MAIZE H3C4 PROMOTER COMBINED WITH THE FIRST INTRON OF RICE ACTIN, CHIMERIC GENE COMPRISING IT AND TRANSFORMED PLANT

Inventors: Richard Derose, Lyon (FR); Georges Freyssinet, Saint-Cyr-Au-Mont-D'or (FR)

Correspondence Address:
CONNOLLY BOVE LODGE & HUTZ, LLP
P O BOX 2207
WILMINGTON, DE 19899 (US)

Related U.S. Application Data
Continuation of application No. 09/037,531, filed on Mar. 10, 1998, now Pat. No. 6,750,378.

Publication Classification
Int. Cl. A01H 1/00; C12N 15/82; C07H 21/04
U.S. Cl. 800/288; 435/468; 536/23.6

Abstract
The present invention relates to a DNA sequence, a 5' regulatory element allowing the expression of a heterologous gene in a plant cell from a monocotyledonous plant, characterized in that it comprises, in the direction of transcription, a first DNA sequence, which is a functional fragment of the sequence of the maize H3C4 promoter, and a second DNA sequence, which is a functional fragment of the sequence of the first intron of rice actin.

The invention also relates to a chimeric gene comprising the said DNA sequence and the plants transformed with the said gene.

pRPA-RD-195 133 base pairs - unique sites
Fig. 1

Act1 5' UTR + intron 1

Histone H3C4 promoter

NcoI

EcoRI, PstI

pRPA-RD-1002

Fig. 2

Act1 5' UTR + intron 1

Histone H3C4 promoter

GUS

NcoI

EcoRI, PstI

pRPA-RD-109

Fig. 3
MAIZE H3C4 PROMOTER COMBINED WITH THE FIRST INTRON OF RICE ACTIN, CHIMERIC GENE COMPRISING IT AND TRANSFORMED PLANT

[0001] The present invention relates to a new 5' regulatory sequence allowing the expression, in monocotyledonous plants, of a sequence heterologous to the said regulatory sequence, encoding a protein of interest. The present invention also relates to a chimeric gene comprising the said regulatory sequence, a heterologous sequence encoding a protein of interest and a 3' regulatory sequence allowing the expression of the protein of interest in a plant cell from a monocotyledonous plant, as well as a transformed monocotyledonous plant comprising the said chimeric gene and the means necessary for the transformation of plant cells and of plants.

[0002] Various promoters allowing the expression of sequences encoding proteins of interest in plants are known, are described in the literature, and have already allowed the development, to a commercial stage, of plants modified by genetic engineering. They are promoter sequences of genes which are expressed naturally in plants, in particular promoters of bacterial, viral or plant origin such as, for example, that of a gene for the ribulose bisphosphate carboxylase/oxygenase small subunit (U.S. Pat. No. 4,962,028) or of a gene of a plant virus such as, for example, that of cauliflower mosaic (U.S. Pat. No. 5,352,605). Promoters allowing the expression of heterologous genes in plants are in particular described in the following patents and patent applications: U.S. Pat. No. 5,486,169, EP 0 353 908, 5,139, 954, 5,378,619, 5,563,328, 5,589,583, 5,633,363, 5,633,439, 5,633,440, 5,633,445, 5,635,618, 5,639,948 and 5,639,952. However, some of these promoters, and more particularly the promoters of plant origin, are not functional in monocotyledonous plants.

[0003] Arabidopsis thaliana promoters described in patent application EP 0,507,698 are for example known which are particularly efficient for allowing the expression of a heterologous gene in dicotyledonous plants such as tobacco, oil seed rape or soya bean, which are not functional in monocotyledonous plants such as maize.

[0004] The rice actin promoter is a promoter known to allow the expression of heterologous genes in monocotyledonous plants (U.S. Pat. No. 5,641,876). However, the problem of identifying new functional 5' regulatory sequences for the expression of heterologous sequences in monocotyledonous plants still remains.

[0005] The present invention relates to a new DNA sequence, a 5' regulatory element allowing the expression of a heterologous gene in a plant cell from a monocotyledonous plant, the said DNA sequence comprising, in the direction of transcription, a first DNA sequence, which is a functional fragment of the sequence of the maize H3C4 promoter, and a second DNA sequence, which is a functional fragment of the sequence of the first intron of rice actin.

[0006] The sequence of the maize H3C4 promoter is in particular described by Brignone et al. (Plant. Mol. Biol., 22: 1007-1015, 1993). It is the Alul fragment of the maize H3C4 promoter, of about 1 kb, corresponding to bases -7 to -1029 relative to the ATG of the sequence encoding the maize histone H3C4.

[0007] The sequence of the first intron of rice actin is in particular described in patent U.S. Pat. No. 5,641,876.

[0008] Functional fragment is understood according to the invention to mean any DNA sequence derived from the sequence of the maize H3C4 promoter or from the sequence of the first intron of rice actin, which reproduces the function of the sequence from which it is derived.

[0009] According to one embodiment of the invention, the functional fragment of the sequence of the maize H3C4 promoter comprises the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO: 1) or a sequence homologous to the said sequence. Preferably, the functional fragment of the sequence of the maize H3C4 promoter consists of the DNA sequence described by the sequence identifier No. 1.

[0010] According to one embodiment of the invention, the functional fragment of the first intron of rice actin comprises the DNA sequence described by the sequence identifier No. 2 (SEQ ID NO: 2) or a sequence homologous to the said sequence. Preferably, the functional fragment of the first intron of rice actin consists of the DNA sequence described by the sequence identifier No. 2.

[0011] The DNA sequence, a 5' regulatory element, according to the invention may comprise, in addition, between the first and second DNA sequences, neutral DNA fragments which are generally necessary for the construction of the sequence according to the invention. These are DNA fragments comprising up to 30 base pairs, preferably up to 20 base pairs. Neutral DNA fragments are understood according to the invention to mean DNA fragments do not substantially modify the respective functions of the first and second DNA sequences of the sequence according to the invention.

[0012] According to a preferred embodiment of the invention, the DNA sequence according to the invention comprises the DNA sequence represented by the sequence identifier No. 3 (SEQ ID NO: 3) or a sequence homologous to the said sequence. More preferably, the sequence according to the invention consists of the DNA sequence represented by the sequence identifier No. 3. “Homologue” is understood according to the invention to mean a DNA sequence representing one or more sequence modifications relative to the reference DNA sequence described by the sequence identifier No. 1, 2 or 3, and reproducing the function of the abovementioned sequences. These modifications may be obtained according to the customary techniques, or alternatively by choosing the synthetic oligonucleotides which may be used in the preparation of the said sequence by hybridization. Advantageously, the degree of homology will be at least 70% relative to the reference sequence, preferably at least 80%, preferably at least 90%.

[0013] The present invention also relates to a chimeric gene (or an expression cassette) comprising a coding sequence as well as heterologous regulatory elements at the 5' and 3' positions capable of functioning in plant cells from monocotyledonous plants, in which the 5' regulatory elements comprise the DNA sequence according to the invention defined above.

[0014] “Plant cell” is understood according to the invention any cell derived from a monocotyledonous plant
and capable of constituting undifferentiated tissues such as calli, differentiated tissues such as embryos, monocotyle-
donous plant portions, monocotyledonous plants or seeds. "Monocotyledonous plant" is understood according to the
invention to mean any differentiated multicellular organism capable of photosynthesis, more particularly crop plants
intended or otherwise as animal feed or for human consumption, such as for example wheat, barley, oats, rice,
maize, sorghum, sugar cane and the like.

According to the invention, it is also possible to use, in combination with the regulatory promoter sequence
according to the invention, other regulatory sequences, which are situated between the promoter and the coding
sequence, such as the sequences encoding transit peptides, either single, or double, and in this case optionally separated
by an intermediate sequence, that is to say comprising, in the
direction of transcription, a sequence encoding a transit
peptide for a plant gene encoding a plastid localization
enzyme, a portion of sequence of the mature N-terminal
portion of a plant gene encoding a plastid localization
enzyme, and then a sequence encoding a second transit
peptide for a plant gene encoding a plastid localization
enzyme consisting of a portion of sequence of the mature
N-terminal portion of a plant gene encoding a plastid localization
enzyme, as described in application EP 0,508,
909. As transit peptide, there may also be mentioned
the signal peptide for the tobacco PR-1a gene described by
Cornelissen et al.

As regulatory terminator or polyadenylation
sequence, there may be used any corresponding sequence of
bacterial origin, such as for example the Agrobacterium
tumefaciens nos terminator, or alternatively of plant origin,
such as for example a histone terminator as described in
application EP 0,633,317.

The coding sequence of the chimeric gene according
to the invention may comprise any sequence encoding
the protein of interest which it is desired to express in a plant
cell or a monocotyledonous plant.

This may be a gene encoding a selectable marker
such as a gene conferring on the transformed monocotyle-
donous plant new agronomic properties, or a gene for
enhancing the agronomic quality of the transformed
monocotyledonous plant.

Among the genes encoding selectable markers,
there may be mentioned genes for resistance to antibiotics,
genes for tolerance to herbicides (bialaphos, glyphosate or
isoxazoles), genes encoding easily identifiable enzymes
such as the enzyme GUS, genes encoding pigments or
enzymes regulating the production of pigments in the
transformed cells. Such selectable marker genes are in particular
described in patent applications WO 91/02071 and WO
95/06128.

Among the genes conferring new agronomic prop-
eties on transformed monocotyledonous plants, there may
be mentioned the genes conferring tolerance to certain
herbicides, those conferring tolerance to certain insects,
those conferring tolerance to certain diseases and the like.
Such genes are in particular described in patent applications
WO 91/02071 and WO 95/06128.

As regulatory terminator or polyadenylation
sequence, there may be used any corresponding sequence of
bacterial origin, such as for example the Agrobacterium
tumefaciens nos terminator, or alternatively of plant origin,
such as for examples histone terminator as described in
application EP 0,633,317.

The present invention is particularly appropriate
for the expression of genes conferring tolerance to certain
herbicides on transformed plant cells and on transformed
monocotyledonous plants. Among the genes conferring
tolerance to certain herbicides, there may be mentioned the Bar
gene conferring tolerance to bialaphos, the gene encoding an
appropriate EPSPS conferring resistance to herbicides hav-
ing EPSPS as target, such as glyphosate and its salts (U.S.
Pat. Nos. 4,535,060, 4,769,061, 5,094,945, 4,940,835,
5,188,642, 4,971,908, 5,145,783, 5,310,667, 5,312,910,
5,627,061, 5,633,435, FR 2,736,926), the gene encoding
glyphosate oxidoreductase (U.S. Pat. No. 5,463,175), or
alternatively a gene encoding an HPPD conferring tolerance
to herbicides having HPPD as target, such as the isoxazoles,
in particular isoxaflutole (FR 95 06800, FR 95 13570), the
diketontetirile (EP 496 630, EP 496 631) or the triketones,
No. 5,506,195). Such genes encoding an HPPD conferring
tolerance to herbicides having HPPD as target are described
in patent application WO 96/38567 and in unpublished
patent application FR 97 14264, filed on 7 Nov. 1997, whose
content is incorporated herein by reference.

Among the genes encoding an appropriate EPSPS
conferring resistance to herbicides having EPSPS as target,
there may be mentioned more particularly the gene encoding
a plant EPSPS, in particular from maize, having two muta-
tions 102 and 106, which is described in patent application
FR 2,736,926, called hereinafter double-mutant EPSPS, or
alternatively the gene encoding an EPSPS isolated from
Agrobacterium which is described by the sequences ID 2
and ID 3 of patent U.S. Pat. No. 5,633,435, called herein-
after CP4.

Among the genes encoding an HPPD conferring
tolerance to herbicides having HPPD as target, there may be
mentioned more particularly the HPPD from Pseudomonas
and that from Arabidopsis, which are described in patent
application WO 96/38567.

In the case of the genes encoding EPSPS or HPPD,
and more particularly for the above genes, the sequence
encoding these enzymes is advantageously preceded by a
sequence encoding a transit peptide, in particular the transit
teptide called optimized transit peptide described in patents
U.S. Pat. No. 5,510,471 or U.S. Pat. No. 5,635,448 whose
content is incorporated herein by reference.

According to a preferred embodiment of the inven-
tion, the chimeric gene according to the invention com-
prises, in the direction of transcription, a 5′ regulatory
sequence according to the invention as defined above, func-
tionally linked to a sequence encoding a fusion protein
transit peptide/protein of interest, functionally linked to a 3′
regulatory sequence, the different elements of the chimeric
gene being defined above, the protein of interest being
preferably an enzyme conferring tolerance to certain herbi-
cides, more preferably enzymes of the EPSPS or HPPD type
defined above.

Sequences encoding fusion proteins transit peptide/
EPSPS, and more particularly OTP double-mutant EPSPS
are in particular described in patents U.S. Pat. Nos. 4,940,835, 5,633,448 and FR 2,736,926.

[0029] For the fusion protein OTP/CP4, persons skilled in the art will know how to construct the corresponding gene by taking the sequence encoding the CP4 described in patent U.S. Pat. No. 5,633,435 and by following the procedure described in patents U.S. Pat. Nos. 4,940,835, 5,633,448 and FR 2,736,926 or in the examples below. The present invention also relates to a chimeric gene comprising, in the direction of transcription, an appropriate 5' regulatory sequence to ensure the expression of a heterologous gene in a plant cell, functionally linked to a sequence encoding a fusion protein OTP/CP4, functionally linked to a 3' regulatory sequence. The 5' regulatory elements comprise not only the 5' regulatory elements according to the invention defined above, but also all the appropriate regulatory elements for allowing the expression of heterologous genes in plant cells from monocotyledonous or dicotyledonous plants which are known to a person skilled in the art or of the future, and in particular those described above.

[0030] The sequences encoding fusion proteins transit peptide/HPPD are described in patent application WO 96/38567.

[0031] The present invention also relates to a cloning or expression vector for the transformation of a plant cell or of a monocotyledonous plant, the transformed plant cells and plants containing at least one chimeric gene as defined above. The vector according to the invention comprises, in addition to the above chimeric gene, at least one replication origin. This vector may consist of a plasmid, a cosmid, a bacteriophage or a virus, which are transformed by introducing the chimeric gene according to the invention. Such vectors for transforming plant cells and monocotyledonous plants are well known to a person skilled in the art and are widely described in the literature. Preferably, the vector for transforming plant cells or plants according to the invention is a plasmid.

[0032] The subject of the invention is also a method of transforming plant cells by integrating at least one nucleic acid fragment or a chimeric gene as defined above, which transformation may be obtained by any appropriate known means with the vector according to the invention.

[0033] A series of methods consists in bombarding cells or cellular tissues with particles to which DNA sequences are attached. Another series of methods consists in using, as means of transfer into the plant, a chimeric gene inserted into an Agrobacterium tumefaciens Ti plasmid or an Agrobacterium rhizogenes Ri plasmid. Other methods may be used, such as microinjection or electroporation, or alternatively direct precipitation by means of PEG.

[0034] Persons skilled in the art will choose the appropriate method according to the nature of the plant cell or of the plant.

[0035] The subject of the present invention is also the plant cells or plants transformed and which contain at least one chimeric gene according to the invention defined above.

[0036] The subject of the present invention is also the plants containing transformed cells, in particular the plants regenerated from transformed cells. The regeneration is obtained by any appropriate method which depends on the nature of the species.


[0038] The present invention also relates to the transformed plants derived from the culture and/or the crossing of the above regenerated plants, as well as the seeds of transformed plants.

[0039] In the case where the chimeric gene according to the invention comprises a sequence encoding an enzyme conferring tolerance to a particular herbicide, the present invention also relates to a method of controlling weed in an area of a field comprising seeds or plants transformed with the said chimeric gene according to the invention, which method consists in applying to the said area of the field a dose of the said particular herbicide which is toxic to the said weed, without, however, substantially affecting the seeds or plants transformed with the said chimeric gene according to the invention comprising the said sequence encoding an enzyme conferring tolerance to the said particular herbicide.

[0040] The present invention also relates to a method of cultivating the plants transformed according to the invention with a chimeric gene according to the invention comprising a sequence encoding an enzyme conferring tolerance to a particular herbicide defined above, which method consists in planting the seeds of the said transformed plants in an area of a field which is appropriate for the culture of the said plants, in applying to the said area of the said field a dose of the said particular herbicide which is toxic to weeds should weeds be present, without substantially affecting the said seeds or the said transformed plants, and then in harvesting the cultivated plants when they reach the desired maturity and optionally in separating the seeds from the harvested plants.

[0041] In the above two methods, the application of the particular herbicide may be made according to the invention before sowing, before emergence and after emergence of the crop.

[0042] Advantageously, the enzyme for tolerance to a herbicide is an appropriate EPSPS, and in this case the herbicide is glyphosate or its salts, or the enzyme is an HPPD and the herbicide is chosen from the isoxazoles, in particular isoxaflutole, the diketonirolites or the triketones, in particular sulcotrine.

[0043] The examples below make it possible to illustrate the invention without seeking to limit its scope.

[0044] 1. Construction of a Chimeric Gene with a Sequence Encoding an HPPD:

[0045] The plasmids below are prepared so as to create an expression cassette comprising a maize H3C4 histone promoter combined with the untranslated 5' region of the first
intron of the rice actin gene (ActI) described by Mc Elroy D. et al. (Plant Molecular Biology 15: 257-268 (1990)) directing the expression of the gene OTP-HPPD of Pseudomonas fluorescens.

[0046] PRPA-RD-195

[0047] The plasmid pRPA-RD-195 is a derivative of the plasmid pUC-19 which contains a modified multiple cloning site. The complementary oligonucleotides 1 and 2 below are hybridized at 65°C. For 5 minutes, followed by a slow cooling down to 30°C over 30 minutes:

[0048] Oligo 4: 5’ AGGGCCCTCT AGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCGGGGGATC CTCCTAGATC GACCTGACG CATGC 3’

[0049] Oligo 5: 5’ CCCTGAACCA GGCTGAGGG CGGCCCTTAA TAT AAA GCTT GCATGCTGCG AGGTCGACTC TAGGG 3’

[0050] The hybridized oligonucleotides are made double-stranded using the Klenow fragment of DNA polymerase I of E. coli to extend the 3’ ends of each oligo using the standard conditions recommended by the manufacturer (New England Biolabs). The double-stranded oligo obtained is then linked in the plasmid pUC-19 previously digested with the restriction enzymes EcoRI and HindIII and made blunt-ended using the Klenow fragment of DNA polymerase I of E. coli. A cloning vector is thus obtained which comprises a multiple cloning site so as to facilitate the introduction of expression cassettes into a plasmid vector of Agrobacterium tumefaciens (FIG. 1).

[0051] pRPA-RD-2010

[0052] Insertion of the sequence “H4A748 promoter-OTP-double mutant EPSPS gene” of pRPA-RD-159 into the plasmid pRPA-RD-195.

[0053] The plasmid pRPA-RD-195 is digested with the restriction enzyme SacI and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). The plasmid pRPA-RD-173 (described in patent FR 2,736,926) is digested with the restriction enzyme SacI and the DNA fragment containing the EPSPS gene is purified and linked into the plasmid pRPA-RD-195 prepared above. The clone obtained contains several unique restriction sites flanking the double-mutant EPSPS gene.

[0054] pRPA-RD-1002

[0055] Creation of an expression cassette OTP-HPPD for use in monocotyledonous plants. The plasmid pRP-P contains the optimized transit peptide (OTP) linked to the HPPD of Pseudomonas fluorescens followed by the polyadenylation site of nopaline synthase as described in patent application WO 96/38567. The components of the plasmid pRP-P are the following:

[0056] the optimized transit peptide (OTP) described in patents U.S. Pat. No. 5,510,471 and No. 5,633, 448; this OTP consisting of 171 bp of the Helianthus annuus ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit transit peptide (Waksman G. et al. 1987. Nucleic Acids Res. 15: 7181) which are followed by the 66 bp of the mature portion of the Zea mays ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (Lebrun et al. 1987. Nucleics Acids Res. 15: 4360) which are themselves followed by the 150 bp of the Zea mays ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit transit peptide (Lebrun et al. 1987. Nucleic Acids Res. 15: 4360); the combination is therefore 387 bp;

[0057] the coding region of the HPPD of Pseudomonas fluorescens described in patent application WO 96/38567; and


[0059] The plasmid pRP-P is digested with the restriction enzyme BstEII, treated with the Klenow fragment of DNA polymerase I of E. coli in order to make the fragment blunt-ended, and followd by digestion with the restriction enzyme NcoI. The DNA fragment obtained, containing the coding region OTP-HPPD about 1.5 kb, is then purified. The plasmid pRPA-RD-2010 obtained above is digested with the restriction enzyme BglII treated with the Klenow fragment of DNA polymerase I of E. coli in order to obtain a blunt-ended fragment, and then digested with the restriction enzyme Neol. The DNA fragment obtained, comprising the sequences of the plasmid vector, the H3C4 promoter combined with the untranslated 5’ region and the first intron of the rice actin gene, is purified and the NOS polyadenylation site is purified. The two DNA fragments purified are linked so as to create an expression cassette OTP-HPPD comprising the maize H3C4 histone promoter (Brignon et al.) combined with the 5’ untranslated region and the first intron of the rice actin gene (ActI) (Act 5’ UTR + intron 1) in order to control the expression of the coding region OTP-HPPD incorporating the NOS polyadenylation site (NOS polyA) (FIG. 2).

[0060] 2. Construction of a Chimeric Gene with a Sequence Encoding the Double-Mutant EPSPS

[0061] pRPA-RD-1010

[0062] Creation of an expression cassette OTP-double mutant EPSPS for use in monocotyledonous plants.

[0063] The plasmid pRPA-RD-109 contains the β-glucuronidase (GUS) gene of E. coli controlled by the maize H3C4 histone promoter (Brignon et al.) combined with the 5’ untranslated region and the first intron of the rice actin gene (ActI) described by Mc Elroy D. et al. (Plant Molecular Biology 15: 257-268, 1990). A diagram of this plasmid is represented in FIG. 3. The plasmid pRPA-RD-109 is digested with the restriction enzymes Neol and EcoRI, and the large DNA fragment (about 5 kb) containing the vector sequence, the GUS gene and the NOS polyadenylation site is purified. The plasmid pRPA-RD-2010 is digested with the restriction enzymes Neol and EcoRI, and the DNA fragment (about 1.6 kb) containing the H3C4 promoter combined with the 5’ untranslated region and the first intron of the rice actin gene (ActI) is purified. The two DNA fragments purified are linked in order to create an expression cassette OTP-double mutant EPSPS comprising the maize H3C4 histone promoter (Brignon et al.) combined with the 5’ untranslated region and the first intron of the rice actin gene (ActI) in order to control the expression of the coding region OTP-double mutant EPSPS incorporating the NOS polyadenylation site.
3. Construction of a Chimeric Gene For Tolerance to Phosphinothricin (Bar Gene)

The phosphinothricin acetyl transferase (PAT) encoded by the bar gene is an enzyme which inactivates a herbicide, phosphinothricin (PPT). PPT inhibits the synthesis of glutamine and causes a rapid accumulation of ammonia in the cells, leading to their death (Tachibana et al. 1986).

The plasmid used to introduce the tolerance to phosphinothricin as selection agent is obtained by inserting the chimeric gene pDM 302 into the vector pSP72 of 2462 bp, marketed by Promega Corp. (Genbank/DDBJ database accession number X65332) and containing the gene for resistance to ampicillin.


The various components of this plasmid are:

- the promoter of the rice actin gene described by Mc Elroy D. et al. Plant Molecular Biology 15: 257-268 (1990) consisting of 840 bp;
- the first exon of the rice actin gene consisting of 80 bp;
- the first intron of the rice actin gene consisting of 450 bp;
- the region encoding the bar gene of 600 bp excised from the plasmid pIJ41404 described by White J. et al. Nuc. Acids Res. 18: 1862 (1990);
- the terminator of the nopaline synthase (nos) gene (polyadenylation zone of the nos gene isolated from pTi 37, 250 bp; (Bevan M. et al. Nucleic Acids Res. 11: 369-385).

4. Transformation of Maize Cells

The particle bombardment technique is used to introduce the genetic construct. The plasmids are purified on a Qiagen column and coprecipitated on M10 tungsten particles according to the Klein method (Nature 327: 70-73, 1987).

A mixture of metal particles, of the plasmid pRPARD-1002 and of the plasmid of Example 3 which are described above, is then bombarded onto embryogenic maize cells according to the protocol described by Gordon-Kamm, W.J. et al. (Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants, The Plant Cell, vol. 2, 603-618, July 1990).

5. Regeneration and Use of the Bar Gene as Selection Agent

The bombarded calli are selected on glufosinate until green sectors appear. The glufosinate-resistant positive calli are then converted to somatic embryos, and then placed under conditions which promote germination according to the operating conditions described by Gordon-Kamm, W.J. et al. (Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants, The Plant Cell, vol. 2, 603-618, July 1990). The young plants are transferred to a greenhouse for the production of seeds.

6. Analysis of the Progeny of the Transformed Plants

The transformed plants obtained above are assumed in part to be transgenic, comprising a heterologous gene encoding OTP/HPPD conferring tolerance to isoxazoles such as isoxaflutole. These transformed plants produced pollen, which fertilized ovules from a nontransgenic wild-type maize. The seeds obtained are selected on sand after treating with isoxaflutole. The selection protocol is the following:

- 800 ml of Fontainebleau sand are placed in a tub of sides 15x20 cm. These tubes are then sprinkled with water and kept moist by supplying a nutrient solution consisting of 5 ml of Quinoligo (Quinoline) per litre of water. Twenty maize seeds are placed in the tubes, which are then treated with isoxaflutole by spraying at a rate of 100 g of active material per hectare (300 µg of active material per tub). The tubs are then cultured in a greenhouse. The phytotoxicity is determined 14 [lacuna] after planting. According to the above conditions, the nontransformed plants exhibit 100% phytotoxicity whereas the transformed plants exhibit no phytotoxicity.

A comparative study was carried out with 20 maize lines transformed according to the invention and 20 maize lines transformed with a corresponding gene for which the sequence encoding the first intron of rice actin has been replaced with the sequence encoding the maize adh I intron. After treating by spraying very high doses of isoxaflutole at a rate of 200 g of active material per hectare (600 µg of active material per tub), the following results are obtained:

- 8/20 lines are tolerant
- 3/20 lines are tolerant

The results above demonstrate that the combination of the maize H3C4 promoter with the first intron of rice actin according to the invention substantially enhances the expression of a protein of interest in transformed monocotyledonous plants compared with the combination of the same maize H3C4 promoter with another intron of the state of the art.
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1021 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

CTTATGCA CCACTTACG TAATGATAA CCACTTAATT GAATACTCAA CTTTTCTATT 60
ACTCTTTAC TAAACATAAT GTGGTTTTTA AAATTCAGTC TCAACATTC ATGGCTCAG 120
TATAAGTCTA GACTCTCAA AATTACATTT TTAATCCTTC ATATTTTTTT TCTTATACA 180
CATTTGGCGC CTCACTAATCC ATGATCTTA CACACCTCCT GTGCTCAACATTCAG 240
CCATCTCGT AACCTATGTC CTCTCAATAA AGCTTCTCTA ATCAGCTGCT CTATAACGAA 300
TACCTATATT AGAGCAATTT TTTATTTTTT GTACATAACAT ATTTGTCAATA CTATCAATA 360
CATTACAT ATTATGATTT ACTAAACCAG TATTTTAAAG TATTCGAAGC GATGAAGAC 420
TGGTGGATATA AATATCAATAT ATAGAGAATC CAGTACGTTT CTCTATATTG AGATGATT 480
T TAGAGAGGC ATCTAGAAA ACGTAAAATC TTTCTATTAT ATTATATTTT AGGGTAGAGT 540
AGGCTTTAGT CTCTTATAGT CTGGTGGGA CCAAGCTTTA TACCGTTAT TTTGTCTTAT 600
GGCGCTCTCA TTTTCACTCC AGGGCCCAAC ATTTTACGCT TTTCCCAGAAA GGGCAGGCC 660
TGCTACCACT ACAATGTGTC AGTGGTGCCC GTTGGTCAAAC AAGATGACGC AACACCATGC 720
ACCCACCACT CATAGCCAGC TGGATCCTCT CCAAGATTAG TCTCAAGCAGA TAGGGCCCA 780
GAACACACCA TCGCCTGCAT CTGCCCTCAG CGTGCACCTC ATCGCGGCAG TCCACCTCAG 840
TCCAACACCT ATGGGGCTAC CTTGCCTCAC CTCCGAAAAT ATTCTGCAGT CGGCTCGGC 900
ACCTACTACA ATACACAGTT CCATACGAGC CATCAGCGCT ACTCCGAAAT CCCCGAGAAA 960
ATCAACACCT CCAAACTCGA CGCTGGCCAC AACTGCGCTG CCTCCGCGGC AAGCACAAA 1020
G 1021

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GTAACCCACC CGCCCCCTCTC CTCTTTTTTT TTGCTGGTTT CGTCTTGAGT 60
CTTCGCTTTC GTAGTCTTGGG GTGCGGCCAGA GGGCGTTCTG GGCAGAGTC GTGCGGCCAG 120
AGGGTGGGAA TCCTGGCGGCT GCCGTCTCGG GCGCTGAGTC GGGCGCGATC CTGCGCGGGA 180
AGGGGCTCTC CGAGCTGAGA CTGGATGGCG AGAGATGAGTGG GGGCGTTTTAA 240
AATTTGCCCA CTCTAACAAG GATCGAGAAG AGGGGAAGAC GCGACTATGCT TTAATATT 300
TATATTCTC TCCTGGCGCT CTTAGGGGTC AGATGTGCTA GTCTTTGCTT TTCTCTTATT 360
GTGGTCTAGAA TTGAACCTCC TCAGATGCTG TGTGCGTGAAT TTTCCTTTT ATGATGATTG 420
GACAAAGCTA GCCTCGTGCG GAGCTTTTTTT GTAG 454
(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2565 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GAATTCCTGC AGTCCAGCG ACTCCCCCTTA TGTCGACCAT TTACTUTAAAT GCATATACT
60
TTAATTGAAT AGCACAATTT CTTATACTCT TTTATCAAAC ATAAATCTTG GTTTTAAAT
120
TCAATCCTCA AAGTTCAATC CTCAAGTATA AGTCTGAACT GCAGAATAAT ACTACATTT
180
TTCTCTATAT TTTTTTTTCT TATACACATT TGGGGCTTTA CAAAACATCA TCTATATCCA
240
TCTTTTCCGG TGGCCTTTAT AAGATTTCAA CTCGTCGAAT TCTACATTTCT CCAAAAGCG
300
TCTCTAAATC AGCTCTCAT ATGAATAATAC TTATTTTAGA ATCACTTTT TTTTTTTTAC
360
ATCATATTG GCTGACTATC CAAAACATT ATACATATTT AGTATTACTA AAAAAAGTT
420
TTAAATTATT CAAACCTCAG TGGCACTTCT TAGAAATATT CTATATATAT CCAAAACT
480
ACGCTTCTAG AATATTATAG GAGCAATCTT TAAACAACTG TAAAAATAT
540
TTGATATTAT AATATTGAGG TAGAATGCC CTTATCCTTT ATGAGATCTT GGTGGAACCA
600
GCCCTATCAG GTTATTTTAC GAGGGCGCT CTCTGATTTT CACTCCAGG CCCGACATT
660
TCACGTTTTT CGGAGGCAGG CCCCATTCCC AAAACCAAAA AAAAACTCT
720
TCAAAAAAG GTAGAGCTCC ATCTCGACCC ACCGACATG TGGCCCTCGG ATCCCGCTCG
780
AATAAGCTCT AGCCATAGG AGCCAGAAC CACCCAGACG GGGATGTCG CTCAGAGCT
840
CACCCATAGG GGCGATCAGC TCTCACTTCA AGCCATATG CACTCAGTGG CCGCCCATCC
900
GAAAAAATTC TGGGCGCGCG CTCTCGCTCT ACTAACATAA CCCATCCCCA CCAGAGCAT
960
GGCCTAGCTG CCAATTCCTCC ATGAAAAAGC ACCATCCCA ATCTCGACCC GCCCAGACT
1020
CGCGGTCCTG CGGCGCGAGG ACCAAAGGA TGGGCGCAGG CGGGCGTTGA CTCCTCCTCC
1080
CTCCCCGCTC CGGCGGGGCC GGAACGCCAC CCCTCTCTCT TTCTTCGGT
1140
TTTTTTCCTG TGCTGTCCAG TCTTGGCTCT TGTTAGTGGG GGGCGGTGAG AGGCGCTTCC
1200
TGGCCCCAGT CGATCCGCGC GAGGCGCGGG ATCTCCGCGC TGAGGCTTCC CGCCGCTGAT
1260
CGCCGCGAGT CCGGCGCGG AAAOGCCCGT TGCACTAGT ATCGAATGCG CCGTGTTGATG
1320
GGGAGTACG GGGCGGTTCAA ATCTCGAGCC AGGACGAAGA GGGCAAAAGG
1380
GGCGACTAG GTTTTATTTT TTATATATTT CTGGCTCTTG TCGAGCGGG TGAAGGATGT
1440
AGAGCTGTCT TCTGTCTGTGT TGGGTGAGA AGTGAATTCC CTGCGAATGG GACACGGTTA
1500
GGTTTTTCTT TCGTGGATCG TGACAAAGTC AGGCTCGGCG GGAACGTTTAG TGGTACGAGA
1560
CCATG
1565

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
1-21 (canceled)
23-35 (canceled)
35. An isolated DNA sequence comprising, in the direction of transcription, a functional fragment of the sequence of the maize H3C4 promoter and a functional fragment of the first intron of rice actin.
36. The DNA sequence according to claim 35 wherein said sequence of the maize H3C4 promoter is the AtuI fragment of the maize H3C4 promoter.
37. The DNA sequence according to claim 35 wherein the functional fragment of the sequence of the maize H3C4 promoter comprises a sequence homologous to SEQ ID NO: 1.
38. The DNA sequence of claim 37 wherein said functional fragment of the first intron of rice actin comprises a sequence homologous to SEQ ID NO: 2.
39. The DNA sequence of claim 35 comprising a neutral DNA fragment between said functional fragment of the sequence of the maize H3C4 promoter and said functional fragment of the first intron of rice actin.
40. An isolated DNA sequence comprising a sequence homologous to SEQ ID NO: 3.
41. An expression cassette comprising a coding sequence and heterologous regulatory elements at the 5' and 3' positions relative to the coding sequence capable of functioning in monocotyledonous plant cells or monocotyledonous plants, wherein the 5' regulatory element comprises the DNA sequence of claim 35.
42. The expression cassette of claim 41 wherein said coding sequence is a DNA sequence encoding a protein of interest.
43. The expression cassette of claim 41, wherein the DNA sequence encoding a protein of interest is a DNA sequence encoding a selectable marker.
44. The expression cassette of claim 41 wherein said protein of interest is selected from the group consisting of a DNA sequence encoding a protein that confers herbicide tolerance, a DNA sequence encoding a protein that confers insect tolerance, and a DNA sequence encoding a protein that confers disease tolerance.
45. The expression cassette of claim 44 wherein said wherein the DNA sequence encoding a protein that confers herbicide tolerance is selected from the group consisting of a DNA sequence encoding PAT conferring tolerance to bialophos, a DNA sequence encoding an EPSPS conferring resistance to herbicides having EPSPS as a target, a DNA sequence encoding glyphosate oxidoreductase, and a DNA sequence encoding an HPPD conferring tolerance to herbicides having HPPD as a target.
46. The expression cassette of claim 45 wherein the DNA sequence encoding a protein that confers herbicide tolerance is a DNA sequence encoding an EPSPS or a DNA sequence encoding an HPPD.
47. The expression cassette of claim 46 wherein the DNA sequence encoding a protein that confers herbicide tolerance is a DNA sequence encoding CP4 or a double-mutant EPSPS.
48. The expression cassette of claim 44 wherein said DNA sequence is preceded by a sequence encoding a transit peptide.
49. The expression cassette according to claim 48 wherein said transit peptide is the optimized transit peptide.
50. An expression cassette comprising, in the direction of transcription, a 5' regulatory element of claim 1 functionally linked to a sequence encoding a fusion protein, functionally linked to a b 3' regulatory sequence, wherein said fusion protein comprises a transit peptide linked to a protein of interest.
51. The expression cassette of claim 50, wherein said protein of interest is a protein that confers herbicide tolerance selected from the group consisting of PAT, EPSPS, glyphosate oxidoreductase, and HPPD.
52. The expression cassette of claim 51, wherein the sequence encoding a protein of interest is a sequence encoding the fusion protein OTP/double-mutant EPSPS or a sequence encoding the fusion protein OTP/CP4.
53. An isolated DNA sequence encoding a fusion protein OTP/CP4.
54. An expression cassette comprising in the direction of transcription, a 5' regulatory sequence functionally linked to a sequence encoding a fusion protein OTP/CP4, optionally linked to a 3' regulatory sequence, wherein said expression cassette functions in plants or plant cells.

55. A cloning or expression vector for the transformation of a plant cell or of a plant, which comprises an expression cassette of claim 41 and at least one replication origin.

56. The vector of 55, wherein said vector is a plasmid.

57. A method of transforming plant cells, comprising integrating the expression vector of claim 41 into plant cells.

58. A plant cell which contains at least one expression cassette of claim 41.

59. A transformed plant which comprises a plant cell of claim 58.

60. A transformed plant which is regenerated from a plant cell of claim 58.

61. A transformed plant produced from the culture of a transformed plant of claim 59 or the crossing of a transformed plant of claim 59 with another plant.

62. A transformed seed of the transformed plant of claim 59.

63. A method of controlling weeds in a field comprising weeds and seeds or plants, said seeds or plants each comprising the expression cassette according to claim 41, which method comprises applying to the field a dose of herbicide which is toxic to the weeds but to which the seeds or plants are tolerant.

64. A method of cultivating plants transformed with the expression cassette of claim 41, which method comprises, sowing seeds comprising said expression cassette in a field comprising weeds; cultivating plants from the seeds; applying to the field a dose of herbicide which is toxic to the weeds but to which the seeds or plants are tolerant prior to sowing the seeds or during cultivation of the seeds; and; harvesting the cultivated plants when they reach maturity.

65. The method of claim 64 wherein said herbicide is applied before sowing the seeds, before emergence of the plants or after emergence of the plants.

* * * * *