PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, 15/52, A01H 5/00, 5/10, C12N 9/02

A1

(11) International Publication Number: **WO 98/33927**

(43) International Publication Date: 6 August 1998 (06.08.98)

(21) International Application Number: PCT/US98/01622

(22) International Filing Date: 29 January 1998 (29.01.98)

(30) Priority Data:
60/036,793 31 January 1997 (31.01.97) US

(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CASPAR, Timothy [US/US]; 2927 Barley Mill Road, Yorklyn, DE 19736 (US). COWAN, Carrie, R. [US/US]; 2411 Carelton Street, Berkeley, CA 94704 (US).

(74) Agent: KING, Karen, K.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GW, HU, ID, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENETICALLY TRANSFORMED PLANTS DEMONSTRATING RESISTANCE TO PORPHYRINOGEN BIOSYNTHESIS–INHIBITING HERBICIDES

(57) Abstract

A bacterial gene (*hemG*) encoding a PROTOX enzyme, resistant to PBI herbicide compounds has been cloned and used to transform plants and seeds. Plant tissue transformed with *hemG* demonstrates a resistance to PBI herbicides and plants grown from transformed seeds possess the PBI herbicide–resistant phenotype.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TITLE

GENETICALLY TRANSFORMED PLANTS DEMONSTRATING RESISTANCE TO PORPHYRINOGEN BIOSYNTHESIS-INHIBITING HERBICIDES

FIELD OF INVENTION

The present invention relates to the field of molecular biology and the genetic transformation of plants with foreign gene fragments. More specifically the invention provides a transformed plant demonstrating resistance to porphyrinogen biosynthesis inhibiting (PBI) herbicides and genes encoding PBI resistant protoporphyrinogen oxidase (PROTOX) enzymes.

BACKGROUND

Rational design of useful herbicides is often dependent on knowledge of plant metabolic enzymatic pathways. One of the most important of these is the plant porphyrin pathway responsible for the synthesis of chlorophyll, heme, and other pigments vital to plant metabolism. To date, thousands of compounds have been developed that exert phytotoxicity through the disruption of the plant porphyrin pathway. These compounds are commonly known as porphyrin biosynthesis inhibiting (PBI) herbicides and represent a significant portion of the herbicide market.

The early steps of porphyrin biosynthesis (Figure 1) occur in plastids, producing the porphyrin intermediate protoporphyrinogen (PROTOGEN). PROTOGEN is converted into protoporphyrin (PROTO) by the enzyme protoporphyrinogen oxidase (PROTOX). PROTOX isozymes are located in both the plastid and the mitochondrion. The PROTOGEN that serves as the substrate for PROTOX in the mitochondrion is transported from the plastid. The vast majority of PBI compounds act as inhibitors of the plastid and mitochondrial PROTOX enzymes (Duke et al., ACS Symp. Ser. (1994), 559(Porphyric Pesticides, 191-204). In the presence of light and molecular oxygen, blockage of the porphyrin pathway at the PROTOX enzyme results in the production of toxic oxygen species including singlet oxygen, superoxide, peroxide and hydroxyl radicals. These toxic species trigger peroxidation of polyunsaturated fatty acid moieties in cell lipid membranes resulting in plant cell death (Komives et al., ACS Symp. Ser. (1994), 559(Porphyric Pesticides), 177-90). This production of toxic oxygen species is believed to be caused by the movement of PROTOGEN to the plasma membrane following blockage of the plastid PROTOX. In the plasma membrane there exists a third form of PROTOX which, unlike the plastid and mitochondrial forms, is resistant to PBI compounds. This PROTOX converts the PROTOGEN into PROTO within the plasma membrane. The plasma membrane lacks enzymes to efficiently further metabolize the PROTO, resulting in its

accumulation. In the presence of light and oxygen, PROTO photooxidizes producing the toxic oxygen intermediates.

Although PBI herbicides are useful, they affect an enzymatic pathway common to all plants and therefore are generally indiscriminate in their action. Consequently, care must be exercised in the application of these compounds around crop plants. Crop plants resistant to PBI herbicide compounds would represent a useful companion to the use of PBI compounds in the field. Because it is the site of action of the majority of the PBI herbicides, the PROTOX enzyme, promises to play an important role in the development of such plants.

PROTOX is a ubiquitous enzyme and has been isolated from plants (Jacobs et al., *Biochem J.* 244, 219, (1987)), yeast (Labbe-Bois et al., (1990) in Biosynthesis of Heme and Chlorophylls, Dailey, H.A. ed., pp 235-285, McGraw-Hill Publishing Co. New York), bacteria (Klemm et al., *J. Bacteriol.*, 169, 5209, (1987)), and mammalian species (Dailey et al., *Biochem.*, 26, 2697, (1987); Proulx et al., *Protein Sci.*, 1, 801, (1992); Siepker et al., *Biochem. Biophys. Acta.*, 913, 349 (1987)). PROTOX deficiencies have also been reported in a variety of species including man (Kappas et al., in The Metabolic Basis Of Inherited Disease, Stanbury et al., ed. (1983) McGraw-Hill, New York, pp 1301-1348) and bacteria (Sasarman et al., *J. Gen. Microbiol.*, 113, 297, (1979)).

Although the enzymatic site and mechanism of action of PBI compounds is known, little progress has been made in developing plants resistant to these herbicides. PBI resistance has been shown in plant tissue culture, but not in whole plants. For example, Pornprom et al. (*Weed Res.*, Japan, 39, 102, (1994) have reported the selection of soybean cell lines resistant to several PBI compounds including oxyfluorfen. Cells were made resistant using suspension cultures grown in increasing concentrations of PBI compound. Cell lines developed from this selection process maintained PBI resistance for six months. No seeds or plants were generated from any cell line.

PBI resistance has also been reported in green algae (Shibata et al., *Res. Photosynth., Proc. Int. Congr. Photosynth.*, 9th (1992), Volume 3, 567-70. Editor(s): Murata, Norio. Publisher: Kluwer, Dordrecht, Netherlands); Sato et al., ACS Symp. Ser. (1994), 559(Porphyric Pesticides), 91-104) where strains of *Chlamydomonas reinhardtii* have been isolated with resistance to certain PBI photo-bleaching herbicides including acifluofenethyl, oxyfluorfen and oxadiazon. These organisms derive their resistance from a PBI-resistant PROTOX produced by a mutant gene.

Finally, WO 9534659 describes the isolation of plant and yeast PROTOX sequences and suggests that the overexpression of those sequences or mutated versions of them in plant tissues will result in resistance to PBI compounds.

WO 9534659 does not teach the transformation of plant tissue with prokaryotic genes that encode a PROTOX resistant to PBI compounds, and they do not demonstrate PBI resistance in any transformed plant.

The *hemG* gene, encoding the wildtype PROTOX gene in *E. coli*, has been isolated and sequenced (Sasarman et al., *J. Gen. Microbiol.*, 113, 297, (1979); Sasarman et al., *Can. J. Microbiol.*, 39, 1155, (1993)). The *E. coli* PROTOX has been found to be resistant to PBI-inhibiting compounds (Jacobs et al., *Arch. Biochem. Biophys.*, 280, 369, (1990).

The problem to be overcome, therefore, is how to modify the PROTOX enzyme to develop plants resistant to PBI herbicides. Applicants have solved this problem by cloning an *E. coli* gene encoding a PBI-resistant PROTOX enzyme into a unique vector and transforming suitable plant tissue with the gene. Transformed plants demonstrate significant PBI resistance. Seed derived from transformed plants give rise to plants carrying the PBI-resistant phenotype, demonstrating the trait is heritable.

The *hemG* PROTOX from *E. coli* differs from the other previously characterized PROTOX enzymes from plant sources not only in its sensitivity to PBI herbicides, but also in its size, sequence and cofactor requirements. These significant differences make the functional expression of the *E. coli* gene in plants less than certain. For example, the *E. coli hemG* gene codes for a protein of 21 kDa (Sasarman et al., *Can. J. Microbiol.*, 39, 1155, (1993)), while the three complete PROTOX genes characterized from plants (two from *Arabidopsis thaliana* [Narita,S., genbank ID g1183006 and WO 9534659] and one from maize [WO 9534659]) encode proteins of 55-59 kDa. Furthermore, homology comparisons using the GCG Bestfit program [Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711] between the *hemG* gene of *E. coli* and one of the *Arabidopsis* genes (genbank ID g1183006) demonstrate only 22% identity over the 181 amino acids of the shorter *hemG* protein. The *E. coli* PROTOX is inactivated by detergent solubilization, whereas the plant enzyme can be detergent-extracted and retain activity (Jacobs et al., *Arch. Biochem. Biophys.*, 229, 312 (1984)). Based on this plus the fact that prokaryotic forms of PROTOX are sensitive to the respiratory inhibitor cyanide whereas plant enzymes are not, it has been suggested that the PROTOX from *E. coli* and other Prokaryotes is obligatorily coupled to the cell's respiratory chain whereas plant enzymes can use molecular oxygen as the terminal electron acceptor (Jacobs et al., *Arch. Biochem. Biophys.*, 211, 305 (1981); (Jacobs et al., *Arch. Biochem. Biophys.*, 229, 312 (1984)). Moreover, the environment in which the *E. coli* PROTOX normally functions in the bacterial cytosol is markedly different than in the plant chlorplast.

Adding to the uncertainty of *hemG* expression in plants are reports of the problematic expression of bacterial proteins in eukaryotic species and plants in particular. For example, the expression of a full-length lepidopteran-specific *Bacillus thuringiensis* toxin has been reported to be unsuccessful in yielding insecticidal levels of expression in some plant species (Vaeck et al., *Nature* (1987) vol. 328, pp. 33-37). Similarly, it has also been reported that the full length gene from *Bacillus thuringiensis kurstaki* HD-73 gave some insecticidal effect in tobacco (Adang et al., *UCLA Symp.Mol.Cell.Biol.*(1987) vol. 48, pp. 345-353). However, the *Bacillus thuringiensis* mRNA detected in these plants was only 1.7 kb compared to the expected 3.7 kb indicating improper expression of the gene.

Finally, if the *E. coli* PROTOX enzyme is expressed in the plant and is able to function in the chloroplast, there is still an uncertainty as to the effect of ectopically expressing a foreign PROTOX enzyme at high, constitutive levels. The plant porphyrin pathway is highly regulated both by allosteric and genetic controls (S. I. Beale and J. D. Weinstein, New Comprehensive Biochemistry (1991) vol. 19, pp. 155-235) suggesting that the proper flux through the pathway is critical to the health of the plant. Altering the activity of PROTOX by introducing the *E. coli* PROTOX gene might disrupt the regulation of the porphyrin pathway and lead to highly deleterious effects on the plant.

Duke et al. (Second International Weed Control Congress, (1996) pp. 775-780, Protoporphyrinogen oxidase inhibitors - their current and future roles) have considered the possibility of engineering resistance to PBI herbicides using a bacterial PROTOX gene. They concluded that this is unlikely to succeed because inhibition of the native plastid PROTOX would still lead to accumulation of PROTO and integration of the bacterial PROTOX into the normal plastid porphyrin biosynthesis will be difficult.

Considering the differences between the bacterial and plant PROTOX genes, the lack of success in the literature in the expression of *Bacillus* genes in plant tissue, and the potential for deleterious effects of expression of a foreign PROTOX enzyme, Applicants' success in the expression of active bacterial PROTOX in plant tissue without deleterious effects is highly unexpected and unusual.

SUMMARY OF THE INVENTION

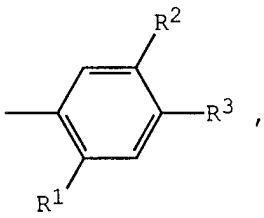
The present invention provides a plant which is resistant to porphyrin biosynthesis-inhibiting herbicides, the plant containing a chimeric gene encoding a herbicide-resistant protoporphyrinogen oxidase activity and wherein the porphyrin biosynthesis-inhibiting herbicides to which the plant is resistant are according to the formula:

J—G

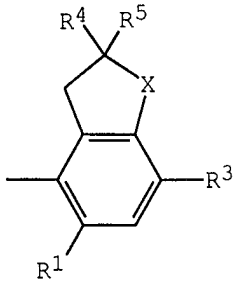
I

wherein

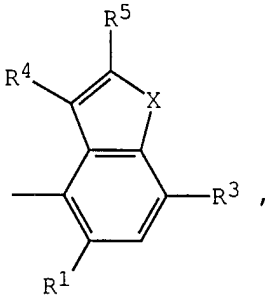
G is



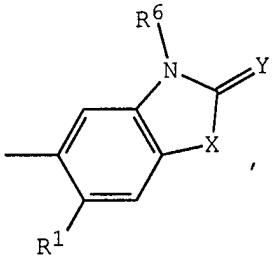
G-1



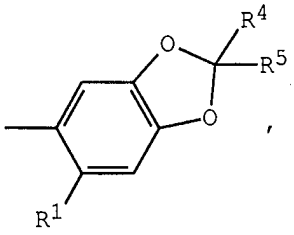
G-2



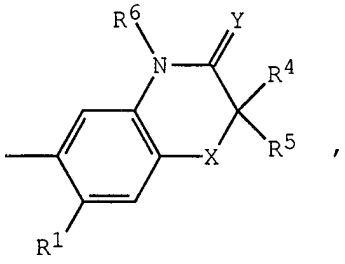
G-3



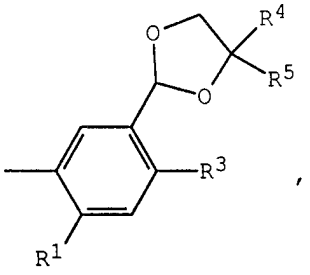
G-4



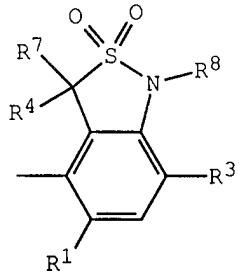
G-5



G-6

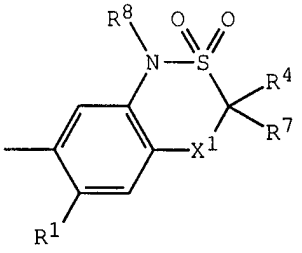


G-7



G-8

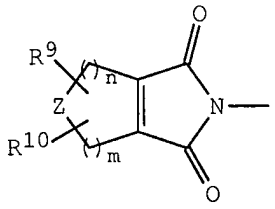
or



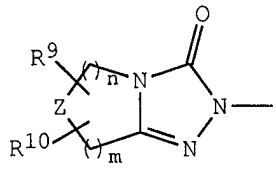
G-9

;

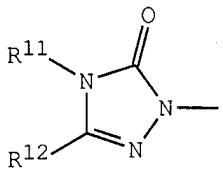
and wherein J is



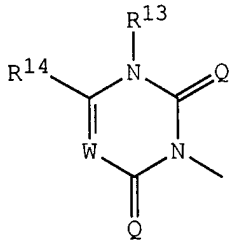
J-1



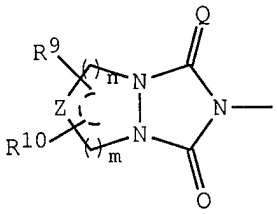
J-2



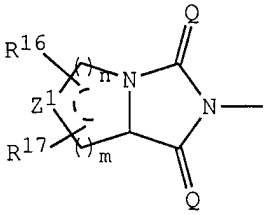
J-3



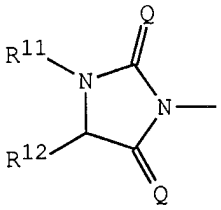
J-4



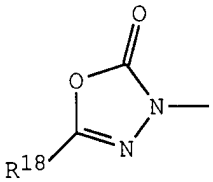
J-5



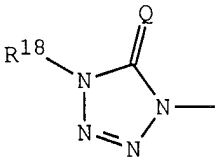
J-6



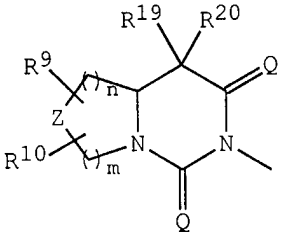
J-7



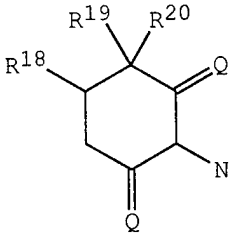
J-8



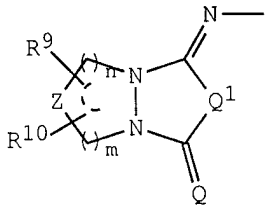
J-9



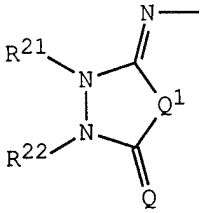
J-10



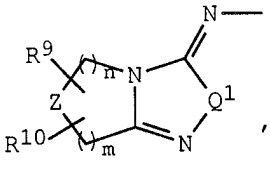
J-11



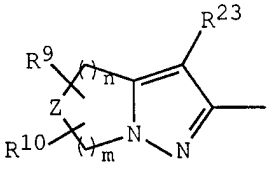
J-12



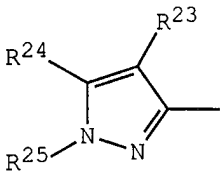
J-13

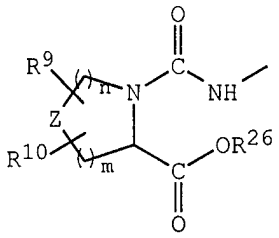


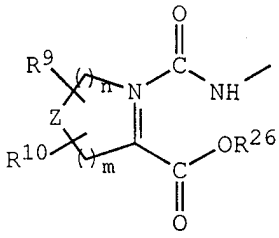
J-14

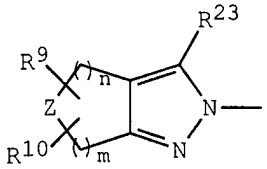


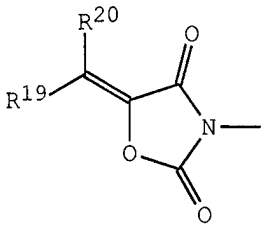
J-15

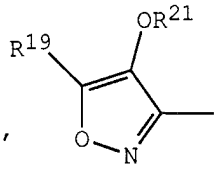

J-16

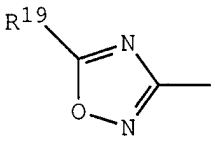

J-17

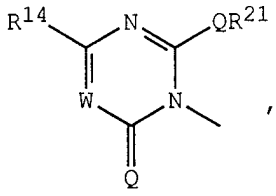

J-18

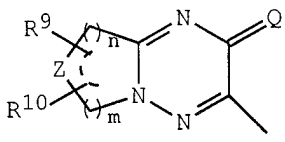

J-19


J-20


J-21


J-22


J-23


J-24

wherein the dashed line in J-5, J-6, J-12 and J-24 indicates that the left-hand ring contains only single bonds or one bond in the ring is a carbon-carbon double bond;

X is O or S;

Y is O or S;

R^1 is hydrogen or halogen;

R^2 is H; C_1-C_8 alkyl; C_1-C_8 haloalkyl; halogen; OH; OR^{27}; SH; $S(O)_pR^{27}$; COR^{27}; CO_2R^{27}; $C(O)SR^{27}$; $C(O)NR^{29}R^{30}$; CHO; CR^{29}=NOR^{36}; CH=$CR^{37}CO_2R^{27}$; $CH_2CHR^{37}CO_2R^{27}$; CO_2N=$CR^{31}R^{32}$; nitro; cyano; $NHSO_2R^{33}$; $NHSO_2NHR^{33}$; $NR^{27}R^{38}$; NH_2; or phenyl optionally substituted with at least one member independently selected from C_1-C_4 alkyl;

p is 0; 1; or 2;

R^3 is C_1-C_2 alkyl; C_1-C_2 haloalkyl; OCH_3; SCH_3; $OCHF_2$; halogen; cyano or nitro;

R^4 is H; C_1-C_3 alkyl; C_1-C_3 haloalkyl; or halogen;

R^5 is H; C_1-C_3 alkyl; halogen; C_1-C_3 haloalkyl; cyclopropyl; vinyl; C_2 alkynyl; cyano; $C(O)R^{38}$; CO_2R^{38}; $C(O)NR^{38}R^{39}$; $CR^{34}R^{35}CN$; $CR^{34}R^{35}C(O)R^{38}$; $CR^{34}R^{35}CO_2R^{38}$; $CR^{34}R^{35}C(O)NR^{38}R^{39}$; $CHR^{34}OH$; $CHR^{34}OC(O)R^{38}$; or $OCHR^{34}OC(O)NR^{38}R^{39}$; or

when G is G-2 or G-6, then R^4 and R^5 can be taken together with the carbon to which they are attached to form C=O;

R^6 is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_2-C_6 alkoxyalkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;

X^1 is a direct bond; O; S; NH; $N(C_1$-C_3 alkyl); $N(C_1$-C_3 haloalkyl); or N(allyl);

R^7 is H; C_1-C_6 alkyl; C_1-C_6 haloalkyl; halogen; $S(O)_2(C_1$-C_6 alkyl); or $C(=O)R^{40}$;

R^8 is H; C_1-C_8 alkyl; C_3-C_8 cycloalkyl; C_3-C_8 alkenyl; C_3-C_8 alkynyl; C_1-C_8 haloalkyl; C_2-C_8 alkoxyalkyl; C_3-C_8 alkoxyalkoxyalkyl; C_3-C_8 haloalkynyl; C_3-C_8 haloalkenyl; C_1-C_8 alkylsulfonyl; C_1-C_8 haloalkylsulfonyl; C_3-C_8 alkoxycarbonylalkyl; $S(O)_2NH(C_1$-C_8 alkyl); $C(O)R^{41}$; or benzyl optionally substituted on the phenyl ring with R^{42};

n and m are each independently 0; 1; 2; or 3; provided that m + n is 2 or 3;

Z is CR^9R^{10}; O; S; S(O); $S(O)_2$; or $N(C_1$-C_4 alkyl);

each R^9 is independently H; C_1-C_3 alkyl; halogen; hydroxy; C_1-C_6 alkoxy; C_1-C_6 haloalkyl; C_1-C_6 haloalkoxy; C_2-C_6 alkylcarbonyloxy; or C_2-C_6 haloalkylcarbonyloxy;

each R^{10} is independently H; C_1-C_3 alkyl; hydroxy; or halogen;

R^{11} and R^{12} are each independently H; halogen; C_1-C_6 alkyl; C_3-C_6 alkenyl; or C_1-C_6 haloalkyl;

R^{13} is H; C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 haloalkenyl; C_3-C_6 alkynyl; C_3-C_6 haloalkynyl; HC(=O); $(C_1$-C_4 alkyl)C(=O); or NH_2;

R^{14} is C_1-C_6 alkyl; C_1-C_6 alkylthio; C_1-C_6 haloalkyl; or $N(CH_3)_2$;

W is N or CR^{15};

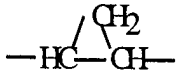
R^{15} is H; C_1-C_6 alkyl; halogen; or phenyl optionally substituted with C_1-C_6 alkyl, 1-2 halogen, C_1-C_6 alkoxy, or CF_3;

each Q is independently O or S;

Q^1 is O or S;

Z^1 is $CR^{16}R^{17}$; O; S; S(O); $S(O)_2$; or $N(C_1$-C_4 alkyl);

each R^{16} is independently H; halogen; hydroxy; C_1-C_6 alkoxy; C_1-C_6 haloalkyl; C_1-C_6 haloalkoxy; C_2-C_6 alkylcarbonyloxy; or C_2-C_6 haloalkylcarbonyloxy;

each R^{17} is independently H; hydroxy; or halogen; or
when R^{16} and R^{17} are bonded to adjacent atoms they can be taken together with the carbons to which they are attached to form  optionally substituted with at least one member selected from 1-2 halogen and 1-2 C_1-C_3 alkyl;

R^{18} is C_1-C_6 alkyl; halogen; or C_1-C_6 haloalkyl;
R^{19} and R^{20} are each independently H; C_1-C_6 alkyl; or C_1-C_6 haloalkyl;
R^{21} and R^{22} are each independently C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 haloalkenyl; C_3-C_6 alkynyl; or C_3-C_6 haloalkynyl;
R^{23} is H; halogen; or cyano;
R^{24} is C_1-C_6 alkylsulfonyl; C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 alkynyl; C_1-C_6 alkoxy; C_1-C_6 haloalkoxy; or halogen;
R^{25} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;
R^{26} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; or phenyl optionally substituted with C_1-C_6 alkyl, 1-2 halogen, 1-2 nitro, C_1-C_6 alkoxy, or CF_3;
R^{27} is C_1-C_8 alkyl; C_3-C_8 cycloalkyl; C_3-C_8 alkenyl; C_3-C_8 alkynyl; C_1-C_8 haloalkyl; C_2-C_8 alkoxyalkyl; C_2-C_8 alkylthioalkyl; C_2-C_8 alkylsulfinylalkyl; C_2-C_8 alkylsulfonylalkyl; C_1-C_8 alkylsulfonyl; phenylsulfonyl optionally substituted on the phenyl ring with at least one substituent selected from the group halogen and C_1-C_4 alkyl; C_4-C_8 alkoxyalkoxyalkyl; C_4-C_8 cycloalkylalkyl; C_6-C_8 cycloalkoxyalkyl; C_4-C_8 alkenyloxyalkyl; C_4-C_8 alkynyloxyalkyl; C_3-C_8 haloalkoxyalkyl; C_4-C_8 haloalkenyloxyalkyl; C_4-C_8 haloalkynyloxyalkyl; C_6-C_8 cycloalkylthioalkyl; C_4-C_8 alkenylthioalkyl; C_4-C_8 alkynylthioalkyl; C_1-C_4 alkyl substituted with phenoxy or benzyloxy, each ring optionally substituted with at least one substituent selected from the group halogen, C_1-C_3 alkyl and C_1-C_3 haloalkyl; C_4-C_8 trialkylsilylalkyl; C_3-C_8 cyanoalkyl; C_3-C_8 halocycloalkyl; C_3-C_8 haloalkenyl; C_5-C_8 alkoxyalkenyl; C_5-C_8 haloalkoxyalkenyl; C_5-C_8 alkylthioalkenyl; C_3-C_8 haloalkynyl; C_5-C_8 alkoxyalkynyl; C_5-C_8 haloalkoxyalkynyl; C_5-C_8 alkylthioalkynyl; C_2-C_8 alkylcarbonyl; benzyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_3 alkyl and C_1-C_3 haloalkyl; $CHR^{34}COR^{28}$; $CHR^{34}CO_2R^{28}$; $CHR^{34}P(O)(OR^{28})_2$; $CHR^{34}P(S)(OR^{28})_2$; $CHR^{34}C(O)NR^{29}R^{30}$; or $CHR^{34}C(O)NH_2$;
R^{28} is C_1-C_6 alkyl; C_2-C_6 alkenyl; C_2-C_6 alkynyl; or tetrahydrofuranyl;

R^{29} and R^{31} are independently hydrogen or C_1-C_4 alkyl;

R^{30} and R^{32} are independently C_1-C_4 alkyl or phenyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_3 alkyl, and C_1-C_3 haloalkyl; or

R^{29} and R^{30} can be taken together to form $-(CH_2)_5-$, $-(CH_2)_4-$ or $-CH_2CH_2OCH_2CH_2-$, each ring thus formed optionally substituted with a substituent selected from the group C_1-C_3 alkyl, phenyl and benzyl; or

R^{31} and R^{32} can be taken together with the carbon to which they are attached to form C_3-C_8 cycloalkyl;

R^{33} is C_1-C_4 alkyl; C_1-C_4 haloalkyl; or C_2-C_6 alkenyl;

R^{34} and R^{35} are independently H or C_1-C_4 alkyl;

R^{36} is H; C_1-C_6 alkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;

R^{37} is H; C_1-C_4 alkyl; or halogen;

R^{38} is H; C_1-C_6 alkyl; C_3-C_6 cycloalkyl; C_3-C_6 alkenyl; C_3-C_6 alkynyl; C_2-C_6 alkoxyalkyl; C_1-C_6 haloalkyl; phenyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_4 alkyl, and C_1-C_4 alkoxy; $-CH_2CO_2(C_1$-C_4 alkyl); or $-CH(CH_3)CO_2(C_1$-C_4 alkyl);

R^{39} is H; C_1-C_2 alkyl; or $C(O)O(C_1$-C_4 alkyl);

R^{40} is H; C_1-C_6 alkyl; C_1-C_6 alkoxy; or $NH(C_1$-C_6 alkyl);

R^{41} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_1-C_6 alkoxy; $NH(C_1$-C_6 alkyl); phenyl optionally substituted with R^{42}; benzyl; or C_2-C_8 dialkylamino; and

R^{42} is C_1-C_6 alkyl; 1-2 halogen; C_1-C_6 alkoxy; or CF_3.

The invention also provides stably transformed plants expressing a chimeric gene where the chimeric gene comprises:

(i) a nucleic acid fragment encoding protoporphyrinogen oxidase enzyme which is resistant to inhibition by porphyrin biosynthesis inhibiting herbicides; and

(ii) a plant regulatory sequence,

wherein the nucleic acid fragment encoding protoporphyrinogen oxidase enzyme is operably linked to the regulatory sequence.

The chimeric gene may optionally further contain various constitutive and inducible plant promoters and plant organelle targeting sequences useful for the expression of the gene and in the translocation of the gene into suitable organelles.

BRIEF DESCRIPTION OF THE FIGURES, BIOLOGICAL DEPOSITS AND SEQUENCE LISTING

Figure 1 is a schematic of the porphyrin biosynthetic pathways in plants.

Figure 2 illustrates leaf damage produced in tobacco transformants treated with 300 μM PBI-1.

Figure 3 illustrates ion leakage from leaves of tobacco transformants incubated in 500 μM PBI-1.

Figure 4 illustrates tobacco leaf spotting damage on transformants in response to varying concentrations of PBI-1.

Figure 5 is a plasmid map of the p35S-PROTOX binary transformation vector which contains a plant nptII selectable marker, right and left T-DNA border fragments, and the *hemG* expression cassette derived from pHGV4.

Figure 6 is a plasmid map of pHGV4 which contains the *hemG* expression cassette including the *hemG* gene under the control of the 35S cauliflower mosaic virus promoter, a *cab* 5' leader, a chloroplast targeting sequence, and a *nos* 3' terminator sequence.

Figure 7 is a plasmid map of pBT455, used in the construction of pHGV4, containing the *dapA* gene under the control of the 35S cauliflower mosaic virus promoter, a *cab* 5' leader,a chloroplast targeting sequence, and a *nos* 3' terminator sequence.

Figure 8 is a plasmid map of pZS199, used in the construction of the binary plasmid p35S-PROTOX, containing a chimeric gene nopaline synthase/neomycin phosphotransferase, the left and right borders of the T-DNA of the Ti plasmid, the *E. coli lacZ* alpha-complementing segment with unique restriction endonuclease sites for EcoRI, KpnI, BamHI, HinDIII, and SalI, the bacterial replication origin from the *Pseudomonas* plasmid pVS1, and the bacterial neomycin phosphotransferase gene from Tn5.

Figure 9 illustrates PBI-1-induced ion leakage damage from leaf disks of tobacco transformants.

Figures 10a-f illustrate leaf spotting assays on primary tobacco transformants with six diverse PBI compounds, including PBI-1, PBI-2, PBI-3, PBI-4, PBI-5 and PBI-6.

Figure 11 illustrates a leaf spotting assay of sensitivity of PROTOX-24 and Binary Control-2 to varying concentrations of PBI-1.

The plasmid pHGV4 containing the *dapA* gene under the control of the 35S cauliflower mosaic virus promoter, a *cab* 5' leader, a chloroplast targeting sequence and a *nos* 3' terminator sequence was deposited on 7 August 1996 with the American Type Culture Collection international depository (12301 Parklawn Drive, Rockville, MD 10852 U.S.A.) under the terms of the Budapest Treaty and is identified by the designation ATCC 97675.

The plasmid p35S-Protox containing a plant nptII selectable marker, right and left T-DNA border fragments, and the *hemG* expression cassette was

deposited on 7 August 1996 with the American Type Culture Collection international depository (12301 Parklawn Drive, Rockville, MD 20852 U.S.A.) under the terms of the Budapest Treaty and is identified by the designation ATCC 97674.

Applicants have provided 7 sequences in conformity with "Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications" (Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992) and with 37 C.F.R. 1.821-1.825 and Appendices A and B ("Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences").

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a chimeric gene encoding a PBI herbicide-resistant PROTOX enzyme. The chimeric gene is linked to a plant chloroplast transit sequence and a constitutive regulatory sequence. Plants transformed with the instant chimerical gene express an active PBI-resistant PROTOX enzyme and are resistant to the toxic effects of PBI herbicides. Seeds produced from transformed plants gave rise to mature plants having the PBI-resistant phenotype.

In the context of this disclosure, the following terms have the meaning set out below.

The term "homologous to" refers to the similarity between the nucleotide sequence of two nucleic acid molecules or between the amino acid sequences of two protein molecules. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art [as described in Hames and Higgins (eds.) Nucleic Acid Hybridisation, IRL Press, Oxford, U.K.]; or by the comparison of sequence similarity between two nucleic acids or proteins.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. "Native" gene refers to the gene as found in nature with its own regulatory sequences.

A "chimeric" gene refers to a gene comprising heterogeneous regulatory and coding sequences.

An "endogenous" gene refers to the native gene normally found in its natural location in the genome.

A "foreign" gene refers to a gene not normally found in the host organism but that is introduced by gene transfer.

A "coding sequence" refers to a DNA sequence that codes for a specific protein and excludes the non-coding sequences.

An "initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides in a coding sequence that specifies initiation and chain termination, respectively, of protein synthesis (mRNA translation).

An "open reading frame" refers to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence.

"Suitable regulatory sequences" refer to nucleotide sequences located upstream (5'), within, and/or downstream (3') to a coding sequence, which control the transcription and/or expression of the coding sequences, potentially in conjunction with the protein biosynthetic apparatus of the cell. These regulatory sequences include promoters, translation leader sequences, transcription termination sequences, and polyadenylation sequences.

An "enhancer" is a DNA sequence which can stimulate promoter activity. It may be an innate element of the promoter or a heterologous element inserted to enhance the level and/or tissue-specificity of a promoter.

The term "promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions. It may also contain enhancer elements.

"Constitutive promoters" refers to those that direct gene expression in all tissues and at all times. "Organ-specific" or "development-specific" promoters as referred to herein are those that direct gene expression almost exclusively in specific organs, such as leaves or seeds, or at specific development stages in an organ, such as in early or late embryogenesis, respectively.

"Inducible promoters" are promoters induced to activity by specific triggers such as light or particular chemical compounds. Examples include light-inducible promoters, ABA inducible promoters, benzenesulfonamide-inducible promoters, and methyl jasmonate-inducible promoters.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structural gene when it is capable of affecting the expression of that structural gene.

The term "expression", as used herein, is intended to mean the production of the protein product encoded by a gene. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms.

The "3' non-coding sequences" refers to the DNA sequence portion of a gene that contains a polyadenylation signal and any other regulatory signal capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

The "translation leader sequence" refers to that DNA sequence portion of a gene between the promoter and coding sequence that is transcribed into RNA and is present in the fully processed mRNA upstream (5') of the translation start codon. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

A "chloroplast targeting signal" or "chloroplast targeting sequence" is an amino acid sequence which is translated in conjunction with a protein and specifically directs the protein to the chloroplast.

A "mitochondrial targeting signal" or "mitochondrial targeting sequence" is an amino acid sequence which is translated in conjunction with a protein and specifically directs the protein to the mitochondria.

"Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast targeting signal.

"Transformation" herein refers to the transfer of a foreign gene into the genome of a host organism and its genetically stable inheritance. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation and particle-accelerated or "gene gun" transformation technology as described in U.S. Patent No. 5,204,253.

The term "transformants" refer to plants which have been through the transformation process and contain a foreign gene integrated into their genome.

The term "primary transformants" or the "T1 generation" are of the same genetic generation as the tissue which was initially transformed, i.e., not having gone through meiosis and fertilization since the transformation.

The term "secondary transformants" or the "T2, T3, T4, etc. generations" are derived from primary transformants through one or more meiotic and fertilization cycles. They may be derived by self-fertilizations of primary or secondary transformants or crosses of primary or secondary transformants with other transformed or untransformed plants.

The term "tolerance" means the heritable ability of a plant to sustain less damage than other individuals of a given species in the presence of an injurious concentration of a toxin or pathogen. "Resistance" refers to a special case of tolerance in which there is a heritable ability to survive (with agronomically acceptable injury) a concentration of toxin or pathogen that is normally lethal or severely injurious to individuals of given species.

The term "*hemG*" will refer to a bacterial gene encoding a PBI herbicide resistant protoporphyrinogen oxidase enzyme.

The term "PROTOX" will refer to protoporphyrinogen oxidase, an enzyme responsible for the conversion of protoporphyrinogen to protoporphyrin.

The terms "PROTOGEN" or "PROTOGEN IX" will refer to compound protoporphyrinogen IX, an intermediate in the porphyrin biosynthetic pathway and the substrate for PROTOX.

The terms "PROTO" or "PROTO IX" will refer to compound protoporphyrin IX, an intermediate in the porphyrin biosynthetic pathway and the product of PROTOX.

The term "PBI herbicide" or "PBI compounds" will refer to herbicides that inhibit the plant porphyrin biosynthetic pathway at the level of PROTOX. Typical PBI compounds fall into six general classes of compounds consisting of the triazolones , cyclic imides (e.g. N-(4-chloro-5(cyclopropenyloxy) -2-fluorophenyl)tetrahydro-2-phthalimide), thiadiazoles , pyrazoles , uracils and diphenylethers (e.g., acifluorfen, nitrofen and oxyfluorfen).

Isolation of genes encoding PBI resistant PROTOX enzymes

Both the *E. coli hemG* (Sasarman et al., *supra*) and the *Bacillus hemY* (Dailey et al., *J. Biol. Chem.* (1994), 269(2), 813-15) genes encode PBI-resistant PROTOX enzymes. Recently mutations in eukaryotic forms of the PROTOX genes (e.g., *Arabidopsis*, maize, and yeast, WO 9534659) have been isolated. It is contemplated that any of these genes will be suitable in the present invention and may be isolated from native sources by methods well known in the art.

The sequence of the *hemG* gene, encoding the PBI resistant PROTOX from *E. coli* is known. The gene may be obtained by a variety of methods, the most direct being by the use of polymerase chain reaction (PCR) using suitable primers. In the present application genomic DNA was amplified using standard PCR protocols [Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)], and isolated and purified by gel electrophoresis. Purified amplification product was then restricted with HaeIII and KpnI to allow for insertion into the appropriate transformation vector.

Construction of chimeric genes for the expression of *hemG* in plants

The expression of foreign genes in plants is well-established [De Blaere et al. (1987) *Meth. Enzymol.* 143:277-291]. Proper level of expression of the *hemG* mRNAs may require the use of different chimeric genes utilizing different promoters. Such chimeric genes can be transferred into host plants either together in a single expression vector or sequentially using more than one vector.

A preferred class of heterologous hosts for the expression of the coding sequence of the *hemG* gene are eukaryotic hosts, particularly the cells of higher plants. Particularly preferred among the higher plants and the seeds derived from them are soybean, rapeseed *(Brassica napus, B. campestris)*, sunflower (*Helianthus annus*), cotton (*Gossypium hirsutum*), corn (*Zea mays*), *tobacco (Nicotiana tabacum)*, alfalfa (*Medicago sativa*), *wheat* (*Triticum sp*), barley (*Hordeum vulgare*), oats (*Avena sativa, L*), sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), *Arabidopsis,* cruciferous vegetables (broccoli, cauliflower, cabbage, parsnips, etc.), melons, carrots, celery, parsley, tomatoes, potatoes, strawberries, peanuts, grapes, grass seed crops, hardwood trees, softwood trees, and forage grasses. Expression in plants will use regulatory sequences functional in such plants.

The origin of the promoter chosen to drive the expression of the coding sequence is not critical as long as it has sufficient transcriptional activity to accomplish the invention by expressing translatable mRNA for the *hemG* gene in the desired host tissue. Preferred promoters for expression in all plant organs, and especially for expression in leaves include those directing the 19S and 35S transcripts in Cauliflower mosaic virus [Odell et al.(1985) *Nature* 313:810-812; Hull et al. (1987) *Virology* 86:482-493], small subunit of ribulose 1,5-bisphosphate carboxylase [Morelli et al.(1985) *Nature* 315:200; Broglie et al. (1984) *Science* 224:838; Hererra-Estrella et al.(1984) *Nature* 310:115; Coruzzi et al.(1984) *EMBO J.* 3:1671; Faciotti et al.(1985) *Bio/Technology* 3:241], and chlorophyll a/b binding protein [Lampa et al.(1986) *Nature* 316:750-752], ferredoxin promoter [Caspar and Quail (1993) Plant J. 3:161], actin promoters [Park et al., *J. Plant Biol.* (1995), 38(4), 365-71], ubiquitin promoters [Garbarino, et al., *Plant Mol. Biol. (1994), 24(1), 119-27*], and opine promoters [von Lintig et al., *J. Bacteriol.* (1994), 176(2), 495-503].

Depending upon the application, it may be desirable to select promoters that are specific for expression in one or more organs of the plant. Examples include the light-inducible promoters of the small subunit of ribulose 1,5-bisphosphate carboxylase if the expression is desired in photosynthetic organs, or promoters active specifically in roots {e.g., subdomains of the CaMV 35S promoter [Benfey et al. (1990) EMBO J. 9:1677]}. Other inducible promoters that may prove useful include those sensitive to various chemical agents such as those induced to an activity by benzenesulfonamides [WO 9513389; U.S. Patent No. 5364780], abscisic acid [Devic et al., *Plant J.* (1996), 9(2), 205-15],and methyl jasmonate [Xu et al., *Plant Mol. Biol.* (1993), 22(4), 573-88].

It is envisioned that the introduction of enhancers or enhancer-like elements into other promoter constructs will also provide increased levels of

primary transcription for the *hemG* gene to accomplish the invention. These would include viral enhancers such as that found in the 35S promoter [Odell et al. (1988) *Plant Mol. Biol.* 10:263-272], enhancers from the opine genes [Fromm et al. (1989) *Plant Cell* 1:977-984], or enhancers from any other source that result in increased transcription when placed into a promoter operably linked to the nucleic acid fragment of the invention.

Any 3' non-coding region capable of providing a polyadenylation signal and other regulatory sequences that may be required for the proper expression of the *hemG* coding regions can be used to accomplish the invention. This would include the 3' end from viral genes such as the 3' end of the 35S or the 19S cauliflower mosaic virus transcripts, the 3' end from the opine synthesis genes, the 3' ends of ribulose 1,5-bisphosphate carboxylase or chlorophyll a/b binding protein, or 3' end sequences from any source such that the sequence employed provides the necessary regulatory information within its nucleic acid sequence to result in the proper expression of the promoter/*hemG* coding region combination to which it is operably linked. There are numerous examples in the art that teach the usefulness of different 3' non-coding regions [for example, see Ingelbrecht et al. (1989) *Plant Cell* 1:671-680].

DNA sequences coding for intracellular localization sequences may be added to the *hemG* coding sequence if required for the proper expression of the proteins to accomplish the invention. One of the plant PROTOX isozymes is localized in the chloroplasts and therefore must be synthesized with a chloroplast targeting signal. Bacterial proteins such as the *E. coli* PROTOX enzyme have no such signal. A chloroplast transit sequence could, therefore, be fused to the *hemG* coding sequences. Preferred chloroplast transit sequences are those of the small subunit of ribulose 1,5-bisphosphate carboxylase, e.g. from soybean [Berry-Lowe et al. (1982) *J. Mol. Appl. Genet.* 1:483-498] for use in dicotyledonous plants and from corn [Lebrun et al. (1987) *Nucleic Acids Res.* 15:4360] for use in monocotyledonous plants.

It is contemplated that the *hemG* gene may be integrated in the chloroplast genome. Methods of incorporating foreign DNA into plant plastid genomes are known [Golds et al., *Bio/Technology* (1993), 11(1), 95-7]. Transformation of the plastid genome requires a method for the translocation of the foreign DNA across the plastid double membrane and subsequent integration of the DNA into the plastid genome. Suitable methods for the introduction of the foreign DNA into the plastid include biolistic bombardment, treatment of the plant tissue with polyethylene glycol and *Agrobacterium* vector transfection. Suitable vectors for plastid genome transformation have been developed [Svab et al., *Proc. Natl. Acad. Sci. U. S. A.* (1993), 90(3), 913-17]. Vectors will typically include the foreign

DNA to be incorporated into the plastid genome flanked 5' by a suitable plastid promoter (often ribosomal RNA operon promoters) and 3' by other regulatory sequences.

In similar fashion the *hemG* gene product may be tageted to the mitochondrion by fusing the *hemG* gene to a mitochondrial targeting sequence such as that found in the F1-ATPase .beta. subunit of *Nicotiana plumbaginifolia*[Chaumont et al., *Plant Molecular Biology* 24:631-641 (1994)]. Alternatively, the *hemG* construct could be integrated into and expressed from the mitochondrial genome.

Transformation of the mitochondrial genome requires a method for the translocation of the foreign DNA across the mitochondrial membrane, and integration of the DNA into the mitochondrial genome. Suitable methods for the introduction of the foreign DNA into the mitochondrial genome include biolistic bombardment, treatment of the plant tissue with polyethylene glycol and *Agrobacterium* vector transfection. Vectors will typically include the foreign DNA to be incorporated into the mitochondrial genome flanked 5' by a suitable mitochondrial promoter and 3' by other regulatory sequences that are effective for the expression of the desired foreign gene.

Expression of *hemG* Chimeric Genes in Plants

Various methods of introducing a DNA sequence (i.e., of transforming) into eukaryotic cells of higher plants are available to those skilled in the art (see EPO publications 0 295 959 A2 and 0 138 341 A1). Such methods include those based on transformation vectors based on the Ti and Ri plasmids of *Agrobacterium spp.* It is particularly preferred to use the binary type of these vectors. Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton, rape, tobacco, and rice [Pacciotti et al. (1985) *Bio/Technology* 3:241; Byrne et al. (1987) *Plant Cell, Tissue and Organ Culture* 8:3; Sukhapinda et al. (1987) *Plant Mol. Biol.* 8:209-216; Lorz et al. (1985) *Mol. Gen. Genet.* 199:178; Potrykus (1985) *Mol. Gen. Genet.* 199:183; Park et al., *J. Plant Biol.* (1995), 38(4), 365-71; Hiei et al., *Plant J.* (1994), 6:271-282].

For introduction into plants, the chimeric genes of the invention can be inserted into binary vectors as described in Example 3.

Other transformation methods are available to those skilled in the art, such as direct uptake of foreign DNA constructs [see EPO publication 0 295 959 A2], techniques of electroporation [see Fromm et al. (1986) *Nature* (London) 319:791] or high-velocity ballistic bombardment with metal particles coated with the nucleic acid constructs [see Kline et al. (1987) *Nature* (London) 327:70, and see

U.S. Patent No. 4,945,050]. Once transformed, the cells can be regenerated by those skilled in the art.

Of particular relevance are the recently described methods to transform foreign genes into commercially important crops, such as rapeseed [see De Block et al. (1989) *Plant Physiol.* 91:694-701], sunflower [Everett et al. (1987) *Bio/Technology* 5:1201], soybean [McCabe et al. (1988) *Bio/Technology* 6:923; Hinchee et al. (1988) *Bio/Technology* 6:915; Chee et al. (1989) *Plant Physiol.* 91:1212-1218; Christou et al. (1989) *Proc. Natl. Acad. Sci USA* 86:7500-7504; EPO Publication 0 301 749 A2], rice [Hiei et al., *Plant J.* (1994), 6:271-282], and corn [Gordon-Kamm et al. (1990) *Plant Cell* 2:603-618; Fromm et al. (1990) *Biotechnology* 8:833-839].

Assay methods

To assay for expression of the chimeric genes in leaves or seeds of the transformed plants, PROTOX enzyme can be extracted, detected and quantitated enzymatically and/or immunologically or visually by methods known to those skilled in the art. In this way lines producing high levels of expressed protein can be easily identified. Levels of active PROTOX in plant tissue may be measured in a variety of ways as described for example in Wang et al., (*Biosci., Biotechnol., Biochem.* (1993), 57(12), 2205-6). Preferred is the method of Camadro, J-M. et al., [(1993), *Fluorometric assay of protoporphyrinogen oxidase in chloroplasts and in plant, yeast, and mammalian mitochondria.* In, Target Assays for Modern Herbicides and Related Phytotoxic Compounds. P. Boger and G. Sandmann, eds., Lewis Publishers, Boca Raton, FL, pp 29-34] more fully described in the GENERAL METHODS.

Methods for determining the resistance of plants to various herbicides are common and well known in the art. Typical methods include leaf disk assays and ion leakage assays such as are described by; Koch et al., [Bull. *Environ. Contam. Toxicol.* (1995), 54(4), 606-13]; and Whitlow et al., [*Plant Physiol.* (1992), 98(1), 198-205]. Ion leakage and leaf spotting assays are more fully described in the GENERAL METHODS.

Porphyrin Biosynthesis-Inhibiting compounds

Porphyrin Biosynthesis-Inhibiting (PBI) compounds are common and have been commercially available since the 1960's. Many common PBI compounds fall into six general classes of compounds consisting of the triazolones , cyclic imides (e.g. N-(4-chloro-5-(cyclopropenyloxy) -2-fluorophenyl)tetrahydro-2-phthalimide), thiadiazoles , pyrazoles , uracils and diphenylethers (e.g., acifluorfen, nitrofen and oxyfluorfen). Methods for synthesis of PBI compounds are common and well known in the art (see, for example, DE 3905916 for the preparation of cyclic imides, U.S. Patent No. 5,446,197 for the preparation of

dipheylethers and FR 2222378 for the preparation of oxadiazoles, JP 05097848 for the preparation of flumioxazin, EP 698604 for the preparation of fluthiacetmethy, and U.S. Patent No. 5,176,735 for the preparation of uracils such as flupropacil).

The instant PBI resistant plants are expected to be tolerant to a wide variety of PBI compounds including but not limited to 1H-Isoindole-1,3(2H)-dione, 2-[4-chloro-5-(cyclopentyloxy)-2- fluorophenyl]-4,5,6,7-tetrahydro- {}; Benzoic acid, 2-chloro-5-[2-chloro-4-(trifluoromethyl)phenoxy]-, 2-ethoxy-1-methyl-2-oxoethyl ester {*HC 252*}; 2H-1,4-Benzoxazin-3(4H)-one, 6-[(6,7-dihydro-6,6-dimethyl-3H,5H-pyrrolo[2,1-c][1,2,4]thiadiazol-3-ylidene)amino]-7-fluoro-4-(2-propynyl){*SN 124085*}; Benzoic acid, 2-chloro-5-[3,6-dihydro-3-methyl-2,6-dioxo-4- (trifluoromethyl)-1(2H)-pyrimidinyl]-, 1-methylethyl ester {*UCC-C 4243*}; Acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H,3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1 ylidene)amino]phenyl]thio]-, methyl ester {*KIH 9201*}; 2,4-Oxazolidinedione, 3-[4-chloro-5-(cyclopentyloxy)-2-fluorophenyl]- 5-(1-methylethylidene){*BW 4*}; 2,4-Oxazolidinedione, 3-[4-chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl]-5-(1-methylethylidene) {*BW 3*}; Acetic acid, [[[1-[5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrophenyl]-2-methoxyethylidene]amino]oxy]-, methyl ester {*AKH 7088*}; 1H-Isoindole-1,3(2H)-dione, 2-[7-fluoro-3,4-dihydro-3-oxo-4-(2-propynyl)-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-{*Flumioxazin; S 53482*}; Acetic acid, [2-chloro-4-fluoro-5-(1,3,4,5,6,7-hexahydro-1,3-dioxo- {*S 23031*};1H-Isoindole-1,3(2H)-dione, 2-[4-chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl]-4,5,6,7-tetrahydro-{*Flumipropyn*}; Furan, 3-[5-[2-chloro-4-(trifluoromethyl)phenoxy]-2 nitrophenoxy]tetrahydro-2H-isoindol-2-yl)phenoxy]-, pentyl ester {*Furyloxyfen*}; Benzoic acid, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitro-,2-ethoxy-2-oxoethyl ester {*RH 0265*}; Benzoic acid, 5-[2-chloro-4-(trifluoro-methyl)phenoxy]-2-nitro-,2-ethoxy-1-methyl-2-oxoethyl ester {*PPG 844*}; Benzenamine, 2-chloro-6-nitro-3-phenoxy-{ CME 127}; Benzamide, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-N-(methylsulfonyl)-2-nitro- {*Fomesafen*}; Methanone, (2,4-dichlorophenyl)[1,3-dimethyl-5-[[(4-methylphenyl)sulfonyl]-oxy]-1H-pyrazol-4-yl]- {*H 468T*}; Benzoic acid, 5-[2-chloro-4-(trifluoromethyl)-phenoxy]-2-nitro- {*Acifluorfen*};Benzene, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)- {*Oxyfluorfen; RH 2915* };Benzene, 2-chloro-1-(4-nitro-phenoxy)-4-(trifluoromethyl)- {*RH 2512*}; Benzoic acid, 5-(2,4-dichloro-phenoxy)-2-nitro-, methyl ester {*MC 4379*}; 1,3,4-Oxadiazol-2(3H)-one, 3-[2,4-dichloro-5-(2-propynyloxy)phenyl]-5-(1,1-dimethylethyl)-; Benzene, 2,4-dichloro-1-(3-methoxy-4-nitrophenoxy)-{*Pl 3468*}; 1,3,4-Oxadiazol-2(3H)-one, 3-[2,4-dichloro-5-(1-methylethoxy)phenyl]-5-(1,1-dimethylethyl)-{*RP 17623*};

Benzene, 2-nitro-1-(4-nitrophenoxy)-4-(trifluoromethyl)-{*C 6989*}; Benzene, 1,5-dichloro-3-fluoro-2-(4-nitrophenoxy)-{*Fluoronitrofen*}; Benzene, 1,3,5-trichloro-2-(4-nitrophenoxy)-{*CNP 1032*}; Benzoic acid, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitro- carboxymethyl ester {*Fluoroglycofen*}; and Benzene, 2,4-dichloro-1-(4-nitrophenoxy)-{*FW 925*}.

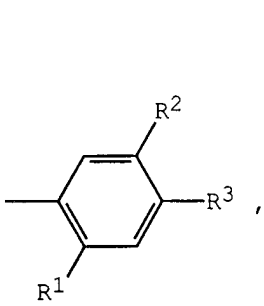
Preferred within the context of the present invention are compounds that correspond to the formula:

J—G

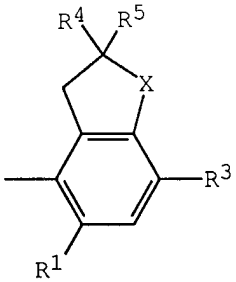
I

wherein

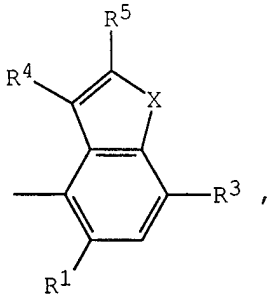
G is



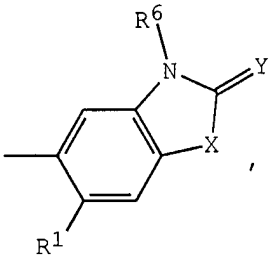
G-1 ,



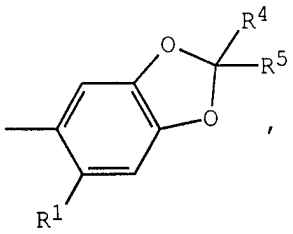
G-2 ,



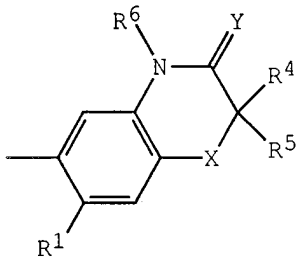
G-3 ,



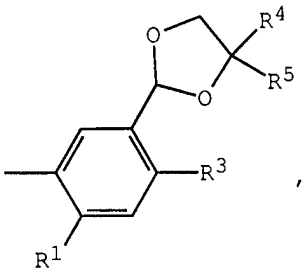
G-4 ,



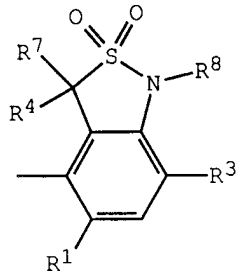
G-5 ,



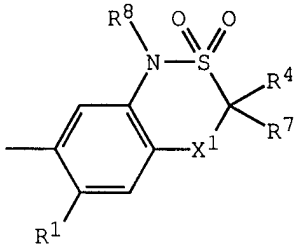
G-6 ,



G-7 ,

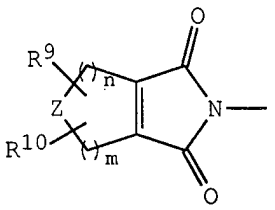


G-8 or

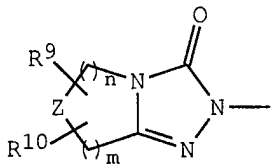


G-9 ;

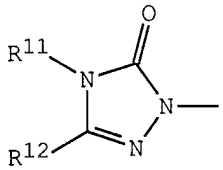
and wherein J is



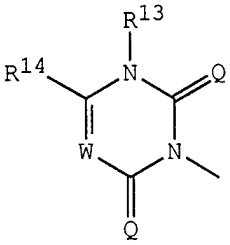
J-1



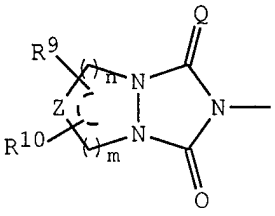
J-2



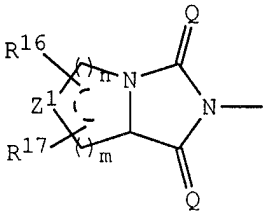
J-3



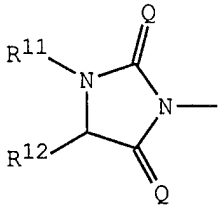
J-4



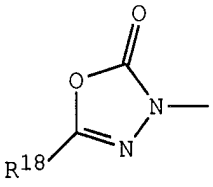
J-5



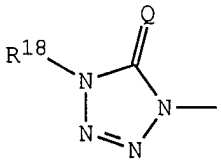
J-6



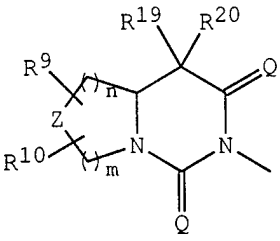
J-7



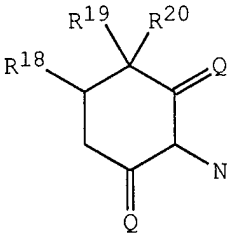
J-8



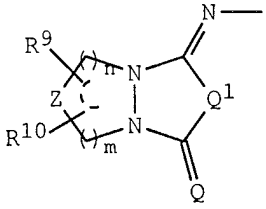
J-9



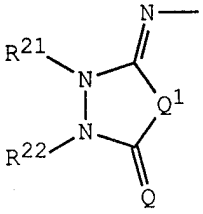
J-10



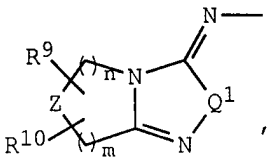
J-11



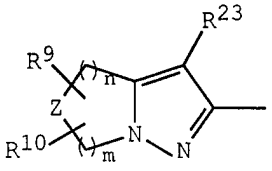
J-12



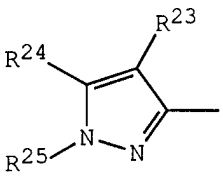
J-13



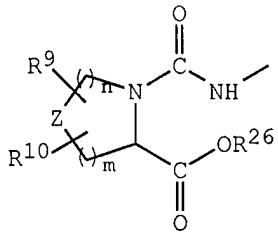
J-14



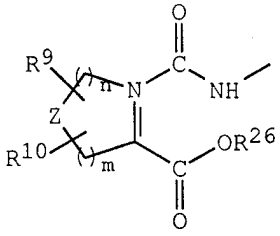
J-15



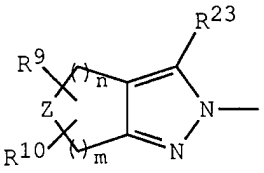
J-16



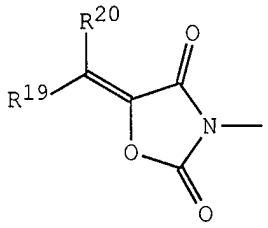
J-17



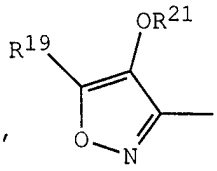
J-18



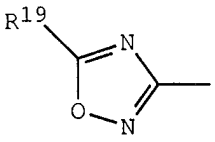
J-19



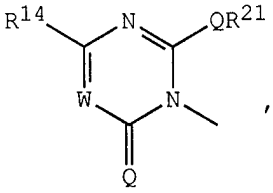
J-20



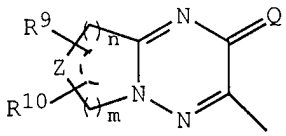
J-21



J-22



J-23



J-24

wherein the dashed line in J-5, J-6, J-12 and J-24 indicates that the left-hand ring contains only single bonds or one bond in the ring is a carbon-carbon double bond;

X is O or S;

Y is O or S;

R^1 is hydrogen or halogen;

R^2 is H; C_1-C_8 alkyl; C_1-C_8 haloalkyl; halogen; OH; OR^{27}; SH; $S(O)_pR^{27}$; COR^{27}; CO_2R^{27}; $C(O)SR^{27}$; $C(O)NR^{29}R^{30}$; CHO; CR^{29}=NOR^{36}; CH=$CR^{37}CO_2R^{27}$; $CH_2CHR^{37}CO_2R^{27}$; CO_2N=$CR^{31}R^{32}$; nitro; cyano; $NHSO_2R^{33}$; $NHSO_2NHR^{33}$; $NR^{27}R^{38}$; NH_2; or phenyl optionally substituted with at least one member independently selected from C_1-C_4 alkyl;

p is 0; 1; or 2;

R^3 is C_1-C_2 alkyl; C_1-C_2 haloalkyl; OCH_3; SCH_3; $OCHF_2$; halogen; cyano or nitro;

R^4 is H; C_1-C_3 alkyl; C_1-C_3 haloalkyl; or halogen;

R^5 is H; C_1-C_3 alkyl; halogen; C_1-C_3 haloalkyl; cyclopropyl; vinyl; C_2 alkynyl; cyano; $C(O)R^{38}$; CO_2R^{38}; $C(O)NR^{38}R^{39}$; $CR^{34}R^{35}CN$; $CR^{34}R^{35}C(O)R^{38}$; $CR^{34}R^{35}CO_2R^{38}$; $CR^{34}R^{35}C(O)NR^{38}R^{39}$; $CHR^{34}OH$; $CHR^{34}OC(O)R^{38}$; or $OCHR^{34}OC(O)NR^{38}R^{39}$; or

when G is G-2 or G-6, then R^4 and R^5 can be taken together with the carbon to which they are attached to form C=O;

R^6 is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_2-C_6 alkoxyalkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;

X^1 is a direct bond; O; S; NH; N(C_1-C_3 alkyl); N(C_1-C_3 haloalkyl); or N(allyl);

R^7 is H; C_1-C_6 alkyl; C_1-C_6 haloalkyl; halogen; $S(O)_2$(C_1-C_6 alkyl); or $C(=O)R^{40}$;

R^8 is H; C_1-C_8 alkyl; C_3-C_8 cycloalkyl; C_3-C_8 alkenyl; C_3-C_8 alkynyl; C_1-C_8 haloalkyl; C_2-C_8 alkoxyalkyl; C_3-C_8 alkoxyalkoxyalkyl; C_3-C_8 haloalkynyl; C_3-C_8 haloalkenyl; C_1-C_8 alkylsulfonyl; C_1-C_8 haloalkylsulfonyl; C_3-C_8 alkoxycarbonylalkyl; $S(O)_2NH$(C_1-C_8 alkyl); $C(O)R^{41}$; or benzyl optionally substituted on the phenyl ring with R^{42};

n and m are each independently 0; 1; 2; or 3; provided that m + n is 2 or 3;

Z is CR^9R^{10}; O; S; S(O); $S(O)_2$; or N(C_1-C_4 alkyl);

each R^9 is independently H; C_1-C_3 alkyl; halogen; hydroxy; C_1-C_6 alkoxy; C_1-C_6 haloalkyl; C_1-C_6 haloalkoxy; C_2-C_6 alkylcarbonyloxy; or C_2-C_6 haloalkylcarbonyloxy;

each R^{10} is independently H; C_1-C_3 alkyl; hydroxy; or halogen;

R^{11} and R^{12} are each independently H; halogen; C_1-C_6 alkyl; C_3-C_6 alkenyl; or C_1-C_6 haloalkyl;

R^{13} is H; C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 haloalkenyl; C_3-C_6 alkynyl; C_3-C_6 haloalkynyl; HC(=O); (C_1-C_4 alkyl)C(=O); or NH_2;

R^{14} is C_1-C_6 alkyl; C_1-C_6 alkylthio; C_1-C_6 haloalkyl; or $N(CH_3)_2$;

W is N or CR^{15};

R^{15} is H; C_1-C_6 alkyl; halogen; or phenyl optionally substituted with C_1-C_6 alkyl, 1-2 halogen, C_1-C_6 alkoxy, or CF_3;

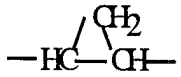
each Q is independently O or S;

Q^1 is O or S;

Z^1 is $CR^{16}R^{17}$; O; S; S(O); $S(O)_2$; or N(C_1-C_4 alkyl);

each R^{16} is independently H; halogen; hydroxy; C_1-C_6 alkoxy; C_1-C_6 haloalkyl; C_1-C_6 haloalkoxy; C_2-C_6 alkylcarbonyloxy; or C_2-C_6 haloalkylcarbonyloxy;

each R^{17} is independently H; hydroxy; or halogen; or
when R^{16} and R^{17} are bonded to adjacent atoms they can be taken together

with the carbons to which they are attached to form 

optionally substituted with at least one member selected from 1-2 halogen and 1-2 C_1-C_3 alkyl;

R^{18} is C_1-C_6 alkyl; halogen; or C_1-C_6 haloalkyl;
R^{19} and R^{20} are each independently H; C_1-C_6 alkyl; or C_1-C_6 haloalkyl;
R^{21} and R^{22} are each independently C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 haloalkenyl; C_3-C_6 alkynyl; or C_3-C_6 haloalkynyl;
R^{23} is H; halogen; or cyano;
R^{24} is C_1-C_6 alkylsulfonyl; C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 alkynyl; C_1-C_6 alkoxy; C_1-C_6 haloalkoxy; or halogen;
R^{25} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;
R^{26} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; or phenyl optionally substituted with C_1-C_6 alkyl, 1-2 halogen, 1-2 nitro, C_1-C_6 alkoxy, or CF_3;
R^{27} is C_1-C_8 alkyl; C_3-C_8 cycloalkyl; C_3-C_8 alkenyl; C_3-C_8 alkynyl; C_1-C_8 haloalkyl; C_2-C_8 alkoxyalkyl; C_2-C_8 alkylthioalkyl; C_2-C_8 alkylsulfinylalkyl; C_2-C_8 alkylsulfonylalkyl; C_1-C_8 alkylsulfonyl; phenylsulfonyl optionally substituted on the phenyl ring with at least one substituent selected from the group halogen and C_1-C_4 alkyl; C_4-C_8 alkoxyalkoxyalkyl; C_4-C_8 cycloalkylalkyl; C_6-C_8 cycloalkoxyalkyl; C_4-C_8 alkenyloxyalkyl; C_4-C_8 alkynyloxyalkyl; C_3-C_8 haloalkoxyalkyl; C_4-C_8 haloalkenyloxyalkyl; C_4-C_8 haloalkynyloxyalkyl; C_6-C_8 cycloalkylthioalkyl; C_4-C_8 alkenylthioalkyl; C_4-C_8 alkynylthioalkyl; C_1-C_4 alkyl substituted with phenoxy or benzyloxy, each ring optionally substituted with at least one substituent selected from the group halogen, C_1-C_3 alkyl and C_1-C_3 haloalkyl; C_4-C_8 trialkylsilylalkyl; C_3-C_8 cyanoalkyl; C_3-C_8 halocycloalkyl; C_3-C_8 haloalkenyl; C_5-C_8 alkoxyalkenyl; C_5-C_8 haloalkoxyalkenyl; C_5-C_8 alkylthioalkenyl; C_3-C_8 haloalkynyl; C_5-C_8 alkoxyalkynyl; C_5-C_8 haloalkoxyalkynyl; C_5-C_8 alkylthioalkynyl; C_2-C_8 alkylcarbonyl; benzyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_3 alkyl and C_1-C_3 haloalkyl; $CHR^{34}COR^{28}$; $CHR^{34}CO_2R^{28}$; $CHR^{34}P(O)(OR^{28})_2$; $CHR^{34}P(S)(OR^{28})_2$; $CHR^{34}C(O)NR^{29}R^{30}$; or $CHR^{34}C(O)NH_2$;
R^{28} is C_1-C_6 alkyl; C_2-C_6 alkenyl; C_2-C_6 alkynyl; or tetrahydrofuranyl;

R^{29} and R^{31} are independently hydrogen or C_1-C_4 alkyl;

R^{30} and R^{32} are independently C_1-C_4 alkyl or phenyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_3 alkyl, and C_1-C_3 haloalkyl; or

R^{29} and R^{30} can be taken together to form $-(CH_2)_5-$, $-(CH_2)_4-$ or $-CH_2CH_2OCH_2CH_2-$, each ring thus formed optionally substituted with a substituent selected from the group C_1-C_3 alkyl, phenyl and benzyl; or

R^{31} and R^{32} can be taken together with the carbon to which they are attached to form C_3-C_8 cycloalkyl;

R^{33} is C_1-C_4 alkyl; C_1-C_4 haloalkyl; or C_2-C_6 alkenyl;

R^{34} and R^{35} are independently H or C_1-C_4 alkyl;

R^{36} is H; C_1-C_6 alkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;

R^{37} is H; C_1-C_4 alkyl; or halogen;

R^{38} is H; C_1-C_6 alkyl; C_3-C_6 cycloalkyl; C_3-C_6 alkenyl; C_3-C_6 alkynyl; C_2-C_6 alkoxyalkyl; C_1-C_6 haloalkyl; phenyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_4 alkyl, and C_1-C_4 alkoxy; $-CH_2CO_2(C_1$-C_4 alkyl); or $-CH(CH_3)CO_2(C_1$-C_4 alkyl);

R^{39} is H; C_1-C_2 alkyl; or $C(O)O(C_1$-C_4 alkyl);

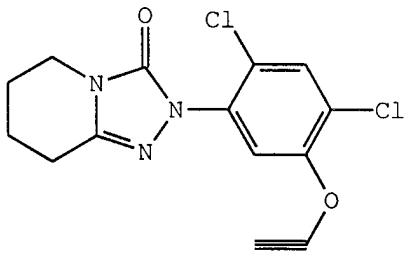
R^{40} is H; C_1-C_6 alkyl; C_1-C_6 alkoxy; or $NH(C_1$-C_6 alkyl);

R^{41} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_1-C_6 alkoxy; $NH(C_1$-C_6 alkyl); phenyl optionally substituted with R^{42}; benzyl; or C_2-C_8 dialkylamino; and

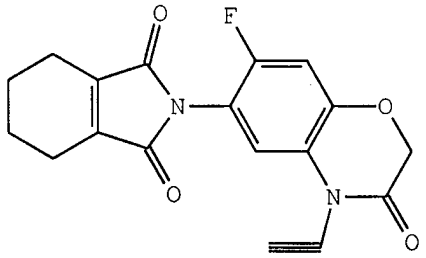
R^{42} is C_1-C_6 alkyl; 1-2 halogen; C_1-C_6 alkoxy; or CF_3.

Within the context of the present invention the compounds six PBI compounds are preferred as described below:

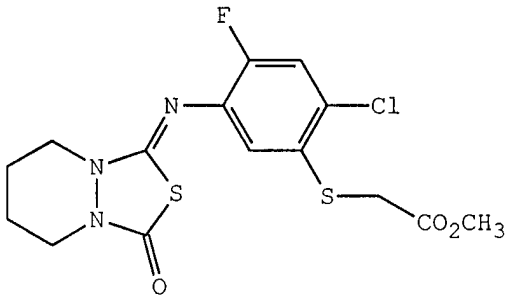
PBI-1 is described by the formula:



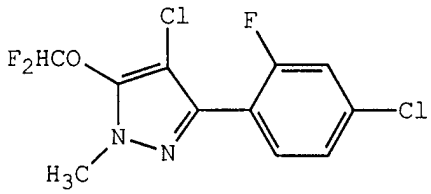
PBI-2 is described by the formula:



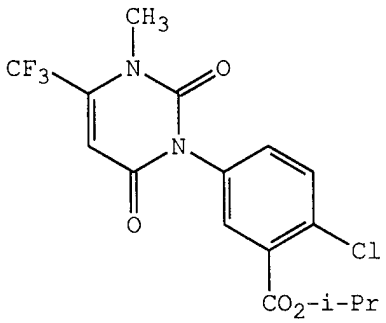
PBI-3 is described by the formula:



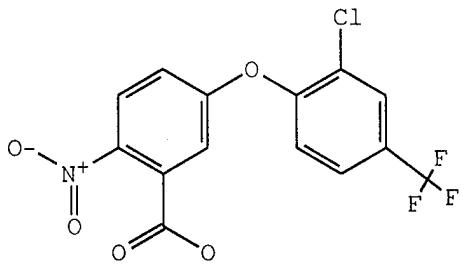
PBI-4 is described by the formula:



PBI-5 is described by the formula:



PBI-6 is described by the formula:



Expression of the *hemG* gene and demonstration of PBI compound resistance in transformed plants

Plant tissue from soybean and tobacco were transformed either by high-velocity biolistic bombardment with metal particles coated with the nucleic acid constructs containing the *hemG* gene or using an *Agrobacterium tumefaciens* containing a binary plasmid. Callus and mature plants were regenerated from the transformed cells and assayed for production of active *E. coli* PROTOX enzyme and resistance to PBI compounds.

Embryonic suspension cultures of soybean tissue, biolistically transformed with the plasmid pHGV4 and expressing the *E. coli hemG* gene were analyzed. Extracts from soybean cultures expressing *hemG* and untransformed cultures were assayed for PROTOX levels in the presence and absence of 3 μM PBI-1. Direct comparisons of the activity from the *hemG* and untransformed cultures in the absence of PBI-1 was somewhat compromised by the fact that transformed soybean tissue was somewhat sick. This is frequently the case with transformed soybean tissue grown under these conditions, irrespective of the identity of the foreign DNA. However, there was sufficient PROTOX activity in both the *hemG* transformed and untransformed cultures to determine their sensitivity to PBI-1. Control cultures showed no PROTOX activity after exposure to 3 μM PBI-1 whereas cultures expressing the *hemG* gene retained 67% of normal PROTOX levels (Table 1, Example 2), indicating that the *hemG* construct expressed a functional PROTOX in plant cells.

Tobacco transformants containing the binary p35S-protox vector demonstrated clear resistance of about 30-fold over controls to the compound PBI-1 at levels up to 1000 μM in leaf spotting assays (Figures 2, 4, and 11). Similar results were seen in ion leakage assays where leaf disks from transformants which were exposed to between 0 and 1000 μM PBI-1 showed about a 100-fold increase in resistance over controls (Figures 3 and 9).

Tobacco transformants were also tested for PBI resistance against a broad range of PBI herbicide compounds including PBI-1, PBI-2, PBI-3, PBI-4, PBI-5, and PBI-6, all defined above. As shown in Figure 10 and Table 2, Example 5

tobacco transformants demonstrated some resistance in leaf spotting assays to all compounds tested with levels of resistance as compared to controls ranging from 3-fold to 300-fold depending on the compound tested.

These data clearly indicate that the expression of the bacterial *hemG* gene is able to confer resistance, both *in vivo* and *in vitro* to a broad range of PBI herbicides.

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLES

Restriction enzyme digestions, phosphorylations, ligations and transformations were done as described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989). Restriction enzymes were obtained from New England Biolabs (Boston, MA), GIBCO/BRL (Gaithersburg, MD), or Promega (Madison, WI). Taq polymerase was obtained from Perkin Elmer (Branchburg, NJ). Growth media was obtained from GIBCO/BRL (Gaithersburg, MD).

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), and "d" means day(s).

GENERAL METHODS

Growing Seeds and Seedlings:

Seedling Growth on Petri plates:

Unless otherwise noted, all seeds grown in Petri plates were sterilized in 50% bleach with 0.1% Tween-20 for 7-10 min and then washed in sterile water 3-5 times before plating. Seeds were then placed onto sterile media containing 1/2 strength Murashige-Skoog salts (Gibco #11117-066) plus 0.7% agar and 1% sucrose. Kanamycin was prepared as a 50 mg/mL stock in water, sterilized by passage through a 0.2 μm filter, and added to the media after it had been autoclaved and cooled to 60°C. Plates containing kanamycin were stored at 4°C and used within one month of preparation. For testing transformants for antibiotic sensitivity, 20-100 seeds per 57 cm^2 Petri plate were used. Plates were incubated in a growth chamber at 23°C with illumination from fluorescent tubes of about 60 μmol/m2/sec photosynthetically active radiation and a 14 h photoperiod.

Growth in soil:

Plants were grown in commercial soil mixes (metro mix or others) in growth chambers at 20-25°C with fluorescent and incandescent illumination of 100-300 μmol/m2/sec photosynthetically active radiation and a photoperiod ranging from 12 h to continuous illumination or in greenhouses at 23-28°C on a natural daylength supplemented with artificial lighting.

Herbicide Stocks:

All porphyrin biosynthesis-inhibiting compounds were obtained from the DuPont chemical library and included representatives of the three major classes of PBI compounds. Compounds used in the following examples included PBI compounds PBI-1 through PBI-6 as described above.

Compounds were dissolved at 10 mg active ingredient/mL in DMSO except for PBI-6 which was dissolved at 2.5 mg active ingredient/mL in 25% DMSO, 75% water, 0.19% X-77®, a mixture of nonionic surfactants (alkylarylpolyoxyethylene glycols), free fatty acids, isopropanol, and water was obtained from Loveland (Loveland Industries Inc., Greeley, CO 80632-289). All herbicide stocks were used immediately or stored at -20°C until use.

Protoporphyrinogen Oxidase Assay:

Protoporphyrinogen oxidase (PROTOX) was assayed fluorometrically essentially as described in the literature with slight modifications [Camadro et al., (1993), *Fluorometric assay of protoporphyrinogen oxidase in chloroplasts and in plant, yeast, and mammalian mitochondria.* In, Target Assays for Modern Herbicides and Related Phytotoxic Compounds. P. Boger and G. Sandmann, eds., Lewis Publishers, Boca Raton, FL, pp 29-34.]

Protoporphyrinogen (PROTOGEN) was obtained by chemical reduction of protoporphyrin (PROTO) (Porphyrin Products, Logan, UT) with 3 percent Na amalgam [Jacobs et al., *Enzyme* 28, 206, (1982)]. The amalgam was prepared by melting Na spheres (Aldrich Chemical Company, Milwaukee, WI) under a nitrogen stream using a heat gun and then adding Hg (triple distilled, J.T. Baker, Inc., Phillipsburg, NJ). PROTO was dissolved in 10 mM KOH, 20 percent ethanol, with the balance water, to make a 1 mM solution. The PROTO stock was diluted 1:1 with 10 mM KOH, 10 mL placed into a 100 mL Pyrex test tube, 150 μL of a 1:100 (v/v) dilution of antifoam A emulsion (Sigma Chemical Company, St. Louis, MO) added, and the solution saturated with argon. Laboratory lights were dimmed and freshly crushed amalgam (approximately 18 grams) was added to the tube which was then vortexed under argon for 4 min. Residual amalgam and particles of unreduced PROTO were removed by passing the solution through a 0.2 μm syringe filter. The filtered solution was sparged with argon, DTT was added (0.0154 grams dissolved in 1 mL of 1 M MOPS), and

the PROTOGEN solution adjusted to approximately pH 8 with 1 M MOPS saturated with nitrogen gas. Aliquots were placed in 1.5 mL amber vials (Wheaton, Millville, NJ), overlaid with mineral oil (J.T. Baker, Phillipsburg, NJ), closed with septa-containing caps, and stored at -80°C until use. A portion of the PROTOGEN was oxidized to PROTO with rat mitochondria to determine the concentration of PROTOGEN.

PROTOX activity was determined in an assay mixture consisting of buffer A (100 mM HEPES pH 7.5, 1 mM EGTA, 5 mM EDTA, 2 mM DTT, 10 percent glycerol, 0.03 percent Tween-80), 250 μL of tissue extract, 3.8 μM PROTOGEN and 1 percent DMSO with or without 3 μM PBI-1 in a total volume of 1 mL. (DMSO was used to dissolve the PBI-1.) The assay was started by addition of PROTOGEN. The oxidation of PROTOGEN to PROTO was followed with a Millipore CytoFluor 2300 multiwell fluorescence plate reader using an excitation filter of 395 nm with a 25 nm bandwidth and emission filter of 620 nm with a 40 nm bandwidth with no temperature control. Assays were conducted in Corning 24 well plates, PBI-1 was added at least 1 min before the assay was started, and the change in fluorescence was monitored for 5 min. The rate of non-enzymatic oxidation of PROTOGEN to PROTO was determined using extracts that were heat killed by boiling for 5 min. The assay volume with the heat-killed enzyme was a total of 500 μL with only 125 μL of extract, due to insufficient plant material for a 1 mL assay. Other assay components were halved so that the final concentration of substrate, etc., remained the same.

Leaf Spotting Tests:

Responses to herbicide damage were assessed by measuring the damage produced by spotting the compounds onto attached leaves of plants grown in soil in a greenhouse. Test compounds were dissolved in DMSO and then diluted into 0.25% X-77 to the appropriate concentration. The DMSO concentrations of the samples spotted onto the leaves was always 3% or below. Preliminary experiments (not shown) demonstrated that 10% DMSO in 0.25% X-77 (with no herbicide) had no affect on tobacco in this assay. Test compounds were applied to leaves in rows of 5 to 10, 1 μL drops for each concentration. Four to 7 rows were placed on each leaf. Damage to the area of the leaf surrounding the drop was scored visually on a 0 (no injury) to 10 (complete death of region surrounding the drop) following spotting. For a given concentration of herbicide, the rate of production of damaged tissue and the final degree of damage depended on the age of the plant and environmental conditions. All experiments were conducted using control plants which lacked the *E. coli* PROTOX gene (*hemG*), but were treated in exactly the same manner as the PROTOX transformants.

Ion leakage assay:

Responses to herbicide damage were also assessed by measuring electrolyte leakage from tobacco leaf discs treated with herbicides. Leaf discs (5 mm) were cut from expanded leaves and washed twice in deionized water for 1-2 h. Leaf discs were placed into 20 mL of deionized water and transferred to the dark. Herbicides were dissolved in DMSO and added to the indicated concentration. [Controls with no herbicide contained the same concentration of DMSO (3.4%) as found in the highest tested herbicide concentration and this amount of DMSO did not affect ion leakage (results not shown).] Plates were incubated in the dark at 25°C for 12-18 h. The plates were then placed in the light (220 μmol/m2/s photosynthetically active radiation) at 25°C. The conductivity of the bathing solution was measured just before the plates were illuminated (time zero) and at intervals afterward using a Cole-Parmer Conductivity meter (model 1481-60). Conductivity results are presented as the increase in conductivity over that at time zero. Preliminary experiments (not presented) indicated that ion leakage caused by PBI treatment in the light was not observed in discs which were kept in the dark. In addition, PBI-induced ion leakage in the light was inhibited by treatment with either 1 mM gabaculine or 1 mM dioxoheptanoic acid which inhibit the early steps of porphyrin synthesis. The results from these preliminary experiments confirm that the ion leakage caused by the PBI treatment is indeed due to inhibition of PROTOX.

EXAMPLE 1

CONSTRUCTION OF A GENE EXPRESSION CASSETTE FOR THE EXPRESSION OF *E. COLI hemG* IN PLANT TISSUE

Construction of parent vector

A unique gene expression cassette was used for construction of chimeric genes for expression of the *E. coli hemG* gene in plants. This cassette was developed for the expression of *E. coli dapA* in plant chloroplasts (WO 9515392). To create vectors for expressing *E. coli* PROTOX, the *E. coli hemG* gene was ligated into the vector in place of the *dapA*.

The *dapA* leaf expression cassette is inserted into the vector pGEM9Z giving pBT455 (Figure 7). The cassette is composed of the 35S promoter of cauliflower mosaic virus [Odell et al. (1985) *Nature* 313:810812; Hull et al. (1987) *Virology* 86:482493], the translation leader from the chlorophyll binding protein (*cab*) gene, [Dunsmuir (1985) *Nucleic Acids Res.* 13:2503-2518], the chloroplast transit sequence (cts) of the small subunit of ribulose 1,5-bisphosphate carboxylase from soybean [Berry-Lowe et al. (1982) *J. Mol. Appl.* Genet. 1:483-498], the *E. coli dapA* coding sequence, and 3' transcription termination region from the nopaline synthase (*nos*) gene [Depicker et al. (1982) *J. Mol. Appl.*

Genet. 1:561-570]. The entire cassette is flanked by SalI sites; there is also a BamHI site upstream of the cassette.

Isolation of the *E. coli hemG* gene and construction of a *hemG* expression vector

The *hemG* gene of *E. coli* has been cloned and sequenced (Sasarman et al., *J. Gen. Microbiol.*, 113, 297, (1979); Sasarman et al., *Can. J. Microbiol.*, 39, 1155, (1993), and was amplified from genomic *E. coli* (strain BAR1091 [Rasmussen et al. (1985) *J. Bact.* 164:665-673]) DNA using PCR with primers SEQ ID NOS:1, 2. These primers were designed to amplify the entire open reading frame of the *hemG* gene plus they contain additional sequences at the 5' ends which add restrictions endonuclease sites (HaeIII/StuI for SEQ ID NO:1 and KpnI for SEQ ID NO:2 at the sites indicated by the ^ symbol).

5' CTGCAGG^CCTCGGTGAAAACATTAATTC 3' (SEQ ID NO:1)
5' GACGTGGTAC^CATTATTTCAGCGTCGG 3' (SEQ ID NO:2)

Amplification was accomplished using the UlTma® DNA polymerase (Perkin-Elmer, Branchburg, NJ) using the conditions suggested by the supplier. UlTma® DNA polymerase was used for its lower error rate relative to Taq® Polymerase. In order to further reduce the chance of PCR-induced mutations occurring in the final *hemG* vector, four amplifications were done in parallel and the products from these reactions were kept separate (but treated identically) throughout all of the following steps. Ultimately, unique *hemG* vectors from three of these four PCR products were identified and characterized. The amplified 570 bp *hemG* fragment was gel-purified and then digested with HaeIII and KpnI. The resultant 552 bp fragment was gel-purified and used for subsequent ligations.

The transformation vector pBT455 (see above) was modified by replacing the *dapA* gene with the PCR amplified *hemG* product. The *dapA* gene was excised from the pBT455 vector using NruI and KpnI in order to create compatible ends for ligation with the *hemG* fragment. NruI cuts 22 bp downstream of the junction between the *rbcS* chloroplast targetting sequence and the beginning of the *dapA* coding sequence. KpnI cuts at the junction between the end of the *dapA* coding sequence and the *nos* 3' terminator. The vector was also digested with BstEII (which cuts inside the *dapA* sequence) to reduce the number of pBT455 parent vectors recovered due to incomplete digestion at the NruI and KpnI sites or religation.

The HaeIII/KpnI digested *hemG* fragment was ligated with the NruI/KpnI/BstEII digested pBT455. (Both HaeIII and NruI produce blunt ends which were ligated together.) The ligation produces an in-frame fusion of the *rbcS* chloroplast targetting signal, the *dapA* coding sequence, and the *hemG*

coding sequence. The NruI site is 22 bp downstream from the *dapA* start codon and, therefore seven amino acids from the *dapA* gene are attached to the N-terminus of the *hemG* protein encoded by this construct. Two additional amino acids, derived from the linker region of primer SEQ ID NO:1, are also in the chimeric *hemG* protein encoded by this construct between the *dapA* and the *hemG* sequences.

E. coli DH5α competent cells (Gibco-BRL) were transformed via heat shock treatment with the pBT455/*hemG* ligation mixture. Transformed cells containing the desired *hemG* construct were differentiated from the pBT455 parent vector by PCR analysis using SEQ ID NOS:3 and 4 and restriction digests. SEQ ID NOS:3 and 4 are shown below.

5'-CATGGTCACGGGAAG-3' (SEQ ID NO:3)
5'-TCAGAAACTTGCGCG-3' (SEQ ID NO:4)

The PCR primers were designed to anneal upstream of the 5' ligation site in both the parent and the ligated vector (SEQ ID NO:3) and in the middle of the *hemG* gene (SEQ ID NO:4).

PCR products were analyzed by gel electrophoresis and confirmed by restriction with EcoRI. Clones giving correct PCR products were confirmed by restriction digests of plasmid DNA. Three clones (pHGV2, pHGV3, and pHGV4), one each from three of the four original, independent PCR reactions, were determined to have the correct restriction digests. Sequence analysis of these clones confirmed their identity and indicated that each contains one or more mutations induced by the PCR within the *hemG* sequence. pHGV4 was chosen for further use. The DNA sequence of the translated part of chimeric hemG protein, including the chloroplast targeting sequence, dapA residues, linker region and hemG coding sequence is shown in SEQ ID NO:6. Within the hemG region, pHGV4 contains a conservative mutation of the threonine at residue 67 of the chimeric *hemG* protein to serine. It also contains a deletion of 1 base pair within the original stop codon which results in the addition 3 amino acids (tyrosine - glycine - threonine) at the carboxy terminus of the protein.

EXAMPLE 2

Transformation of Soybean and Expression of *E. coli hemG* conferring resistance to PBI-1

Soybean was transformed by biolistic bombardment using the pHGV4 plasmid described in Example 1. Embryogenic cultures of soybean were used as the recipient tissue for bombardment by DNA-coated particles. These cultures were initiated according to methods described by Finer and Nagasawa [Finer et

al., (1988) Development of an embryogenic suspension culture of soybean (*Glycine max Merrill*) *Plant Cell Tissue Organ Cult.* 15:125-136] by placing immature zygotic cotyledons about 2 to 4 mm in length on agarose-solidified MS medium containing 2,4-dichlorophenoxyacetic acid. After about 6 weeks of incubation in the light (16 h daylength, 30 μEinsteins) at 28°C, masses of globular stage somatic embryos form on the surface of the cotyledons. These somatic embryos are excised and transferred to liquid medium containing modified MS medium as described by Finer et al. (see above) with 10 μg/mL 2,4-dichloro-phenoxyacetic acid. The tissue proliferates in this medium and is subcultured by transferring green, globular stage somatic embryos to fresh medium every two weeks. This tissue can be used for transformation using modifications of published procedures [Parrott et al., (1994), Recovery and evaluation of soybean (*Glycine max [L.] Merr.*) plants transgenic for a *Bacillus thuringiensis var. kurstaki* insecticidal gene, *In Vitro Cell. Dev. Biol.* 30P:144-149; Finer et al., (1991), Transformation of soybean via particle bombardment of embryogenic suspension culture tissue *In Vitro Cell. Dev. Biol.* 27P:175-182.]

Gold particles (1 μm in diameter) (Bio-Rad Labs, Hercules, CA)were coated with DNA using the following technique. pHGV4 plasmid DNA was co-precipitated with a plasmid containing a hygromycin phosphotransferase gene (HPTII) for use in selecting transformed cells. The HPTII protein inactivates the antibiotic hygromyicn and acts as a selectable marker for plant transformation (Waldron et al., (1985), Resistance to hygromycin B: a new marker for plant transformation studies, *Plant Mol. Biol.* 18:189-200). Any of a number of plasmids containing a hygromycin phosphotransferase gene suitably engineered for high level expression in soybean tissue cultured cells can be used as the selective plasmid. The actual selective plasmid used was pML151, which harbors the hygromycin phosphotransferase gene [Gritz et al., (1983) *Gene* 25:179-188] under the control of the 35S promoter [Odell (1985) *Nature* 313:810812] and the *nos* 3' end (Depicker et al., (1982), *J. Mol. Appl. Genet.*, 1:561-574). pML151 was made by deleting the ampicillin resistance gene from the plasmid pSP72 (Promega Biotech, Madison, WI) and inserting the HPTII cassette described above. Plasmid DNA (1 μg of pML151 and 9 μg of the pHGV4 plasmid) were added to 50 μL of a suspension of gold particles (60 mg/mL). Calcium chloride (50 μL of a 2.5 M solution) and spermidine free base (20 μL of a 1.0 M solution) were added to the particles. The suspension was vortexed during the addition of these solutions. After 10 min, the tubes were briefly centrifuged and the supernatant removed. The particles were then rinsed with 200 μL of 100% ethanol, the ethanol rinse was performed again and the particles resuspended in a final volume of 30 μL of

ethanol. An aliquot (5 μL) of the DNA-coated particles was placed in the center of a Kapton™ flying disc (Bio-Rad Labs, Hercules, CA).

For bombardment, about 0.5 g of suspension culture (covering an area of about 3 cm^2) was placed in the center of a petri dish. The tissue was bombarded 3 times with gold particles 1-μm in diameter using a Bio-Rad Biolistic(TM) gene gun (Model #PDS-1000/He). The tissue was placed about 5 cm from the stopping screen and bombarded under a vacuum of 28 in Hg. The particles were accelerated using a flying disc propelled by a shock wave generated with a 1100 psi rupture disc.

Following bombardment, the tissue was transferred to liquid medium and incubated on a rotary shaker for 10 d. Hygromycin was then added to the media at a concentration of 50 mg/L. Fresh hygromycin-containing medium was then added at weekly intervals for 6 weeks. After about 4 to 6 weeks, sectors of surviving green tissue was transferred to fresh medium without hygromycin. The transgenic soybean tissue was proliferated in SB55 medium.

Soybean tissue was transformed, as described above, with the pHGV4 construct. DNA was isolated (Edwards et al., *Nucl. Acids Res.* 19:1349) from part of this tissue or from control, untransformed tissue-culture grown tissue and used as template in PCR assays using primers specific for *pHGV4* (SEQ ID NOS:3 and 5) and standard PCR conditions (Sambrook, *supra*) with a 43°C annealing temperature. When DNA from the transformed tissue was used as template, a single strong product of about 600 bp was amplified as predicted from the sequence of the pHGV4 plasmid. A second product of about 700 bp of much lower abundance was also produced. Products of the same size and relative abundance were also produced when the PCR was conducted with the pHGV2 plasmid. No products were produced from DNA made from control, untransformed soybean tissue. These results indicate that the soybean tissue had been successfully transformed with the pHGV4 plasmid.

Transformed Tissue Synthesizes a PROTOX Resistant to the PBI Compound PBI-1

Protein extracts were made from both the transformed and control soybean tissues and were tested for PROTOX activity. Callus was removed from agar growth media, frozen at -78°C, and transferred to a glass homogenizer containing 1 mL of buffer A (100 mM HEPES pH 7.5, 1 mM EGTA, 5 mM EDTA, 2 mM DTT, 10 percent glycerol, 0.03 percent Tween-80) on ice. The plant tissue was homogenized on ice and then poured through one layer of miracloth. PROTOX activity was determined as described in the GENERAL METHODS. PROTOX activity was determined both in the presence and absence of 3 μM PBI-1. This concentration of PBI-1 is approximately 1000-fold above the level needed to

inhibit 50% of the activity of a corn chloroplast PROTOX (Maxwell et al., unpublished results). The results (Table 1) show that in the absence of inhibitor both lines had substantial PROTOX activity. However, the transformed line had only about 40% of the PROTOX activity of the untransformed control, probably due to the poor vigor of the pHGV4 transformed tissue. Soybean tissue grown in these types of tissue culture conditions is frequently sick, irrespective of the presence or absence of inserted foreign DNA. In the presence of 3 μM PBI-1, the untransformed control tissue had no activity above the background found in the heat-killed control samples indicating that this concentration of PBI-1 completely inhibits normal soybean PROTOX activity. By contrast, the transformant retained about 67% of its activity in the presence of the inhibitor. These results demonstrate that the pHGV4 construct directs the production of a protein in plant cells which has PROTOX activity and, moreover, this activity is resistant to high levels of PBI-1.

TABLE 1
PROTOX activity in transformed and control soybean tissue

Sample	Treatment	PROTOX Activity (Units/sec)
Control	none	19.8
pHGV4 Transformant	none	8.0
Control	3 μM PBI-1	1.0
pHGV4 Transformant	3 μM PBI-1	5.4
Control	Heat denatured	1.1
pHGV4 Transformant	Heat denatured	0.2

Tissue-culture grown soybean tissue that had been transformed with the pHGV4 construct or untransformed control tissue was assayed for PROTOX activity. Activity was measured in the absence of any treatment (total activity), in the presence of 3 μM PBI-1 (resistant activity) and after heat denaturation of the extracts (background activity).

EXAMPLE 3

Construction of an *Agrobacterium tumefaciens* Binary Plasmid p35S-PROTOX for Plant Transformation

A vector for transformation of the chimeric *hemG* construction described above into plants using *Agrobacterium tumefaciens* was produced by constructing a binary Ti plasmid vector [Bevan et al., (1984) *Nucl. Acids Res.*, 12:8711-8720]. The starting vector used for this work (pZS199, Figure 8) is based on a vector

which contains: (1) the chimeric gene nopaline synthase/neomycin phosphotransferase as a selectable marker for transformed plant cells [Bevan et al., (1984) *Nature*, 304:184-186], (2) the left and right borders of the T-DNA of the Ti plasmid [Bevan et al., (1984) *Nucl. Acids Res.*, 12:8711-8720], (3) the *E. coli* lacZ α-complementing segment (Vieria and Messing (1982), *Gene*, 19:259-267) with unique restriction endonuclease sites for Eco RI, Kpn I, Bam HI, Hin DIII, and Sal I, (4) the bacterial replication origin from the *Pseudomonas* plasmid pVS1 (Itoh et al., (1984) *Plasmid* 11:206-220), and (5) the bacterial neomycin phosphotransferase gene from Tn5 (Berg et al., (1975) *Proc. Natnl. Acad. Sci. U.S.A.*, 72:3628-3632) as a selectable marker for transformed *A. tumefaciens*. The nopaline synthase promoter in the plant selectable marker was replaced by the 35S promoter (Odell et al. (1985) *Nature*, 313:810-813) by a standard restriction endonuclease digestion and ligation strategy. The 35S promoter is required for efficient tobacco transformation as described below.

pZS199 was digested with XbaI and SalI. The *hemG* chimeric gene from pHGV4 (i.e., 35S promoter, *cab* leader, *rbcS* chloroplast transit sequence, residual *dapA* sequences, *hemG* coding region, and *nos* 3' terminator) was excised by digestion with XbaI and SalI. The *hemG* fragment was ligated with the XbaI/SalI digested pZS199 vector yielding p35S-PROTOX (Figure 5). The ligation mixture was transformed into *E. coli* and candidate plasmids were confirmed by restriction analysis. The p35S-protox plasmid was introduced by tri-parental mating [Ruvkin et al., (1981), *Nature*, 289:8588] to *Agrobacterium* strain LBA4404/pAL4404 [Hockema et al., (1983) *Nature*, 303:179180 (1983)) using the *E. coli* helper strain PRK2013 and selected for kanamycin resistance.

EXAMPLE 4

Transformation of Tobacco with p35S-protox and Resistance of Transformants to PBI-1

Cultures of *Agrobacterium* containing the binary vector p35S-protox were used to transform tobacco (*cultivar Xanthi*) leaf disks [Horsch et al., (1985) *Science* 227:12291231]. Forty-eight independently transformed tobacco plants were generated and are termed PROTOX-1 through PROTOX-48. In addition, the pZS199 binary vector was also used for parallel transformation experiments giving three control lines termed Binary Control 1 through Binary Control 3. These control lines were treated in the same manner as the PROTOX transformants and contain the same exogenous DNA except they lack the *hemG* transgene.

The sensitivity of the PROTOX and Binary Control primary transformants to PBI-1 were tested using the leaf spotting and ion leakage assays described above and the results are given in Figures 2, 3, 4, 9, and 11.

Figure 2 shows the results from a leaf spotting experiment using 1 μL drops of 300 μM PBI-1. The damage produced 5 d after the herbicide was spotted onto the leaves of the tobacco transformants is plotted. The results indicate that many of the *hemG*-containing lines are more resistant to 300 μM PBI-1 than the controls. Other lines have a similar level of sensitivity as the controls. Similar results were also observed when 30 μM PBI-1 was spotted onto these leaves (results not shown).

Figure 3 shows the results from an ion leakage experiment in which the conductivity increase for 50 leaf discs incubated in 500 μM PBI-1 for 29 hours in the light is plotted. These results confirm that, by this second criteria, many of the lines are more resistant to 500 μM PBI-1 than the controls. Other *hemG*-containing lines have a similar level of sensitivity as the controls. Similar results were also obtained when leaf discs were incubated in 10 μM PBI-1 (results not shown).

Based on these experiments, smaller subsets of PROTOX plants, which appeared most resistant to PBI-1, were chosen for further characterization using the leaf spotting and ion leakage assays as described below.

Concentrations of PBI-1, ranging from 0.3 to 900 μM were spotted onto leaves of 2 Binary Control and 13 PROTOX transformants. (The PROTOX lines were not spotted at the 0.3, 0.9 and 3 μM concentrations.) PBI-1-induced damage was scored visually after 10 d. The results are illustrated in Figure 4 which shows the average of the scores of the two Binary Controls and of PROTOX-23. From this analysis, PROTOX-23 appears about 30-fold more resistant to PBI-1 than the control lines.

Figure 11 shows the results of a third leaf spotting experiment in which PBI-1 was spotted onto Binary Control-2 and PROTOX-24 leaves. Five, 1 μL drops of PBI-1 of the indicated concentrations were spotted onto each leaf at the positions marked by the black dots. After 12 d of growth in the greenhouse, visual observations of the damage produced were made and photographs were taken. Only very minor damage was produced in PROTOX-24 by 900 μM PBI-1, the highest concentration tested. By contrast, severe damage was produced in the Binary Control-2 leaf at concentrations as low as 30 μM. Based on these results, PROTOX-24 is at least 30-fold more resistant to PBI-1 than the control plant.

Concentrations of PBI-1, ranging from 0-1000 μM, were used in an ion leakage experiment using leaf disks from 2 Binary Control and 5 PROTOX primary tobacco transformants. PBI-1-induced ion leakage was measured after incubation of the disks for 17 h in the dark and 25 h in the light and the results are illustrated in Figure 9 which shows the average scores of the two Binary Controls and of PROTOX-22. Under the conditions of this experiment, PROTOX-22

shows no consistent PBI-1-induced ion leakage in the light even at the highest tested concentration, 1000 μM. By contrast, ion leakage in the Binary Controls is clearly detectable at 10 μM. From this analysis, PROTOX-22 appears at least 100-fold more resistant to PBI-1 than the control lines.

EXAMPLE 5

Resistance of Transformants to PBI compounds PBI-2, PBI-3, PBI-4, PBI-5, PBI-6

In order to determine whether the PROTOX transformants are resistant to all PBIs, a representative set of diverse PBIs was tested at a range of concentrations on Binary Control and PROTOX primary tobacco transformants using the leaf spotting procedure. PBI compounds tested were PBI-1, PBI-2, PBI-3, PBI-4, PBI-5 and PBI-6 and are fully described in the details of the invention.

Leaves were spotted with five, 1 μL drops of the indicated PBI herbicides at the concentrations indicated. Plants were maintained in a greenhouse and the damage produced by the herbicides was scored visually 4 d (6 d for the slower acting PBI-6) after spotting. The results with one of the most resistant lines, PROTOX-23 are shown in Figure 10. In this figure, the results labeled Binary Control represent the average of those from the Binary Control-2 and Binary Control-3 plants. Overlapping data points in this figure have been offset by 0.05 vertical units to improve visual clarity.

As seen in Figure 10, the PROTOX-23 line showed no response to 5 of the 6 PBIs, whereas the Binary Control plants were strongly affected by all of the compounds. Because of the lack of response for PROTOX-23, even at the highest concentrations, only a minimum level of resistance for most of these compounds can be determined (Table 2). The actual level of resistance is likely to be higher than these estimates.

TABLE 2
Resistance to a variety of PBIs in tobacco PROTOX-23 transformant

PBI Test Compound	Fold Increase in Resistance in PROTOX-23
PBI-1	>30-fold
PBI-2	>300-fold
PBI-3	>3-fold
PBI-4	>3-fold
PBI-5	>300-fold
PBI-6	15-fold

The fold increase in resistance of the PROTOX-23 line, relative to the Binary Control-2 and -3 lines was calculated using the leaf spotting assay with the results shown in Figure 10. For PBI-6, the estimate is based on the response at 840 μM in PROTOX-23 compared with the rate that would be required to produce a similar level of damage in the Binary Control by interpolating the dose response curve shown in Figure 10. For the other PBIs, since PROTOX-23 did not show any response to even the highest level of PBI tested, the fold increase in resistance is a minimal estimate based on the lowest concentration that produced a response in the Binary Controls, compared to the highest dose tested in PROTOX-23.

EXAMPLE 6

Characterization of Transgene Loci Conferring Resistance to PBI Herbicides

In order to determine the number of transgene loci present in each tobacco transformant, progeny from the primary transformants were analyzed for their resistance to kanamycin, since the T-DNA also carries the nptII gene as a transformation marker. Segregation data indicated that 70% of the PROTOX tobacco transformants and 4 of the 5 selected for more detailed study (Table 3) segregated approximately 75% kanamycin resistant to 25% kanamycin sensitive, indicative of a single transgene locus. The fifth line (PROTOX-18) segregated with a ratio indicative of 2 transgene loci.

Genomic DNA was isolated [see Reiter et al. (1992), *Proc. Nat. Acad. Sci.* 89:1477] from leaves of progeny of the same 5 Protox lines and restricted with either BamHI or EcoRI. DNA was blotted onto Hybond N+ (Amersham, Arlington Heights, Illinois) in 0.4 N NaOH and probed with a DNA fragment containing the *hemG* coding region using procedures recommended by the manufacturer. Based on these results (Table 3), three of the five lines (PROTOX-23, 24, and 36) contain 2 apparently intact copies of the T-DNA. A fourth line (PROTOX-18) contains 4 apparently intact copies and the fifth line (PROTOX-26) contains 1 or 2 apparently rearranged copies of the T-DNA.

The combined Southern and kanamycin segregation results indicate that only a small number of chimeric *hemG* genes (from 2 to 4) are sufficient to produce the high levels of PBI resistance observed in these tobacco transformants.

TABLE 3

Number of genetically defined T-DNA loci and T-DNA inserts in tobacco Protox transformants

Line	# Kan^r	# Kan^S	Kan^r/Kan^S	# loci	Transgene copy #
PROTOX-18	114	9	12.7	2	4
PROTOX-23	280	82	3.4	1	2
PROTOX-24	142	60	2.4	1	2
PROTOX-26	322	72	4.5	1	1 or 2
PROTOX-36	108	36	3.0	1	2

Kanamycin resistance was determined on progeny of primary transformants by scoring growth of seedlings on standard media supplemented with 200 μg/mL kanamycin sulfate. Transgene copy number in each line was determined by Southern blotting of DNA from 1 or 2 progeny from each primary transformant. The T-DNA was rearranged in Protox 26 so the copy number could not be determined precisely.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: U.S.A.
(F) ZIP: 19898
(G) TELEPHONE: 302-892-7229
(H) TELEFAX: 302-773-0164
(I) TELEX: 6717325

(ii) TITLE OF INVENTION: GENETICALLY TRANSFORMED PLANTS DEMONSTRATING RESISTANCE TO PORPHYRINOGEN BIOSYNTHESIS INHIBITING HERBICIDES

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.5 INCH
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: MICROSOFT FOR WINDOWS '95
(D) SOFTWARE: MICROSOFT WORD 7.0

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/036,793
(B) FILING DATE: JANUARY 31, 1997

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: KING, KAREN K.
(B) REGISTRATION NO.: 34,850
(C) REFERENCE/DOCKET NUMBER: CR-9854

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCAGGCCT CGGTGAAAAC ATTAATTC 28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GACGTGGTAC CATTATTTCA GCGTCGG 27

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATGGTCACG GGAAG 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCAGAAACTT GCGCG 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTGGATCTCA CTATT 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGGCTTCCT CAATGATCTC CTCCCCAGCT GTTACCACCG TCAACCGTGC CGGTGCCGGC 60

ATGGTTGCTC CATTCACCGG CCTCAAATCC ATGGCTGGCT TCCCCACGAG GAAGACCAAC 120

AATGACATTA CCTCCATTGC TAGCAACGGT GGAAGAGTAC AATGCATGGT CACGGGAAGT 180

ATTGTCGCCT CGGTGAAATC ATTAATTCTT TTCTCAACAA GGGACGGACA AACGCGCGAG 240

ATTGCCTCCT ACCTGGCTTC GGAACTGAAA GAACTGGGGA TCCAGGCGGA TGTCGCCAAT 300

GTGCACCGCA TTGAAGAACC ACAGTGGGAA AACTATGACC GTGTGGTCAT TGGTGCTTCT 360

ATTCGCTATG GTCACTACCA TTCAGCGTTC CAGGAATTTG TCAAAAAACA TGCGACGCGG 420

CTGAATTCGA TGCCGAGCGC CTTTTACTCC GTGAATCTGG TGGCGCGCAA ACCGGAGAAG 480

CGTACTCCAC AGACCAACAG CTACGCGCGC AAGTTTCTGA TGAACTCGCA ATGGCGTCCC 540

GATCGCTGCG CGGTCATTGC CGGGGCGCTG CGTTACCCAC GTTATCGCTG GTACGACCGT 600

TTTATGATCA AGCTGATTAT GAAGATGTCA GGCGGTGAAA CGGATACGCG CAAAGAAGTT 660

GTCTATACCG ATTGGGAGCA GGTGGCGAAT TTCGCCCGAG AAATCGCCCA TTTAACCGAC 720

AAACCGACGC TGAAATATGG TACCTAA 747

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 184 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Val Lys Ser Leu Ile Leu Phe Ser Thr Arg Asp Gly Gln Thr Arg Glu
1               5                  10                  15

Ile Ala Ser Tyr Leu Ala Ser Glu Leu Lys Glu Leu Gly Ile Gln Ala
            20                  25                  30

Asp Val Ala Asn Val His Arg Ile Glu Glu Pro Gln Trp Glu Asn Tyr
        35                  40                  45

Asp Arg Val Val Ile Gly Ala Ser Ile Arg Tyr Gly His Tyr His Ser
    50                  55                  60

Ala Phe Gln Glu Phe Val Lys Lys His Ala Thr Arg Leu Asn Ser Met
65                  70                  75                  80

Pro Ser Ala Phe Tyr Ser Val Asn Leu Val Ala Arg Lys Pro Glu Lys
                85                  90                  95

Arg Thr Pro Gln Thr Asn Ser Tyr Ala Arg Lys Phe Leu Met Asn Ser
            100                 105                 110

Gln Trp Arg Pro Asp Arg Cys Ala Val Ile Ala Gly Ala Leu Arg Tyr
        115                 120                 125

Pro Arg Tyr Arg Trp Tyr Asp Arg Phe Met Ile Lys Leu Ile Met Lys
    130                 135                 140

Met Ser Gly Gly Glu Thr Asp Thr Arg Lys Glu Val Val Tyr Thr Asp
145                 150                 155                 160

Trp Glu Gln Val Ala Asn Phe Ala Arg Glu Ile Ala His Leu Thr Asp
                165                 170                 175

Lys Pro Thr Leu Lys Tyr Gly Thr
            180
```

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description
on page 11, line 34-37

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit	Accession Number
7 August 1996	ATCC97675

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☑ This sheet was received with the international application

29/7/98

Authorized officer

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 12, line 1-5

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit	Accession Number
7 August 1996	ATCC97674

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☑ This sheet was received with the international application

Authorized officer Paralegal Specialist

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

Form PCT/RO/134 (July 1992)

WHAT IS CLAIMED IS:

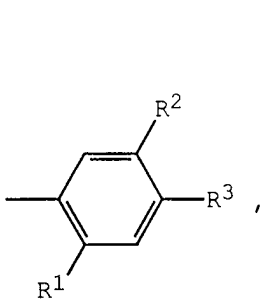
1. A plant resistant to porphyrin biosynthesis-inhibiting herbicides comprising a chimeric gene encoding a herbicide-resistant protoporphyrinogen oxidase activity, wherein the porphyrin biosynthesis-inhibiting herbicides to which the plant is resistant are according to the formula:

J—G

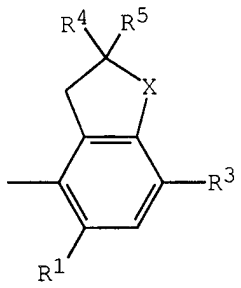
I

wherein

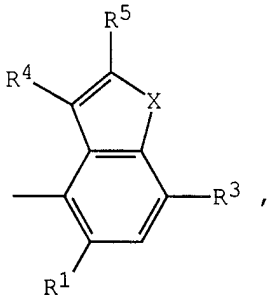
G is



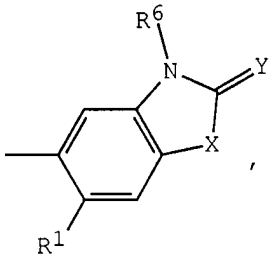
G-1



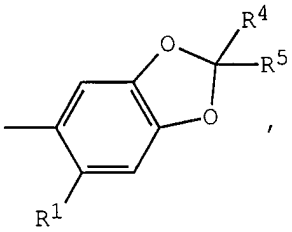
G-2



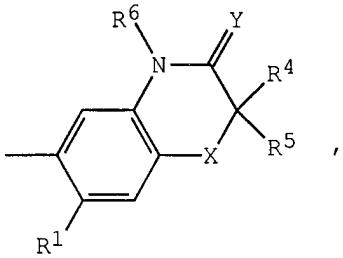
G-3



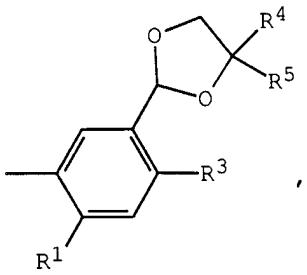
G-4



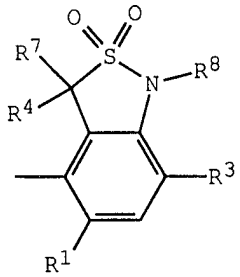
G-5



G-6

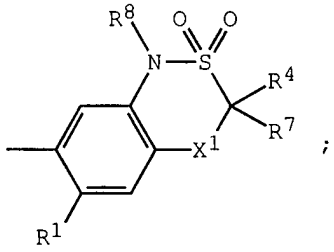


G-7



G-8

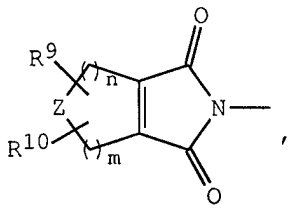
or



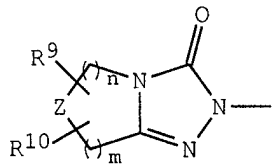
G-9

;

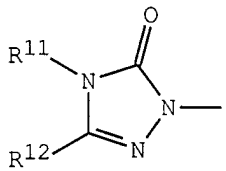
and wherein J is



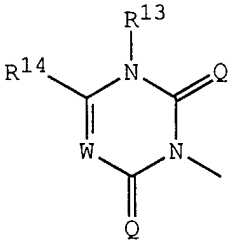
J-1



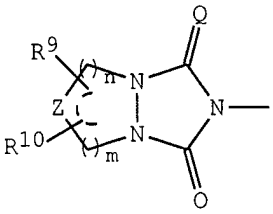
J-2



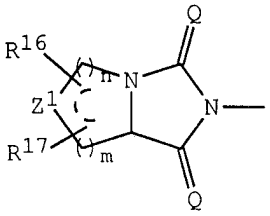
J-3



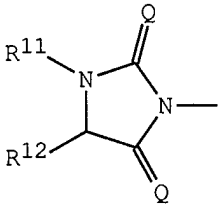
J-4



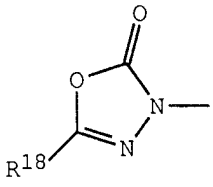
J-5



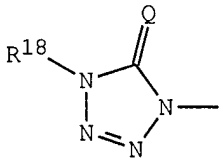
J-6



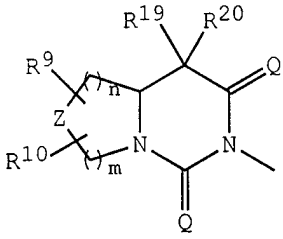
J-7



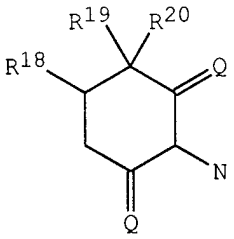
J-8



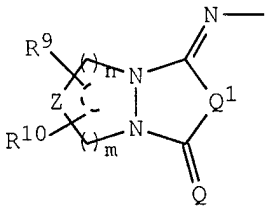
J-9



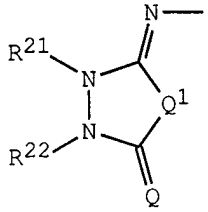
J-10



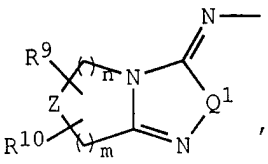
J-11



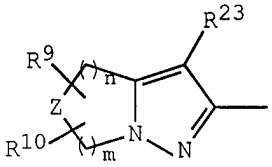
J-12



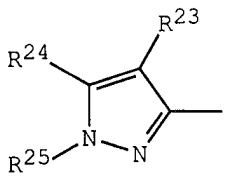
J-13



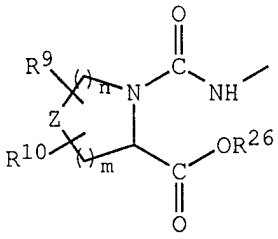
J-14



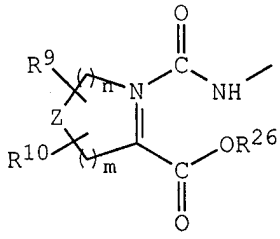
J-15



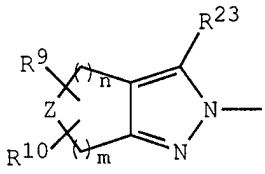
J-16



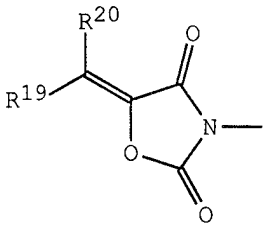
J-17



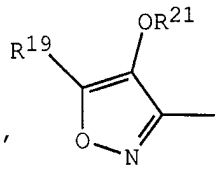
J-18



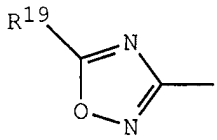
J-19



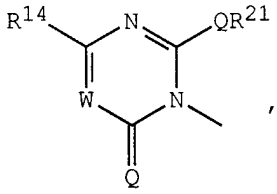
J-20



J-21

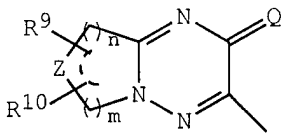


J-22



J-23

, or



J-24

wherein the dashed line in J-5, J-6, J-12 and J-24 indicates that the left-hand ring contains only single bonds or one bond in the ring is a carbon-carbon double bond;

X is O or S;

Y is O or S;

R^1 is hydrogen or halogen;

R^2 is H; C_1-C_8 alkyl; C_1-C_8 haloalkyl; halogen; OH; OR^{27}; SH; $S(O)_pR^{27}$; COR^{27}; CO_2R^{27}; $C(O)SR^{27}$; $C(O)NR^{29}R^{30}$; CHO; CR^{29}=NOR^{36}; CH=$CR^{37}CO_2R^{27}$; $CH_2CHR^{37}CO_2R^{27}$; CO_2N=$CR^{31}R^{32}$; nitro; cyano; $NHSO_2R^{33}$; $NHSO_2NHR^{33}$; $NR^{27}R^{38}$; NH_2; or phenyl optionally substituted with at least one member independently selected from C_1-C_4 alkyl;

p is 0; 1; or 2;

R^3 is C_1-C_2 alkyl; C_1-C_2 haloalkyl; OCH_3; SCH_3; $OCHF_2$; halogen; cyano, or nitro;

R^4 is H; C_1-C_3 alkyl; C_1-C_3 haloalkyl; or halogen;

R^5 is H; C_1-C_3 alkyl; halogen; C_1-C_3 haloalkyl; cyclopropyl; vinyl; C_2 alkynyl; cyano; $C(O)R^{38}$; CO_2R^{38}; $C(O)NR^{38}R^{39}$; $CR^{34}R^{35}CN$; $CR^{34}R^{35}C(O)R^{38}$; $CR^{34}R^{35}CO_2R^{38}$; $CR^{34}R^{35}C(O)NR^{38}R^{39}$; $CHR^{34}OH$; $CHR^{34}OC(O)R^{38}$; or $OCHR^{34}OC(O)NR^{38}R^{39}$; or

when G is G-2 or G-6, then R^4 and R^5 can be taken together with the carbon to which they are attached to form C=O;

R^6 is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_2-C_6 alkoxyalkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;

X^1 is a direct bond; O; S; NH; $N(C_1$-C_3 alkyl); $N(C_1$-C_3 haloalkyl); or N(allyl);

R^7 is H; C_1-C_6 alkyl; C_1-C_6 haloalkyl; halogen; $S(O)_2(C_1$-C_6 alkyl); or $C(=O)R^{40}$;

R^8 is H; C_1-C_8 alkyl; C_3-C_8 cycloalkyl; C_3-C_8 alkenyl; C_3-C_8 alkynyl; C_1-C_8 haloalkyl; C_2-C_8 alkoxyalkyl; C_3-C_8 alkoxyalkoxyalkyl; C_3-C_8 haloalkynyl; C_3-C_8 haloalkenyl; C_1-C_8 alkylsulfonyl; C_1-C_8 haloalkylsulfonyl; C_3-C_8 alkoxycarbonylalkyl; $S(O)_2NH(C_1$-C_8 alkyl); $C(O)R^{41}$; or benzyl optionally substituted on the phenyl ring with R^{42};

n and m are each independently 0; 1; 2; or 3; provided that m + n is 2 or 3;

Z is CR^9R^{10}; O; S; S(O); $S(O)_2$; or $N(C_1$-C_4 alkyl);

each R^9 is independently H; C_1-C_3 alkyl; halogen; hydroxy; C_1-C_6 alkoxy; C_1-C_6 haloalkyl; C_1-C_6 haloalkoxy; C_2-C_6 alkylcarbonyloxy; or C_2-C_6 haloalkylcarbonyloxy;

each R^{10} is independently H; C_1-C_3 alkyl; hydroxy; or halogen;

R^{11} and R^{12} are each independently H; halogen; C_1-C_6 alkyl; C_3-C_6 alkenyl; or C_1-C_6 haloalkyl;

R^{13} is H; C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 haloalkenyl; C_3-C_6 alkynyl; C_3-C_6 haloalkynyl; HC(=O); $(C_1$-C_4 alkyl)C(=O); or NH_2;

R^{14} is C_1-C_6 alkyl; C_1-C_6 alkylthio; C_1-C_6 haloalkyl; or $N(CH_3)_2$;

W is N or CR^{15};

R^{15} is H; C_1-C_6 alkyl; halogen; or phenyl optionally substituted with C_1-C_6 alkyl, 1-2 halogen, C_1-C_6 alkoxy, or CF_3;

each Q is independently O or S;

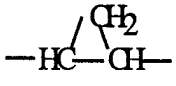
Q^1 is O or S;

Z^1 is $CR^{16}R^{17}$; O; S; S(O); $S(O)_2$; or $N(C_1$-C_4 alkyl);

each R^{16} is independently H; halogen; hydroxy; C_1-C_6 alkoxy; C_1-C_6 haloalkyl; C_1-C_6 haloalkoxy; C_2-C_6 alkylcarbonyloxy; or C_2-C_6 haloalkylcarbonyloxy;

each R^{17} is independently H; hydroxy; or halogen; or

when R^{16} and R^{17} are bonded to adjacent atoms they can be taken together with the carbons to which they are attached to form

 optionally substituted with at least one member selected from 1-2 halogen and 1-2 C_1-C_3 alkyl;

R^{18} is C_1-C_6 alkyl; halogen; or C_1-C_6 haloalkyl;

R^{19} and R^{20} are each independently H; C_1-C_6 alkyl; or C_1-C_6 haloalkyl;

R^{21} and R^{22} are each independently C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 haloalkenyl; C_3-C_6 alkynyl; or C_3-C_6 haloalkynyl;

R^{23} is H; halogen; or cyano;

R^{24} is C_1-C_6 alkylsulfonyl; C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; C_3-C_6 alkynyl; C_1-C_6 alkoxy; C_1-C_6 haloalkoxy; or halogen;

R^{25} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;

R^{26} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; or phenyl optionally substituted with C_1-C_6 alkyl, 1-2 halogen, 1-2 nitro, C_1-C_6 alkoxy, or CF_3;

R^{27} is C_1-C_8 alkyl; C_3-C_8 cycloalkyl; C_3-C_8 alkenyl; C_3-C_8 alkynyl; C_1-C_8 haloalkyl; C_2-C_8 alkoxyalkyl; C_2-C_8 alkylthioalkyl; C_2-C_8 alkylsulfinylalkyl; C_2-C_8 alkylsulfonylalkyl; C_1-C_8 alkylsulfonyl; phenylsulfonyl optionally substituted on the phenyl ring with at least one substituent selected from the group halogen and C_1-C_4 alkyl; C_4-C_8 alkoxyalkoxyalkyl; C_4-C_8 cycloalkylalkyl; C_6-C_8 cycloalkoxyalkyl; C_4-C_8 alkenyloxyalkyl; C_4-C_8 alkynyloxyalkyl; C_3-C_8 haloalkoxyalkyl; C_4-C_8 haloalkenyloxyalkyl; C_4-C_8 haloalkynyloxyalkyl; C_6-C_8 cycloalkylthioalkyl; C_4-C_8 alkenylthioalkyl; C_4-C_8 alkynylthioalkyl; C_1-C_4 alkyl substituted with phenoxy or benzyloxy, each ring optionally substituted with at least one

substituent selected from the group halogen, C_1-C_3 alkyl and C_1-C_3 haloalkyl; C_4-C_8 trialkylsilylalkyl; C_3-C_8 cyanoalkyl; C_3-C_8 halocycloalkyl; C_3-C_8 haloalkenyl; C_5-C_8 alkoxyalkenyl; C_5-C_8 haloalkoxyalkenyl; C_5-C_8 alkylthioalkenyl; C_3-C_8 haloalkynyl; C_5-C_8 alkoxyalkynyl; C_5-C_8 haloalkoxyalkynyl; C_5-C_8 alkylthioalkynyl; C_2-C_8 alkylcarbonyl; benzyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_3 alkyl and C_1-C_3 haloalkyl; $CHR^{34}COR^{28}$; $CHR^{34}CO_2R^{28}$; $CHR^{34}P(O)(OR^{28})_2$; $CHR^{34}P(S)(OR^{28})_2$; $CHR^{34}C(O)NR^{29}R^{30}$; or $CHR^{34}C(O)NH_2$;

R^{28} is C_1-C_6 alkyl; C_2-C_6 alkenyl; C_2-C_6 alkynyl; or tetrahydrofuranyl;

R^{29} and R^{31} are independently hydrogen or C_1-C_4 alkyl;

R^{30} and R^{32} are independently C_1-C_4 alkyl or phenyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_3 alkyl, and C_1-C_3 haloalkyl; or

R^{29} and R^{30} can be taken together to form $-(CH_2)_5-$, $-(CH_2)_4-$ or $-CH_2CH_2OCH_2CH_2-$, each ring thus formed optionally substituted with a substituent selected from the group C_1-C_3 alkyl, phenyl and benzyl; or

R^{31} and R^{32} can be taken together with the carbon to which they are attached to form C_3-C_8 cycloalkyl;

R^{33} is C_1-C_4 alkyl; C_1-C_4 haloalkyl; or C_2-C_6 alkenyl;

R^{34} and R^{35} are independently H or C_1-C_4 alkyl;

R^{36} is H; C_1-C_6 alkyl; C_3-C_6 alkenyl; or C_3-C_6 alkynyl;

R^{37} is H; C_1-C_4 alkyl; or halogen;

R^{38} is H; C_1-C_6 alkyl; C_3-C_6 cycloalkyl; C_3-C_6 alkenyl; C_3-C_6 alkynyl; C_2-C_6 alkoxyalkyl; C_1-C_6 haloalkyl; phenyl optionally substituted with at least one substituent selected from the group halogen, C_1-C_4 alkyl, and C_1-C_4 alkoxy; $-CH_2CO_2(C_1$-C_4 alkyl); or $-CH(CH_3)CO_2(C_1$-C_4 alkyl);

R^{39} is H; C_1-C_2 alkyl; or $C(O)O(C_1$-C_4 alkyl);

R^{40} is H; C_1-C_6 alkyl; C_1-C_6 alkoxy; or $NH(C_1$-C_6 alkyl);

R^{41} is C_1-C_6 alkyl; C_1-C_6 haloalkyl; C_1-C_6 alkoxy; $NH(C_1$-C_6 alkyl); phenyl optionally substituted with R^{42}; benzyl; or C_2-C_8 dialkylamino; and

R^{42} is C_1-C_6 alkyl; 1-2 halogen; C_1-C_6 alkoxy; or CF_3.

2. A plant according to Claim 1 wherein the chimeric gene comprises:

(i) a nucleic acid fragment encoding protoporphyrinogen oxidase enzyme which is resistant to inhibition by porphyrin biosynthesis-inhibiting herbicides; and

(ii) a plant regulatory sequence,

wherein the nucleic acid fragment encoding protoporphyrinogen oxidase enzyme is operably linked to the plant regulatory sequence.

3. A plant according to Claim 2 wherein the chimeric gene further comprises a chloroplast targeting sequence for the specific localization of the gene to the chloroplast.

4. A plant according to Claim 3 wherein the chimeric gene further comprises a mitochondrial targeting sequence for the specific localization of the gene to the mitochondria.

5. A plant according to Claim 2 wherein the plant regulatory sequence contains a constitutive or inducible promoter.

6. A plant according to Claim 5 wherein the constitutive promoter is selected from the group consisting of the 35S promoter of cauliflower mosaic virus promoter, the chlorophyll a/b binding protein promoter, ferredoxin promoter, actin promoters, ubiquitin promoters, and opine promoters.

7. A plant according to Claim 5 wherein the inducible promoter is selected from the group consisting of light-inducible promoters, ABA-inducible promoters, benzenesulfonamide-inducible promoters, and methyl jasmonate inducible promoters.

8. A plant according to Claim 2 wherein the nucleic acid fragment encoding protoporphyrinogen oxidase enzyme is derived from a prokaryote.

9. A plant according to Claim 8 wherein the nucleic acid fragment encoding protoporphyrinogen oxidase enzyme is selected from the group consisting of *Escherichia* and *Bacillus*.

10. A plant according to Claim 9 wherein the nucleic acid fragment encoding protoporphyrinogen oxidase enzyme is the *hemG* gene.

11. A plant according to Claim 10 wherein the nucleic acid fragment encoding protoporphyrinogen oxidase enzyme has the amino acid sequence encoded by the mature functional *hemG* gene which corresponds to the nucleic acid sequence of SEQ ID NO:6 or to any nucleotide sequence encoding the protoporphyrinogen oxidase enzyme in which one or more amino acid substitutions, additions or deletions have been made that do not affect the functional properties of the protoporphyrinogen oxidase enzyme.

12. A plant according to Claim 1 wherein the porphyrin biosynthesis-inhibiting herbicides are selected from the group consisting of PBI-1, PBI-2, PBI-3, PBI-4, PBI-5, and PBI-6.

13. A plant according to Claim 1 wherein the plant is selected from the group consisting of soybean, rapeseed *(Brassica napus, B. campestris)*, sunflower (*Helianthus annus*), cotton (*Gossypium hirsutum*), corn, tobacco *(Nicotiana tabacum)*, alfalfa (*Medicago sativa*), wheat *(Triticum sp)*, barley (*Hordeum vulgare*), oats (*Avena sativa, L*), sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), *Arabidopsis,* cruciferous vegetables (broccoli, cauliflower, cabbage, parsnips, etc.), melons, carrots, celery, parsley, tomatoes, potatoes, strawberries, peanuts, grapes, grass seed crops, sugar beets, sugar cane, beans, peas, rye, flax, hardwood trees, softwood trees, and forage grasses.

14. A plant according to Claim 13 wherein the plant is selected from the group consisting of soybean, tobacco and *Arabidopsis*.

15. Seed obtained from the plant of Claim 1.

16. A plant according to Claim 1 wherein the plant demonstrates a resistance to the porphyrin biosynthesis-inhibiting compounds at levels of between 30 to 100 fold greater than resistance of plants lacking a chimeric gene encoding protoporphyrinogen oxidase enzyme which is resistant to inhibition by porphyrin biosynthesis-inhibiting herbicides.

17. A nucleic acid fragment encoding protoporphyrinogen oxidase enzyme having the amino acid sequence encoded by the mature *hemG* gene which corresponds to the nucleic acid sequence SEQ ID NO:6 or to any nucleotide sequence encoding the protoprophyrinogen oxidase enzyme in which one or more amino acid substitutions, additions or deletions have been made that do not affect the functional properties of the protoporphyrinogen oxidase enzyme.

FIG. 1

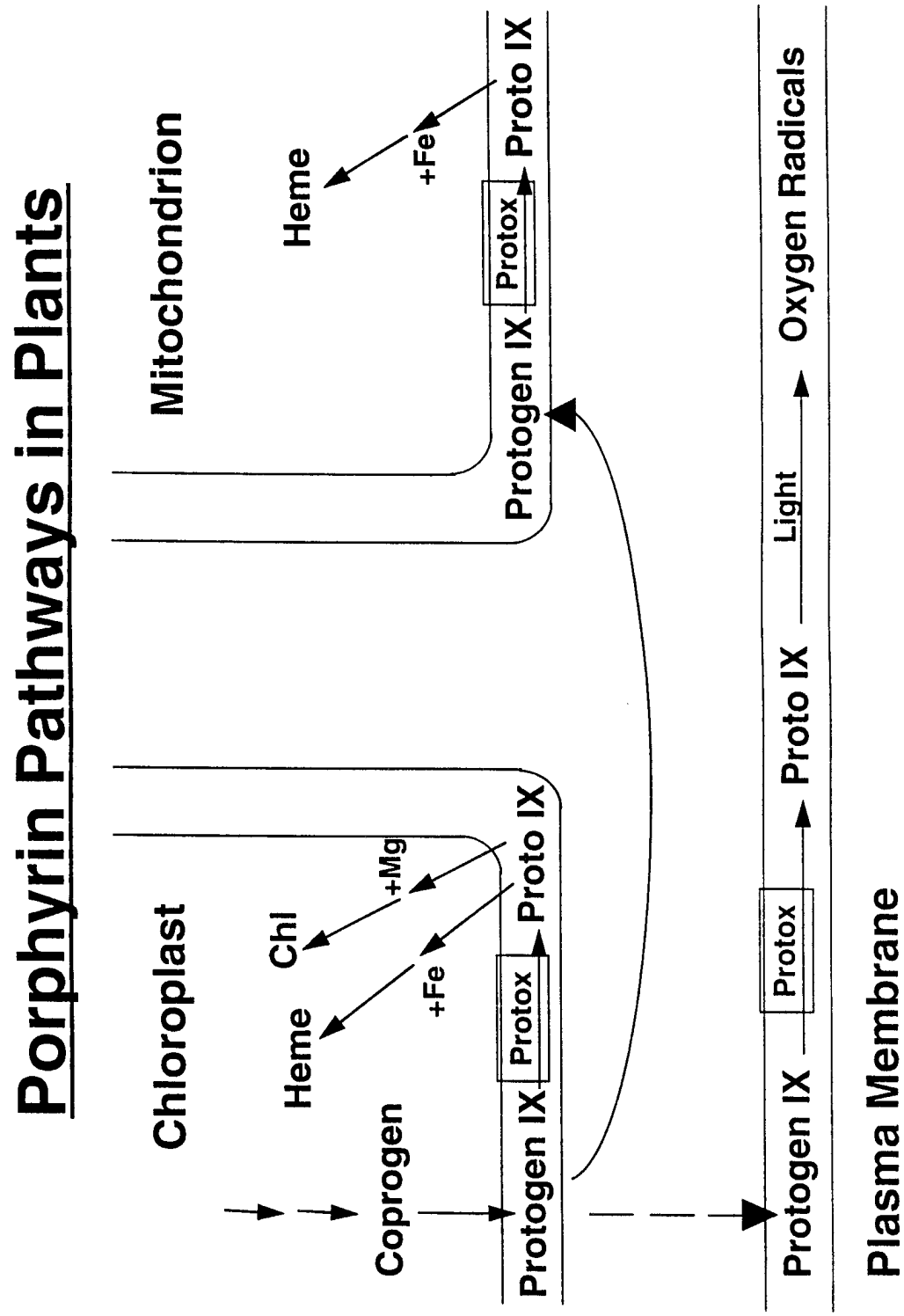

Porphyrin Pathways in Plants
Mitochondrion
Heme
+Fe
Protox
Proto IX
Protogen IX
Chloroplast
Chl
+Mg
Heme
+Fe
Protox
Proto IX
Coprogen
Protogen IX
Protox
Proto IX
Light
Oxygen Radicals
Protogen IX
Plasma Membrane

FIG.2

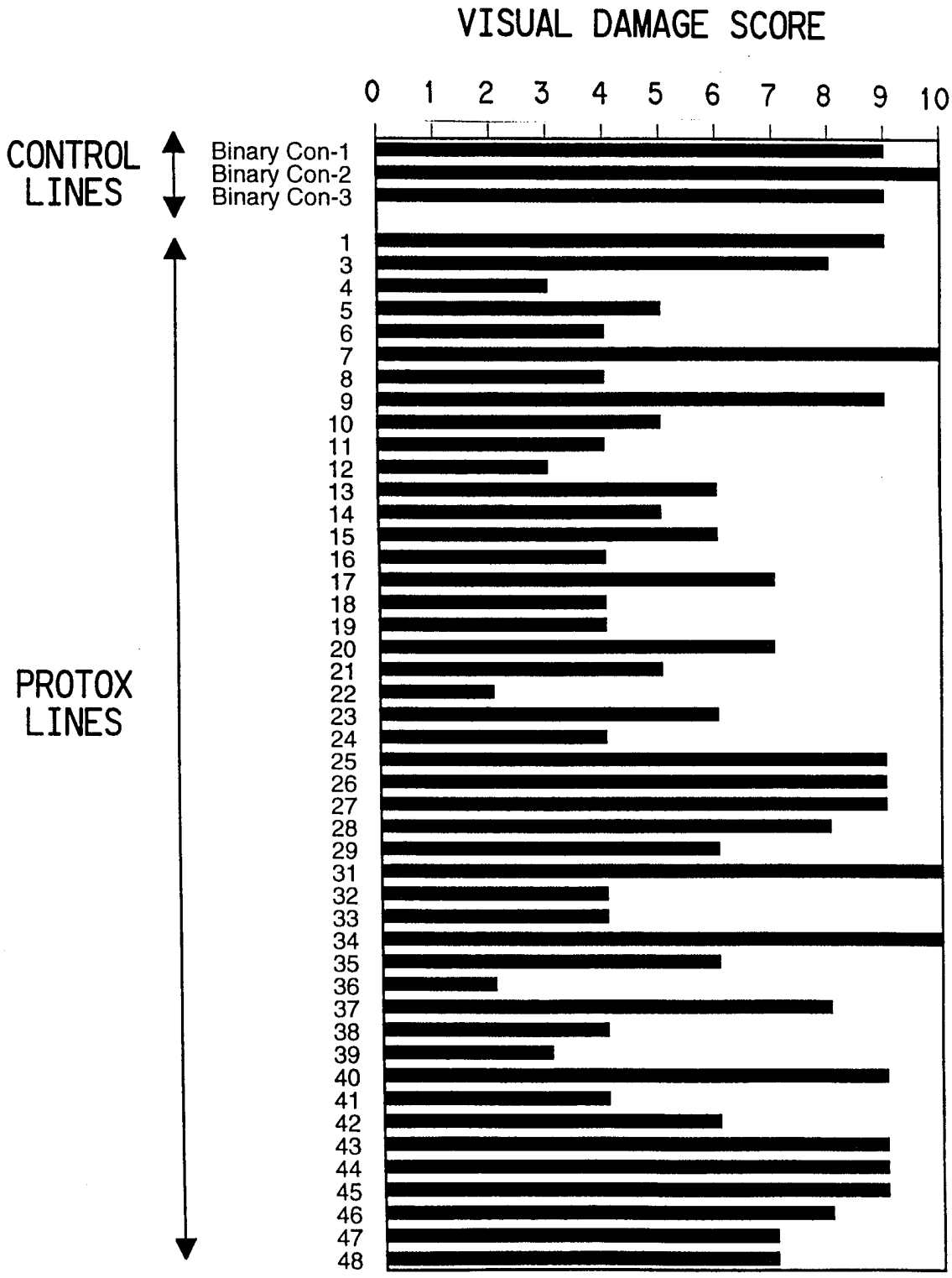

VISUAL DAMAGE SCORE
0 1 2 3 4 5 6 7 8 9 10
CONTROL LINES
Binary Con-1
Binary Con-2
Binary Con-3
PROTOX LINES
1
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48

FIG.3

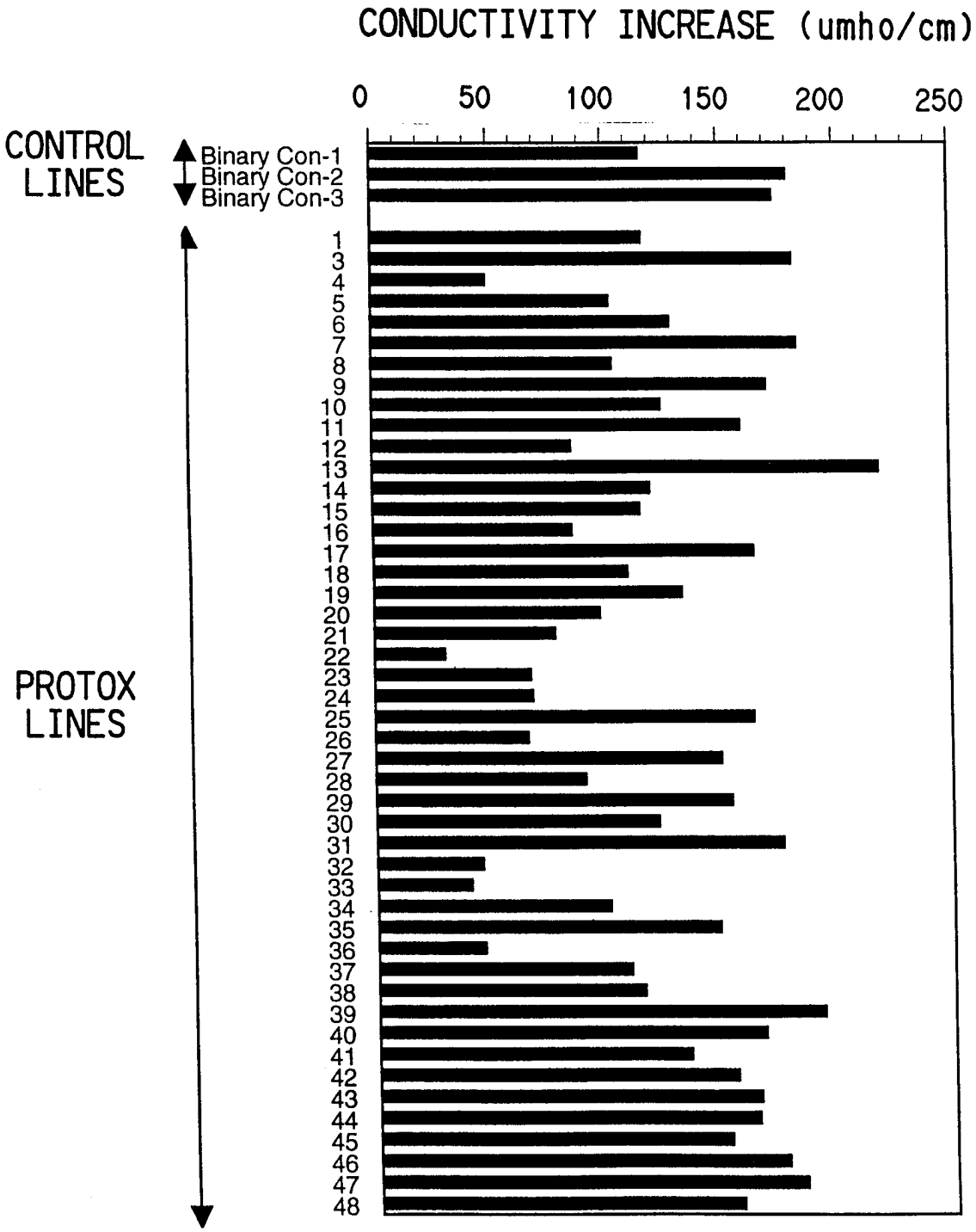
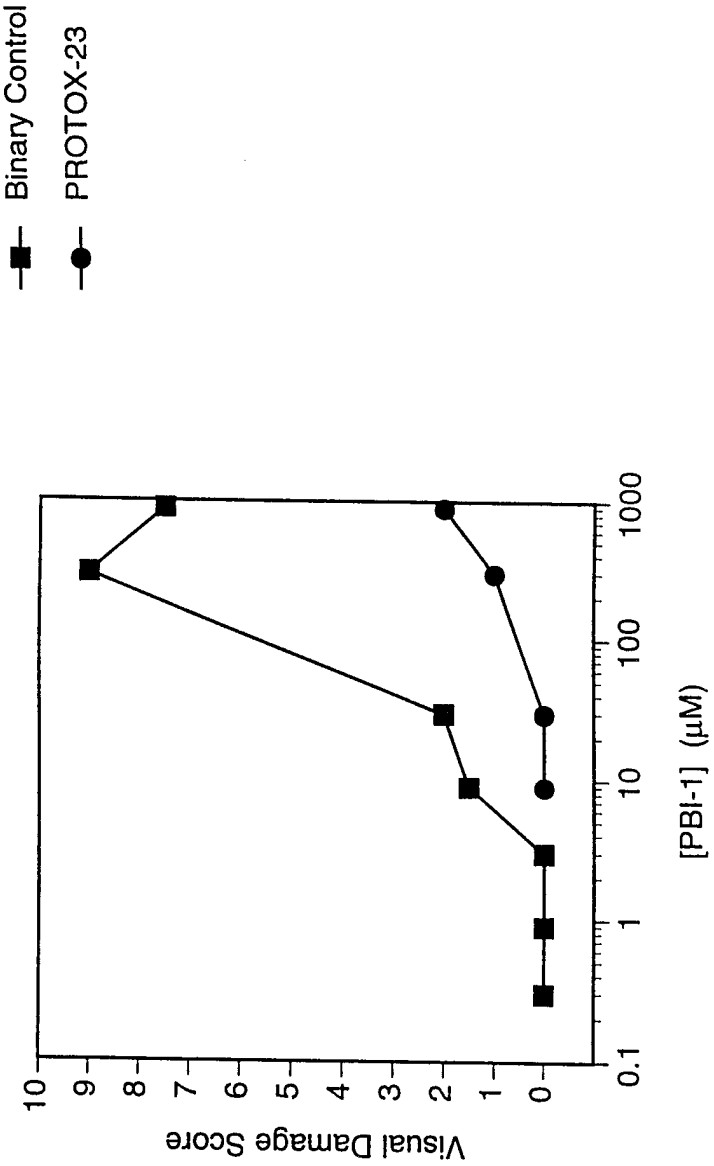
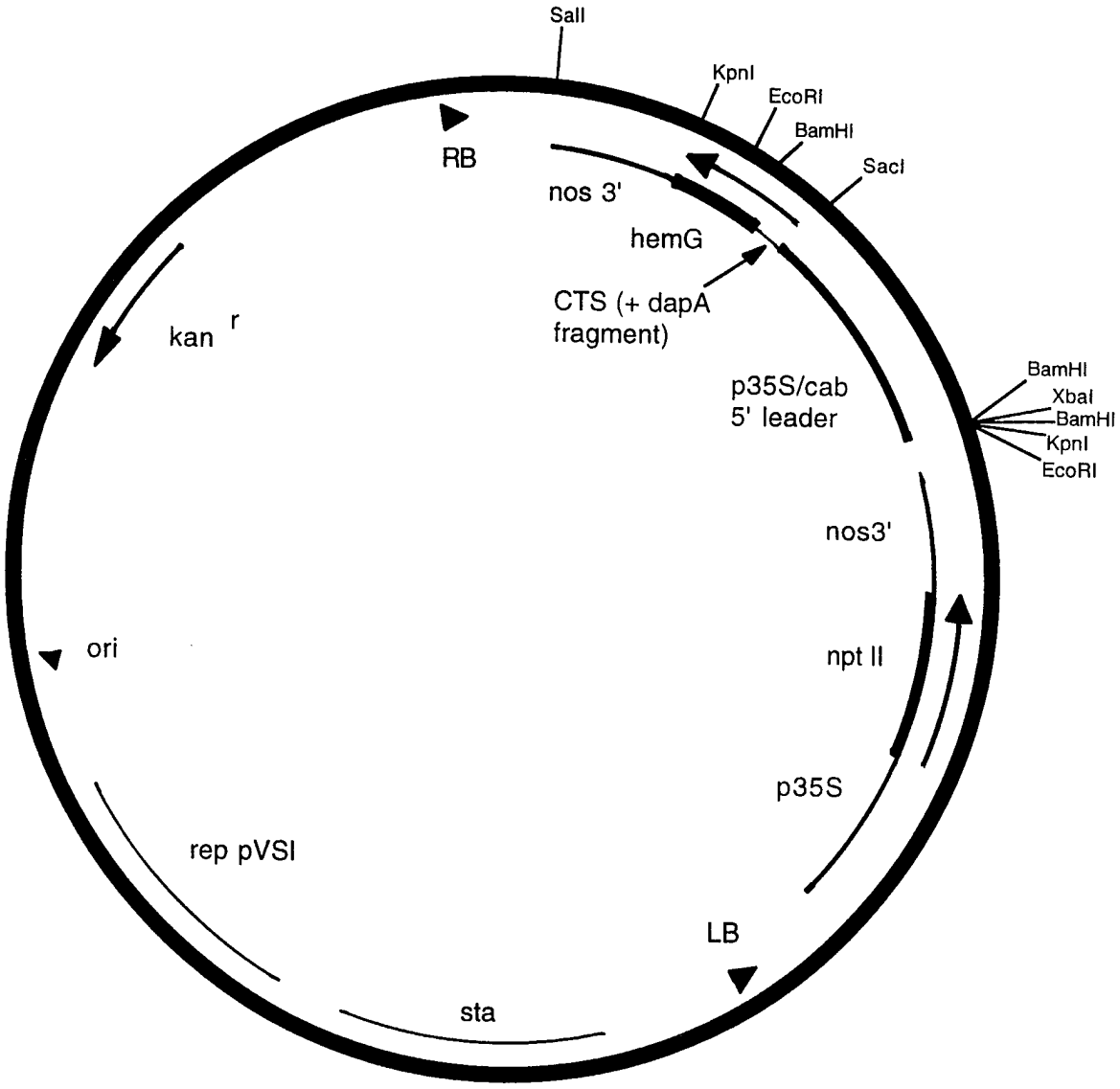

CONDUCTIVITY INCREASE (umho/cm)
0
50
100
150
200
250
CONTROL LINES
Binary Con-1
Binary Con-2
Binary Con-3
PROTOX LINES
1
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48

FIG.4


Binary Control
PROTOX-23
Visual Damage Score
10
9
8
7
6
5
4
3
2
1
0
0.1
1
10
100
1000
[PBI-1] (μM)

5/13

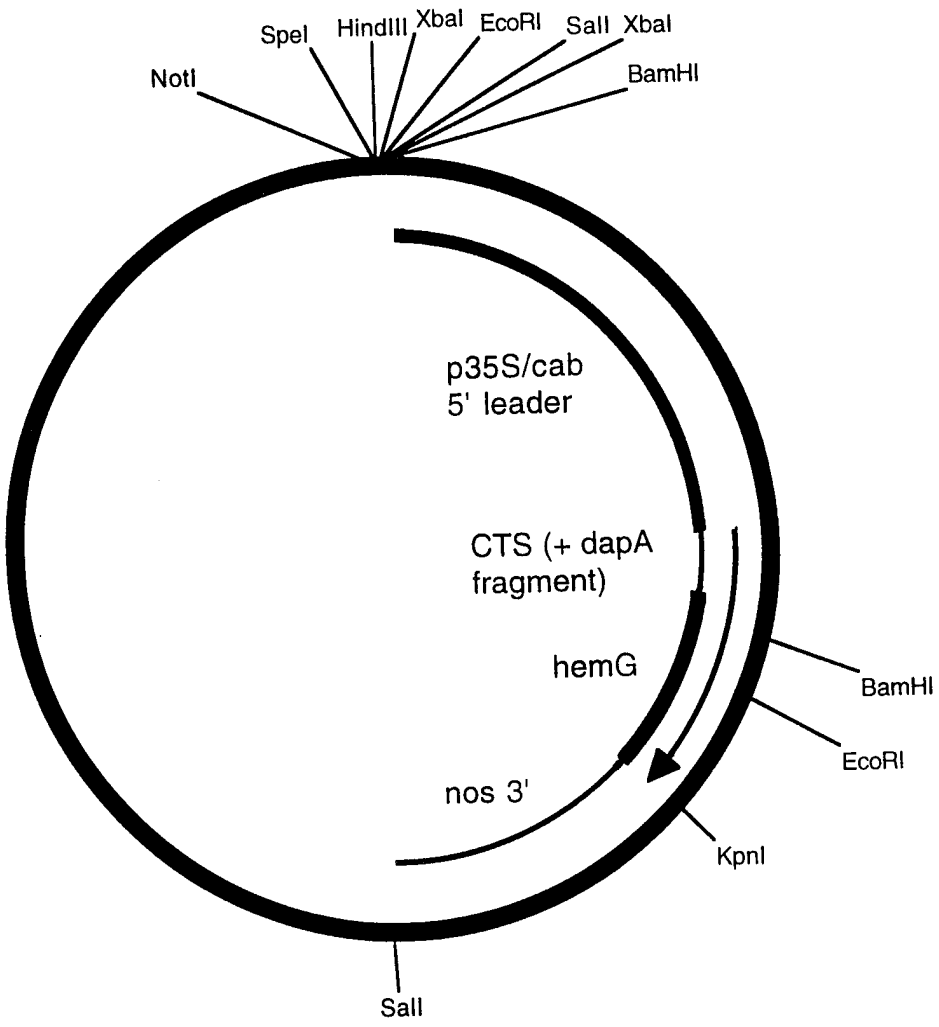
FIG.5



p35S-PROTOX
(16.2 kb)

6/13

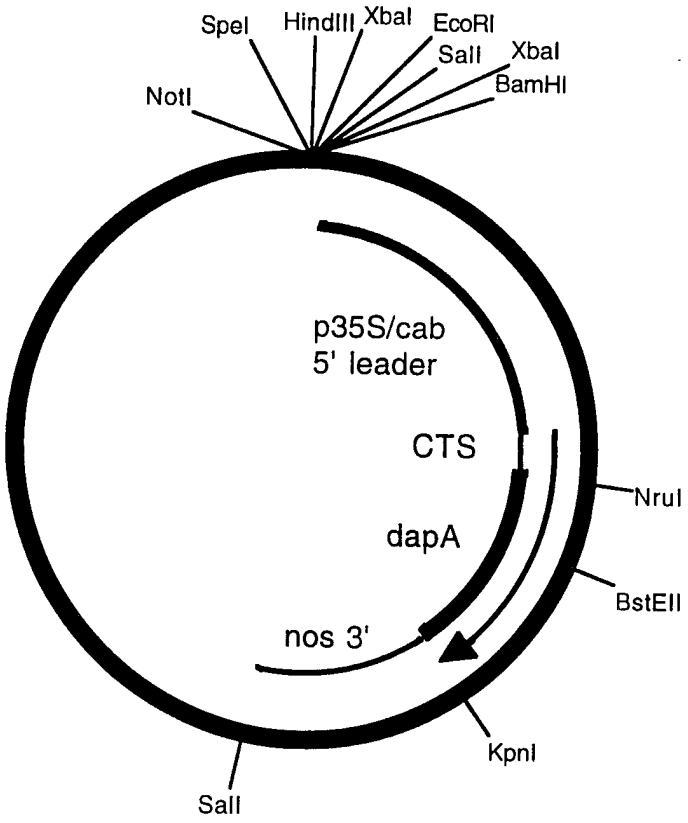
FIG.6



pHGV4
(5867 bp)

7/13

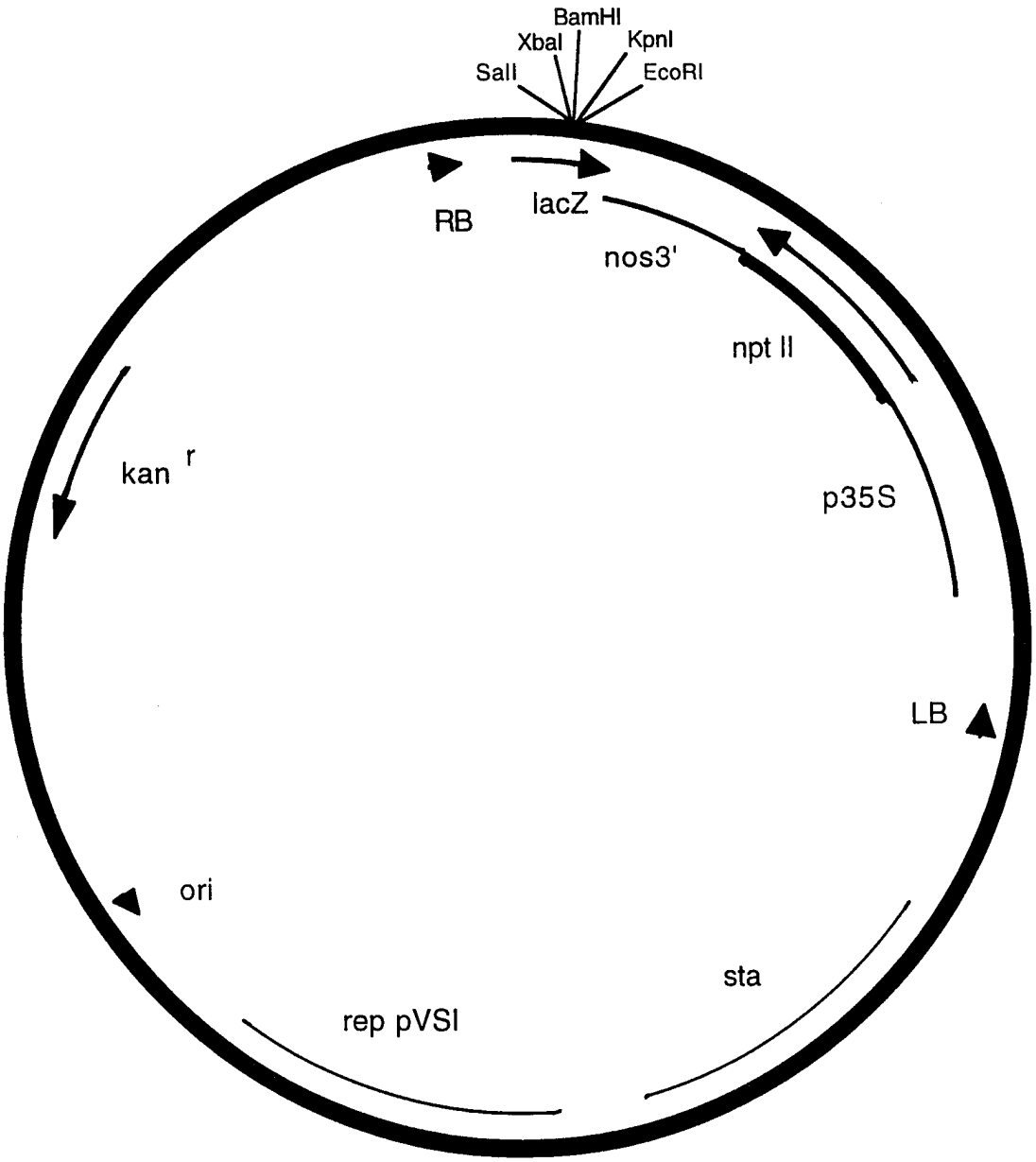
FIG.7



pBT455
(6174 bp)

8/13

FIG.8



pZS199
13.2 kb

FIG.9

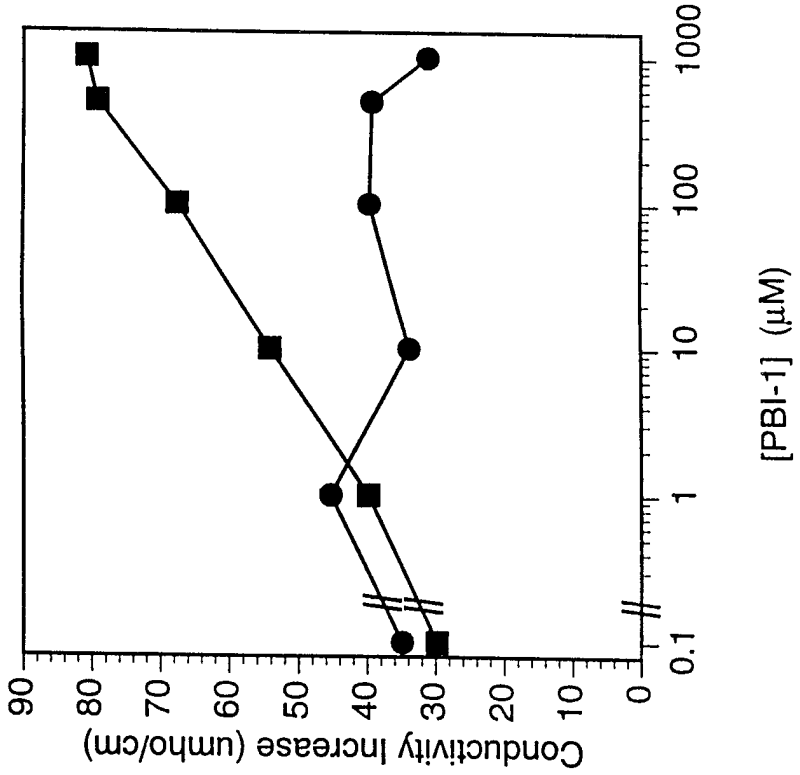

Binary Control
PROTOX-22
Conductivity Increase (umho/cm)
0
10
20
30
40
50
60
70
80
90
0.1
1
10
100
1000
[PBI-1] (μM)

FIG.10B

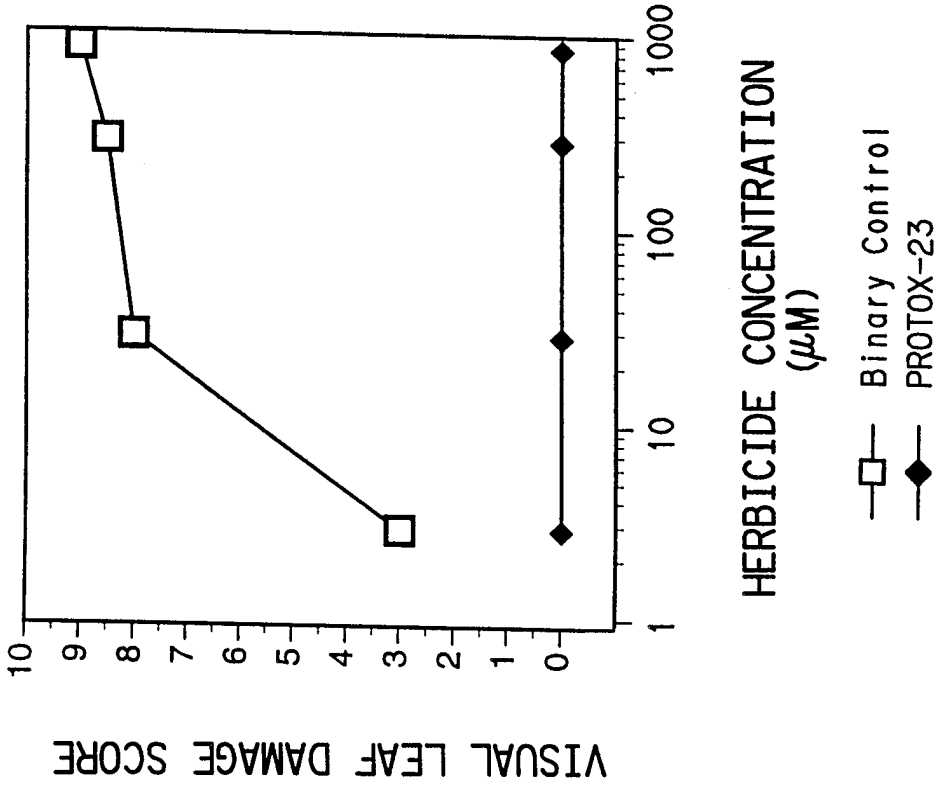


FIG.10A

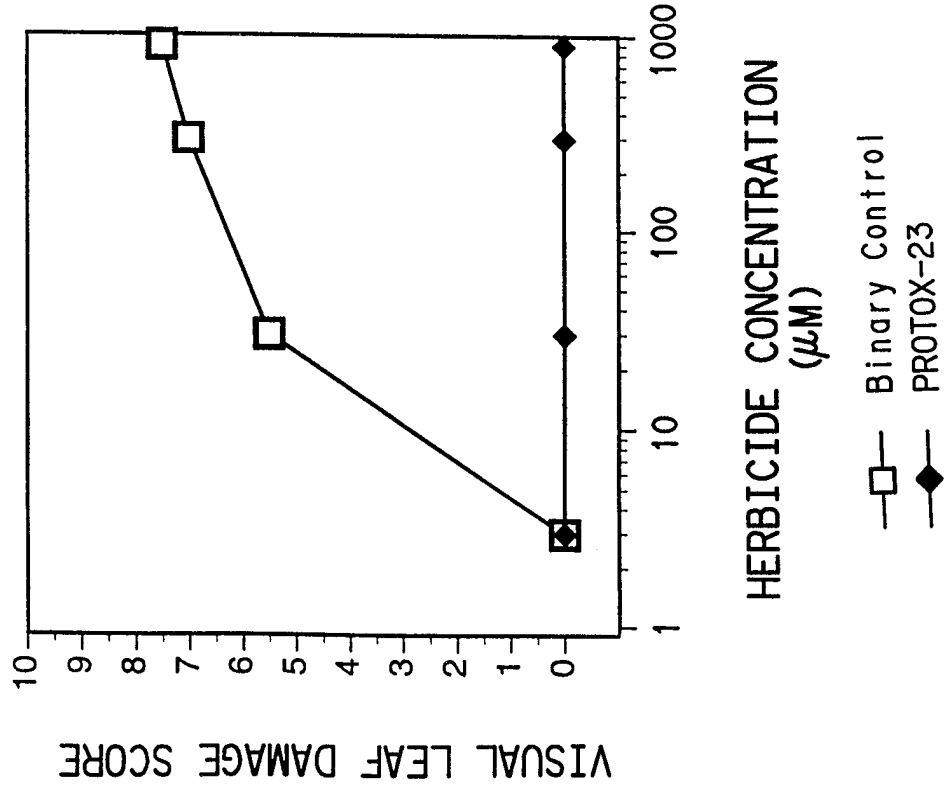


FIG.10D

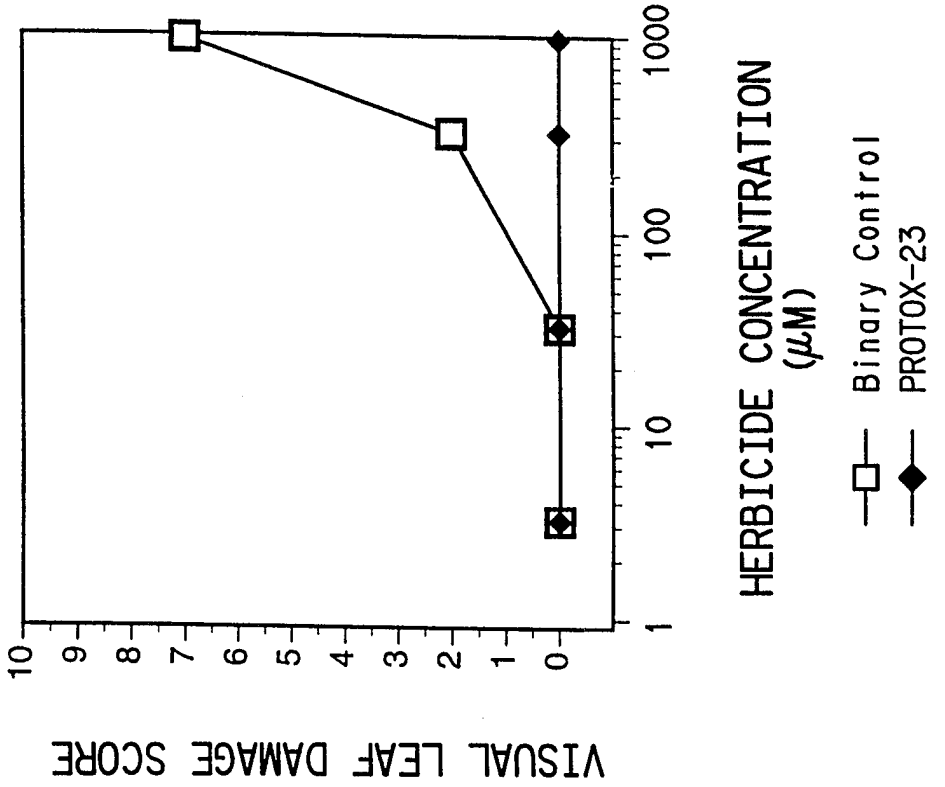


FIG.10C

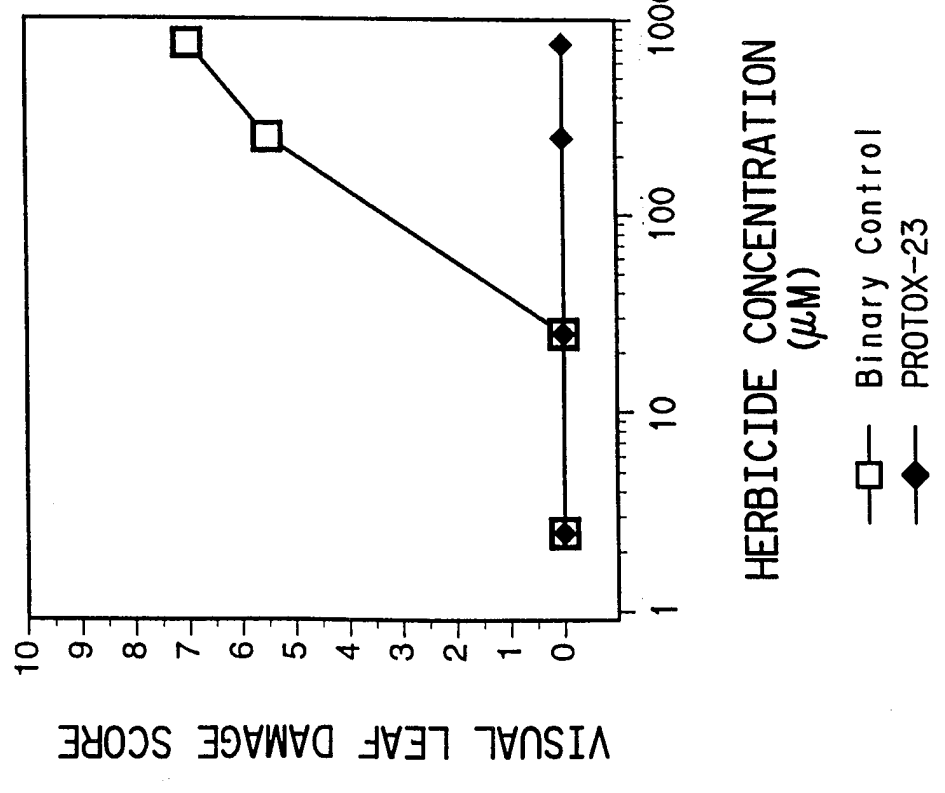


FIG.10F

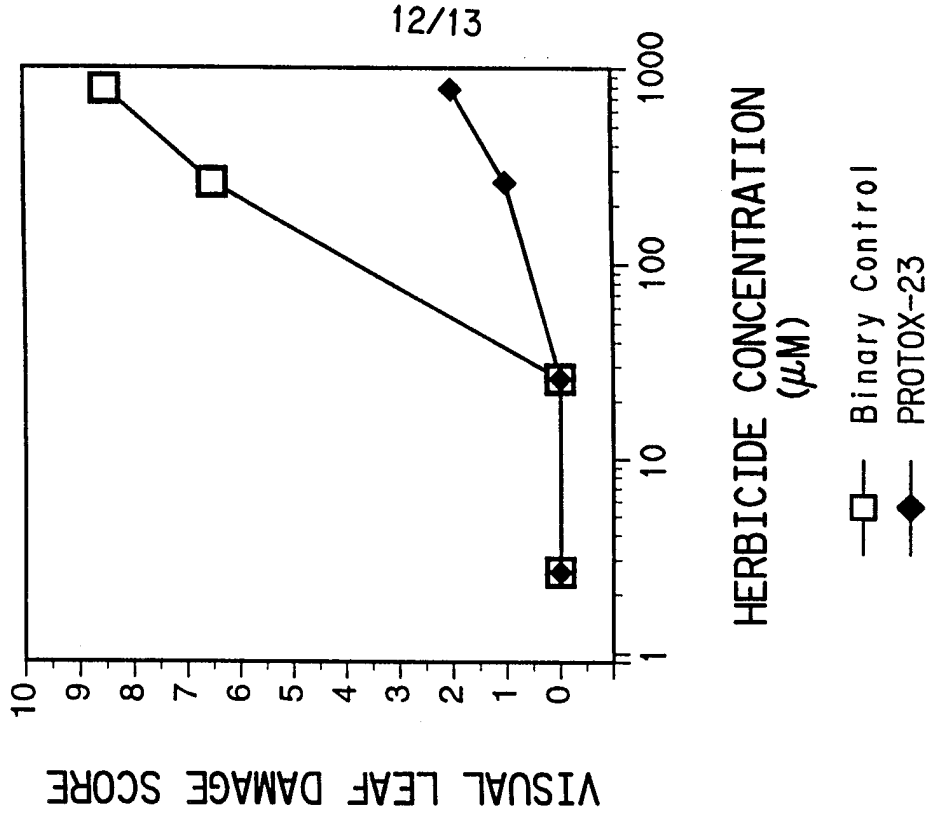


FIG.10E

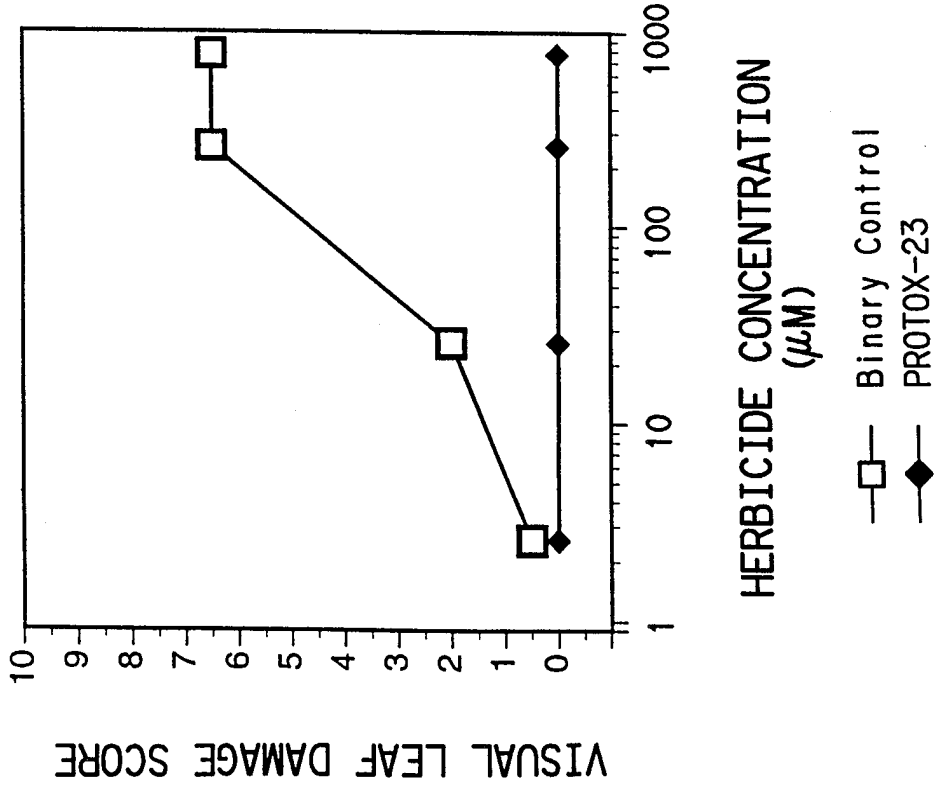
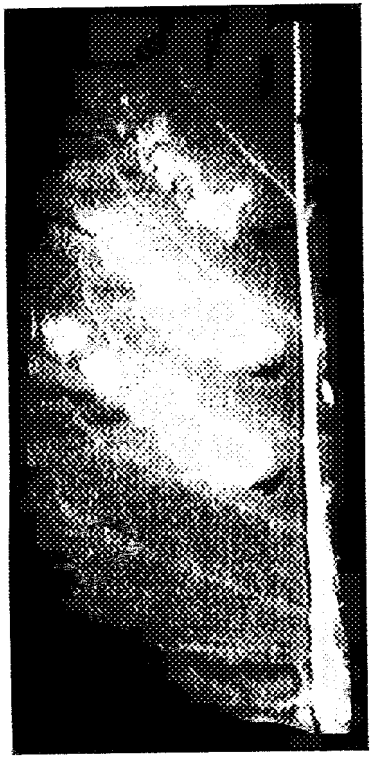


FIG. 11

Tobacco Leaf PBI Spotting Assay

Binary Control-2



[PBI-1] (μM)	Damage Score
3	0
30	5
300	10
900	10

PROTOX-24

[PBI-1] (μM)	Damage Score
3	0
30	1
300	1
900	1

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/US 98/01622

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/52 A01H5/00 A01H5/10 C12N9/02

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 6 C12N

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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP 0 770 682 A (JINRO LIMITED SEOUL) 2 May 1997 see page 3, line 14 - line 44; examples 2,4 ---	1,2,5,6, 8,9, 12-14
P,X	WO 97 32011 A (NOVARTIS AG) 4 September 1997 see page 4, line 15 - page 5, line 20 see page 25, line 23 - page 26, line 29 see page 30, line 17 - page 31, line 16 see page 38, line 10 - page 39, line 7 see page 41, line 4 - page 50, line 2; examples 19-27,34-38 --- -/--	1-5, 12-16

[X] Further documents are listed in the continuation of box C.

[X] 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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 May 1998	16/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int itional Application No
PCT/US 98/01622

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 34659 A (CIBA-GEIGY AG) 21 December 1995 cited in the application	1-10, 12-15
Y	see page 6, line 1 - page 8, line 22 see page 15, line 23 - page 21, line 28 see page 23, line 9 - page 24, line 19; examples 7,8,21-23,27	11

X	SASARMAN A. ET AL.: "Nucleotide sequence of the hemG gene involved in the protoporphyrinogen oxidase activity of Escherichia coli K12" CANADIAN JOURNAL OF MICROBIOLOGY, vol. 39, no. 12, December 1993, pages 1155-1161, XP002065603 cited in the application	17
Y	see the whole document	11

A	NARITA S.-I. ET AL.: "Molecular cloning and characterization of a cDNA that encodes protoporphyrinogen oxidase of Arabidopsis thaliana" GENE, vol. 182, no. 1-2, 5 December 1996, pages 169-175, XP002065604 see the whole document	1-17

A	NISHIMURA K. ET AL.: "Cloning of a human cDNA for protoporphyrinogen oxidase by complementation in Vivo of a hemG mutant of Escherichia coli" JOURNAL OF BIOLOGICAL CHEMISTY, vol. 270, no. 14, 7 April 1995, pages 8076-8080, XP002065605 see the whole document	1-17

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 98/01622

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0770682 A	02-05-1997	AU 686035 B	29-01-1998
		AU 4091596 A	17-04-1997
		CA 2167228 A	12-04-1997
		DE 770682 T	26-03-1998
		JP 9107833 A	28-04-1997
WO 9732011 A	04-09-1997	AU 1984697 A	16-09-1997
		AU 2065497 A	16-09-1997
		WO 9732028 A	04-09-1997
WO 9534659 A	21-12-1995	AU 2453895 A	05-01-1996
		BG 101115 A	31-10-1997
		EP 0769059 A	23-04-1997
		FI 964958 A	11-12-1996
		HU 76353 A	28-08-1997
		JP 10502524 T	10-03-1998
		PL 317759 A	28-04-1997
		SK 161096 A	08-10-1997