



USOORE36449E

**United States Patent** [19]

[11] E

**Patent Number: Re. 36,449****Lebrun et al.**[45] **Reissued Date of Patent: Dec. 14, 1999****[54] CHIMERIC GENE FOR THE TRANSFORMATION OF PLANTS**5,719,046 2/1998 Guerineau et al. .... 435/468  
5,728,925 3/1998 Estrella et al. .... 800/300  
5,750,875 5/1998 Stalker ..... 800/284**[75] Inventors: Michel Lebrun, Montpellier, France; Bernard Leroux, Raleigh, N.C.; Alain Sailland, Lyons, France****FOREIGN PATENT DOCUMENTS**0 189 707 B1 8/1986 European Pat. Off. .  
0 218 571 B1 4/1987 European Pat. Off. .  
WO 88/02402 4/1988 WIPO .**[73] Assignee: Rhone-Poulenc Agro, Lyons, France****OTHER PUBLICATIONS****[21] Appl. No.: 09/025,082**Keegstra et al., *Annu Rev Plant Physiol Plant Mol Biol* 40:471–501, 1989.**[22] Filed: Feb. 17, 1998**Ostrem et al., *J. Biol. Chem.* 264:3662 (1989).**Related U.S. Patent Documents**Chan et al., *EMBO Journal* 9:333 (1990).

Reissue of:

**[64] Patent No.: 5,510,471**  
**Issued: Apr. 23, 1996**  
**Appl. No.: 08/251,621**  
**Filed: May 31, 1994**B. Reiss et al (1989), "Effect of Mutations on the Binding and Translocation Functions of a Chloroplast Transit Peptide", *Proc. Natl Acad Sci USA*, 86:886–890.

U.S. Applications:

B. Mazur et al (1989), "The Development of Herbicide Resistant Crops", *Annu. Rev. Plant Physiol Plant Mol. Biol* 40:441–470.**[63] Continuation of application No. 07/846,211, Mar. 4, 1992, abandoned.**S. Smeekens et al (1990) "Protein Transport into and Within Chloroplasts", *TIBS* 15:73–76.**[30] Foreign Application Priority Data**

Mar. 5, 1991 [FR] France ..... 9102872

LeBrun et al "Nucleotide Sequence of a Gene Encoding Corn Ribulose–1,5 Bisphosphate Carboxylase/Oxygenase Small Subunit (rbcs)", *Nucleic Acids Research*, vol. 15, No. 10 (1987), p. 4360.**[51] Int. Cl.<sup>6</sup> ..... A01H 5/00; C07H 17/00; C12N 15/00; C12N 15/29**Waksman "Nucleotide sequence of a gene encoding sunflower ribulose–1,5Bisphosphate carboxylase/oxygenase small subunit (rbcs)", *Nucleic Acids Research*, vol. 15, No. 17 (1987), p. 7181.**[52] U.S. Cl. .... 800/298; 800/317.3; 800/320.1; 800/322; 800/278; 536/23.4; 536/23.6; 435/69.1; 435/69.7**L. Comai, et al (1988), "Chloroplast Transport of a Ribulose Bisphosphate Carboxylase Small Subunit–5–Enolpyruvyl 3–Phosphoshikimate Synthase Chimeric Protein Requires Part of the Mature Small Subunit in Addition to the Transit Peptide", *The Journal of Biological Chemistry* vol. 263, No. 29 Issue of Oct. 15, pp. 15104–15109.**[58] Field of Search ..... 800/298, 278, 800/320.1, 322, 317.3; 536/23.4, 23.6; 435/69.1, 419**Wasmann, Reiss, Bartlett and Bohnert, (1986) "The Importance of the Transit Peptide and the Transported Protein for Protein Import into Chloroplasts", *Mol Gen Genet*, 205: 446–453.**[56] References Cited****U.S. PATENT DOCUMENTS**4,535,060 8/1985 Comai ..... 435/252.33  
4,801,540 1/1989 Hiatt et al. .... 435/411  
4,940,835 7/1990 Shah et al. .... 800/288  
4,962,028 10/1990 Bedbrook et al. .... 800/278  
5,073,677 12/1991 Heimer et al. .... 800/300  
5,188,642 2/1993 Shah et al. .... 47/58.1  
5,254,799 10/1993 De Greve et al. .... 800/302  
5,258,300 11/1993 Glassman et al. .... 800/288  
5,312,910 5/1994 Kishore et al. .... 536/23.2  
5,349,123 9/1994 Shewmaker ..... 800/284  
5,349,127 9/1994 Dean et al. .... 800/288  
5,367,110 11/1994 Galili et al. .... 800/300  
5,463,175 10/1995 Barry et al. .... 800/300  
5,489,520 2/1996 Adams et al. .... 800/293  
5,498,544 3/1996 Gengenbach et al. .... 435/320.1  
5,498,830 3/1996 Barry et al. .... 800/281  
5,530,188 6/1996 Ausich et al. .... 800/282  
5,538,878 7/1996 Thomas et al. .... 800/288  
5,554,798 9/1996 Lundquist et al. .... 800/300.1  
5,559,024 9/1996 Leroux et al. .... 435/252.3  
5,597,717 1/1997 Guerineau et al. .... 800/288  
5,608,149 3/1997 Barry et al. .... 800/284  
5,618,988 4/1997 Hauptmann et al. .... 800/282  
5,633,444 5/1997 Guerineau et al. .... 800/300  
5,646,024 7/1997 Leemans et al. .... 435/6  
5,684,238 11/1997 Ausich et al. .... 800/298  
5,717,084 2/1998 Estrella et al. .... 536/23.4J. Bennett, (1982), "Sic Transit Peptide", *Trends in Biotechnology, Elsevier Biomedical Press*.

(List continued on next page.)

*Primary Examiner*—Elizabeth F. McElwain  
*Attorney, Agent, or Firm*—Connolly and Hutz**[57] ABSTRACT**

Chimeric gene for conferring to plants an increased tolerance to a herbicide having as its target EPSPS comprises, in the direction of transcription, a promoter region, a transit peptide region, a coding sequence for glyphosate tolerance and a polyadenylation signal region, wherein the transit peptide region comprises, in the direction of translation, at least one transit peptide of a plant gene encoding a plastid-localised enzyme, a partial sequence of the N-terminal mature part of a plant gene encoding a plastid-localised enzyme and then a second transit peptide of a plant gene encoding a plastid-localised enzyme. Production of glyphosate-tolerant plants is disclosed.

**17 Claims, No Drawings**

## OTHER PUBLICATIONS

- J. Bryant, (1985), "Targetting Proteins Into Subcellular Organelles", *Trends in Biotechnology, Elsevier Biomedical Press*, p. 13.
- E. Bell & L. McIntosh, (1984) "Molecular Basis of Transit Peptide Function", *Annual Mtg. of the Am.Soc.Plant Phys., Davis*.
- S. Smith et al., (1983), "Characterization of three cDNA clones encoding different mRNAs for the precursor to the small subunit of wheat ribulose biphosphate carboxylase", *Nucleic Acids Research*, vol. 11 No. 24 pp. 8719–34.
- A. Cashmore et al (1985) "Import of Polypeptides into Chloroplasts", *Bio/Technology*, vol. 3 Sep. 1985 pp. 803–07.
- G. Van de Broeck et al, (1985), "Targeting of a Foreign Protein to Chloroplasts by Fusion to the Transit Peptide from the Small Subunit of Ribulose 1,5-bisphosphate Carboxylase", *Nature (Lond)*, vol. 313 pp. 358–63.
- R. Broglie et al., (1983), "Structural Analysis of Nuclear Genes Coding for the Precursor to the Small Subunit of Wheat Ribulose-1,5 Bisphosphate Carboxylase", *Bio/Technology*, Mar. 1983 pp. 55–61.
- B. Reiss et al, (1985), "Transport of Foreign Proteins into Chloroplasts; Functional Domains of the Rbcs Transit Peptide", *3d Joint Meeting of Biochemical Societies, Basel*, vol. 36 p. 839.
- G. Coruzzi et al, (1983) "Expression of Nuclear Genes Encoding the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase", *NATO Adv. Sci. Inst. Serv. A*, pp. 47–59.
- B. Leroux et al, (1988), "A New Selectable Marker to Study Gene Expression in Plants", *Journal of Cellular Biochemistry*, Abstract L513 at p. 201.
- B. Martineau et al, (1988), "Expression of a C3 Plant Rubisco SSU Gene in Regenerated C4 Plants", *Journal of Cellular Biochemistry*, Abstract L514 at p. 201.
- P.H. Schreier et al, (1985), "The Use of Nuclear-Encoded Sequences to Direct the Light-Regulated Synthesis and Transport of a Foreign Protein into Plant Chloroplasts", *MBO Journal*, vol. 4, No. 1, pp. 25–32.
- Reiss, B.; Wasmann C. C; Bohnert H.J.; *Mol. Gen. Genet* 209, (1), pp. 116–121; 1987\* Abstract 1.
- Kuntz M; Simons A., Schell J.; Schreier P.H., *Mol. Gen. Genet* 205 (3); 454–460 1986 (Recd. 1987), 454–460, Coden Meggeae Issn: 0026–8925\* Abstract 2.
- Schreier, P.H., Philos. Trans. R. Soc. London B. Biol. Sci. 313 (1162), 429–432, 1986, "Use of Chimeric Genes Harboring . . . Chloroplasts"\* Abstract 3.
- Kloesgen R. B., et al., *Mol. Gen Genet* 203 (2), 237–244, 1986 "Molecular Analysis of the Waxy Locus of *Zea Mays*" \* Abstract 4.
- Schreier P. H. et al.; Impact Gene Transfer Tech. Eukaryotic Cell Biol; (1984) 91–102, The chloroplast genome, its interaction with the nucleus and the modification of chloroplast metabolism \*Abstract 6.
- Cioppa, G. della, et al., *Bio/Technology*, (1987) vol. 5, No. 6, pp. 579–584, 18 ref. "Targeting a herbicide-resistant enzyme . . . higher plants"\* Abstract 7.
- Della-Cioppa G., et al, *Proc. Natl. Acad. Sci USA* 83 (18), 6873–6877, 1986 "Translocation of the Precursor . . . Higher Plants In-Vitro"\* Abstract 8.
- Della-Cioppa G., et al; *Fed. Proc. Fed. Am. Soc. Exp. Biol.*; (1987) 46 (6): 2109 "Targeting of a glyphosate-resistant . . . chloroplasts of higher plants"\* Abstract 9.
- Della-Cioppa G. et al.; *Bio/Technology*; (1987) 5 (6): 579–84 Targeting a Herbicide-Resistant . . . Higher Plants\* Abstract 10.
- Scherer D. E., et al., *Plant Mol Biol* 9 (2) 127–134, 1987 "Isolation of a Complementary DNA . . . Spinach"\* Abstract 11.
- Rose R. E. et al; *Nucleic Acids Res.*, 15 (17): 7197 "The Nucleotide Sequence of a cDNA . . . Compestris Seeds"\* Abstract 12 (1987).
- Pichersky E.; et al., *Gene* 40 (2–3), 247–258, (1985) "Molecular Characterization and Genetic . . . Esculentum Tomato"\* Abstract 13.
- Lamppa G. K., et al., *Mol. Cell Biol* 5 (6) 1370–1378, 1985 "Structure and Developmental Regulation . . . Polypeptide"\* Abstract 14.
- Brogliè, R., et al., *Bio/Technology*, (1983) vol. 1, No. 1, pp. 55–61, "Structural Analysis of Nuclear Genes . . . Carboxylase"\* Abstract 16.
- Coruzzi, Gloria, et al., *J. Biol. Chem.* (1983), 258(3), 1399–402, "Nucleotide Sequences of two pea cDNA . . . Polypeptide"\* Abstract 17.
- Timko, Michael, P., et al., *UCLA Symp. Mol. Cell. Biol., New Ser.* (1983), 12 (Plant Mol. Biol). 403–12, "Nuclear Genes Enclosing . . . Complex From Pea"\* Abstract 18.
- Smeeckens, S., et al., *Plant Mol Biol.* 7 (6), 433–440, 1986 "Silene-Pratensis Complementary DNA . . . Carboxylase"\* Abstract 20.
- Smeeckens S., et al., *Plant Molecular Biology*, (1987), vol. 9, No. 4, pp. 377–388, "Import Into Chloroplasts . . . Plastocyanin Transit Peptides"\* Abstract 21.
- Hageman J., et al., *Symposium on Plant Systems . . .*, Feb. 2–8, 1987, *J. Cell Biochem Suppl* 0 (11 Part B), 64, "Import of Plastocyanin . . . Chloroplasts"\* Abstract 22.
- Smeeckens S., et al., *Cell* 46 (3) 365–376, 1986, "The Role of the Transit Peptide in the Routing of Precursors Toward Different Chloroplast Compartments"\* Abstract 23.
- Smeeckens S., et al., *Nature (Lond)* 317 (6036) 456–468, 1985 "Sequence of the Precursor . . . Protein Plastocyanin"\* Abstract 24.
- Smeeckens, Sjef, et al., *Nucleic Acids Res.* (1985), 13(9), 3179–94, "The Plant Ferredoxin precursor . . . cDNA clone"\* Abstract 25.
- Herrera-Estrella, L. et al., *Second European Congress on Cell Biology*, Jul. 6–11, 1986, *ACTA Biol. Hung* 37 (Suppl.), "The Roll of the Transit Peptide . . . Chloroplasts"\* Abstract 26.
- Dunsmuir, Pamela, *Nucleic Acids Res.* (1985), 13(7) 2503–18, "The Petuna chlorophyll a/b/ . . . different gene families"\* Abstract 41.
- Mayfield S.P., *Proc. Natl Acad Sci USA* 84 (3) 749–753, 1987, "Expression of the Nuclear Gene Encoding . . . Chlamydomonas-Reinhardtii"\* Abstract 43.
- Rochaix, J.D., et al., *Gene Manipulation Plant Improv., Stadler Genet., Symp.*, 16<sup>th</sup> (1984) 577–603, "Chlamydomonas reinhardtii, a potential model . . . gene manipulation"\* Abstract 44.
- Bell, Erin, *Michigan State Univ.*, (1987) 121 pp. Avail.: *Univ. Microfilms Int.*, Order No. DA8714299, from: *Diss. Abstr. Int. B* 1987, 48(3), 654, "A Molecular Analysis of ribulose . . . peptide function"\* Abstract 46.
- Tyagi A.k. et al., *Mol. Gen Genet* 207 (2–3), 288–293, 1987, "Nucleotide Sequence of complementary DNA . . . Complex From Spinach"\* Abstract 47 (1987).

- Hodspeth R.L., et al., Proc Natl Acad Sci USA 83 (9), 2884–2888, 1986, “Genomic and Complementary DNA Clones . . . Members in leaves and roots”\* Abstract 48.
- Hurt E. C., et al., EMBO (Eur Mol Biol Organ) J 5 (6), 1343–1350, 1986, “The Cleavable Pre-Sequence . . . Polypeptides into yeast mitochondria”\* Abstract 49.
- Anderson, S., et al., Biochem J 240 (3), 709–716, 1986, “Synthesis of the Small Subunit . . . SP6 Promoter”\* Abstract 50.
- Nierzwicki-Bauer S.A., et al., Proc. Natl. Acad Sci USA 81 (19), 5961–5965, 1984, “Contranscription of Genes Encoding . . . Cyanobacterium Anabaena”\* Abstract 51.
- Pinck M., et al., Biochimie (Paris) 66 (7–8), 539–546, 1984, “Complete Sequence of One of the Messenger RNA . . . Carboxylase EC-4.1.1.39 of Nicotiana-Sylvestris”\* Abstract 52.
- Belford H.S., et al., J. Biol Chem 258 (7), 4503–4510, 1983, “Phycobilli Protein synthesis . . . Peptides of Phycocyanin”\* Abstract 53.
- Martin P.G., et al., Aust J. Bot 31 (4), 395–410, 1983, “The Study of Plant Phylogeny Using Amino-Acid . . . Variability”\* Abstract 54.
- Berry-Lowe S.L., et al., J. Mol. Appl. Genet 1 (6), 483–498, 1982, “The Nucleotide Sequence Expression . . . Glycine-Max”\* Abstract 55.
- Ishiy M., et al., FEBS (Fed Eurn Biochem Soc) Lett 124 (2), 233–236, 1981, “The High Iso Electric Point . . . Transit Peptide”\* Abstract 56.
- Bennett J., Trends in Biochemical Sciences, (1982) vol. 7, No. 8, pp. 269, “Transit Peptide”\* Abstract 58.
- Kaderbhai, N., et al., Biochem. Soc. Trans. (1987), 15(6), 1143–4, “Expression of the fusion protein . . . *Escherichia coli*”\* Abstract 60.
- Gatenby, Anthony A., et al., Eur. J. Biochem. (1987), 168(1), 227–31, “Coexpression of both the maize . . . in *escherichia coli*”\* Abstract 61.
- Stayton, Mark, M., et al., Nucleic Acids Res. (1986), 14(24), 9781–96; “A novel chlorophyll a/b/binding . . . precursor protein”\* Abstract 62.
- Broeck, G. van den et al., Nature, UK, (1985) vol. 313, No. 6001, pp. 358–363, “Targeting of a foreign protein . . . carboxylase”\* Abstract 27.
- Van Montagu, et al., Prod. Agents Ther. Genie Genet., Symp Satell. (1986), Meeting Date 1985, 59–63, “Plant Genetic Engineering, Present Techniques & prospects”\* Abstract 29.
- Herrera-Estralla, Mol. Biol. Photosynth. Appar. (1985), 397–405. “Use of chimeric genes to study light-inducible . . . Carboxylase”\* Abstract 30.
- Wasmann C. C., et al., Annual Meeting of the American Society of Plant Physiologists, St. Louis, Missouri, Jul. 19–23, 1987, Plant Physiol 83 (4 Suppl). 1987, 55., “The Transit Peptide of the Precursor . . . Functional Regions”\* Abstract 34.
- Lubben, T.H., Proc Natl. Acad. Sci, USA 83 (15):5502–06 1986, “Efficient In-Vitro Import of a Cytosolic . . . Chloroplasts”\* Abstract 35.
- Keegstra K., et al., Symposium on Plant Membranes: Structure, Function, Biogenesis Held at the 16<sup>th</sup> Annual UCLS Meeting on Molecular and Cellular Biology, Feb. 8–13, 1987, J. Cell. Biochem Suppl 0 (11 Part B), 1987, 75, “Targeting of Proteins into Chloroplasts”\* Abstract 36.
- Kohorn B. D., et al., Plant physiol (Bethesda) 82 (4), 1172–1174, 1986, “Chloroplast Import of Light-Harvesting . . . Transit Peptides”\* Abstract 38.
- Karlin-Neuman, G.A., et al., EMBO (Eur. Mol. Biol. Organ) J, 5 (1) 9–14, 1986, “Transit Peptides of Nuclear-Encoded . . . Acid Framework”\* Abstract 39.
- Stiekema, Willem J., et al. Nucleic Acids Res. (1983), 11(22), 8051–61, “Nucleotide Sequence Encoding . . . from *Lemna gibba* L.G-3”\* Abstract 40.

## CHIMERIC GENE FOR THE TRANSFORMATION OF PLANTS

**Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.**

This is a continuation of application Ser. No. 846,211 filed on Mar. 24, 1992, now abandoned.

### BACKGROUND OF THE INVENTION

The present invention relates to novel transit peptide DNA sequences, to novel chimeric genes and to their use in plants for conferring to them an increased tolerance to herbicides in general especially to those of the phosphonomethylglycine family. It also relates to the plant cells transformed by these genes, to the transformed plants regenerated from these cells as well as to the plants derived from crossbreedings using these transformed plants.

Glyphosate, sulfosate or fosametine are broad-spectrum systemic herbicides of the phosphonomethyl-glycine family. They act essentially as competitive inhibitors of 5-(enolpyruvyl)shikimate-3 phosphate synthase (EC 2.5.1.19) or EPSPS in relation to PEP (phosphoenolpyruvate). After their application to the plant, they are translocated inside the plant where they accumulate in the rapidly growing parts, in particular the caulinary and root apices, causing the deterioration and even the destruction of sensitive plants.

Plastidial EPSPS, the main target of these products, is an enzyme of the aromatic amino acid biosynthesis pathway which is encoded by one or more nuclear genes and synthesised in the form of a cytoplasmic precursor and then imported into the plastids where it accumulates in its natural form.

The tolerance of plants to glyphosate and to products of the family is obtained by the stable introduction inside their genome of an EPSPS gene of plant or bacterial origin mutant or nonmutant with respect to the characteristics of the inhibition of the product of these gene by glyphosate. Given the mode of action of glyphosate and the degree of tolerance to glyphosate of the product of the genes used, it is useful to be able to express the product of translation of this gene so as to permit its substantial accumulation in plastids.

It is known, for example from U.S. Pat. No. 4,535,060, to confer to a plant a tolerance to a herbicide of the above-mentioned type, in particular N-(phosphonomethyl)glycine or glyphosate, by introducing into the plant genome a gene encoding an EPSPS carrying at least one mutation making this enzyme more resistant to its competitive inhibitor (glyphosate), after localisation of the enzyme in the plastidial compartment. However, these techniques need to be improved in order to achieve greater reliability in the use of these plants under agronomic conditions.

### SUMMARY OF THE INVENTION

In the present invention, "plant" is understood as meaning any differentiated multicellular organism capable of photosynthesis and "plant cell" any cell derived from a plant and capable of forming undifferentiated tissues such as calluses or differentiated tissues such as embryos or plant sections, plants or seeds.

The subject of the present invention is the production of transformed plants having an increased tolerance to herbicides in general and especially to those of the phosphonomethylglycine family by regenerating cells transformed by means of novel chimeric genes comprising a gene for tolerance to these herbicides. The invention also relates to these novel chimeric genes, to the novel transit peptides which they contain as well as to the plants containing them which are made more tolerant by an accumulation of the mutant enzyme, in its mature form, in the plants.

More particularly, the subject of the invention is a chimeric gene for conferring to plants an increased tolerance to a herbicide whose target is EPSPS, comprising, in the direction of transcription, a promoter region, a transit peptide region, a sequence of a gene encoding a glyphosate tolerance enzyme and an untranslated polyadenylation signal region at the 3' terminus, where the transit peptide region comprises, in the direction of transcription, a transit peptide of a plant gene encoding a plastid-localised enzyme, a partial sequence of the N-terminal mature part of a plant gene encoding a plastid-localised enzyme and then a second transit peptide of a plant gene encoding a plastid-localised enzyme.

The invention also relates to any DNA sequence of the transit peptide region defined above.

The transit peptides which can be used in the transit peptide region may be known per se and may be of plant origin, for example, derived from maize, sunflower, peas, tobacco or the like. The first and the second transit peptides may be identical, analogous or different. They may in addition each comprise one or more transit peptide units. A sequence derived from the SSU of the ribulose 1,5-diphosphate carboxylate oxygenase (RuBisCo) gene is preferably used.

The partial sequence of the N-terminal mature part is derived from a plant gene encoding a plastid-localised enzyme, such as for example a maize, sunflower or pea gene or the like, it being possible for the original plant species to be identical, analogous or different from that from which the first and second transit peptides are derived respectively. Furthermore, the partial sequence of the mature part may comprise a varying number of amino acids, generally from 10 to 40, preferably from 18 to 33. A sequence derived from the SSU of the ribulose 1,5-diphosphate carboxylase oxygenase (RuBisCO) gene is preferably used.

Construction of the entire transit region may be carried out in a manner known per se, in particular by fusion or any other suitable means. The role of this characteristic region is to enable the release of a mature, native protein with a maximum efficiency.

The coding sequence for herbicide tolerance which may be used in the chimeric gene according to the invention encodes a mutant EPSPS having a degree of glyphosate tolerance. This sequence, obtained in particular by mutation of the EPSPS gene, may be of bacterial origin, for example derived from *Salmonella typhimurium* (and called in the text which follows "AroA gene"), or of plant origin, for example from petunia or from tomatoes. This sequence may comprise one or more mutations, for example the Pro 101 to Ser mutation or alternatively the Gly 96 to Ala mutations.

The promoter region of the chimeric gene according to the invention may consist advantageously of at least one promoter on a fragment thereof of a gene which is expressed naturally in plants, that is to say promoters of viral origin such as that of 35S RNA of the cauliflower mosaic virus (CaMV35S) or of plant origin such as the small subunit of the ribulose 1,5-diphosphate carboxylate (RuBisCO) gene of a crop such as maize or sunflower.

The untranslated polyadenylation signal region at the 3' terminus of the chimeric gene according to the invention

may be any origin, for example bacterial, such as the nopaline synthase gene, or of plant origin, such as the small subunit of the maize or sunflower RuBisCO.

The chimeric gene according to the invention may comprise, in addition to the above essential parts, an untranslated intermediate region (linker) between the promoter region and the coding sequence which may be of any origin, bacterial, viral or plant.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### EXAMPLE 1

##### Construction of a Chimeric Gene

The construction of the chimeric gene according to the invention is carried out using the following elements:

1) "Double CaMV" promoter (that is to say part of which has been duplicated): The CaMV35S promoter was isolated by Odell et al (1985). A clone, pJ05-2, containing about 850 bp upstream of the site of initiation of transcription was cut with EcoRI-HindIII, the ends of this isolated fragment were made blunt using Klenow polymerase and the fragment inserted at the HindII site of the vector pUC19 (Yannish-Perron et al., 1985). This promoter was digested with ClaI, the ends filled using Klenow polymerase and then redigested with HindIII. A HindIII-EcoRV fragment, isolated from the same initial promoter, was introduced between these two sites. The promoter thus obtained possesses a double amplification region upstream of the regulatory elements of the CaMV35S promoter. It was introduced in the form of a HindIII-EcoRI fragment into the vector pRPA-BL 150 A alpha 2, described in French Patent Application 88/04130, cut with HindIII and EcoRI.

2) Transfer region: the two transit peptides as well as the mature protein elements used are derived from the cloned cDNA of the small subunit of the gene of maize RuBisCO whose gene has been described by Lebrun et al. (1987), and from the cloned cDNA of the small subunit of the gene of sunflower RuBisCO, isolated by Waksman et al. (1987). More specifically, the transit region, called optimised transit peptide, comprises, in the direction of translation.

a transit peptide of the small subunit of sunflower RuBisCO,

an N-terminal sequence of 22 amino acids of the mature part of the small subunit of maize RuBisCO,

a transit peptide of the small subunit of maize RuBisCO.

The construct using this optimised transfer peptide is called pRPA-BL 410.

Other similar sequences may be used which contain sequences of 10 to 40 and preferably 18 and 33 amino acids respectively.

In order to provide a comparative element, another construction was carried out using a first transit peptide and the same mature sequence part but without a second transit peptide, according to the prior art (pRPA-BL 294).

3) Structural gene: it is derived from the mutant gene at the position (Pro 101 to Ser) of EPSPS of *Salmonella typhimurium* isolated by Stalker et al. (1985). The pMG34-2 clone (provided by Calgene) was linearised with XbaI and then treated with *Vigna radiata* nuclease. After recutting with SmaI, the two blunt ends were ligated. The clone obtained possess an NcoI site in the initiator ATG as well as a 17-bp Sall site downstream of the stop codon. This clone was called pRPA-BL 104.

4) Polyadenylation signal region: the fragment is derived from the nopaline synthase gene of pTi37 (Bevan et al.,

1983). This site is contained in a 260-bp MboI fragment (Fraley et al., 1983; Patent Application PCT 84/02913) which was treated with Klenow polymerase and cloned in the SmaI site of M13 mp 18 in order to introduce the BamHI and EcoRI sites at the 5' and 3' ends respectively.

After cutting with BamHI and treating with *Vigna radiata* nuclease followed by cutting with EcoRI and treating with Klenow polymerase, the resulting fragment was introduced in the vector p-BL 20 (cf. French Patent Application 88/04130), cut by XbaI and BamHI and treated with Klenow polymerase. After recutting with Sall and SstI, a fragment of about 0.4 kbp containing the 3' nos sequence on the side of the Sall site and the right end on the T-DNA side of the SstI site is obtained.

The assembly of the various elements was carried out in the following manner:

"Transit peptide of the SSU of the maize RuBiCO/AroA gene" fusion:

The transit peptide of the SSU of the maize RuBisCO gene is derived from a 192-bp EcoRI-SphI fragment obtained from the cDNA corresponding to the SSU gene of the maize RuBisCO gene, described by Lebrun et al. (1987), possessing an NcoI site spanning the initiation codon for translation and an SphI site corresponding to the cleavage site of the transit peptide.

Translational fusion is obtained between the maize transit peptide and the bacterial EPSPS gene by treating the SphI end with bacteriophage T4 polymerase and by ligating it with the Klenow polymerase-treated NcoI end of the AroA gene from pRPA-BL 104, recut with EcoRI.

Transit peptide of the SSU of maize RuBisCO/sequence of 22 amino acids of the mature part of the SSU of maize RuBisCO/AroA gene fusion.

Similarly, a 228-bp EcoRI-HindIII fragment of the cDNA of the SSU of the maize RuBisCO gene is ligated with the Klenow polymerase-treated NcoI end of the AroA gene from pRPA-BL 104 and recut with EcoRI. A translational fusion is obtained between the transient peptide of the SSU of maize RuBisCO, the 22 amino acids of the mature part of the SSU of maize RuBisCO and the bacterial EPSPS gene.

Transit peptide of the SSU of sunflower RuBisCO:

The fragment is derived from the cDNA isolated by Waksman and Freyssinet (1987). An SphI site was created at the cleavage site of the transit peptide according to the method of Zoller and Smith (1984). The transit peptide of the SSU of sunflower RuBisCO thus obtained is a 171-bp EcoRI-SphI fragment.

Transit peptide of the SSU of sunflower RuBisCO/sequence of 22 amino acids of the mature part of the SSU of maize RuBisCO/AroA gene fusion:

The construct containing the transit peptide of the SSU of maize RuBisCO/sequence of 22 amino acids of the SSU of maize RuBisCO of the mature part of the maize gene fusion was cut with 171-bp EcoRI-SphI corresponding to the transit peptide of the SSU of sunflower RuBisCO. A resulting construct exhibits a substitution of the EcoRI-SphI fragments and is a translational fusion "transit peptide of the SSU of sunflower RuBisCO/sequence of 22 amino acids of the mature part of the SSU of maize RuBisCO/AroA gene".

The EcoRI-Sall fragment was ligated with the Sall-SstI fragment containing the 3' nos sequence and the right end of the T-DNA. The resulting EcoRI-SstI fragment, comprising "transit peptide of the SSU of sunflower RuBisCO/sequence of 22 amino acids of the mature part of the SSU of maize RuBisCO/AroA gene/3' nos/T-DNA right end", is substituted for the EcoRI-SstI fragment containing the right end of the T-DNA of the plasmid 150 A alpha 2 containing the

double CaMV promoter. The transcriptional fusion “double CaMV/transit peptide of the SSU of sunflower RuBisCO/sequence of 22 amino acids of the mature part of the SSU of maize RuBisCO/AroA gene/3' nos” in the vector 150 A alpha 2 was called pRPA-BL 294. “Transit peptide of the SSU of sunflower RuBisCO/sequence of 22 amino acids of the SSU of maize RuBisCO/transit peptide of the SSU of maize RuBisCO/AroA gene” fusion.

The above construct is cut with NcoI-HindIII, releasing the AroA gene. Next it is ligated with a 1.5 kbp NcoI-HindIII fragment containing the “transit peptide of the SSU of maize RuBisCO/AroA gene” fusion. A resulting construct exhibits a substitution of the NcoI-HindIII fragments and is a translational fusion “transit peptide of the SSU of sunflower RuBisCO/sequence of 22 amino acids of the SSU of the RuBisCO of the mature part of the maize gene/transit peptide of the SSU of maize RuBisCO/AroA gene”.

The EcoRI-SstI fragment was ligated with the Sall-SstI fragment containing the 3' nos sequence and the right end of the T-DNA. The resulting EcoRI-SstI fragment comprising “transit peptide of the SSU of sunflower RuBisCO/sequence of 22 amino acids of the SSU of the RuBisCO of the mature part of the maize gene/transit peptide of the SSU of maize RuBisCO/AroA gene/3' nos/T-DNA right end” is substituted for the EcoRI-SstI fragment containing the right end of the T-DNA of the plasmid 150 A alpha 2 containing the double CaMV promoter. The transcriptional fusion “double CaMV/transit peptide of the SSU of sunflower RuBisCO/sequence of 22 amino acids of the SSU of the RuBisCO of the mature part of the maize gene/transit peptide of the SSU of maize RuBisCO/AroA gene/ 3' nos” in the vector 150 A alpha 2 was called pRPA-BL 410.

#### EXAMPLE 2

##### Resistance of the Transformed Plants

###### 1. Transformation:

The vector is introduced into the nononcogenic agrobacterium strain EHA 101 (Hood et al., 1987) carrying the cosmid pTVK 291 (Komari et al., 1986). The transformation method is based on the procedure of Horsh et al. (1985).

###### 2. Regeneration:

The regeneration of the tobacco PBD6 (source SEITA France) using foliar explants is carried out on a Murashige and Skoog (MS) basic medium containing 30 g/l of sucrose and 200 g/ml of kanamycin. The foliar explants are removed from greenhouse- or in vitro-grown plants and transformed according to the foliar disc method (Science 1985, Vol. 227, p. 1229-1231) in three successive stages: the first comprises the induction of shoots on an MS medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (ANA) and 2 mg/l of benzylaminopurine (BAP), for 15 days. The shoots formed during this stage are then developed by culturing on an MS medium supplemented with 30 g/l of sucrose, but not containing hormone, for 10 days. The developed shoots are then removed and they are cultured on an MS planting medium containing half the content of salts, vitamins and sugars and not containing hormone. After about 15 days, the deeply-rooted shoots are placed in soil.

###### 3. Measurement of the glyphosate tolerance:

a) In vitro: the tolerance is measured by weighting the mass of calluses extrapolated to 100 foliar discs of 0.5 cm in diameter, after 30 days of growth on an MS medium supplemented with 30 g/l of sucrose, 0.05 mg/l of naphthaleneacetic acid and 2 mg/l of BAP containing 35 ppm of glyphosate and 200 micrograms/ml of kanamycin. Under

these conditions, it is observed that for the tobacco plants modified by the chimeric gene of pRPA BL 410 according to the invention, the mass of calluses is 34 g whereas for the plants modified by the chimeric gene without a second transit peptide, the mass is only 12 g.

b) In vivo: 30 plants derived from the regeneration of the tobaccos transformed using pRPA-BL 294 and pRPA-BL 410 respectively are transferred to a greenhouse and treated at the 5-leaf stage by spraying with an aqueous suspension at a dose corresponding to 0.6 kg/ha of glyphosate (Round up). After 21 days, a phenotypic examination is carried out of the plants relative to untransformed control plants. Under these conditions, it is observed that the plants transformed using pRPA-BL 410 possess a negligible phytotoxicity whereas the control plants are completely destroyed; moreover, the plants transformed using a chimeric gene, which differs from the preceding one by the absence of a second transit peptide, possess a phytotoxicity of not less than 30% destruction.

These results clearly show the improvement brought by the use of a chimeric gene according to the invention for the same gene encoding the glyphosate tolerance.

The transformed plants according to the invention may be used as parents for producing lines and hybrids having an increased tolerance to glyphosate.

#### EXAMPLE 3

Spring colzas, Westar cultivar, resistant to glyphosate, were obtained using the method of BOULTER et al., 1990 (Plant Science, 70; 91-99), with pRPA-BL 410. These plants were resistant to a greenhouse treatment with glyphosate at 400

g a.s/ha, a treatment which destroys nontransgenic plants.

We claim:

1. A nucleic acid construct which codes for a polypeptide sufficient for localization of a gene product in a chloroplast of a plant cell which polypeptide comprises a fusion which in the direction of translation comprises a first chloroplast transit peptide from a ribulose-1,5-bisphosphate carboxylate small subunit, an N-terminal domain of a mature ribulose-1,5-bisphosphate carboxylate small and subunit protein and a second chloroplast transit peptide from a ribulose-1,5-bisphosphate carboxylate small subunit.

2. The nucleic acid construct of claim 1 wherein said first chloroplast transit peptide and said second chloroplast transit peptide have identical amino acid sequences.

3. The nucleic acid construct of claim 1 wherein said first chloroplast transit peptide and said second chloroplast transit peptides each have different amino acid sequences.

4. The nucleic acid construct of claim 1 wherein said first or second transit peptide is a transit peptide of a maize ribulose-1,5-bisphosphate carboxylate small subunit.

5. The nucleic acid construct of claim 1 wherein said N-terminal domain is an N-terminal domain of a mature maize ribulose-1,5-bisphosphate carboxylate small subunit.

6. The nucleic acid construct of claim 1 wherein said first or second transit peptide and said N-terminal domain are from the same 1,5-bisphosphate carboxylase small subunit protein.

7. The nucleic acid construct of claim 1 wherein said first or second transit peptide is a transit peptide of a sunflower ribulose-1,5-bisphosphate carboxylate small subunit.

8. The nucleic acid construct of claim 1 wherein said N-terminal domain is an N-terminal domain of mature sunflower ribulose-1,5-bisphosphate carboxylase small subunit.

9. The nucleic acid construct [of any] of claim[s] 1, [5, 6 or 8] wherein said N-terminal domain comprises about 10 to about 40 amino acids.

10. The nucleic acid construct [of any] of claim [1,] 5, [6 or 8] wherein said N-terminal domain comprises about 18 to about 33 amino acids.

11. A nucleic acid construct which codes for a polypeptide sufficient for localization of a gene product in a chloroplast of a plant cell which polypeptide comprises a fusion which in the direction of translation comprises a first chloroplast transit peptide from a sunflower ribulose-1,5-bisphosphate carboxylase small subunit, approximately 22 amino acids from the N-terminal region of a mature maize ribulose-1,5-bisphosphate carboxylate small subunit and a second chloroplast transit peptide from a maize ribulose-1,5-bisphosphate carboxylate small subunit.

12. A plant which comprises in its genome a nucleic acid sequence encoding a transit peptide region, wherein the nucleic acid sequence encoding the transit peptide region comprises, in the direction of transcription,

- a) a transit peptide sequence of a ribulose-1,5-bisphosphate carboxylase oxygenase small subunit,
- b) a sequence encoding an N-terminal domain from a mature ribulose-1,5-bisphosphate carboxylase oxygenase small subunit, and
- c) a transit peptide sequence of a ribulose-1,5-bisphosphate carboxylase oxygenase small subunit.

13. A plant as claimed in claim 12, wherein the N-terminal domain comprises 10–40 amino acids.

14. A plant as claimed in claim 13, wherein the N-terminal domain is from maize, sunflower, pea or tobacco.

15. A plant which contains in its genome a nucleic acid sequence encoding a transit peptide region, wherein the nucleic acid sequence encoding the transit peptide region comprises in the direction of transcription,

- a) a transit peptide sequence of sunflower ribulose-1,5-bisphosphate carboxylase oxygenase small subunit,
- b) a sequence encoding an N-terminal domain of a mature maize ribulose-1,5-bisphosphate carboxylase oxygenase small subunit, and
- c) a transit peptide sequence of maize ribulose-1,5-bisphosphate carboxylase oxygenase small subunit.

16. A plant transformation vector comprising nucleic acid sequence encoding a transit peptide region, wherein the nucleic acid sequence encoding the transit peptide region comprises, in the direction of transcription,

- a) a transit peptide sequence of a ribulose-1,5-bisphosphate carboxylase oxygenase small subunit,
- b) a sequence encoding an N-terminal domain from a mature ribulose-1,5-bisphosphate carboxylase oxygenase, and
- c) a transit peptide sequence of a ribulose-1,5-bisphosphate carboxylase oxygenase small subunit.

17. A plant transformation vector as claimed in claim 16, wherein the transit peptide sequence a) is a sunflower transit peptide sequence, the N-terminal domain comprises 10–40 amino acids from a mature ribulose-1,5-bisphosphate carboxylase oxygenase, and the transit peptide sequence c) is a maize transit peptide sequence.

\* \* \* \* \*