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(54) Title: GLYPHOSATE TOLERANT CORN EVENT VCO-Ø1981-5 AND KIT AND METHOD FOR DETECTING THE SAME

(57) Abstract: The present invention relates to the field of plant transformation with genes conferring tolerance to glyphosate. The invention particularly relates to a maize (corn) plant transformed with a gene encoding an EPSPS providing the plant tolerance to an application of glyphosate under conditions where this herbicide is effective in killing weeds. The invention particularly concerns an elite transformation event VCO-Ø1981-5 comprising the gene construct and means, kits and methods for detecting the presence of the said elite event.

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Glyphosate tolerant corn event VCO-Ø1981-5 and kit and method for detecting the same

The present invention relates to the field of plant transformation with genes conferring tolerance to glyphosate. The invention particularly relates to a maize (corn) plant transformed with a gene encoding an EPSPS providing the plant tolerance to an application of glyphosate under conditions where this herbicide is effective in killing weeds.

The invention particularly concerns an elite transformation event comprising the gene construct and means, kits and methods for detecting the presence of the said elite event.

BACKGROUND OF THE INVENTION

Glyphosate tolerant plants are known in the art and well studied in the past two decades. Glyphosate is an herbicide inhibiting EPSPS which is an enzyme whose activity is upstream of the aromatic amino acids pathway leading to the synthesis of the amino acids tyrosine, tryptophan and phenylalanine. Since glyphosate is a systemic total herbicide, tolerance in the plant when the herbicide is sprayed under usual agronomic conditions may only be achieved by genetic modification of all cells of the plants with an heterologous gene coding for a glyphosate insensitive EPSPS enzyme, either mutated or selected from microorganisms known to have evolved such insensitive EPSPS enzyme.

Glyphosate insensitive EPSPS, gene constructs and plants transformed with said gene constructs are disclosed inter alia in EP 507 698, EP 508 909, US 4 535 060, US 5 436 389, WO 92/04449, WO 92/06201, WO 95/06128, WO 97/04103, WO 2007/064828 and WO 2008/100353, and in references cited herein.

The biophysical characteristics of the EPSPS protein are essential to achieve a good level of tolerance to glyphosate. However, the choice of regulatory elements providing an adequate expression level of the insensitive protein in the plant is also important, as well as the selection of a transformation event, corresponding to a stable line with a stable and limited number of copies of the gene being inserted in the genome of the plant, as well as its stability in the locus where the gene has been inserted is also important to obtain glyphosate tolerance at a commercial level, sufficient for the plant to be used for the preparation of seeds to be planted in a field with a level of tolerance to glyphosate under agronomic conditions sufficient to allow use of the herbicide at effective concentrations to

kill the weeds without affecting growing conditions and yields of the crop transformed with the gene encoding EPSPS protein.

Transformation events selected for the preparation of commercial varieties of glyphosate tolerant maize (corn) are known in the art, particularly disclosed in US 6 040 497 and EP 1 167 531.

These varieties of the first generation used for the preparation of commercial plants currently used in the field have some drawbacks.

The event GA21 disclosed in US 6 040 497 comprise multiple copies of a gene construct comprising a rice actin promoter and intron, a sequence coding for an optimized transit peptide, as disclosed in EP 505 909 and a sequence coding for a mutated plant EPSPS comprising two mutations as disclosed in WO 97/04103. The commercially required level of tolerance in the transformation event is obtained with a complex transit peptide and multiple copies of the chimeric gene construct.

The event NK603 disclosed in EP 1 167 531, is also a complex event with the combination of two gene constructs in one locus. The first gene construct comprises a rice actin promoter and intron, with a sequence coding for an *Arabidopsis* EPSPS transit peptide and a sequence coding for a type II EPSPS resistant to inhibition by glyphosate, isolated from *Agrobacterium* strain CP4. The second gene construct comprises the CaMV 35S promoter and the rice actin intron, with a sequence coding for an *Arabidopsis* EPSPS transit peptide and a sequence coding for a type II EPSPS resistant to inhibition by glyphosate, isolated from *Agrobacterium* strain CP4.

There is a need for a new generation of transformation events allowing a high glyphosate tolerance to maize (corn) plants grown under agronomic conditions with a single copy of the foreign gene construct in the plant genome.

SUMMARY OF THE INVENTION

The invention concerns a maize (corn) plant comprising the event VCO-Ø1981-5 representative seeds deposited with NCIMB with accession number 41842.

The invention also concerns a maize (corn) plant comprising the VCO-Ø1981-5 event characterized by the presence of a genomic flanking sequence-gene construct junctions comprising the sequences of SEQIDN°1 and/ or SEQIDN°2 or SEQIDN°3.

The invention also concerns corn plants progenies comprising the VCO-Ø1981-5 event of the invention, characterized by the presence of the said junctions sequences.

Probes to identify the presence of said junction sequences in a maize (corn) plant genome, as well as kits and methods for such identification comprising said probes and their uses, particularly a method for the detection of the VCO-Ø1981-5 event and primers, probes and a kit for such a detection are also part of the invention.

DETAILED DESCRIPTION OF THE INVENTION

"Transformation event" means a product of plant cell transformation with a heterologous DNA construct, the regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion of the gene construct into a particular genome location.

"Gene construct" means, according to the invention, a gene constructed from different nucleotide sequences, comprising regulatory elements controlling the expression and translation of a coding sequence in a host organism. The host organism in the invention is particularly maize (corn), cells, tissues and whole plants. The gene construct comprises a promoter region, operably linked to a coding sequence and a terminator region. It may comprise enhancers, such as introns, generally linked downstream the promoter region and upstream the coding region. In the case of glyphosate tolerance, the coding sequence comprise a sequence coding for a chloroplast transit peptide, linked to the sequence coding for an EPSPS enzyme selected for its resistance to inhibition by glyphosate, either mutated or selected or selected and mutated from microorganism having developed resistance to glyphosate.

The gene construct in the event of the invention comprises a DNA molecule of a sugarcane ubiquitin promoter and intron, operably linked to a DNA molecule coding for the maize acetohydroxyacid synthase (AHAS) transit peptide, operably linked to a DNA molecule coding for the *Arthrobacter globiformis* EPSPS GRG23ACE5. The gene construct also comprises a terminator sequence, particularly the terminator sequence of the 35S CaMV transcript.

The various elements of the gene construct of the invention are isolated and operably linked according to usual techniques of molecular biology known and available to the person skilled in the art.

"Ubiquitin promoter and intron" means the promoter from sugarcane ubiquitin-4 gene and the intron from sugarcane ubiquitin-4 gene, from the non-coding 5' region of the

ubiquitin-4 gene of *Saccharum officinarum* L. as disclosed in Albert and Wei (US Patent 6,638,766) and set forth in SEQ ID NO 4 and 5, respectively.

"Maize AHAS chloroplast transit peptide" is the N-terminal transit peptide sequence derived from the *Zea mays* L. (maize) acetohydroxyacid synthase (AHAS) gene, as disclosed in Fang et al (1992) and set forth in SEQ ID NO 6.

"*Arthrobacter globiformis epspsgrg23ace5*" means the nucleotide sequence as set forth in SEQ ID NO 28 of WO 2008/100353. (SEQ ID N°7).

"35 CaMV terminator sequence" is the non-coding 3' end from the cauliflower mosaic virus which terminates mRNA transcription and induces polyadenylation as disclosed in Gardner et al (1981) and set forth in SEQ ID NO 8.

"Plant transformation" and selection of transformed plants is widely disclosed in the art, and more particularly corn transformation. Techniques for corn transformation and breeding are now well known in the art, and particularly disclosed in laboratory notebooks and manuals such as "Transgenic Plants: Methods and Protocols (Methods in Molecular Biology)" (Leandro Peña, Humana Press Inc., 2005), "Heterosis and Hybrid Seed Production in Agronomic Crops" (Amarjit Basra, The Harwoth Press Inc., 1999) and "The Maize Handbook" (Michael Freeling and Virginia Walbot, Springer Lab Manuals, 1994). The transformation of corn is more particularly performed with an *Agrobacterium* mediated transformation comprising a transformation vector (Hiei and Komari, 1997, US Patent 5591616).

The transformation of a plant with a gene construct generally comprises the steps of

a) inoculating a plant cell with a strain of *Agrobacterium tumefaciens* comprising a transformation vector comprising the gene construct;

b) selecting the plant cells having integrated into their genome the gene construct of the invention;

c) regenerating a fertile plant from the selected plant cell;

d) pollinating the regenerated plant, and;

e) selecting progeny plants tolerant to high doses of glyphosate, then;

f) selecting the plants having stably integrated one unique copy of the gene construct of the invention.

"Transformation vectors" means a DNA molecule comprising the gene construct and additional DNA elements allowing introduction of the gene construct into a plant cell and integration of said gene construct into the genome of the plant cell. Transformation is

an *Agrobacterium* mediated transformation, wherein the transformation vector comprises right and left borders of a T-DNA plasmid from *Agrobacterium tumefaciens* flanking the gene construct to be inserted. Such transformation vectors are well disclosed in the art and readily available to the person skilled in the art of plant molecular and cellular biology and plant transformation.

"Right and left borders of a T-DNA plasmid from *Agrobacterium tumefaciens*" are DNA sequences of the right and left border sequences from Ti plasmids and well known and disclosed in the art of plant transformation. More particularly, the right border (RB) sequence is used as the initiation point of T-DNA transfer from *Agrobacterium tumefaciens* to the plant genome, it is particularly the right border sequence of nopaline type T-DNA derived from plasmid pTiT37. (Depicker *et al.* 1982; Komari *et al.*, 1996). The left border (LB) sequence defines the termination point of T-DNA transfer from *A. tumefaciens* to the plant genome, it is particularly the left border sequence from Ti plasmid pTiC58. (Komari *et al.*, 1996; Otten *et al.*, 1999).

"Transformed plants" mean plants having integrated into their genome the gene construct flanked with the full or a fragment of the sequence of the right and left borders of a T-DNA plasmid from *Agrobacterium tumefaciens*. All cells of the transformed plants have integrated into their genome the gene construct. The transformed plant is a fertile plant and more particularly a plant which agronomic properties (yield, grain quality, drought tolerance, etc.) are not impaired compared to the same plant not transformed.

"Insert DNA" is the gene construct flanked by RB and LB sequences and inserted in the plant genome at a specific locus.

The event is defined by a stable integration of the insert T-DNA of the invention at a specific locus in the maize (corn) genome.

The insertion defines two unique junctions DNA sequence wherein the insert T-DNA sequence joins the flanking maize genomic sequences. By reference to the insert T-DNA, there is a 5' junction DNA localized in the 5' part of the insert T-DNA and a 3' junction DNA localized in the 3' part of the insert T-DNA. Non limiting examples of the event VCO-Ø1981-5 junctions DNA (or so called "event VCO-Ø1981-5 DNA") are set forth in SEQIDN°1, SEQIDN°2 or SEQIDN°3.

The term "event" refers to the original transformed plant and progeny of the transformed plant that include the heterologous DNA. The term "event" also refers to

progeny produced by a sexual outcross between the transformed plant and another variety in that the progeny includes the heterologous DNA.

The term « event » also refers to progeny produced by sexual backcrosses between a donor inbred line (the original transformed line and the progeny) comprising the insert DNA and the adjacent flanking genomic sequences and a recipient inbred line (or recurrent line) that does not contains the said insert DNA. After repeated back-crossing, the insert DNA is present in the recipient line at the same locus in the genome as in the donor line.

The term "event" or event sequence of VCO-Ø1981-5 also refers to the insert DNA from the original transformed plant comprising part or all of the insert DNA and adjacent flanking genomic sequences that would be transferred from the donor line to the recipient line.

The last backcross progeny would be selfed to produce progeny which are homozygous for the introgressed insert DNA.

These progeny would then be used as inbred parent line to produce hybrids.

A glyphosate tolerant maize (corn) VCO-Ø1981-5 (also named 6981 maize (corn)) can be bred by first sexually crossing a donor parental maize (corn) plant consisting of a maize (corn) plant grown from the transgenic maize (corn)
plant VCO-Ø1981-5 (also named 6981 maize (corn)); representative seeds deposited with NCIMB with accession number 41842 and progeny thereof derived from transformation with the expression cassettes of the present invention that tolerates application of glyphosate herbicide, and a recipient parental maize (corn) plant that lacks the tolerance to glyphosate herbicide, thereby producing a plurality of first progeny plants; and then selecting a first progeny plant that is tolerant to application of glyphosate herbicide; and selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants a glyphosate herbicide tolerant plant. These steps can further include the back-crossing of the first glyphosate tolerant progeny plant or the second glyphosate tolerant progeny plant to the recipient parental (or recurrent) maize (corn) plant or a third parental maize (corn) plant, thereby producing a maize (corn) plant that tolerates the application of glyphosate herbicide.

Methods for producing a hybrid maize (corn) seed are well known in the art. The method comprises crossing the plant comprising the VCO-Ø1981-5 event deposited on 13 May 2011 by GEMSTAR , rue Limagrain, BP-1 , 63720 Chappes, FRANCE, with NCIMB with accession number 41842 or said plant progeny comprising the VCO-Ø1981-5 event

with a different maize (corn) plant and harvesting the resultant hybrid maize (corn) seed comprising the VCO-Ø1981-5 event.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two or more independently segregating added, transgenes. A method for producing a maize (corn) plant that contains in its genetic material two or more transgenes, wherein the method comprises crossing the maize (corn) plant comprising the VCO-Ø1981-5 event deposited with NCIMB with accession number 41842 or said plant progeny comprising the VCO-Ø1981-5 event with a second plant of maize (corn) which contains at least one transgene so that the genetic material of the progeny that results from the cross contains the transgene(s) operably linked to a regulatory element and wherein the transgene is selected from the group consisting of male sterility, male fertility, insect resistance, disease resistance and water stress tolerance and herbicide resistance (wherein the transgene confers resistance to an herbicide selected from the group consisting of imidazolinone, sulfonylurea, glyphosate, glufosinate).

Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Said maize (corn) plant comprising two or more transgenes would be used to produce hybrid maize (corn) seeds wherein the method comprises crossing the said maize (corn) plant with a different maize (corn) plant and harvesting the resultant hybrid maize (corn) seeds comprising two or more transgenes.

Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several references, e.g., A. Hallauer and J.B. Miranda in Quantitative genetics in maize breeding. (2nd edition, Iowa State University press) and R. Bernardo in Breeding for quantitative traits in plants. (Stemma press.com).

The term « event » also refers to a maize (corn) plant produced by vegetative reproduction from the maize (corn) plant comprising the VCO-Ø1981-5 event deposited with NCIMB with accession number 41842 or said plant progeny comprising the VCO-Ø1981-5 event. Vegetative reproduction can be initiated from a plant part as for example cells, tissues such as leaves, pollen, embryos, roots, root tips, anthers, silks, flowers, kernels, ears, cobs, husks, stalks or tissue culture initiated from said plant part. The term event also refers to said plant part.

The term event concerns a glyphosate tolerant corn, comprising in its genome the nucleotide sequences that are at least 95%, preferably at least 96, 97, 98, or 99% identical to SEQ ID NO 1 or SEQ ID NO 2 or SEQ ID NO 3.

The invention also concerns the polynucleotide sequences comprising SEQIDN°1 or SEQIDN°2 or SEQIDN°3 and having any length from 25 nucleotides to 5092 nucleotides.

Particularly the invention concerns the polynucleotide sequences of SEQIDN°1, SEQIDN°2 and SEQIDN°3 specific to event VCO-Ø1981-5. These polynucleotide sequences are suitable for selectively identifying the event VCO-Ø1981-5 in different biological samples. By biological samples, it is to be understood a plant, plant part or plant material such as cells, tissues as leaves, pollen, embryos, roots, root tips, anthers, silks, flowers, kernels, ears, cobs, husks, stalks or seeds. It is also to be understood a processed products comprising or derived from plant part or plant material.

Methods for the detection of the presence or absence of specific DNA elements in a plant genome are well known in the art. Main techniques comprise DNA sequence amplification, particularly with Polymerase Chain Reaction, with specific primers allowing amplification of the DNA sequence, and hybridization with a probe specific for the DNA sequence.

The invention comprises a method for the identification of the presence or the absence of the transformation event VCO-Ø1981-5 of the invention, particularly with one of the known techniques.

In a particular embodiment of the invention, the method comprises the steps of:

a) extracting DNA from a biological sample obtained from a maize (corn) plant, tissue or cell;

b) contacting said extracted DNA with a first and second primers of appropriate length selected to allow production of an amplicon DNA molecule comprising all or part of the event sequence of VCO-Ø1981-5;

c) performing an amplification reaction to produce amplicon DNA molecules, and;

d) detecting the presence or the absence of a nucleotide sequence comprising all or part of the event sequence of VCO-Ø1981-5 in the amplicon molecule.

Primers have generally a length comprised between 10 and 30 nucleotides, and are selected and prepared according to techniques well known to the person skilled in the art of molecular biology.

In a particular embodiment of the invention, the amplicon molecule comprising all or part of the event sequence of VCO-Ø1981-5 comprises the event junction sequence set forth in SEQ ID NO 1 and/or the event junction sequence set forth in SEQ ID NO 2 and/ or a sequence that is at least 95%, preferably at least 96, 97, 98, or 99% identical to SEQIDN°1 or SEQIDN°2.

Advantageously, the first and second primers comprises sequences homologous to a sequence fragment of the event sequence set forth in SEQ ID NO 3, and are selected to be flanking the event VCO-Ø1981-5 sequence and to generate an amplicon comprising the DNA sequences set forth in SEQ ID NO 1 and SEQ ID NO 2.

Preferred primers comprise the DNA sequences set forth in SEQ ID NO 11 and SEQ ID NO 12.

In another embodiment of the invention, the method comprises the steps of:

a) extracting DNA from a biological sample obtained from a maize (corn) plant, tissue or cell;

b) contacting said extracted DNA with probe(s) of sufficient length to hybridize under stringent conditions with a nucleotide sequence that specifically detect at least one of VCO-Ø1981-5 junction sequence;

c) subjecting the extracted DNA and probe(s) to stringent hybridization conditions, and;

d) detecting the hybridization of the probe(s) to the extracted DNA, wherein detection indicates the presence of an event VCO-Ø1981-5 sequence.

The invention also concerns a method for producing a glyphosate tolerant plant comprising breeding a plant of the invention, comprising the event VCO-Ø1981-5 sequence, and selecting progenies by detecting the presence of the event VCO-Ø1981-5 sequence, particularly with the detection method of the invention.

"Amplicon" refers to the product obtained by amplification with a specific pair of primers of a target nucleotide sequence comprised in a nucleotide template sequence.

Primers, probes and methods for the identification of the presence or absence of a specific DNA or amplicon sequence in a corn genome are well known in the art, particularly disclosed in paragraphs [0027] to [0043] of EP 1 167 531 which are incorporated herein by reference, as well as publications cited herein.

Stringent conditions are defined as following. For sequences comprising more than 30 bases, Tm is defined by the equation: Tm = 81.5 + 0.41 (%G+C) + 16.6 Log

(concentration in cations) – 0.63 (%formamide) – (600/number of bases) (Sambrook et al., 1989).

For sequences shorter than 30 bases, Tm is defined by the equation: Tm = 4(G+C) + 2(A+T).

Under appropriate stringency conditions, in which non-specific (aspecific) sequences do not hybridize, the temperature of hybridization is approximately between 5 and 30°C, preferably between 5 and 10°C below Tm and hybridization buffers used are preferably solutions of higher ionic force like a solution 6*SSC for example.

The invention also concerns a kit for detecting the presence or absence of the VCO-Ø1981-5 event of the invention in a biological sample, wherein it comprises primers and/or probes amplifying or hybridizing to a polynucleotide sequence comprising an event VCO-Ø1981-5 DNA sequence.

The invention particularly comprises a first primer of 10 to 30 nucleotides, comprising a sequence homologous to a sequence fragment of SEQ ID NO 3 and a second primer of 10 to 30 nucleotides comprising a sequence having complementarity to a sequence fragment of SEQ ID NO 3, the first and the second primers flanking an event VCO-Ø1981-5 DNA sequence and generating an amplicon molecule comprising SEQ ID NO 1 or SEQ ID NO2.

Particularly, said first and second primers comprise the sequences set forth in SEQ ID NO 11 and SEQ ID NO 12, respectively.

The invention also concerns an isolated nucleotide sequence comprising, or consisting essentially of, a sequence set forth in SEQ ID NO 11 and/or SEQ ID NO 12.

The invention also concerns an isolated nucleotide sequence comprising a sequence set forth in SEQ ID NO 1 and/or SEQ ID NO 2, particularly comprising, or consisting essentially of, the sequence set forth in SEQ ID NO 3 or a fragment thereof and/or a sequence that is at least 95%, preferably at least 96, 97, 98, or 99% identical to SEQIDN°1 or SEQIDN°2 or SEQIDN°3.

Techniques for gene constructions as well as techniques for gene identification using amplification techniques such as PCR or hybridization techniques are well known in the art, and particularly disclosed in laboratory notebooks and manuals such as Sambrook & Russel (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.).

FIGURES

Figure 1 represents the transformation vector pAG3541.

Figure 2 represents the schematic diagram of the selection of event VCO- Ø1981-5.

Figure 3 describes the breeding diagram for event VCO- Ø1981-5 .

Figure 4 represents the EPSPS GRG23ACE5 expression cassette within the T-DNA region.

Figure 5 represents a segregation analysis carried out in the following generation for the B110 and B109 crosses, and in the next 4 generations for line AAX3.

EXAMPLES

Abbreviations, Acronyms, and Definitions

AHAS	Acetohydroxyacid synthase
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CaMV	Cauliflower mosaic virus
CHI-test	Pearson's chi-square test
CTP	Chloroplast transit peptide
DNA	Deoxyribonucleic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase (protein)
epsps	5-enolpyruvylshikimate-3-phosphate synthase (DNA sequence)
FST	Flanking sequence tag
GRG23ACE5	Modified EPSPS from *Arthrobacter globiformis*
kbp	kilobase pairs
LB	Left border
PCR	Polymerase chain reaction
RB	Right border
T-DNA	Transferred-DNA
Ti	Tumor-inducing
Vir	Virulence genes of *Agrobacterium*

I. Production of glyphosate tolerant Event VCO-Ø1981-5

Maize event VCO-Ø1981-5 was generated using a standard *Agrobacterium* mediated transformation protocol (Hiei and Komari, 1997). *Agrobacterium* contains a tumour-inducing (Ti) plasmid, which includes virulence (*vir*) genes and a transferred-DNA (T-DNA) region. Genes of interest can be inserted into the T-DNA region and thereafter transferred to the plant nuclear genome. The use of a Ti plasmid with the tumor-inducing genes deleted is commonly known as disarmed *Agrobacterium*-mediated plant transformation. Wounded plant cells produce phenolic defense compounds, which trigger the expression of the *Agrobacterium vir* genes. The encoded virulence (Vir) proteins process the T-DNA region from the Ti-plasmid, producing a 'T-strand'. After the bacterium attaches to a plant cell, the T-strand and several types of Vir proteins are transferred to the plant through a transport channel. Inside the plant cell, the Vir proteins interact with the T-strand, forming a T-complex. This complex targets the nucleus, allowing the T-DNA to integrate into the plant genome and express the encoded genes (Gelvin, 2005).

The recipient organism is the dent type of *Zea mays*, which belongs to the genus *Zea* of the family *Gramineae* (Hi-II stock material). This material is supplied in the form of two separate lines Hi-IIA and Hi-IIB. These lines are then crossed and the resulting embryos are used as target tissue for transformation. Hi-IIA and Hi-IIB are partially inbred lines selected out of a cross between corn inbred lines A188 and B73. As the recipient organism, hybrid Hi-II of *Zea mays* was produced by crossing the partially inbred Hi-IIA and Hi-IIB lines which were obtained from Maize Genetics COOP Stock Center (Urbana, IL, USA). The T-DNA region in transformation vector pAG3541 was introduced using *Agrobacterium* into the hybrid Hi-II by co-cultivation (approximately 72 hours at 22°C in the dark) with immature maize embryos. Transformed callus was selected on glyphosate-containing medium as a selective agent. The antibiotic timentin (200 ppm) was included in tissue culture media to eliminate *Agrobacterium* cells from the callus after transformation (Cheng *et al.,* 1998).

The transformation vector pAG3541 (Figure 1) was used to transfer the epsps grg23ace5 expression cassette to maize. Only the T-DNA existing between the right and left border (RB and LB) sequences respectively is integrated into the maize genome. The DNA regions outside the T-DNA borders are not transferred. Outside these borders

bacterial antibiotic resistant marker genes are required for the introduction and maintaining of the vector in the *Agrobacterium* cells. The *vir* genes are required for the production of the T-DNA transfer complex (De la Riva *et al.*, 1998).

Out of 100 events generated in T0, VCO-Ø1981-5 event was selected through multiple evaluation field trials for glyphosate tolerance and agronomic performances like germination, vegetative characteristics (such as plant height, grain weight) and reproductive characteristics (such as days to 50% pollen shed, days to 50% silking, yield).

The schematic diagram of the selection of event VCO-Ø1981-5 is provided on figure 2.

Event VCO-Ø1981-5 was also selected for good molecular characteristics based on the unicity and integrity of the insert and the stability of the genomic insertion locus and its inheritance.

More specifically, event VCO-Ø1981-5 was selected for its low level of allergenicity risk. Twelve Open Reading Frames (ORFs), created by the insertion of the T-DNA in the genome, have been identified at the junctions between the T-DNA and the maize genome. For this analysis, we consider that ORFs are any potential coding region between two stop codons as defined by the European Food safety Authority (EFSA) . Bioanalysis of ORFs was first performed, followed by analysis for putative allergenic motifs in the determined ORFs using an 80 amino acids (AA) sliding window and 8 AA exact match. Analysis was performed according to Codex Alimentarius (2003) and using AllergenOnline Database Version 11 from February 2011 (http://www.allergenonline.org/databasefasta.shtml). Two potential hits were identified using the 80 AA sliding window, but it is highly unlikely that the identified genetic sequence would generate a translatable mRNA sequence and since these sequences were identified from the native maize genome, there is no impact to the allergenicity risk assessment.Finally, event VCO-Ø1981-5 was selected due to its advantageous location in a genomic region harboring a good recombination rate. This characteristic is notably important for the conversion program in which the event will be further used.

Figure 3 describes the breeding diagram for event VCO-Ø1981-5.

II. Donor genes and regulatory sequences

A. Transformation vector map

Event VCO-Ø1981-5 was produced by disarmed *Agrobacterium*-mediated transformation using the plasmid pAG3541. This transformation vector contains the epsps grg23ace5 expression cassette within the T-DNA region (Figure 4).

B. Description of the genes and regulatory sequences

A synthetic coding region sequence comprising a maize chloroplast transit peptide (acetohydoxyacid synthase) (Fang *et al.*, 1992) and a gene encoding EPSPS GRG23ACE5 enzyme was generated. The synthetic gene was subcloned downstream from the ubiquitin-4 promoter from *Saccharum officinarum* L. (Albert and Wei, 2003) and upstream from the terminator 35S of Cauliflower mosaic virus (Gardneret al, 1981) to create plasmid pAX3541. The promoter::gene::terminator fragment from this intermediate plasmid (based on pSB11, Japan Tobacco, Inc. (Hiei and Komari, 1997)) was mobilized into *Agrobacterium tumefaciens* strain LBA4404, which also harbors the plasmid pSB1, using triparental mating and plating on media containing spectinomycin, streptomycin, tetracycline and rifampicin to form a final plasmid, pAG3541. Rifampicin is included as an additional selection for *Agrobacterium* as the rifampicin resistance marker gene is present in the *Agrobacterium* chromosomal DNA. The integrity of cointegrate product of pSB1 and pAX3541 - plasmid pAG3541 was verified by Southern hybridization.

The amino acid sequence of the wild-type EPSPS isolated from *Arthrobacter globiformis* was altered using a directed evolution technique resulting in the EPSPS GRG23ACE5 protein described herein and expressed in event VCO-Ø1981-5. The deduced amino acid sequence of the EPSPS GRG23ACE5 protein is shown below (SEQ ID NO 23).

```
metdrlvipg sksitnrall laaaakgtsv lvrplvsadt safktaiqal ganvsadgdd
wvveglgqap nldadiwced agtvarflpp fvaagqgkft vdgseqlrrr plrpvvdgir
hlgarvsseq lpltieasgl aggeyeieah qssqfasgli maapyarqgl rvkipnpvsq
pyltmtlrmm rdfgietstd gatvsvppgr ytarryeiep dastasyfaa asavsgrrfe
fqglgtdsiq gdtsffnvlg rlgaevhwas nsvtirgper ltgdievdmg eisdtfmtla
aiapladgpi titnigharl kesdrisame snlrtlgvqt dvghdwmriy pstphggrvn
chrdhriama fsilglrvdg itlddpqcvg ktfpgffdyl grlfpekalt lpg
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III. Transgene Copy Number Analysis

Maize genomic DNA was isolated (Dellaporta *et al.*, 1983) and quantified by fluorimetry. DNA restriction, gel electrophoresis, Southern blotting and hybridization with radiolabeled probes were carried out according to standard procedures (Sambrook *et al.*, 1989). Total genomic DNA was purified from event VCO-Ø1981-5 and digested with appropriate restriction endonucleases to determine both insert copy number and insert integrity.

Templates for radioactive probes synthesis were prepared using standard PCR methods. Oligonucleotide primers specific to promoter and terminator sequences in the T-DNA were used to generate a DNA probe specific for the T-DNA insert. The DNA probe was labeled with ^{32}P α-dCTP using Ready-To-Go DNA labeling beads (GE Health). The labeled probe was purified over Micro Bio-Spin P-30 Tris-Chromatography Columns (BioRad). Hybridizations were carried out at 65°C (Church, 1984). After hybridization, blots were washed at 65°C, with the final wash containing 1% (w/v) sodium dodecyl sulfate at pH 7.0. Blots were exposed to Kodak AR X-OMAT film using a Kodak intensifying screen at -80°C.

Genomic DNA from event VCO-Ø1981-5 corn, BC1 negative segregant corn, and B110 inbred corn was digested with the restriction enzymes *Hind*III and *Nde*I (New England Biolabs, Ipswich, MA) independently. Each of these restriction enzymes cuts once within the T-DNA region. When hybridized with the *epsps grg23ace5* gene probe, the resulting number of hybridization products would indicate the insert copy number within the maize genome. Both digests produced a single band indicating a single copy of the insert present.

Genomic DNA from event VCO-Ø1981-5 corn, BC1 negative segregant corn, and B110 inbred corn was digested with a combination of *Hind*III and *Eco*RI, and independently with *Mfe*I (New England Biolabs, Ipswich, MA). A set of four independent probes (ScUbi4 promoter, ScUbi4 intron, *epsps grg23ace5* gene, and 35S terminator) were used to confirm the integrity of the expression cassette structure The results of the analysis indicated that the *epsps grg23ace5* expression cassette was intact and the functional components were found and verified in the expected order in the inserted DNA.

Southern blot analysis was conducted to verify the absence of the transformation plasmid components outside of the transferred T-DNA region. Maize genomic DNA (VCO-Ø1981-5 event and appropriate negative controls) was digested with a combination

of *Hind*III and *Eco*RI, and independently with *Mfe*I (New England Biolabs, Ipswich, MA). The *Agrobacterium* plasmid pAG3541 was included as a positive control for hybridization of the transformation plasmid components. The probes used were designed to hybridize to the functional components of the plasmid including the sequence of *aad*, *tet*R, *tet*A, oriT, *vir*C, *vir*G, and *vir*B.

Southern blot analysis results indicate that none of the vector probes hybridized to VCO-Ø1981-5 genomic DNA confirming the absence of the sequences of the functional components of the plasmid in event VCO-Ø1981-5. These same probes however did show hybridization with the plasmid vector control on each blot indicating that if the vector sequences were inadvertently transferred to event VCO-Ø1981-5 corn, they would have been detected in this analysis.

Southern blot analysis was conducted on multiple generations of event VCO-Ø1981-5 progeny to evaluate the stability of the T-DNA sequence insertion. Genomic DNA isolated from leaf material of VCO-Ø1981-5 plants from four successive breeding generations (BC0, BC1, BC3, and BC4) and negative controls were digested with the restriction enzyme *Hind*III (New England Biolabs, Ipswich, MA) which, as noted earlier, cuts once within the T-DNA region. When hybridized with the probe specific for the *epsps grg23ace5* gene, VCO-Ø1981-5 produces a single band approximately 4.0 kb in size. The transformation plasmid pAG3541 was included as a hybridization control. All four generations analyzed showed an identical hybridization pattern producing the identical 4.0 kb band. If the genetic insert were unstable within the maize genome through successive breeding of the event, one would expect to detect changes in the banding pattern produced. The data indicates a stable insertion site in event VCO-Ø1981-5.

IV. Sequencing of the Insert and Flanking Genomic DNA

Southern blot analysis has demonstrated that event VCO-Ø1981-5 contains a single intact T-DNA insert containing a single expression cassette. The sequence of the transgenic locus including 5' and 3' FSTs (flanking sequence tags) and the sequence of the pre-insertion locus (locus in the corn genome where the transgene was inserted) have been determined.

The maize genomic sequences flanking the T-DNA insertion in event VCO-Ø1981-5 were obtained by Genome Walker™ (Clontech) (5'FST) and direct PCR (3'FST). Using the DNA sequences generated, a BLAST search (Altschul *et al.*, 1997) was performed

against the Maize Genetics and Genomics Database (Lawrence *et al.*, 2004). Both the 5' and 3' FST sequences mapped to chromosome 1.

700bp were obtained for the 5' FST and 700bp for the 3' FST. The enzyme *Ssp*I was used for generating the library. The T-DNA specific primers used are listed in the following Table 1.

Table 1

	Primer	5'-->3' sequence	SEQ ID
3'FST	Ace5-1	ACAGGATCGCTATGGCGTTTTCAATCC	17
	Ace5-2	ATGCGTCGGGAAGACCTTTCCTGGCTTC	18
	O39	CACCAGGGAGGAGGCAACAACAAGTAG	19
5'FST	Scubi-NewR	AGAAAGAGTCCCGTGAGGCTACGGCAC	20
	Scubi2-Rev	CTGGGATTTGGATGGATGAGGCAAGGAG	21
	Scubi1-Rev	AGAGGTCGCCGCGGAGATATCGAGGAG	22

The insertion site could be mapped using a BLAST search against the Maize Genetics and Genomics Database (http://www.maizegdb.org/). It is located in the chromosome 1, more precisely on the BAC: AC185611,.

To confirm the FST result, primers were deduced from the sequence obtained by the Genome Walker strategy and used to directly amplify the 5' and 3' FST sequence from Hi-II and VCO-Ø1981-5 (6981). The expected PCR products were obtained and sequenced. The sequences obtained were found identical as the one obtained from the Genome Walker which is thus considered as accurate.

The Map of inserted T-DNA, gene construct of the invention flanked with the right and left border and the flanking sequences (SEQ ID NO 9 and SEQ ID NO 10) is described on figure 4.

The 3' flanking sequence (SEQ ID NO 9) has the following sequence:

gttctcagagggagatgggcggcaagggcggcgggggtggtggcaagggcggcggcgggggtggtggcaa
gggcggaggaggttttggtggcaagagcggcggcgggggtggtggcaagggcggaggaggtgttggtggc
aagagcggcggcggcaagtcaggcggcggcggcggtgggggctatggtggtggagggaagtcaggctccg
gcggcagtggcggcgacggaatgatgaaggcgcccggcggcagtggcgagtacatctcccgctctgtctt
cgaggccagcccgcaggtgttcttccatggcctccaccagggaggaggcaacaacaagtagatccatcta
gctagactgctgctgctacttcacaagcttgggacgatgtgtgatcatgcatgcttggactggcatcagt
ctctatgtagcttctgaataaaataaaatgtaacgatgctcgattgtgtttcacttgctcgcttgtttca
gccaagttattatatatcatcaggctcgtacgtcagctatatatatatatatatatatatatatatatat

atatatatatatatatatatatatatatatatatatatatatatatatatatatacacacacacatat
gcaggtgcatggattgtgcaacgcgaatgtgtgattgtgctaatccgttagttgatgccgtttgttgctt

The 5' flanking sequence (SEQ ID NO 10) has the following sequence:

tttcctcattttctttttcccgcttttgtttcaatttttcttgggtaatgtacagtgagtatatttttt
cttgttctttttctcatggccaaaatccacaatggatcgatgaattagctgtcgttgttgccaacaaca
acaacagaacaaaatcacgtgacgtactagcacaatgcaagtagccaaactgagcttccgggcaccgac
gaacggttgcacgccatcggcgggaaggaacaggccgggctgtcaatggacaaacgggccgccaagctg
gagggagtgtcatgggctttgagaaccatcgtcagggtccagtttattcttttgtttttattaaaggcg
gtaaactcggggaacgaatatactaggaaaaacactagccagtcagagtcagtcaaagtggactgagtt
aaaattgcaacgacacacacgcagcagtcagggcgtcgggaatgaacaatggatgaatttattataatc
tgaagaaaacgaagggacacagccactacgaacactggggagtggggagtgaatgaatgaatgcattcc
actggaccgttccagcgcttcgtgtgcctcgctagatgcgctgaacactcgaacgccatggacctcgct
ccgctctctatatatagagggaaggccttcagtctactcctcgggatataccactgaacgtcaccaaga
agatcagtac

Additionally, the entire T-DNA insert in event VCO-Ø1981-5 was sequenced and verified to be identical to that in transformation vector pAG3541. During the transformation integration process, the right and left border sequences do not typically remain intact and minor deletions in both were identified in event VCO-Ø1981-5.

A complete sequence comprising the entire T-DNA insert sequence and the flanking genomic sequence is listed as SEQ ID NO 3.

V. Inheritance of the Glyphosate Tolerant Trait

During performance evaluation of event VCO-Ø1981-5, the locus containing *epsps grg23ace5* was crossed with 3 inbred lines (B110, B109, AAX3). Progeny plants for each line were then sprayed with glyphosate to identify plants that inherited and expressed *epsps grg23ace5* and assess the segregation ratio into each of the lines. The progenitor line for testing was generated by pollinating line B110 with the parental T0 plant for event VCO-Ø1981-5, which yielded a BC0 line (B110 x VCO-Ø1981-5). These BC0 seeds were germinated and plants were crossed simultaneously with lines B110, B109 and AAX3. Segregation analysis was carried out in the following generation for the B110 and B109 crosses, and in the next 4 generations for line AAX3 (Figure 5).

All glyphosate sprays were carried out at either 1x, 4x, or 8x the spray rate in outdoor field plots (1x was 540g of glyphosate, acid form/ha). Positive segregants that survived the spray were scored as "tolerant", while negative segregants did not survive the spray and were scored as "sensitive".

Table 2

Generation (line)	No	Gly. S.R.	Obs. Tol.	Obs. Sens.	Exp. Tol.	Exp. Sens.	% Tol.	CHI test value
BC1 (B110)	9	4x	7	2	4.5	4.5	77.8%	0.096
BC1 (B110)	7	8x	2	5	3.5	3.5	28.6%	0.257
BC0 (B109)	10	4x	5	5	5	5	50.0%	1.000
BC0 (B109)	11	8x	5	6	5.5	5.5	45.5%	0.763
BC0 (AAX3)	28	1x	12	16	14	14	42.9%	0.450
BC1 (AAX3)	227	1x	100	127	113.5	113.5	44.1%	0.073
BC2 (AAX3)	58	1x	29	29	29	29	50.0%	1.000
BC3 (AAX3)	74	1x	38	36	37	37	51.4%	0.816

Abbreviations: Gen.: Generation; No: Number of plants; Gly. S.R.: glyphosate spray rate; Obs. Tol: observed tolerant; Obs. Sens.: observed sensitive; Exp. Tol.: expected tolerant; Obs. Tol: expected sensitive; % Tol.: % Tolerant.

All plants were evaluated two weeks after spraying. A segregation ratio of 1:1 was expected in each generation because *epsps grg23ace5* is present at single and hemizygous copy in the donor parental line crossed with the lines B109, B110 or AAX3.

Observed segregation patterns were compared to the expected patterns and these data were compared using a chi-squared (X^2) distribution analysis, as follows:

$X^2 = \Sigma [(|o - e|)^2/e]$, where o = observed frequency of tolerance, and e = expected frequency of tolerance.

A chi-square value of ≥ 0.05 was treated as the cutoff for statistical support of a 1:1 segregation in each generation, and this value was exceeded for each of the segregation analysis groups. The results of this analysis are consistent with the inheritance of a single copy of *epsps grg23ace5* into each of the inbred lines tested (B110, B109, AAX3).

Transformation event VCO-Ø1981-5 contains a single genetic insertion of the *epsps grg23ace5* gene, and that gene is inherited through successive breeding generations in the predictable Mendelian fashion.

VI. Method of detection of the VCO-Ø1981-5 event:

This example describes an event-specific real-time quantitative TaqMan® PCR method for determination of the relative content of event VCO-Ø1981-5 DNA to total maize (*Zea mays*) DNA in a biological sample.

The PCR assay has been optimized for use in an ABI Prism® 7900 sequence detection system.

For specific detection of event VCO-Ø1981-5 genomic DNA, a 85-bp fragment of the region that spans the 5' TDNA insert and flanking genomic junction in maize event VCO-Ø1981-5, is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with a fluorescent dye: FAM as a reporter dye at its 5′ end and MGBmolecule as a quencher at its 3′ end. The 5′-nuclease activity of the Taq DNA polymerase is exploited, which results in the specific cleavage of the probe, leading to increased fluorescence, which is then monitored. For relative quantification of event VCO-Ø1981-5 DNA, a maize specific reference system amplifies a 70-bp fragment of aldolase (Kelley et al. ,1986), a maize endogenous sequence, using a pair of aldolase gene-specific primers and an aldolase gene-specific probe labelled with VIC and TAMRA.

Two types of quantification are simultaneously performed in this method: one for the endogenous gene aldolase and one for the event VCO-Ø1981-DNA region.

The following sets of primers and probes are used.

Table 3

	Sequence (5' to 3')
VCO-Ø1981-5 primer F	Ccactgaacgtcaccaagaaga (SEQ ID NO 11)
VCO-Ø1981-5 primer R	Gccgctactcgagggattta (SEQ ID NO 12)
VCO-Ø1981-5 probe	6-FAM-cagtactcaaacactgatag-MGB (SEQ ID NO 13)
Aldolase primer F	Agggaggacgcctccct (SEQ ID NO 14)
Aldolase primer R	Accctgtaccagaagaccaagg (SEQ ID NO 15)
Aldolase probe	6-VIC-tgaggacatcaacaaaaggcttgcca-TAMRA (SEQ ID NO 16)

The master-mix for the aldolase reference gene system is prepared as followed in Table 4:

Table 4

Component	Final concentration in PCR	µl/reaction
TaqMan® Universal Master Mix 2X	1x	12.5
Primer F (5 µM)	300 nM	1.5
Primer R (5 µM)	300 nM	1.5
Probe (5 µM)	200 nM	1.0
Nuclease free water	#	6.0
Template DNA (maximum 200 ng)	#	2.5
Total volume:		25µl

The master-mix for VCO-Ø1981-5 event is prepared as followed in Table 5:

Table 5

Component	Final concentration in PCR	µl/reaction
TaqMan® Universal Master Mix 2X	1x	12.5
Primer F (5 µM)	300 nM	1.5
Primer R (5 µM)	300 nM	1.5
Probe (5 µM)	200 nM	1.0
Nuclease free water	#	6.0
Template DNA (maximum 200 ng)	#	2.5
Total volume:		25µl

Run the PCR with cycling conditions listed below for both VCO-Ø1981-5 event and aldolase assays in the Applied Biosystems 7900 system.

Table 6

Step	Stage		T°C	Time (sec)	Data collection	Cycles
1	Uracil-DNA-N Glycosylase (UNG)		50 °C	120”	no	1x
2	Initial denaturation		95°C	600”	no	1x
3	Amplification	Denaturation	95°C	15”	no	40x
		Annealing & Extension	60°C	60”	yes	

VII. Evaluation of Agronomic Performance of event VCO-Ø1981-5

In order to evaluate agronomic performance characteristics of event VCO-Ø1981-5 as compared to an appropriate negative isoline, two experimental varieties were produced and seed used for multiple location evaluation. The experimental varieties are hybrid maize obtained by crossing the event VCO-Ø1981-5 (BC2S2) with two different lines (B116 and CH01). Negative segregants crossed with the lines B116 and CH01 were used as comparators (see table 5 and figure 3 for breeding diagram).

Table 7: Maize hybrids tested in agronomic evaluations.

Line Tested	Pedigree
VCO-Ø1981-5 (A)	BC0S2 VCO-Ø1981-5 x B116
Control : Negative isoline (A)	BC0S2 null x B116
VCO-Ø1981-5 (B)	BC0S2 VCO-Ø1981-5 x CH01
Control : Negative isoline (B)	BC0S2 null x CH01

These hybrids were characterized under diverse environmental and growing conditions similar to those used in maize production. The study was conducted using a Randomized Complete Block design with three replications (plots) of each entry per location. Each plot consisted of four, 30-inch rows by 17.5 to 20 ft. long. Plants were thinned prior to reaching the V8 leaf stage resulting in a uniform number of plants in each row. Weeds outside of the plots (in alleyways and borders) managed as to not confound measures of agronomic characteristics. Weeds within the plots were managed by conventional herbicides and cultural practices (hand hoeing). No broad spectrum herbicides were applied to the study or borders rows except as a pre-plant or pre-emergence application. Data on all traits was collected on the middle two rows of each four row plot. Data collected over season is summarized in Tables 8 and 9.

Table 8: Agronomic performance results – vegetative characteristics

Agronomic Characteristic (unit)	Genetic Background	VCO-Ø1981-5 Corn	Number of plants	Control	Number of plants	
Plant height (inches)	B116	116.9	49	113.5	44	Mean
		32.0-136.5	36-72	26.7-138.5	27-72	Range
		0.7918	0.0067		0.0067	p-value
	CH01	110.4	48	106.8	46	Mean
		32.7-124.8	36-72	21.7-130.7	31-72	Range
		0.7632	0.01797		0.01797	p-value
Grain weight (pounds per plot)	B116	19.5	49	18.2	44	Mean
		3.8-27.0	36-72	4.0-37.4	27-72	Range
		0.2292	0.0067		0.0067	p-value
	CH01	19.9	48	18.8	46	Mean
		6.0-30.6	36-72	2.0-31.1	31-72	Range
		0.3662	0.01797		0.01797	p-value

Table 9: Agronomic performance results – reproductive parameters

Agronomic Characteristic (unit)	**Genetic Background** (same as in Table 8)	**VCO-Ø1981-5 Corn**	**Control**	
Days to 50% pollen shed (# days)	B116	72.6	73.3	Mean
		59-95	59-94	Range
		0.6978		p-value
	CH01	72.3	72.9	Mean
		57-93	56-94	Range
		0.7365		p-value
Days to 50% silking (# days)	B116	74.6	75.0	Mean
		59-97	59-95	Range
		0.8087		p-value
	CH01	72.6	72.8	Mean
		57-96	56-96	Range
		0.9003		p-value
Yield (bushel per acre)	B116	143.0	130.7	Mean
		35.6-218.9	26.5-228.7	Range
		0.1584		p-value
	CH01	150.4	138.6	Mean
		52.4-259.7	18.3-222.1	Range
		0.1896		p-value

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1-3-2	Address of depositary institution	Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB21 9YA, United Kingdom
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1-3-4	Accession Number	NCIMB 41842
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CLAIMS

1. A maize (corn) plant comprising the event VCO-Ø1981-5, representative seeds deposited with NCIMB with accession number 41842.

2. A glyphosate tolerant maize (corn) plant comprising the event VCO-Ø1981-5, obtainable by breeding a maize (corn) plant with a maize (corn) plant grown from seeds deposited with NCIMB with accession number 41842.

3. The glyphosate tolerant maize (corn) of claim 2, comprising in its genome the nucleotide sequences as set forth in SEQ ID NO 1 or SEQ ID NO 2 and/or SEQ ID NO 3.

4. The glyphosate tolerant maize (corn) of one of claims 1 to 3, wherein it is an hybrid maize (corn) plant.

5. The part of the plant, or cells of the plant or seeds of the plants of one any of the claims 1 to 4, wherein said part of the plant, cells or seeds comprise the event VCO-Ø1981-5.

6. An isolated nucleotide sequence comprising a sequence that is at least 95% identical to SEQ ID NO 1 and/or SEQ ID NO 2 and/or SEQ ID NO 3.

7. The isolated nucleotide sequence of claim 6 comprising a sequence set forth in SEQ ID NO 1 and/or SEQ ID NO 2.

8. The isolated nucleotide sequence of claim 6 wherein it consists of the sequence set forth in SEQ ID NO 3.

9. A kit for detecting the presence or absence of the VCO-Ø1981-5 event of claim 1 to 5 in a biological sample, wherein it comprises primers or probes amplifying or hybridizing to a polynucleotide sequence comprising an event VCO-Ø1981-5 DNA sequence.

10. The kit of claim 9 wherein it comprises a first primer of 10 to 30 nucleotides, comprising a sequence homologous to a sequence fragment of SEQIDN°3 and a second primer of 10 to 30 nucleotides comprising a sequence fragment having complementarity to a sequence fragment of SEQ ID NO 3, the first and the second primers flanking an event VCO-Ø1981-5 DNA sequence and capable of generating an amplicon molecule comprising SEQ ID NO 1 and/or SEQ ID NO2.

11. The kit of any one of claims 9 or 10, wherein said first and second primers comprise the sequences set forth in SEQ ID NO 11 and SEQ ID NO 12, respectively.

12. A method for detecting the presence or the absence of the maize (corn) transformation VCO-Ø1981-5 event of claim 1 to 5 in a biological sample comprising:

a) extracting DNA from a biological sample;

b) contacting said extracted DNA with a first and a second primer of length comprised between 10 nucleotides to 30 nucleotides;

c) performing an amplification reaction to produce an amplicon molecule comprising an event VCO-Ø1981-5 DNA sequence, and;

d) detecting the presence or the absence of a nucleotide sequence comprising an event VCO-Ø1981-5 DNA sequence in the amplicon molecule.

13. The method of claim 12 wherein the amplicon molecule comprising an event VCO-Ø1981-5 DNA sequence comprises SEQ ID NO 1 or a sequence that is at least 95% identical to SEQIDN°1.

14. The method of claim 12 wherein the amplicon molecule comprising an event VCO-Ø1981-5 DNA sequence comprises SEQ ID NO 2 or a sequence that is at least 95% identical to SEQIDN°2.

15. The method of one of claims 12 to 14, wherein said first primer comprises a sequence homologous to a sequence fragment of SEQIDN°3 and the second primer comprises a sequence having complementarity to a sequence fragment of SEQIDN° 3, the first and the second primers flanking an event VCO-Ø1981-5 DNA sequence to generate an amplicon molecule comprising SEQIDN°1 or SEQIDN°2 or a sequence that is at least 95% identical to SEQIDN°1 or SEQIDN°2.

16. The method of one of claims 12 to15, wherein said first and second primers comprise the sequences set forth in SEQ ID NO 11 and SEQ ID NO 12, respectively.

17. A method for detecting the presence or the absence of the maize (corn) plant VCO-Ø1981-5 event in a biological sample comprising:

a) extracting DNA from a biological sample;

b) contacting said extracted DNA with probe(s) of sufficient length to hybridize under stringent conditions with a nucleotide sequence that specifically detects a VCO-Ø1981-5 event junction sequence;

c) subjecting the extracted DNA and probe(s) to stringent hybridization conditions, and;

d) detecting the hybridization of the probe(s) to the extracted DNA, wherein detection indicates presence of an event VCO-Ø1981-5 DNA sequence.

18. A method of producing a glyphosate tolerant plant comprising breeding a plant of one of claims 1 to 4, and selecting progeny by detecting the presence of polynucleotide sequence comprising the event VCO-Ø1981-5 DNA sequence.

19. The method of claim 18 wherein said polynucleotide comprises a sequence set forth SEQ ID NO 1 , SEQ ID NO 2 and/or SEQ ID NO 3.

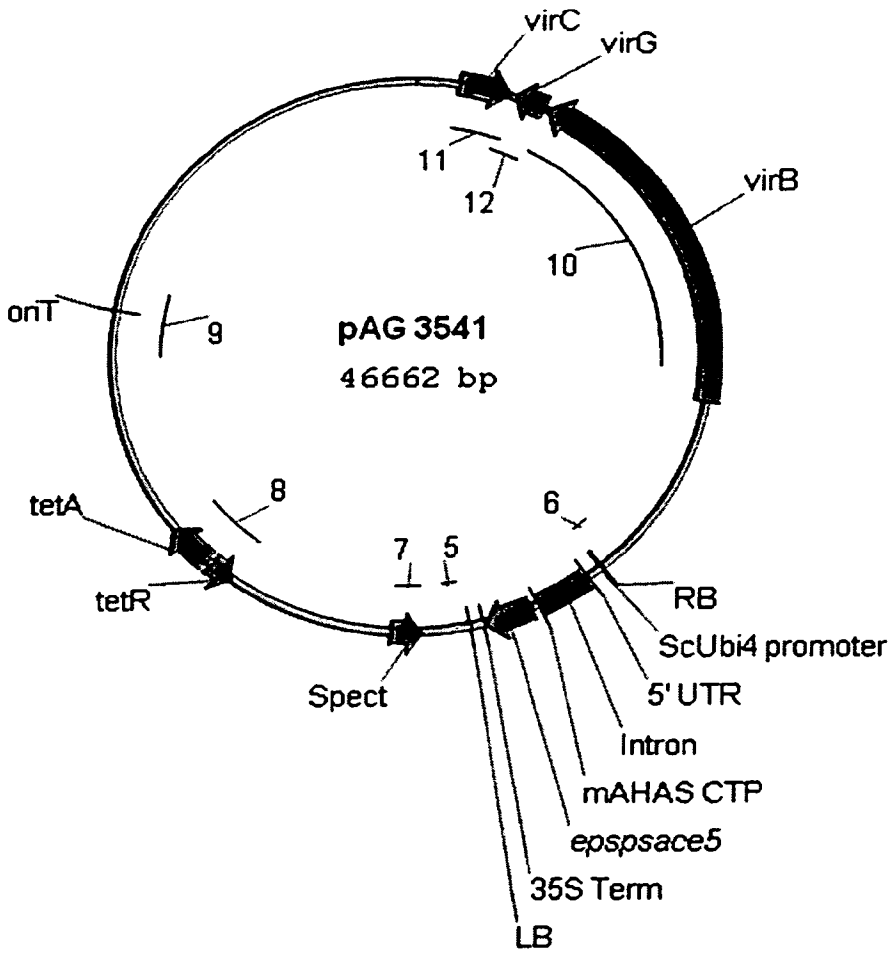
virC
virG
11
12
virB
10
onT
9
pAG 3541
46662 bp
8
6
tetA
7
5
tetR
RB
ScUbi4 promoter
Spect
5' UTR
Intron
mAHAS CTP
epspsace5
35S Term
LB

Fig. 1

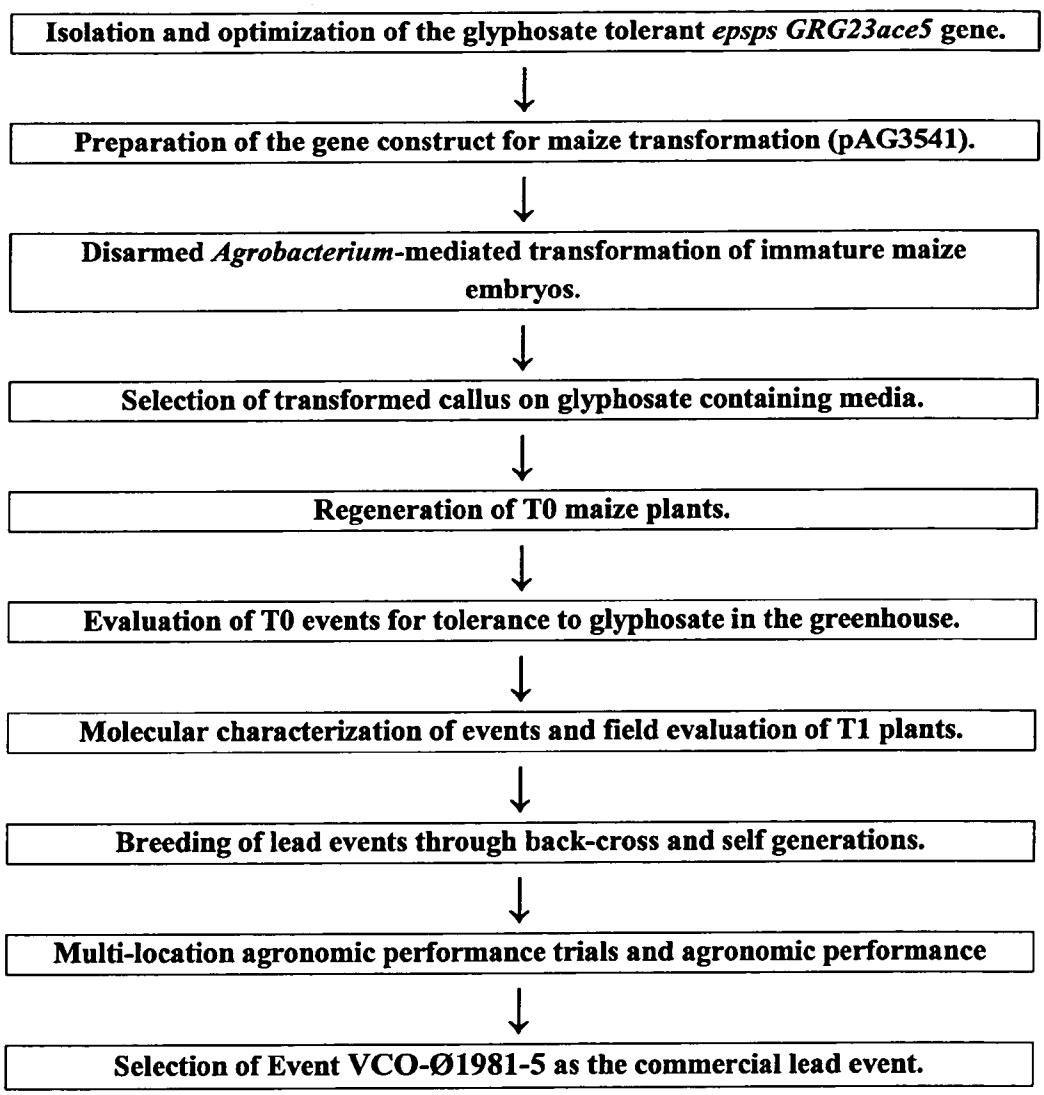
Isolation and optimization of the glyphosate tolerant *epsps GRG23ace5* gene.
Preparation of the gene construct for maize transformation (pAG3541).
Disarmed *Agrobacterium*-mediated transformation of immature maize embryos.
Selection of transformed callus on glyphosate containing media.
Regeneration of T0 maize plants.
Evaluation of T0 events for tolerance to glyphosate in the greenhouse.
Molecular characterization of events and field evaluation of T1 plants.
Breeding of lead events through back-cross and self generations.
Multi-location agronomic performance trials and agronomic performance
Selection of Event VCO-Ø1981-5 as the commercial lead event.

Fig. 2

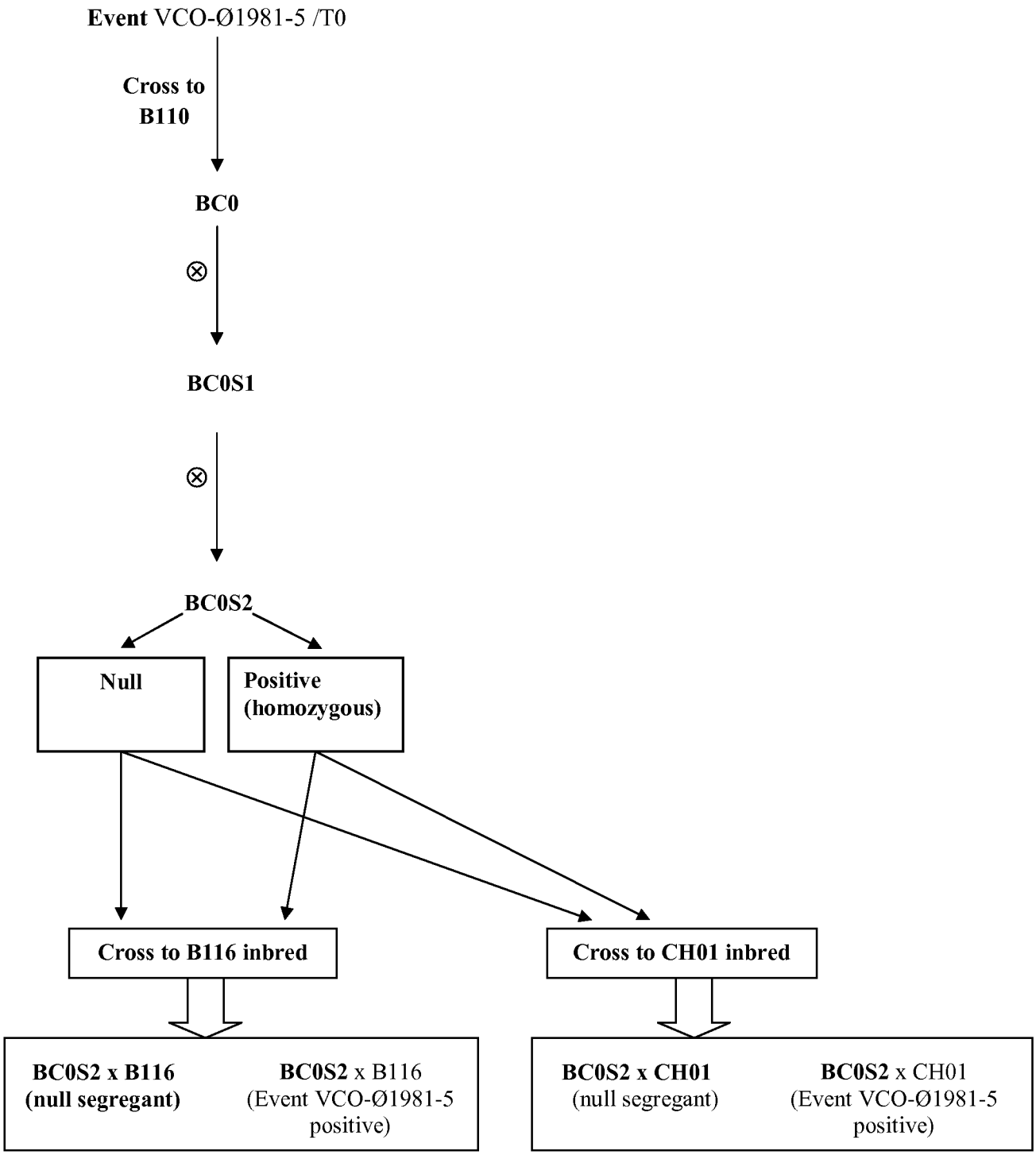
Event VCO-Ø1981-5 /T0
Cross to B110
BC0
⊗
BC0S1
⊗
BC0S2
Null
Positive (homozygous)
Cross to B116 inbred
Cross to CH01 inbred
BC0S2 x B116 (null segregant)
BC0S2 x B116 (Event VCO-Ø1981-5 positive)
BC0S2 x CH01 (null segregant)
BC0S2 x CH01 (Event VCO-Ø1981-5 positive)

Fig. 3

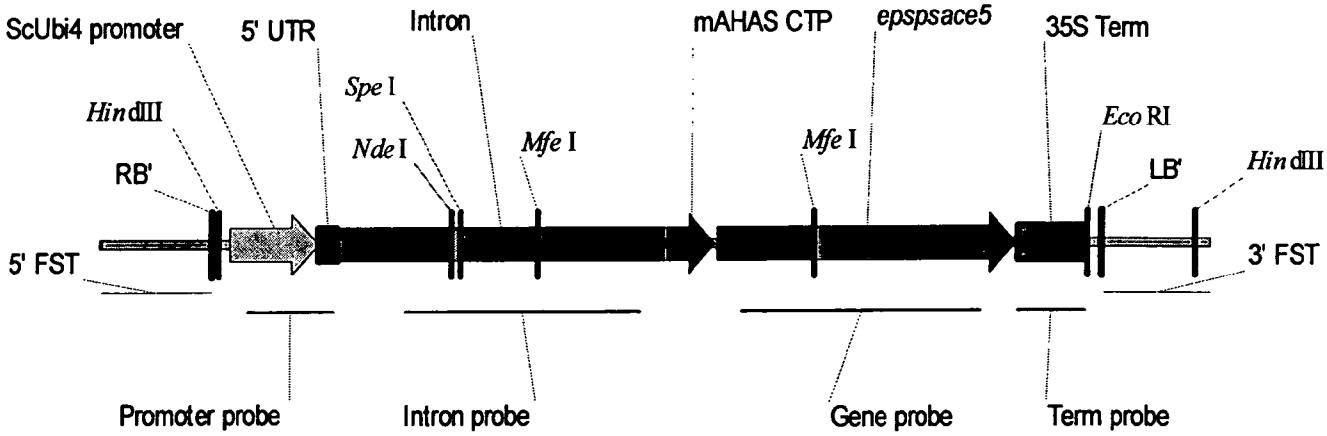
ScUbi4 promoter
5' UTR
Intron
mAHAS CTP
epspsace5
35S Term
Spe I
HindIII
Nde I
Mfe I
Mfe I
Eco RI
LB'
HindIII
RB'
5' FST
3' FST
Promoter probe
Intron probe
Gene probe
Term probe

Fig. 4

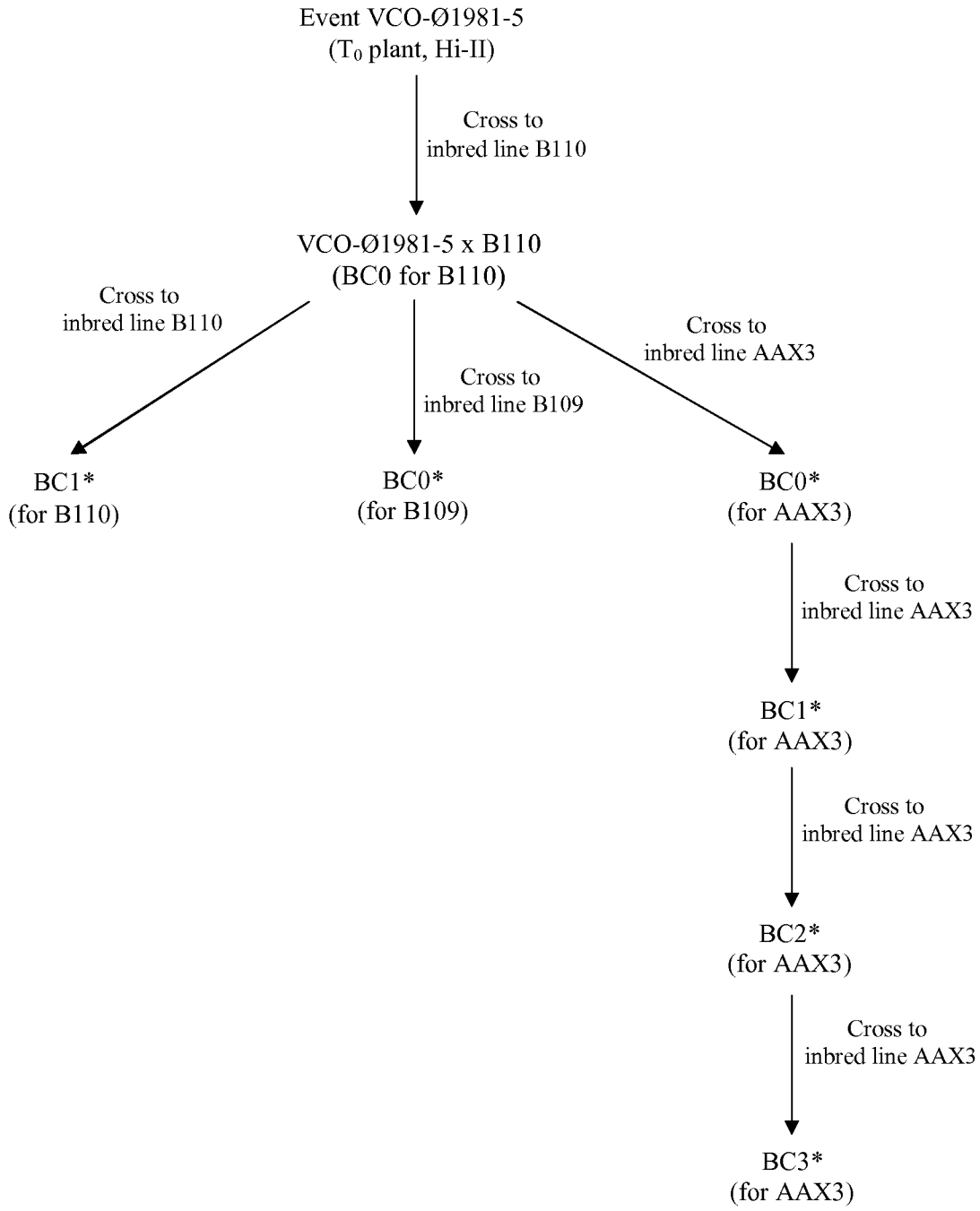
Event VCO-Ø1981-5
(T_0 plant, Hi-II)
Cross to
inbred line B110
VCO-Ø1981-5 x B110
(BC0 for B110)
Cross to
inbred line B110
Cross to
inbred line B109
Cross to
inbred line AAX3
BC1*
(for B110)
BC0*
(for B109)
BC0*
(for AAX3)
Cross to
inbred line AAX3
BC1*
(for AAX3)
Cross to
inbred line AAX3
BC2*
(for AAX3)
Cross to
inbred line AAX3
BC3*
(for AAX3)

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/064712

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/82 C12N9/10
ADD.

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)
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 practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/100353 A2 (ATHENIX CORP [US]; SCHOUTEN LAURA COOPER [US]; PETERS CHERYL [US]; VAN) 21 August 2008 (2008-08-21) cited in the application claims 1-10, 15, 19,23; examples 12, 13; sequence 28 -----	2-17
X	HECK G R ET AL: "Development and characterization of a CP4 EPSPS-based, glyphosate-tolerant corn event", CROP SCIENCE, vol. 45, no. 1, January 2005 (2005-01), pages 329-339, XP002687299, ISSN: 0011-183X abstract ----- -/--	2-17

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

[X] See patent family 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 application or patent 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
16 November 2012	03/12/2012

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, Fax: (+31-70) 340-3016	Krüger, Julia

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/064712

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 040 497 A (SPENCER MICHAEL [US] ET AL) 21 March 2000 (2000-03-21) cited in the application column 2 - column 8; claims 1-10,; example 9	2-17
A	WO 2008/112019 A2 (PIONEER HI BRED INT [US]; DU PONT [US]; CHICOINE TIMOTHY K [US]; DERRY) 18 September 2008 (2008-09-18) pages 5, 10,	2-17

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/064712

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1, 18, 19
because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(ii) PCT - Plant variety
Rule 39.1(ii) PCT - Essentially biological process for the production of plants

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/064712

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008100353 A2	21-08-2008	AR 064004 A1	04-03-2009
		AT 509113 T	15-05-2011
		AU 2007347119 A1	21-08-2008
		CA 2671203 A1	21-08-2008
		EP 2087124 A2	12-08-2009
		HK 1134108 A1	30-12-2011
		HR P20110587 T1	30-09-2011
		NZ 577310 A	30-09-2011
		PT 2087124 E	29-07-2011
		US 2008127372 A1	29-05-2008
		US 2011030091 A1	03-02-2011
		US 2011232179 A1	29-09-2011
		US 2011236950 A1	29-09-2011
		US 2011252502 A1	13-10-2011
		WO 2008100353 A2	21-08-2008
US 6040497 A	21-03-2000	NONE	
WO 2008112019 A2	18-09-2008	AR 063442 A1	28-01-2009
		CA 2666323 A1	18-09-2008
		CL 30642007 A1	16-05-2008
		EP 2069506 A2	17-06-2009
		TW 200825180 A	16-06-2008
		US 2008108072 A1	08-05-2008
		US 2010240059 A1	23-09-2010
		WO 2008112019 A2	18-09-2008