

(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,

4.2.2 Documents **D8**, **D9**, **D10**, **D11**, and **D12** cited by the Opponent as relevant for novelty relate to transgenic plants comprising the *tfdA* gene from *Ralstonia eutropha*. The TfdA protein encoded by said gene is described as possessing degrading activity on achiral phenoxy auxins, i.e. 2,4-D. The TfdA protein cannot enzymatically degrade R-enantiomers of chiral phenoxy auxin herbicides as required by alternative (i) of amended claim 2. Further, the TfdA protein is not capable of degrading aryloxyphenoxypropionate herbicides as required by alternatives (ii) and (iii) of amended claim 2.

4.2.3 In summary: the protein encoded by the *tfdA* gene of *Ralstonia eutropha* is described as possessing achiral phenoxy auxin degrading activity in **D8**, **D9**, **D10**, **D11**, and **D12**. However, the *tfdA* gene of *Ralstonia eutropha* does not possess the enzymatic activities recited in alternatives (i), (ii) and (iii) of amended claim 2. Therefore, the transgenic plants disclosed in **D8**, **D9**, **D10**, **D11**, and **D12** do not anticipate the subject-matter of amended claim 2. Hence the subject-matter of amended claim 2 is novel over the prior art.

For the sake of completeness, we would like to repeat that **D12** does not even constitute prior art for the subject-matter of amended claim 2 of the Main Request (see section 3.2 above).

4.3 The subject-matter of amended claims 3 to 17 is novel over the prior art

Amended claims 3 to 17 of the Main Request are directly or indirectly dependent on claim 1 or 2. Accordingly, the subject-matter of claims 3 to 17 derives its novelty from this dependency.

5. Inventive Step: Articles 56 and 100(a) EPC

5.1 The Opponent has alleged that claims 1, 11 and 13 as granted are anticipated by one or more of documents **D8** to **D12**, all of which are concerned with the known 2,4-D-herbicide degrading enzyme TfdA. An objection of lack of inventive step is raised against the subject-matter of granted claims 3, 5-9, 12, 14 and 16 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**), when considered in combination with **D19** (or any one of **D20**, **D21**, **D22**, or **D23**); against the subject-matter of granted claims 2 and 10 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**) taken alone; against the subject-matter of granted claim 4 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**), when considered in combination with **D15**; and against the subject-matter of granted claim 15 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**), when considered in combination with **D18**.

5.2 In this respect the Patentee reiterates that the claims of the Main Request can be considered entitled to the priority date of April 30, 2004 and thus **D12** and **D15** are not citable prior art.

Further, while not acquiescing to the Opponent's objection of lack of novelty over D8 to D11, the independent claims of the Main Request presented herewith are amended to clearly recite additional features not disclosed in those documents.

For example, claim 1 now includes the feature that the encoded protein:

"is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5".

Claim 2 now includes the feature that the encoded protein enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins;*
- (ii) an aryloxyphenoxypropionate herbicide; or*
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.*

It is submitted that claims 1 to 17 of the Main Request are both novel and inventive over all of the art cited by the Opponent and in the absence of any submissions to the contrary, the Patentee makes no further comments at this time. However, the Patentee reserves the right to make further submissions concerning the inventive step of the Main Request as necessary.

6. Sufficiency of Disclosure: Articles 83 and 100(b) EPC

- 6.1 The Opponent asserted that the invention as claimed in the granted claims is insufficiently disclosed, contrary to the provisions of Art. 83 EPC. The Opponent held the view that the specification provides support and disclosure only for polynucleotides having SEQ ID NOs: 3 to 5 but not for the broad definition based on stringent hybridization which allegedly covers polynucleotides having as little as 32% sequence identity to SEQ ID NO: 9. Allegedly, the broad scope of the granted claims is not congruent with the limited disclosure of the application.
- 6.2 The enclosed Main Request contains two independent claims that are each based on claim 1 as granted. Amended claim 1 is further limited in structural terms and amended claim 2 is further limited in functional terms (see sections 2.1.5 and 2.2.1, respectively). In view of these limitations, the Opponent's objections under Art. 83 EPC are moot.

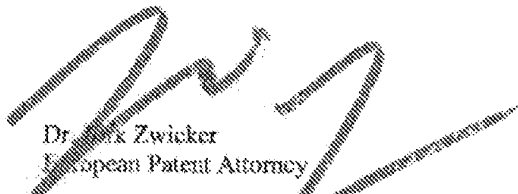
7. Double Patenting

- 7.1 In a remark on page 17 of the Opposition Brief, the Opponent alleged that granted claim 4, which corresponds to claim 5 of the Main Request claims the same subject-matter as claim 1 granted in the parent patent EP 1 740 039 B1.
- 7.2 According to Art. 100 EPC, an opposition may only be filed on the grounds recited in paragraphs (a), (b) and (c) of Art. 100 EPC. In other words, the list of grounds for opposition in Art. 100 EPC is exhaustive. Double patenting is not listed as a ground for opposition and, thus, cannot be a basis to revoke this patent (see for example T 936/84). Consequently, Patentee sees no reason to further address Opponent's allegation.

8. Summary

From the above discussion it is evident that the subject-matter claimed in the Main Request fulfils the requirements of Art. 54, 56, 83, 76(1), and 123(2) EPC.

Therefore, our request to maintain the Opposed Patent on the basis of the Main Request is fully justified.



Dr. Dirk Zwicker
European Patent Attorney

Enclosures:

Main Request (marked-up copy and as clean copy)

I. Summary of Facts and Submissions

- 1 The European patent EP 2 308 976 B1, Application number 10 012 200.1, titled "Novel herbicide resistance gene (hereinafter: "the patent") in the name of Dow AgroSciences, LLC (US) was granted on 27 March 2013.
- 2 The Opponent (Bayer CropScience NV (BE)), represented by Mr. J. Desomer of Bayer CropScience NV (BE), opposed the patent in its whole extent under Articles 100(a), 100(b) and 100(c) EPC. The opposition was filed in electronic form on 20 December 2013.
- 3 The Proprietor, represented by Dr. J. Zwicker of ZSP Patentanwälte (DE), requested that the opposition be rejected and the patent be maintained on the basis of amended claims as 1 to 19 of the Main Request provided in electronic form on 8 August 2014.
- 4 Both parties requested oral proceedings.
- 5 On 7 December 2015 the opposition division issued a summons to attend oral proceedings on 5 April 2016.
- 6 On 29 January 2016 the Opponent informed the Division that no further submissions will be made and withdrew his request for oral proceedings.
- 7 On 29 January the Proprietor conditionally withdrew his request for oral proceedings.
- 8 In the brief communication dated 11 February 2016 the Division informed the parties that the summons to attend oral proceedings have been cancelled, that the procedure will be continued in writing and further of the preliminary intention to grant a patent on the basis of the Main Request, filed in electronic form on 8 August 2014.

- 9 In reply to the communication the Proprietor submitted an amended page 3 of the description on 16 February 2016. The Opponent was given the chance to provide comments within two months which he did not.
- 10 The numbering of the cited **documents** follows the numbering of references from the Opponent's submissions:
- D1 Autoradiographs from hybridisation experiments between AAD-1 and tfdA nucleic acids
- D2 WOLFGANG R STREBER ET AL: "Analysis, Cloning, and High-Level Expression of 2,4-Dichlorophenoxyacetate Monooxygenase Gene tfdA of *Alcaligenes eutrophus* JMP134", JOURNAL OF BACTERIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 169, no. 7, 1 July 1987 (1987-07-01), pages 2950-2955, XP001315263, ISSN: 0021-9193
- D3 FUKUMORI FUMIYASU ET AL: "Alcaligenes eutrophus JMP134 "2,4-dichlorophenoxyacetate monooxygenase" is an alpha-ketoglutarate-dependent dioxygenase", JOURNAL OF BACTERIOLOGY, vol. 175, no. 7, 1993, pages 2083-2086, ISSN: 0021-9193
- D4 Amino acid sequence alignment between TFDA and AAD-1 proteins
- D5 HULSE J D ET AL: "CARNITINE BIOSYNTHESIS BETA HYDROXYLATION OF TRI METHYL LYSINE BY AN ALPHA KETO GLUTARATE DEPENDENT MITOCHONDRIAL DI OXYGENASE", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 253, no. 5, 1978, pages 1654-1659, ISSN: 0021-9258
- D6 EICHHORN ERIC ET AL: "Characterization of alpha-ketoglutarate-dependent taurine dioxygenase from *Escherichia coli*", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 37, 1997, pages 23031-23036, ISSN: 0021-9258
- D7 Priority document USSN 60/567,052

- D8 STREBER W R ET AL: "TRANSGENIC TOBACCO PLANTS EXPRESSING A BACTERIAL DETOXIFYING ENZYME ARE RESISTANT TO 2,4-D",
BIOTECHNOLOGY. THE INTERNATIONAL MONTHLY FOR INDUSTRIAL BIOLOGY, NATURE PUBLISHING GROUP, US,
vol. 7, no. 8, 1 January 1989 (1989-01-01), pages 811-816,
XP008062554,
ISSN: 0733-222X, DOI: 10.1038/NBT0889-811
- D9 LYON B R ET AL: "EXPRESSION OF A BACTERIAL GENE IN TRANSGENIC TOBACCO PLANTS CONFERS RESISTANCE TO THE HERBICIDE 2 4-D",
PLANT MOLECULAR BIOLOGY,
vol. 13, no. 5, 1989, pages 533-540,
ISSN: 0167-4412
- D10 LYON BRUCE R ET AL: "Cotton plants transformed with a bacterial degradation gene are protected from accidental spray drift damage by the herbicide 2,4-dichlorophenoxyacetic acid",
TRANSGENIC RESEARCH,
vol. 2, no. 3, 1993, pages 162-169,
ISSN: 0962-8819
- D11 BAYLEY C ET AL: "ENGINEERING 2,4-D RESISTANCE INTO COTTON",
THEORETICAL AND APPLIED GENETICS, SPRINGER, BERLIN, DE,
vol. 83, no. 5, 1 January 1992 (1992-01-01), pages 645-649,
XP002037287,
ISSN: 0040-5752, DOI: 10.1007/BF00226910
- D12 BISHT NAVEEN CHANDRA ET AL: "Development of 2,4-D-resistant transgenics in Indian oilseed mustard (Brassica juncea)",
CURRENT SCIENCE (BANGALORE),
vol. 87, no. 3, 10 August 2004 (2004-08-10), pages 367-370,
ISSN: 0011-3891
- D13 Alignment of the nucleotide sequence of SEQ ID NO: 3 of the patent with the nucleotide sequence of entry AF516751 (Rhodoferax sp P230 rdpA gene)
- D14 AF516751 nucleotide sequence, version of June 26, 2003

- D15 SCHLEINITZ KATHLEEN M ET AL: "Localization and characterization of two novel genes encoding stereospecific dioxygenases catalyzing 2(2,4-dichlorophenoxy)propionate cleavage in *Delftia acidovorans* MC1", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 70, no. 9, 1 September 2004 (2004-09-01), pages 5357-5365, XP002549093, ISSN: 0099-2240, DOI: 10.1128/AEM.70.9.5357-5365.2004
- D16 Alignment of the nucleotide sequence of SEQ ID NO: 3 of the patent with the nucleotide sequence entry AJ628859 (*Sphingomonas herbicidovorans* tnpA gene)
- D17 AJ628859 nucleotide sequence, version of March 3, 2004
- D18 WO 97/13402 A1 (DOWELANCO [US]) 17 April 1997 (1997-04-17)
- D19 WO 87/05629 A1 (PLANT GENETIC SYSTEMS NV [BE]; BIOGEN NV [NL]) 24 September 1987 (1987-09-24)
- D20 WO 03/013224 A2 (BAYER BIOSCIENCE NV [BE]) 20 February 2003 (2003-02-20)
- D21 US 5 463 175 A (BARRY GERARD F [US] ET AL) 31 October 1995 (1995-10-31)
- D22 US 2003/135879 A1 (WEEKS DONALD P [US] ET AL) 17 July 2003 (2003-07-17)
- D23 WO 96/33270 A1 (AMERICAN CYANAMID CO [US]; KAKEFUDA GENICHI [US]; OTT KARL HEINZ [US];) 24 October 1996 (1996-10-24)
- D24 PRINS M: "Broad virus resistance in transgenic plants", TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 21, no. 9, 1 September 2003 (2003-09-01), pages 373-375, XP004450444, ISSN: 0167-7799, DOI: 10.1016/S0167-7799(03)00183-5
- D25 DELLAPORTA S L ET AL: "A PLANT DNA MINIPREPARATION: VERSION II", PLANT MOLECULAR BIOLOGY REPORTER, SPRINGER SCIENCE

+BUSINESS MEDIA B.V, NL,
vol. 1, no. 4, 1 January 1983 (1983-01-01), pages 19-21, XP001016105,
ISSN: 0735-9640

II. Reasons for the Decision

11 Admissibility

The opposition is admissible since it fulfills the requirements of Article 99(1) and Rule 76 EPC.

12 Article 100(c) EPC in conjunction with Articles 76(1) and 123(2) EPC

12.1 The requirements of Rule 80 EPC are respected.

12.2 The Opponent states that the subject matter of the granted claims goes beyond the disclosure of the application as originally filed. The Opponent further mentions that the current divisional application is an exact copy of the parental application. Accordingly, any such subject matter gives rise to objections under Article 76(1) EPC.

The following claims are objected to:

1. The combination of the specified enzymatic activity with the hybridisation conditions in claim 1 resulting in a wide range of nucleic acids claimed,
2. The combination of "an asexual propagate" in claim 17 with the plant of claim 1,
3. The ground tissue of claim 18 which is taken from particular tissue of maize calli, rice plantlets or canola plants in specific Examples which does not allow to derive the further combination with the generalized subject matter of claim 1,
4. The further insect-resistance or agronomic trait genes of claims 14 and 15 in combination with the plants of claim 1,
5. The specific combination of a glufosinate with a glyphosate tolerance trait of claim 3 taken out of a longer list (singling out of a specific combination) or from an Example relating to specific combination of a plant species and the AAD1 (v3) gene.

12.3 The Proprietor confirms that the opposed patent was granted for a divisional application which was filed as an exact copy of the parent application as originally filed. Accordingly, any subject matter having a basis in the parent

application as originally filed (Article 76(1) EPC) has also a basis under Article 123(2) EPC in the application as originally filed. He submits the following with regard to the amended claims of the Main Request.

ad 1. The combination of technical features of claim 1 is derivable from original claim 5 and paragraphs [0112], [0118], [0124]-[0131] in combination with paragraphs [020], [087], [0149], [0151], [0095], [0112] and [0142] of the application as originally filed and published as WO 2005/107437.

ad 2. Claim 59 of the parent application refers to claim 5 which in turn refers to claim 1. As indicated in point "ad 1." claims 1 and 5 together with the paragraphs mentioned provide a basis for said claims 18 (granted claim 17) and 16 (granted claim 15).

ad 3. Claim 19 (granted claim 18) refers to claim 9 which in turn refers to claims 1 and 2. Paragraph [0320] discloses grinding within the context of general methods and therefore the extracted feature is not inextricably linked with the other features of the specific embodiment/example.

ad 4. Claims 61 and 62 of the parent application refer to claim 5 which in turn refers to claim 1. As indicated in point "ad 1.", claims 1 and 5 together with the paragraphs mentioned provide a basis for said claims 15 (granted claim 14) and 16 (granted claim 15).

ad 5. Paragraph [0016] provides a list of three herbicide resistance genes from which one of the preferred embodiments was deleted in claim 4 (granted claim 3). This deletion is in agreement with the principles of decision G 2/10 relating to disclaimers.

Furthermore, the Patentee indicates paragraphs [0074] and the parts of the description mentioned above in conjunction with claim 1 as basis for the subject matter of newly introduced claim 2 in which the protein is defined in functional terms.

- 12.4 In agreement with the parties, the Opposition Division notes that the current divisional application is an exact copy of the parental application as originally filed. Consequently, any subject matter having a basis in the parent application as originally filed (Article 76(1) EPC) also a basis under Article 123(2) EPC in the application as originally filed and *vice versa*.

With regard to a basis in the application as originally filed the Opposition Division observes the following.

First, concerning claim 1, the feature "a transgenic plant" is based on claim 5 and paragraph [0149]. Such plants may comprise a polynucleotide "encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide", see for

example paragraph [0020]. "Wherein said protein is at least 70% identical" is derivable from paragraph [0142]: "Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having 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". Paragraphs [0045] to [0047] disclose "a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5" as follows: "SEQ ID NO: 3 is the nucleotide sequence of *AAD-1* (v1) from *Sphingobium herbicidovorans*. 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. 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". In paragraph [0050] it is stated that "SEQ ID NO: 9 is the native amino acid sequence encoded by the *AAD-1* (v1) gene from *Sphingobium herbicidovorans*". A polynucleotide may "hybridize under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO: 9", see paragraph [0112]: "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" and paragraph [0124]: "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" and paragraph [0126]: "Hybridisation 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".

Second, concerning claim 2, the feature "a transgenic plant" is based on claim 5 and paragraph [0149]. Such plants may comprise a polynucleotide "encoding a protein that enzymatically degrades (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins", see paragraph [0074],

"(ii) an aryloxyphenoxypropionate herbicide, or

(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide", see paragraph [0020]. The feature "wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9" is derivable from the wording in paragraph [0112]: "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" plus paragraph [0124]: "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" and paragraph [0126]: "Hybridisation 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".

Third, concerning claim 18, the feature an "asexual propagate of the plant of claim 1 or 2" is once referred to in the parent application, namely in claim 5 which in turn refers to claim 1, see claim 59 "A part, progeny, or asexual propagate of the plant of claim 5" and 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".

Fourth, concerning claim 19, the feature "ground tissue from the plant of claim 9" is derivable from paragraph [0320]: "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". Said paragraph is labelled "7.10.1 Tissue harvesting DNA isolation and quantification" and is a subsection of paragraph [0319] titled "7.10 Molecular Analysis: Maize Materials and Methods". The grinding of plant material is considered a general method applicable to material originating from a wide range of species and tissue types. From the description it is furthermore not evident that the feature "ground tissue" would be inextricably linked with the other features of the specific embodiment or example.

Fifth, concerning claims 15 and 16, the wording "insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis*, *Photorhabdus*, and *Xenorhabdus*" and "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" is derivable from claims 61 and 62 as originally filed. Said claims refer to original claim 5 which in turn refers to claim 1.

Sixth, concerning claim 4, the feature "glyphosate tolerance trait and a glufosinate tolerance trait" is derivable from i. a. paragraph [0016]: "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" (emphasis added by the Division). According to the Guidelines for Examination at the EPO, Part H - Chapter V-2, 3.1 "The replacement or removal of a feature from a claim does not violate Art. 123(2) if the skilled person would directly and unambiguously recognise that:

- (i) the feature was not explained as essential in the disclosure;
- (ii) the feature is not, as such, indispensable for the function of the invention in the light of the technical problem the invention serves to solve; and
- (iii) the replacement or removal requires no real modification of other features to compensate for the change".

The Opposition Division notes that from the description it is not evident that the feature "imidazolinone tolerance/resistance" is to be regarded as being essential or indispensable or its removal requires a modification of the other features, i.e. "glyphosate tolerance" and "glufosinate tolerance" to compensate.

For the reasons above, the Opposition Division considers that the claimed subject-matter is directly and unambiguously derivable from the application as originally filed.

The wording of new claim 2 does not extend the scope of protection conferred by the patent as granted. The Opposition therefore considers that the requirements of Article 123(3) EPC are fulfilled.

13 **Priority entitlement (Article 87 EPC)**

- 13.1 The Opponent raises the objection that the claims as granted are not entitled to the priority date of 30 April 2004 (US60/567,052; document D7) and the relevant date being the international filing date of 2 May 2005. Thereby, documents D12 and D15 are prior art within the meaning of Article 54(2) EPC. According to the Opponent, the priority application contains less teaching than the subsequently filed parent and divisional application, i. a. no claims were filed with the priority document. In addition, a number of sequences, figures and examples are missing from said document. More specifically, the concept of "a protein that enzymatically degrades a herbicide selected from the group of a phenoxy auxin and arylophenoxypropionate" of claim 1 can only be found in originally filed claim 47 in the context of three exactly defined hybridizing polynucleotides encoding AAD1 but not in the priority application. Claims 2-18 are dependent on or referred to claim 1 and thus deficient as well. Additionally, the generic terms of claims 14 (insect resistance gene), 15 (agronomic trait genes) and 17 (asexual propagate) can not be found in the priority application.
- 13.2 With respect to the claims that are in his view objected to by the Opponent under novelty or inventive step using documents D12 and D15, the Proprietor submits that the subject matter of claim 1 has a basis in D7, particularly in paragraphs [0019], [0035]-[0037], [0039], [0094], [0100], [0101], [0116], [0123] and [0124] of the priority document. In addition thereto, he indicates paragraphs [0019], [0051] and [0061] as basis for claim 2. Claim 10 (granted claim 9) finds a basis in paragraphs [0015], [0017], [0051], [0064] and [0074] whereas claim 11 (granted claim 10) is based on paragraphs [0040], [0109] and [0116]. Furthermore, claim 13 (granted claim 12) finds a basis in paragraphs [0014], [0015], [0019], [0243] and [0313] and claim 18 (granted claim 17) in paragraphs [0125] and [0130].

- 13.3 The Opposition Division refers to the Guidelines for Examination at the EPO, Part F - Chapter VI-2, 1.3 which state "the expression **"the same invention"** in Art. 87(1) means that the subject-matter of a claim in a European application may enjoy the priority of a previous application only if the skilled person can derive the subject-matter of the claim **directly and unambiguously**, using common general knowledge, from the previous application **as a whole**. This means that the specific combination of features present in the claim must at least **implicitly** be disclosed in the previous application (see F-VI, 2.2 and G 2/98)" (emphasis added by the Division).

First, concerning claim 1, the following features are disclosed in the respective paragraphs of D7. Paragraph [0019]: plants comprising a, e.g. recombinantly expressed, AAD-1 gene, i.e. transgenic plants (see paragraphs [0123] and [0124]). The gene encodes an aryloxyalkanoate dioxygenase enzyme that is capable of degrading phenoxy auxin and arylphenoxypropionate herbicides. Paragraph [0094] mentions that "the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing", i.e. AAD-1 nucleotide (SEQ ID NO: 3-5) and amino acid (SEQ ID NO: 9) sequences, see paragraphs [0035]-[0037] and [0040]. According to paragraph [0100] "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", for example "stringent" conditions for hybridization, see paragraph [0101]. According to paragraph [0116] "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".

Second, concerning claim 2, the following features are disclosed in the respective paragraphs of D7. In addition to the paragraphs indicated above in conjunction with claim 1, the enzymatic activities of the claimed AAD-1 protein are specified in paragraphs [0019] and [0061]: degradation of phenoxy acid and aryloxyphenoxypropionate herbicides (claim 2 (ii) and (iii)), paragraph [0051]: degradation of the R-enantiomer of the chiral phenoxy auxins in addition to achiral phenoxy auxins (claim 2 (i)).

Third, concerning claim 10, the feature "plants resistant to a 2,4-D herbicide" is disclosed in paragraphs [0015] to [0019], [0051] and [0060] to [0062] of D7.

Forth, concerning claim 11, the following features are disclosed in the respective paragraphs of D7. "SEQ ID NO: 9" encoding native AAD-1: paragraph [0040], "a variant thereof": paragraph [0109]: "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". Paragraph [0116] specifies that "the variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) have the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%".

Fifth, concerning claim 13, the following features are disclosed in the respective paragraphs of D7. Paragraphs [0008] and [0014] list MCPA, diclorprop and further aryloxyalkanoate herbicides including 2,4-D, haloxyfop, quizalofop and diclofop; paragraphs [0051] and [0313] further disclose mecoprop and fluazifop. However, the herbicides fenoxaprop, metamifop, cyhalofop and clodinoxop are not mentioned in the description of D7.

Sixth, concerning claim 14, for example paragraph [0015] mentions the stacking with one or more other herbicide resistance genes.

Seventh, concerning claim 15, paragraph [0072] mentions insect resistance traits, e.g. *Cry1F*, *Cry1Ab*, *Cry34/35*. There is, however, no specific disclosure in D7 of the organisms listed in claim 15, namely *Bacillus thuringiensis*, *Photorhabdus* and *Xenorhabdus*.

Eighth, concerning claim 17, paragraph [0058] specifies that "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".

Ninth, concerning claim 18, it is noted that plant parts, seeds and tissues are referred to in paragraphs [0125] and [0126]. The term "progeny" is used in paragraph [0130], however, not in conjunction with claimed subject matter. The term "asexual propagate" is not mentioned in the description of D7.

Taken together, the Opposition Division considers that claims 13, 15 and 18 are not entitled to priority. The relevant date is therefore the international filing date of 2 May 2005. Accordingly, documents D12 and D15 might become relevant for the assessment of novelty and/or inventive step of said claims.

14 Article 100(a) EPC in conjunction with Article 54 EPC

- 14.1 The Opponent raises objections concerning lack of novelty of the subject matter of granted claims 1, 9, 12 and 17 in view of the disclosure of D8. The document describes transgenic plants expressing *tfdA* from *Alcaligenes eutrophus* under control of the CaMV 35 S promoter resulting in resistance to 2,4-D herbicide. The *tfdA* coding region, disclosed in D2, hybridizes under stringent conditions to the nucleic acid encoding a protein of SEQ ID NO: 9, as derivable from the experimental data provided in document D1. TfdA possesses a 2,4-D degrading activity, see page 3, paragraph 11 of the application. A similar argumentation is formulated for said claims in view of the disclosure of documents D9 to D11 and late document D12.
- 14.2 The Proprietor submits that granted claim 1 was split into two independent claims, namely claims 1 and 2. Claim 1 was amended and further defined by structural terms, i. e. by including a reference to 70% identity. Since the *tfdA* encoding gene and the nucleic acid in SEQ ID NO: 9 share only 32% identity, the objection is overcome. Claim 2 is defined by functional terms, i.e. enzymatic activities, which are not assigned to *tfdA*. Thereby the novelty objection is overcome.
- 14.3 With regard to claim 1 the Opposition Division notes that documents D8 to D12 disclose transgenic plants (tobacco, cotton or mustard) comprising an isolated polynucleotide (*tfdA* gene from *Alcaligenes eutrophus*/*Ralstonia eutropha*) that encodes a protein (2,4-dichlorophenoxyacetate monooxygenase, DPAM) that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide (2,4-D) and an aryloxyphenoxypropionate herbicide wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9, as evidenced by experimental data shown in D1, wherein said plant expresses said polynucleotide and produces said protein. The following technical feature is not disclosed in D8 to D12: "wherein said protein is at least 70% identical with a protein encoded by a nucleic acid

molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5". Indeed, as derivable from sequence alignments between the TFDA protein of D8 (see also D2) and the AAD-1 protein of the patent presented in document D4, the identity of the claimed proteins encoded by SEQ ID NO: 3 (protein with SEQ ID NO: 9) with the protein disclosed in document D8 is merely 32% which lies far out of the claimed range of "at least 70% identity". The same holds true *mutatis mutandis* for nucleic acids in SEQ ID NO: 4 and 5 which encode proteins that only differ in one or two amino acid positions from the protein in SEQ ID NO: 9 by that resulting in comparable identities.

Accordingly, claim 1 is novel over documents D8 to D12.

With regard to claim 2 the Opposition Division notes that documents D8 to D12 disclose transgenic plants (tobacco, cotton or mustard) comprising an isolated polynucleotide (tfdA gene from *Alcaligenes eutrophus*/*Ralstonia eutropha*) that encodes a protein (2,4-dichlorophenoxyacetate monooxygenase, DPAM) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9, as evidenced by experimental data shown in D1, wherein said plant expresses said polynucleotide and produces said protein. The following technical features are not disclosed in D8 to D12:

"the protein enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
- (ii) an aryloxyphenoxypropionate herbicide, or
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide".

Accordingly, claim 2 is novel over documents D8 to D12.

Claims 3-19 are dependent on or make direct reference to claim 1 or 2 and are therefore novel as well.

Taken together, the Opposition Division considers that the subject matter of claims 1 to 19 is novel.

15 Article 100(a) EPC in conjunction with Article 56 EPC

- 15.1 The Opponent raises a first objection against the subject matter of granted claims 2-7, 11 and 13 in view of document D8 in combination with any of documents D19-D22. Said claims make reference to other herbicide genes, particularly glufosinate, glyphosate, dicamba and ALS inhibitor herbicide tolerance genes. Since the application does not claim an interaction between

the presence of the phenoxy and arylphenoxypropionate herbicide degrading transgene and said other herbicide tolerance genes, the technical problems can be separated and amount to the provision of plants with these other tolerance genes in addition to the transgenic plants of claim 1. Said other herbicide genes are described in documents D19, D20 (glufosinate), D21 (glyphosate), D22 (dicamba) and D23 (ALS inhibitor). A second objection is raised against claim 8 in view of document D8. According to the Opponent, the choice of a particular plant can not provide an inventive step. A third objection is raised against claim 10 pertaining to the rdpA gene. The technical problem, provision of transgenic plants with an alternative phenoxy or arylphenoxypropionate herbicide degrading enzyme encoding transgenes, is obvious starting from document D8 as closest prior art in view in combination with document D15. A fourth objection arises for claims 14 and 15 starting from document D8 as closest prior art. The other insect-resistant genes of claim 14 and agronomic trait genes of claim 15 are known from documents D18 and D24, respectively. Again, since the application does not claim an interaction between the presence of the phenoxy and arylphenoxypropionate herbicide degrading transgene and said other transgenes, the technical problems can be separated and amount to the provision of plants with these other tolerance genes in addition to the transgenic plants of claim 1. A fifth objection is formulated against claim 18 in view of document D8 as closest prior art. Said document discloses the isolation of DNA which involves grinding of tissues as derivable from document D25. The difference in source (maize plant) has no specific advantage and therefore the choice of a particular plant can not provide an inventive step.

- 15.2 The Proprietor submits that the structural feature "70% identity" introduced into granted claim 1 and the functional features relating to specific enzymatic activities specified in claim 2 recite additional features not disclosed in any of the documents D8 to D11 and D18 to D24. In this context, the Proprietor further reiterates his view that documents D12 and D15 are not citable prior art.
- 15.3 In this respect, the Opposition Division refers to the "problem and solution approach" that is usually used to assess inventive step. According to the Case Law of the Boards of Appeal of the EPO, 6th edition, 2010 (page 162), this "consists essentially of
- (a) identifying the "closest prior art",
 - (b) assessing the technical results (or effects) achieved by the claimed invention when compared with the "closest state of the art" established,

(c) defining the technical problem to be solved as the object of the invention to achieve these results, and

(d) examining whether or not a skilled person, having regard to the state of the art within the meaning of Art. 54(2) EPC, would have suggested the claimed technical features in order to obtain the results achieved by the claimed invention”.

Claim 1 is directed to a transgenic plant comprising a polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate (AOPP) herbicide, wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5, wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO: 9.

In accordance with the Opponent and the Proprietor, the Opposition Division considers document D8 to represent prior art disclosing subject matter conceived for the same purpose or aiming at the same objective.

Said document discloses transgenic plants (tobacco) comprising an isolated polynucleotide (tfdA) that encodes a protein (DPAM) that enzymatically degrades a phenoxy auxin herbicide (2,4-D) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The subject-matter of claim 1 differs from these transgenic plants in that said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5 (AAD-1, rpdA gene).

The objective problem to be solved by the present application resides in the provision of transgenic plants resistant to a phenoxy auxin herbicide and an AOPP herbicide.

The solution provided are plants expressing AAD-1 (rpdA) from *Sphingobium herbicidovorans*.

Document D14 discloses the rpdA gene and protein sequences from *Sphingobium herbicidovorans*. The document is, however, silent on the use of the AAD-1 encoding gene to generate transgenic plants with herbicide resistance.

As stated above, document D8 describes transgenic plants with 2,4-D resistance resulting from expression of a 2,4-D degrading enzyme (tfdA) from *Alcaligenes eutrophus*. A person skilled in the art could in principle have

combined the teaching of D8 and D14 and arrive at the claimed solution. However, there is no suggestion in any of the cited document that would have incited him to do so. In fact, there is no indication in document D14 that AAD-1 would degrade AOPP herbicides and thereby generating plants that are resistant to both phenoxy auxin **and** AOPP herbicides.

For these reasons, the solution proposed in claim 1 involves an inventive step.

Claim 2 is directed to a transgenic plant comprising a polynucleotide that encodes a protein that enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
 - (ii) an aryloxyphenoxypropionate herbicide, or
 - (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,
- wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The Opposition Division considers document D8 to represent prior art disclosing subject-matter conceived for the same purpose or aiming at the same objective.

Said document discloses transgenic plants (tobacco) comprising an isolated polynucleotide (lfdA) that encodes a protein (DPAM) that enzymatically degrades a phenoxy auxin herbicide (2,4-D) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The subject-matter of claim 2 differs from these transgenic plants in that said protein enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
- (ii) an aryloxyphenoxypropionate herbicide, or
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

The objective problem to be solved by the present application resides in the provision of transgenic plants resistant to an R-enantiomer of chiral phenoxy auxin herbicides; and to a phenoxy auxin and an AOPP herbicide.

The solution provided are plants expressing AAD-1 (rpdA) from *Sphingobium herbicidovorans*.

Document D14 discloses the rpdA gene and protein sequences from *Sphingobium herbicidovorans*. The documents is, however, silent on the use of the AAD-1 encoding gene to generate transgenic plants with herbicide resistance.

As stated above, document D8 describes transgenic plants with 2,4-D resistance resulting from expression of a 2,4-D degrading enzyme (tfdA) from *Alcaligenes eutrophus*. A person skilled in the art could in principle have combined the teaching of D8 and D14 and arrive at the claimed solution. However, there is no suggestion in any of the cited document that would have incited him to do so. In fact, there is no indication in document D14 that AAD-1 would degrade an R-enantiomer of chiral phenoxy auxin herbicides or AOPP herbicides and thereby generating plants that are resistant to phenoxy auxin and AOPP herbicides specified in claim 2.

For these reasons, the solution proposed in claim 2 involves an inventive step.

The same applies *mutatis mutandis* to claims 3-19 which are dependent on or directly referring to claims 1 and 2.

The same reasoning applies *mutatis mutandis* to claims 13, 15 and 18 for the combination of document D8 with document D15 taking into consideration the comments concerning priority of said claims, see section 7.2.3 above.

Considering that the objective technical problem stated above is solved by the claimed transgenic plants as well as their non-obviousness, the Opposition Division considers that the claimed subject matter is inventive.

16 Article 100(b) EPC in conjunction with Article 83 EPC

- 16.1 The Opponent raises the objection that the granted claimed subject-matter is insufficiently disclosed. The application provides support and disclosure only for a limited number of methods and polynucleotides, i.e. those having SEQ ID NO: 3-5 which encode a polypeptide having SEQ ID NO: 9 and transgenic plants comprising these whereas the hybridizing polynucleotides encompass polynucleotides encoding a protein with only 32% amino acid sequence identity, thereby not being congruent with the disclosure of the application.
- 16.2 The Proprietor submits that by including the structural term into claim 1 and the functional terms into claim 2 said claims are further limited and thereby the Opponent's objections are rendered moot.
- 16.3 Indeed, the proteins of claim 1 are on one hand defined by their ability to degrade a phenoxy auxin or an AOPP herbicide and on the other hand limited by the ability of the corresponding polynucleotides to hybridise under stringent conditions with the nucleic acid encoding the protein with SEQ ID NO: 9 and their identity of at least 70% to the nucleic acids of SEQ ID NO: 3, 4 or 5. Consequently, the proteins referred to in claim are both functionally and structurally limited. The proteins can be determined by routine experimentation, i. e. by determination of the specified enzyme activities and

sequence identity using alignment, thereby not involving any undue burden. The claimed subject matter is thus congruent with the disclosure of the application.

The same holds true *mutatis mutandis* for claim 2 in which the proteins are defined by their ability to degrade specific R-enantiomers, an AOPP herbicide and optionally a phenoxy auxin and on the other hand limited by the ability of the corresponding polynucleotides to hybridise under stringent conditions with the nucleic acid encoding the protein with SEQ ID NO: 9.

Consequently, the proteins referred to in claim 2 are both functionally and structurally limited. The proteins can be determined by routine experimentation, i. e. by determination of the specified enzyme activities and sequence identity using alignment, thereby not involving any undue burden. The claimed subject matter is thus congruent with the disclosure of the application.

Taken together, the Opposition Division considers that the subject matter of claims 1 and 2 is disclosed in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

17 Double patenting

- 17.1 The Opponent remarks that granted claim 10 claims the same subject matter as claim 5 of EP 1 740 039 granted for the parent application whereas according to an accepted principle in most patent systems two patents can not be granted to the same applicant for one invention. That principle of prohibition of double patenting was accepted obiter dictum by the Enlarged Board of Appeal in decisions G 1/05 and G1/06.
- 17.2 The Proprietor submits that double patenting is not listed as a ground for opposition in Article 100 EPC and therefore not a basis to revoke the patent.
- 17.3 The Opposition Division agrees that double patenting is indeed not listed as a ground for opposition in Article 100 EPC and therefore not a basis to revoke the patent.

18 Decision

The grounds of opposition under Article 100(a), (b) and (c) EPC do not prejudice maintenance of the patent in amended form.

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Taking into account the amendments made by the Proprietor the patent and the invention, to which it relates, meet the requirements of the EPC, Article 101(3)(a) EPC.

According to Article 106 EPC, this decision is subject to appeal. If such appeal is filed, it shall have suspensive effect. In accordance with Article 107 EPC, any party to the proceedings adversely affected by the decision may appeal. Notice of appeal must be filed at the EPO within two months after the date of notification of the decision appealed. The notice shall not be deemed to have been filed until the appeal fee has been paid. In case of an appeal, a written statement setting out the grounds for appeal must be filed within four months from notification of this decision (Article 108 EPC).

I. Summary of Facts and Submissions

- 1 The European patent EP 2 298 901 B1, Application number 10 012 201.9, titled "Novel herbicide resistance genes (hereinafter: "the patent") in the name of Dow AgroSciences, LLC (US) was granted on 6 March 2013.
- 2 The Opponent (Bayer CropScience NV (BE)), represented by Mr. J. Desomer of Bayer CropScience NV (BE), opposed the patent in its whole extent under Articles 100(a), 100(b) and 100(c) EPC. The opposition was filed in electronic form on 6 December 2013.
- 3 The Proprietor, represented by Dr. J. Zwicker of ZSP Patentanwälte (DE), requested that the opposition be rejected and the patent be maintained on the basis of amended claims as 1 to 11 of the Main Request provided in electronic form on 28 July 2014.
- 4 Both parties requested oral proceedings.
- 5 On 7 December 2015 the opposition division issued a summons to attend oral proceedings on 5 April 2016.
- 6 On 29 January 2016 the Opponent informed the Division that no further submissions will be made and withdrew his request for oral proceedings.
- 7 On 29 January the Proprietor conditionally withdrew his request for oral proceedings.
- 8 In the brief communication dated 11 February 2016 the Division informed the parties that the summons to attend oral proceedings have been cancelled, that the procedure will be continued in writing and further of the preliminary intention to grant a patent on the basis of the Main Request, filed in electronic form on 28 July 2014.

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- 9 In reply to the communication the Proprietor submitted an amended page 3 of the description on 16 February 2016. The Opponent was given the chance to provide comments within two months which he did not.
- 10 The numbering of the cited **documents** follows the numbering of references from the Opponent's submissions and will be adhered to in the rest of the procedure:
- D1 Autoradiographs from hybridisation experiments between AAD-1 and tfdA nucleic acids
- D2 WOLFGANG R STREBER ET AL: "Analysis, Cloning, and High-Level Expression of 2,4-Dichlorophenoxyacetate Monooxygenase Gene tfdA of *Alcaligenes eutrophus* JMP134", JOURNAL OF BACTERIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 169, no. 7, 1 July 1987 (1987-07-01), pages 2950-2955, XP001315263, ISSN: 0021-9193
- D3 FUKUMORI FUMIYASU ET AL: "Alcaligenes eutrophus JMP134 "2,4-dichlorophenoxyacetate monooxygenase" is an alpha-ketoglutarate-dependent dioxygenase", JOURNAL OF BACTERIOLOGY, vol. 175, no. 7, 1993, pages 2083-2086, ISSN: 0021-9193
- D4 Amino acid sequence alignment between TFDA and AAD-1 proteins
- D5 HULSE J D ET AL: "CARNITINE BIOSYNTHESIS BETA HYDROXYLATION OF TRI METHYL LYSINE BY AN ALPHA KETO GLUTARATE DEPENDENT MITOCHONDRIAL DI OXYGENASE", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 253, no. 5, 1978, pages 1654-1659, ISSN: 0021-9258
- D6 EICHHORN ERIC ET AL: "Characterization of alpha-ketoglutarate-dependent taurine dioxygenase from *Escherichia coli*", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 37, 1997, pages 23031-23036, ISSN: 0021-9258
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- D7 Priority document USSN 60/567,052
- D8 STREBER W R ET AL: "TRANSGENIC TOBACCO PLANTS EXPRESSING A BACTERIAL DETOXIFYING ENZYME ARE RESISTANT TO 2,4-D",
BIOTECHNOLOGY. THE INTERNATIONAL MONTHLY FOR INDUSTRIAL BIOLOGY, NATURE PUBLISHING GROUP, US, vol. 7, no. 8, 1 January 1989 (1989-01-01), pages 811-816, XP008062554,
ISSN: 0733-222X, DOI: 10.1038/NBT0889-811
- D9 LYON B R ET AL: "EXPRESSION OF A BACTERIAL GENE IN TRANSGENIC TOBACCO PLANTS CONFERS RESISTANCE TO THE HERBICIDE 2 4-D",
PLANT MOLECULAR BIOLOGY,
vol. 13, no. 5, 1989, pages 533-540,
ISSN: 0167-4412
- D10 LYON BRUCE R ET AL: "Cotton plants transformed with a bacterial degradation gene are protected from accidental spray drift damage by the herbicide 2,4-dichlorophenoxyacetic acid",
TRANSGENIC RESEARCH,
vol. 2, no. 3, 1993, pages 162-169,
ISSN: 0962-8819
- D11 BAYLEY C ET AL: "ENGINEERING 2,4-D RESISTANCE INTO COTTON",
THEORETICAL AND APPLIED GENETICS, SPRINGER, BERLIN, DE, vol. 83, no. 5, 1 January 1992 (1992-01-01), pages 645-649, XP002037287,
ISSN: 0040-5752, DOI: 10.1007/BF00226910
- D12 BISHT NAVEEN CHANDRA ET AL: "Development of 2,4-D-resistant transgenics in Indian oilseed mustard (Brassica juncea)",
CURRENT SCIENCE (BANGALORE),
vol. 87, no. 3, 10 August 2004 (2004-08-10), pages 367-370,
ISSN: 0011-3891
- D13 Alignment of the nucleotide sequence of SEQ ID NO: 3 of the patent with the nucleotide sequence of entry AF516751 (Rhodospirillum rubrum sp P230 rdpA gene)

- D14 AF516751 nucleotide sequence, version of June 26, 2003
- D15 SCHLEINITZ KATHLEEN M ET AL: "Localization and characterization of two novel genes encoding stereospecific dioxygenases catalyzing 2(2,4-dichlorophenoxy)propionate cleavage in *Delftia acidovorans* MC1", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 70, no. 9, 1 September 2004 (2004-09-01), pages 5357-5365, XP002549093, ISSN: 0099-2240, DOI: 10.1128/AEM.70.9.5357-5365.2004
- D16 Alignment of the nucleotide sequence of SEQ ID NO: 3 of the patent with the nucleotide sequence entry AJ628859 (*Sphingomonas herbicidovorans* InpA gene)
- D17 AJ628859 nucleotide sequence, version of March 3, 2004
- D18 WO 97/13402 A1 (DOWELANCO [US]) 17 April 1997 (1997-04-17)

II. Reasons for the Decision

11 Admissibility

The opposition is admissible since it fulfills the requirements of Article 99(1) and Rule 76 EPC.

12 Article 100(c) EPC in conjunction with Articles 76(1) and 123(2) EPC

12.1 The requirements of Rule 80 EPC appear to be respected.

12.2 The Opponent states that the subject matter of the granted claims goes beyond the disclosure of the application as originally filed. The Opponent further mentions that the current divisional application is an exact copy of the parental application. Accordingly, any such subject matter also gives rise to objections under Article 76(1) EPC.

The following claims are objected to:

1. The exchange of the teleological functional definition in claim 1 "that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide" with the biochemical functional definition "having alpha-ketoglutarate-dependent dioxygenase activity" and further absence of an indication of the substrate of the enzyme.

2. Claims 2 and 4 which depend on claim 1 were indicated to be based on originally filed claims 45 and 46 which in turn depend on claim 44 requiring "aryloxyalkanoate dioxygenase activity"; this activity is, however, no longer present in said claims.

- 12.3 The Proprietor confirms that the opposed patent was granted for a divisional application which was filed as an exact copy of the parent application as originally filed. Accordingly, any subject matter having a basis in the parent application as originally filed (Article 76(1) EPC) has also a basis under Article 123(2) EPC in the application as originally filed. He submits the following with regard to the amended claims of the Main Request.

ad 1. The functional characterization objected to by the opponent is re-introduced into claim 1. Further, paragraphs [0018], [0045] to [0047], [0085], [0086], [0120] and [0142] are indicated as basis for claim 1.

ad 2. Due to their dependency on claims 1 and 2, and because aryloxyalkanoate is a more generic term encompassing phenoxy auxins and AOPPs, aryloxyalkanoate dioxygenase activity is present in claims 3 and 5 (granted claims 2 and 4). Moreover, reference is made to paragraph [0015] which comprise a definition of the term "aryloxyalkanoate". Additionally, paragraph [0074] is indicated as a basis for claim 4 (granted claim 3).

Furthermore, the Patentee indicates originally filed claim 47, paragraphs [0020], [0074] and [0084] in addition to the parts of the description mentioned above in conjunction with claim 1 as a basis for the subject matter of newly introduced claim 2 in which the protein is defined in functional terms including the re-introduced stated feature.

- 12.4 In agreement with the parties, the Opposition Division notes that the current divisional application is an exact copy of the parental application as originally filed. Consequently, any subject matter having a basis in the parent application as originally filed (Article 76(1) EPC) would have also a basis under Article 123 (2) EPC in the application as originally filed and *vice versa*.

With regard to a basis in the application as originally filed the Opposition Division observes the following.

First, concerning claim 1, the teleological functional feature "that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide" was re-introduced into said claim. Basis for the wording of said feature can be found for example in paragraphs [0018] and [0086].

Second, concerning claims 3 and 5, the description specifies in paragraphs [0085] and [0086] that "[t]he **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**" and "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 (**Aryloxy Alkanoate Dioxygenase**) genes and proteins. 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" (emphasis added by the division). Accordingly, the enzyme of the invention is a α -ketoglutarate-dependent dioxygenase, more specifically an aryloxyalkanoate dioxygenase (AAD-1) which degrades phenoxy auxin and aryloxyphenoxypropionate herbicides. The feature "wherein a nucleic acid molecule hybridizes under stringent conditions with the full complement of a sequence selected from SEQ ID NO: 5, 3 or 4" is derivable from the wording in paragraph [0112]: "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" plus paragraph [0124]: "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" and paragraph [0126]: "Hybridisation 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".

"Wherein said protein is at least 70% identical" is derivable from paragraph [0142]: "Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having 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". Paragraphs [0045] to [0047] disclose "a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5" as follows: "SEQ ID NO: 3 is the nucleotide sequence of *AAD-1 (vi)* from *Sphingobium herbicidovorans*. 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. SEQ ID NO: 5 is a "plant-optimized" DNA sequence *AAD-1 (v3)*."

Third, concerning claim 2, the feature "a polynucleotide" is based on claim 44. The polynucleotide "encoding a protein that enzymatically degrades (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins", see paragraph [0074],

"(ii) an aryloxyphenoxypropionate herbicide, or

(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide", see paragraph [0020]. The feature "wherein a nucleic acid molecule hybridizes under stringent conditions with the full complement of a sequence selected from SEQ ID NO: 5, 3 or 4" is derivable from the wording in paragraph [0112]: "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" plus paragraph [0124]: "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" and paragraph [0126]: "Hybridisation 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".

For the reasons above, the Opposition Division considers that the claimed subject-matter is derivable from the application as originally filed.

The wording of new claim 2 does not to extend the scope of protection conferred by the patent as granted. The Opposition therefore considers that the requirements of Article 123(3) EPC are fulfilled.

13 **Priority entitlement (Article 87 EPC)**

13.1 The Opponent raises the objection that the claims as granted are not entitled to the priority date of 30 April 2004 (US60/567,052; document D7) and the relevant date being the international filing date of 2 May 2005. Thereby, documents D12 and D15 are prior art within the meaning of Article 54(2) EPC. According to the Opponent, the priority application contains less teaching than the subsequently filed parent and divisional application, for example no claims were filed with the priority document. In addition, a number of sequences, figures and examples are missing from said document.

More specifically the Opponent raises the following objections. With regard to claim 1: the priority document merely discloses proteins having α -ketoglutarate dependent dioxygenase activity in connection with degradation of phenoxy auxins or aryloxyphenoxyalkanoates, there is no specific link between the stringency conditions for hybridization and the sequences of SEQ ID NO: 3-5 and additionally, there is no connection between plant promoters and said hybridising nucleic acids. Claim 2 mentions codon bias optimized for plants whereas paragraphs [0111] and [0222] indicate codon bias "more closely aligned to the plant target sequence". Claim 3 specifies the proteins have R-specific α -ketoglutarate dependent dioxygenase activity to 2,4-D which is an achiral phenoxy auxin. The priority application does not provide for R-specific dioxygenases relative to 2,4-D. Claim 4 refers to nucleotide of SEQ ID NO: 5, said sequence differs in the 3' end in the priority application and the patent. The general language of claim 6 is not derivable from the priority application which uses a more limited language on page 56.

13.2 With respect to the claims that are in his view objected to by the Opponent under novelty or inventive step using documents D12 and D15, the Proprietor submits that the subject matter of claim 1 had a basis in D7, particularly in paragraphs [0092] to [0106], [0059], [0019], [100], [0101], [0035]-[0037], [0116], [0094], [0093] and [0132]. Furthermore, the 3' end of the sequence in SEQ ID NO: 5 is identical in both the priority document and the patent. In addition to the parts referred to in conjunction with claim 1, the Proprietor indicates paragraphs [0019], [0051] and [0061] as a basis for claim 2. The amended enzymatic activity in claim 4 (granted claim 3) has a basis in paragraph [0051], whereas claim 6 (granted claim 5) is based on paragraph [140], pages 49 and 51 and Table 14. Claim 8 (granted claim 7) finds a basis in paragraph [0019] and claim 10 (granted claim 9) in paragraph [0092] of the priority document D7.

13.3 The Opposition Division refers to the Guidelines for Examination at the EPO, Part F - Chapter VI-2, 1.3 which state "the expression **"the same invention"** in Art. 87(1) means that the subject-matter of a claim in a European application may enjoy the priority of a previous application only if the skilled person can derive the subject-matter of the claim **directly and unambiguously**, using common general knowledge, from the previous application **as a whole**. This means that the specific combination of features present in the claim must at least **implicitly** be disclosed in the previous application (see F-VI, 2.2 and G 2/98)" (emphasis added by the Division).

First, concerning claim 1, the following features are disclosed in the respective paragraphs of D7. Paragraphs [0092] to [0106]: polynucleotides encoding an α -ketoglutarate-dependent dioxygenase (paragraph [0059], which is capable of degrading phenoxy auxin and arylphenoxypropionate herbicides (paragraph [0019]). Paragraph [0094] mentions that "the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing", i.e. AAD-1 nucleotide (SEQ ID NO: 3-5) and amino acid (SEQ ID NO: 9) sequences, see paragraphs [0035]-[0037] and [0040]. Comparing the sequence with SEQ ID NO: 5 in the priority document D7 with that of the patent it appears that both comprise an identical 3' end consisting of CTTAATCACCTAGAGCTC. According to paragraph [0100] "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", for example "stringent" conditions for hybridization, see paragraph [0101]. According to paragraph [0116] "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". The polynucleotide "can be placed under the control of a promoter in a host cell of interest", see paragraph [0093], "either by constitutive or inducible promoters in the plant cell", see paragraph [0132].

Second, concerning claim 2, the following features are disclosed in the respective paragraphs of D7. In addition to the paragraphs indicated above in conjunction with claim 1, the enzymatic activities of the claimed AAD-1 protein are specified in paragraphs [0019] and [0061]: degradation of phenoxy acid and aryloxyphenoxypropionate herbicides (claim 2 (ii) and (iii)), paragraph [0051]: degradation of the R-enantiomer of the chiral phenoxy auxins in addition to achiral phenoxy auxins (claim 2 (i)).

Third, concerning claim 4, the amended wording "R-specific alpha-ketoglutarate dependent dioxygenase activity relative to phenoxy auxins" is based on paragraph [0051].

Fourth, concerning claim 6, paragraph [140] mentions plant promoter and promoters of viral origin. Furthermore, paragraphs [0193] and [0199] make reference to the "CsVMV promoter". However, the person skilled in the art is aware of the full name "cassava vein mosaic virus promoter" as defined in the application as originally filed on page 66 in legend of Table 14.

Fifth, concerning claim 8, paragraph [0019] mentions "methods of using an AAD-1 gene as selectable marker for identifying plant cells and whole plants transformed with AAD-1". Although the paragraph does not make reference to the steps of collecting and assaying a sample from the plant their presence in the claimed method is considered common knowledge.

Sixth, concerning claim 10, paragraph [0092] mentions "methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity" using primers for PCR or probes.

Taken together, the Opposition Division considers that the claims are entitled to priority. Accordingly, documents D12 and D15 do not form prior art within the meaning of Article 54(2) EPC.

- 14.1 The Opponent raises objections concerning lack of novelty of the subject matter of granted claims 1, 5, 7, 8 and 9 in view of the disclosure of D8. The document describes transgenic plants expressing tfdA from *Alcaligenes eutrophus* under control of the CaMV 35 S promoter resulting in resistance to 2,4-D herbicide. The tfdA coding region, disclosed in D2, hybridizes under stringent conditions to the nucleic acid encoding a protein of SEQ ID NO: 9, as derivable from the experimental data provided in document D1. TfdA possesses a 2,4-D degrading activity, see page 3, paragraph 11 of the application. A similar argumentation is formulated for said claims in view of the disclosure of document D9 for claims 1 and 5; and further in view of documents D10 and D11 for claims 1, 5, 7 and 9.
- 14.2 The Proprietor submits that granted claim 1 was split into two independent claims, claims 1 and 2. Claim 1 was amended and further defined by structural terms, i. e. by including a reference to 70% identity. Since the tfdA encoding gene and the nucleic acid in SEQ ID NO: 9 share only 32% identity the objection is overcome. Claim 2 was defined by functional terms, i.e. enzymatic activities, which are not assigned to tfdA. Thereby the novelty objection is overcome.
- 14.3 With regard to claim 1 the Opposition Division notes that documents D8 to D11 disclose transgenic plants (tobacco, cotton or mustard) comprising an isolated polynucleotide (tfdA gene from *Alcaligenes eutrophus*/*Ralstonia eutropha*) that encodes a protein (2,4-dichlorophenoxyacetate monooxygenase, DPAM) that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide (2,4-D) and an aryloxyphenoxypropionate herbicide wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9, as evidenced by experimental data shown in D1, wherein said plant expresses said polynucleotide and produces said protein. The following technical feature was not disclosed in D8 to D12: "wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5". Indeed, as derivable from sequence alignments between the TFDA protein of D8 (see also D2) and the AAD-1 protein of the patent presented in document D4, the identity of the claimed proteins encoded by SEQ ID NO: 3 (protein with SEQ ID NO: 9) with the protein disclosed in document D8 is merely 32% which lies far out of the claimed range of "at least 70% identity". The same holds true *mutatis mutandis* for nucleic acids in SEQ ID NO: 4 and 5 which encode proteins that only differ in one or two amino acid positions from the protein in SEQ ID NO: 9 by that resulting in comparable identities.

Accordingly, claim 1 is novel over documents D8 to D11.

With regard to claim 2 the Opposition Division notes that documents D8 to D11 disclose transgenic plants (tobacco, cotton or mustard) comprising an isolated polynucleotide (tfdA gene from *Alcaligenes eutrophus*/*Ralstonia eutropha*) that encodes a protein (2,4-dichlorophenoxyacetate monooxygenase, DPAM) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9, as evidenced by experimental data shown in D1, wherein said plant expresses said polynucleotide and produces said protein. The following technical features were not disclosed in D8 to D11:

"the protein enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
- (ii) an aryloxyphenoxypropionate herbicide, or
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide".

Accordingly, claim 2 is novel over documents D8 to D11.

Claims 3-11 are dependent on or make direct reference to claim 1 or 2 and are therefore novel as well.

Taken together, the Opposition Division considers that the subject matter of claims 1 to 11 is novel.

15 Article 100(a) EPC in conjunction with Article 56 EPC

- 15.1 The Opponent raises a first objection against the subject matter of granted claim 2 in view of document D8 (or any of documents D9 to D12) in combination with document D18. Said claim merely specifies that the polynucleotide comprises a codon bias that is optimized for expression in plants. Such codon optimization is however common knowledge, as derivable from document D18 cited on page 58 of the application; further the optimization of AAD-1 was outsourced and therefore a workshop adaption. A second objection is raised against claim 3 in view of document D8 (or any of documents D9 to D12) in combination with D15. The difference, stereospecific (R-specific) activity towards a phenoxy auxin results in the technical problem of provision of stereospecific (R-specific) activity towards a phenoxy auxin. Document D15 discloses such a coding region identical to SEQ ID NO: 3 (with one exception) and encoding an identical protein. The skilled person would substitute the tfdA gene of document D8 with that of document D15 and thereby arrive at the subject matter of claim 3. A third objection is raised against claim 4 with document D8 (or any of documents D9 to D12) as closest

art. The technical problem is the provision of alternative phenoxy auxin herbicide degrading enzyme encoding polynucleotide constructs. Document D14 discloses a nucleotide sequence being codon optimized according to general knowledge to arrive at the nucleotide sequence of SEQ ID NO: 5. A fourth objection is raised against claim 10 starting from document D8 (or any of documents D9 to D12) as closest prior art in combination with common general knowledge. Said claim further specifies the use of an antibody in the detection method. The specification already acknowledges the preparation of antibodies by standard procedures, thereby lacking an inventive step.

- 15.2 The Proprietor submits that the structural feature "70% identity" introduced into granted claim 1 and the functional features relating to specific enzymatic activities specified in claim 2 recite additional features not disclosed in any of the documents cited by the Opponent. In this context, the Proprietor further reiterates his view that documents D12 and D15 are not citable prior art.

In this respect, the Opposition Division refers to the "problem and solution approach" that is usually used to assess inventive step. According to the Case Law of the Boards of Appeal of the EPO, 6th edition, 2010 (page 162), this "consists essentially of

- (a) identifying the "closest prior art",
- (b) assessing the technical results (or effects) achieved by the claimed invention when compared with the "closest state of the art" established,
- (c) defining the technical problem to be solved as the object of the invention to achieve these results, and
- (d) examining whether or not a skilled person, having regard to the state of the art within the meaning of Art. 54(2) EPC, would have suggested the claimed technical features in order to obtain the results achieved by the claimed invention".

Claim 1 is directed to a polynucleotide that encodes a protein having α -ketoglutarate-dependent dioxygenase activity and enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate (AOPP) herbicide, wherein said polynucleotide hybridizes under stringent conditions with the full complement of a sequence in SEQ ID NO: 3, 4 or 5, and wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.

In accordance with the Opponent and the Proprietor, the Opposition Division considers document D8 to represent prior art disclosing subject matter conceived for the same purpose or aiming at the same objective.

Said document discloses an isolated polynucleotide (tfdA) that encodes a protein (DPAM) that enzymatically degrades a phenoxy auxin herbicide (2,4-D) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The subject-matter of claim 1 differs from these transgenic plants in that said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5 (AAD-1, rpdA gene).

The objective problem to be solved by the present application resides in the provision of a polynucleotide encoding a protein that confers resistance to a phenoxy auxin herbicide and an AOPP herbicide in plants.

The solution provided is a polynucleotide encoding AAD-1 (rpdA) from *Sphingobium herbicidovorans*.

Document D14 discloses the rpdA gene and protein sequences from *Sphingobium herbicidovorans*. The document is, however, silent on the expression of the AAD-1 encoding gene under control of a promoter functional in plant cells to generate transgenic plants with herbicide resistance.

As stated above, document D8 describes transgenic plants with 2,4-D resistance resulting from expression of a 2,4-D degrading enzyme (tfdA) from *Alcaligenes eutrophus*. A person skilled in the art could in principle have combined the teaching of D8 and D14 and arrive at the claimed solution. However, there is no suggestion in any of the cited document that would have incited him to do so. In fact, there was no indication in document D14 that AAD-1 would degrade AOPP herbicides and thereby generating plants that are resistant to both phenoxy auxin **and** AOPP herbicides.

For these reasons, the solution proposed in claim 1 involves an inventive step.

Claim 2 is directed to a polynucleotide that encodes a protein that enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
- (ii) an aryloxyphenoxypropionate herbicide, or
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide, wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The Opposition Division considers document D8 to represent prior art disclosing subject-matter conceived for the same purpose or aiming at the same objective.

Said document discloses an isolated polynucleotide (tfdA) that encodes a protein (DPAM) that enzymatically degrades a phenoxy auxin herbicide (2,4-D) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The subject-matter of claim 2 differs from these transgenic plants in that said protein enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
- (ii) an aryloxyphenoxypropionate herbicide, or
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

The objective problem to be solved by the present application resides in the provision of a polynucleotide which when expressed under the control of a promoter functional in plant cells results in resistance to an R-enantiomer of chiral phenoxy auxin herbicides; and to a phenoxy auxin and an AOPP herbicide.

The solution provided are plants expressing AAD-1 (rpdA) from *Sphingobium herbicidovorans*.

Document D14 discloses the rpdA gene and protein sequences from *Sphingobium herbicidovorans*. The documents is, however, silent on the use of the AAD-1 encoding gene to generate transgenic plants with herbicide resistance.

As stated above, document D8 describes transgenic plants with 2,4-D resistance resulting from expression of a 2,4-D degrading enzyme (tfdA) from *Alcaligenes eutrophus*. A person skilled in the art could in principle have combined the teaching of D8 and D14 and arrive at the claimed solution. However, there is no suggestion in any of the cited document that would have incited him to do so. In fact, there is no indication in document D14 that AAD-1 would degrade an R-enantiomer of chiral phenoxy auxin herbicides or AOPP herbicides and whereby its expression under control of a promoter functional in plant cells generates plants that are resistant to phenoxy auxin and AOPP herbicides specified in claim 2.

For these reasons, the solution proposed in claim 2 involves an inventive step.

The same applies *mutatis mutandis* to claims 3-11 which are dependent on or directly referring to claims 1 and 2.

Considering that the objective technical problem stated above is solved by the claimed transgenic plants as well as their non-obviousness, the Opposition Division considers that the claimed subject matter is inventive.

16 **Article 100(b) EPC in conjunction with Article 83 EPC**

16.1 The Opponent raises the objection that granted claims 1, 3 and 6 are insufficiently disclosed. The application provides support and disclosure only for a limited number of methods and polynucleotides, i.e. those having SEQ ID NO: 3-5 which encode a polypeptide having SEQ ID NO: 9 and transgenic plants comprising these whereas the hybridizing polynucleotides encompass polynucleotides encoding a protein with only 32% amino acid sequence identity, thereby not being congruent with the disclosure of the application. Moreover, claim 3 pertains to proteins with R-specific alpha-ketoglutarate-dependent dioxygenase activity relative to a 2,4 D herbicide whereas according to the application 2,4 D is an achiral phenoxy auxin; no disclosure is provided for R-specific dioxygenases relative to 2,4-D.

16.2 The Proprietor submits that by including the structural term into claim 1 and the functional terms into claim 2 said claims are further limited. In addition, claim 4 (granted claim 3) was amended by replacing the reference to 2,4-D with chiral phenoxy auxins. With the amendments made, the Opponent's objections are rendered moot.

16.3 Indeed, the proteins of claim 1 are on one hand defined by their ability to degrade a phenoxy auxin or an AOPP herbicide and on the other hand limited by the ability of the corresponding polynucleotides to hybridise under stringent conditions with the nucleic acid of SEQ ID NO: 3, 4 or 4 and the identity of said protein of at least 70% with a protein encoded by the nucleic acids of SEQ ID NO: 3, 4 or 5. Consequently, the proteins referred to in claim are considered both functionally and structurally limited. The proteins can be determined by routine experimentation, i. e. by determination of the specified enzyme activities and sequence identity using alignment, thereby not involving any undue burden. The claimed subject matter is thus congruent with the disclosure of the application.

The same holds true *mutatis mutandis* for claim 2 in which the proteins are on the hand functionally defined by their ability to degrade specific R-enantiomers, an AOPP herbicide and optionally a phenoxy auxin and on the other hand limited by the ability of the corresponding polynucleotides to hybridise under stringent conditions with the nucleic acid encoding the protein with SEQ ID NO: 9.

Consequently, the proteins referred to in claim 2 are both functionally and structurally limited. The proteins can be determined by routine experimentation, i. e. by determination of the specified enzyme activities and

sequence identity using alignment, thereby not involving any undue burden. The claimed subject matter is thus congruent with the disclosure of the application.

Claim 4 now specifies the encoded proteins as having R-specific alpha-ketoglutarate-dependent dioxygenase activity relative to chiral phenoxy auxins which is congruent with the disclosure of the application, see paragraph [0074] of the application as originally filed.

Taken together, the Opposition Division considers that the subject matter of claims 1, 2 and 4 is disclosed in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

17 Decision

The grounds of opposition under Article 100(a), (b) and (c) EPC do not prejudice maintenance of the patent in amended form.

Taking into account the amendments made by the Proprietor the patent and the invention, to which it relates, meet the requirements of the EPC, Article 101(3)(a) EPC.

According to Article 106 EPC, this decision is subject to appeal. If such appeal is filed, it shall have suspensive effect. In accordance with Article 107 EPC, any party to the proceedings adversely affected by the decision may appeal. Notice of appeal must be filed at the EPO within two months after the date of notification of the decision appealed. The notice shall not be deemed to have been filed until the appeal fee has been paid. In case of an appeal, a written statement setting out the grounds for appeal must be filed within four months from notification of this decision (Article 108 EPC).

I. Summary of Facts and Submissions

- 1 The European patent EP 2 308 977 B1, Application number 10 012 202.7, titled "Novel herbicide resistance gene (hereinafter: "the patent") in the name of Dow AgroSciences, LLC (US) was granted on 6 March 2013.
- 2 The Opponent (Bayer CropScience NV (BE)), represented by Mr. J. Desomer of Bayer CropScience NV (BE), opposed the patent in its whole extent under Articles 100(a), 100(b) and 100(c) EPC. The opposition was filed in electronic form on 6 December 2013.
- 3 The Proprietor, represented by Dr. J. Zwicker of ZSP Patentanwälte (DE), requested that the opposition be rejected and the patent be maintained on the basis of amended claims as 1 to 22 of the Main Request provided in electronic form on 28 July 2014.
- 4 Both parties requested oral proceedings.
- 5 On 7 December 2015 the opposition division issued a summons to attend oral proceedings on 5 April 2016.
- 6 On 29 January 2016 the Opponent informed the Division that no further submissions will be made and withdrew his request for oral proceedings.
- 7 On 29 January the Proprietor conditionally withdrew his request for oral proceedings.
- 8 In the brief communication dated 11 February 2016 the Division informed the parties that the summons to attend oral proceedings have been cancelled, that the procedure will be continued in writing and further of the preliminary intention to grant a patent on the basis of the Main Request, filed in electronic form on 28 July 2014.

- 9 In reply to the communication the Proprietor submitted an amended page 3 of the description on 16 February 2016. The Opponent was given the chance to provide comments within two months which he did not.
- 10 The numbering of the cited **documents** follows the numbering of references from the Opponent's submissions and will be adhered to in the rest of the procedure:
- D1 Autoradiographs from hybridisation experiments between AAD-1 and tfdA nucleic acids
- D2 WOLFGANG R STREBER ET AL: "Analysis, Cloning, and High-Level Expression of 2,4-Dichlorophenoxyacetate Monooxygenase Gene tfdA of *Alcaligenes eutrophus* JMP134", JOURNAL OF BACTERIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 169, no. 7, 1 July 1987 (1987-07-01), pages 2950-2955, XP001315263, ISSN: 0021-9193
- D3 FUKUMORI FUMIYASU ET AL: "Alcaligenes eutrophus JMP134 "2,4-dichlorophenoxyacetate monooxygenase" is an alpha-ketoglutarate-dependent dioxygenase", JOURNAL OF BACTERIOLOGY, vol. 175, no. 7, 1993, pages 2083-2086, ISSN: 0021-9193
- D4 Amino acid sequence alignment between TFDA and AAD-1 proteins
- D5 HULSE J D ET AL: "CARNITINE BIOSYNTHESIS BETA HYDROXYLATION OF TRI METHYL LYSINE BY AN ALPHA KETO GLUTARATE DEPENDENT MITOCHONDRIAL DI OXYGENASE", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 253, no. 5, 1978, pages 1654-1659, ISSN: 0021-9258
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- D14 AF516751 nucleotide sequence, version of June 26, 2003
- D15 SCHLEINITZ KATHLEEN M ET AL: "Localization and characterization of two novel genes encoding stereospecific dioxygenases catalyzing 2(2,4-dichlorophenoxy)propionate cleavage in *Delftia acidovorans* MC1", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 70, no. 9, 1 September 2004 (2004-09-01), pages 5357-5365, XP002549093, ISSN: 0099-2240, DOI: 10.1128/AEM.70.9.5357-5365.2004
- D16 Alignment of the nucleotide sequence of SEQ ID NO: 3 of the patent with the nucleotide sequence entry AJ628859 (*Sphingomonas herbicidovorans* InpA gene)
- D17 AJ628859 nucleotide sequence, version of March 3, 2004
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II. Reasons for the Decision

11 Admissibility

The opposition is admissible since it fulfills the requirements of Article 99(1) and Rule 76 EPC.

12 Article 100(c) EPC in conjunction with Articles 76(1) and 123(2) EPC

- 12.1 The requirements of Rule 80 EPC are respected.
- 12.2 The Opponent states that the subject matter of the granted claims goes beyond the disclosure of the application as originally filed. The Opponent further mentions that the current divisional application is an exact copy of the parental application. Accordingly, any such subject matter gives rise to objections under Article 76(1) EPC.

The following claims are objected to:

1. Claim 1 pertains to a method of controlling a weed in "an area" whereas originally filed claim 13 mentioned "a field". The feature "area" is only mentioned in connection with glyphosate use and occurrence of weed tolerant thereto in paragraphs [0005] and [0007].

2. The combination of the specified enzymatic activity with the hybridisation conditions in claim 1 resulting in a wide range of nucleic acids claimed.

Thus originally filed claim 13 is much more limited as granted claim 1, namely to a method of controlling a weed in a field using AAD-1 or a highly related variant thereof.

3. The same argumentation applies *mutatis mutandis* to claim 19.

- 12.3 The Proprietor confirms that the opposed patent was granted for a divisional application which filed as an exact copy of the parent application as originally filed. Accordingly, any subject matter having a basis in the parent application as originally filed (Article 76(1) EPC) has also a basis under Article 123(2) EPC in the application as originally filed. He submits the following with regard to the amended claims of the Main Request.

ad 1. The technical feature "area" is found in paragraphs [0005], [0007] and [0017]; "glyphosate" is only mentioned by way of example. Therefore the method is not limited to these two feature.

ad 2. The combination of further technical features of claim 1 is derivable from paragraphs [0089], [0149], [0087], [0045]-[0047] and [0120] in combination with paragraphs [0050], [0095], [0112], [0124], [0126], [0127], [0149] and [0151] plus paragraphs [0150] and [0017] of the application as originally filed and published as WO 2005/107437.

ad 3. Claim 20 (granted claim 19) relates to "a field" not to an area. Basis for the claim is found in paragraphs [0020], [0078], [0089] and [0095] and the parts indicated in conjunction with claim 1 plus paragraphs [0078], [0119] and [0158].

Furthermore, the Patentee indicates originally filed claim 13 and paragraphs [0020], [0074] and [0087] and the parts of the description mentioned above in conjunction with claim 1 as basis for the subject matter of newly introduced claims 2 and 21 in which the protein is defined in functional terms.

- 12.4 In agreement with the parties, the Opposition Division notes that the current divisional application is an exact copy of the parental application as originally filed. Consequently, any subject matter having a basis in the parent application as originally filed (Article 76(1) EPC) also has a basis under Article 123(2) EPC in the application as originally filed and *vice versa*.

With regard to a basis in the application as originally filed the Opposition Division observes the following.

First, concerning claim 1, a method of controlling weed is disclosed in paragraphs [0020] and [0089]: "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 feature "area" is derivable from paragraph [0017]: "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)". The Opposition Division further notes that according to the Guidelines for Examination at the EPO, Part H - Chapter V-2, 3.1 "The replacement or removal of a feature from a claim does not violate Art. 123(2) if the skilled person would directly and unambiguously recognise that:

- (i) the feature was not explained as essential in the disclosure;
- (ii) the feature is not, as such, indispensable for the function of the invention in the light of the technical problem the invention serves to solve; and
- (iii) the replacement or removal requires no real modification of other features to compensate for the change".

From the description it is not evident that the feature "field" would be essential or indispensable or its removal would require a modification of the other features to compensate.

"Transgenic plants" are based on claim 5 and paragraph [0149]. Such plants may comprise a polynucleotide "encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide", see for example paragraph [0020].

"Wherein said protein is at least 70% identical" is derivable from paragraph [0142]: "Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having 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". Paragraphs [0045] to [0047] disclose "a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5" as follows: "SEQ ID NO: 3 is the nucleotide sequence of *AAD-1* (v1) from *Sphingobium herbicidovorans*. 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. 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". In paragraph [0050] it is stated that " SEQ ID NO: 9 is the native amino acid sequence encoded by the *AAD-1* (v1) gene from *Sphingobium herbicidovorans*". A polynucleotide may "hybridize under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO: 9", see paragraph [0112]: "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" and paragraph [0124]: "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" and

paragraph [0126]: "Hybridisation 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".

Second, claim 2 is based on granted claim 1; a polynucleotide "encoding a protein that enzymatically degrades (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins", see paragraph [0074],

"(ii) an aryloxyphenoxypropionate herbicide, or

(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide", see paragraph [0020]. The feature "wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9" is derivable from the wording in paragraph [0112]: "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" plus paragraph [0124]: "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" and paragraph [0126]: "Hybridisation 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".

Third, it is noted that the feature "area" is absent in claim 20 whereas the wording "a method of controlling at least one weed in a field" is based on originally filed claim 13 and paragraphs [0020] and [0078]. The other technical features of the claim were already identified in conjunction with claim 1.

Forth, the technical features of claim 21 were already identified in conjunction with claim 2.

For the reasons above, the Opposition Division considers that the claimed subject-matter is derivable from the application as originally filed.

The wording of new claim 2 does not to extend the scope of protection conferred by the patent as granted. The Opposition therefore considers that the requirements of Article 123(3) EPC are fulfilled.

13 **Priority entitlement (Article 87 EPC)**

- 13.1 The Opponent raises the objection that the claims as granted are not entitled to the priority date of 30 April 2004 (US60/567,052; document D7) and the relevant date being the international filing date of 2 May 2005. Thereby, documents D12 and D15 are prior art within the meaning of Article 54(2) EPC. According to the Opponent, the priority application contains less teaching than the subsequently filed parent and divisional application, i. a. no claims were filed with the priority document. In addition, a number of sequences, figures and examples are missing from said document. More specifically, a method of controlling weeds as broadly claimed are more specifically limited in the priority document to plants comprising the AAD-1 gene. Additionally, claim 19 refers to a nucleotide of SEQ ID NO: 5, said sequence differs in the 3' end in the priority application and the patent.
- 13.2 With respect to the claims that were in his view objected to by the Opponent under novelty or inventive step using documents D12 and D15, the Proprietor submits that the subject matter of claim 1 has a basis in D7, particularly in paragraphs [0019], [0063] in combination with paragraphs [0004], [0006] and [0016] plus paragraphs [0035]-[0037], [0039], [0040], [0061], [0069], [0086], [0094], [0098], [0100], [0101], [0116], and [0123]-[0125] of the priority document. In addition to these parts of the description, he indicates paragraphs [0019] and [0051] as basis for new claim 2 and paragraph [0053] as basis for claim 20. The same parts are indicated as basis for newly filed claim 21.
- 13.3 The Opposition Division refers to the Guidelines for Examination at the EPO, Part F - Chapter VI-2, 1.3 which state "the expression **"the same invention"** in Art. 87(1) means that the subject-matter of a claim in a European application may enjoy the priority of a previous application only if the skilled person can derive the subject-matter of the claim **directly and unambiguously, using common general knowledge**, from the previous application **as a whole**. This means that the specific combination of features present in the claim must at least **implicitly** be disclosed in the previous application (see F-VI, 2.2 and G 2/98)" (emphasis added by the Division).
- First, concerning claim 1, the following features are disclosed in the respective paragraphs of D7. Paragraphs [0019] and [0063]: "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", paragraph [0017]: "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)". These plants comprise a, e.g. recombinantly expressed, *AAD-1* gene, i.e. transgenic plants (see paragraphs [0123] and [0124]). The gene encodes an aryloxyalkanoate dioxygenase enzyme that is capable of degrading phenoxy auxin and aryloxyphenoxypionate herbicides. Paragraph [0094] mentions that "the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing", i.e. *AAD-1* nucleotide (SEQ ID NO: 3-5) and amino acid (SEQ ID NO: 9) sequences, see paragraphs [0035]-[0037] and [0040]. According to paragraph [0100] "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", for example "stringent" conditions for hybridization, see paragraph [0101]. According to paragraph [0116] "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".

Second, concerning claim 2, the following features are disclosed in the respective paragraphs of D7. In addition to the paragraphs indicated above in conjunction with claim 1, the enzymatic activities of the claimed *AAD-1* protein are specified in paragraphs [0019] and [0061]: degradation of phenoxy acid and aryloxyphenoxypionate herbicides (claim 2 (ii) and (iii)), paragraph [0051]: degradation of the R-enantiomer of the chiral phenoxy auxins in addition to achiral phenoxy auxins (claim 2 (i)).

Third, concerning claims 20 and 21, the feature "field" is disclosed in paragraph [0053] of D7 which states: "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".

Taken together, the Opposition Division considers that the claims are entitled to priority. Accordingly, documents D12 and D15 do not form prior art within the meaning of Article 54(2) EPC.

14 Article 100(a) EPC in conjunction with Article 54 EPC

- 14.1 The Opponent raises objections concerning lack of novelty of the subject matter of granted claims 1-3, 11, 12 and 18 in view of the disclosure of D8. The document describes transgenic plants expressing *tfdA* from *Alcaligenes eutrophus* under control of the CaMV 35 S promoter resulting in resistance to 2,4-D herbicide, i.e. transgenic plants were sprayed with 2,4-D (page 813, right column). The *tfdA* coding region, disclosed in D2, hybridizes under stringent conditions to the nucleic acid encoding a protein of SEQ ID NO: 9, as derivable from the experimental data provided in document D1. TfdA possesses a 2,4-D degrading activity, see page 3, paragraph 11 of the application. A similar argumentation is formulated for said claims in view of the disclosure of documents D9 to D11 and late document D12.
- 14.2 The Proprietor submits that granted claim 1 was split into two independent claims, claims 1 and 2. Claim 1 was amended and further defined by structural terms, i. e. by including a reference to 70% identity. Since the *tfdA* encoding gene and the nucleic acid in SEQ ID NO: 9 share only 32% identity, the objection was overcome. The same limitation was introduced into claim 20 which therefore is novel as well. Claim 2 is defined by functional terms, i.e. enzymatic activities, which are not assigned to *tfdA*. Thereby the novelty objection is overcome. The same limitation is introduced into claim 21 which therefore is novel as well.
- 14.3 With regard to claims 1 and 20 the Opposition Division notes that documents D8 to D11 disclose transgenic plants (tobacco, cotton or mustard) comprising an isolated polynucleotide (*tfdA* gene from *Alcaligenes eutrophus*/*Ralstonia eutropha*) that encodes a protein (2,4-dichlorophenoxyacetate monooxygenase, DPAM) that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide (2,4-D) and an aryloxyphenoxypropionate herbicide wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9, as evidenced by experimental data shown in D1,

wherein said plant expresses said polynucleotide and produces said protein. The following technical feature was not disclosed in D8 to D12: "wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO: 5". Indeed, as derivable from sequence alignments between the TFD protein of D8 (see also D2) and the AAD-1 protein of the patent presented in document D4, the identity of the claimed proteins encoded by SEQ ID NO: 3 (protein with SEQ ID NO: 9) with the protein disclosed in document D8 is merely 32% which lies far out of the claimed range of "at least 70% identity". The same holds true *mutatis mutandis* for nucleic acids in SEQ ID NO: 4 and 5 which encode proteins that only differ in one or two amino acid positions from the protein in SEQ ID NO: 9 by that resulting in comparable identities.

Accordingly, claims 1 and 20 are novel over documents D8 to D11.

With regard to claims 2 and 21 the Opposition Division notes that documents D8 to D11 disclose transgenic plants (tobacco, cotton or mustard) comprising an isolated polynucleotide (tfdA gene from *Alcaligenes eutrophus*/*Ralstonia eutropha*) that encodes a protein (2,4-dichlorophenoxyacetate monooxygenase, DPAM) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9, as evidenced by experimental data shown in D1, wherein said plant expresses said polynucleotide and produces said protein. The following technical features are not disclosed in D8 to D11:

"the protein enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
- (ii) an aryloxyphenoxypropionate herbicide, or
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide".

Accordingly, claims 2 and 21 are novel over documents D8 to D11.

Claims 3-19 and 22 are dependent on or make direct reference to claim 1 or 2 and are therefore novel as well.

Taken together, the Opposition Division considers that the subject matter of claims 1 to 22 is novel.

- 15.1 The Opponent raises a first objection against the subject matter of granted claims 5-9 in view of document D8 in combination with any of documents D19-D22. Said claims make reference to other herbicide genes, particularly glufosinate, glyphosate, dicamba and ALS inhibitor herbicide tolerance genes. Since the application does not claim an interaction between the presence of the phenoxy and arylphenoxypropionate herbicide degrading transgene and said other herbicide tolerance genes, the technical problems can be separated and amount to the provision of plants with these other tolerance genes in addition to the transgenic plants of claim 1. Said other herbicide genes are described in documents D19, D20 (glufosinate), D21 (glyphosate), D22 (dicamba) and D23 (ALS inhibitor). A second objection is raised against claim 13 in view of document D8. According to the Opponent, the choice of a particular plant can not provide an inventive step. A third objection is raised against claim 14 pertaining to the *rdpA* gene. The technical problem, provision of transgenic plants with an alternative phenoxy or arylphenoxypropionate herbicide degrading enzyme encoding transgenes, is obvious starting from document D8 as closest prior art in view in combination with document D15.
- 15.2 The Proprietor submits that the structural feature "70% identity" introduced into granted claims 1 and 20 and the functional features relating to specific enzymatic activities specified in claims 2 and 21 recite additional features not disclosed in any of the documents D8 to D11 and D18 to D24. In this context, the Proprietor further reiterates his view that documents D12 and D15 are not citable prior art.
- 15.3 In this respect, the Opposition Division refers to the "problem and solution approach" that is usually used to assess inventive step. According to the Case Law of the Boards of Appeal of the EPO, 6th edition, 2010 (page 162), this "consists essentially of
- (a) identifying the "closest prior art",
 - (b) assessing the technical results (or effects) achieved by the claimed invention when compared with the "closest state of the art" established,
 - (c) defining the technical problem to be solved as the object of the invention to achieve these results, and
 - (d) examining whether or not a skilled person, having regard to the state of the art within the meaning of Art. 54(2) EPC, would have suggested the claimed technical features in order to obtain the results achieved by the claimed invention".

Claim 1 is directed to method of controlling weed in an area comprising a transgenic plant comprising a polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate (AOPP) herbicide, wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5, wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO: 9.

In accordance with the Opponent and the Proprietor, the Opposition Division considers document D8 to represent prior art disclosing subject matter conceived for the same purpose or aiming at the same objective.

Said document discloses a method of controlling weed comprising transgenic plants (tobacco) comprising an isolated polynucleotide (tfdA) that encodes a protein (DPAM) that enzymatically degrades a phenoxy auxin herbicide (2,4-D) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The subject-matter of claim 1 differs from this method in that the transgenic plants comprise a protein that is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO: 3, SEQ ID NO:4 and SEQ ID NO:5 (AAD-1, rpdA gene).

The objective problem to be solved by the present application resides in the provision of a method of controlling weed comprising transgenic plants resistant to a phenoxy auxin herbicide and an AOPP herbicide.

The solution provided is a method comprising plants expressing AAD-1 (rpdA) from *Sphingobium herbicidovorans*.

Document D14 discloses the rpdA gene and protein sequences from *Sphingobium herbicidovorans*. The document is, however, silent on the use of the AAD-1 encoding gene to generate transgenic plants with herbicide resistance to be used in a method of controlling weed.

As stated above, document D8 describes transgenic plants with 2,4-D resistance resulting from expression of a 2,4-D degrading enzyme (tfdA) from *Alcaligenes eutrophus*. A person skilled in the art could in principle have combined the teaching of D8 and D14 and arrive at the claimed solution. However, there was no suggestion in any of the cited document that would have incited him to do so. In fact, there is no indication in document D14 that AAD-1 would degrade AOPP herbicides and thereby generating plants that are resistant to both phenoxy auxin **and** AOPP herbicides.

The same reasoning applies *mutatis mutandis* to claim 20.

For these reasons, the solution proposed in claims 1 and 20 involves an inventive step.

Claim 2 is directed to method of controlling a weed comprising a transgenic plant comprising a polynucleotide that encodes a protein that enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
- (ii) an aryloxyphenoxypropionate herbicide, or
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide, wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The Opposition Division considers document D8 to represent prior art disclosing subject-matter conceived for the same purpose or aiming at the same objective.

Said document discloses a method of controlling weed comprising transgenic plants (tobacco) comprising an isolated polynucleotide (tfdA) that encodes a protein (DPAM) that enzymatically degrades a phenoxy auxin herbicide (2,4-D) wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9.

The subject-matter of claim 2 differs from this method in that the transgenic plants comprise a protein that enzymatically degrades

- (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
- (ii) an aryloxyphenoxypropionate herbicide, or
- (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

The objective problem to be solved by the present application resides in the provision a method of controlling a weed comprising transgenic plants resistant to an R-enantiomer of chiral phenoxy auxin herbicides; and to a phenoxy auxin and an AOPP herbicide.

The solution provided is a method comprising plants expressing AAD-1 (rpdA) from *Sphingobium herbicidovorans*.

Document D14 discloses the rpdA gene and protein sequences from *Sphingobium herbicidovorans*. The documents is, however, silent on the use of the AAD-1 encoding gene to generate transgenic plants with herbicide resistance to be used in a method of controlling weed.

As stated above, document D8 describes transgenic plants with 2,4-D resistance resulting from expression of a 2,4-D degrading enzyme (tfdA) from *Alcaligenes eutrophus*. A person skilled in the art could in principle have combined the teaching of D8 and D14 and arrive at the claimed solution. However, there is no suggestion in any of the cited document that would have incited him to do so. In fact, there is no indication in document D14 that AAD-1 would degrade an R-enantiomer of chiral phenoxy auxin herbicides or AOPP herbicides and thereby generating plants that are resistant to phenoxy auxin and AOPP herbicides specified in claim 2.

For these reasons, the solution proposed in claim 2 involves an inventive step.

The same reasoning applies *mutatis mutandis* to claim 21.

The same applies *mutatis mutandis* to claims 3-19 and 22 which are dependent on or directly referring to claims 1, 2, 20 and 21.

Considering that the objective technical problem stated above is solved by the claimed transgenic plants as well as their non-obviousness, the Opposition Division considers that the claimed subject matter is inventive.

16 Article 100(b) EPC in conjunction with Article 83 EPC

- 16.1 The Opponent raises the objection that the granted claimed subject-matter is insufficiently disclosed. The application provides support and disclosure only for a limited number of methods and polynucleotides, i.e. those having SEQ ID NO: 3-5 which encode a polypeptide having SEQ ID NO: 9 and transgenic plants comprising these whereas the hybridizing polynucleotides encompass polynucleotides encoding a protein with only 32% amino acid sequence identity, thereby not being congruent with the disclosure of the application. Additionally, claim 17 requires the application of a "parent herbicide". The term "herbicide" is however merely used in connection with the metabolism of 2,4-D by AAD-1. It is therefore unclear how the term "parent herbicide" connects with the claimed method.
- 16.2 The Proprietor submits that by including the structural term into claims 1 and 20 and the functional terms into claims 2 and 21 said claims are further limited and thereby the Opponent's objections are rendered moot. The Proprietor further submits that the Opponent's objection against claim 17 is a clarity objection which is not a ground for opposition.
- 16.3 Indeed, the proteins of claims 1 and 20 are on one hand defined by their ability to degrade a phenoxy auxin or an AOPP herbicide and on the other hand limited by the ability of the corresponding polynucleotides to hybridise under stringent conditions with the nucleic acid encoding the protein with SEQ ID

NO: 9 and their identity of at least 70% to the nucleic acids of SEQ ID NO: 3, 4 or 5. Consequently, the proteins referred to in claim are both functionally and structurally limited. The proteins can be determined by routine experimentation, i. e. by determination of the specified enzyme activities and sequence identity using alignment, thereby not involving any undue burden. The claimed subject matter is thus congruent with the disclosure of the application.

The same holds true *mutatis mutandis* for claims 2 and 21 in which the proteins are defined by their ability to degrade specific R-enantiomers, an AOPP herbicide and optionally a phenoxy auxin and on the other hand limited by the ability of the corresponding polynucleotides to hybridise under stringent conditions with the nucleic acid encoding the protein with SEQ ID NO: 9.

Consequently, the proteins referred to in claims 2 and 21 are both functionally and structurally limited. The proteins can be determined by routine experimentation, i. e. by determination of the specified enzyme activities and sequence identity using alignment, thereby not involving any undue burden. The claimed subject matter is thus congruent with the disclosure of the application.

The Division notes that the term "parent herbicide" is referred to in paragraph [0088]: "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" and paragraph [0197]: "Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR₅₀ 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)". According to Table 6 the parent herbicides 2,4-D, DCP, Dichlorprop, Triclopyr and Fluoroxypyr were tested. The term is thus used in the description in connection with AAD-1 but not limited to the herbicide 2,4-D. Accordingly, the Opposition Division considers that the application teaches how the term connects with the method claimed, namely by applying such "parent herbicide" to an area and thereby controlling weed.

Taken together, the Opposition Division considers that the subject matter of the claims is disclosed in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

- 17.1 The Opponent remarks that granted claim 14 claimed the same subject matter as claim 16 of EP 1 740 039 granted for the parent application whereas according to an accepted principle in most patent systems two patents can not be granted to the same applicant for one invention. That principle of prohibition of double patenting was accepted obiter dictum by the Enlarged Board of Appeal in decisions G 1/05 and G1/06.
- 17.2 The Proprietor submits that double patenting is not listed as a ground for opposition in Article 100 EPC and therefore not a basis to revoke the patent.
- 17.3 The Opposition Division notes that double patenting is indeed not listed as a ground for opposition in Article 100 EPC and therefore in principle not a basis to revoke the patent.

18 **Decision**

The grounds of opposition under Article 100(a), (b) and (c) EPC do not prejudice maintenance of the patent in amended form.

Taking into account the amendments made by the Proprietor the patent and the invention, to which it relates, meet the requirements of the EPC, Article 101(3)(a) EPC.

According to Article 106 EPC, this decision is subject to appeal. If such appeal is filed, it shall have suspensive effect. In accordance with Article 107 EPC, any party to the proceedings adversely affected by the decision may appeal. Notice of appeal must be filed at the EPO within two months after the date of notification of the decision appealed. The notice shall not be deemed to have been filed until the appeal fee has been paid. In case of an appeal, a written statement setting out the grounds for appeal must be filed within four months from notification of this decision (Article 108 EPC).

I. Summary of Facts and Submissions

- 1 European patent 2 319 932 entitled "Novel herbicide resistance gene" is based upon European patent application No. 10 012 199.5 filed on 02-05-2005. It claims priority of US 567052 filed on 30-04-2004.

The mention of the grant of the patent was published in the European Patent Bulletin of 18-09-2013. Proprietor of the patent is Dow AgroSciences, LLC.

The opposed patent is a divisional of European patent 1 740 039 with Application number 05 771 746.
- 2 Notice of opposition was filed by Bayer CropScience NV on 18-06-2014. Grounds of opposition are lack of novelty (Art. 100(a) and 54 EPC); lack of inventive step (Art. 100(a) and 56 EPC); insufficiency of disclosure (Art. 100(b) and 83 EPC) and added subject-matter (Art. 100(c) and 123(2) EPC). Revocation of the patent as a whole is requested on these grounds (Art.101(2) EPC).
- 3 With the reply to the notice of opposition dated 24.11.2014, the proprietor requested the patent to be maintained on the basis of claims 1-17 of the new main request as filed therein.
- 4 Both parties requested oral proceedings in case their respective requests could not be fully met (Art.116 EPC).
- 5 On 26-06-2015 the opposition division issued a summons to attend oral proceedings on 08.12.2015, together with an annex comprising division's preliminary and non binding opinion with regard to the opposition.
- 6 The proprietor replied to the summons with letter of 05.11.2015 reiterating the request of maintaining the patent on the basis the main request submitted on 24.11.2014, or on the basis of auxiliary requests I, II or III submitted on 05.11.2015 and in this order.
- 7 The opponent replied to the summons to attend oral proceeding with letter of 05.11.2015 confirming objections under R. 80, Arts. 123(2)/76(1) and 83 EPC for the main request on file, and the observation regarding invalidity of the claimed priority.
- 8 The oral proceedings were held on 08.12.2015. During the oral proceedings, the proprietor submitted a new auxiliary request II while II while maintaining his main request and auxiliary request I. The previous auxiliary requests II and III were renumbered III and IV, respectively.

9 At the end of the oral proceedings the opposition division announced its decision that the claims of the auxiliary request II filed during said proceedings met the requirements of the EPC. Amended description page 3 were agreed among the parties.

10 Claim 1 of new auxiliary request II reads as follows:

A transgenic plant cell comprising an isolated polynucleotide that encodes a protein that enzymatically degrades:

(i) a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide, wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ 10 NO:3, SEQ 10 NO:4 and SEQ 10 NO:5,

(ii) an aryloxyphenoxypropionate herbicide, or

(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide, wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9, wherein said plant expresses said polynucleotide and produces said protein.

11 At the end of the oral proceedings the opposition division announced its decision that the claims of the auxiliary request II filed during said proceedings met the requirements of the EPC. Amendments to the description on page 3 were agreed among the parties.

12 The numbering of the cited documents follows the numbering in the consolidated list of references of the Opponent (D1a-D1c, D2-D23).

Reasons for the decision

13 The Opposition is regarded admissible since it fulfils the requirements of Articles 99(1) and Rule 76 EPC.

MAIN REQUEST

14 Rule 80 EPC

14.1 The main request comprises an amended independent claim 1 and a new independent claim 2. With reply to the objection under R. 80 EPC raised in the summons to attend oral proceedings, the proprietor referred to the Guidelines for examination of the EPO (part H, chapter II, section 3.1) and decision of the boards of appeal T223/97 and explained that claims 1 and 2 were each directed to a respective specific embodiment of claim 1 as granted wherein novelty objections raised with the notice of oppositions were addressed.

- 14.2 The opposition division finds that claims 1 and 2 of the main request encompass embodiments of claim 1 as granted, attempting to address novelty objections. In an overlapping manner, i.e.: claim 1 restricts granted claim 1 using a structural limitation based on percentage of identity with regards to three variants of AAD-1; whereas claim 2 tries to restrict the scope of granted claim 1 by functional limitations wherein the sole activity of degrading phenoxy auxins in general is replaced by the more specific "an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins". The opposition division sees no reason why the present case should require two independent claims to overcome an objection based on Art. 100 EPC raised against a single granted independent claims, that could be done solely by modifying the granted claim 1 once. The scope of both claims overlaps in common functional and structural limitations that are not directly related to two specific embodiments of granted claim 1, as could be accepted in exceptional cases as held in T181/02. The addition of a second independent claim is not at present needed to avoid revocation. The proposed amendments do not seem to be an appropriate and necessary response in order to avoid revocation and cannot thus be regarded as occasioned by the grounds of opposition as indicated in decision T263/05.
- 14.3 The main request therefore does not fulfil the requirements of Rule 80 EPC.
- 15 **Article 100(c), 76(1) and 123(2) EPC.**
- 15.1 With the notice of opposition the opponent argued that the subject-matter of claim 15 as granted (i.e. claim 16 of the main request) did not comply with Arts. 100(c) and 76(1) EPC. According to the opponent, only claim 61 as originally filed with the parent related to insect-resistance genes as in claim 15 as granted, but having a narrower scope of protection compared to granted claim 15.
- 15.2 The proprietor disagreed and pointed to claims 61 and 1 as originally filed in the parent application as basis for claim 16 of the main request (corresponding to claim 15 as granted) together with item 47 (see page 165 of the application as published).
- 15.3 The opposition division considers that the subject-matter of claim 16 of the main request goes beyond the application as originally filed and in the parent application as originally filed contrary to Article 100(c), 76(1) and 123(2) EPC. Claim 61 in the parent application (i.e.: item 61 of the application as originally filed), depends on claim 5 and ultimately on claim 1, also of the parent application. The scope of claim 1 of the parent application relates to AAD-1 proteins and closely related variants able to degrade aryloxyalkanoate

herbicides. Claim 16 of the main request depends on claim 1, having a broader scope both in the percentage of identity encompassed in the variants and in the functional limitation.

AUXILIARY REQUEST I

- 16 This request also contains the same two independent new claims as the main request. Following the same reasoning as above (see point 15), the opposition division hence considers that the auxiliary request I does not fulfil the requirements of Rule 80 EPC.

AUXILIARY REQUEST II

17 Priority (Art. 87 EPC)

- 17.1 The opponent argued that the claims as granted were not entitled to the priority date of 30.04.2004 because in the absence of item 47 (p. 165) of the application as filed, the terms of the claim 1 could not be clearly and unambiguously derived from the priority document (D7). The opponent concluded that the relevant date to establish the state of the art regarding the patent is the international filing date, i.e. 02.05.2005, and that D12 and D15 were therefore prior art documents within the meaning of Art. 54(2) EPC.
- 17.2 During the written procedure, the proprietor referred to paragraphs [0123], [0019], [0034]-[0037], [0040], [0124] and page 28, I. 2 of D7 for the basis and, in addition, during the oral proceedings the proprietor pointed to paragraphs [51] to [53] and [216] of D7.
- 17.3 The opposition division considers that the subject-matter claimed can be unambiguously derived from the priority document, specially from paragraphs [216] and [63]. Paragraph 63 teaches that the invention also includes methods of controlling weeds wherein an aryloxyalkanoate and/or a phenoxy auxin herbicides can be applied, i.e. the use of alternative enzymatic activities is disclosed in the priority document. The opposition division concludes that the opposed patent is entitled to the priority date claimed. It follows that D12 and D15 cannot be considered as prior art documents in the sense of Article 54(2) EPC.
- ##### **18 Novelty (Art. 100(a) in conjunction with 54 EPC)**
- 18.1 The opponent argued with the notice of opposition that D8 disclosed transgenic plants expressing *tfdA* gene from *Alcaligenes eutrophus*, presently renamed *Ralstonia eutropha* being tolerant to herbicide 2,4-D. The opponent argued that in view of the test submitted with D1, wherein *tfdA* DNA hybridizes with SEQ ID NO:9 of the present patent, the subject-matter of claim 1 as

granted was not novel. The same is argued for dependent claims 11 and 13. The same reasoning is followed with respect to prior art documents D9 to D12.

- 18.2 With the reply to the notice of opposition, the proprietor commented on the results of the experiments submitted by the opponent with D1 without acquiescence, but addressed the objection by amendment.

The proprietor noted that the opponent stated that lfdA polypeptide sequence is 32% identical over the whole length to the polypeptide encoded by aad-1 gene of the opposed patent.

Since claim 1 of the main request requires that the protein is at least 70% identical to aad-1, prior art documents D8 to D12 did not anticipate new Claim 1.

This amendment is maintained for feature (i) of claim 1 of the auxiliary request II. Regarding features (ii) and (iii) the proprietor added that they are restricted to enzymatic activities that lfdA lacks, and are therefore also novel over the prior art.

- 19 With regards to claim 1 of the auxiliary request II, the opposition division finds that the amendments introduced restore novelty over all D1 and D8 to D12.

20 **Inventive step (Art. 100(a) in conjunction with 56 EPC)**

- 20.1 According to the opponent, the subject-matter of granted claims 3, 5-9, 12, 14 and 16 lacks an inventive step from the combination of any of D8 to D12 with D19, D20, D21, D22 or D23; granted claims 2 and 10 are not inventive in the light of any of D8 to D12 alone; granted claim 4 lacks inventive step over any of D8 to D12 combined with D15; and that granted claim 15 lacks inventive step over any of D8 to D12 in combination with D18.

- 20.2 Following the conclusions on Article 87 EPC above, D12 and D15 are not citable prior art for the auxiliary request II. In addition, claim 1 is considered novel over D8 (or D9 to D11). It follows that the inventive step objections of the opponent are therefore void.

- 20.3 The opposition division considers that the subject-matter of claims 1 to 15 complies with Article 100(a) together with 56 EPC.

21 **Sufficiency of disclosure (Arts.100(b) and 83 EPC).**

- 21.1 The opponent contended that the patent lacked a sufficient disclosure over its entire scope in so far as claim 1(i) encompasses plant cells expressing genes only 32% identical to the sequences disclosed and supported in the opposed patent, that according to the opponent, is limited to polynucleotides of SEQ ID

NOs:3-5 encoding the polypeptide of SEQ ID NO:9. In addition, during the oral proceedings the opponent argued that the only guidance to work the subject-matter of points (ii) and (iii) of claim 1 was the enzymatic activity and a hybridization test that could encompass sequences with low percentage of identity, as seen with gene *tfdA*. According to the opponent this did not suffice to carry out the invention over its entire scope without undue burden.

- 21.2 The proprietor disagreed and notes that the opponent has the burden of proof when sufficiency of disclosure is at stake.
- 21.3 The opposition division agrees with the proprietor in considering that the opponent has not proven that the invention cannot be carried out without undue burden. Claim 1 requires that the transgene express by the plant cell degrades either aryloxyalkanoate or phenoxy auxin herbicides or both, and that the polynucleotide encoding the enzyme hybridizes with stringent conditions to SEQ ID NO:9 of the patent. Both features can be routinely tested by the skilled person and in combination it does not seem to the opposition division to represent an undue burden. It should be noted that *tfdA*, although it is only 32% identical to SEQ ID NO:9, also encodes a dioxygenase enzyme, as AAD-1 does. It is concluded that the subject-matter of the auxiliary request II complies with Arts. 100(b) and 83 EPC.

CONCLUSIONS

- 22 The proprietor indicated some amendments in the description in line with the claims of the auxiliary request II. The opponent did not object to this. The opposition division finds that the amendments properly reflects the claims of the auxiliary request II and therefore is acceptable according to Article 84 EPC, Guidelines F.IV 4.3 (iii).
- 23 The patent as amended according to the auxiliary request II and the invention to which it relates meet the requirements of the Convention (Article 101(3)(a) EPC). The opposition division has therefore decided to maintain the patent in amended form, on the basis of the currently valid auxiliary request II.

Electronic Patent Application Fee Transmittal				
Application Number:		15288406		
Filing Date:		07-Oct-2016		
Title of Invention:		NOVEL HERBICIDE RESISTANCE GENES		
First Named Inventor/Applicant Name:		Terry WRIGHT		
Filer:		John P. Breen/Garla Twyman		
Attorney Docket Number:		14764-258662		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
Total in USD (\$)				260

Electronic Acknowledgement Receipt	
EFS ID:	41407480
Application Number:	15288406
International Application Number:	
Confirmation Number:	5402
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Customer Number:	107540
Filer:	John P. Breen/Garla Twyman
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Authorized User	Garla Twyman
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)					
37 CFR 1.20 (Post Issuance fees)					
37 CFR 1.21 (Miscellaneous fees and charges)					
File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Amendment_and_Response_under_37_C_F_R_1_116.pdf	51520	yes	9
			e3a2638a480fd0f4ca45782373c886a9e740284		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Response After Final Action		1	1	
	Claims		2	7	
	Applicant Arguments/Remarks Made in an Amendment		8	9	
Warnings:					
Information:					
2	Transmittal Letter	Supplemental_IDS.pdf	91730	no	8
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3	Foreign Reference	WO2003013224-PAMPH-20030220-570b.pdf	2194922	no	54
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4	Foreign Reference	WO1987005629-PAMPH-19870924-1554.pdf	3588285	no	98
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20	Other Reference-Patent/App/Search documents	Opposition_for_EP_10012199_5.pdf	5761624	no	18
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27	Other Reference-Patent/App/Search documents	Opposition_Decision_for_EP10012201_9.pdf	8544294 f035f9e343bcb4fe903409692de01687f4e70fdc5	no	17
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Warnings:					
Information:					
Total Files Size (in bytes):			170300399		
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(57) Abstract			
The present invention provides, <i>inter alia</i> , an isolated polynucleotide comprising a region encoding a chloroplast transit peptide and a glyphosate resistant 5-enolpyruvylshikimate phosphate synthase (EPSPS) 3' of the peptide, the said region being under expression control of a plant operable promoter, with the proviso that the said promoter is not heterologous with respect to the said region, and the chloroplast transit peptide is not heterologous with respect to the said synthase.			

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HERBICIDE RESISTANT PLANTS

The present invention relates to recombinant DNA technology, and in particular to the production of transgenic plants which exhibit substantial resistance or substantial
5 tolerance to herbicides when compared with non transgenic like plants. The invention also relates, *inter alia*, to the nucleotide sequences (and expression products thereof) which are used in the production of, or are produced by, the said transgenic plants.

Plants which are substantially "tolerant" to a herbicide when they are subjected to it provide a dose/response curve which is shifted to the right when compared with that
10 provided by similarly subjected non tolerant like plants. Such dose/response curves have "dose" plotted on the x-axis and "percentage kill", "herbicidal effect" etc. plotted on the y-axis. Tolerant plants will typically require at least twice as much herbicide as non tolerant like plants in order to produce a given herbicidal effect. Plants which are substantially "resistant" to the herbicide exhibit few, if any, necrotic, lytic, chlorotic or other lesions when
15 subjected to the herbicide at concentrations and rates which are typically employed by the agricultural community to kill weeds in the field in which crops are to be grown for commercial purposes.

It is particularly preferred that the plants are substantially resistant or substantially tolerant to herbicides (hereinafter "glyphosate") which have 5-enol pyruvyl shikimate
20 phosphate synthetase (hereinafter "EPSPS") as their site of action, of which N-phosphonomethylglycine (and its various salts) is the pre-eminent example.

The herbicide may be applied either pre- or post emergence in accordance with usual techniques for herbicide application to fields comprising crops which have been rendered resistant to the herbicide. The present invention provides, *inter alia*, nucleotide sequences
25 useful in the production of such herbicide tolerant or resistant plants.

According to the present invention there is provided an isolated polynucleotide comprising the sequence depicted in SEQ ID No. 43. The invention also provides a polynucleotide, excluding the cDNA encoding the rice and corn EPSPS, which encodes an EPSPS and which is complementary to one which when incubated at a temperature of
30 between 65 and 70°C in 0.1 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.1 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 43. An EPSPS encoding

polynucleotide according to the invention may, however, be obtained by screening plant genomic DNA libraries with a nucleotide constituting an intron within the SEQ ID No. 43 sequence, and the invention also includes such a sequence obtainable from that screening.

The invention also includes an isolated polynucleotide comprising a region encoding a chloroplast transit peptide and a glyphosate resistant 5-enolpyruvylshikimate phosphate synthase (EPSPS) 3' of the peptide, the said region being under expression control of a plant operable promoter, with the *provisos* that the said promoter is not heterologous with respect to the said region, and the chloroplast transit peptide is not heterologous with respect to the said synthase.

By "heterologous" is meant from a different source, and correspondingly "non-heterologous" means derived from the same source - but at a gene rather than organism or tissue level. For example the CaMV35S promoter is clearly heterologous with respect to a petunia EPSPS coding sequence insofar as the promoter is derived from a virus and the sequence - the expression of which it controls - from a plant. The term "heterologous" according to the present invention has a still narrower meaning, however. For example "heterologous" as it relates to the present invention means that the petunia EPSPS coding sequence is "heterologous" with respect to, for example, a promoter also derived from petunia - other than that which controls expression of the EPSPS gene. In this sense the petunia promoter derived from the petunia EPSPS gene then used to control expression of an EPSPS coding sequence likewise-derived from petunia is "non-heterologous" with respect to the said coding sequence. "Non-heterologous" does not mean, however, that the promoter and coding sequence must necessarily have been obtained from one and the same (original or progenitor) polynucleotide. Likewise with respect to transit peptides. For example, a rubisco chloroplast transit peptide derived from sunflower is "heterologous" with respect to the coding sequence of an EPSPS gene likewise derived from sunflower (the same plant, tissue or cell). A rubisco transit peptide encoding sequence derived from sunflower is "non-heterologous" with respect to a rubisco enzyme encoding-sequence also derived from sunflower even if the origins of both sequences are different polynucleotides which may have been present in different cells, tissues or sunflower plants.

A preferred form of the polynucleotide comprises the following components in the 5' to 3' direction of transcription:-

- (i) At least one transcriptional enhancer being that enhancing region which is upstream from the transcriptional start of the sequence from which the enhancer is obtained and which enhancer *per se* does not function as a promoter either in the sequence in which it is endogenously comprised or when present heterologously as part of a construct;
- (ii) The promoter from the rice EPSPS gene;
- (iii) The rice genomic sequence which encodes the rice EPSPS chloroplast transit peptide;
- (iv) The genomic sequence which encodes the rice EPSPS;
- (v) A transcriptional terminator;
- wherein the rice EPSPS coding sequence is modified in that a first position is mutated so that the residue at this position is Ile rather than Thr and a second position is mutated so that the residue at this position is Ser rather than Pro, the mutations being introduced into EPSPS sequences which comprise the following conserved region GNAGTAMRPLTAAV in the wild type enzyme such that modified sequence reads GNAGIAMRSLTAAV.

The enhancing region preferably comprises a sequence the 3' end of which is at least 40 nucleotides upstream of the closest transcriptional start of the sequence from which the enhancer is obtained. In a further embodiment of the polynucleotide, the enhancing region comprises a region the 3' end of which is at least 60 nucleotides upstream of the said closest start, and in a still further embodiment of the polynucleotide the said enhancing region comprises a sequence the 3' of which is at least 10 nucleotides upstream from the first nucleotide of the TATA consensus of the sequence from which the enhancer is obtained.

The polynucleotide according to the invention may comprise two or more transcriptional enhancers, which in a particular embodiment of the polynucleotide may be tandemly present.

In the present inventive polynucleotide the 3' end of the enhancer, or first enhancer if there is more than one present, may be between about 100 to about 1000 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide or the first nucleotide of an intron in the 5' untranslated region in the case that the said region contains an intron. In a more preferred embodiment of the polynucleotide, the 3' end of the enhancer, or first enhancer, is between about 150 to about 1000 nucleotides upstream of the

codon corresponding to the translational start of the EPSPS transit peptide or the first nucleotide of an intron in the 5' untranslated region, and in a still more preferred embodiment the 3' end of the enhancer, or first enhancer, may be between about 300 to about 950 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide or the first nucleotide of an intron in the 5' untranslated region. In a yet more preferred embodiment, the 3' end of the enhancer, or first enhancer, may be located between about 770 and about 790 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide or the first nucleotide of an intron in the 5' untranslated region.

10 In an alternative inventive polynucleotide, the 3' end of the enhancer, or first enhancer, may be located between about 300 to about 380 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide or the first nucleotide of an intron in the 5' untranslated region, and in a preferred embodiment of this alternative polynucleotide the 3' end of the enhancer, or first enhancer, is located between about 320 to
15 about 350 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide, or the first nucleotide of an intron in the 5' untranslated region.

In the polynucleotide according to the invention, the region upstream of the promoter from the rice EPSPS gene may comprise at least one enhancer derived from a sequence which is upstream from the transcriptional start of either the CaMV35S or FMV35S
20 promoters.

Accordingly the polynucleotide may comprise in the 5' to 3' direction a first enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the GOS 2 promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the
25 transcriptional start of either the CaMV35S or FMV35S promoters.

Alternatively, the polynucleotide may comprise in the 5' to 3' direction a first enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the rice actin promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream
30 from the transcriptional start of either of the FMV35S or CaMV35S promoters.

Alternatively, the polynucleotide may comprise in the 5' to 3' direction a first enhancer comprising a transcriptional enhancing region derived from a sequence which is

upstream from the transcriptional start of the barley plastocyanin promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of either the CaMV35S or FMV35S promoters.

Alternatively, the polynucleotide may comprise in the 5' to 3' direction a first
5 enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the maize polyubiquitin promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of either the CaMV35S or FMV35S promoters.

Alternatively, the polynucleotide may comprise in the 5' to 3' direction a first
10 enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the FMV35S promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the CaMV35S promoter.

Whatever the identity and juxtaposition of the various enhancers present in the
15 polynucleotide, the nucleotides 5' of the codon which constitutes the translational start of the rice EPSPS chloroplast transit peptide may be Kozack preferred. What is meant by this is well known to the skilled man and will be further apparent from the examples below.

Particularly preferred embodiments of the present inventive polynucleotide have a non-translated region which comprises a sequence which functions as an intron located 5' of
20 the rice genomic sequence which encodes the rice EPSPS chloroplast transit peptide. The non-translated region may comprise the sequence depicted in SEQ ID No. 55. In particular, the non-translated region may comprise the maize ADHI intron which has the sequence of SEQ ID No. 55.

The polynucleotide of the invention may comprise a virally derived translational
25 enhancer located within the non translated region 5' of the rice genomic sequence which encodes the rice EPSPS chloroplast transit peptide. The man skilled in the art is aware of the identity of such suitable translational enhancers - such as the Omega and Omega prime sequences derived from TMV and that derived from the tobacco etch virus, and how such translational enhancers can be introduced into the polynucleotide so as to provide for the
30 desired result.

The polynucleotide according to the invention may further comprise regions encoding proteins capable of conferring upon plant material containing it at least one of the following

agronomically desirable traits: resistance to insects, fungi, viruses, bacteria, nematodes, stress, dessication, and herbicides. Whilst such a polynucleotide contemplates the herbicide resistance conferring gene being other than an EPSPS, such as glyphosate oxido-reductase (GOX) for example, the herbicide may be other than glyphosate in which case the resistance

5 conferring genes may be selected from the group encoding the following proteins: phosphinothricin acetyl transferase (PAT), hydroxyphenyl pyruvate dioxygenase (HPPD), glutathione S transferase (GST), cytochrome P450, Acetyl-CoA carboxylase (ACCase), Acetolactate synthase (ALS), protoporphyrinogen oxidase (PPO), dihydropteroate synthase, polyamine transport proteins, superoxide dismutase (SOD), bromoxynil nitrilase, phytoene

10 desaturase (PDS), the product of the *tfdA* gene obtainable from *Alcaligenes eutrophus*, and known mutagenised or otherwise modified variants of the said proteins. In the case that the polynucleotide provides for multiple herbicide resistance such herbicides may be selected from the group consisting of a dinitroaniline herbicide, triazolo-pyrimidines, uracil, a phenylurea, triketone, isoxazole, acetanilide, oxadiazole, triazinone, sulfonanilide, amide,

15 anilide, RP201772, flurochloridone, norflurazon, and triazolinone type herbicide and the post-emergence herbicide is selected from the group consisting of glyphosate and salts thereof, glufosinate, asulam, bentazon, bialaphos, bromacil, sethoxydim or another cyclohexanedione, dicamba, fosamine, flupoxam, phenoxy propionate, quizalofop or another aryloxy-phenoxypropanoate, picloram, fluometron, atrazine or another triazine, metribuzin,

20 chlorimuron, chlorsulfuron, flumetsulam, halosulfuron, sulfometron, imazaquin, imazethapyr, isoxaben, imazamox, metosulam, pyriproxyfen, rimsulfuron, bensulfuron, nicosulfuron, fomesafen, fluroglycofen, KIH9201, ET751, carfentrazone, ZA1296, sulcotrione, paraquat, diquat, bromoxynil and fenoxaprop.

In the case that the polynucleotide comprises sequences encoding insecticidal

25 proteins, these proteins may be selected from the group consisting of crystal toxins derived from Bt, including secreted Bt toxins; protease inhibitors, lectins, Xenorhabdus/Photobacterium toxins; the fungus resistance conferring genes may be selected from the group consisting of those encoding known AFPs, defensins, chitinases, glucanases, Avr-CE9. Particularly preferred insecticidal proteins are cryIAC, cryIAb, cry3A, Vip 1A, Vip

30 1B, cysteine protease inhibitors, and snowdrop lectin. In the case that the polynucleotide comprises bacterial resistance conferring genes these may be selected from the group consisting of those encoding cecropins and tachyplesin and analogues thereof. Virus

resistance conferring genes may be selected from the group consisting of those encoding virus coat proteins, movement proteins, viral replicases, and anti-sense and ribozyme sequences which are known to provide for virus resistance; whereas the stress, salt, and drought resistance conferring genes may be selected from those that encode Glutathione-S-transferase and peroxidase, the sequence which constitutes the known CBF1 regulatory
5 sequence and genes which are known to provide for accumulation of trehalose.

The polynucleotide according to the invention may be modified to enhance expression of the protein encoding sequences comprised by it, in that mRNA instability motifs and/or fortuitous splice regions may be removed, or crop preferred codons may be
10 used so that expression of the thus modified polynucleotide in a plant yields substantially similar protein having a substantially similar activity/function to that obtained by expression of the unmodified polynucleotide in the organism in which the protein encoding regions of the unmodified polynucleotide are endogenous. The degree of identity between the modified polynucleotide and a polynucleotide endogenously contained within the said plant and
15 encoding substantially the same protein may be such as to prevent co-suppression between the modified and endogenous sequences. In this case the degree of identity between the sequences should preferably be less than about 70%.

The invention still further includes a biological or transformation vector comprising the present inventive polynucleotide. Accordingly, by "vector" is meant, *inter alia*, one of the
20 following: a plasmid, virus, cosmid or a bacterium transformed or transfected so as to contain the polynucleotide.

The invention still further includes plant material which has been transformed with the said polynucleotide or vector, as well as such transformed plant material which has been, or is, further transformed with a polynucleotide comprising regions encoding proteins
25 capable of conferring upon plant material containing it at least one of the following agronomically desirable traits: resistance to insects, fungi, viruses, bacteria, nematodes, stress, dessication, and herbicides.

The invention still further includes morphologically normal, fertile whole plants which have been regenerated from the material disclosed in the immediately preceding
30 paragraph, their progeny seeds and parts, which progeny comprises the polynucleotide or vector of the invention stably incorporated into its genome and heritable in a Mendelian manner.

The invention still further includes morphologically normal fertile whole plants which contain the present inventive polynucleotide and which result from the crossing of plants which have been regenerated from material transformed with the present inventive polynucleotide or vector, and plants which have been transformed with a polynucleotide
5 comprising regions encoding proteins capable of conferring upon plant material containing it at least one of the following agronomically desirable traits: resistance to insects, fungi, viruses, bacteria, nematodes, stress, dessication, and herbicides, the progeny of the resultant plants, their seeds and parts.

Plants of the invention may be selected from the group consisting of field crops,
10 fruits and vegetables such as canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, cabbage, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax and oilseed rape, and nut producing plants insofar as they are not already specifically mentioned, their progeny, seeds and parts.

15 Particularly preferred such plants include maize, soybean, cotton, sugar beet and canola.

The invention still further comprises a method of selectively controlling weeds in a field, the field comprising weeds and plants of the invention or the herbicide resistant progeny thereof, the method comprising application to the field of a glyphosate type
20 herbicide in an amount sufficient to control the weeds without substantially affecting the plants. According to this method, one or more of a herbicide, insecticide, fungicide, nematicide, bactericide and an anti-viral may be applied to the field (and thus the plants contained within it) either before or after application of the glyphosate herbicide.

The invention still further provides a method of producing plants which are
25 substantially tolerant or substantially resistant to glyphosate herbicide, comprising the steps of:

- (i) transforming plant material with the polynucleotide or vector of the invention;
- (ii) selecting the thus transformed material; and
- (iii) regenerating the thus selected material into morphologically normal fertile
30 whole plants.

The transformation may involve the introduction of the polynucleotide into the material by any known means, but in particular by: (i) biolistic bombardment of the material

with particles coated with the polynucleotide; (ii) by impalement of the material on silicon carbide fibres which are coated with a solution comprising the polynucleotide; or (iii) by introduction of the polynucleotide or vector into *Agrobacterium* and co-cultivation of the thus transformed *Agrobacterium* with plant material which is thereby transformed and is subsequently regenerated. Plant transformation, selection and regeneration techniques, which may require routine modification in respect of a particular plant species, are well known to the skilled man. The thus transformed plant material may be selected by its resistance to glyphosate.

The invention still further provides the use of the present inventive polynucleotide or vector in the production of plant tissues and/or morphologically normal fertile whole plants which are substantially tolerant or substantially resistant to glyphosate herbicide.

The invention still further includes a method of selecting biological material transformed so as to express a gene of interest, wherein the transformed material comprises the polynucleotide or vector of the invention, and wherein the selection comprises exposing the transformed material to glyphosate or a salt thereof, and selecting surviving material. The said material may be of plant origin, and may in particular be derived from a monocot selected from the group consisting of barley, wheat, corn, rice, oats, rye, sorghum, pineapple, sugar cane, banana, onion, asparagus and leek.

The invention still further includes a method for regenerating a fertile transformed plant to contain foreign DNA comprising the steps of:

- (a) producing regenerable tissue from said plant to be transformed;
- (b) transforming said regenerable tissue with said foreign DNA, wherein said foreign DNA comprises a selectable DNA sequence, wherein said sequence functions in a regenerable tissue as a selection device;
- (c) between about one day to about 60 days after step (b), placing said regenerable tissue from step (b) in a medium capable of producing shoots from said tissue, wherein said medium further contains a compound used to select regenerable tissue containing said selectable DNA sequence to allow identification or selection of the transformed regenerated tissue;
- (d) after at least one shoot has formed from the selected tissue of step (c) transferring said shoot to a second medium capable of producing roots from said

shoot to produce a plantlet, wherein the second medium optionally contains the said compound; and

- (e) growing said plantlet into a fertile transgenic plant wherein the foreign DNA is transmitted to progeny plants in Mendelian fashion, characterised in that the foreign DNA is, or the selectable DNA sequence comprised by the foreign DNA comprises, the polynucleotide according to the invention, and the said compound is glyphosate or a salt thereof. The plant may be a monocot as indicated above - more preferably selected from banana, wheat, rice, corn and barley and the said regenerable tissue may consist of embryogenic calli, somatic embryos, immature embryos etc.

- 10 The present invention will be further apparent from the following description taken in conjunction with the associated drawings and sequence listings.

List of Sequences

- SEQ ID NO. 1-42 PCR primers.
 SEQ ID NO. 43 Rice genomic EPSPS sequence (from ATG).
 15 SEQ ID NO. 44 Rice genomic EPSPS sequence containing double mutation.
 SEQ ID NO. 45 FMV enhancer.
 SEQ ID NO. 46 CaMV35S enhancer 1.
 SEQ ID NO. 47 CaMV35S enhancer 2.
 SEQ ID NO. 48 Maize polyubiquitin enhancer.
 20 SEQ ID NO. 49 Rice actin enhancer
 SEQ ID NO. 50 Rice GOS2 enhancer
 SEQ ID NO. 51 Barley Plastocyanin enhancer
 SEQ ID NO. 52 G1 rice EPSPS promoter + 5' upstream region.
 SEQ ID NO. 53 G3 rice EPSPS promoter + 5' upstream region.
 25 SEQ ID NO. 54 G2 rice EPSPS promoter + Adh 1 intron in 5' upstream region.
 SEQ ID NO. 55 Maize Adh1 intron

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 Figure 2 Vector pCR4-OSEPPS (rice dmEPSPS gene in vector pCR4-Blunt)
 30 Figure 3 Schematic representation of strategy used to introduce the double mutation.
 Figure 4 Vector pTCV1001
 Figure 5 Vector pTCV1001OSEPPS (comprises rice dmEPSPS gene in vector pTCV1001).

Figure 6 Vector pTCV1001EPSPSPAC (comprises rice dmEPSPS gene in vector pTCV1001).

Figure 7 Vector pBluSK+EPSPS (comprises rice dmEPSPS gene in vector pBluescript SK+).

Figure 8 Vector pPAC1

5 Figure 9 Vector pTCVEPSPSPH

Figure 10 Vector pTCVEPSPSADH

Figure 11 Vector pBluSKEPSPSADH (comprises rice dmEPSPS gene and Adh1 intron)

Figure 12 Vector pIGPD9

Figure 13 Vector Zen 9

10 Figure 14 Vector Zen 12

Figure 15 Vector Zen 18

Figure 16 Introduction of Zen vectors into superbinary vectors

Production of plants tolerant to glyphosate treatment through over-expression of a mutated EPSPS under the control of a non-heterologous promoter.

15 The term 'enhancer' as used throughout this specification means those sequences upstream of the promoter which do not comprise the promoter itself but which act to enhance or regulate transcription initiation from the promoter. The term "EPSPS promoter deletion" as used throughout this specification refers to the EPSPS promoter together with nucleotides from the enhancer which is native to the EPSPS gene - i. e. nucleotides which are upstream
20 (5') of the promoter.

In respect of the transformation of plant material, those skilled in the art will recognise that although particular types of target material (e.g. embryogenic cell suspension culture or de-differentiating immature embryos) and particular methods of transformation (e.g. using *Agrobacterium* or particle bombardment) are specified in the examples below, the
25 present invention is not limited to these particular embodiments and such target materials and methods may be used interchangeably. Furthermore, the term "plant cells" as used throughout this description of the invention can refer to isolated cells, including suspension cultures as well as to cells in an intact or partly intact tissue such as embryo, scutella, microspore, microspore-derived embryo or somatic cells from plant organs. Similarly,
30 although the specific examples are limited to maize, wheat and rice, the invention is equally applicable to any of a broad range of agricultural crops and amenity plants which can be transformed using suitable methods of plant cell transformation.

General molecular biological methods are carried out according to Sambrook *et al* (1989) * Molecular cloning: A laboratory Manual, 2nd Edn. Cold Spring Harbour Lab. Press.

EXAMPLE 1. Generation of a cDNA probe for Rice EPSPS

A partial length cDNA encoding rice EPSPS is obtained using reverse transcriptase PCR (RT-PCR). Total RNA is isolated from two-week-old rice plants (*Oryza sativa* L. indica var. Koshihikari) using the TRI-ZOL™ method (Life Technologies). First-strand cDNA synthesis is performed using Superscript II reverse transcriptase (Life Technologies) with 200 ng of EPSPS degenerate reverse 10 primer (SEQ ID NO.1) and 2 µg of total RNA according to the supplied protocols. Second strand synthesis and cDNA amplification by PCR is performed using EPSPS degenerate primers 10 and 4 (SEQ ID NO.1 and SEQ ID NO.2) and PCR beads (Pharmacia) according to the manufacturers instructions. All letter codes are standard abbreviations (Eur. J. Biochem. (1985) 150:15)

SEQ ID NO.1

EPSPS degenerate reverse 10 5' GCACARGCTGCAAGIGARAAIGCCATIGCCAT 3'

SEQ ID NO.2

EPSPS degenerate forward 4 5' GCGGGAACWGCMTGCGICCRYTIACIGC 3'

The products are cloned into vector pCR2.1 (Invitrogen) using a TA Cloning kit™ as recommended by the supplier. Plasmid is recovered from selected colonies and the sequence analysed by a process involving computer based homology searches (BLAST) to confirm that the cloned RT-PCR product shows high homology to known plant EPSPS sequences.

EXAMPLE 2. Isolation of Rice EPSPS genomic sequence and cloning of the rice EPSPS gene

A region of genomic DNA containing the full rice EPSPS gene and 5' upstream region is isolated from a λ EMBLSP6/T7 genomic library constructed from five-day-old etiolated rice shoots (*Oryza sativa* L. Indica var. IR36) (Clontech). 1x10⁶ plaque forming units (pfu) are screened using the ³²P-labelled rice EPSPS cDNA probe (example 1) using protocols provided by the manufacturer. Positive plaques are subjected to subsequent rounds of hybridisation screening until plaque purity of a cross-hybridising plaque is obtained. λ-DNA is prepared from the phage pure stock, according to the method described by Sambrook *et al.*, 1989. The DNA obtained is analysed by restriction digest and Southern blotting, using the same ³²P-labelled rice EPSPS cDNA as a probe. Restriction fragments that cross-hybridise are, where applicable, blunt-ended using a method such as Perfectly Blunt™

(Novagen), and cloned into a suitable vector such as pSTBlue (Novagen). The DNA is then sequenced using an ABI 377A PRISM automated DNA sequencer. Figure 1 shows a schematic of the rice EPSPS gene with some of the restriction sites marked.

A 3.86 kb fragment of the rice EPSPS gene, containing the coding region, the EPSPS promoter, some of the 5' upstream region and the terminator is obtained by PCR. Oligonucleotide primer OSGRA1 (SEQ ID NO.3) is used in conjunction with OSEPSPS3 (SEQ ID NO. 4) to amplify the desired region. OSEPSPS3 contains additional *Sac* I and *Sma* I restriction enzyme sites to facilitate the subcloning of the gene during the later stages of vector construction. A schematic location of these primers is given in Figure 1.

10 SEQ ID NO. 3

OSSGRA1 5' ATT TCT TCT TCT TCC TCC CTT CTC CGC CTC 3'

SEQ ID NO. 4

OSEPSPS3 5' GAG CTC CCC GGG CGA GTG TTG TTG TGT TCT GTC TAA TG 3'

High fidelity Pfu Turbo™ polymerase (Stratagene) is used to perform the PCR reaction with DNA obtained from λ preparation (described above) as the amplification template. The PCR product of expected size is cloned into pCRblunt 4-TOPO™ (Invitrogen) and sequenced to check integrity.

EXAMPLE 3. Mutation of T to I and P to S in the rice EPSPS.

The T to I and P to S mutation is obtained by the introduction of two point mutations. These mutations are introduced into the rice genomic EPSPS gene by PCR using oligonucleotide primers containing the desired mutation. A schematic diagram, indicating the binding sites of the primers used, is shown in Figure 3. Two separate PCR reactions are performed (both using the λ DNA as template).

1) EcoRVEnd (SEQ ID NO.5) + OSMutBot (SEQ ID NO. 6)

25 2) OsMutTop (SEQ ID NO. 7) + SalIEnd (SEQ ID NO. 8)

SEQ ID NO.5

EcoRVEnd 5' GCCTACGAAGGTATGATATCCTCCTACATGTCAGGC 3'

SEQ ID NO.6

OSMutBot 5' GCAGTCACGGCTGCTGTCAATGATCGCATTGCAATTCCAGCGTTCC 3'

30 SEQ ID NO.7

OsMutTop 5' GGAACGCTGGAATTGCAATGCAATCATTGACAGCAGCCGCTGACTGC 3'

SEQ ID NO.8

SalIEnd 5' GGTGGGCATTTCAGTCCCAAGGAACAGTCGACATCCGACCAAGTTGTTTCAACC 3'

The resulting PCR products are joined by using equimolar concentrations of each PCR product as template with the two oligos SalIEnd and EcoRVEnd in a new PCR reaction. An aliquot of the reaction product is analysed by agarose gel electrophoresis and cloned into pCR-Blunt II™ (Invitrogen). Plasmid DNA is recovered and sequenced to detect the successful incorporation of the double mutation.

The DNA fragment containing the double mutation is incorporated into the rice EPSPS genomic clone (Figure 1) as follows. The clone containing the double mutant is digested with *Eco* RV and *Sal* I. The plasmid containing the rice EPSPS DNA PCR product is similarly digested and the *Eco* RV/*Sal* I fragment containing the double mutant ligated into the rice EPSPS gene in pCR4Blunt -TOPO™ using standard cloning methods described in Sambrook *et al.*, 1989 and transformed into competent *E. coli*. Plasmid is recovered from resultant colonies and sequenced in order to confirm the presence of the double mutation with no further alterations. This plasmid, pCR4-OSEPSPS, is shown in Figure 2.

The genomic rice EPSPS gene containing the double mutant (Figure 2) is excised from pCR4-Blunt-TOPO™ using *Pst* I and *Not* I and ligated into pTCV1001 (Figure 4), to generate pTCV10010SEPSPS (Figure 5) and this is transformed into *E. coli* for amplification. Next, the *Pac* I/*Eco* RV restriction fragment is excised from the λ DNA (Figure 1) and inserted into pTCV10010SEPSPS (Figure 5) to generate pTCV1001EPSPSPAC (Figure 6). The rice EPSPS gene, now containing sequence from *Pac* I to *Sac* I (Figure 6), is excised from pTCV1001EPSPSPAC (Figure 6) as an *Eag* I/*Sac* I fragment and ligated into similarly digested pBluescript SK+ to make pBluSK+EPSPS (Figure 7). Further rice EPSPS upstream regions and desired enhancers are assembled (as described below) and ligated into the pBluescript SK+ vector using *Xba* I/*Pac* I.

EXAMPLE 4. Generation of singly enhanced : rice EPSPS promoter fusions

Figure 1 indicates the binding sites of the primers G1 and G2 used to generate a series of deletions at the 5' end of the rice EPSPS gene. The G1 and G2 primers are used in combination with the RQCR10 primer using the rice EPSPS lambda DNA template and Pfu Turbo™ polymerase (Stratagene) using protocols provided by the supplier.

SEQ ID NO.9

G1 5' CGCCTGCAGCTCGAGGTTGGTTGGTTSAGAGTGGAGACACC 3'

SEQ ID NO.10

G2 5' CGCCTGCAGCTCGAGGCCACACCAATCCAGCTGGTGTGG 3'

SEQ ID NO.11

RQCR10 5' GAACCTCAGTTATATCTCATCG 3'

The products obtained are analysed by agarose gel electrophoresis and cloned into
 5 pCR-Blunt II-TOPO™ vector (Invitrogen). The sequence of the resulting products is
 determined to ensure that there is no alteration in the sequence of the rice genomic EPSPS
 clone. Clones to progress are selected based on their orientation within the vector by
 establishing whether or not *Xho* I digestion removes only the polylinker sequence rather than
 the whole insert from the vector.

10 The sequences of the CaMV35 S and FMV 35S genes and their associated 5'
 upstream regions are published in the EMBL database (for example accession v00141 and
 x06166). Primers are designed so as to amplify only the upstream enhancer regions of the
 said genes. The CaMV35S enhancer1 (SEQ ID NO. 36) is thus obtained by PCR using
 primers SEQ ID NO. 12 and SEQ ID NO. 13 in conjunction with Pfu Turbo™ polymerase
 15 and pMJB1 DNA (A59870) as the template. Alternatively a different region of the
 CaMV35S enhancer is obtained using similar methods (SEQ ID NO. 37). The FMV35S
 enhancer is synthesised chemically (SEQ ID NO. 35).

The following oligonucleotide primers are used.

SEQ ID NO. 12

20 35S5 5' CGCTCTAGAGGCCGGCCAAACATGCTGGAGCAGCAGCACTTGTCTAC 3'

SEQ ID NO. 13

35S3 5' CGCTGCAGCTCGAGCATCAATCCACTTGCTTTGAAGACG 3'

The sequence of the amplified and cloned molecules is confirmed following cloning
 into the PCR Blunt-II-TOPO vector (Invitrogen). The pCR Blunt-II-TOPO vector, containing
 25 the EPSPS 5'UTR deletion is digested with *Xba* I/*Xho* I. The Enhancer is removed from its
 respective pCR Blunt-II-TOPO vector also using *Xba* I/*Xho* I digestion and ligated into the
 first vectors containing the EPSPS promoter deletions generated by PCR.

EXAMPLE 5. Generation of doubly enhanced : rice EPSPS promoter fusions.

In order to further increase expression from the rice EPSPS promoter an additional
 30 enhancer may be used in conjunction with either the 35S or FMV enhancer. In one example
 of a cloning strategy to achieve this, enhancer/EPSPS fusions are initially made (similar to
 example 4) comprising a single (first) enhancer. Such first enhancers are selected from the

upstream regions 5' of the promoters of the rice GOS2, rice actin 1, maize polyubiquitin, CaMV 35S, FMV 35S and barley plastocyanin genes. The nucleotide sequences of these regions are published in the EMBL database (accession number x51910, x15865, u29159, v00141, x06166 and z28347 respectively). Primers are designed so as to amplify only the
 5 desired transcriptional enhancer regions 5' to these genes (SEQ ID NO. 15 to 22).

SEQ ID NO.15

Barley5 5' CGCTCTAGAGGCGCGCCCAAAATCTCCCATGAGGAGCACC 3'

SEQ ID NO.16

Barley3 5' CGCTGCAGCTCAGCGCGCTCTTCATCCGGATGAGG 3'

10 SEQ ID NO.17

OSGOS5 5' CGCTCTAGAGGCGCGCGCAATCCGAAAGTTTCTGCACCGTTTTCACC 3'

SEQ ID NO.18

OSGOS3 5' CGCTGCAGCTCAGCGCGCTCTCTCCGTTAGATCATEG 3'

SEQ ID NO.19

15 MPU5 5' GAC TAG TGG CCG GCC ATC AGC GGC CAG CTT TTG TTC 3'

SEQ ID NO.20

MPU3 5' TTA ACT AGT GAG GAG GGC GCC TGC CRT GC 3'

SEQ ID NO.21

RA5 5' CGCTCTAGAGGCGCGCGGATATCCCTCAGCCGCGCTTCACTATC 3'

20 SEQ ID NO.22

RA3 5' CGCTGCAGTGTCTCGCGATCTCTCTGCTTTTCC 3' pst?

DNA is isolated from greenhouse grown plants (maize, barley or rice) using the DNAeasy protocol (Qiagen) and used as the starting template for PCR amplification. The PCR products are cloned into pCR-Blunt-II-TOPO and sequenced to check authenticity. The
 25 enhancer EPSPS fusion is made as described previously in Example 4 (using Xba/Xho1 swap) except in the case of rice actin 1, where the enhancer is inserted as an Xba 1/Pst 1 fragment (a Xho1 site is replaced with a Pst1 site due to the presence of a Xho 1 site in the Rice actin enhancer) and maize polyubiquitin, where the enhancer is inserted as a Spe 1/Xho 1 fragment (the Xba 1 site is replaced with a Spe 1 site due to the presence of a Xba 1 site in
 30 the maize polyubiquitin enhancer). Note that an internal Xho1 site is used with respect to the maize polyubiquitin enhancer region. The second enhancer, which can be either the CaMV35S enhancer or the FMV enhancer is amplified using the primers 35SXho and 35S3

(SEQ ID NO 23 and SEQ ID NO. 13) (35S) or FMVXho and FMV3 (SEQ ID NO 25 and SEQ ID NO 26) respectively. These primers facilitate the introduction of a *Xho* I site (or *Pst* I site) at the 5' and 3' termini of the enhancer. Alternatively a *Pac* I site is introduced at the 3' end in place of the *Xho* I site using primers 35SPac (SEQ ID NO. 24) or FMVPAC3 (SEQ ID. 27).

SEQ ID NO.23

35Sxho 5' CTGCACTCTGAGAACATGGTGGAGCAGACACACTTGTCTAC 3'

SEQ ID NO.24

35SPAC 5' TTAATTAAACATCAATCCACTTGCCTTGAAGAGG 3'

10 SEQ ID NO.25

FMVXho1 5' CTCGAGGGCCGGCCGCGAGCTGCTTG 3'

SEQ ID NO.26

FMV3 5' CTCGACTTTTGTGGTGGTCACTGCGTTGG 3'

SEQ ID NO.27

15 FMV3Pac 5' TTAATTAAATTTGTGGTGGTCACTGCGTTGG 3'

Once sequenced, the PCR product, either *Xho* I : *Xho* I, *Pst* I/*Pac* I (when rice actin is the first enhancer) or *Xho* I/*Pac* I, is introduced into the construct which comprises the first enhancer : EPSPS gene fusion either at the *Xho* I site or between the *Xho* I and *Pac* I or *Pst* I and *Pac* I restriction sites as required. The introduction at the *Xho* I site is used to construct the double enhancer fusion constructs comprising either the G1 or G2 EPSPS promoter deletions. Both the *Xho* I/*Pac* I and *Pst* I/*Pac* I fragments are used to construct double enhancer fusions comprising the G3 EPSPS promoter deletion. Where the second enhancer is introduced as an *Xho* I fragment the orientation of the enhancer is determined by PCR.

25 **EXAMPLE 6. Insertion of Adh1 intron into the 5' UTR of the rice EPSPS gene**

The insertion of the Maize Adh1 intron 1 into the desired rice EPSPS promoter deletion (e.g. made as described in Example 4) is performed prior to the generation of the fusion construct with the desired enhancer(s). In this particular example the Adh1 intron is introduced into the G2 EPSPS promoter deletion. The skilled man will appreciate that similar methodology can be adopted to incorporate the Adh1 intron into other EPSPS promoter deletions. The maize Adh1 intron is inserted into the constructs by PCR. The Adh I intron is

amplified from a suitable source, such as maize genomic DNA or a vector such as pPAC1 (Figure 8) using primers Adh5 (SEQ ID NO. 28) and Adh3 (SEQ ID NO. 29):

SEQ ID NO. 28

Adh5 CCGATCCTCCCGACCTCCACGCGGTCGCGAGGATCAAGTGAAGGTCGGCCTTGTTCCTCTCTG

5 SEQ ID NO. 29

Adh3 GACGCGATGGTCGCGCCCATCCGAGCTGCACGGGTCCAGGAAAGCAATC

The resulting PCR product is denatured and used as a primer in conjunction with Adh5Pac (SEQ ID NO. 30) to amplify the desired product using the vector pTCV1001EPSPSPAC (Figure 6) as template.

10 SEQ ID NO. 30

Adh5Pac CGAGTTCTTATAGTAGATTTCACCTTAATTAAAC

The resulting PCR product is cloned into PCR-Blunt II (Invitrogen). The *Pac* I:*Hind* III fragment is excised from the rice genomic clone (Figure 1) and inserted into pTCV1001 to generate pTCVEPSPSPH (Figure 9). Next, the *Pac* I/*Nco* I PCR product obtained above and comprising the Adh 1 intron is inserted into pTCVEPSPH as shown by the schematic (Figure 9). The *Pac* I:*Eco* RV fragment present in the cloned EPSPS gene containing the double mutant (Figure 10) is excised and replaced with the *Pac* I/*Eco* RV fragment from pTCVEPSPSPH that comprises the Adh 1 intron sequence (Figure 9). Finally the full EPSPS gene comprising the Adh 1 sequence is excised from pTCVEPSPSADH (Figure 10) as an *Eag* I/*Sac* I fragment and cloned into pBluescript SK+ to give pBluSKEPSPSADH (Figure 11).

EXAMPLE 7. Introduction of optimised pre ATG consensus sequence (Kozak) via site directed mutagenesis for constructs comprising the maize Adh1 intron.

Optionally, site directed mutagenesis is performed on constructs containing the Adh1 intron using the QuickChange Site Directed Mutagenesis kit (Stratagene). This is performed on the *Pac* I/*Sac* I EPSPS fragment in pBluescript SK+ (Figure 11) prior to fusion with the enhancer : EPSPS promoter fusions. The following oligonucleotide are used according to the supplied protocols to optimise the KOZAK sequence.

SEQ ID NO. 31

30 Oskozak 5' GGACCCGTGCAGCTGCGGTACCATGCGGGGACCATGGC 3'

SEQ ID NO. 32

OSkozakrev 5' GCCATGGTCGCCGCCATGGTACCGCAGCTGCACGGGTCC 3'

Clones are analysed by restriction analysis, using *Kpn* I, on recovered plasmid. The correctly altered DNA is characterised by an additional *Kpn* I restriction site compared to the un-altered DNA. The sequence is then verified by automated DNA sequencing.

The altered DNA sequence may be transferred original constructs using the unique restriction enzyme sites of *Sph* I or *Pac* I at the 5' end and *Avr* II or *Eco* RV at the 3' end as appropriate for each vector.

EXAMPLE 8. Completion of EPSPS expression cassettes comprising, in the 5' to 3' direction, Enhancer region(s), rice EPSPS promoter upstream region, EPSPS promoter, EPSPS 5'UTR + (optional) maize Adh1 intron 1, rice EPSPS plastid transit peptide coding region, rice mature EPSPS coding region and rice EPSPS gene terminator region.

The singly and doubly enhanced rice EPSPS promoter fusions (Examples 4 and 5) contained within the pCR Blunt-II-TOPO vectors are excised using *Xba* I and *Pac* I and inserted into the similarly digested pBluescript SK+ clone containing the remainder of the rice EPSPS sequence (Figures 7/11). However, when the maize polyubiquitin enhancer:EPSPS fusion is to be used this is firstly cloned into pBluescript using *Spe*I/*Pac* I. The remainder of the rice EPSPS sequence is then inserted as *Pac* I/*Sac* I to complete the expression cassette. This final cloning step results in the required gene constructs. Examples of constructs obtainable using the above strategies are given below in Table 1. Schematic maps are given in Figures 13-15.

Clone	First enhancer	Second enhancer	EPSPS Promoter deletion	Adh1 Intron	EPSPS genomic coding region	EPSPS Terminator
ZEN9	CaMV35S	None	G1	No	Yes	Yes
ZEN11	RA	35S	G1	No	Yes	Yes
ZEN14	RA	35S	G3	No	Yes	Yes
ZEN15	MPU	35S	G3	No	Yes	Yes
ZEN20	BPC	35S	G2	Yes	Yes	Yes
ZEN18	GOS2	35S	G2	Yes	Yes	Yes
ZEN12	GOS2	35S	G3	No	Yes	Yes
ZEN23	FMV	None	G2	Yes	Yes	Yes
ZEN24	FMV	35S	G2	Yes	Yes	Yes
ZEN25	35S	FMV	G2	Yes	Yes	Yes

EXAMPLE 9. Subcloning of EPSPS expression cassettes from Bluescript into pIGPD9

Where desired and especially for transformation of plants by direct DNA methods (whiskers, bombardment and protoplasts), the EPSPS expression constructs are excised from pBluescript using *Xma* I and cloned into pIGPD9 (Figure 12). The use of this vector for transformation avoids transfer of antibiotic resistance genes to the plant since selection relies on complementation of an auxotrophic his B *E.coli* mutant with the gene expressing IGPD (the his B product). The pIGPD9-derived plasmids deriving from insertion of the *Xma* I fragments of pZEN9, 11, 12, 14, 15, 16, 18, 20, 23, 24 and 25 are termed pZEN9i, pZEN11i, pZEN12i ...etc

Large DNA preparations for use in plant transformation are obtained using the Maxi-prep procedure (Qiagen) using protocols provided by the Manufacturer

EXAMPLE 10. Preparation of DNA for plant transformation

The above procedure describes the assembly of 'EPSPS expression cassettes' comprising, in a 5' to 3' direction, an enhancer sequence(s), an EPSPS promoter from rice, a region encoding a rice EPSPS transit peptide, a region encoding a mature rice EPSPS enzyme which is resistant to glyphosate through having T to I and P to S changes at the specified positions and a rice EPSPS gene terminator.

Optionally the desired cassettes also further comprise a drug selection marker gene (e.g. ampicillin resistance, kanamycin resistance etc.) a T-DNA Left or Right Border region and (optionally) a scaffold attachment region added 5' and/or 3' to the above described construct. The skilled man will recognise that similar methods to those described above can be used to obtain these added components and clone them into the desired positions.

EXAMPLE 11. Transformation of corn lines using an *Agrobacterium* strain containing a superbinary vector which includes an EPSPS expression cassette between the right and left borders of the T-DNA; selection and regeneration of plant cells and plants which are resistant to glyphosate

Construction of *Agrobacterium* strain

Bluescript plasmid DNA (e.g. ZEN9, ZEN11, ZEN14, ZEN15, ZEN18, ZEN20, ZEN12, ZEN23, ZEN24 or ZEN25) is digested with either *Xma* I or with *Xba* I/ *Sac* I and the thus-obtained (~ 5-6.5 kb) EPSPS-encoding fragment ligated into a position within the cloning site located between the right and left T-DNA borders of similarly restricted pSB1. In the case, for example, of using the *Xma* I fragment of pZEN 18 this ligation creates the

plasmid pZEN18SB11 (Figure 16). The construction of plasmid pSB11 and the construction of its parent, pSB21, is described by Komari et al (1996, Plant J. 10: 165-174). The T-DNA region of pZEN18 is integrated into the superbinary pSB1 vector. (Saito *et al* EP 672 752 A1) by a process of homologous recombination (Figure 16) to create the plasmid, pSB1ZEN18. To achieve this the plasmid pZEN18SB11 is transformed into *E. coli* strain HB101 which is then, according to the triple cross method of Ditta et al (1980, Proc. Natl. Acad. Sci. USA 77: 7347-7351), mated with an *Agrobacterium* LBA4404 harbouring pSB1 to create the transformed strain of *Agrobacterium*, LBA4404 (pSB1ZEN18) in which the presence of the cointegrate plasmid pSB1ZEN18 is selected for on the basis of resistance to spectinomycin. The identity of pSB1ZEN18 is also confirmed on the basis of Sal I restriction analysis (Figure 16). LBA4404 strains containing the directly analogous constructs pSB1ZEN9, pSB1ZEN11, pSB1ZEN12, pSB1ZEN14 etc are similarly constructed starting from the XmaI fragments of pZEN9, ZEN11, ZEN12, ZEN 14 etc..

Alternatively, using similar methods to those described above, a similar fragment of pZEN9, ZEN 11 etc is homologously recombined into a position between the right and left borders of the superbinary vector pTOK162 (Fig 1 in US 5591616) to generate a similar set of cointegrate plasmids selected for in *Agrobacterium* on the basis of combined resistance to kanamycin and spectinomycin.

Agrobacterium strain LBA4404 which has a helper plasmid PAL4404 (having a complete *vir* region) is available from the American Type Culture Collection (ATCC 37349). An alternative useful strain is *Agrobacterium* EHA101 (1986, Hood *et al*, J. Bacteriol., 168(3): 1283-1290) which has a helper plasmid having the *vir* region from the strongly virulent strain *Agrobacterium tumefaciens* A281.

Preparation of *Agrobacterium* suspensions

Agrobacterium strains LBA4404(pSB1ZEN9), LBA4404 (pSB1ZEN11) etc are each streaked onto plates containing 'PHI-L' solid medium and cultured at 28 C in the dark for 3 to 10 days.

PHI-L medium is as described on page 26 (Example 4) of WO 98/32326. PHI-L medium made up in double-distilled water comprises 25 ml/l of stock solution A, 25 ml/l of stock solution B, 450.9 ml/l of stock solution C and 50 mg/l of spectinomycin. Stock solutions are sterilised by autoclaving or filtration. Stock solution A is 60 g/l K₂HPO₄ and 20 g/l NaH₂PO₄ adjusted to pH 7.0 with KOH; stock solution B is 6 g/l Mg SO₄.7H₂O, 3 g/l

1 KCl, 20 g/l NH₄Cl, 0.2 g/l CaCl₂ and 50 mg/l FeSO₄ · 7H₂O : stock solution C is 5.56 g/l of glucose and 16.67 g/l of agar (A-7049, Sigma Chemicals, St Louis, Mo, USA)

Alternatively the *Agrobacterium* are cultured for 3 -10 d on a plate containing YP medium (5 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, 15 g/l agar at pH 6.8) as described
5 by Ishida *et al* (1996, Nature Biotechnology, 14, 745-750) or, alternatively, as described by Hei *et al* in US 5591616 (AB medium (Drlica and Kado, 1974; Proc. Natl. Acad. Sci. USA 71:3677-3681)) but, in each case, modified to provide the appropriate antibiotic selection (e.g. containing 50 mg/ml spectinomycin in the case of *Agrobacterium* strain LBA4404(pSB1ZEN9) etc. or containing both 50 mg/ml spectinomycin and 50 mg/ml
10 kanamycin in the case that *Agrobacterium* containing a pTOK 162-derived superbinary vector is used).

Plates of *Agrobacterium* made as described above are stored at 4 C and used within a month of preparation. For preparation of suspensions a single colony from the master plate is streaked out onto a plate containing, at pH 6.8, 5 g/l yeast extract (Difco), 10 g/l peptone
15 (Difco), 5 g/l NaCl, 15 g/l agar (Difco) and 50 mg/l of spectinomycin (or as appropriate for the particular strain of *Agrobacterium*) . Plates are incubated at 28 C, in the dark for 2d.

Suspensions of *Agrobacterium* for transformation of plant material are prepared in a similar manner to described in US 5591616. (Using good microbiological practice to avoid contamination of aseptic cultures) 3 X 5 mm loopfuls of *Agrobacterium* are removed from
20 plates, transferred and suspended in 5 ml of sterile AA liquid medium in a 14 ml Falcon tube. As used here, AA liquid medium at pH 5.2 contains the major inorganic salts, amino acids and vitamins defined by Toriyama and Hinata (1985) in Plant Science 41, 179-183), the minor inorganic salts of Murashige and Skoog medium (Murashige and Skoog, 1962 in Physiol. Plant 15, 473-497), 0.5 g/l of casamino acids (casein hydrolysate), 1 mg/l of 2,4 -
25 dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l of kinetin, 0.1 mg/l of gibberellin, 0.2M glucose, 0.2M sucrose and 0.1 mM acetosyringone.

Alternatively, suspensions of *Agrobacterium* for transformation of plant material are prepared in a similar manner to described in WO 98/32326. 3 X 5 mm loopfuls of
Agrobacterium are removed from plates, transferred and suspended in 5 ml of the sterile
30 PHI-A basic medium as described in Example 4 on page 26 of WO 98/32326 or, alternatively, suspended in 5 ml of the sterile PHI-I combined medium also described in Example 4 on page 26 of WO 98/32326. In either case 5 ml of 100 mM 3'-5'-Dimethoxy-

4'-hydroxyacetophenone is also added. PHI-A basic medium at pH 5.2 comprises 4 g/l of CHU(N6) basal salts (Sigma C-1416), 1.0 ml/l of Eriksson's vitamin mix (1000X, Sigma E-1511), 0.5 mg/l thiamine. HCl, 1.5 mg/ml of 2,4-D, 0.69g/l L-proline, 68.5 g/l sucrose and 68.5 g/l glucose. PHI-I combined medium, also adjusted to pH 5.2 with KOH and filter sterilized, comprises 4.3 g/l of MS salts (GIBCO-BRL), 0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine. HCl, 1.0 mg/ml thiamine. HCL, 100 mg/l myo-inositol, 1 g/l vitamin assay casamino acids (Difco), 1.5 mg/ml of 2,4-D, 0.69g/l L-proline, 68.5 g/l sucrose and 36 g/l glucose.

Alternatively, suspensions of *Agrobacterium* for transformation of plant material are prepared in a similar manner to described by Ishida et al (1996) Nature Biotechnology, 14, 745-750. 3 X 5 mm loopfuls of *Agrobacterium* are removed from plates, transferred and suspended in 5 ml of LS-inf medium. LS-inf medium (Linsmaier and Skoog, 1965, Physiol. Plant 18, 100-127) adjusted to pH 5.2 with KOH contained LS major and minor inorganic salts, 0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine. HCl, 1.0 mg/ml thiamine. HCL, 100 mg/l myo-inositol, 1 g/l vitamin assay casamino acids (Difco), 1.5 mg/ml of 2,4-D, 68.5 g/l sucrose and 36 g/l glucose.

However produced, the suspension of *Agrobacterium* is vortexed to make an even suspension and the cell population adjusted to between 0.5×10^9 and 2×10^9 cfu/ml (preferably the lower). 1×10^9 cfu/ml corresponds to an OD (1 cm) of ~ 0.72 at 550 nm.

Agrobacterium suspensions are aliquoted into 1 ml lots in sterile 2 ml microcentrifuge tubes and used as soon as possible

Corn lines for transformation

Suitable maize lines for transformation include but are not restricted to, A188, F1 P3732, F1 (A188 x B73Ht), F1 (B73Ht x A188), F1 (A188 x BMS). Varieties A188, BMS (Black Mexican Sweet) and B73 Ht are obtained from the Ministry of Agriculture, Forestry and Fisheries, which is well known to the skilled man. P3732 is obtained from IWATA RAKUNOU KYODOKUMIAI. Suitable maize lines also include a variety of A188 x inbred crosses (e.g. PHJ90 x A188, PHN46 x A188, PHPP8 x A188 in table 8 of WO98/ 32326) as well as elite inbreds from different heterotic groups (e.g. PHN46, PHP28 and PHJ90 in table 9 of WO98/ 32326).

For example immature embryos are produced from "Hi-II" corn. "Hi-II" is a hybrid between inbreds (A188 x B73) generated by reciprocal crosses between Hi-II parent A and

Hi-II parent B available from the Maize Genetic Cooperation Stock Center, University of Illinois at Champaign, Urbana, Illinois). Seeds, termed 'Hi-II' seeds obtained from these crosses are planted out in a greenhouse or field. The resulting Hi-II plants are self or cross-pollinated with sister plants.

5 Preparation of immature embryos, infection and co-cultivation

Transformation of immature embryos of corn is carried out by contacting the immature embryos with the suitable recombinant strains of *Agrobacterium* described above. An immature embryo means the embryo of an immature seed which is in the stage of maturing following pollination. Immature embryos are an intact tissue that is capable of cell
10 division to give rise to callus cells that can then differentiate to produce the tissues and organs of a whole plant. Preferred material for transformation also includes the scutella of embryos which is also capable of inducing dedifferentiated calli with the ability to regenerate normal fertile plants having been initially transformed. Preferred material for transformation thus also includes callus derived from such dedifferentiated immature zygotic embryos or
15 scutella.

Immature corn embryos are isolated aseptically from developing ears as described by Green and Phillips (1976, Crop. Sci. 15: 417-421) or, alternatively, by the methods of Neuffer et al (1982, "Growing Maize for genetic purposes" in *Maize for biological research*, W.F. Sheridan ed., University Press, University of North Dakota, Grand Forks, North
20 Dakota, USA). For example, immature corn embryos between 1-2 mm (preferably 1-1.2 mm) long are aseptically isolated from female spikes at 9-12 (preferably 11) d after pollination using a sterile spatula. Typically ears are surface sterilised with 2.63% sodium hypochlorite for 20 min before washing with sterile deionized water and aseptic removal of immature embryos. Immature embryos (preferably ~ 100 in number) are dropped directly
25 into a 2 ml microcentrifuge tube containing about 2 ml of the same medium as used for preparing the suspension of *Agrobacterium* (the alternatives for which are described above). The cap of the tube is closed and the contents mixed by vortexing for a few seconds. The medium is decanted off, 2 ml of fresh medium are added and vortexing is repeated. All of the medium is then drawn off to leave the washed immature embryos at the bottom of the
30 tube.

Having prepared the immature maize embryos the next phase of the process, the infection step, is to contact them in with the transformed strain of *Agrobacterium*.

In one example of this process, the infection step takes place in a liquid medium which includes the major inorganic salts and vitamins of N6 medium (1987, Chu C.C. Proc. Symp. Plant Tissue Culture, Science Press Peking, Pp 43-50) as described in example 4 of WO 98/32326. 1.0 ml of suspension of *Agrobacterium*, prepared as described above in PHI-
 5 A medium is added to the embryos in the microcentrifuge tube and vortexed for about 30s. Alternatively, 1.0 ml of suspension of *Agrobacterium* prepared, also as described above, in either PHI-I medium or in LS-inf medium is added.

After standing for 5 minutes the suspension of *Agrobacterium* and embryos is poured out into a Petri plate containing either 1) PHI-B medium or 2) PHI-J medium or 3) LS-AS
 10 medium according to whether the original suspension of *Agrobacterium* had been prepared in PHI-A medium, PHI-I medium or LS-inf medium, respectively. The *Agrobacterium* suspension is drawn off using a Pasteur pipette, the embryos manipulated so that they sit axis-side downwards onto the medium, the plate sealed with parafilm and incubated in the dark at 23-25 C for 3 days of cocultivation. PHI-B medium at pH 5.8 comprises 4 g/l of
 15 CHU(N6) basal salts (Sigma C-1416), 1.0 ml/l of Eriksson's vitamin mix (1000X, Sigma E-1511), 0.5 mg/l thiamine. HCl, 1.5 mg/ml of 2,4-D, 0.69g/l L-proline, 0.85 mg/l silver nitrate, 30 g/l sucrose, 100 µM acetosyringone and 3 g/l gelrite (Sigma). PHI-J medium, also adjusted to pH 5.8 comprises 4.3 g/l of MS salts (GIBCO-BRL), 0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine. HCl, 1.0 mg/ml thiamine. HCL, 100 mg/l myo-inositol, 1.5
 20 mg/ml of 2,4-D, 0.69g/l L-proline, 20 g/l sucrose, 10 g/l glucose, 0.5 g/l MES (Sigma), 100 mM acetosyringone and 8 g/l purified agar (Sigma A-7049). LS-AS medium (Linsmaier and Skoog, 1965, Physiol. Plant 18, 100-127) adjusted to pH 5.8 with KOH contains LS major and minor inorganic salts, 0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine. HCl, 1.0 mg/ml thiamine. HCL, 700 mg/l L-proline, 100 mg/l myo-inositol, 1.5 mg/ml of 2,4-D, 20
 25 g/l sucrose, 10 g/l glucose, 0.5 g/l MES, 100 mM acetosyringone and 8 g/l purified agar (Sigma A-7049).

Following the preparation of immature embryos, as described above, an alternative method of achieving transformation is to infect them during and after a period of dedifferentiation as described in US 5591616. Immature embryos are placed on LSD 1.5
 30 solid medium containing LS inorganic salts and vitamins along with 100 mg/ml casamino acids, 700 mg/l L-proline, 100 mg/l myo-inositol, 1.5 mg/ml of 2,4-D, 20 g/l sucrose and 2.3 g/l of gelrite. After 3 weeks at 25 C, calli originating from the scutella are collected in a

2 ml microcentrifuge tube and immersed in 1 ml of *Agrobacterium* suspension prepared, as described above, in AA medium. After standing for 5 minutes, the resultant calli are transferred to 2N6 solid medium containing 100 mM acetosyringone and incubated in the dark at 25 C for a 3 day period of cocultivation. 2N6 solid medium comprises the inorganic salts and vitamins of N6 medium (Chu C.C., 1978; Proc. Symp. Plant Tissue Culture, Science Press Peking, pp 43-50) containing 1 g/l casamino acids, 2 mg/l 2,4-D, 30 g/l sucrose and 2 g/l of gelrite.

'Resting and Selection of transformants'

Following cocultivation, embryos are, optionally, transferred to a plate containing PHI-C medium, sealed over with parafilm and incubated in the dark for 3 days for a 'resting step' prior to selection. PHI-C medium at pH 5.8 comprises 4 g/l of CHU(N6) basal salts (Sigma C-1416), 1.0 ml/l of Eriksson's vitamin mix (1000X, Sigma E-1511), 0.5 mg/l thiamine. HCl, 1.5 mg/ml of 2,4-D, 0.69g/l L-proline, 0.85 mg/l silver nitrate, 30 g/l sucrose, 0.5 g/l MES, 100 mg/l carbenicillin and 8 g/l purified agar (Sigma A-7049). As disclosed in WO 98/32326, the desirability of including this resting step in the overall transformation process varies according to corn line and is a matter of experiment.

For the selection step, about 20 embryos are transferred onto each of a number of fresh plates containing PHI-D selection medium or LSD 1.5 selection medium, sealed with parafilm and incubated in the dark at 28 C. PHI-D selection medium, adjusted to pH 5.8 with KOH, comprises 4 g/l of CHU(N6) basal salts (Sigma C-1416), 1.0 ml/l of Eriksson's vitamin mix (1000X, Sigma E-1511), 0.5 mg/l thiamine. HCl, 1.5 mg/ml of 2,4-D, 0.69g/l L-proline, 0.85 mg/l silver nitrate, 30 g/l sucrose, 0.5 g/l MES, 100 mg/l carbenicillin, 8 g/l purified agar (Sigma A-7049) and between 0.1 mM and 20 mM of tissue culture grade N-(Phosphonomethyl)-glycine (Sigma P-9556). LSD 1.5 selection medium, adjusted to pH 5.8 with KOH, comprises LS major and minor inorganic salts (Linsmaier and Skoog, 1965, Physiol. Plant 18, 100-127), 0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine. HCl, 1.0 mg/ml thiamine. HCL, 700 mg/l L-proline, 100 mg/l myo-inositol, 1.5 mg/ml of 2,4-D, 20 g/l sucrose, 0.5 g/l MES, 250 mg/l cefotaxime, 8 g/l purified agar (Sigma A-7049) and between 0.1 mM and 20 mM of tissue culture grade N-(Phosphonomethyl)-glycine (Sigma P-9556).

Alternatively, in the case that the starting material for selection are calli-derived from immature embryos as disclosed in WO 5591616 then such calli are washed with sterilised water containing 250 mg/ l cefotaxime before culturing on LSD 1.5 selection medium.

The embryos or clusters of cells that proliferate from the immature embryos are
5 transferred (if necessary using a sterile scalpel) to plates containing fresh selection medium at 2 weekly intervals over a total period of about 2 months. Herbicide-resistant calli are then bulked by continued growth on the same medium until the diameter of the selected callus exceeds about 1.5 cm

The concentration of N-(Phosphonomethyl)-glycine in the selection medium is
10 chosen appropriately to select a desirable number of genuine transformants and is preferably within the range 0.3- 5 mM. Preferably the concentration of N-(Phosphonomethyl)-glycine used in the selection medium is about 1 mM for the first two weeks of selection and about 3 mM thereafter.

Regeneration of transformants/ propagation and analysis of transformed plant material

15 The selected calli are regenerated into normal fertile plants according to, for example, the methods described by Duncan *et al* (1985, *Planta*, 165, 322-332) by Kamo *et al* (1985, *Bot. Gaz.* 146(3), 327-334) and/or by West *et al* (1993, *The Plant Cell*, 5, 1361-1369) and/or by Shillito *et al* (1989) *Bio/ Technol.* 7, 581-587.

For example, selected calli of diameter 1.5- 2 cm are transferred to regeneration/
20 maturation medium and incubated in the dark for about 1-3 weeks to allow the somatic embryos to mature. A suitable regeneration medium, PHI-E medium (WO 98/ 32326) is adjusted to pH 5.6 with KOH and comprises 4.3 g/l of MS salts (GIBCO-BRL), 0.5 mg/ ml nicotinic acid, 0.5 mg/ ml pyridoxine. HCl, 0.1 mg/ ml thiamine. HCL, 100 mg/ l myo-inositol, 2 mg/ l glycine, 0.5 mg/ l zeatin, 1.0 mg/ ml of indoleacetic acid, 0.1 mM abscisic
25 acid, 100 mg/ l carbenicillin, 60 g/ l sucrose, 8 g/ l purified agar (Sigma A-7049) and, optionally, between 0.02 mM and 1 mM of tissue culture grade N-(Phosphonomethyl)-glycine (Sigma P-9556).

The calli are then transferred to rooting/ regeneration medium and grown at 25 C under either a schedule of 16 h daylight ($270 \text{ mE m}^{-2} \text{ s}^{-1}$) and 8 h of darkness or under
30 continuous illumination ($\sim 250 \text{ mE m}^{-2} \text{ s}^{-1}$) until such a time as shoots and roots develop. Suitable rooting/ regeneration media are either LSZ medium as described in the following paragraph (optionally containing no phosphonomethylglycine) or PHI-F medium at pH 5.6

which comprises 4.3 g/l of MS salts (GIBCO-BRL), 0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine. HCl, 0.1 mg/ml thiamine. HCL, 100 mg/l myo-inositol, 2 mg/l glycine, 40 g/l sucrose and 1.5 g/l gelrite.

Alternatively, selected calli are transferred directly to LSZ regeneration medium
5 adjusted to pH 5.8 with KOH and comprising LS major and minor inorganic salts (Linsmaier and Skoog, 1965, *Physiol. Plant* 18, 100-127), 0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine. HCl, 1.0 mg/ml thiamine. HCL, 700 mg/l L-proline, 100 mg/l myo-inositol, 5 mg/ml of zeatin, 20 g/l sucrose, 0.5 g/l MES, 250 mg/l cefotaxime, 8 g/l purified agar (Sigma A-7049) and, optionally, between 0.02 mM and 1 mM of tissue culture grade N-
10 (Phosphonomethyl)-glycine (Sigma P-9556) is used. After a period of incubation in the dark plates are subject to illumination (continuous or light/day as above) and plantlets regenerated.

Small plantlets are transferred to individual glass tubes containing either PHI-F medium or half strength LSF medium at pH 5.8 comprising LS major salts (Linsmaier and Skoog, 1965, *Physiol. Plant* 18, 100-127) at half strength, LS minor salts, 0.5 mg/ml
15 nicotinic acid, 0.5 mg/ml pyridoxine. HCl, 1.0 mg/ml thiamine. HCL, 100 mg/l myo-inositol, 20 g/l sucrose, 0.5 g/l MES, 8 g/l purified agar (Sigma A-7049) and grown on for about another week. Plantlets are then transferred to pots of soil, hardened off in a growth chamber (85% relative humidity, 600 ppm CO₂ and 250 mE m⁻² s⁻¹) and grown to maturity in a soil mixture in a greenhouse.

20 The first (T₀) generation of plants obtained as above are self fertilised to obtain second generation (T₁) seeds. Alternatively (and preferably) the first generation of plants are reciprocally crossed with another non-transgenic corn inbred line in order to obtain second generation seeds. The progeny of these crosses (T₁) are then expected to segregate 1:1 for the herbicide resistance trait. T₁ seeds are sown, grown up in the glass house or field and the
25 level of resistance, inheritance of resistance and segregation of resistance to the herbicide glyphosate through this and subsequent generations assessed by the observation of differential plant survival, fertility, and symptoms of necrosis in tissue following spray treatment of with glyphosate (suitably formulated and, optionally, as a salt) at a range of rates between 25 and 2000 g/ha and at a range of growth stages between and including V₂ and V₈
30 (or, alternatively, at 7-21 days post germination). These assessments are made relative to susceptible segregants and relative to similar, untransformed lines of corn which do not comprise genes of the present invention or prior art sequences capable of conferring resistance

to glyphosate. Transgenic lines which exhibit resistance to glyphosate are selected and again selfed or backcrossed to a non-transgenic inbred.

At all stages in the above process tissue samples of transformed callus, plantlets, T0 and T1 plant material are optionally taken and analysed by 1) Southern and PCR in order to indicate the presence, copy number and integrity of transgenes, 2) Northern (or similar) analysis in order to measure expression of mRNA from transgenes, 3) quantitative Western analysis of SDS gels in order to measure expression levels of EPSPS and 4) measurement of EPSPS enzyme activity levels in the presence and absence of glyphosate in order to assess more accurately how much of the EPSPS which is expressed derives from the transgene.

Such methods of analysis are well known in the art. Suitable methods to test for the presence, integrity and expression of the transgene by PCR, for carrying out Southern analysis, for the cloning and expression of mature rice EPSPS in *E.coli*, for the purification of rice EPSPS, for the generation of polyclonal antibodies to purified rice EPSPS, for Western analysis of EPSPS levels in callus and in plant tissues and for the measurement of EPSPS activity levels in plant-derived extracts at a concentration of glyphosate which discriminates between the endogenous glyphosate-susceptible EPSPS and the glyphosate-resistant product of the EPSPS-encoding transgene are described in more detail below in Examples 17 -20.

TABLE 2 Transgene expression in calli originating from transformation of immature embryos of A188 X B73 (Hi-II) corn with Agrobacterium strain LBA4404 containing a superbinary vector carrying the EPSPS expression cassettes of bluescript vectors (pZEN 11, 12, 14 or 15) within the T-DNA borders

The table shows EPSPS enzyme assay (+/- 100 μ M glyphosate at 100 μ M PEP) results based upon enzyme assays of extracts of stably transformed callus of regenerable A188 x B73 regenerable corn, transformed using Agrobacterium containing a superbinary vector carrying the EPSPS expression cassettes of the pZEN11, ZEN12, ZEN14 or ZEN15 Bluescript plasmids. Each callus line represents a single event which is assayed in duplicate. The ratio of the true (allowing for ~ 8% inhibition) tolerant enzyme activity expressed by the transgene to endogenous susceptible activity (> 98% inhibited + glyphosate) is calculated. The mutant EPSPS is expressed relatively strongly in some lines, notably lines 5 and 12, where, allowing for the reduced Vmax of the tolerant enzyme relative to the w/t (about a third) it can be estimated that the tolerant enzyme is expressed at 2-6 X the normal level of

the endogenous EPSPS (this calculation is complicated by the fact that, in some selected calli, expression of the transgene occurs alongside an apparent ~ 2-2.5X increase in the background expression of the susceptible endogenous enzyme).

5

Event. Line#	DNA Construct	Measured activity (nmol/ min/ mg) + 100uM glyphosate (true tolerant activity = measured x 1.08)	Total Activity (nmole / min / mg) in absence of glyphosate	Ratio of sensitive/ true tolerant EPSPS activity
1	ZEN15	3.4	22.69	6:1
		2.5	21.72	
5	ZEN15	6.6	21.72	1.9:1
		7.7	23.94	
7	ZEN14	5.11	18.13	2.8:1
		4.8	22.33	
10	ZEN14	6.89	28.13	2.7:1
		7.17	29.13	
12	ZEN11	20.31	50.16	1.6:1
		16.63	53.66	
13	ZEN11	4.54	20.92	3: 1
		5.31	21.57	
14	ZEN12	3.74	22.44	6:1
		2.55	23.64	
15	ZEN12	12.74	39.79	2:1
		11.40	39.13	
21	control	0.0	11.85	-
		0.1	12.29	
22	control	-0.5	11.59	-
		-0.02	11.97	

EXAMPLE 12. Transformation of corn lines by bombardment with particles coated with DNA which includes an EPSPS expression cassette; selection and regeneration of plant cells and plants which are resistant to glyphosate

In a further example, friable embryogenic callus derived from immature maize embryos is initiated on a solid medium and transformed biolistically. Similar to the process described in example 11, transformed callus is then selected on the basis of differential growth rate in medium containing a range of concentrations of glyphosate. Resistant callus is selected and regenerated to provide plantlets which are transferred to pots, grown to maturity and self or cross fertilised in the glasshouse. The progeny seed (T1) are then grown up to provide further generations of plants which are assessed for resistance to glyphosate and analysed for transgene presence, integrity and expression as described in example 11.

Initiation of callus from immature embryos

Friable embryogenic Type II callus suitable for transformation is derived from immature embryos of, for example, A188 X B73 corn. Alternative inbred such as B73-derived and hybrid lines of corn can be also used including, for example, those listed in Example 11. Immature embryos of maize between 1-2 mm long are isolated aseptically from female spikes at, typically, about 11 d after pollination using the methods indicated in example 11.

Immature embryos are plated onto, for example, onto a N6-based medium (Chu et al, 1975, Scientia Sinica, 18, 659-668) adjusted with KOH to pH 5.8 containing 1mg/l 2,4-D, 2.9g/l L-proline, 2 mg/l L-glycine, 100 mg/l of casein hydrolysate, N6 major salts, N6 minor salts, N6 vitamins, 2.5 g/l gelrite (or 2 g/l 'Gelgro') and 20 g/l sucrose. Alternative suitable media include, for example, a similar medium but containing MS salts (Murashige and Skoog, 1962, Physiol. Plant, 15, 473-497) in place of N6 salts. Alternatively, the medium may contain ~ 10 mg/l dicamba in place of 2,4-D.

Immature embryos are incubated in the dark on the above medium at ~ 25 C in order to initiate callus. Type II callus material is selected by visual selection of fast growing friable embryogenic cells by methods known in the art and as described for example in WO 98/44140. For example, suitable recipient cells are selected manually by choosing preferred cells which may be at the surface of a cell cluster and further identifiable by their lack of differentiation, small size and high nucleus/ cytoplasm volume ratio. A suspension culture is initiated from tissue within the callus which appears the least differentiated, softest and

most friable. Tissue with this morphology is transferred to fresh plates of media about 8- 16 d after the initial plating of the immature embryos. The tissue is then routinely subcultured every 14- 21 d by taking on ~ 10% of pieces which reach approximately a gram. At each step only material with the desired type II or type III morphology is subcultured on.

5 Preparation of cell suspension cultures

Preferably within 6 months of the above-described callus initiation, dispersed suspension cultures are initiated in liquid media containing suitable hormones such as 2,4-D and NAA optionally supplied in the form of slow-release hormone capsule treatments as described for example in examples 1 and 2 of US 5550318. Optionally, hormone levels
10 within the cultures are maintained by occasional spiking with fresh hormone supplement. Suspension cultures are initiated, for example, by adding approximately 0.5 g of callus tissue to a 100 ml flask containing 10 ml of suspension culture medium. Every 7 days, the culture is further subcultured by transferring, by use of a sterile wide-ended pipette, 1 ml of settled cells and 4 ml of conditioned medium to a fresh flask containing fresh medium. Large
15 aggregates of cells unable to pass through the pipette tip are excluded at each subculturing step. Optionally, suspension cultures are passed through a suitable sieve (e.g. ~ 0.5-1.0 mm mesh) at each subculturing step. After 6- 12 weeks the culture becomes dispersed. Suitable cell suspension culture media include for example, a medium adjusted to pH 6.0 containing Murashige and Skoog (1962) major and minor salts (optionally modified to contain a reduced
20 level, 1.55 g/l, of ammonium nitrate), 30 g/l sucrose, 0.25 mg/l thiamine, 10 mg/l dicamba, 25 mM L-proline, 200 mg/l casein hydrolysate, 100 mg/l myo-inositol, 500 mg/l potassium sulphate and 400 mg/l potassium hydrogen phosphate. Alternatively, in place of dicamba, cell suspension medium contains a 2,4-D and/or NAA.

Cryopreservation of cell suspension cultures

25 Optionally, suspension cultures obtained as described above, are cryopreserved using cryoprotectants and methods described for example in example 2 of US 5550318. Cryopreservation entails adding cryoprotectant at ice temperature to pre-cooled cells, also at ice temperature, in a stepwise manner over a period of one to two hours. The mixture is maintained at ice temperature and the eventual volume of cryoprotectant is equal to the
30 volume of cell suspension. The final concentrations of cryoprotectants are, for example, 10% dimethylsulfoxide, 10% polyethylene glycol (6000 Mw), 0.23 M L-proline and 0.23 M glucose. After a 30 min period of equilibration at ice temperature the mixture is divided into

~ 0.5 ml aliquots, transferred to 2 ml microcentrifuge tubes, and cooled slowly at a rate of 0.5 C/ min down to a temperature of -8 C. Following a period for ice nucleation, the sample is further cooled slowly down to -35 C and then plunged into liquid nitrogen. When required for use, frozen samples are thawed by first bathing them in their containers in water at ~ 40 C
5 for 2 min and then allowing them to slowly thaw completely. The mixture of cells and cryoprotectants is then pipetted onto a filter laid over a layer of BMS 'feeder' cells at 25 C. Once the thawed tissue begins to grow it is transferred back to fresh solid culture medium and, once established (within 1 to 2 weeks) is further transferred into cell suspension culture medium. Once growth in liquid suspension culture is re-established the cells are used for
10 transformation.

Particle-mediated transformation

Plasmid pIGPD9-derived DNA (Figure 12) containing XmaI EPSPS expression cassettes (i.e. pZEN9i, ZEN11i, ZEN12i, ZEN14i etc.) is purified, bulked up (e.g by anion exchange chromatographic or CsCl₂ gradient densitometric isolation of plasmid DNA from
15 cells of a suitable HisB-, Rec A- host strain of *E.coli* (e.g. DH5 α : hisB-) after growth to stationary phase in a minimal 5xA medium (K₂HPO₄ 52.5g, KH₂PO₄ 22.5g, (NH₄)₂SO₄ 5g and sodium citrate.2H₂O 2.5g per litre) and provided as a concentrated solution (preferably ~ 1 mg/ ml) in sterile water. DNA is provided as a circular plasmid DNA or, alternatively is restricted with Xma I to provide a linear EPSPS-expression cassette-containing fragment and
20 used following purification by agarose gel electrophoresis and electroelution.

Suitable apparatus for bombardment is, for example, the Biorad PDS1000 Helium gun. The dish is placed 5-6 cm below the stopping screen used to stop the capton macroprojectile. The DNA construct is precipitated onto tungsten or gold particles with an average diameter of ~ 1.0 μ m in a similar manner to that described by Klein *et al* 1987,
25 Nature, 327, 70-73. For example, 1.25 mg of tungsten or gold particles (pre-washed in ethanol at 65 C for 12 h) are mixed, in successive order, with ~ 20-30 mg of DNA, 1.1 M CaCl₂ and 8.7 mM spermidine to a final volume of ~ 0.6 ml. The mixture is vortexed for 10 min at 0-4 C, subject to low speed centrifugation (~ 500g) for 5 min and the bulk of the supernatant decanted off to leave the tungsten particles suspended in a final volume of ~ 30
30 ml. 1- 10 μ l aliquots are pipetted onto the macroprojectile of the particle gun.

Suspension cultures derived from type II and/or type III callus are maintained in culture for 3-5 months (or, alternatively, recovered from cryopreservation) , freshly

subcultured and then sieved through a ~ 0.5-1.0 mm stainless steel mesh. Approximately 0.5 ml packed cell volume of cells recovered from the filtrate is then pipetted onto 5 cm paper filters and vacuum dried before transfer to a petri dish containing a stack of three 7 cm paper filters moistened with suspension culture medium. Each plate of suspension cells is centred
5 onto the sample plate tray, the petri dish lid removed and bombarded twice at a vacuum of 28 inches of mercury. 0.1 or 1.0 mm screens are placed about 2.5 cm below the stop plate in order to ameliorate injury to the bombarded tissue. After bombardment the plant cells are removed from the filter, resuspended back into cell suspension culture medium and cultured for 2-21 days. Alternatively, the bombarded callus is transferred, plate to plate, onto to a
10 plate containing a similar solid medium (for example containing 8g/l of purified agar) and similarly cultured at ~ 25 C in the dark.

Selection of transformants

Following transformation, cells growing unselected in liquid or solid culture are transferred to filters and overlayed onto solid medium containing a range (0.1 - 20 mM) of
15 selecting concentrations of tissue culture grade N-(phosphonomethyl) glycine (Sigma). Suitable solid selection media include media, adjusted to pH 5.8 or 6.0 with KOH, containing either MS or N6 salts (such as those described above for callus initiation or, with suitable addition of agar, those described above for growth of cells in liquid suspension) and N-(phosphonomethyl) glycine. Suitable selection media also include, for example, the
20 selection media described in example 11 but, in this case, modified so as to lack antibiotics. Transformed calli expressing the resistant EPSP synthase enzyme are selected on the basis of their growth at concentrations inhibitory to similar preparations of untransformed cells. Growing clumps are subcultured on to fresh selective medium. Preferably the concentration of N-(Phosphonomethyl)-glycine used in the selection medium is about 1 mM for the first
25 two weeks of selection and about 3 mM thereafter. After 6-18 weeks putative resistant calli are identified and selected.

Regeneration of transformants/ Propagation and Analysis of transformed plant material

The selected calli are regenerated into normal fertile plants according to, for example, the methods described by Duncan *et al* (1985, *Planta*, 165, 322-332) by Kamo *et al* (1985,
30 *Bot. Gaz.* 146(3), 327-334) and/or by West *et al* (1993, *The Plant Cell*, 5, 1361-1369) and/or by Shillito *et al* (1989) *Bio/ Technol.* 7, 581-587.

For example, plants are efficiently regenerated by transferring the embryogenic callus to Murashige and Skoog medium adjusted to pH 6.0 containing 0.25 mg/l 2,4-D, 10 mg/l 6-benzyl-aminopurine and, optionally, 0.02 to 1 mM N-(phosphonomethyl) glycine. After ~ 2 weeks tissue is transferred to a similar medium but lacking hormones. Optionally the hormone level is decreased step wise through more transfers and over a longer period of time up to 6-8 weeks. Shoots which develop after 2-4 weeks are transferred to MS medium containing 1% sucrose and solidified with 2g/l Gelgro into which they then root.

Alternatively methods and media used for regeneration are as in example 1 except that the media used do not contain antibiotic.

Methods for growing plants to maturity, for the further propagation of plants through generations, for analysis of the inheritance of resistance to glyphosate and for analysis of the presence, integrity and expression of the EPSPS transgene are as described in example 11.

EXAMPLE 13. Transformation of corn lines with DNA which includes an EPSPS expression cassette coated onto silicon carbide whiskers; selection and regeneration of plant cells and plants which are resistant to glyphosate

In a further example, maize lines including, for example, hybrid lines having the genotype A188 x B73 are prepared as cell suspensions and transformed by contacting the cells with silicon carbide whiskers coated with DNA using methods essentially as described by Frame et al (1994, Plant J. 6, 941-948). As described in the previous examples, the transformed callus so generated is selected on the basis of differential growth rate in medium containing a range of concentrations of glyphosate, regenerated into plantlets (T₀) which are grown to maturity and either self or cross fertilised to provide progeny seed (T₁) for further breeding. Plants and plant material is assessed for resistance to glyphosate and analysed for transgene presence, integrity and expression as described in the previous examples.

Initiation of callus from immature embryos, preparation of cell suspension cultures

Maize cell suspensions suitable for transformation are optionally cryopreserved and provided in the same manner as described in example 12

Transformation

Plasmid pIGPD9-derived DNA (Figure 12) containing XmaI EPSPS expression cassettes (i.e. pZEN9i, ZEN11i, ZEN12i, ZEN14i etc.) is purified, bulked up (e.g. by anion exchange chromatographic or CsCl₂ gradient densitometric isolation of plasmid DNA from cells of a suitable HisB⁻, Rec A⁻ host strain of *E. coli* (e.g. DH5α: hisB⁻) after growth to

stationary phase in a minimal 5xA medium (K_2HPO_4 52.5g, KH_2PO_4 22.5g, $(NH_4)_2SO_4$ 5g and sodium citrate.2H₂O 2.5g per litre) and provided as a concentrated solution (preferably ~ 1 mg/ ml) in sterile water. DNA is provided as a circular plasmid DNA or, alternatively is restricted with Xma 1 to provide a linear EPSPS-expression cassette-containing fragment an
5 used following purification by agarose gel electrophoresis and electroelution.

Transformation is carried out exactly as described by Frame et al 1994. Alternatively the procedure is somewhat modified as described below.

Cells grown in liquid culture in cell suspension medium one day after subculturing are allowed to settle out in a shake flask. Spent medium is decanted and drawn off and 12 ml
10 of N6 medium at pH 6.0 (Chu et al 1975) modified to contain 6 mM L-proline, 20 g/l sucrose, 2 mg/l 2,4-D, 0.25 M sorbitol and 0.25M mannitol is added per 4 ml packed cell volume. The flask is returned to the shaker (rotary shaken at 125 rpm and incubated at 26-28 C) for 45 min. At the end of this period 1 ml aliquots of cell suspension are removed, using a wide bore pipette, into a series of sterile microcentrifuge tubes. After allowing the cells in
15 each tube to settle out, 0.6 ml of the spent medium supernatant is then removed to leave most of the remaining content as settled cells. 50 mg of silicon carbide whiskers (Silar SC-9 whiskers, Advanced Composite Materials Corp., Greer, SC, USA) are suspended by vortexing in 1 ml of the modified N6 medium described above. 40 ml of these suspended whiskers and 25 mg of the plasmid or linear DNA including the EPSPS expression cassette
20 are then added to each tube of settled cells. The tubes are finger vortexed 2-3 times, mixomated (in a Mixomat dental amalgam mixer (Degussa, Ontario, Canada) for 1 second and then 0.3 ml of N6 medium (modified as described above) is added to each microcentrifuge tube. The suspended cells are then plated (200 µl/ plate) out onto a filter disc overlying solid N6 medium (the same as the modified N6 medium described above but
25 lacking sorbitol, lacking mannitol and containing 30 g/l sucrose and 3 g/l of gelrite). Each plate is then wrapped with Urgopore tape (Stelrico, Brussels) and left to incubate in the dark for 1 week at 26-28 C.

Selection of transformants

Transformed callus is selected as described in example 12 or, alternatively, as
30 described in Frame et al 1994 except that N-(phosphonomethyl)glycine is used, at a range of concentrations between 1 and 5 mM in place of the bialaphos specified in the Frame et al publication.

Regeneration of transformants/ propagation and analysis of transformed plant material

Plants are regenerated, propagated, and bred as described in example 12. Plants are analysed for resistance to glyphosate and plant material is analysed for transgene presence, integrity and expression as described in example 12

5 **EXAMPLE 14. Transformation of rice lines using an *Agrobacterium* strain containing a superbinary vector which includes an EPSPS expression cassette between the right and left borders of the T-DNA; selection and regeneration of plant cells and plants which are resistant to glyphosate**

In a further example, scutella are isolated from mature seeds of suitable lines of rice
10 (including, for example, varieties Koshihikari, Tsukinohikari and Asanohikari) dedifferentiated and the callus thus-obtained transformed by infection with *Agrobacterium*. Following selection and regeneration, transgenic plantlets (T₀) are obtained which are grown to maturity and either self or cross fertilised to provide progeny seed (T₁) for further breeding. Plants and plant material are assessed for resistance to glyphosate and analysed for
15 transgene presence, integrity and expression as described in the previous examples. As an alternative to the methods described below the methods described in example 1 of US 5591616, suitable adapted so that glyphosate rather than hygromycin is used for selection, are used.

Construction of *Agrobacterium* strain: Preparation of *Agrobacterium* suspension

20 A strain of *Agrobacterium* containing superbinary vector having the desired EPSPS expression cassette between the right and left borders is constructed (using electroporation to transform *Agrobacterium* with plasmid DNA) as described in example 1. Suspensions are prepared according to the methods described in example 1. Alternatively, the transformed strain of *Agrobacterium* is grown for 3 days on AB medium (Chilton *et al*, 1974, Proc. Natl.
25 Acad. Sci. USA, 71, 3672-3676) containing appropriate antibiotic selection (e.g. 50 mg/l spectinomycin in the case of LBA4404 (pSB1ZEN18 etc)) and looped off of the plate to form a suspension in AAM medium (Hiei *et al*, 1994, The Plant Journal, 6(2), 271-282) at a density of $1-5 \times 10^9$ cells/ml.

Rice cultivars, preparation of callus from scutella

30 Rice cultivars are, for example *Oryza sativa* L. Tsukinohikari, Asanohikari and Koshihikari.

Mature seeds are dehusked, surface sterilized by washing in 70% ethanol and then soaked for 30 minutes in 1.5% NaOCl. After rinsing in sterile water they are cultured at 30 C, in darkness for 3 weeks on 2N6 medium at pH 5.8 which contains the major salts, minor salts and vitamins of N6 medium (Chu 1978 in Proc. Symp. Plant Tissue Culture., Peking: Science Press, pp 43-50) 30 g/l sucrose, 1 g/l casein hydrolysate, 2 mg/l 2,4-D and 2 g/l gelrite. Proliferated callus derived from the seed scutella is subcultured for 3-7 days on fresh 2N6 medium. Growing callus (1-2 mm in diameter) is selected, suspended in 2N6 liquid medium (without gelrite) and cultured in flasks, in darkness on a rotary shaker at 125 rpm and at 25 C. The medium is changed every 7 days. Cells growing in log phase after 3- 4 transformations are used for transformation.

Infection, transformation and selection

Suspended rice callus cells are allowed to settle out of suspension and then resuspended in the suspension of *Agrobacterium*, left in contact for several minutes and then, again, allowed to settle out and, without rinsing, plated out onto 2N6-AS medium (2N6 medium adjusted to pH 5.2 and containing 10 g/l D-glucose and 100 mM acetosyringone) and incubated in the dark at 25 C for 3-5 days. Growing material is rinsed thoroughly with 250 mg/l cefotaxime in sterile water and then transferred onto 2N6-CH medium (2N6 medium adjusted to pH 5.8 with KOH containing 250 mg/l cefotaxime and 0.5 - 5 mM tissue culture grade N-(phosphonomethyl) glycine) or, alternatively, 2N6K-CH medium (2N6 medium modified as described by Hiei et al 1994 but, in place of hygromycin, containing 0.5 - 5 mM tissue culture grade N-(phosphonomethyl) glycine) and cultured for 3 weeks in the dark at 25C. Proliferating colonies are subcultured onto a second plate of selective medium for a further period of 7-14 days.

Regeneration and analysis of plants

Growing colonies are plated onto a regeneration medium at pH 5.8 containing half strength N6 major salts, N6 minor salts, N6 amino acids, vitamins of AA medium (Chilton *et al* 1974), 1 g/l casein hydrolysate, 20 g/l sucrose, 0.2 mg/l naphthaleneacetic acid, 1 mg/l kinetin, 3 g/l gelrite and, optionally, 0.04-0.1 mM N-(phosphonomethyl)glycine. These plates are incubated at 25 C and kept under continuous illumination (~ 2000 lux). As described in example 11 regenerated plants are eventually transferred to soil in pots and matured in a greenhouse.

Plants are propagated, and bred (for example the transgenic plants are selfed) essentially as described in example 11. Plants are analysed for resistance to glyphosate and plant material is analysed for transgene presence, integrity and expression essentially as described in example 11.

5 **EXAMPLE 15. Transformation of wheat lines with DNA which includes an EPSPS expression cassette by use of microprojectile bombardment; selection and regeneration of plant cells and plants which are resistant to glyphosate**

In a further example, immature embryos are isolated from suitable lines of wheat (including, for example, spring wheat cv BobWhite, or Jaggar) incubated on hormone(2,4-
10 D)-containing medium for 2 days and transformed by bombardment with DNA-coated particles. Following a period for recovery and continued growth of callus, callusing embryos are subcultured through a series of media containing a fixed level of glyphosate and (serially diluted) decreasing levels of 2,4-D such that somatic embryogenesis is induced. The selected material is regenerated to form shoots on a medium also containing glyphosate,
15 transferred to rooting medium and, as in the previous maize-related examples, regenerated into plantlets (To) which are grown to maturity and either self or cross fertilised to provide progeny seed (T1) for further breeding. Plants and plant material are assessed for resistance to glyphosate and analysed for transgene presence, integrity and expression as described in the previous examples. As an alternative to the methods described below the methods
20 described in example 1 of US 5631152 are used.

Preparation of immature embryos

Wheat plant lines (for example spring wheat *Triticum aestivum* cv BobWhite) are grown to maturity in the greenhouse and caryopses isolated at 11 - 15 postanthesis. Caryopses are surface sterilised by treatment for 15 minutes in 5%NaOCl and then washed
25 repeatedly in sterile water. Immature embryos are aseptically isolated onto 3cm squares of nylon netting (mesh size 1.5 mm) overlying A2 medium . A2 medium adjusted to pH 5.8 is 4.32 g/l Murashige and Skoog salts, 20 g/l sucrose, 0.5 g/l L-glutamine, 2 mg/l 2,4-D, 100 mg/l casein hydrolysate, 2 mg/l glycine, 100 mg/l myo-inositol, 0.5 mg/l nicotinic acid, 0.1 mg/l thiamine.HCl and 2.5 g/l gelrite. Embryos are arranged into a solid 2.5 cm disc,
30 comprising approx. 50 in number. Plates are sealed with leukopore tape and incubated at 25°C in the dark for 2 days. Four hours prior to bombardment embryos are transferred onto plates containing fresh A2 medium supplemented with 36.44 g/l D-sorbitol and 36.44 g/l D-

mannitol. The embryos are transferred from plate to plate by means of the nylon net upon which they sit. The embryos sit on this increased osmotic strength medium for 4 h at 25°C in the dark before being bombarded.

Particle-mediated transformation

5 Plasmid pIGPD9-derived DNA (Figure 12) containing XmaI EPSPS expression cassettes (i.e. pZEN9i, ZEN11i, ZEN12i, ZEN14i etc.) is purified, bulked up (e.g. by anion exchange chromatographic or CsCl₂ gradient densitometric isolation of plasmid DNA from cells of a suitable HisB⁻, Rec A⁻ host strain of *E. coli* (e.g. DH5 α : hisB⁻) after growth to stationary phase in a minimal 5xA medium (K₂HPO₄ 52.5g, KH₂PO₄ 22.5g, (NH₄)₂SO₄ 5g
10 and sodium citrate.2H₂O 2.5g per litre) and provided as a concentrated solution (preferably ~ 1 mg/ ml) in sterile water. DNA is provided as a circular plasmid DNA or, alternatively is restricted with Xma I to provide a linear EPSPS-expression cassette-containing fragment and used following purification by agarose gel electrophoresis and electroelution.

Particles are prepared and coated with DNA in a similar manner to that described by
15 Klein *et al* 1987, Nature, 327, 70-73. Preparation of DNA-coated particles and operation of the particle gun is as described in example 2. Alternatively, the details are as follows. For example, 60 mg of gold or tungsten particles (~ 1.0 μ m) in a microcentrifuge tube are washed repeatedly in HPLC-grade ethanol and then, repeatedly, in sterile water. The particles are resuspended in 1 ml of sterile water and dispensed into 50 μ l aliquots in microcentrifuge
20 tubes. Gold particles are stored at 4 C, tungsten particles at - 20 C. 3 mg of DNA are added to each aliquot of (defrosted) particles and the tubes are vortexed at top speed. Whilst maintaining near continuous vortexing, 50 μ l of 2.5M CaCl₂ and 20 μ l of 0.1M spermidine is added. After 10 minutes of further vortexing, samples are centrifuged for 5 seconds in an eppendorf microcentrifuge, the supernatant is drawn off and the particles washed in
25 successive additions of HPLC-grade ethanol. The particles are thoroughly resuspended in 60 μ l of ethanol and then dispensed in 10 μ l aliquots onto the surface of each macrocarrier to be used in the PDS1000 particle gun

Components of the PDS1000 particle gun are surface sterilised by immersion in 70% ethanol and air-drying. Target plates prepared, as described above, with ~ 50 embryos
30 arranged into an ~ 2.5 cm disc are placed 6 cm from the stopping screen. 1100 psi rupture discs are then used for bombardment. Each plate is bombarded once or twice.

Bombarded plates are sealed with pore tape and maintained at 25 C in the dark for ~ 16h. Embryos dislodged from the surface of the medium by the helium shock wave are recovered and also incubated overnight on fresh plates of the same mannitol and sorbitol-supplemented A2 medium. The bombarded embryos are then transferred to fresh plates of A2 medium and incubated for 1 week at 25 C in the dark prior to selection.

Selection and Regeneration of Transformants

After this recovery period callusing embryos are removed from the nets and transferred to A2 2P medium (A2 medium, adjusted to pH 5.8 containing 2 mM N-(phosphonomethyl)glycine), at a density of 20 explants / plate. After one week on A2 2P medium, calli are removed to A1 2P medium (A2 medium containing only 1.0 mg / l 2,4-D and 2 mM N-(phosphonomethyl)glycine) for 2 weeks and thence to A 0.5 2P medium (A2 medium containing only 0.5 mg / l 2,4-D and 2 mM N-(phosphonomethyl)glycine) for a further two weeks. Optionally, the 2 week incubation periods are reduced to 1 week and/or the middle step of incubation on A1 2P medium is omitted. Optionally, the selecting concentration of N-phosphonomethylglycine is between 0.5 and 10 mM although 2 mM is preferred. The overall time for this period of callus induction with descending levels of 2,4-D in the medium is 2- 10 weeks, preferably 3-6 weeks and most preferably ~ 4 weeks.

To encourage maximum shoot growth and to discourage root development the calli are then transferred to Z medium. Z medium is A2 medium but containing 10 mg/ l zeatin in place of 2,4-D and also containing 0.1 mM N-(phosphonomethyl)glycine. Optionally, N-(phosphonomethyl)glycine is in the range 0.04 - 0.25 mM. Regenerating calli are maintained on this medium for a period of 3 weeks before subculture, at which point well developed shoots are excised. As only one event is likely to be produced on a single callus (which represents a single embryo), the entire callus is removed to a fresh plate and maintained with the excised shoot(s) to ensure multiple clones arising from the same callus do not get counted as separate events. Calli with only partially developed shoots or without regenerating sectors are returned to Z medium for a further 3 weeks. At the end of this period non regenerating calli are discarded.

Shoots are maintained on Z medium until 4 or more well-developed leaves (extending to ~ 2 cm in length) have formed. The regenerating plant material is then carefully transferred to plastic tubs containing 0.5MS medium. 0.5 MS medium at pH 5.8 is 2.16 g/ l of Murashige and Skoog salts, 15 g/ l sucrose, 2.5 g activated charcoal, 2.5 g/ l gelrite, 1 mg/

1 g glycine, 50 mg/l myo-inositol, 0.25 mg/l nicotinic acid, 0.25 mg/l pyridoxine.HCL, 0.05 mg/l thiamine.HCl and 0.1 mM N-(phosphonomethyl)glycine (optionally 0.0-0.25 mM).

Once plants have rooted they may be potted into soil and weaned, or removed to individual glass boiling tubes containing 0.5MS (with no N-(phosphonomethyl)glycine) and 2.5 g/l charcoal. It is preferred to have charcoal present in the rooting medium to adsorb any remaining PGRs or selection chemical transferred with the plantlet, and to create a dark rooting environment thereby avoiding physiologically aberrant green roots.

Callus induction and the first week of regeneration occurs at 25°C in the dark. The second week of regeneration occurs at low light at 25°C, then subsequent weeks at approx. 2500 lux on a 16 hour photo period.

Propagation, breeding and analysis of transformed plant material

Methods of producing T1 and further progeny are all well known in the art and essentially as described in the previous examples. Methods for analysis of the inheritance of resistance to glyphosate and the presence, integrity and expression of transgenes are as in the previous examples

EXAMPLE 16. Transformation of wheat lines with DNA which includes an EPSPS expression cassette by electroporation of protoplasts; selection and regeneration of plant cells and plants which are resistant to glyphosate

In a further example, plasmid or linear DNA comprising an EPSPS expression cassette and identical to that used in examples 12, 13 and 15 is used for direct transformation of protoplasts of a line of wheat capable of regeneration into fertile plants (*cf* US 5231019). Isolated protoplasts of wheat, preferably from leaf tissue or cells in culture (*cf* Gamborg, O.L. and Wetter, L.R., Plant Tissue Culture Methods, 1975, 11-21) are prepared at ~ ca 2×10^6 protoplasts/ml in 0.4M mannitol at pH 5.8. To this suspension are added first, 0.5 ml of 40% w/v polyethylene glycol (PEG) of molecular weight 6000 in modified F medium (Nature (1982), 296, 72-74) at pH 5.8 and, second, 65 ml of water containing 15 mg of the desired plasmid or linear DNA and 50 mg of calf thymus DNA. The mixture is incubated together for 30 min at 26 C, occasionally agitated and subsequently diluted into F medium (Nature (1982), 296, 72-74). The protoplast are isolated by low-speed centrifugation, taken up in 4 ml of CC culture medium (Potrykus, Harms, Lorz, (1979) Theor. Appl. Genet., 54, 209-214) and incubated in the dark at 24 C.

Alternatively, and in addition to treatment with PEG, transformation of cereal protoplasts is carried out using further steps of heat shock and/or electroporation (Neumann, E. *et al* (1982), the EMBO J. , 7, 841-845). Thus, for example, wheat protoplasts are incubated in an aqueous solution of DNA and mannitol, heated to 45 C for 5 min and then
5 cooled to 0 C over a period of 10 seconds. Then polyethylene glycol is added (Mr 3K -8K) until the final concentration is ~ 8% w/v. After gentle but thorough mixing treatment is carried out in an electroporator. The chamber of a Dialog 'Porator' (Dialog, Dusseldorf, Germany) is sterilised by washing with 70% ethanol and then drying in sterile air. Suspensions of protoplasts (~ ca 2×10^6 protoplasts/ml in 0.4M mannitol + the DNA) are
10 adjusted with manganese chloride to a measured electrical resistance of ~ 1.4 k ohm. Samples of volume ~ 0.4 ml are subjected, at 10 second intervals, to three pulses of applied voltages of between 1000 and 2000 V. The, thus transformed protoplasts are then collected and diluted back out into CC culture medium.

Those skilled in the art will recognise that many permutations and variations of these
15 transformation procedures are possible and that, for example, transformation may also be improved by raising the pH to 9.5 and/or increasing the concentration of calcium ions in the solution within which transformation is carried out.

After 3-14 days aliquots of the developing cell cultures are transferred to medium containing alternative selecting concentrations of tissue-culture grade N-(phosphonomethyl)
20 glycine (Sigma) between 1 and 5 mM (preferably 2 mM). Resistant cell colonies so identified (exhibiting growth on concentrations of glyphosate at least 2 fold greater than tolerated by untransformed controls) are transferred to fresh agar medium also containing a range of selecting concentrations of glyphosate and, as described, in example 15, subcultured between plates containing successively declining concentrations of 2,4-D. Growing resistant
25 colonies may be analysed (by PCR etc) for the presence of the recombinant DNA. It may or may not be possible to effect much selection at the callus step. In any case all growing calli will be taken forward.

Growing calli are then transferred to shoot regeneration medium containing zeatin and N-(phosphonomethyl)glycine and thence to rooting medium exactly as described in
30 example 15. Fertile transgenic plants expressing glyphosate-resistant EPSP synthase are then regenerated, selected and tested as known in the art and as described in example 15 and using the analytical methods described in example 11.

EXAMPLE. 17. Method for assaying EPSPS activity and determination of kinetic constants. Method for assaying EPSPS activities in crude plant materials and discriminating the proportion of the total which is resistant to glyphosate.

EPSPS Enzyme assay

5 Assays are carried out generally according to the radiochemical method of Padgett *et al* 1987 (Archives of Biochemistry and Biophysics, 258(2) 564-573) with K⁺ ions as the major species of cationic counterion. Assays in a total volume of 50µl, in 50mM Hepes(KOH) pH 7.0 at 25°C, contain purified enzyme or plant extract (see below) diluted appropriately in Hepes pH 7.0 containing 10% glycerol, and 5mM DTT, ¹⁴C PEP either as
10 variable substrate(for kinetic determinations) or fixed at 100 or 250 µM and shikimate 3 Phosphate (K⁺ salt) at 2 or 0.75 mM as indicated. Optionally, for assays of crude plant extracts, assays also contain 5 mM KF and/or 0.1 mM ammonium molybdate. Assays are started with the addition of ¹⁴C phosphoenolpyruvate (cyclohexylammonium+ salt) and stopped after 2-10 minutes (2 minutes is preferable) with the addition of 50µl of a solution of
15 1 part 1M acetic acid and 9 parts ethanol. After stopping, 20µl is loaded onto a synchropak AX100 (25cm x 4.6mm) column and chromatographed using isocratic elution with a 0.28M potassium phosphate pH 6.5 mobile phase flowing at 0.5 ml/min over 35 minutes. Under these conditions the retention times for PEP and EPSP are ~ 19 and 25 minutes respectively. A CP 525TR scintillation counter is connected to the end of the AX 100
20 column. It is fitted with a 0.5ml flow cell, and the flow rate of scintillant (Ultima Flo AP) is set at 1ml/min. Relative peak areas of PEP and EPSP are integrated to determine the percentage conversion of labelled PEP to EPSP. Apparent K_m and Vmax values are determined by least squares fit to a hyperbola with simple weighting using the Grafit 3.09b from Erithacus Software Ltd. Km values are generally ascertained using 8-9 concentrations
25 of variable substrate ranging from K_m / 2 - 10 K_m and triplicate points. Except where specifically noted, data points are only included in the analysis where there is < 30% conversion of substrate to EPSP.

 Shikimate-3-Pi (S3P) is prepared as follows. To 7mls of 0.3M TAPS pH 8.5 containing 0.05M Shikimate, 0.0665M ATP (Na salt), 10mM KF, 5mM DTT, and 0.05M
30 MgCl₂.6H₂O, 75µl of a 77 unit (µmol min⁻¹) ml⁻¹ solution of shikimate kinase is added. After 24hrs at room temperature, the reaction is stopped by brief heating to 95°C. The reaction solution is diluted 50 fold in 0.01M Tris HCl pH 9, and chromatographed by anion

exchange on Dowex 1 X 8 - 400, using a 0 - 0.34M LiCl₂ gradient. The S3P fractions are combined, freeze dried, and then redissolved in 7mls distilled H₂O. 28mls of 0.1M Ba(CH₃COOH)₂ and 189mls of absolute ethanol are then added. This solution is left to stir overnight at 4°C. The resulting precipitate of tri-Barium S3P is collected and washed in 5 30mls of 67% ethanol. The washed precipitate is then dissolved in ~ 30mls distilled H₂O. By adding either K₂SO₄ the K⁺ or TMA⁺ salt of S3P is produced as required. Great care is taken to add a minimal excess of sulphate. The BaSO₄ precipitate is removed and the supernatant containing the required salt of S3P freeze dried. Each salt is weighed and analysed by proton NMR. S3P preparations so-prepared are > 90% pure according to proton NMR and 10 (according to their weights and integration of ³¹P NMR) contain only low residues of potassium sulphate.

Preparation of extracts of plant material suitable for EPSPS assay

Callus or plantlet material (0.5 - 1.0 g) is ground to a fine frozen powder in a liquid nitrogen-chilled mortar and pestle. This powder is taken up in an equal volume of a suitable 15 chilled extraction buffer (for example, 50 mM Hepes/ KOH buffer at pH 7.5 containing 1 mM EDTA, 3 mM DTT, 1.7 mM 'pefabloc' (serine protease inhibitor), 1.5 mM leupeptin, 1.5 mM pepstatin A, 10% v/v glycerol and 1% polyvinylpyrrolidone), resuspended, mixed and centrifuged in a chilled centrifuge to bring down debris. The supernatant is exchanged down a chilled PD10 column of Sephadex G25 into 25 mM Hepes/ KOH buffer at pH 7.5 20 containing 1 mM EDTA, 3 mM DTT and 10% v/v glycerol. Protein is estimated by the Bradford method standardised using bovine serum albumen. A portion of the extract is frozen in liquid nitrogen; a portion is assayed immediately.

EPSPS assays of plant extracts are standardly carried out, as described above, with 0.1 mM ¹⁴C-PEP and 0.75 mM shikimate-3-Pi either in the absence or the presence of 0.1 25 mM N-(phosphonomethyl)glycine. Under these assay conditions, the resistant form of EPSPS (see below) is estimated to be inhibited by < 8.5% whilst the sensitive w/t form is essentially fully inhibited (> 98%). Thus, the level of activity observed in the presence of glyphosate (A) is taken to represent ~ 92% of the level of resistant enzyme derived from expression of the transgene whilst the level of susceptible w/t EPSPS is taken to be the total 30 level of EPSPS activity observed in the absence of glyphosate minus the value of A x ~ 1.08. Because the V_{max} of the mutant enzyme is estimated to be only about a third of the V_{max} of the w/t enzyme (and because the K_m values for PEP of both w/t and mutant forms are

estimated to be about 20 μ M or less), the level of expression of the mutant enzyme polypeptide relative to the level of expression of the endogenous w/t EPSPS is taken to be about three fold higher than the ratio calculated on the basis of the ratio of their relative observed activities. The total level of EPSPS polypeptide expression (mutant + w/t) is also
 5 estimated by using Westerns (see below).

EXAMPLE. 18. Cloning and expression of w/t and mutated cDNA encoding mature rice EPSPS in *E.coli*. Purification and characterisation of w/t and mutant rice EPSPS
Expression, purification and characterisation of w/t mature rice EPSPS

Rice EPSPS cDNA is amplified using RT-PCR from RNA isolated from rice variety
 10 Koshigari using Superscript RT from BRL according to the recommendation supplied by the manufacturer. PCR is performed using Pfu turbo polymerase from Stratagene according to the methods supplied by the manufacturer. The oligonucleotides below are used in the amplification reaction and the reverse transcription steps.

SEQ. ID. NO. 33

15 Rice 3' oligo GCGCTCGAGTCAGTTCTCTGACGAAAGTGGCTTAGAACTCG

SEQ. ID. NO. 34

Rice 5' oligo GCGCATATGAAGCGGAGGAGATCGTGC

The PCR product is cloned into pCRBlunt II using Invitrogens Zero Blunt TOPO kit. The sequence of the insert is confirmed by sequencing and it is verified that the predicted open
 20 reading frame corresponds to that of the predicted mature chloroplastic rice EPSPS protein with the exception of the presence of an initiating Met. The cloned and verified rice epsps sequence is excised using Nde I and Xho I and the purified fragment is cloned into pET24a (Novagen) digested similarly. The recombinant clones are introduced into BL21 (DE3) a codon-optimised RP strain of *E.coli* supplied by Stratagene. The EPSPS protein is expressed
 25 in this strain following addition of 1 mM IPTG to the fermenter medium (LB supplemented with 100ug/ml Kanamycin). The recombinant protein of the correct predicted mass is identified i) on the basis of Coomassie staining of SDS gels of cell extracts and side by side comparison with Coomassie-stained gels of extracts of similar *E.coli* cells transformed with an empty pET24a vector and ii) by western analysis using a polyclonal antibody raised to
 30 previously-purified plant EPSPS protein. The mature rice EPSPS protein is purified at ~ 4 C as follows. 25 g wet weight of cells are washed in 50 ml of 0.1M HEPES/ KOH buffer at pH 7.5 containing 5 mM DTT, 2 mM EDTA and 20% v/v glycerol. Following low-speed

centrifugation, the cell pellet is resuspended in 50 ml of the same buffer but also containing 2 mM of 'Pefabloc' a serine protease inhibitor. Cells are evenly suspended using a glass homogenizer and then disrupted at 10000 psi using a Constant Systems (Budbrooke Rd, Warwick, U.K.) Basic Z cell disrupter. The crude extract is centrifuged at ~ 30,000 g for 1 h and the pellet discarded. Protamine sulphate (salmine) is added to a final concentration of 0.2% , mixed and the solution left to stand for 30 min. Precipitated material is removed by centrifugation for 30 min at ~ 30,000 g. Aristar grade ammonium sulfate is added to a final concentration of 40% of saturation, stirred for 30 min and then centrifuged at ~ 27,000 g for 30 min. The pellet is resuspended in ~ 10 ml of the same buffer as used for cell disruption, further ammonium sulfate is added to bring the solution to ~ 70% of saturation, the solution is stirred for 30 min and centrifuged again to yield a pellet which is resuspended in ~ 15 ml of S200 buffer (10 mM Hepes/ KOH (pH 7.8) containing 1 mM DTT, 1 mM EDTA and 20% v/v glycerol). This is filtered (0.45 micron) loaded and chromatographed down a K26/ 60 column containing Superdex 200 equilibrated with S200 buffer. EPSPS-containing fractions detected on the basis of EPSPS enzyme activity are combined and loaded onto an xk16 column containing 20 ml of HP Q-Sepharose equilibrated with S200 buffer. The column is washed with S200 buffer and then EPSPS eluted within a linear gradient developed from 0.0M to 0.2M KCl in the same buffer. EPSPS elutes within a single peak corresponding to a salt concentration at or below 0.1 M. EPSPS-containing fractions detected on the basis of EPSPS enzyme activity are combined and loaded onto a HiLoad xk26/60 column of Superdex 75 equilibrated with Superdex 75 buffer (25 mM Hepes/ KOH (pH 7.5) containing 2 mM DTT, 1 mM EDTA and 10% v/v glycerol). EPSPS elutes later from the column that might be expected on the basis of the molecular weight of the presumed dimer. This may be due to interaction of the protein with the gel matrix at low ionic strength. EPSPS-containing fractions identified on the basis of enzyme activity are combined and loaded onto a 1ml column of MonoQ equilibrated with the same, Superdex 75 buffer. The column is washed with starting buffer and EPSPS eluted as a single peak over the course of a 15 ml linear gradient developed between 0.0 and 0.2M KCl. EPSPS is obtained near (>90% pure) at this stage in the purification. Optionally, EPSPS is further purified by exchange into Superdex 75 buffer containing 1.0 M (Aristar) ammonium sulphate and loading onto a 10 ml column of phenyl sepharose equilibrated in the same buffer. EPSPS is eluted as a single peak early

during the course of a linear gradient of declining ammonium sulphate developed between 1.0 and 0.0 M ammonium sulphate.

Cloning, expression, purification and characterisation of glyphosate-resistant mutant rice EPSPS

- 5 The rice EPSPS cDNA in pCRBlunt is used as a template for two further PCR using the following primer pairs designed to introduce specific changes

SEQ. ID. NO. 35

Rice 5' oligo GCGCATATGAAGGCGGAGGAGATCGTGC

SEQ. ID. NO. 36

- 10 Rice mutant reverse to RV GCAGTCACGGCTGCTGTCAATGATCGCATTGCAATTCAGCGTTCC

SEQ. ID. NO. 37

Rice 3' oligo GCGCTCGAGTCAGTTCTCTGACGAAAGTGTCTTGAACGTCG

SEQ. ID. NO. 38

Rice mutant forward to sal GGAACGCTGGGAAATGCAATGCGATCATTGACAGCAGCCGTGAATGC

- 15 The resultant products are gel purified and placed into a tube in equi-molar concentrations to serve as a template for another round of PCRs with the rice 5' and 3' oligos. The resultant products are cloned into pCRBlunt II using Invitrogens Zero Blunt TOPO kit. It is confirmed that the DNA sequence of the insert and its predicted open reading frame correspond to that of the predicted mature chloroplastic rice EPSPS protein (with the exception of the presence
- 20 of an initiating Met) and also that the desired changes (the specific mutation of T to I and P to S at specific positions in the EPSPS sequence) are encoded. The thus cloned and verified rice epsps sequence is excised using Nde I and Xho I and the purified fragment cloned into pET24a (Novagen) digested similarly. The recombinant clones are introduced into BL21 (DE3), a codon optimised RP strain of *E. coli* supplied by Stratagene. The EPSPS protein is
- 25 expressed in this strain following addition of 1 mM IPTG to the fermenter medium (LB supplemented with 100ug/ml Kanamycin). The recombinant protein of the correct predicted mass is identified i) on the basis of Coomassie staining of SDS gels of cell extracts and side by side comparison with Coomassie-stained gels of extracts of similar *E. coli* cells transformed with an empty pET24a vector and ii) by western analysis using a polyclonal
- 30 antibody raised to previously-purified plant EPSPS protein. This mutant form of rice EPSPS is purified and characterised in a similar manner to the method described above for w/t rice EPSPS.

The so-obtained mutant form of rice EPSPS, assayed as described above in the presence of 2 mM shikimate-3-Pi, has an apparent V_{max} of $\sim 10 \mu\text{mol}/\text{min}/\text{mg}$ and a K_m for PEP of 22 μM . At 40 μM PEP, the IC_{50} value for the potassium salt of glyphosate is $\sim 0.6 \text{ mM}$. The estimated K_i value for potassium glyphosate of the mutant EPSPS is ~ 0.2 mM.

EXAMPLE. 19. Preparation of antibodies to purified rice EPSPS and methods for Western analysis

Standard methods for generation of polyclonal antisera in rabbits are used. Rabbits are young female New Zealand Whites. Immunisation courses consist of 4 doses, each ~ 100 mg, administered at monthly intervals. Each dose in phosphate buffered saline is administered as an emulsion with Freund's Complete adjuvant (dose 1) or Incomplete adjuvant (doses 2-4) and is injected at four sub-cutaneous sites. A pre-bleed is taken before dose 1 is administered. A test bleed is taken 14 days after the second dose to confirm the immune response. A term bleed is taken 14 days after the fourth dose and used for experimentation. A fifth and final dose is given when at least 6 weeks has elapsed since the fourth dose, and the final bleed (also used for experimentation) is taken 14 days later. Antibodies are raised to both (i) purified native mature w/t rice EPSPS (example 8) and also (ii) to SDS-denatured rice EPSPS polypeptide which is eluted from a band cut out of a 12% SDS gel (the correct position of the protein being accurately marked by side by side Coomassie staining of the band).

12% polyacrylamide gels are used for SDS gel electrophoresis and Western blotting. Electrophoresis is performed at a constant current of 100V for 90 minutes. Gels are blotted against nitrocellulose sheets overnight at 4 C at a constant 30V. Sheets are blocked in 5% Marvel phosphate buffered saline containing 0.1% Tween (PBS-tween) for 1 or 2 hours, washed three times in PBS-tween and incubated in rice EPSPS-Rb1 primary antibody at $\sim 1.3 \text{ mg IgG/ml}$ (normally equivalent to a 1:4000 to 1: 20,000 dilution of term bleed). These antibody dilutions are applied in PBS (phosphate-buffered saline) containing 1% Marvel and 0.05% Tween 20 for 2 hours. Secondary antibody, Goat anti Rabbit HRP (Sigma A6154) is applied at 1:10,000 or 1:20,000 in PBS containing 0.05% Tween and 1% Marvel. Incubation with secondary antibody is continued for 1 hour, the blott is washed three times in PBS (0.05% tween), ECL substrate is applied for usually 1 minute and film exposed for 10-60 seconds. Negative control blots are blots with (1) preimmune serum at a dilution calculated

to yield the same [IgG] as in test immune sera (IgG is routinely purified from an aliquot of each serum and quantitated so that these dilutions can be calculated directly) and also (2) immune serum raised against Freund's adjuvant alone. IgG concentration in control immune sera is adjusted so that controls are at an appropriate concentration of IgG. In order to make this calculation, IgG is purified from crude antiserum by filtration through a 0.45µm syringe filter and passed down a 1ml HiTrap protein G column (Pharmacia cat no:17-0404-01). The bound IgG is eluted from the column with 0.1M glycine HCl pH 2.9 and dialysed vs PBS overnight. The IgG concentration is estimated by UV determination. (a 1 cm pathlength of a 1 mg ml⁻¹ solution of pure IgG has an absorbance at 280nm wavelength of 1.35). From the IgG yield a calculation can be made of IgG concentration in the crude antiserum, and correct dilutions in western blots calculated accordingly.

Plant tissue samples are prepared for example as described in example 17. Alternatively, for Western analysis, a much simpler procedure is used. 100-200 mg of plant tissue to be analysed is rapidly homogenized (for example using an ultra turrax, bead beater or glass homogenizer) in an equal volume of buffer (similar to in example 7), centrifuged for 5 minutes in a chilled eppendorf microcentrifuge and the supernatant a) a small aliquot is analysed for protein using the Bradford method and b) for the most part mixed 1:1 with Laemli SDS 'sample buffer', heated and then stored ready for loading onto gels. Typically SDS slab gels are loaded with 10 protein samples in 10 wells. Typically these are 1 to 10 µg of crude extracts of plant material for analysis alongside a standard curve of between 0 and 20 ng of pure rice EPSPS. In some cases Westerns are run using antibodies raised to purified w/t EPSPS from *Brassica napus* (expressed and purified using similar methods to those described above). In this case the strength of the cross reaction of the antibodies is less strong with rice EPSPS (or with endogenous plant wheat or maize EPSPS) than in the case that antibodies raised to rice EPSPS be used but still, nevertheless, provide sufficient reaction for useful quantitative information in relation to the standard curve to be derivable.

EXAMPLE 20. Isolation of genomic DNA from transgenic plant material. PCR analysis. DNA probe preparation and hybridisation. Copy number and integrity of transgene.

Genomic DNA is isolated from plants, plantlets and callus material using ,for example, the method of Dellporta et al (1983) in Chromosome Structure and Function: Impact of New Concepts, 18'th Stadler Genetics Symposium. J.P. Gustafson and R . Appels,

eds). New York: Plenum Press) pp 263-282 or, alternatively, using a DNAeasy kit (Qiagen). Transgenic callus and plant segregants that contain the mutated rice EPSPS transgene are identified using fluorescence PCR using oligonucleotide primers SEQ. ID NO. 39 and 40 that are specific to the mutations within the rice EPSPS genomic sequence. The fluorescent dye SYBR green, which intercalates with double stranded DNA, is included in the PCR so that samples containing the mutated rice EPSPS gene are identified by an increase in fluorescence in the sample, which is detected using an ABI 3377 machine. Alternatively, those skilled in the art will know that the primers may be fluorescently labelled and technologies such as molecular beacons and 'Scorpions' are available.

10 SEQ. ID NO. 39

RiceDM Fwd2-3A 5'- gtg gaa cgc tgg aat tgc aat gca at -3'

SEQ. ID. NO. 40

Universal Reverse 5'- gtt gca ttt cca cca gca gca gt -3'

A typical PCR reaction consists of, prepared in 96 well Optical plates and sealed

15 with Optical lids (PE Biosystems), is as follows:

5.0µl gDNA template, (Qiagen DNeasy prep)

12.5µl 2x SYBR Green premix

2.5µl 5pmol/µl Stock Forward Primer

2.5µl 5pmol/µl Stock Reverse Primer

20 2.5µl ddH₂O

25.0µl Total volume

The following cycling parameters are followed:

Stage 1: 50oC x 2 mins

Stage 2: 95oC x 10 mins

25 Stage 3: 50 cycles of 95oC x 15secs
60oC x 45secs

Changes in fluorescence within the samples is recorded every seven seconds from stage 3 of the reaction.

For Southern blotting approximately 10 µg of DNA is used for each restriction digest.

30 Genomic DNA is digested with suitable restriction enzymes (e.g Hind III) according to the manufacturer's instructions (e.g Promega). Restriction enzymes are chosen that cut both within and outside the mutant EPSPS sequence. DNA is separated using TAE (0.04M tris-acetate, 1 mM EDTA) 0.8% agarose gels. Southern blotting is carried out according to

methods giving by Sambrook *et al.*, 1989 using HyBond N+ nitrocellulose blotting membrane (AmershamPharmacia). The DNA is cross-linked to the membrane by exposure to UV illumination.

DNA fragments used for generating specific probes are isolated by purification on
5 gels of restriction digests of plasmid DNA or generated by PCR. For example, a 700 bp fragment containing intron 1 of the rice EPSPS gene, is generated by PCR using primers as shown below.

SEQ. ID. NO. 41

INT1/5 5' ccccttctctcttgctgaattccatttc 3'

10 SEQ. ID. NO. 42

INT1/3 5' gttgtgccccataataaccagag 3'

Such probes are labelled with ³²P using the random priming method, for example Ready-To-Go DNA labelling beads (AmershamPharmacia) and purified using, for example, MicroSpin G-25 columns (AmershamPharmacia).

15 Blots of DNA gels are prehybridized at 65 C in 5x SSC, 0.5% SDS, 2xDenhardt's solution, 0.15 mg/ ml denatured salmon sperm DNA for at least one hour. The blot is then hybridized with denatured probe for 16-24 h at 65 C in fresh pre-hybridisation solution. Membranes are blotted dry and visualised using autoradiography.

Where Southern blotting indicates a single integration event of the transgene at a
20 single locus, indicted by the probe hybridising with only a single specific sized restriction fragment, then the result is confirmed through a rehybridisation of the blot using an alternative probe. For controls, untransformed material is used. Additionally the blot may be probed further with hybridisation probes specific to other regions of the transgenic construct (for example the promoter, 5'UTR intron or upstream enhancer sequences) in order to verify the
25 integrity of the construct. Additionally, in the case that Agrobacterium transformation is used, specific probes are used to indicate the presence or absence of any DNA extending from beyond the RB and LB of the super-binary vector.

COR-AAD1-00004652

SEQ ID NO. 44 Rice EPSPS genomic (from ATG including double mutant - shown)

atggcggcgaccatggcgtccaaagccggcgtggcggcgcggtgtccctggaccaggccgtggcggcggtggcggcggttctc
 gtgcgggaagcagctgcggctgcccgcgcggcgcgcggggggatgcgggtgcgggtgcggcgcgkcgggcgcgggaggcgg
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SEQ ID NO. 45 FMV enhancer

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5

SEQ ID NO. 46 CaMV35S enhancer1

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SEQ ID NO. 47 CaMV35S enhancer2

tgagacttttcaacaaagggttaatatccggaaacctctctcggtatccattgcccagcttatctgtcactttattgtgaagatag
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15

SEQ ID NO. 48 Maize Polyubiquitin Enhancer

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SEQ ID NO. 49 Rice Actin Enhancer

gataccctcagccgcctttcactatcttttttgcgcgagtcattgtcacgtgaaccttggcatgtataatcgggtgaattgcg
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 gogagca

SEQ ID NO. 50 Rice GOS2 Enhancer

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 20 cacaggaacaaacagagtggtgctccacagaaacacccaacaaaacagatgatctaacggaggacagc

SEQ ID NO. 51 Barley Plastocyanin Enhancer

ccaaaatctccatgaggagcacctcaatgcctcgggtgcctgttagatctccacccgacacatatctgttaactccttcogt
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 atatactatgatgagacatgcctgtggagatatatatataggtgggatgatattgggtacccacactcatccggatgga
 gggcg

SEQ ID NO. 52 Rice Genomic G1 EPSPS (to ATG)

35 gttgggtgggtgagagtgagacacccgacggaaacggaggagaaacccgcgcgttggtattcttctttttaccttttcaaaat
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[illegible]

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CLAIMS

1. An isolated polynucleotide comprising the sequence depicted in SEQ ID No. 43.
- 5 2. A polynucleotide encoding an EPSPS, excluding the cDNA encoding the rice and corn EPSPS, which polynucleotide is complementary to one which when incubated at a temperature of between 65 and 70°C in 0.1 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.1 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence
10 depicted in SEQ ID No. 43.
3. A polynucleotide encoding an EPSPS obtainable by screening plant genomic DNA libraries with a polynucleotide constituting an intron within the SEQ ID No. 43
15 sequence.
4. An isolated polynucleotide comprising a region encoding a chloroplast transit peptide and a glyphosate resistant 5-enolpyruvylshikimate phosphate synthase (EPSPS) 3' of the peptide, the said region being under expression control of a plant operable promoter, with the *provisos* that the said promoter is not heterologous with respect to
20 the said region, and the chloroplast transit peptide is not heterologous with respect to the said synthase.
5. A polynucleotide according to any one of claims 1-4, comprising the following components in the 5' to 3' direction of transcription:-
25 (i) At least one transcriptional enhancer being that enhancing region which is upstream from the transcriptional start of the sequence from which the enhancer is obtained and which enhancer *per se* does not function as a promoter either in the sequence in which it is endogenously comprised or when present heterologously as part of a construct;
30 (ii) The promoter from the rice EPSPS gene;
(iii) The rice genomic sequence which encodes the rice EPSPS chloroplast transit peptide;

(iv) The genomic sequence which encodes the rice EPSPS;

(v) A transcriptional terminator;

wherein the rice EPSPS coding sequence is modified in that a first position is mutated so that the residue at this position is Ile rather than Thr and a second position is mutated so that the residue at this position is Ser rather than Pro, the mutations being introduced into EPSPS sequences which comprise the following conserved region GNAGTAMRPLTAAV in the wild type enzyme such that modified sequence reads GNAGIAMRSLTAAV.

6. A polynucleotide according to claim 5, wherein the said enhancing region comprises a sequence, the 3' end of which is at least 40 nucleotides upstream of the closest transcriptional start of the sequence from which the enhancer is obtained.
7. A polynucleotide according to either of claim 5 or 6, wherein the enhancing region comprises a region the 3' end of which is at least 60 nucleotides upstream of the said closest start.
8. A polynucleotide according to claim 5, wherein the said enhancing region comprises a sequence the 3' end of which is at least 10 nucleotides upstream from the first nucleotide of the TATA consensus of the sequence from which the enhancer is obtained.
9. A polynucleotide according to any one of claims 5-8, comprising first and second transcriptional enhancers.
10. A polynucleotide according to claim 9, wherein the first and second enhancers are tandemly present in the polynucleotide.
11. A polynucleotide according to any one of claims 5-10, wherein the 3' end of the enhancer, or first enhancer, is between about 100 to about 1000 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide, or the first nucleotide of an intron in the 5' untranslated region

12. A polynucleotide according to any one of claims 5-11, wherein the 3' end of the enhancer, or first enhancer, is between about 150 to about 1000 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide, or the first nucleotide of an intron in the 5' untranslated region.
13. A polynucleotide according to any one of claims 5-12, wherein the 3' end of the enhancer, or first enhancer, is between about 300 to about 950 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide, or the first nucleotide of an intron in the 5' untranslated region.
14. A polynucleotide according to any one of claims 2-13, wherein the 3' end of the enhancer, or first enhancer, is between about 770 and about 790 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide, or the first nucleotide of an intron in the 5' untranslated region.
15. A polynucleotide according to any one of claims 2-13, wherein the 3' end of the enhancer, or first enhancer, is between about 300 and about 380 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide, or the first nucleotide of an intron in the 5' untranslated region.
16. A polynucleotide according to any one of claims 2-13 and 15, wherein the 3' end of the enhancer, or first enhancer, is between about 320 and about 350 nucleotides upstream of the codon corresponding to the translational start of the EPSPS transit peptide, or the first nucleotide of an intron in the 5' untranslated region.
17. A polynucleotide according to any one of claims 5-16, wherein the region upstream of the promoter from the rice EPSPS gene comprises at least one enhancer derived from a sequence which is upstream from the transcriptional start of either the CaMV35S or FMV35S promoters.

18. A polynucleotide according to claim 17, comprising in the 5' to 3' direction a first enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the GOS 2 promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of either the CaMV35S or FMV35S promoters.
19. A polynucleotide according to claim 18, comprising in the 5' to 3' direction a first enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the rice actin promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of either of the FMV35S or CaMV35S promoters.
20. A polynucleotide according to claim 17, comprising in the 5' to 3' direction a first enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the barley plastocyanin promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of either the CaMV35S or FMV35S promoters.
21. A polynucleotide according to claim 17, comprising in the 5' to 3' direction a first enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the maize polyubuitin promoter and a second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of either the CaMV35S or FMV35S promoters.
22. A polynucleotide according to claim 17, comprising in the 5' to 3' direction a first enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the FMV35S promoter and a

second enhancer comprising a transcriptional enhancing region derived from a sequence which is upstream from the transcriptional start of the CaMV35S promoter.

23. A polynucleotide according to any one of claims 1-22, wherein the nucleotides 5' of
5 the codon which constitutes the translational start of the rice EPSPS chloroplast transit peptide are Kozack preferred.
24. A polynucleotide according to any one of claims 5-23, wherein 5' of the rice genomic
10 sequence which encodes the rice EPSPS chloroplast transit peptide there is located a non-translated region which comprises a sequence which functions as an intron.
25. A polynucleotide according to claim 24 wherein the non-translated region comprises an intron, wherein the intron is the maize ADHI intron.
- 15 26. A polynucleotide according to either of claims 24 or 25, wherein the intron comprises the sequence depicted in SEQ ID NO. 55.
27. A polynucleotide according to any one of claims 5-26, which comprises a virally
20 derived translational enhancer or other non-viral translational enhancer located within the non translated region 5' of the rice genomic sequence which encodes the rice EPSPS chloroplast transit peptide.
28. A polynucleotide according to any one of claims 5-27, further comprising regions
25 encoding proteins capable of conferring upon plant material containing it at least one of the following agronomically desirable traits: resistance to insects, fungi, viruses, bacteria, nematodes, stress, dessication, and herbicides.
29. A polynucleotide according to claim 28, wherein the herbicide is other than
30 glyphosate.
30. A polynucleotide according to either of claims 28 or 29, wherein the insect resistance conferring regions encode crystal toxins derived from Bt, including secreted Bt

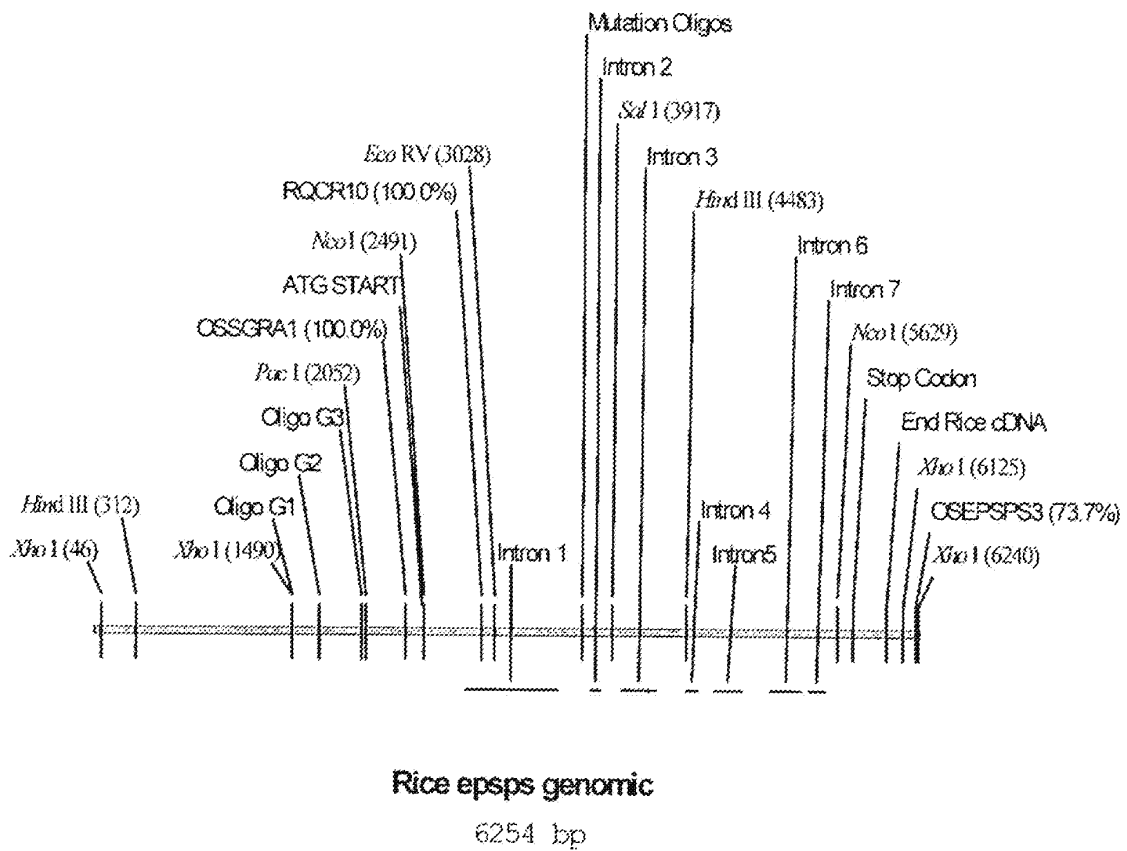
- toxins; protease inhibitors, lectins, *Xenorhabdus/Photorhabdus* toxins; the fungus resistance conferring regions are selected from the group consisting of those encoding known AFPs, defensins, chitinases, glucanases, Avr-*Cf9*; the bacterial resistance conferring regions are selected from the group consisting of those encoding cecropins and techyplesin and analogues thereof; the virus resistance regions are selected from the group consisting of genes encoding virus coat proteins, movement proteins, viral replicases, and antisense and ribozyme sequences which are known to provide for virus resistance; the stress, salt, and drought resistance conferring regions are selected from those that encode Glutathione-S-transferase and peroxidase, the sequence which constitutes the known CBF1 regulatory sequence and genes which are known to provide for accumulation of trehalose.
- 5
- 10
31. A polynucleotide according to claim 30, wherein the insect resistance conferring regions are selected from the group consisting of cryI*Ac*, cryI*Ab*, cry3*A*, Vip 1*A*, Vip 1*B*, cystein protease inhibitor, and snowdrop lectin genes.
- 15
32. A polynucleotide according to any preceding claim, which is modified in that mRNA instability motifs and/or unwanted splice regions are removed, or crop preferred codons are used so that expression of the thus modified polynucleotide in a plant yields substantially similar protein having a substantially similar activity/function to that obtained by expression of the protein encoding regions of the unmodified polynucleotide in the organism in which they are endogenous.
- 20
33. A polynucleotide according to the preceding claim, wherein the degree of identity between the modified polynucleotide and a polynucleotide endogenously contained within the said plant and encoding substantially the same protein is such as to prevent co-suppression between the modified and endogenous sequences.
- 25
34. A polynucleotide according to the preceding claim, wherein the said degree is less than about 70%.
- 30
35. A vector comprising the polynucleotide of any preceding claim.

36. Plant material which has been transformed with the polynucleotide of any one of claims 1-34, or the vector of claim 35.
37. Plant material which has been transformed with the polynucleotide of any one of claims 1-34 or the vector of claim 35, and which has been, or is, further transformed with a polynucleotide comprising regions encoding proteins capable of conferring upon plant material containing it at least one of the following agronomically desirable traits: resistance to insects, fungi, viruses, bacteria, nematodes, stress, desiccation, and herbicides.
38. Morphologically normal, fertile whole plants which have been regenerated from the material according to either of claims 36 or 37, their progeny seeds and parts.
39. Morphologically normal fertile whole plants which comprise the polynucleotide of any one of claims 1-34 and which result from the crossing of plants which have been regenerated from material transformed with the polynucleotide of any one of claims 1-34 or the vector of claim 35, and plants which have been transformed with a polynucleotide comprising regions encoding proteins capable of conferring upon plant material containing it at least one of the following agronomically desirable traits: resistance to insects, fungi, viruses, bacteria, nematodes, stress, desiccation, and herbicides, the progeny of the resultant plants, their seeds and parts.
40. Plants according to either of claims 38 or 39, selected from the group consisting of field crops, fruits and vegetables such as canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, cabbage, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax and oilseed rape, and not producing plants insofar as they are not already specifically mentioned, their progeny, seeds and parts.
41. Maize, wheat or rice plants according to any one of claims 38-40.

42. A method of selectively controlling weeds in a field, the field comprising weeds and plants or progeny according to any one of claims 38-41, the method comprising application to the field of a glyphosate type herbicide in an amount sufficient to control the weeds without substantially affecting the plants.
- 5 43. A method according to the preceding claim, further comprising application to the field either before or after application of the glyphosate herbicide of one or more of the following: a herbicide, insecticide, fungicide, nematocide, bacteriocide and an anti-viral.
- 10 44. A method of producing plants which are substantially tolerant or substantially resistant to glyphosate herbicide, comprising the steps of:
- (i) transforming plant material with the polynucleotide of any one of claims 1 to 34 or the vector of claim 35;
- 15 (ii) selecting the thus transformed material; and
- (iii) regenerating the thus selected material into morphologically normal fertile whole plants.
45. A method according to the preceding claim, wherein the transformation involves the introduction of the polynucleotide into the material by: (i) biolistic bombardment of the material with particles coated with the polynucleotide; or (ii) impalement of the material on silicon carbide fibres which are coated with a solution comprising the polynucleotide; or (iii) introduction of the polynucleotide or vector into *Agrobacterium* and co-cultivation of the thus transformed *Agrobacterium* with plant material which is thereby transformed and is subsequently regenerated.
- 20 46. A method according to the preceding claim, wherein the transformed material is selected by its resistance to glyphosate.
- 25 47. Use of the polynucleotide of any one of claims 1 to 34, or the vector of claim 35, in the production of plant tissues and/or morphologically normal fertile whole plants which are substantially tolerant or substantially resistant to glyphosate herbicide.
- 30

48. Use of the polynucleotide of any one of claims 1 to 34, or the vector of claim 35, in the production of a herbicidal target for the high throughput *in vitro* screening of potential herbicides.
- 5
49. A method of selecting biological material transformed so as to express a gene of interest, wherein the transformed material comprises the polynucleotide of any one of claims 1 to 34 or the vector of claim 35 and wherein the selection comprises exposing the transformed material to glyphosate or a salt thereof, and selecting surviving material.
- 10
50. A method according to the preceding claim, wherein the biological material is of plant origin.
- 15
51. A method according to the preceding claim, wherein the plant is a monocot.
52. A method according to the preceding claim, wherein the monocot is selected from group consisting of barley, wheat, corn, rice, oats, rye, sorghum, pineapple, sugar cane, banana, onion, asparagus, leek,
- 20
53. A method for regenerating a fertile transformed plant to contain foreign DNA comprising the steps of:
- (a) producing regenerable tissue from said plant to be transformed;
- (b) transforming said regenerable tissue with said foreign DNA, wherein said foreign DNA comprises a selectable DNA sequence, wherein said sequence functions in a regenerable tissue as a selection device;
- 25
- (c) between about one day to about 60 days after step (b), placing said regenerable tissue from step (b) in a medium capable of producing shoots from said tissue, wherein said medium further contains a compound used to select regenerable tissue containing said selectable DNA sequence to allow identification or selection of the transformed regenerated tissue;
- 30

- (d) after at least one shoot has formed from the selected tissue of step (c) transferring said shoot to a second medium capable of producing roots from said shoot to produce a plantlet, wherein the second medium optionally contains the said compound; and
- 5 (e) growing said plantlet into a fertile transgenic plant wherein the foreign DNA is transmitted to progeny plants in Mendelian fashion, wherein between step (b) and step (c) there is an optional step of placing the transformed material onto callus inducing medium, characterised in that the foreign DNA is, or the selectable DNA sequence comprised by the foreign DNA comprises, the polynucleotide according to
- 10 any one of claims 1 to 34, and the said compound is glyphosate or a salt thereof.
54. A method according to the preceding claim, wherein the plant is a monocot selected from the group consisting of banana, wheat, rice, corn and barley.
- 15 55. A method according to either of claims 53 or 54, wherein the said regenerable tissue is selected from the group consisting of embryogenic calli, somatic embryos, immature embryos etc.



Rice epsps genomic

6254 bp

Figure 1

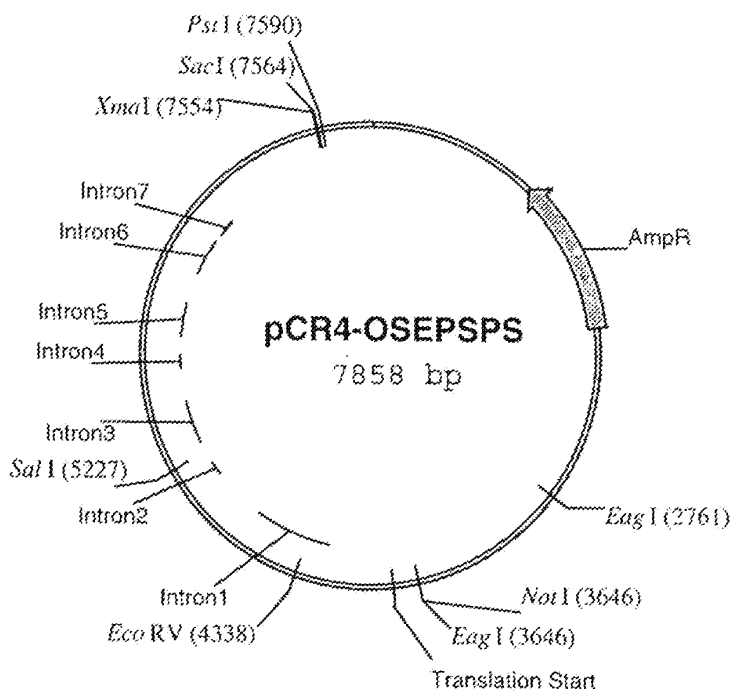


Figure 2

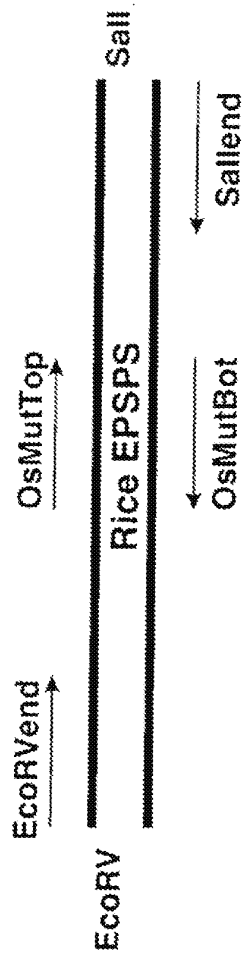


Figure 3

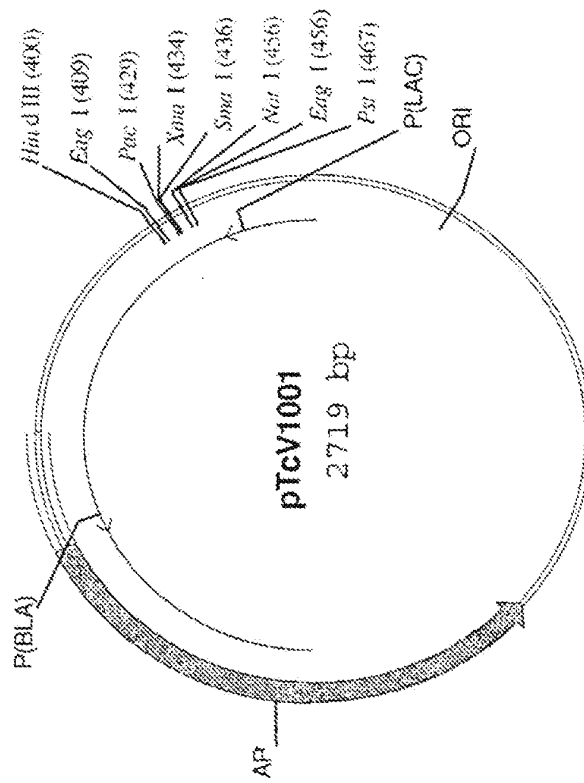


Figure 4

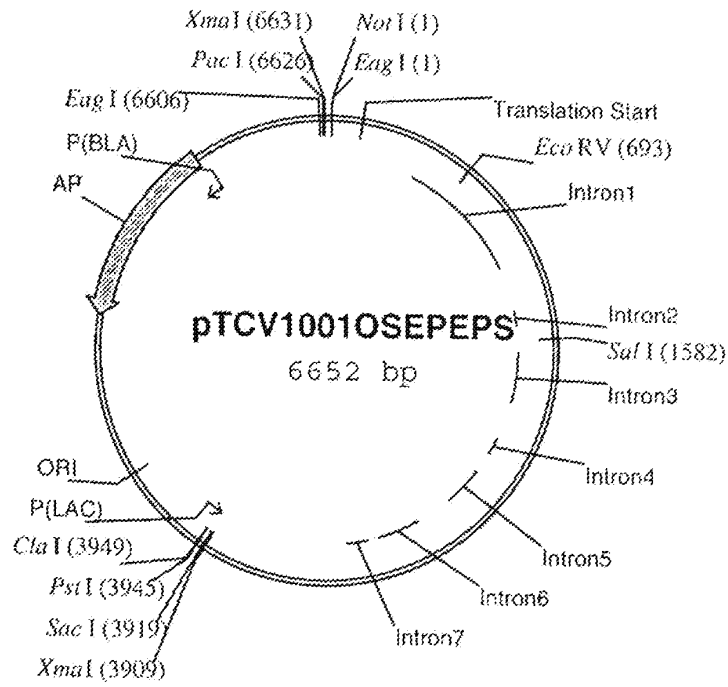


Figure 5

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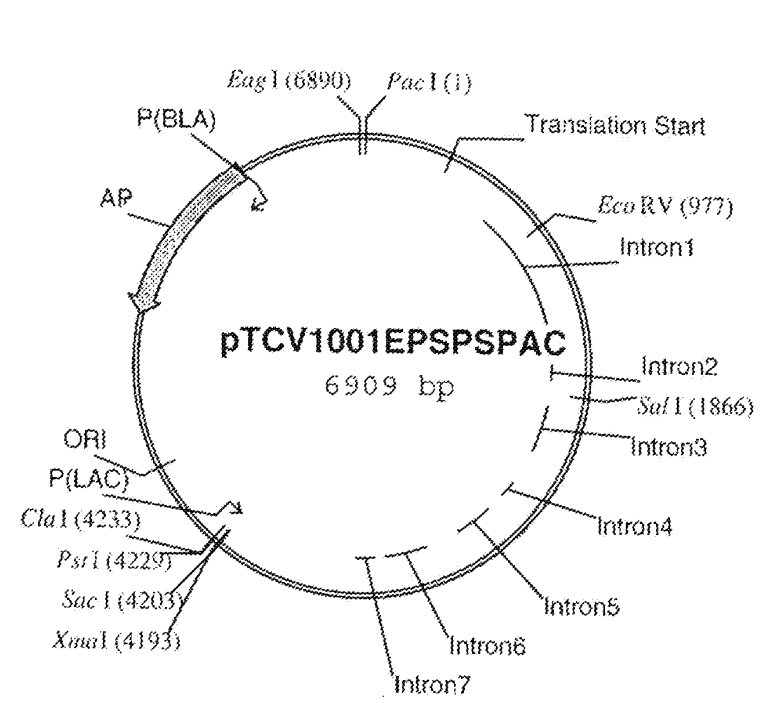


Figure 6

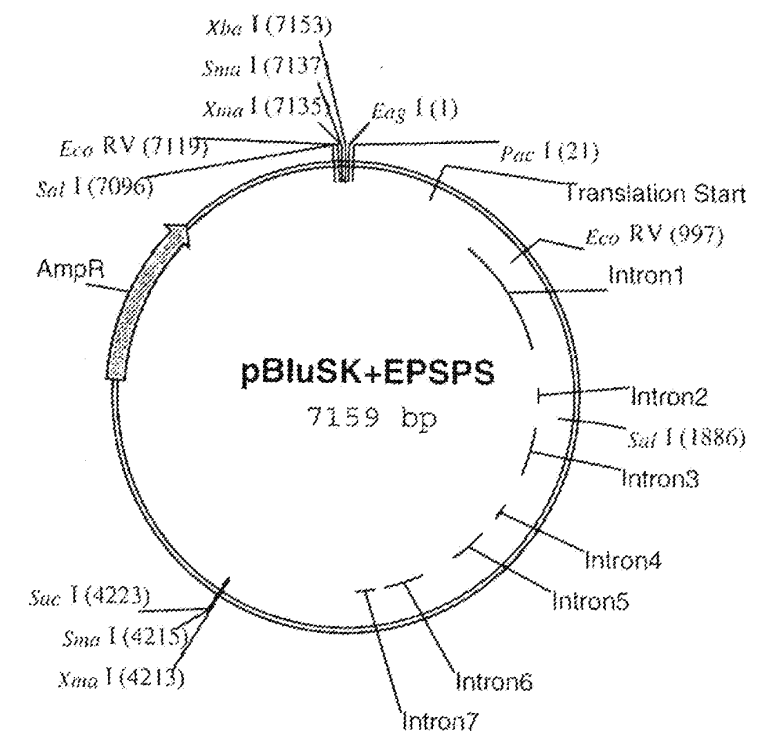


Figure 7

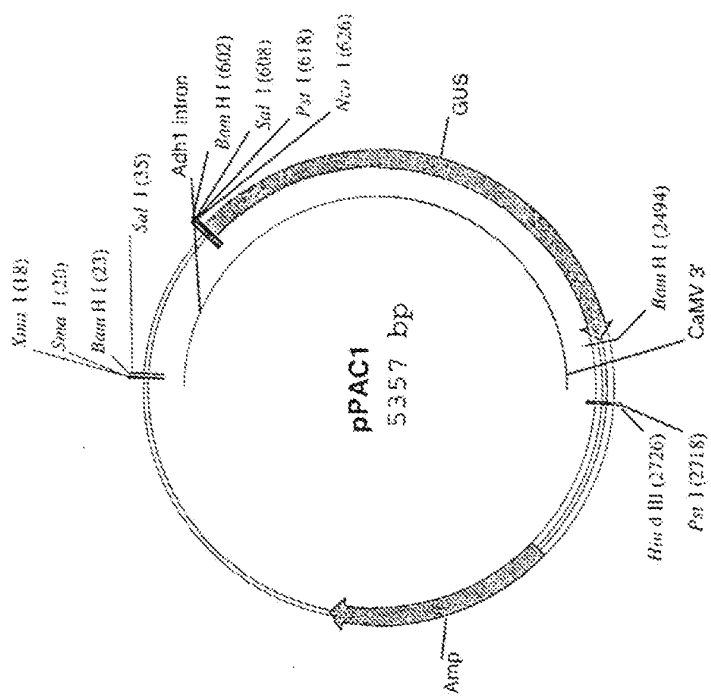


Figure 8

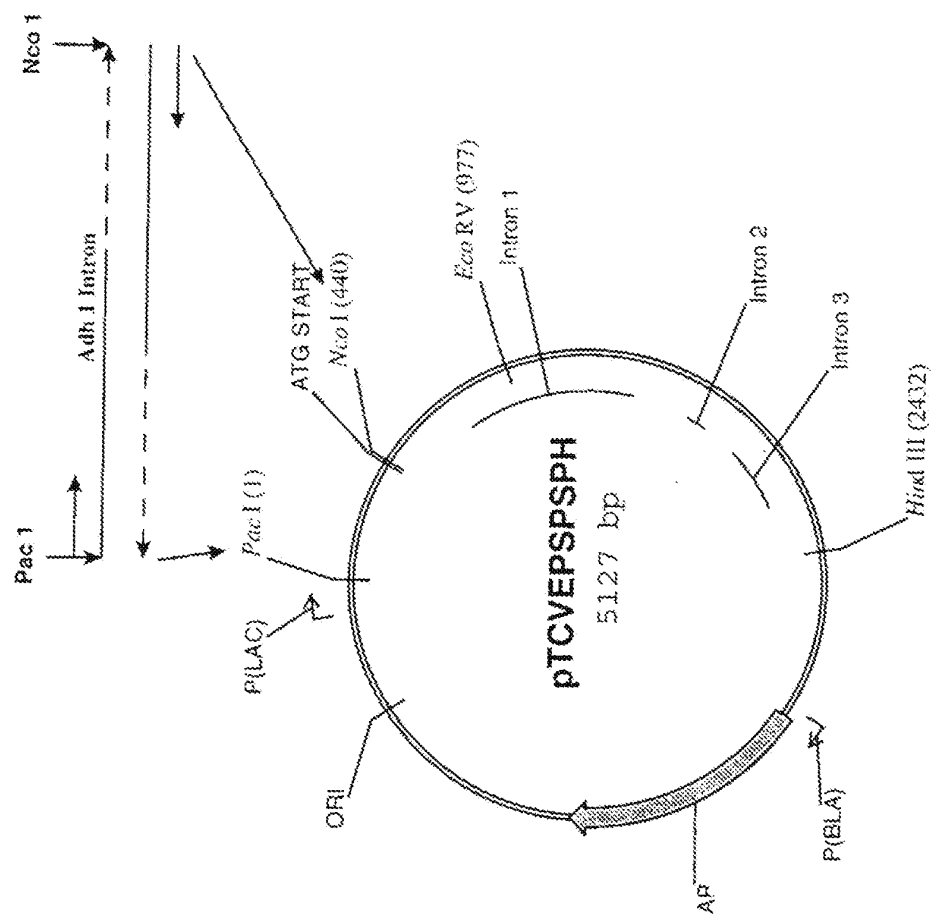


Figure 9

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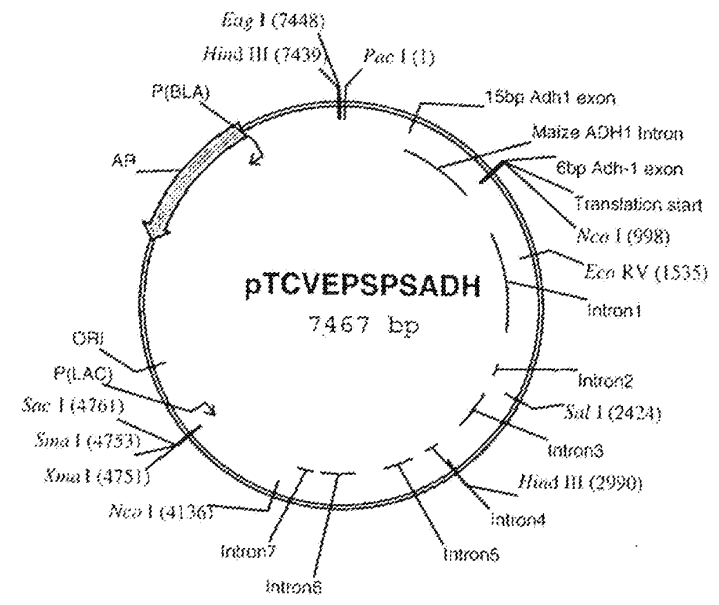


Figure 10

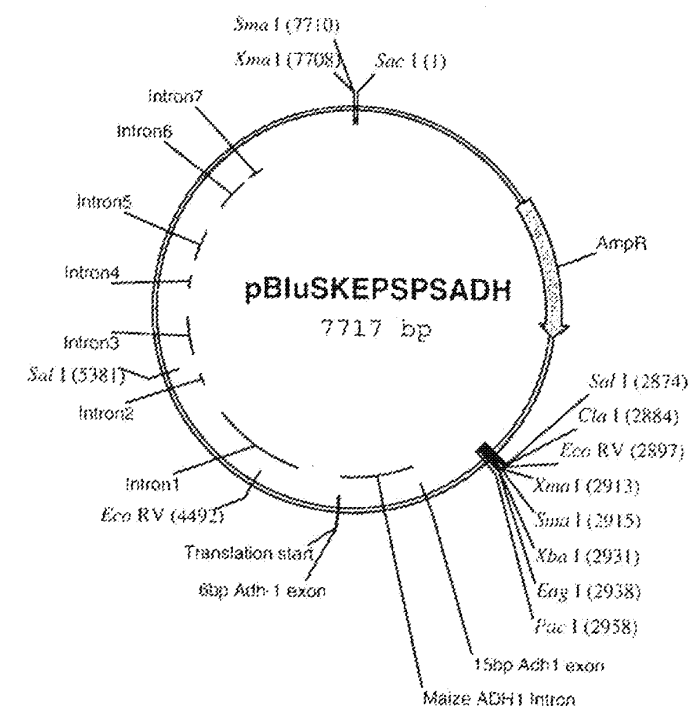


Figure 11

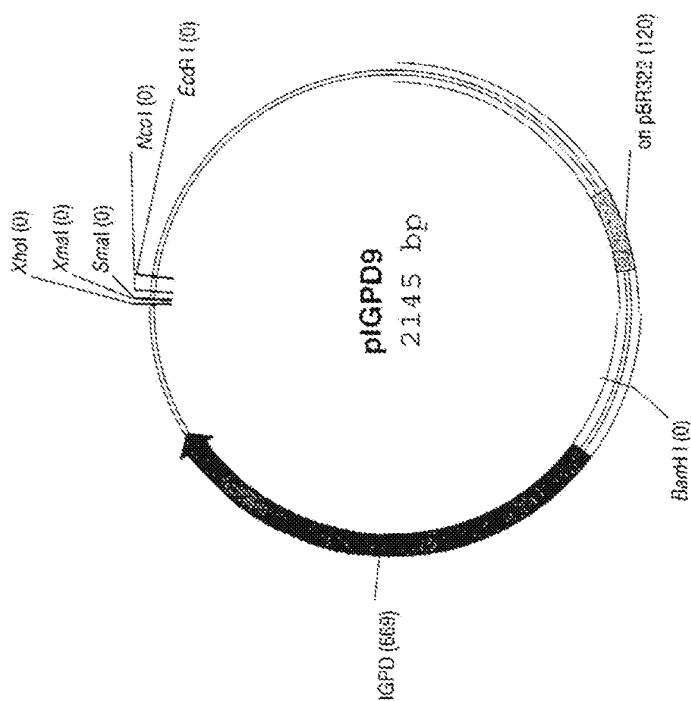


Figure 12

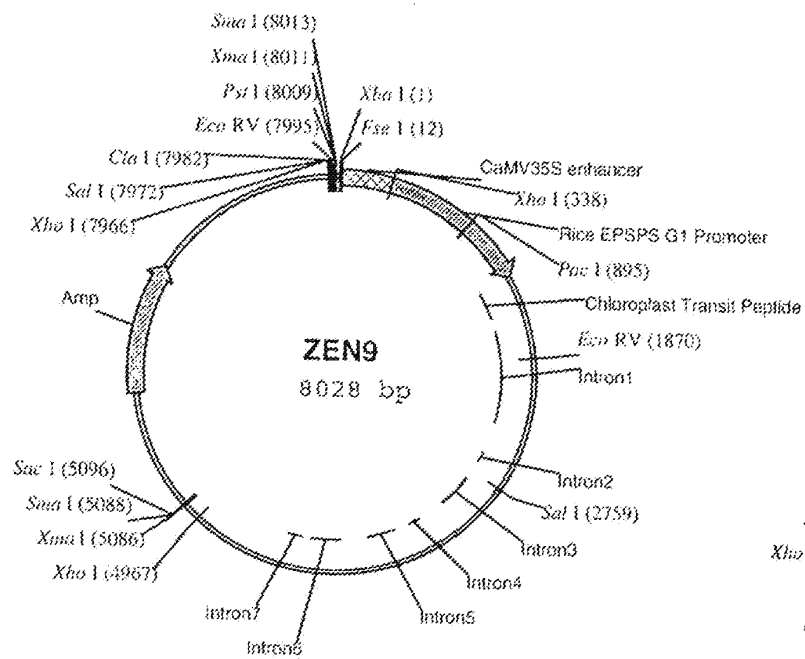


Figure 13

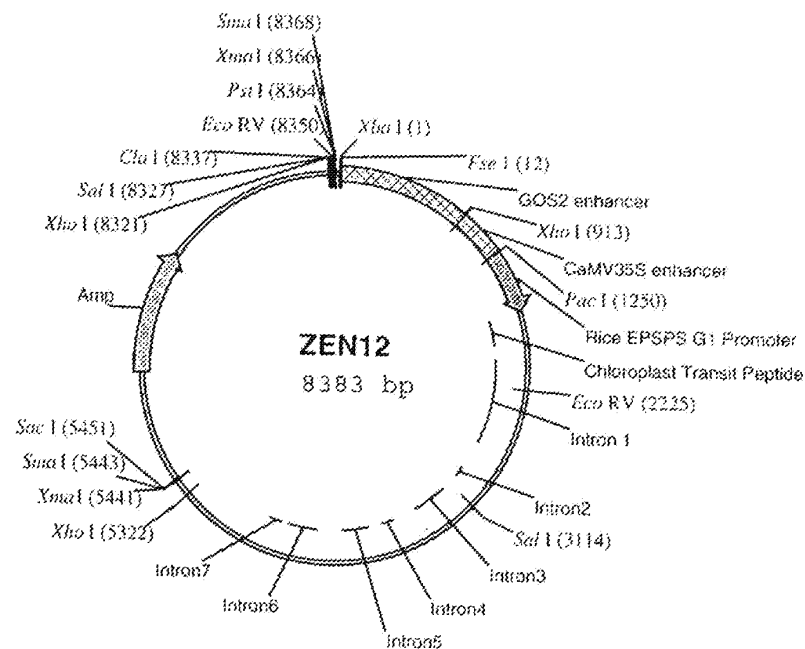


Figure 14

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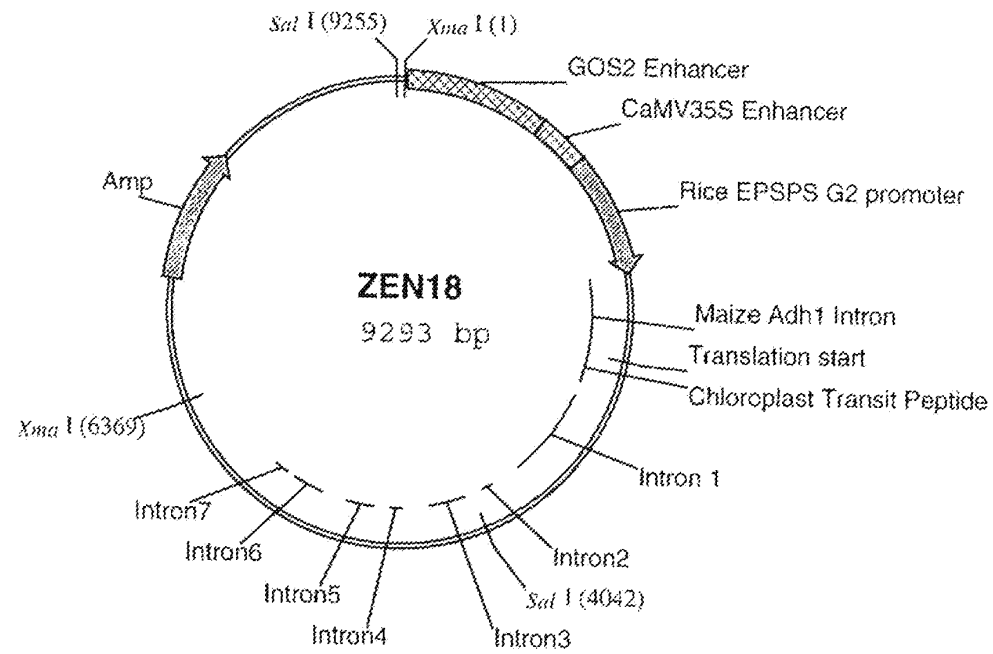


Figure 15

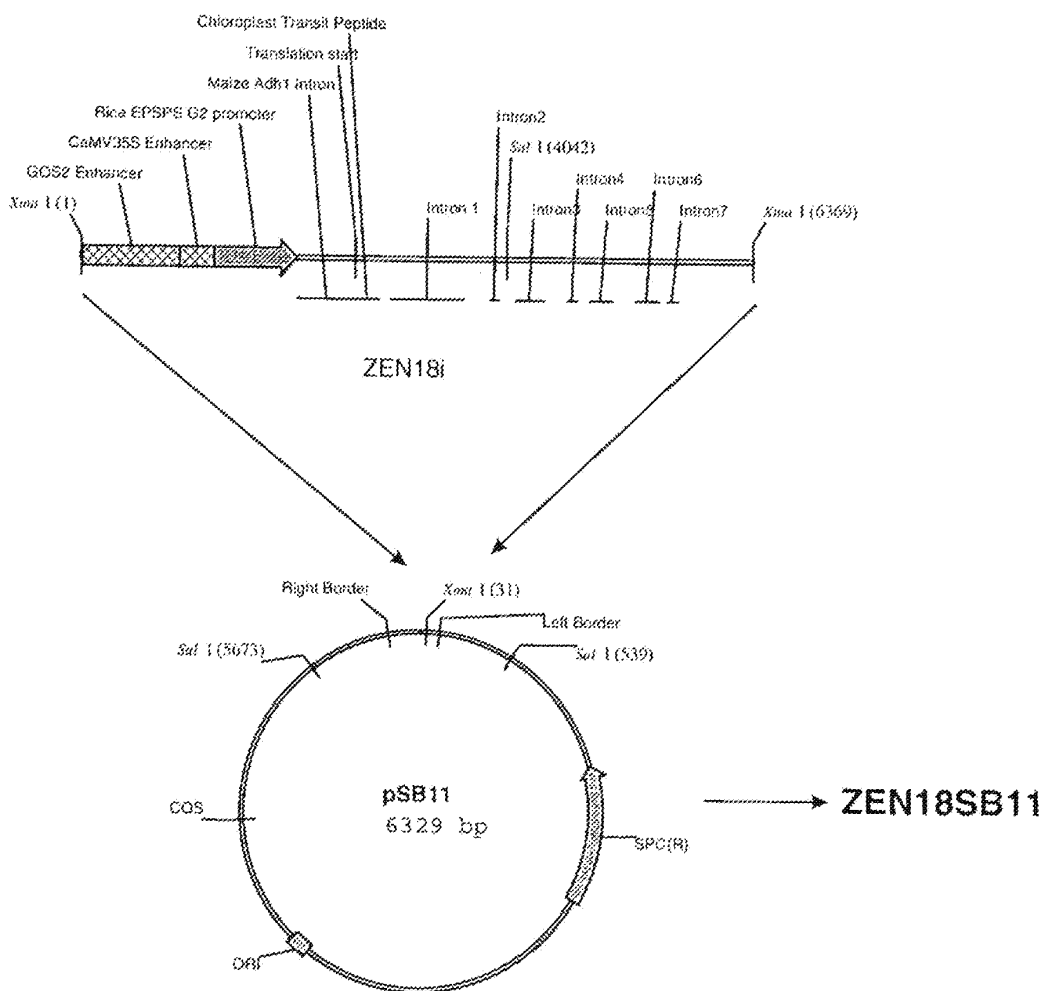


Figure 16a

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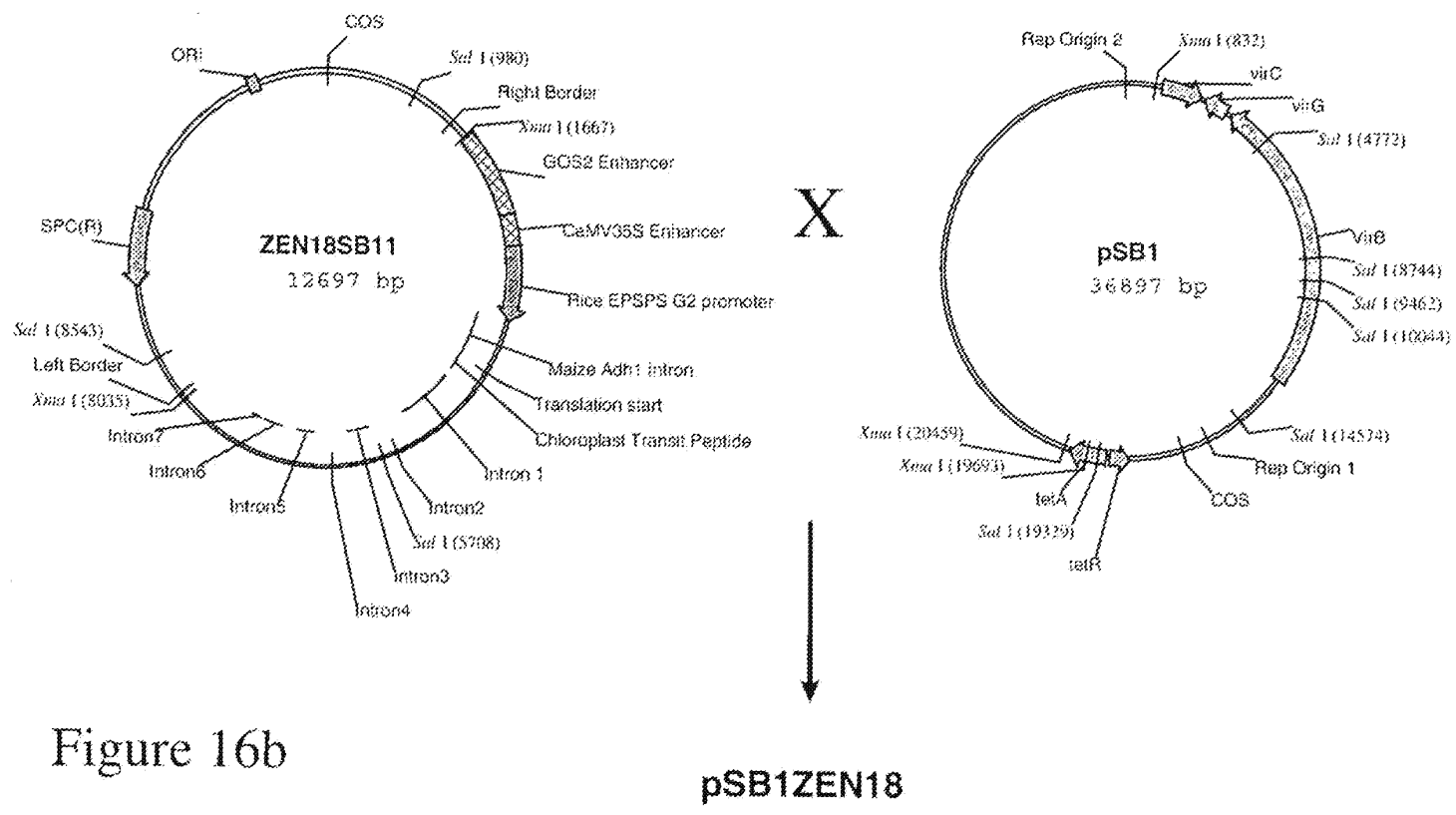


Figure 16b

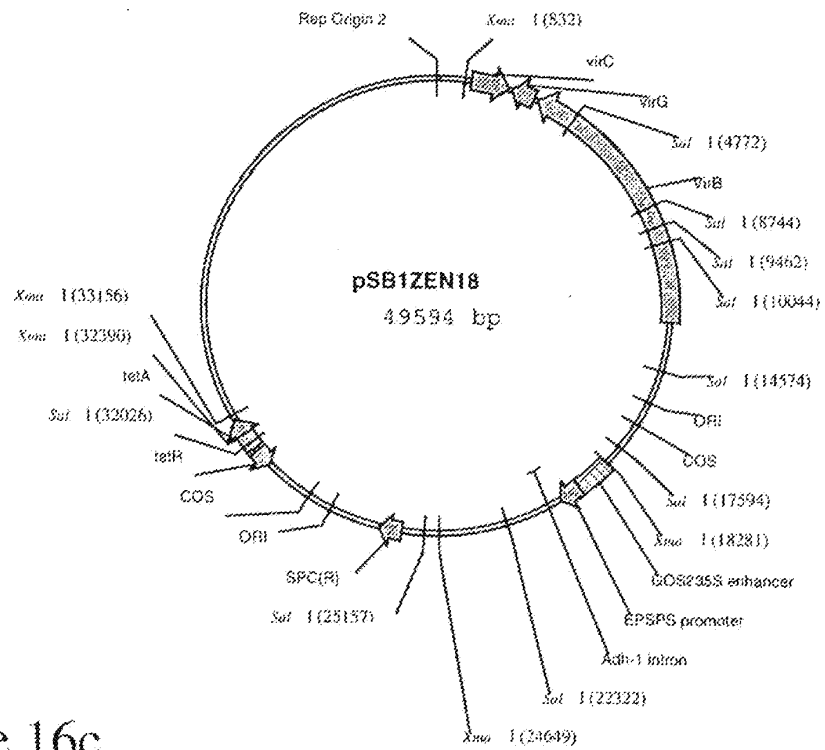


Figure 16c

INTERNATIONAL SEARCH REPORT

Int. nat. Application No.
PCT/GB 00/01573

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N15/54 C12N15/82 C12N9/10 A01H5/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N A01H		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GASSER C S ET AL: "STRUCTURE EXPRESSION AND EVOLUTION OF THE 5 ENOLPYRUVYL SHIKIMATE-3-PHOSPHATE SYNTHASE GENES OF PETUNIA AND TOMATO" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 9, 1988, pages 4280-4287, XP002145760 ISSN: 0021-9258 see esp. p.4283, fig.3	4,23,35
X	WO 98 44140 A (MCELROY DAVID; STEPHENS MICHAEL A; SPENCER T MICHAEL; MUMM RITA) 8 October 1998 (1998-10-08) the whole document	4,23,35
☒ Further documents are listed in the continuation of box C. ☒ Patent family members are listed in annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family		
Date of the actual completion of the international search 25 August 2000		Date of mailing of the international search report 12/09/2000
Name and mailing address of the ISA European Patent Office, P.O. 5818 Patentaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-9016		Authorized officer Kania, T

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/GB 00/01573

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	WO 92 06201 A (MONSANTO CO) 16 April 1992 (1992-04-16) see the whole document; esp. p.9 l.26 - p.10 l.10; p.32 l.20 - p.33 l.4; p.65 l.12-19; examples -----	4,23,35, 36,38, 40-42, 44-47, 49-55 24-26
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A	WO 97 20056 A (CAMBRIDGE ADVANCED TECH ;GRAY JOHN CLINTON (GB); SANDHU JAGDEEP SI) 5 June 1997 (1997-06-05) the whole document -----	5-26
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A	WO 98 39419 A (ASGROW SEED CO) 11 September 1998 (1998-09-11) see esp. example 1 -----	28,29,37
A	KISHIMA Y AND SANO Y. : "Oryza sativa mRNA for EPSP-synthase; AC AB016765" EBI DATABASE, 12 August 1998 (1998-08-12), XP002145761 the whole document -----	1-3

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Form PCT/ISAQ12 (patent family annex) (July 1992)

COR-AAD1-00004684

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												
		(Column 1)		(Column 2)								
FOR		NUMBER FILED		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	12/16/2020	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR		PRESENT EXTRA		RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	* 34	Minus	** 34	= 0	x \$100 =		0				
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0	x \$480 =		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		
AMENDMENT			(Column 1)		(Column 2)		(Column 3)					
			CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR		PRESENT EXTRA		RATE (\$)		ADDITIONAL FEE (\$)	
	Total (37 CFR 1.16(i))		*		Minus		**		=		x \$0 =	
	Independent (37 CFR 1.16(h))		*		Minus		***		=		x \$0 =	
	☐ Application Size Fee (37 CFR 1.16(s))											
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))												
								TOTAL ADD'L FEE		0		
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.								SLIE				
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".								/DESHONNE T. MARTINO/				
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".												
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.												

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

107540 7590 01/04/2021
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

EXAMINER

KRUSE, DAVID H

ART UNIT

PAPER NUMBER

1663

DATE MAILED: 01/04/2021

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

TITLE OF INVENTION: NOVEL HERBICIDE RESISTANCE GENES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1200	\$0.00	\$0.00	\$1200	04/05/2021

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

107540 7590 01/04/2021
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

	(Typed or printed name)
	(Signature)
	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

TITLE OF INVENTION: NOVEL HERBICIDE RESISTANCE GENES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1200	\$0.00	\$0.00	\$1200	04/05/2021

EXAMINER	ART UNIT	CLASS-SUBCLASS
KRUSE, DAVID H	1663	800-300000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively, 1 _____
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed. 2 _____
- 3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☐ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment: (Please first reapply any previously paid fee shown above)

- ☐ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)
- ☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. Change in Entity Status (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402
107540	7590	01/04/2021	EXAMINER	
Barnes & Thornburg LLP (Dow)			KRUSE, DAVID H	
11 South Meridian Street			ART UNIT	
Indianapolis, IN 46204			PAPER NUMBER	
			1663	
DATE MAILED: 01/04/2021				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. 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 30 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, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

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

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

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

Notice of Allowability	Application No. 15/288,406	Applicant(s) WRIGHT et al.	
	Examiner DAVID H KRUSE	Art Unit 1663	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to the Reply filed 16 December 2020.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are 15-16 and 18-49. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some *c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. ____.

3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: ____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date ____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)	5. ☒ Examiner's Amendment/Comment
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date ____.	6. ☐ Examiner's Statement of Reasons for Allowance
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material ____.	7. ☐ Other ____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. ____.	

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	
--	--

Examiner's Comment

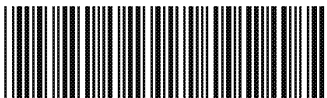
Terminal Disclaimer

1. The terminal disclaimer filed on 8 December 2020 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of each of U.S. Patent 7,838,733 and 9,127,289 and 10,174,337 has been reviewed and is accepted. The terminal disclaimer has been recorded.
2. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

Search Notes 	Application/Control No. 15/288,406	Applicant(s)/Patent Under Reexamination WRIGHT et al.
	Examiner DAVID H KRUSE	Art Unit 1663

CPC - Searched*		
Symbol	Date	Examiner
C12N 9/0069	11/07/2018	/DK/

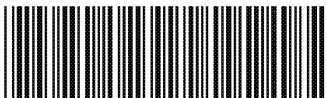
CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

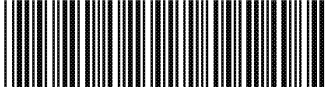
Search Notes		
Search Notes	Date	Examiner
Inventor Name Search	11/07/2018	/DK/
EAST: USPAT, USPGPUB, EPO, JPO, DERWENT	11/07/2018	/DK/
STN: AGRICOLA, BIOSIS, CAPLUS, EMBASE, CABA	11/07/2018	/DK/
Sequence Search of SEQ ID NO: 9	10/30/2018	/DK/
Update EAST	07/05/2019	/DK/
Update EAST	03/04/2020	/DK/
Update EAST	11/25/2020	/DK/
Sequence Search of SEQ ID NO: 9, included interference search	12/01/2020	/DK/
Update EAST	12/21/2020	/DK/

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	
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<i>Search Notes</i> 	Application/Control No. 15/288,406	Applicant(s)/Patent Under Reexamination WRIGHT et al.
	Examiner DAVID H KRUSE	Art Unit 1663

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
EAST	EAST	12/21/2020	/DK/

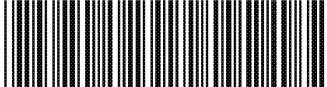
/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	
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Issue Classification 	Application/Control No. 15/288,406	Applicant(s)/Patent Under Reexamination WRIGHT et al.
	Examiner DAVID H KRUSE	Art Unit 1663

CPC						
Symbol					Type	Version
C12N	/	15	/	8274	F	2013-01-01
C12N	/	9	/	0069	I	2013-01-01
C12N	/	9	/	0071	I	2013-01-01
C12N	/	15	/	8209	I	2013-01-01

CPC Combination Sets					
Symbol				Type	Set
	/		/		

NONE (Assistant Examiner) _____ (Date)		Total Claims Allowed: 34	
/DAVID H KRUSE/ Primary Examiner, Art Unit 1663 (Primary Examiner) _____ (Date)		21 December 2020 (Date)	O.G. Print Claim(s) 1
			O.G. Print Figure NONE

<i>Issue Classification</i> 	Application/Control No. 15/288,406	Applicant(s)/Patent Under Reexamination WRIGHT et al.
	Examiner DAVID H KRUSE	Art Unit 1663

INTERNATIONAL CLASSIFICATION			
CLAIMED			
C12N	/	15	/ 82
NON-CLAIMED			
/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	34	
/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	21 December 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

Issue Classification 	Application/Control No. 15/288,406	Applicant(s)/Patent Under Reexamination WRIGHT et al.
	Examiner DAVID H KRUSE	Art Unit 1663

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☒ T.D. ☐ R.1.47															
CLAIMS															
Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		10	4	19	13	28	22	37	31	46				
	2		11	5	20	14	29	23	38	32	47				
	3		12	6	21	15	30	24	39	33	48				
	4		13	7	22	16	31	25	40	34	49				
	5		14	8	23	17	32	26	41						
	6	1	15	9	24	18	33	27	42						
	7	2	16	10	25	19	34	28	43						
	8		17	11	26	20	35	29	44						
	9	3	18	12	27	21	36	30	45						

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 34	
/DAVID H KRUSE/ Primary Examiner, Art Unit 1663 (Primary Examiner) _____ (Date) 21 December 2020		O.G. Print Claim(s) 1	O.G. Print Figure NONE

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	17	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/12/21 12:57
L2	60	aad-1.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/12/21 12:57
S1	688	aad-1	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:20
S2	48	aad-1.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:21
S3	14	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2018/11/07 08:36
S4	15	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2019/07/05 10:38
S5	1	("10323287").PN.	USPAT; USOCR	OR	OFF	2019/07/05 10:39
S6	1	("10174337").PN.	USPAT; USOCR	OR	OFF	2019/07/05 12:38
S7	54	aad-1.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/03/04 12:46
S8	16	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/03/04 12:46
S9	859	aad-1	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/03/04 12:46

S10	1	("20030041357").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/03/05 12:37
S11	60	aad-1.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/11/25 10:56
S12	17	dichlorophenoxypropionate with dioxygenase	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/11/25 10:56
S13	6	((("9062284") or ("8916752") or ("8748700") or ("8278500") or ("10167483") or ("10450549")).PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/12/02 12:56

EAST Search History (Interference)

<This search history is empty>

12/21/2020 12:57:52 PM

C:\Users\dkruse\Documents\EAST\Workspaces\15288406.wsp

BARNES & THORNBURG

OK TO ENTER: /D.H.K/

11 South Meridian Street
Indianapolis, Indiana 46204
(317) 236-1313
(317) 231-7433 Fax

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No.:	107540	}
		}
Group:	1663	}
		}
Confirmation No.:	5402	}
		}
Application No.:	15/288,406	}
		}
Invention:	Novel Herbicide Resistance Genes	}
		}
Inventor:	Terry Wright et al.	}
		}
Filed:	October 7, 2016	}
		}
Attorney Docket:	14764-258662	}
		}
Examiner:	David Kruse	}

FILED ELECTRONICALLY:

December 16, 2020

AMENDMENT AND RESPONSE UNDER 37 CFR 1.116

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

Sir:

In response to the final Office Action dated December 8, 2020, Applicant requests entry of the following amendments and consideration of the accompanying remarks. Applicant believes that no fees are required for submission of this response. If any fees are required to effect this filing, the Commissioner is authorized to charge the same to the account of Barnes & Thornburg, Deposit Account No. 10-0435 with reference to our file 14764-258662.

Amendments to the Claims are submitted herewith beginning on page 2.

Remarks begin on page 8 of this paper.

BARNES & THORNBURG LLP

11 South Meridian Street
Indianapolis, Indiana 46204
(317) 236-1313

PATENT APPLICATION*IN THE UNITED STATES PATENT AND TRADEMARK OFFICE*

<i>Customer</i>	107540	}
<i>No.:</i>		}
		}
<i>Group:</i>	1663	}
		}
<i>Confirmation</i>	5402	}
<i>No.:</i>		}
		}
<i>Application</i>	15/288,406	}
<i>No.:</i>		}
		}
<i>Invention:</i>	NOVEL HERBICIDE RESISTANCE GENES	}
		}
<i>Applicant:</i>	Terry Wright et al.	}
		}
<i>Filed:</i>	October 7, 2016	}
		}
<i>Attorney</i>	14764-258662	}
<i>Docket:</i>		}
		}
<i>Examiner</i>	David Kruse	}

**FILED
ELECTRONICALLY:**

December 16, 2020

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

This Statement is filed pursuant to 37 C.F.R. § 1.56. No representation is intended that a complete search has been made of the prior art or that no better art references than the references cited in this IDS are available. The filing of this IDS shall not be construed to be an admission that the information cited in the IDS is, or is considered to be, material to patentability as defined in §1.56(b).

Pursuant to 37 C.F.R. § 1.98(a)(2)(ii), copies of cited U.S. patents and patent publications are not provided herewith; a copy of each cited foreign and non-patent reference is provided herewith.

Applicant submits herewith the fee set forth in 37 CFR 1.17 (p) and authorize the Commissioner to charge any additional fee necessary to affect this filing to our Deposit Account No. 10-0435, with reference to our Matter No. 14764-258662.

Consideration of the claims, leading to their allowance and passage of the application to issuance, is respectfully requested. If any further discussion of this matter would speed prosecution of this application, the Examiner is invited to call the undersigned at (434) 220-2866.

Respectfully submitted,

A handwritten signature in cursive script, appearing to read "John P. Breen", written in dark ink.

John P. Breen
Registration No. 38,833
Attorney for Applicants

JPB:gl
(317) 261-7940
Indianapolis, Indiana 46204

Doc code: IDS

PTO/SB/08a (02-18)

Doc description: Information Disclosure Statement (IDS) Filed

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
(Not for submission under 37 CFR 1.99)

Application Number	15288406
Filing Date	2016-10-07
First Named Inventor	Terry Wright
Art Unit	1663
Examiner Name	KRUSE, David H.
Attorney Docket Number	14764-258662

U.S.PATENTS						Remove
Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
/D.H.K./	1	5463175		1995-10-31	Barry et al.	
/D.H.K./	2	6518222		2003-02-11	Arndt et al	
If you wish to add additional U.S. Patent citation information please click the Add button.						Add
U.S.PATENT APPLICATION PUBLICATIONS						Remove
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
/D.H.K./	3	2003135879		2003-07-17	Weeks et al.	
If you wish to add additional U.S. Published Application citation information please click the Add button.						Add

/DAVID H KRUSE/

12/21/2020

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
(Not for submission under 37 CFR 1.99)

Application Number	15288406
Filing Date	2016-10-07
First Named Inventor	Terry Wright
Art Unit	1663
Examiner Name	KRUSE, David H.
Attorney Docket Number	14764-258662

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K./

FOREIGN PATENT DOCUMENTS								
Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ²	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T ⁵
	4	03/013224	WO		2003-02-20	BAYER BIOSCIENCE N.V.		☐
	5	87/05629	WO		1987-09-24	PLANT GENETIC SYSTEMS N.V.		☐
	6	97/13402	WO		1997-04-17	DOW AGROSCIENCES LLC		☐
	7	96/33270	WO		1996-10-24	AMERICAN CYANAMID COMPANY		☐
	8	1167531	EP		2002-01-02	Monsanto Technology LLC		☐
	9	0009727	WO		2000-02-24	MAXYGEN, INC.		☐
	10	0066748	WO		2000-11-09	SYNGENTA LIMITED		☐
	11	1471533	CN		2004-01-28	DOW AGROSCIENCES LLC	English Abstract	☐

/DAVID H KRUSE/

12/21/2020

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
(Not for submission under 37 CFR 1.99)

Application Number	15288406
Filing Date	2016-10-07
First Named Inventor	Terry Wright
Art Unit	1663
Examiner Name	KRUSE, David H.
Attorney Docket Number	14764-258662

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K/

If you wish to add additional Foreign Patent Document citation information please click the Add button			Add
NON-PATENT LITERATURE DOCUMENTS			Remove
Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T ⁵
	12	HULSE J D ET AL: "CARNITINE BIOSYNTHESIS BETA HYDROXYLATION OF TRI METHYL LYSINE BY AN ALPHA KETO GLUTARATE DEPENDENT MITOCHONDRIAL DI OXYGENASE", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 253, no. 5, 1978, pages 1654-1659, ISSN: 0021-9258	☐
	13	EICHHORN ERIC ET AL: "Characterization of alpha-ketoglutarate-dependent taurine dioxygenase from Escherichia coli", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 37, 1997, pages 23031-23036, ISSN: 0021-9258	☐
	14	BISHT NA VEEN CHANDRA ET AL: "Development of 2,4-O-resistant transgenics in Indian oilseed mustard (Brassica juncea)", CURRENT SCIENCE (BANGALORE), vol. 87, no. 3, 10 August 2004 (2004-08-10), pages 367-370, ISSN: 0011-3891	☐
	15	PRINS M: "Broad virus resistance in transgenic plants", TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 21, no. 9, 1 September 2003 (2003-09-01), pages 373-375, XP004430444, ISSN: 0167-7799, DOI: 10.1016/S0167-7799(03)00183-5	☐
	16	DELLAPORTA S L ET AL: "A PLANT DNA MINIPREPARATION: VERSION 11", PLANT MOLECULAR BIOLOGY REPORTER, SPRINGER SCIENCE +BUSINESS MEDIA B.V, NL, vol. 1, no. 4, 1 January 1983 (1983-01-01), pages 19-21, XP001016105, ISSN: 0735-9640	☐
	17	Opposition filed against EP 10012200.1, filed on 2013-12-20	☐
	18	Opposition filed against EP 10012201.9, filed on 2013-12-06	☐
	19	Opposition filed against EP 10012202.7, filed on 2013-12-20	☐
	20	Opposition filed against EP 10012199.5, filed on 2014-06-18	☐

/DAVID H KRUSE/

12/21/2020

21	Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, SECOND EDITION., 1989, Vol.2, p.11.45	☐
22	Applicant Response to Opposition filed against EP 10012200.1, filed on 2014-08-08	☐
23	Applicant Response to Opposition filed against EP 10012201.9, filed on 2014-07-28	☐
24	Applicant Response to Opposition filed against EP 10012202.7, filed on 2014-07-28	☐
25	Applicant Response to Opposition filed against EP 10012199.5, filed on 2014-11-22	☐
26	Final Decision in Opposition filed against EP 10012200.1, filed on 2016-08-01	☐
27	Final Decision in Opposition filed against EP 10012201.9, filed on 2016-08-01	☐
28	Final Decision in Opposition filed against EP 10012202.7, filed on 2016-08-01	☐
29	Final Decision in Opposition filed against EP 10012199.5, issued on 2016-02-09	☐

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K/

/DAVID H KRUSE/

12/21/2020

CERTIFICATION STATEMENT

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Breen/	Date (YYYY-MM-DD)	2020-12-16
Name/Print	John P. Breen	Registration Number	38833

This collection of information is required by 37 CFR 1.97 and 1.98. 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 1 hour 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.**

Receipt date: 12/16/2020

15/288,406 - GAU: 1663

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Jan 04, 2021 03:49:57 AM

Dear PAIR Customer:

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:

The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15288406	NOA	01/04/2021	14764-258662
	1449	01/04/2021	14764-258662

To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

107540 7590 01/04/2021
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

TITLE OF INVENTION: NOVEL HERBICIDE RESISTANCE GENES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1200	\$0.00	\$0.00	\$1200	04/05/2021

EXAMINER	ART UNIT	CLASS-SUBCLASS
KRUSE, DAVID H	1663	800-300000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Barnes & Thornburg LLP

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY AND STATE OR COUNTRY)

Dow AgroSciences LLC

Indianapolis, IN

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment: (Please first reapply any previously paid fee shown above)

- ☒ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 10-0435

5. Change in Entity Status (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /John P. Breen/

Date January 7, 2021

Typed or printed name John P. Breen

Registration No. 38833

Electronic Patent Application Fee Transmittal				
Application Number:		15288406		
Filing Date:		07-Oct-2016		
Title of Invention:		NOVEL HERBICIDE RESISTANCE GENES		
First Named Inventor/Applicant Name:		Terry WRIGHT		
Filer:		John P. Breen/Garla Twyman		
Attorney Docket Number:		14764-258662		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1200	1200

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1200

Electronic Acknowledgement Receipt	
EFS ID:	41585325
Application Number:	15288406
International Application Number:	
Confirmation Number:	5402
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry WRIGHT
Customer Number:	107540
Filer:	John P. Breen/Garla Twyman
Filer Authorized By:	John P. Breen
Attorney Docket Number:	14764-258662
Receipt Date:	07-JAN-2021
Filing Date:	07-OCT-2016
Time Stamp:	17:03:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	\$1200
RAM confirmation Number	E202117H05287976
Deposit Account	100435
Authorized User	Garla Twyman
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)
 37 CFR 1.20 (Post Issuance fees)
 37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	Issue_Fee_Transmittal.pdf	272329	no	1
			f66061abf111113907da5cb53560b9d89b5e1bb1		

Warnings:

Information:

2	Fee Worksheet (SB06)	fee-info.pdf	30361	no	2
			b5ffddbf7558405551c5e33651631aa299998e42		

Warnings:

Information:

Total Files Size (in bytes):	302690
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

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

National Stage of an International Application under 35 U.S.C. 371

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

New International Application Filed with the USPTO as a Receiving Office

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



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
15/288,406	10/07/2016	1663	3120	14764-258662	34	1

107540
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

CONFIRMATION NO. 5402
CORRECTED FILING RECEIPT



0000000122585533

Date Mailed: 01/11/2021

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

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Inventor(s)

Terry WRIGHT, Westfield, IN;
Justin LIRA, Zionsville, IN;
Donald MERLO, Carmel, IN;
Nicole ARNOLD, Indianapolis, IN;

Applicant(s)

Dow AgroSciences LLC, Indianapolis, IN;

Power of Attorney: The patent practitioners associated with Customer Number 107540

Domestic Priority data as claimed by applicant

This application is a CON of 14/820,893 08/07/2015 PAT 10174337
which is a CON of 12/951,813 11/22/2010 PAT 9127289
which is a CON of 11/587,893 05/22/2008 PAT 7838733
which is a 371 of PCT/US2005/014737 05/02/2005
which claims benefit of 60/567,052 04/30/2004

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 10/17/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/288,406**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL HERBICIDE RESISTANCE GENES

Preliminary Class

800

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Jan 11, 2021 03:51:10 AM

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Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
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Application	Document	Mailroom Date	Attorney Docket No.
15288406	APP.FILE.REC	01/11/2021	14764-258662

To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>.

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	03/16/2021	10947555	14764-258662	5402

107540 7590 02/24/2021
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 257 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

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Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Terry WRIGHT, Westfield, IN;
Dow AgroSciences LLC, Indianapolis, IN;
Justin LIRA, Zionsville, IN;
Donald MERLO, Carmel, IN;
Nicole ARNOLD, Indianapolis, IN;

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To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Feb 25, 2021 03:47:29 AM

Dear PAIR Customer:

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11 South Meridian Street
Indianapolis, IN 46204
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Application	Document	Mailroom Date	Attorney Docket No.
15288406	ISSUE.NTF	02/24/2021	14764-258662

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