_0)^b]^{-1}$, and parameters (asymptote [a], time at which the maximum rate is reached [t_1], and maximum rate) were compared for each sampling time and treatment (Fig. 4). The t_1 and rate parameters were calculated from the function as described previously (42). Generally, cumulative mineralization curves were sigmoidal, except for that of 2,4-D in fertilized nonplanted soil (day 116 [Fig. 3E]), where mineralization was minimal (<2.5% mineralized in 36 days). For soils not previously exposed to 2,4-D, 2,4-D mineralization curves were characterized by a lag phase prior to the onset of exponential $^{14}CO_2$ evolution, during which time mineralization occurred at a low and linear rate (~0.2% day^{-1}). This is in contrast to results for soil preexposed to 2,4-D (containing an enriched 2,4-D-degrading population of 6×10^5 MPN g^{-1}) (Fig. 2B), where exponential $^{14}CO_2$ evolution was immediate (Fig. 3A). Nevertheless, independently of whether the soil had previously been exposed to 2,4-D, the percent $^{14}CO_2$ evolution always approached an asymptote of ~70 (Fig. 3 and 4A). The mineralization asymptote was also independent of planting treatment (Fig. 3 and 4A). However, there was a pronounced reduction in the length of the lag phase for *T. pratense*-planted versus *L. perenne*-planted or nonplanted soil, particularly on days 25 and 60 (Fig. 3B and C). The reduction in the lag phase length is reflected by the parameter t_1 (expressed in days). For example, t_1 is 22.0 ± 0.6 or 15.3 ± 0.7 days for day-60-harvested nonplanted or *T. pratense*-planted soil, respectively (Fig. 4B). The rhizosphere of *T. pratense* also had a significant ($P < 0.05$ by ANOVA) effect on the maximum rate of 2,4-D mineralization on harvest days 25 and 60 (e.g., for day 25, the maximum rate for the *T. pratense* rhizosphere was 10.0% day^{-1} while that for nonplanted soil was 7.6% day^{-1} [Fig. 4C]). By day 116, the *T. pratense* rhizosphere effect persisted only in fertilized treatments (Fig. 4B and C).

In contrast to that of 2,4-D, 2,4-dichlorophenol mineralization (Fig. 3B) proceeded after a shorter lag phase (t_1, 5.8 to 6.4 days [Fig. 4B]), but there was no significant effect ($P > 0.05$ by ANOVA) of either *L. perenne* or *T. pratense* planting on any of the parameters describing mineralization (Fig. 4D to F).

Quantification of the fate of the 2,4-D ^{14}C (as $^{14}CO_2$, water-extractable ^{14}C, methanol-extractable ^{14}C, and nonextractable residues released by wet oxidation) at the end of the experiment revealed, except for the fertilized nonplanted soil, no significant difference ($P > 0.1$) in the partitioning of the ^{14}C among the four fractions tested (data not shown). Less than 3% of the ^{14}C could be water or methanol extracted, and the remainder of the ^{14}C (15 to 27%) could be recovered only by wet oxidation. Thus, it is likely that the 2,4-D was completely degraded and that the remaining nonextractable ^{14}C fraction was associated with the soil organic matter and microbial biomass. For nonplanted fertilized soils (day 116), where mineralization was inhibited, the ^{14}C could be accounted for as

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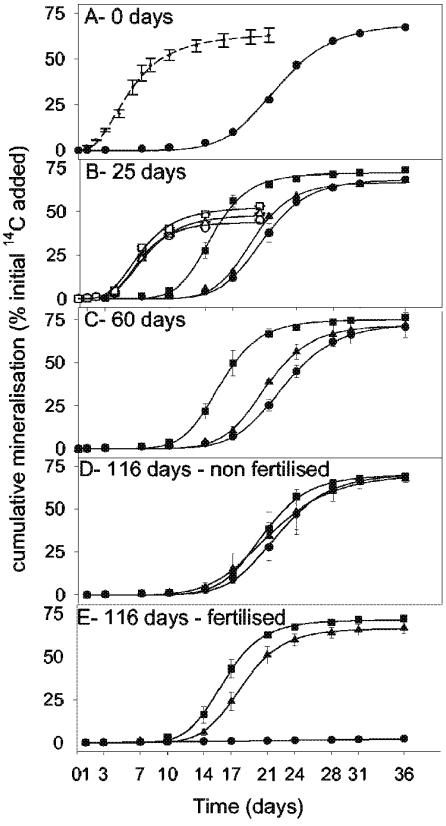


FIG. 3. Effects of plant species, plant age at harvest (0, 25, 60, or 116 days old), and NPK fertilization on mineralization of 2,4-D (solid symbols) or 2,4-dichlorophenol (open symbols) at 50 μg g^{-1} in soil sampled from the rhizosphere of *L. perenne* (▲), the *T. pratense* rhizosphere (■), or nonplanted controls (●). Dashed line in panel A shows mineralization of 2,4-D in nonplanted soil previously exposed to 50 μg of 2,4-D g^{-1} for 36 days. Soils were fertilized on days 87 and 106 with NH_4NO_3 and KH_2PO_4 to give 35 μg of N g^{-1}, 25 μg of P g^{-1}, and 30 μg of K g^{-1}. Error bars, standard errors.

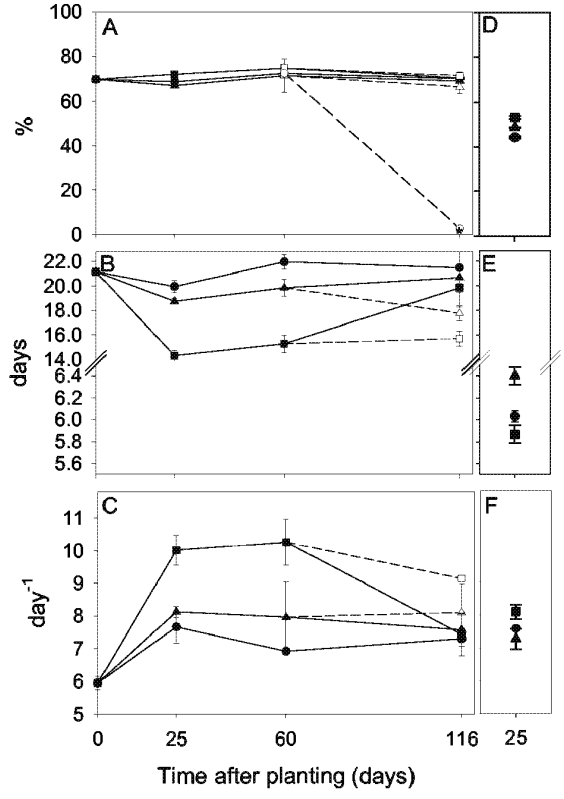


FIG. 4. Effects of plant species and plant age on 2,4-D (A to C) and 2,4-dichlorophenol (D to F) mineralization parameters in soil sampled from the rhizosphere of *L. perenne* (▲), *T. pratense* (■), or nonplanted controls (●). Effects are expressed as asymptotic percentage mineralized (A and D), time point at which the maximum mineralization rate is reached (t_1) (B and E), or calculated maximum rate (C and F). Soils were fertilized on days 87 and 106 with NH_4NO_3 and KH_2PO_4 to give 35 μg of N g^{-1}, 25 μg of P g^{-1}, and 30 μg of K g^{-1}. Open symbols represent fertilized treatments. Error bars, standard errors.

follows: water extractable, 36.7% ± 2%; methanol extractable, 20.2% ± 2%; nonextractable but released by wet oxidation, 50.2% ± 2%. Subsequent HPLC analysis revealed that the water- and methanol-extractable ^{14}C was 2,4-D associated. The reason for the inhibition of 2,4-D transformation and mineralization by NPK fertilization in nonplanted soil is not known, although we speculate that addition of relatively high concentrations of inorganic salts caused osmotic stress. In planted soil, NPK did not have the same detrimental effect, presumably because the plants took up a significant proportion of the fertilizer.

PCR-SSCP analysis of diversity of *tfdA*-like genes. For PCR of DNA extracted from soil, we initially used primers designed by sequence alignment of *tfdA* genes from the β-proteobacterial degraders *R. eutropha* JMP134(pJP4) and *Burkholderia* sp. strain RASC (52). However, we were not able to obtain any PCR products with these primers, even for soil samples that had previously been exposed to 2,4-D (50 μg g^{-1}, 36 days) and, according to the $MPN_{2,4\text{-}D}$ analysis, contained approximately 10^6 degraders g^{-1} (Fig. 2B). In preliminary experiments using template DNA extracted from soil inoculated with a 10-fold dilution series of *Burkholderia* sp. strain RASC (50), we determined that the detection limit for the PCR was approximately 10^4 *tfdA* copies g^{-1} of soil (data not shown). Therefore, if the 2,4-D-degrading population present in the soil possessed the β- and γ-proteobacterial *tfdA*-like gene, we would expect to be able to obtain a PCR product in the 2,4-D-exposed soil. Even when we used DNA extracted from positive $MPN_{2,4\text{-}D}$ tubes as a template, we did not achieve amplification. However, PCR of DNA extracted from soil using the primers (*tfd*Aα1 and -2) designed to target *tfdA*-like genes from α-*Proteobacteria* yielded faint PCR products of the expected size (359 bp) from soil not previously exposed to 2,4-D and more-distinct products from soil exposed to 2,4-D (50 μg g^{-1}) for 36 days (Fig. 5A). Furthermore, DNA was extracted from the highest-dilution (10^{-5} and 10^{-6}) positive $MPN_{2,4\text{-}D}$ tubes set up with 2,4-D-exposed soil sampled at the end of the mineralization experiments. This DNA was used as a basis for PCR with the *tfd*Aα primers; of the 26 samples tested, 22 yielded a product of the correct size, suggesting that the α-proteobacterial *tfdA*-like gene was numerically important in soil exposed to 2,4-D.

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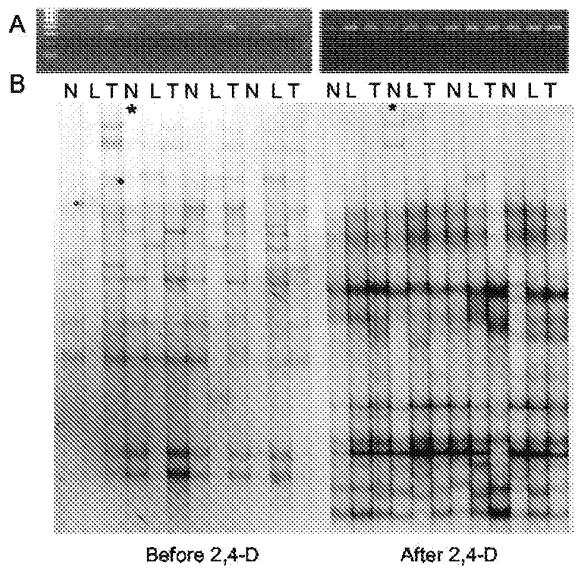


FIG. 5. PCR amplification using *tfd*Aα primers (A) and subsequent SSCP analysis (B) of *tfdA*-like genes in Sourhope soil before and after 2,4-D treatment. Each lane contains a PCR product obtained from DNA extracted from an independent, destructively sampled soil sample that either was not planted (N) or was planted with *L. perenne* (L) or *T. pratense* (T) and was harvested after 25 days. Asterisked lanes contain putative partial *tfdA*-like fragments that were cloned subsequently; clones were sequenced to check primer fidelity.

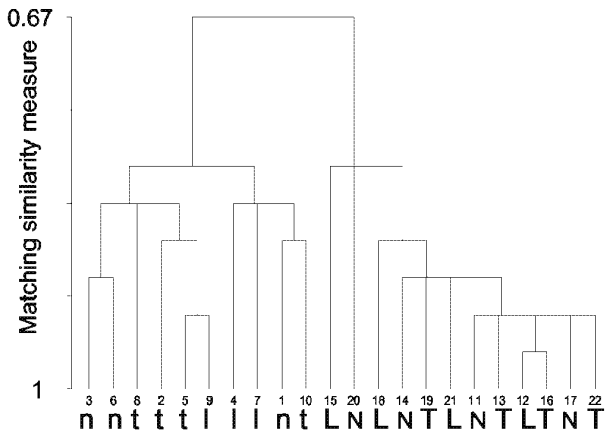


FIG. 7. Hierarchical cluster analysis (using matching binary similarity distances and the single linkage method) of the *tfdA*-like SSCP banding patterns shown in Fig. 5B. N, L, and T, replicate sample lanes from nonplanted, *L. perenne*-planted, and *T. pratense*-planted soils, respectively. Lowercase letters, non-2,4-D-exposed samples; capital letters, 2,4-D-exposed samples.

To check primer fidelity, putative soil *tfdA*-like PCR products were cloned and six clones were sequenced. Phylogenetic comparisons with reference strain *tfdA* genes (representatives of β- and α-proteobacterial degraders) placed the clones on the α-proteobacterial branch of the *tfdA* tree (Fig. 6). The partial *tfdA*-like DNA sequences cloned from soil had 83.2 to 99.4% identity to the common partial *tfdA*-like sequence of the α-proteobacterial strain RD5-C2 (25) (GenBank accession number AB074490).

SSCP analysis of the resulting PCR products revealed differences in the position and abundance of *tfdA*-like conformer bands between non-2,4-D-exposed and 2,4-D-exposed soils which were discernible by eye (Fig. 5B). Indeed, hierarchical cluster analysis of SSCP band patterns (Fig. 7) confirmed that the samples clustered according to 2,4-D exposure history but that plant treatment had no effect on the diversity of *tfdA*-like genes either in non-2,4-D-exposed or in 2,4-D-exposed soils.

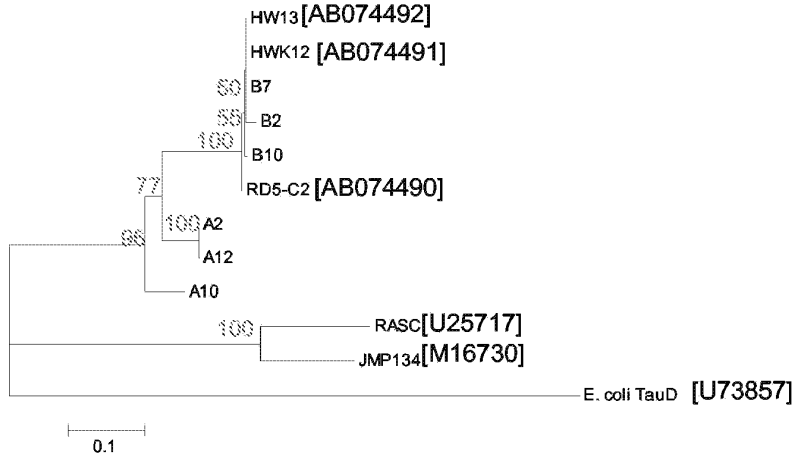


FIG. 6. Phylogenetic positions of *tfdA* sequences cloned from Sourhope soil among *tfdA* sequences from *R. eutropha* JMP134(pJP4), *Burkholderia* sp. strain RASC, and reference strains HW12, HW13, and RD2-C5. Strains HW12, HW13, and RD2-C5 are members of the BANA cluster of the α-*Proteobacteria* most closely related to *Bradyrhizobium* spp. *E. coli tauD* encodes taurine/α-ketoglutarate dioxygenase. The neighbor-joining dendrogram (Jukes-Cantor distances) was constructed from reference sequences and common partial sequences (357 bp) of *tfdA* PCR amplified by using primers *tfd*Aα1 and *tfd*Aα2. Soil clone designations begin with "B" (for "before 2,4-D application") or "A" (for "after 2,4-D application"). Bootstrap confidence limits (percentages) are given at each branch. Scale bar represents a Jukes-Cantor distance of 0.1. GenBank accession numbers are given in brackets after strain designations.

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DISCUSSION

After only 25 days of plant growth, culturable microbial numbers and activity were increased in soil planted with *T. pratense* or *L. perenne*. This is known as the "rhizosphere effect," documented by many authors who report the 2- to 10-fold increases in microbial number and activity in planted soil over nonplanted soil seen in our present study (4, 43). The rhizosphere effect was more pronounced in the *T. pratense* than in the *L. perenne* rhizosphere despite the fact that *L. perenne* produced a root system that was more extensive and had greater biomass than that of *T. pratense* (data not shown). The reason for the difference between the microbial properties of *L. perenne*- and *T. pratense*-planted soils is unknown. We were not able to detect any effect of the rhizospheres on the organic carbon content of the soil (Table 1). However, it can be supposed that *T. pratense* produced exudates either in greater quantity (although this was not detected by the wet oxidation organic carbon method) or of superior quality to those of *L. perenne*, or both.

Aside from the rhizodeposit-mediated impact on gross microbial parameters, such as culturable heterotrophs or activity of a global respiratory enzyme (dehydrogenase), our main aim was to dissect the impact of plant roots on 2,4-D mineralization and numbers and diversity of 2,4-D degraders. Our hypothesis was that plant roots deposit compounds to the rhizosphere that are natural analogs of the 2,4-D pathway and enhance 2,4-D biodegradation by serving as either (i) a substrate for 2,4-D catabolic enzymes, (ii) a cooxidized substrate during cometabolism, or (iii) an effector molecule in the induction the pathway. The results of the mineralization assays show that there was indeed an effect of the rhizosphere on 2,4-D biodegradation but that the effect was dependent on plant species (*T. pratense*) and plant age (for nonfertilized soil, the effect was confined to younger [<60-day] plants).

If enhanced 2,4-D mineralization was due to the presence of rhizodeposit 2,4-D analogs that could be broken down by Tfd enzymes and used as a source of carbon for growth, then we would expect *T. pratense* to have an impact on the numbers and diversity of 2,4-D degraders. Although activities and culturable numbers increased in the rhizosphere, $MPN_{2,4\text{-}D}$ in the pristine soil were low (<100 g^{-1}) and were not affected by plant treatment. In contrast, soil previously exposed to 2,4-D (50 $\mu g\ g^{-1}$) gave $MPN_{2,4\text{-}D}$ estimates of $\sim 1 \times 10^6\ g^{-1}$, showing that the $MPN_{2,4\text{-}D}$ method used was effective at detecting increases in 2,4-D degrader numbers in response to a selective pressure. Other workers (8) have also shown that MPN assays can sensitively detect the enrichment of 2,4-D degraders in aquifer sediments exposed to phenoxy acid herbicides. If rhizodeposition was selectively enriching the pristine soil, then the rhizosphere-to-nonrhizosphere ratio (R:S) for 2,4-D degraders would exceed that for the total heterotrophs. In an early study, Sandmann and Loos (40) used an MPN method to determine R:S values for 2,4-D degraders in the rhizospheres of sugarcane and African clover. They found that rhizosphere soil sampled from sugarcane, with no previous exposure to phenoxyacetic acid herbicides, had an enriched 2,4-D degrading population ($R:S_{2,4\text{-}D}$ = 105; $R:S_{heterotroph}$ = 6). Similarly, Fang et al. (12) reported an enrichment of phenanthrene degraders in soil planted with various grass species. The results of those studies are in contrast to our findings, since we calculate an $R:S_{2,4\text{-}D}$ of 4.27 and a $R:S_{heterotroph}$ of 7.44 based on day-25 and -60 mean (heterotroph) or median ($MPN_{2,4\text{-}D}$) values for *T. pratense*-planted and nonplanted soils.

The soil used for our experiments was collected from an upland grassland with no history of 2,4-D application. The field site was remote from areas used for arable agriculture and therefore could not have received 2,4-D inputs from short-range spray drift. Since 2,4-D is nonvolatile and hydrophilic, it is unlikely to undergo long-range atmospheric transport and global distillation such as has been reported for other more volatile and hydrophobic xenobiotics (58). Thus, the fact that that this pristine soil had intrinsic 2,4-D mineralization ability (an observation common to other pristine soils [17]) is consistent with the idea that naturally occurring analogs of 2,4-D do exist, can be used directly as substrates for growth (17), and are responsible for the maintenance of the low numbers of 2,4-D-mineralizing microorganisms recorded here. However, the MPN evidence suggests that the putative analog was not an exclusive property of rhizosphere soil and therefore did not result directly from rhizodeposition.

Evidence from PCR analysis of DNA extracted from soil and positive $MPN_{2,4\text{-}D}$ tubes with two different primer sets (that targeting the β- and γ-proteobacterial *tfdA* and that targeting α-proteobacterial *tfdA*) suggests that 2,4-D degraders possessing the α-proteobacterial *tfdA*-like genes were present and numerically important in Sourhope soil. As indicated earlier, *tfdA*-like genes have been characterized only recently (25) in oligotrophic α-proteobacterial 2,4-D degraders isolated from pristine soils of Hawaii, Saskatchewan, and Japan (25, 27). 2,4-D mineralizers have proved difficult to isolate from pristine soils (17, 27), probably because of their slow-growing habits and sensitivity to high concentrations of organic nutrients (27). Indeed, despite many attempts, we were not able to resolve 2,4-D mineralizers from active enrichment cultures. However, using culture-independent PCR and subsequent SSCP analysis of *tfdA*-like PCR products, we were able to demonstrate the presence and also the diversity of *tfdA*-like genes in a pristine Scottish soil, adding further weight to the claim of Kamagata et al. (27) that this new class of 2,4-D degraders is widespread in nature.

We used SSCP analysis to examine the effect of the rhizosphere on the sequence diversity of *tfdA*-like genes. In our analysis, we also included soil from the three plant treatments that had previously been exposed to 2,4-D. We found that prior 2,4-D exposure had a pronounced effect on the diversity of 2,4-D-degradative genes, thus demonstrating the ability of the method to detect the impact of a selective pressure on the 2,4-D-degrading population. However, no plant treatment effect on diversity was detectable either in nonexposed or in 2,4-D-exposed soils. Taken together, the $MPN_{2,4\text{-}D}$ and PCR-SSCP evidence suggests that the rhizosphere does not exert a selective pressure on 2,4-D-degrading microorganisms. Rather the 2,4-D degraders present in Sourhope soil form a near-constant proportion of the soil community (both in numbers and in composition) and, in the absence of 2,4-D, are oblivious to the presence of a plant root. When 2,4-D is added to the soil, $MPN_{2,4\text{-}D}$ and PCR-SSCP evidence suggests that a subpopulation of 2,4-D degraders initially present in soil utilizes 2,4-D for growth; however, the composition of this population

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of 2,4-D degraders also is not affected by plant treatment. In other words, the same subpopulation is degrading 2,4-D in the three treatments, but it is able to mineralize with a shorter lag time and at a higher rate in soil previously planted with *T. pratense*.

If the plant root (at least in the plant-soil system used in the present study) does not exert a direct selective pressure as a growth substrate on either the number or the diversity of 2,4-D degraders, an alternative explanation for the enhanced mineralization of 2,4-D in soil harvested from the *T. pratense* rhizosphere is required. Alternative explanations (alluded to earlier) include the involvement of an analog as an inducer or cometabolite, rhizodeposit-mediated promotion of the growth of 2,4-D degraders, or the operation of less-specific mechanisms such as rhizosphere-enhanced horizontal transfer of 2,4-D catabolic genes or the action of phytosurfactants to increase the concentration and bioavailability of 2,4-D in the soil aqueous phase. We think that some of these explanations are unlikely. First, the exponential kinetics of 2,4-D mineralization and the recorded increase in $MPN_{2,4\text{-}D}$ point toward a growth-linked, not a cometabolic process. Second, mating experiments with α-proteobacterial 2,4-D-degrading strains (HW13, HWK12, HWK13, and BTH) have shown that the *tfdA*-like gene is not transmissible (27). Third, there was no evidence of a bioavailability effect, as we could detect no difference in the parameters describing the partitioning of 2,4-dichlorophenol between the soil solid and aqueous phases (Table 1).

Therefore, one explanation not discounted relates to rhizodeposit-mediated promotion of the growth of 2,4-D degraders. For example, since it has been shown that TfdA has affinity for nonchlorinated substrates (11, 25), it may be that, once induced by the presence of 2,4-D, TfdA (or other 2,4-D pathway enzymes) was able to accept *Trifolium* rhizodeposits as substrates, thereby enhancing the growth rate of 2,4-D degraders. However, the explanation we favor is that a component of the rhizodeposits is able to act as an inducer of the *tfd* pathway. The regulation of the pathway in the newly discovered *tfdA*-like-gene-possessing α-proteobacterial degraders has not yet been characterized. Until we know more, we can only discuss the possibility of a rhizodeposit inducer in the context of our knowledge of the canonical *tfd* pathway in β-*Proteobacteria*. We know this pathway to be regulated by a LysR-type transcriptional regulatory protein encoded by *tfdR* (and, in JMP134, the identical *tfdS* gene) (31, 33, 54). The inducing effector molecule of the pathway is thought to be the intermediate 2,4-dichloromuconate (14). Presumably, on initial exposure to 2,4-D, low-level constitutive expression of the *tfd* pathway results in intracellular accumulation of the effector molecule and full induction of the pathway. In the present study, the low linear rate of $^{14}CO_2$ evolution during the lag phase and prior to the onset of exponential mineralization (Fig. 3) would be consistent with this process. It is reasonable to hypothesize that muconate derivatives will be present in the rhizosphere as a result of microbial metabolism of aromatic rhizodeposits. Leigh et al. (30) have shown that a variety of phenolic compounds are produced in the rhizosphere, particularly during the decay of dead roots. *cis,cis*-Muconate is the product of catechol metabolism via the central *ortho*-cleavage pathway (37), and many substituted phenols and aromatic acids (produced by rhizodeposition) could be degraded through this route, thus producing nonchlorinated *cis,cis*-muconates (21). Ogawa et al. (36) have reported that the LysR-type regulator of chlorocatechol degradation in *R. eutropha* can be activated by both chlorinated and nonchlorinated muconate (36). Thus, we consider it likely that an analog of 2,4-dichloromuconate produced in the *T. pratense* rhizosphere may activate transcription of the *tfd* genes by interaction with the regulatory TfdR-like protein.

2,4-D mineralization kinetics were both plant species and plant age specific. Boyle and Shann (3) also reported a specific plant effect on 2,4-D biodegradation: mineralization was enhanced to a greater extent in soil collected from a monocotyledon (e.g., *L. perenne*) rhizosphere than in soil from a dicotyledon (e.g., *T. pratense*) rhizosphere. This is the inverse of the findings of our present study. Because Boyle and Shann (3) did not determine the numbers or diversity of 2,4-D degraders in their study, it is difficult to explain the discrepancy in the results. However, in agreement with our work, Boyle and Shann found that the rhizosphere effect was specific for the first (*tfdA*-encoded) step of 2,4-D degradation, as there was no effect of either the monocot or dicot rhizosphere on the mineralization of the first metabolite of 2,4-D breakdown, 2,4-dichlorophenol. The quality and quantity of rhizodeposition are known to differ between plant species and also to be dependent on the growth stage of the plant (18). Thus, the rhizosphere effect of *T. pratense* may be due not solely to root death, as suggested by Leigh et al. (30), but also to more actively extruded components of the rhizodeposits. In order to narrow down the identity of the active compound(s) in the *T. pratense* rhizodeposits, we are currently conducting experiments to identify in which fraction of the rhizodeposits it predominates (exudates versus root structural compounds) and to examine what edaphic factors (e.g., pH, nitrogen content) control its production.

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ORIGINAL PAPER

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Substrate specificity of chlorophenoxyalkanoic acid-degrading bacteria is not dependent upon phylogenetically related *tfdA* gene types

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Abstract The phenoxyalkanoic acid herbicides constitute a group of chemically related molecules that have been widely used for over 50 years. A range of bacteria have been selected from various locations for their ability to degrade these compounds. Previously reported strains able to utilise 2,4-dichlorophenoxyacetic acid (2,4-D) include, *Ralstonia eutropha* JMP134, *Burkholderia* sp. RASC and *Variovorax paradoxus* TV1 and *Sphingomonas* sp. AW5 able to utilise 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). In addition a novel set of mecoprop-degrading strains including *Alcaligenes denitrificans*, *Alcaligenes* sp. CS1 and *Ralstonia* sp. CS2 are here described. It has been reported recently that TfdA enzymes, initially reported to have a role in 2,4-D catabolism are also involved in the first-step cleavage of related phenoxyalkanoate herbicides. However, a diversity of *tfdA* gene sequences have been reported. We relate the *tfdA* gene type to the metabolic ability of these strains. The *tfdA*-like genes were investigated by polymerase chain reaction amplification using a set of specific *tfdA* primers. Degradation ability was observed via phenol production from a range of unsubstituted and substituted phenoxyalkanoics including, 2,4-D, 2-methyl 4-chlorophenoxyacetic acid (MCPA), racemic mecoprop, (*R*)-mecoprop, 2-(2,4-dichlorophenoxy) propionic acid (racemic 2,4-DP), 2,4,5-T, 2,4-dichlorophenoxybutyric acid (2,4-DB), 4-chloro-2-methylphenoxybutyric acid (MCPB) and phenoxyacetate. Mecoprop-degrading strains showed partial *tfdA* sequences identical to the one described for *V. paradoxus* TV1 (a strain isolated on 2,4-D). However, substrate specificity was not identical as *V. paradoxus* exhibited greatest activity towards 2,4-D and MCPA only, whereas the mecoprop-degrading strains showed intense activity towards 2,4-D, MCPA, racemic mecoprop and (*R*)-mecoprop as substrates. However, *Sphingomonas* sp. AW5 which has been shown to carry a very different *tfdA*-like gene was the only strain to utilise the phenoxybutyric acid MCPB as a sole carbon source. In this study, we thus demonstrate that sequence diversity is not related to substrate specificity within the *tfdA*-like gene family. However, phylogenetically unrelated sequences may govern substrate specific activity.

Keywords Mecoprop · 2,4-Dichlorophenoxyacetic acid · *tfdA* · Biodegradation · α-Ketoglutarate-dependent dioxygenase

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Introduction

The structurally related herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl 4-chlorophenoxyacetic acid (MCPA), 2-(2-methyl 4-chlorophenoxy) propionic acid (mecoprop), 2-(2,4-dichlorophenoxy) propionic acid (2,4-DP), 2,4-dichlorophenoxybutyric acid (2,4-DB), 4-chloro-2-methylphenoxybutyric acid (MCPB) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have been widely applied to cereal crops to control dicotyledonous weeds worldwide. The microbial metabolism of 2,4-D is well understood and is a paradigm for the degradation of other chloroaromatic compounds (Häggblom 1992). The first 2,4-D degradation pathway was established in an *Arthrobacter* species and subsequently demonstrated in numerous 2,4-D-degrading strains (Bollag et al. 1968; Evans et al. 1971; Gaunt and Evans 1971).

The catabolism of 2,4-D by *Ralstonia eutropha* JMP134 (formerly *Alcaligenes eutrophus* JMP134) involves a number of steps including side chain removal, hydroxylation of the resulting 2,4-dichlorophenol (2,4-DCP), opening of the 3,5-dichlorocatechol ring and then conversion of 2,4-dichloro-*cis*,*cis*-muconate to succinate for cell metabolism. In *R. eutropha* JMP134, these activities are encoded by the 2,4-D degradation genes (*tfdA-F*) located on the well-characterised plasmid pJP4

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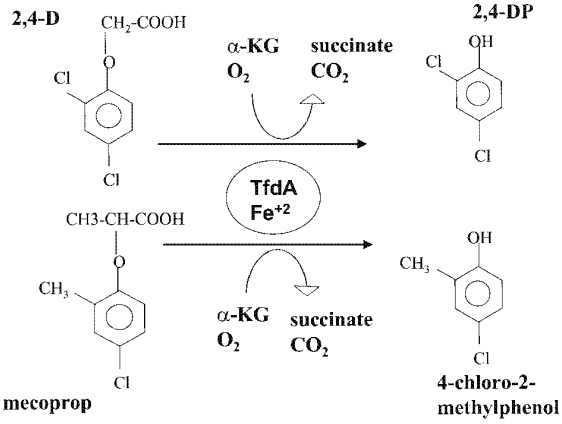


Fig. 1 The catabolic activity of the TfdA enzyme in 2,4-Dichlorophenoxyacetic (*2,4-D*) and mecoprop degradation. *KG* α-Ketoglutarate, *2,4-DCP* 2,4-dichlorophenol

(Don and Pemberton 1981; Don et al. 1985). The *tfdA* gene that encodes the first step of the pathway (i.e. the removal of the side chain of the 2,4-D molecule) is a key feature both functionally and evolutionarily. Fukumori and Hausinger (1993) suggested that the *tfdA* gene may have evolved from a parent gene involved in the metabolism of taurine (*tauD*) (Van der Ploeg et al. 1996).

The mecoprop degradation pathway has been shown to be convergent with the 2,4-D pathway (Tett et al. 1997) and *tfd*-like genes have been shown to be found in mecoprop degraders (Smejkal et al. 2000a). This has also been reported for the metabolism of 2,4-DP (Saari et al. 1999). However, on the basis of the reaction sequence for 2,4-D degradation, the first metabolite detected in mecoprop metabolism by *Alcaligenes denitrificans* is 4-chloro-2-methylphenol (MCP) as opposed to 2,4-DCP (Fig. 1). This activity may be facilitated by an enzyme characteristic of an α-ketoglutarate-dependent dioxygenase (TfdA) (Tett et al. 1997).

It is known that an α-ketoglutarate-dependent dioxygenase from *R. eutropha* JMP134 can utilise MCPA in addition to 2,4-D as a substrate. It also utilises the unsubstituted phenoxyacetic acid, although with a weak induction of the enzyme (Pieper et al. 1988). Many 2,4-D-degrading strains do not have wide substrate specificity for the chlorophenoxyalkanoic herbicides and are often limited to 2,4-D as a growth substrate (Ka et al. 1994; Hoffman et al. 1996). Conversely, mecoprop-degrading strains are frequently able to degrade alternative chlorophenoxyalkanoic acids such as 2,4-D, MCPA and 2,4-DP (Tett et al. 1994; Zipper et al. 1996; Müller et al. 1999; Marriott et al. 2000). However, their ability to utilise a range of phenoxyalkanoates has not previously been described.

Recently *tfdA*-specific PCR primers were developed (Vallaeys et al. 1996), allowing specific amplification of different families of the *tfdA* gene within many 2,4-D-degrading strains. Studies have shown that there are four, diverse types of *tfdA* genes among 2,4-D-degrading bacteria (McGowan et al. 1998; Vallaeys et al. 1996, 1999). The *tfdA* gene type was also found to be incongruent from the species type, indicating that this gene must have originated as a result of gene transfer between bacterial species (McGowan et al. 1998). Likewise, the *tfdA* gene has been found to be incongruent with analyses of the *tfdB* and *tfdC* genes (Vallaeys et al. 1999). The functional role of *tfdA* gene sequence diversity and its overall activity in nature is not yet fully understood. Therefore the question arises; is there a link between the *tfdA* gene type and particular substrate specificity towards a range of related substrates?

Here we present the phylogenetic relationship of *tfdA*-like genes in mecoprop-degrading strains to the four types of *tfdA* gene found in 2,4-D-degrading bacteria. Subsequent determination of gene sequence and type will permit comparisons of *tfdA* gene type and substrate specificity of its encoded α-ketoglutarate-dependent dioxygenase to different chlorophenoxyalkanoic substrates. We provide evidence that there is no apparent phylogenetic functional relationship between substrate specificity and *tfdA* gene type. Conversely, substrate utilisation range may be governed by phylogenetically distinct genes that function in the same way as the *tfdA* gene. Equally this specialisation may be due to a permease gene which encodes an enzyme involved in substrate-specific uptake, or even enzyme activities at subsequent steps of the degradation pathway as encoded by the *tfdB-tfdF* genes.

Materials and methods

Chemicals

Mecoprop, (*R*)-mecoprop, 2,4-DP, MCPA, 2,4-D, MCPB and 2,4-DB were generously donated by A. H. Marks (Bradford, UK). 2,4,5-T was obtained from Sigma (UK), phenoxyacetic acid from Merck, and 4-nitrophenoxyacetic acid from T.C.I. (Tokyo).

Bacterial strains and culture conditions

Bacterial strains are listed in Table 1. The strains were routinely grown at 30°C in 100-ml batch cultures containing (g l^{-1} distilled water): $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5; Na_2EDTA, 0.12; NaOH, 0.02; $ZnSO_4$, 0.004; $CuSO_4.5H_2O$, 0.001; Na_2SO_4, 0.0001; Na_2MoO_4, 0.001; $CoCl_2$, 0.0001; $MnSO_4$, 0.0004; and 0.5 ml concentrated H_2SO_4. The pH of the medium was adjusted to 7.0 and the respective isolation substrate was added at a concentration of 3 mM (2,4-D, mecoprop, 2,4,5-T).

Partial sequencing of the *tfdA*-like gene and phylogenetic analyses

Plasmid DNA was extracted from the mecoprop-degrading strains (Wheatcroft and Williams 1981) then amplified with specific primer sets for the *tfdA* gene as described by Vallaeys et al. (1996). Aliquots of 5–10 µl of the PCR solution were analysed on horizontal 1% agarose gels (BDH) in TBE buffer (40 mM TRIS-HCl, 4 mM sodium acetate, 1 mM EDTA, pH 7.9) using the Biorad DNA sub cell. Electrophoresis was carried out for 30 min at 60 V. Ethidium bromide-stained gels were then photographed on an Alpha Innotech Corporation UV transilluminator connected to ChemiImager 4000i v4.04. Sequencing reactions were performed by MWG Bio-

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Table 1 Bacterial strains used in this study

Strain		Plasmid	Isolated on	Origin	*tfdA* gene type[b]	*tfdA* Accession number/reference	Reference
Ralstonia eutropha	JMP134	pJP4 (85 kb)	2,4-D	Australia	1	M16730	Don and Pemberton (1981)
Variovorax paradoxus	TV1	pTV1	2,4-D	France	4	U65531	Vallaeys et al. (1998)
Burkholderia sp.	RASC	–	2,4-D	USA	2	U25717	Fulthorpe et al. (1995)
Sphingomonas sp.	AW5	pAW5 (50 kb)[a]	2,4,5-T	UK	ND	–	Weightman (unpublished data)
Alcaligenes denitrificans		pCS3 (approx. 225 kb)	Mecoprop	UK	4	Saari et al. (1999)	Tett et al (1994)
Alcaligenes sp.	CS1	pCS1 (approx. 225 kb)	Mecoprop	UK	4	AF181982	(This study)
Ralstonia sp.	CS2	pCS2 (approx. 225 kb)	Mecoprop	UK	4	AF182758	(This study)
Escherichia coli		pUC19::*tfdA*	–	–	1	–	Holben et al. (1995)

[a] Location of degradation genes on this plasmid have not yet been determined

[b] The tfdA gene type is as defined in Vallaeys et al. (1996)
ND not determined

tech (Ebersberg, Germany). The forward and reverse *tfdA* primer consensus sequences were submitted to Genbank under accession numbers AF181982 (*Alcaligenes* sp. CS1) and AF182758 (*Ralstonia* sp. CS2) (Smejkal et al. 2000b, 2000c). Fasta and Blast subroutines (Pearson and Lipman 1988) were used to search for related sequences in the GenBank, EMBL, and RDP databases. The CLUSTAL W program (Thompson et al. 1994) was used for multiple sequence alignments. The PHYLIP package (Felsenstein 1989) was used for distance estimation, using the Kimura (Kimura 1980) method, and dendogram construction, using neighbor-joining (Saitou and Nei 1987). Bootstrap values were estimated according to Zharkikh and Li (1992) with 100 replicates.

The following organisms were included in the phylogenetic analysis (accession numbers in parentheses). *β-proteobacterium* B6–9 (U43196), *Rhodoferax* sp. P230 (AF176240), *Achromobacter xylosoxidans* (pEST4011) (U32188), *Variovorax paradoxus* (pTV1) (U65531), *Halomonas* sp I-18 (U22499), *Burkholderia* sp. RASC (U25717), *Burkholderia* sp. TFD39 (U43197), *Ralstonia eutropha* JMP134 (M16730), *β-proteobacterium* TFD23 (U43276) and *Escherichia coli* K-12 (U00096).

Use of 4-nitrophenoxyacetic acid to detect TfdA activity

The production of a bright yellow 4-nitrophenol chromophore was used to detect whole-cell TfdA activities in the strains described in Table 1, when grown on minimal medium plates containing a range of substituted and unsubstituted phenoxy compounds. Colonies were wetted with a freshly prepared assay solution containing 10 mM-4-NPAA, 50 μM $Fe(NH_4)_2(SO_4)_2$, and 50 μM ascorbate in 20 mM TRIS-HCl; pH 7.4, allowed to dry and then rewetted with an atomiser (Sassanella et al. 1997). Plates were incubated at 30°C with observation at 5 min to 6 h. Control nutrient agar (Oxoid, UK) plates and minimal agar plates containing only 3 mM sodium acetate were employed.

Colorimetric assay spray technique for detection of derivatised phenols

This method relies on the condensation of 4-aminoantipyrine and liberated phenol in the presence of alkaline potassium ferricyanide to produce a red antipyrine dye (AAPPC) and is known as the Emerson reaction (King et al. 1991).

Pre-grown cultures of the strains shown in Table 1 were grown in 10 ml of minimal salts medium containing their respective isolation substrate (2,4-D, Mecoprop, 2,4,5-T) at concentrations of 3 mM. Late log-phase samples were serial diluted in 1/4 strength Ringers solution (Oxoid, UK), the 10^{-5} dilution of each strain was plated onto separate minimal selective agar plates of the following composition (g l^{-1} distilled water): $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5; Na_2EDTA, 0.12; NaOH, 0.02; $ZnSO_4$, 0.004; $CuSO_4$, 0.001; Na_2SO_4, 0.0001; Na_2MoO_4, 0.001; $CoCl_2$, 0.0001; $MnSO_4$, 0.0004; agar, 15.0; and 0.5 ml of concentrated H_2SO_4. The pH of the medium was adjusted to 7.0 and substituted and unsubstituted phenoxyalkanoate substrates were added at concentrations of 3 mM. Plates were incubated at 30°C for 24 h, except for *V. paradoxus*, which required 5 days due to a slower growth rate. The colonies were wetted with an 8% potassium ferricyanide solution (in distilled water). The plates were allowed to air dry before being sprayed with a solution of pH-10.0 buffer solution (3.09 g boric acid, 3.73 g potassium chloride, 44 ml of 1 N sodium hydroxide and made up to 1 l with distilled water), 2% 4-aminoantipyrine (in distilled water) and 8% potassium ferricyanide (5:1:1, v/v/v) (King et al. 1991). Triplicate plates were observed for each substrate. Nutrient agar (Oxoid, UK) plates and minimal agar plates containing only 3 mM sodium acetate were used as negative controls. All plates were observed after 10 min incubation at room temperature.

Results

Partial sequencing of the *tfdA*-like gene and phylogenetic analyses

PCR amplification of the plasmid DNA from *Alcaligenes* sp. CS1 and *Ralstonia* sp. CS2 using the *tfdA* primer set, gave rise to fragments of approximately 300 bp (Fig. 2). Partial nucleotide sequences were undertaken for these strains and these were compared to existing *tfdA* sequences in Genbank, EMBL, and RDP using Fasta and Blast subroutines. Sequence comparison showed that CS1 and CS2 have *tfdA*-like sequences with

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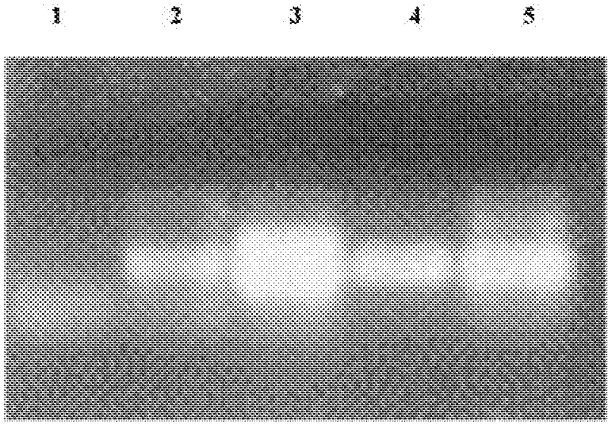


Fig. 2 Polymerase chain reaction amplification of the approximately 300-bp *tfdA* gene fragments from mecoprop-degrading strains. *Variovorax paradoxus* TV1 and *Sphingomonas* sp. AW5 were used as positive and negative controls. *Lane 1* pAW5, *lane 2* pCS1, *lane 3* pCS2, *lane 4* pCS3, *lane 5* pTV1

100% similarity to the 2,4-D-degrading *V. paradoxus* TV1, which has a type 4 *tfdA* gene, and to the mecoprop-degrading *A. denitrificans*. The dendogram produced by neighbor-joining shows the phylogenetic relationship between the *tfdA* gene with 2,4-D-degrading strains and the *tfdA*-like gene found in mecoprop-degrading strains (Fig. 3). The sequences showed 77% similarity to the canonical *tfdA* gene from *R. eutropha* JMP134 (type 1 *tfdA*), 93% to *Burkholderia* sp. RASC (type 2 *tfdA*) and 99% to *Halomonas* sp. I-18 (type 4 *tfdA*). No *tfdA* amplification was achieved with the 2,4,5-T-degrading *Sphingomonas* sp. AW5.

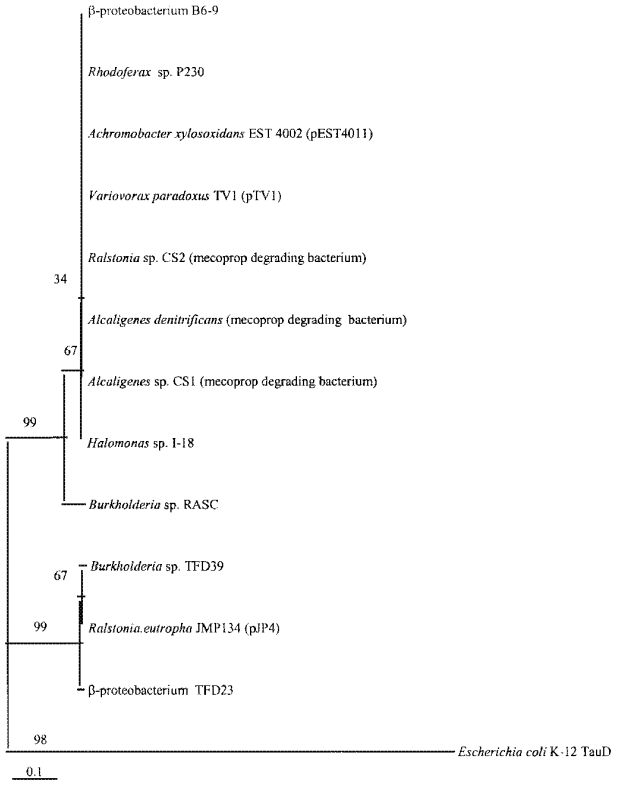


Fig. 3 Neighbor-joining dendogram (Saitou and Nei 1987) of *tfdA* genes from 2,4-D- and mecoprop-degrading bacteria. Bootstrap confidence limits (percentages) are indicated above each branch. A reference outgroup is signified by the taurine (*tauD*)-degrading *Escherichia coli* K-12 (Van der Ploeg et al. 1996). *Scale bar* represents a Jukes-Cantor distance of 0.1

Use of 4-nitrophenoxyacetic acid to detect TfdA activity

All strains studied showed potential TfdA activity as indicated by production of an intense yellow 4-nitrophenol chromophore after 5–10 min on all substrate types tested except control media, thus demonstrating all strains could actively utilise 4-nitrophenoxyacetic acid.

Colorimetric assay spray technique for detection of derivatised phenols

The 2,4-D strains studied exhibited similar specificity for the substrates 2,4-D and MCPA with intense AAPPC production being observed (Table 2). Weak AAPPC was

Table 2 AAPPC production from a set of substituted and unsubstituted phenoxy compounds using the method of King et al. (1991). Observations were performed with all strains pre-grown on their respective isolation media [2,4-dichlorophenoxyacetic acid (*2,4-D*); 2,4,5-trichlorophenoxyacelic acid (*2,4,5-T*); mecoprop]. *MCPA* 2-Methyl 4-chlorophenoxyacetic acid; *2,4-DP* 2-(2,4-dichlorophenoxy)propionic acid; *2,4-DB* 2,4-dichlorophenoxybutyric acid; *MCPB* 2-methyl 4-chlorophenoxybutyric acid; *PA* phenoxyacetate; + intense phenol production on the compounds; +/– weak phenol production; – no phenol production

Strains	Compounds										
	2,4-D	MCPA	Racemic mecoprop	(R)-mecoprop	Racemic 2,4-DP	2,4,5-T	2,4-DB	MCPB	PA	Sodium acetate	Nutrient media
R. eutropha JMP134	+	+	+/–	–	+/–	+/–	–	–	–	–	–
V. paradoxus TV1	+	+	+/–	–	+/–	–	–	–	+	–	–
Burkholderia sp. RASC	+	+	+/–	–	+/–	–	–	–	+	–	–
Sphingomonas sp. AW5	+/–	–	+	–	–	+	–	+	+	–	–
A. denitrificans	+	+	+	+	+/–	–	–	–	–	–	–
Alcaligenes sp. CSI	+	+	+	+	+	+/–	+/–	+/–	–	–	–
Ralstonia sp. CS2	+	+	+	+	+	–	–	–	+	–	–
E. coli	+	+	–	–	–	–	–	–	+	–	–

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produced after these strains were grown on racemic mecoprop and 2,4-DP but not the (*R*)-mecoprop enantiomer. Additionally *R. eutropha* JMP134 expressed weak TfdA activity towards 2,4,5-T but no activity towards phenoxyacetate as substrates. Conversely, intense AAPPC was produced when *Sphingomonas* sp. AW5 was grown on racemic mecoprop, 2,4,5-T, MCPB and phenoxyacetate as growth substrates (Table 2).

Mecoprop-degrading strains, *A. denitrificans*, CS1 and CS2 displayed intense AAPPC production when grown on 2,4-D, MCPA, racemic mecoprop and (*R*)-mecoprop as substrates (Table 2). Intense AAPPC was produced with CS1 and CS2 on racemic 2,4-DP as a substrate whereas there was only weak AAPPC produced for *A. denitrificans* on racemic 2,4-DP (Table 2). Weak AAPPC production was observed for CS1 when 2,4,5-T, 2,4-DB and MCPB were used as substrates (Table 2). Of the mecoprop-degrading strains only CS2 expressed TfdA activity towards phenoxyacetate. No AAPPC was produced on sodium acetate and nutrient media.

Discussion

In many species of bacteria which degrade 2,4-D, methods including *tfdA* gene disruption using transposon mutagenesis (Streber et al. 1987; Suwa et al. 1996; Vallaeys et al. 1998) have shown that divergent *tfdA* genes can play a similar or identical role in the 2,4-D catabolic pathway. *tfdA* gene diversity is not yet understood. Moreover, chlorophenoxyalkanoic acid herbicides were not introduced until after World War II, which is very recent in terms of evolution. This gene diversity is in marked contrast to the degradation of other xenobiotic herbicides, such as atrazine, where there is only one type of *atzA* gene from geographically diverse atrazine-degrading bacteria (De Souza et al. 1998).

Although the role of the *tfdA*-like gene has not been demonstrated in the mecoprop and 2,4-DP degradation pathways, many convergent results suggest that it is indeed involved (Nickel et al. 1997; Saari et al. 1999; Smejkal et al. 2000a). Tett et al. (1997) reported that the enzyme involved in the first step of mecoprop degradation in *Alcaligenes denitrificans* is iron dependent and this is in accordance with the study by Fukumori and Hausinger (1993) suggesting that the first step of mecoprop degradation involves a TfdA. More recently, Saari et al. (1999) showed that in 2,4,-DP catabolism, the first enzyme produced by *A. denitrificans* was characteristic of TfdA because it was α-ketoglutarate and ferrous iron dependent. All of these reports suggest that the first step of mecoprop degradation involves a TfdA enzyme. However, it is necessary to further demonstrate the role of TfdA in both the mecoprop and 2,4-DP degradation pathways by protein sequencing and transposon- or site-directed mutagenesis; this is currently underway.

It has been reported that there are four families of *tfdA* genes in 2,4-D-degrading strains (Vallaeys et al. 1996; McGowan et al. 1998); however, all of the mecoprop strains used in this study have type 4 *tfdA* genes with 100% sequence similarity to *V. paradoxus* TV1 (isolated on 2,4-D). It would therefore be expected that these strains should exhibit identical substrate specificity; however, we report that they all have different specificities that are not influenced by the *tfdA* gene type. Among 2,4-D-degrading strains with distinctive *tfdA* gene types, similar substrate specificity was observed. For example, *R. eutropha* JMP134 (type 1 *tfdA*) (Streber et al. 1987), *Burkolderia* sp. RASC (type 2), chromosomally located *tfdA* gene (Matheson et al. 1996) and *V. paradoxus* TV1 (Vallaeys et al. 1998) all showed greatest activity towards 2,4-D and MCPA with weak activity towards racemic mecoprop and 2,4-DP but no activity towards the (*R*)-enantiomers. These weak activities towards the racemic compounds may be as a result of these strains producing a TfdA which can utilise only one of these enantiomers. This has been demonstrated previously where it was reported that *R. eutropha* JMP134 and *Burkholderia* sp. RASC do have specific activity for the (*S*)-enantiomers of these compounds (Nickel et al. 1997; Saari et al. 1999).

Sphingomonas sp. AW5, isolated on 2,4,5-T, has been reported not to express the *tfdA* gene using *tfdA* primer sets (Vallaeys et al. 1997). However, weak TfdA-like activity was detected with 2,4-D as a substrate. Moreover, intense AAPPC was produced when this strain was grown on 2,4,5-T, racemic mecoprop, MCPB and phenoxyacetate. This may be indicative of trichlorophenoxyacetic acid oxygenase (TftA) activity in the first-step cleavage of these compounds. This enzyme is encoded by the *tftA* gene in the 2,4,5-T-degrading *Burkholderia cepacia* AC1100 (Danganan et al. 1995) and expresses similar properties to TfdA. To date this is the first reported strain with the ability to utilise MCPB as a sole carbon source. In addition, *tfdB*-like and *tfdC*-like genes related to the *tfdB* and *tfdC* genes from 2,4-D-degrading strains have been amplified from AW5 (Vallaeys et al. 1997), suggesting that independent gene recruitment may have occurred.

Although there are several types of *tfdA* gene, it is apparent that substrate specificity must be governed by other unrelated factors. The *tfdB* gene which has a similar sequence to the phenol hydroxylase gene *PheA* (Nurk et al. 1991) may be involved in substrate specificity because there are several types of *tfdB* gene amongst 2,4-D-degrading strains (Vallaeys et al. 1999). The down-stream *tfdC* gene encodes a relaxed substrate chlorocatechol 1,2-dioxygenase and possesses significant sequence similarity to *clcA* (Frantz and Chakrabarty 1987) and the *tcbC* gene (Van der Meer et al. 1991). Further studies are needed to determine any link between the *tfdC* gene and substrate specificity.

A recent study by Leveau et al. (1998) has demonstrated that a gene designated *tfdK* is involved in the uptake of 2,4-D by *R. eutropha* JMP134. This encodes a protein belonging to the major facilitator superfamily (MFS) group of transporter proteins and is related to pro-

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teins responsible for the transport of carboxylated aromatic substances. It could be that the TfdK protein in *R. eutropha* JMP134 is specific to uptake of 2,4-D and MCPA only. This could also be true for the other strains where they have transport proteins exhibiting specificity to particular compounds.

Sequence divergence of the related *tfdA* gene family is not associated with the specific degradation of particular phenoxyalkanoic acid herbicides by the strains studied. It is likely, therefore, that *tfdA* gene diversity pre-existed the post-war "pesticide era" and is not linked to the introduction of different types of related compounds.

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TRANSGENIC TOBACCO PLANTS EXPRESSING A BACTERIAL DETOXIFYING ENZYME ARE RESISTANT TO 2,4-D

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We have used a gene from the soil bacterium *Alcaligenes eutrophus* JMP134 to introduce a herbicide degrading mechanism into plants. The gene, *tfdA*, which encodes a 2,4-dichlorophenoxyacetate monooxygenase (DPAM) catalyzing the first step in the bacterial 2,4-D degradative pathway, has recently been cloned and sequenced. The coding sequence, fused with plant expression sequences was introduced into plants via *Agrobacterium*-mediated gene transfer. The chimeric gene was expressed in tobacco (*Nicotiana tabacum*) under the control of either a constitutive or a light-inducible promoter. Transgenic tobacco lines tolerated elevated levels of 2,4-D in tissue culture, and regenerated plants showed resistance when sprayed with the herbicide.

Phenoxyacetic acids, such as 2,4-D and MCPA (4-chloro-2-methylphenoxyacetic acid) show an auxin-like hormone action when applied to plants. It is postulated they mimic the function of the intrinsic auxin, indole-3-acetic acid, on an internal auxin receptor of the plant. The fact that higher concentrations of the synthetic auxins are toxic to many dicotyledonous, but not to monocotyledonous, plants, has been widely used for their application as herbicides with high selectivity to control broadleaf weeds in cereal and grass crops.

Considerable effort has been spent to create herbicide resistant crop plants. Although various attempts to isolate such mutants or variants by conventional breeding and tissue culture methods have met so far with limited success, the methods of genetic engineering opened up the way to transfer effective resistance mechanisms to plants[1]. Resistance to atrazine[2], glyphosate[3], and sulfonylurea herbicides[4] have been achieved by the introduction of foreign genes encoding modified insensitive target proteins. Resistance to phosphinotricin[5] and bromoxynil[6] were based on the expression of detoxifying enzymes originally isolated from microorganisms.

Attention has also been given to the possibility of generating dicotyledonous plants resistant to 2,4-D. In addition to its agricultural value, the study of 2,4-D resistant plants may improve our knowledge about the site of action and the molecular mechanisms by which auxins exert their effects. 2,4-D-resistant mutants have been isolated after mutagenesis of *Arabidopsis thaliana*[7]. Similar to 2,4-D resistant tobacco cell lines[8], these mutants showed a severely altered phenotype. Genetic and biochemical studies suggested that resistance was due to a mutation affecting a function involved in the action and not in the metabolism of 2,4-D.

A potentially useful approach to the production of 2,4-D resistant plants that show a normal phenotype would therefore be to introduce a gene into plant cells known to encode the metabolism of 2,4-D into a less toxic compound. A gene fulfilling these needs has recently been cloned and sequenced[9]. The *tfdA* gene from the soil bacterium *Alcaligenes eutrophus* JMP134 encodes the first enzyme involved in a 2,4-D degradative pathway: A single polypeptide that is able to remove the acetate side chain from 2,4-D in a monooxygenase-like reaction.

It was not known if the bacterial degradative monooxygenase would be enzymatically active in plants, or if its broad substrate specificity would interfere with the plant metabolism. Here we report the expression of *tfdA*, encoding a 2,4-dichlorophenoxyacetate monooxygenase (DPAM) in genetically engineered tobacco plants. We show that transgenic plants that express DPAM are resistant to the herbicide 2,4-D, and we describe the phenotypic response of the transgenic plants to differently substituted phenoxy herbicides in tissue culture.

RESULTS

Contruction of plasmids for constitutive and light-induced DPAM expression in plants. To create a plant/bacterial hybrid gene for DPAM the procaryotic translation initiation codon GTG of *tfdA* was replaced by an ATG codon. This was achieved by a synthetic double stranded oligonucleotide designed to fit a 5′ protruding end generated by FokI 8 bp downstream from the translation initiation codon. The oligonucleotide contained a sequence reconstituting the original open reading frame for DPAM, the consensus sequence for plant translational start sites upstream from the ATG start codon[10] and a 5′ protruding end compatible with BamHI cloning sites. The oligonucleotide was linked with the coding sequence of *tfdA* truncated at its N-terminal end by a partial digest with FokI (Fig. 1). The correct ligation of the oligonucleotide was verified by sequence analysis.

The new sequence was fused at its 3′ end to a 200 bp fragment containing the polyadenylation signal of the octopine synthase gene (OCS-polyA)[11] and at its 5′ end to a 540 bp fragment of the cauliflower mosaic virus (CaMV) 35S promoter[12,13]. The resulting plasmid, designated pMCJC1007 (Fig. 1), was based on the intermediate vector pMPK110 which is able to form cointegrates with disarmed Ti-plasmids of *A. tumefaciens* such as pGV3850kan[14]. In a similar construction the constitutive CaMV 35S promoter was replaced by the light-inducible promoter of gene ST-LS1[15] to form a plasmid designated pMLJC1005 (data not shown).

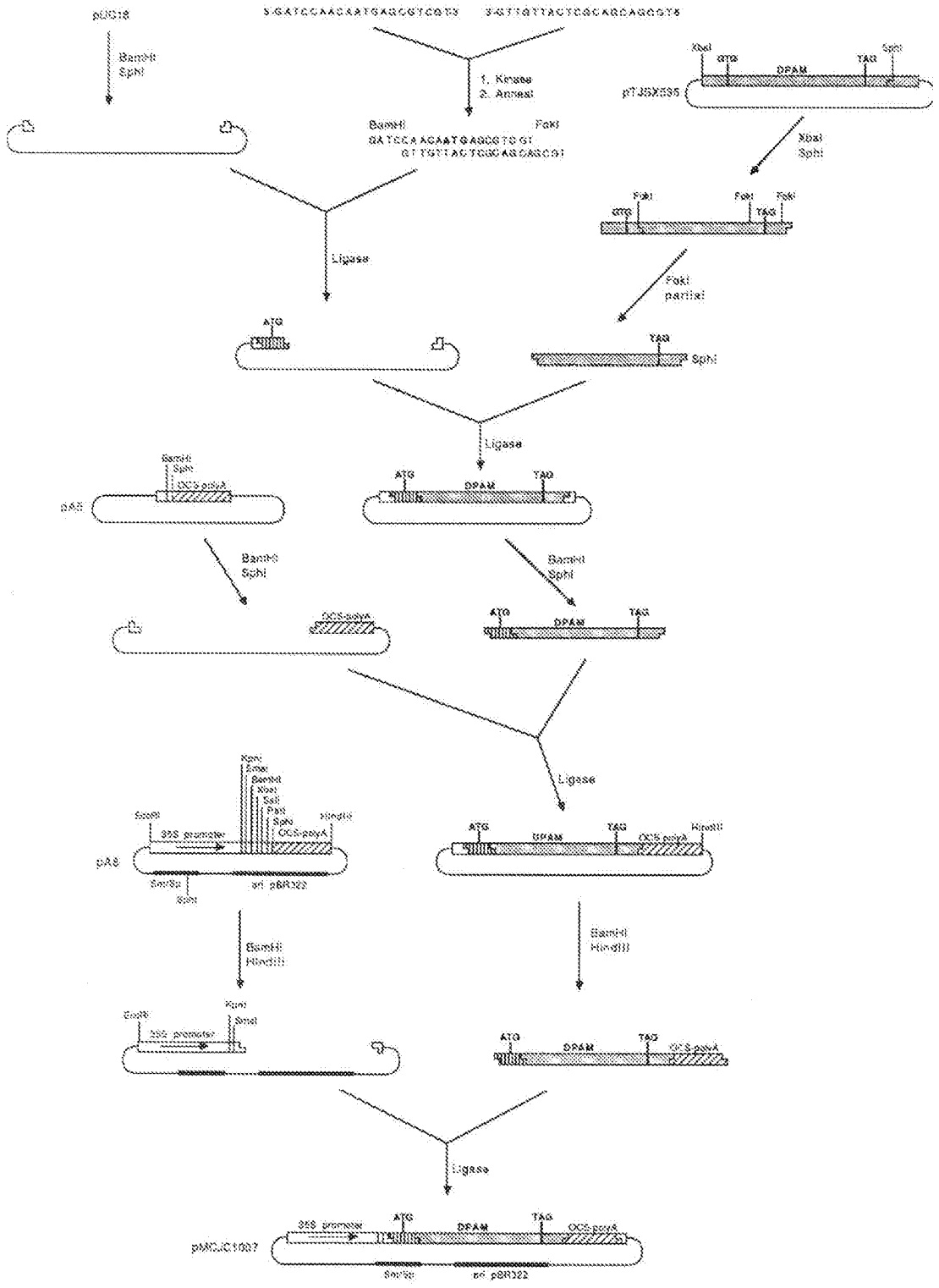
Toxicity of the DPAM reaction product. Gas chromatography and mass spectrometry of *E. coli* pUJC1001 expressing a DPAM-fusion gene showed that 2,4 dichlorophenol (2,4-DCP) was the reaction product thus confirming previous indirect results[9].

In order to evaluate the possible toxicity of 2,4-DCP to

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FIGURE 1 Construction of plasmid pMCJC1007 for stable integration and expression of the DPAM gene in plants. In a first cloning step the procaryotic translational start codon GTG of gene *tfdA* was replaced by a synthetic oligonucleotide. To avoid dimerisation at the BamHI site the oligonucleotide was linked in a two step ligation first to the BamHI end of linearised pUC18 and then to the DPAM coding sequence. In two following cloning steps, the BamHI-SphI fragment containing the hybrid construct was inserted into the plant expression vector plasmid pA8, between the CaMV 35S promoter and the OCS 3' end. As pA8 contains a second SphI site outside of its polylinker, pA5 was used to form an intermediate plasmid based on pUC18.



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