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(71) Applicant: **PIONEER HI-BRED INTERNATIONAL, INC.** [US/US]; 7100 NW 62nd Avenue, PO Box 1014, Johnston, Iowa 5013 1-1014 (US).

(72) Inventors: **CHRISTENSEN, Heather Marie;** 417 Se 5th Street, Ankeny, Iowa 50021 (US). **CONG, Bin;** 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 5013 1-0552 (US). **CRANE, Virginia;** 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 5013 1-0552 (US). **HU, Xu;** 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 5013 1-0552 (US). **LU, Albert L.;** 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 5013 1-0552 (US). **MABRY, Timothy;** 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 5013 1-0552 (US). **RINEHART KREBS, Kristen Denise;** 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 5013 1-0552 (US). **SANDAHL, Gary A.;** 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 5013 1-0552 (US).

(74) Agent: **BECKER, James E.;** 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 5013 1-0552 (US).

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(54) Title: MAIZE EVENT DP-02321 1-2 AND METHODS FOR DETECTION THEREOF

(57) Abstract: Embodiments disclosed herein relate to the field of plant molecular biology, specifically to DNA constructs for conferring insect resistance to a plant. Embodiments disclosed herein relate to insect resistant corn plant containing event DP-023211-2, and to assays for detecting the presence of event DP-023211-2 in samples and compositions thereof.

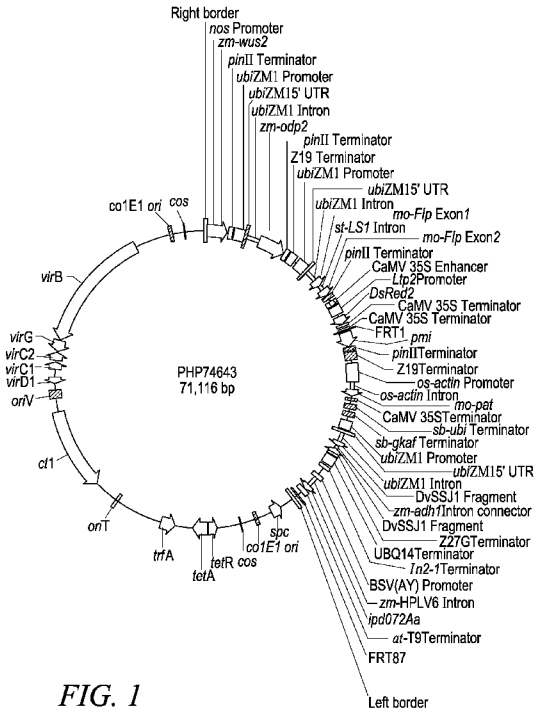

FIG. 1

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[Continued on next page]

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— *with sequence listing part of description (Rule 5.2(a))*

MAIZE EVENT DP-023211-2 AND METHODS FOR DETECTION THEREOF

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named "7493_SeqList.txt" created on April 16, 2018 and having a size of 157 kilobytes and is filed concurrently with the specification. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/663,832 filed April 27, 2018, U.S. Provisional Application No. 62/678,579 filed May 31, 2018, and U.S. Provisional Application No. 62/776,018 filed December 6, 2018, which are each herein incorporated by reference in their entirety.

FIELD

Embodiments disclosed herein relate to the field of plant molecular biology, including to DNA constructs for conferring insect resistance to a plant. Embodiments disclosed herein also include insect resistant corn plant containing event DP-023211-2 and assays for detecting the presence of event DP-023211-2 in a sample and compositions thereof.

BACKGROUND

Corn is an important crop and is a primary food source in many areas of the world. Damage caused by insect pests is a major factor in the loss of the world's corn crops, despite the use of protective measures such as chemical pesticides. In view of this, insect resistance has been genetically engineered into crops such as corn in order to control insect damage and to reduce the need for traditional chemical pesticides. One group of genes which have been utilized for the production of transgenic insect resistant crops is the delta-endotoxin group from *Bacillus thuringiensis (Bt).* Delta-endotoxins have been successfully expressed in crop plants such as cotton, potatoes, rice, sunflower, as well as corn, and in certain circumstances have proven to provide excellent control over insect pests. (Perlak, F.J *et al.* (1990) *Bio/Technology* 8:939-943; Perlak, F.J. *et al.* (1993) *Plant Mol. Biol.*

22:313-321; Fujimoto, H. *et al.* (1993) *Bio/Technology* 11:1151-1155; Tu *et al.* (2000) *Nature Biotechnology* 18:1101-1104; PCT publication WO 01/13731; and Bing, J.W. *et al.* (2000) Efficacy of CrylF Transgenic Maize, 14th Biennial International Plant Resistance to Insects Workshop, Fort Collins, CO).

The expression of transgenes in plants is known to be influenced by many different factors, including the orientation and composition of the cassettes driving expression of the individual genes of interest, and the location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site (Weising *et al.* (1988) *Ann. Rev. Genet.* 22:421-477).

It would be advantageous to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene of interest.

It is possible to detect the presence of a transgene by a nucleic acid detection method by, e.g., a polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc., because for many DNA constructs, the coding region is interchangeable. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known

SUMMARY

The embodiments relate to the insect resistant corn (*Zea mays*) plant event DP-0232! 1-2, also referred to as "maize line DP-023211-2," "maize event DP-023211-2," and "DP-02321 1-2 maize," to the DNA plant expression construct of corn plant event DP-0232! 1-2, and to methods and compositions for the detection of the transgene construct, flanking, and insertion (the target locus) regions in corn plant event DP-0232 11-2 and progeny thereof.

In one aspect compositions and methods relate to methods for producing and selecting an insect resistant monocot crop plant. Compositions include a DNA construct that when expressed in plant cells and plants confers resistance to insects. In one aspect, a DNA construct, capable of introduction into and replication in a host cell, is provided that when expressed in plant cells and plants confers insect resistance to the plant cells and plants. Maize event DP-02321 1-2 was produced by *Agrobacterium*- mediated

transformation with plasmid PHP74643. As described herein, these events include the DvSSJl (SEQ ID NO: 6) and IPD072 (polynucleotide SEQ ID NO: 4 and amino acid SEQ ID NO: 5) cassettes, which confer resistance to certain Coleopteran plant pests. The insect control components have demonstrated efficacy against western corn rootworm (WCR), northern corn rootworm (NCR), and southern com rootworm (SCR).

A first cassette is expressed as a transcript that contains two RNA fragments of the smooth septate junction protein 1 (DvSSJl) gene from *Diabrotica virgifera* (Western corn rootworm) separated by an intron connector sequence derived from the intron 1 region of the *Zea mays* alcohol dehydrogenase (*zm-Adhl*) gene to form an inverted repeat configuration. Expression of the DvSSJl fragments is controlled by a third copy of the *iibiZM* 1 promoter, the 5' ETTR, and intron, in conjunction with the terminator region from the *Zea mays* W64 line 27-kDa gamma zein (Z27G) gene. Two additional terminators are present to prevent transcriptional interference: the terminator region from the *Arabidopsis thaliana* ubiquitin 14 (*UBQ*14*)* gene (Callis *el al,* 1995) and the terminator region from the *Zea mays In2-1* gene (Hershey and Stoner, 1991).

A second cassette contains the insecticidal protein gene, *ipd072Aa,* from *Pseudomonas chlororaphis* (SEQ ID NO: 4). Expression of the IPD072Aa protein (SEQ ID NO: 5) in plants is effective against certain coleopteran pests involves disruption of the midgut epithelium. The IPD072Aa protein is 86 amino acids in length and has a molecular weight of approximately 10 kDa. Expression of the *ipd072Aa* gene is controlled by the promoter region from the banana streak virus of acuminata Yunnan strain (BSV [AY]) (Zhuang *el al,* 2011) and the intron region from the *Zea mays* ortholog of an *Oryza sativa* (rice) hypothetical protein (zm-HPLV9), in conjunction with the terminator region from the *Arabidopsis thaliana at*-T9 gene (GenBank accession NM_00l202984).

A third gene cassette (*mo-pat* gene cassette) contains the phosphinothricin acetyl transferase gene (*mo-pat*) from *Streptomyces viridochromogenes* (Wohlleben *et al,* 1988). The *mo-pat* gene expresses the phosphinothricin acetyl transferase (PAT) enzyme that confers tolerance to phosphinothricin. The PAT protein is 183 amino acids in length and has a molecular weight of approximately 2 1 kDa. Expression of the *mo-pat* gene is controlled by the promoter and intron region of the *Oryza sativa* (rice) actin (as-actin) gene (GenBank accession CP018159), in conjunction with a third copy of the CaMV35S terminator. Two additional terminators are present to prevent transcriptional interference: the terminator regions from the *Sorghum bicolor* (sorghum) ubiquitin (*sb-ubi*) gene

(Phytozome gene ID Sobic.004G049900.l) and γ-kafarin (*sb-gkaf)* gene (de Freitas *el al.*, 1994), respectively.

A fourth gene cassette *(pmi* gene cassette) contains the phosphomannose isomerase (*pmi*) gene from *Escherichia coli* (Negrotto *el al,* 2000). Expression of the PMI protein in plants servers as a selectable marker which allows plant tissue growth with mannose as the carbon source. The PMI protein is 391 amino acids in length and has a molecular weight of approximately 43 kDa. As present in the T-DNA region of PHP74643, the *pmi* gene lacks a promoter, but its location next to the flippase recombination target site, FRT1, allows post-recombination expression by an appropriately-placed promoter. The terminator for the *pmi* gene is a fourth copy of the *pinII* terminator. An additional Z19 terminator present is intended to prevent transcriptional interference between cassettes.

According to some embodiments, compositions and methods are provided for identifying a novel com plant designated DP-02321 1-2 (ATCC Deposit Number PTA-124722). The methods are based on primers or probes which specifically recognize 5' and/or 3' flanking sequence of DP-0232 11-2. DNA molecules are provided that comprise primer sequences that when utilized in a PCR reaction will produce amplicons unique to the transgenic event DP-0232 11-2. In one embodiment, the com plant and seed comprising these molecules is contemplated. Further, kits utilizing these primer sequences for the identification of the DP-0232 11-2 event are provided.

Some embodiments relate to specific flanking sequences of DP-023211-2 as described herein, which can be used to develop identification methods for DP-0232 11-2 in biological samples. More particularly, the disclosure relates to 5' and/or 3' flanking regions of DP-0232 11-2, which can be used for the development of specific primers and probes. Further embodiments relate to identification methods for the presence of DP-0232 11-2 in biological samples based on the use of such specific primers or probes.

According to some embodiments, methods of detecting the presence of DNA corresponding to the corn event DP-0232 11-2 in a sample are provided. Such methods comprise: (a) contacting the sample comprising DNA with a DNA primer set, that when used in a nucleic acid amplification reaction with genomic DNA extracted from corn comprising event DP-0232 11-2 produces an amplicon that is diagnostic for com event DP-0232! 1-2, respectively; (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon. In some aspects, the primer set comprises SEQ ID NOs: 7 and 8, and optionally a probe comprising SEQ ID NO: 9.

According to some embodiments, methods of detecting the presence of a DNA molecule corresponding to the DP-0232 11-2 event in a sample comprise: (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that hybridizes under stringent hybridization conditions with DNA extracted from com event DP-0232 11-2 and does not hybridize under the stringent hybridization conditions with a control com plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA extracted from com event DP-0232 11-2. More specifically, a method for detecting the presence of a DNA molecule corresponding to the DP-0232 11-2 event in a sample consist of (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that comprises of sequences that are unique to the event, e.g. junction sequences, wherein said DNA probe molecule hybridizes under stringent hybridization conditions with DNA extracted from corn event DP-0232 11-2 and does not hybridize under the stringent hybridization conditions with a control com plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.

In addition, a kit and methods for identifying event DP-0232 11-2 in a biological sample which detects a DP-0232 11-2 specific region are provided.

DNA molecules are provided that comprise at least one junction sequence of DP-0232! 1-2; wherein a junction sequence spans the junction located between heterologous DNA inserted into the genome and the DNA from the maize cell flanking the insertion site, and may be diagnostic for the DP-0232 11-2 event.

According to some embodiments, methods of producing an insect resistant corn plant comprise the steps of: (a) sexually crossing a first parental com line comprising the expression cassettes disclosed herein, which confer resistance to insects, and a second parental corn line that lacks such expression cassettes, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that is insect resistant. Such methods may optionally comprise the further step of back-crossing the progeny plant to the second parental corn line to produce a true-breeding com plant that is insect resistant.

Some embodiments provide a method of producing a corn plant that is resistant to insects comprising transforming a corn cell with the DNA construct PHP74643, growing the transformed corn cell into a corn plant, selecting the corn plant that shows resistance to insects, and further growing the com plant into a fertile corn plant. The fertile com plant

can be self-pollinated or crossed with compatible corn varieties to produce insect resistant progeny.

Some embodiments further relate to a DNA detection kit for identifying maize event DP-02321 1-2 in biological samples. The kit comprises a first primer which specifically recognizes the 5' or 3' flanking region of DP-0232 11-2, and a second primer which specifically recognizes a sequence within the non-native target locus DNA of DP-0232 11-2, respectively, or within the flanking DNA, for use in a PCR identification protocol. A further embodiment relates to a kit for identifying event DP-0232 11-2 in biological samples, which kit comprises a specific probe having a sequence which corresponds or is complementary to, a sequence having between about 80% and 100% sequence identity with a specific region of event DP-0232 11-2. The sequence of the probe corresponds to a specific region comprising part of the 5' or 3' flanking region of event DP-0232 11-2. In some embodiments, the first or second primer comprises any one of SEQ ID NOs: 7-8, 10-11, 13-14, 16-17, 19-20, or 22-23.

The methods and kits encompassed by the embodiments disclosed herein can be used for different purposes such as, but not limited to the following: to identify event DP-0232! 1-2 in plants, plant material or in products such as, but not limited to, food or feed products (fresh or processed) comprising, or derived from plant material; additionally or alternatively, the methods and kits can be used to identify transgenic plant material for purposes of segregation between transgenic and non-transgenic material; additionally or alternatively, the methods and kits can be used to determine the quality of plant material comprising maize event DP-0232 11-2. The kits may also contain the reagents and materials necessary for the performance of the detection method.

A further embodiment relates to the DP-02321 1-2 maize plant or its parts, including, but not limited to, pollen, ovules, vegetative cells, the nuclei of pollen cells, and the nuclei of egg cells of the corn plant DP-0232 11-2 and the progeny derived thereof. In another embodiment, the DNA primer molecules targeting the maize plant and seed of DP-0232 11-2 provide a specific amplicon product

DESCRIPTION OF THE DRAWINGS

FIG. 1. shows a schematic diagram of plasmid PHP74643 with genetic elements indicated (SEQ ID NO: 1). Plasmid size is 71,116 bp.

FIG. 2. shows a schematic diagram of the insert T-DNA region of plasmid PHP74643 (SEQ ID NO: 2 is the T-DNA insert and SEQ ID NOs: 3 is the insert T-DNA including the

landing pads) indicating eight gene cassettes. The T-DNA was used to transform a pre-characterized line containing FRT1 and FRT87 sites. The region between the FRT1 and FRT87 sites in the T-DNA containing *pmi* gene, *mo-pat* gene, DvSSJl fragments and *ipd072Aa* gene was integrated into the maize line in a site-specific manner.

FIG. 3. shows a schematic map of the insertion of DP-0232 11-2 maize based on the Southem-by- Sequencing ("SbS") analysis described. A single copy of the integrated PHP74643 T-DNA between FRT1 and FRT87 sites is shown by the middle box. The site-specific landing pad sequence is shown by the outer boxes, and the 5' and 3' flanking maize genome is represented by the horizontal black bar. Representative individual sequencing reads across the FRT1 and FRT87 junctions are shown as stacked lines for each junction. The FRT1 and FRT87 sequences are highlighted with in each read. For the FRT1 site, black lines within each individual read on the left side of the highlighted FRT1 sequence represent the adjacent site-specific landing pad sequence and black on the right side of the FRT1 sequence indicates the integrated PHP74643 sequence. For the FRT87 site, black lines on the left side of the highlighted FRT87 sequence represent the integrated PHP74643 sequence and black on the right side of the FRT87 sequence indicates the adjacent site-specific landing pad sequence. The numbers below the map indicated the bp location of the FRT elements in reference to the sequence of the PHP74643 T-DNA (FIG. 2).

FIG. 4. shows a schematic Diagram of the Transformation and Development of DP-0232! 1-2.

FIG. 5 is a table showing the hybrid performance of five construct designs compared to a base entry for non-yield agronomic traits.

FIG. 6 is a table showing hybrid performance of event DP-0232 11-2 compared to a base entry for non-yield agronomic traits.

FIG. 7 is a table showing inbred performance of construct designs compared to a base entry for all agronomic traits.

FIG. 8 is a table showing inbred performance of event DP-0232 11-2 compared to a base entry for all agronomic traits.

DETAILED DESCRIPTION

As used herein the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the protein" includes reference to one or more proteins and equivalents thereof, and so forth. All technical and scientific terms used

herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs unless clearly indicated otherwise.

Compositions of this disclosure include seed deposited as ATCC Patent Deposit No. PTA-124722 and plants, plant cells, and seed derived therefrom. Applicant(s) deposited at least 2500 seeds of maize event DP-023211-2 (Patent Deposit No. PTA-124722) with the American Type Culture Collection (ATCC), Manassas, VA 20110-2209 USA, on January 18, 2018. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The seeds deposited with the ATCC on January 18, 2018 were taken from the deposit maintained by Pioneer Hi-Bred International, Inc., 7250 NW 62nd Avenue, Johnston, Iowa 50131-1000. Access to this deposit will be available during the pendency of the application to the Commissioner of Patents and Trademarks and persons determined by the Commissioner to be entitled thereto upon request. Upon allowance of any claims in the application, the Applicant(s) will make available to the public, pursuant to 37 C.F.R. § 1.808, sample(s) of the deposit of at least 2500 seeds of hybrid maize with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209. This deposit of seed of maize event DP-02321 1-2 will be maintained in the ATCC depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the enforceable life of the patent, whichever is longer, and will be replaced if it becomes nonviable during that period. Additionally, Applicant(s) have satisfied all the requirements of 37 C.F.R. §§1.801 - 1.809, including providing an indication of the viability of the sample upon deposit. Applicant(s) have no authority to waive any restrictions imposed by law on the transfer of biological material or its transportation in commerce. Applicant(s) do not waive any infringement of their rights granted under this patent or rights applicable to event DP-0232 11-2 under the Plant Variety Protection Act (7 USC 2321 et seq.). Unauthorized seed multiplication is prohibited. The seed may be regulated.

As used herein, the term "com" means *Zea mays* or maize and includes all plant varieties that can be bred with com, including wild maize species.

As used herein, the terms "insect resistant" and "impacting insect pests" refers to effecting changes in insect feeding, growth, and/or behavior at any stage of development, including but not limited to: killing the insect; retarding growth; reducing reproductive capability; inhibiting feeding; and the like.

As used herein, the terms “pesticidal activity” and “insecticidal activity” are used synonymously to refer to activity of an organism or a substance (such as, for example, a protein) that can be measured by numerous parameters including, but not limited to, pest mortality, pest weight loss, pest attraction, pest repellency, and other behavioral and physical changes of a pest after feeding on and/or exposure to the organism or substance for an appropriate length of time. For example, “pesticidal proteins” are proteins that display pesticidal activity by themselves or in combination with other proteins.

As used herein, “insert DNA” refers to the heterologous DNA within the expression cassettes used to transform the plant material while “flanking DNA” can exist of either genomic DNA naturally present in an organism such as a plant, or foreign (heterologous) DNA introduced via the transformation process which is extraneous to the original insert DNA molecule, e.g. fragments associated with the transformation event. A “flanking region” or “flanking sequence” as used herein refers to a sequence of at least 20 bp (in some narrower embodiments, at least 50 bp, and up to at least 5000 bp), which is located either immediately upstream of and contiguous with and/or immediately downstream of and contiguous with the original non-native insert DNA molecule. Transformation procedures of the foreign DNA may result in transformants containing different flanking regions characteristic and unique for each transformant. When recombinant DNA is introduced into a plant through traditional crossing, its flanking regions will generally not be changed. It may be possible for single nucleotide changes to occur in the flanking regions through generations of plant breeding and traditional crossing. Transformants will also contain unique junctions between a piece of heterologous insert DNA and genomic DNA, or two (2) pieces of genomic DNA, or two (2) pieces of heterologous DNA. A "junction" is a point where two (2) specific DNA fragments join. For example, a junction exists where insert DNA joins flanking DNA. A junction point also exists in a transformed organism where two (2) DNA fragments join together in a manner that is modified from that found in the native organism. “Junction DNA” refers to DNA that comprises a junction point. Junction sequences set forth in this disclosure include a junction point located between the maize genomic DNA and the 5’ end of the insert, which range from at least -5 to +5 nucleotides of the junction point (SEQ ID NO: 31), from at least -10 to +10 nucleotides of the junction point (SEQ ID NO: 32), from at least -15 to +15 nucleotides of the junction point (SEQ ID NO: 33), and from at least -20 to +20 nucleotides of the junction point (SEQ ID NO: 34); and a junction point located between the 3’ end of the insert and maize genomic DNA, which range from at least -5 to +5 nucleotides of the junction point (SEQ

ID NO: 35), from at least -10 to +10 nucleotides of the junction point (SEQ ID NO: 36), from at least -15 to +15 nucleotides of the junction point (SEQ ID NO: 37), and from at least -20 to +20 nucleotides of the junction point (SEQ ID NO: 38). Junction sequences set forth in this disclosure also include a junction point located between the target locus and the 5' end of the insert. In some embodiments, SEQ ID NOs: 9 or 25 for DP-023211-2 represent the junction point located between the target locus and the 5' end of the insert.

As used herein, "heterologous" in reference to a nucleic acid sequence is a nucleic acid sequence that originates from a different non-sexually compatible species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous nucleotide sequence can be from a species different from that from which the nucleotide sequence was derived, or, if from the same species, the promoter is not naturally found operably linked to the nucleotide sequence. A heterologous protein may originate from a foreign species, or, if from the same species, is substantially modified from its original form by deliberate human intervention.

The term "regulatory element" refers to a nucleic acid molecule having gene regulatory activity, i.e. one that has the ability to affect the transcriptional and/or translational expression pattern of an operably linked transcribable polynucleotide. The term "gene regulatory activity" thus refers to the ability to affect the expression of an operably linked transcribable polynucleotide molecule by affecting the transcription and/or translation of that operably linked transcribable polynucleotide molecule. Gene regulatory activity may be positive and/or negative and the effect may be characterized by its temporal, spatial, developmental, tissue, environmental, physiological, pathological, cell cycle, and/or chemically responsive qualities as well as by quantitative or qualitative indications.

"Promoter" refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence comprises proximal and more distal upstream elements, the latter elements are often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that

different regulatory elements may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters that cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, nucleic acid fragments of different lengths may have identical promoter activity.

The "translation leader sequence" refers to a nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect numerous parameters including, processing of the primary transcript to mRNA, mRNA stability and/or translation efficiency.

The "3' non-coding sequences" refer to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

A DNA construct is an assembly of DNA molecules linked together that provide one or more expression cassettes. The DNA construct may be a plasmid that is enabled for self replication in a bacterial cell and contains various endonuclease enzyme restriction sites that are useful for introducing DNA molecules that provide functional genetic elements, i.e., promoters, introns, leaders, coding sequences, 3' termination regions, among others; or a DNA construct may be a linear assembly of DNA molecules, such as an expression cassette. The expression cassette contained within a DNA construct comprises the necessary genetic elements to provide transcription of a messenger RNA. The expression cassette can be designed to express in prokaryotic cells or eukaryotic cells. Expression cassettes of the embodiments are designed to express in plant cells.

The DNA molecules disclosed herein are provided in expression cassettes for expression in an organism of interest. The cassette includes 5' and 3' regulatory sequences operably linked to a coding sequence. "Operably linked" means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. Operably linked is intended to indicate a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the

second sequence. The cassette may additionally contain at least one additional gene to be co-transformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes or multiple DNA constructs.

The expression cassette may include in the 5' to 3' direction of transcription: a transcriptional and translational initiation region, a coding region, and a transcriptional and translational termination region functional in the organism serving as a host. The transcriptional initiation region (*e.g.*, the promoter) may be native or analogous, or foreign or heterologous to the host organism. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation.

It is to be understood that as used herein the term "transgenic" generally includes any cell, cell line, callus, tissue, plant part, or plant, the genotype of which has been altered by the presence of a heterologous nucleic acid including those initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic and retains such heterologous nucleic acids.

A transgenic "event" is produced by transformation of plant cells with a heterologous DNA construct(s), including a nucleic acid expression cassette that comprises a transgene of interest, 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 into a particular genome location. An event is characterized phenotypically by the expression of the transgene. At the genetic level, an event is part of the genetic makeup of a plant. The term "event" also refers to progeny produced by a sexual outcross between the transformant and another variety, wherein the progeny includes the heterologous DNA. After back-crossing to a recurrent parent, the inserted DNA and the linked flanking genomic DNA from the transformed parent is present in the progeny of the cross at the same chromosomal location. A progeny plant may contain sequence changes to the insert arising as a result of conventional breeding techniques. The term "event" also refers to DNA from the original transformant comprising the inserted DNA and flanking sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

An insect resistant DP-0232 11-2 corn plant may be bred by first sexually crossing a first parental corn plant having the transgenic DP-0232 11-2 event plant and progeny thereof derived from transformation with the expression cassettes of the embodiments that confers insect resistance, and a second parental corn plant that lacks such expression cassettes, thereby producing a plurality of first progeny plants; and then selecting a first progeny plant that is resistant to insects; and selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants an insect resistant plant. These steps can further include the back-crossing of the first insect resistant progeny plant or the second insect resistant progeny plant to the second parental corn plant or a third parental com plant, thereby producing a com plant that is resistant to insects. The term “selfing” refers to self-pollination, including the union of gametes and/or nuclei from the same organism.

As used herein, the term "plant" includes reference to whole plants, parts of plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and progeny of same. In some embodiments, parts of transgenic plants comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fmits, leaves, and roots originating in transgenic plants or their progeny previously transformed with a DNA molecule disclosed herein, and therefore consisting at least in part of transgenic cells.

As used herein, the term "plant cell" includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants that may be used is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

“Transformation” refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host plants containing the transformed nucleic acid fragments are referred to as “transgenic” plants.

As used herein, the term "progeny," in the context of event DP-02321 1-2, denotes an offspring of any generation of a parent plant which comprises corn event DP-0232 11-2.

Isolated polynucleotides disclosed herein may be incorporated into recombinant constructs, typically DNA constructs, which are capable of introduction into and replication in a host cell. Such a construct may be a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels *et*

al., (1985; Supp. 1987) *Cloning Vectors: A Laboratory Manual,* Weissbach and Weissbach (1989) *Methods for Plant Molecular Biology,* (Academic Press, New York); and Flevin *et al.,* (1990) *Plant Molecular Biology Manual,* (Kluwer Academic Publishers). Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

During the process of introducing an insert into the genome of plant cells, it is not uncommon for some deletions or other alterations of the insert and/or genomic flanking sequences to occur. Thus, the relevant segment of the plasmid sequence provided herein might comprise some minor variations. The same is possible for the flanking sequences provided herein. Thus, a plant comprising a polynucleotide having some range of identity with the subject flanking and/or insert sequences is within the scope of the subject disclosure. Identity to the sequence of the present disclosure may be a polynucleotide sequence having at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity at least 80% identity, or at least 85% 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity with a sequence exemplified or described herein. Hybridization and hybridization conditions as provided herein can also be used to define such plants and polynucleotide sequences of the subject disclosure. A sequence comprising the flanking sequences plus the full insert sequence can be confirmed with reference to the deposited seed.

In some embodiments, two different transgenic plants can also be crossed to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation

A "probe" is an isolated nucleic acid to which is attached a conventional, synthetic detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. Such a probe is complementary to a strand of a target nucleic acid, for example, to a strand of isolated DNA from corn event DP-0232 11-2 whether from a corn plant or from a sample that includes DNA from the event. Probes may include not only

deoxyribonucleic or ribonucleic acids but also polyamides and other modified nucleotides that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

"Primers" are isolated nucleic acids that anneal to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs refer to their use for amplification of a target nucleic acid sequence, e.g., by PCR or other conventional nucleic-acid amplification methods. "PCR" or "polymerase chain reaction" is a technique used for the amplification of specific DNA segments (see, U.S. Patent Nos. 4,683,195 and 4,800,159; herein incorporated by reference).

Probes and primers are of sufficient nucleotide length to bind to the target DNA sequence specifically in the hybridization conditions or reaction conditions determined by the operator. This length may be of any length that is of sufficient length to be useful in a detection method of choice. Generally, 11 nucleotides or more in length, 18 nucleotides or more, and 22 nucleotides or more, are used. Such probes and primers hybridize specifically to a target sequence under high stringency hybridization conditions. Probes and primers according to embodiments may have complete DNA sequence similarity of contiguous nucleotides with the target sequence, although probes differing from the target DNA sequence and that retain the ability to hybridize to target DNA sequences may be designed by conventional methods. Probes can be used as primers, but are generally designed to bind to the target DNA or RNA and are not used in an amplification process.

Specific primers may be used to amplify an integration fragment to produce an amplicon that can be used as a "specific probe" for identifying event DP-0232 11-2 in biological samples. When the probe is hybridized with the nucleic acids of a biological sample under conditions which allow for the binding of the probe to the sample, this binding can be detected and thus allow for an indication of the presence of event DP-0232! 1-2 in the biological sample. In an embodiment of the disclosure, the specific probe is a sequence which, under appropriate conditions, hybridizes specifically to a region within the 5' or 3' flanking region of the event and also comprises a part of the foreign DNA contiguous therewith. The specific probe may comprise a sequence of at least 80%, from 80 and 85%, from 85 and 90%, from 90 and 95%, and from 95 and 100% identical (or complementary) to a specific region of the event.

Methods for preparing and using probes and primers are described, for example, in Sambrook *et al, Molecular Cloning: A Laboratory Manual, 2nd ed.*, vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989 (hereinafter, "Sambrook *et al,* 1989"); Ausubel *et al.* eds., *Current Protocols in Molecular Biology*, , Greene Publishing and Wiley-Interscience, New York, 1995 (with periodic updates) (hereinafter, "Ausubel *et al.,* 1995"); and Innis *et al., PCR Protocols: A Guide to Methods and Applications,* Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as the PCR primer analysis tool in Vector NTI version 6 (Informax Inc., Bethesda MD); PrimerSelect (DNASTAR Inc., Madison, WI); and Primer (Version 0.5®, 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). Additionally, the sequence can be visually scanned and primers manually identified using guidelines known to one of skill in the art.

A "kit" as used herein refers to a set of reagents, and optionally instructions, for the purpose of performing method embodiments of the disclosure, more particularly, the identification of event DP-02321 1-2 in biological samples. A kit may be used, and its components can be specifically adjusted, for purposes of quality control (e.g. purity of seed lots), detection of event DP-02321 1-2 in plant material, or material comprising or derived from plant material, such as but not limited to food or feed products. "Plant material" as used herein refers to material which is obtained or derived from a plant.

Primers and probes based on the flanking DNA and insert sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed sequences by conventional methods, e.g., by re-cloning and sequencing such sequences. The nucleic acid probes and primers hybridize under stringent conditions to a target DNA sequence. Any conventional nucleic acid hybridization or amplification method may be used to identify the presence of DNA from a transgenic event in a sample.

A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity or minimal complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one

another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook *et al*, 1989, and by Haymes *el al.*, In: *Nucleic Acid Hybridization, a Practical Approach*, IRL Press, Washington, D.C. (1985), departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

In hybridization reactions, specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. The thermal melting point (T_m) is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: T_m = 81.5 °C + 16.6 (log M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. T_m is reduced by about 1 °C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the T_m can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the T_m for the specific sequence and its complement at a defined ionic strength and pH. However, in some embodiments, other stringency conditions can be applied, including severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the T_m; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the T_m; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the T_m.

Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution), a user may choose to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid*

Probes, Part I, Chapter 2 (Elsevier, New York); and Ausubel *el al*, eds. (1995) and Sambrook *et al.* (1989).

In some embodiments, a complementary sequence has the same length as the nucleic acid molecule to which it hybridizes. In some embodiments, the complementary sequence is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides longer or shorter than the nucleic acid molecule to which it hybridizes. In some embodiments, the complementary sequence is 1%, 2%, 3%, 4%, or 5% longer or shorter than the nucleic acid molecule to which it hybridizes. In some embodiments, a complementary sequence is complementary on a nucleotide-for-nucleotide basis, meaning that there are no mismatched nucleotides (each A pairs with a T and each G pairs with a C). In some embodiments, a complementary sequence comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or less mismatches. In some embodiments, the complementary sequence comprises 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% or less mismatches.

"Percent (%) sequence identity" with respect to a reference sequence (subject) is determined as the percentage of amino acid residues or nucleotides in a candidate sequence (query) that are identical with the respective amino acid residues or nucleotides in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any amino acid conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (e.g., percent identity of query sequence = number of identical positions between query and subject sequences/total number of positions of query sequence xlOO).

Regarding the amplification of a target nucleic acid sequence (e.g., by PCR) using a particular amplification primer pair, stringent conditions permit the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and optionally to produce a unique amplification product, the amplicon, in a DNA thermal amplification reaction.

As used herein, “amplified DNA” or “amplicon” refers to the product of nucleic acid amplification of a target nucleic acid sequence that is part of a nucleic acid template. Lor example, to determine whether a corn plant resulting from a sexual cross contains

transgenic event genomic DNA from the corn plant disclosed herein, DNA extracted from a tissue sample of a com plant may be subjected to a nucleic acid amplification method using a DNA primer pair that includes a first primer derived from flanking sequence adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA. Alternatively, the second primer may be derived from the flanking sequence. The amplicon is of a length and has a sequence that is also diagnostic for the event. The amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. Alternatively, primer pairs can be derived from flanking sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence of the PHP74643 expression construct as well as a portion of the sequence flanking the transgenic insert. A member of a primer pair derived from the flanking sequence may be located a distance from the inserted DNA sequence, this distance can range from one nucleotide base pair up to the limits of the amplification reaction. The use of the term “amplicon” specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

Nucleic acid amplification can be accomplished by any of the various nucleic acid amplification methods known in the art, including PCR. A variety of amplification methods are known in the art and are described, inter alia, in U.S. Pat. Nos. 4,683,195 and 4,683,202 and in Innis *et al,* (1990) supra. PCR amplification methods have been developed to amplify up to 22 Kb of genomic DNA and up to 42 Kb of bacteriophage DNA (Cheng *et al, Proc. Natl. Acad. Sci. USA* 91:5695-5699, 1994). These methods as well as other methods known in the art of DNA amplification may be used in the practice of the embodiments of the present disclosure. It is understood that a number of parameters in a specific PCR protocol may need to be adjusted to specific laboratory conditions and may be slightly modified and yet allow for the collection of similar results. These adjustments will be apparent to a person skilled in the art.

The amplicon produced by these methods may be detected by a plurality of techniques, including, but not limited to, Genetic Bit Analysis (Nikiforov, *et al. Nucleic Acid Res.* 22:4167-4175, 1994) where a DNA oligonucleotide is designed which overlaps both the adjacent flanking DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (for example, using one primer in the inserted sequence and one in the adjacent

flanking sequence) a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Another detection method is the pyrosequencing technique as described by Winge (2000) *Innov. Pharma. Tech.* 00:18-24. In this method an oligonucleotide is designed that overlaps the adjacent DNA and insert DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (for example, one primer in the inserted sequence and one in the flanking sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. dNTPs are added individually and the incorporation results in a light signal which is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Fluorescence polarization as described by Chen *et al.*, (1999) *Genome Res.* 9:492-498 is also a method that can be used to detect an amplicon. Using this method an oligonucleotide is designed which overlaps the flanking and inserted DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (for example, one primer in the inserted DNA and one in the flanking DNA sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Quantitative PCR (qPCR) is described as a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by commercially available manufacturers. Briefly, in one such qPCR method, a FRET oligonucleotide probe is designed which overlaps the flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

Molecular beacons have been described for use in sequence detection as described in Tyangi *et al.* (1996) *Nature Biotech.* 14:303-308. Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (for example, one primer in the insert DNA sequence and one in the flanking sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

A hybridization reaction using a probe specific to a sequence found within the amplicon is yet another method used to detect the amplicon produced by a PCR reaction.

Insect pests include insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera.

Of interest are larvae and adults of the order Coleoptera including weevils from the families Anthribidae, Bruchidae, and Curculionidae including, but not limited to: *Anthonomus grandis* Boheman (boll weevil); *Cylindrocopturus adspersus* LeConte (sunflower stem weevil); *Diaprepes abbreviatus* Linnaeus (Diaprepes root weevil); *Hypera punctata* Fabricius (clover leaf weevil); *Lissorhoptrus oryzophilus* Kuschel (rice water weevil); *Metamasius hemipterus hemipterus* Linnaeus (West Indian cane weevil); *M. hemipterus sericeus* Olivier (silky cane weevil); *Sitophilus granarius* Linnaeus (granary weevil); *S. oryzae* Linnaeus (rice weevil); *Smicronyx fulvus* LeConte (red sunflower seed weevil); *S. sordidus* LeConte (gray sunflower seed weevil); *Sphenophorus maidis* Chittenden (maize billbug); *S. livis* Vaurie (sugarcane weevil); *Rhabdoscelus obscurus* Boisduval (New Guinea sugarcane weevil); flea beetles, cucumber beetles, rootworms, leaf beetles, potato beetles, and leafminers in the family Chrysomelidae including, but not limited to: *Chaetocnema ectypa* Horn (desert com flea beetle); C. *pulicaria* Melsheimer (corn flea beetle); *Colaspis brunnea* Fabricius (grape colaspis); *Diabrotica barberi* Smith & Lawrence (northern com rootworm); *D. undecimpunctata howardi* Barber (southern com rootworm); *D. virgifera virgifera* LeConte (western corn rootworm); *Leptinotarsa decemlineata* Say (Colorado potato beetle); *Oulema melanopus* Linnaeus (cereal leaf

beetle); *Phyllotreta cruciferae* Goeze (corn flea beetle); *Zygogramma exclamationis* Fabricius (sunflower beetle); beetles from the family Coccinellidae including, but not limited to: *Epilachna varivestis* Mulsant (Mexican bean beetle); chafers and other beetles from the family Scarabaeidae including, but not limited to: *Antitrogus parvulus* Britton (Childers cane grub); *Cyclocephala borealis* Arrow (northern masked chafer, white grub); *C. immaculata* Olivier (southern masked chafer, white grub); *Dermolepida albohirtum* Waterhouse (Greyback cane beetle); *Euetheola humilis rugiceps* LeConte (sugarcane beetle); *Lepidiota frenchi* Blackburn (French's cane grub); *Tomarus gibbosus,* De Geer (carrot beetle); *T. subtropicus* Blatchley (sugarcane grub); *Phyllophaga crinita* Burmeister (white grub); *P. latifrons* LeConte (June beetle); *Popillia japonica* Newman (Japanese beetle); *Rhizotrogus majalis* Razoumowsky (European chafer); carpet beetles from the family Dermestidae; wireworms from the family Elateridae, *Eleodes* spp., *Melanotus* spp. including *M. communis* Gyllenhal (wireworm); *Conoderus* spp.; *Eimonius* spp.; *Agriotes* spp.; *Ctenicera* spp.; *Aeolus* spp.; bark beetles from the family Scolytidae; beetles from the family Tenebrionidae; beetles from the family Cerambycidae such as, but not limited to, *Migdolus fry anus* Westwood (longhorn beetle); and beetles from the Buprestidae family including, but not limited to, *Aphanisticus cochinchinae seminulum* Obenberger (leaf-mining buprestid beetle).

In some embodiments the DP-0232 11-2 maize event may further comprise a stack of additional traits. Plants comprising stacks of polynucleotide sequences can be obtained by either or both of traditional breeding methods or through genetic engineering methods. These methods include, but are not limited to, breeding individual lines each comprising a polynucleotide of interest, transforming a transgenic plant comprising a gene disclosed herein with a subsequent gene and co- transformation of genes into a single plant cell. As used herein, the term "stacked" includes having the multiple traits present in the same plant (i.e., both traits are incorporated into the nuclear genome, one trait is incorporated into the nuclear genome and one trait is incorporated into the genome of a plastid or both traits are incorporated into the genome of a plastid).

In some embodiments the DP-0232 11-2 maize event disclosed herein, alone or stacked with one or more additional insect resistance traits can be stacked with one or more additional input traits (e.g., herbicide resistance, fungal resistance, virus resistance, stress tolerance, disease resistance, male sterility, stalk strength, and the like) or output traits (e.g., increased yield, modified starches, improved oil profile, balanced amino acids, high lysine

or methionine, increased digestibility, improved fiber quality, drought resistance, and the like). Thus, the embodiments can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

In a further embodiment, the DP-0232 11-2 maize event may be stacked with one or more additional *Bt* insecticidal toxins, including, but not limited to, a Cry3B toxin disclosed in US Patent Numbers 8,101,826, 6,551,962, 6,586,365, 6,593,273, and PCT Publication WO 2000/011185; a mCry3B toxin disclosed in US Patent Numbers 8,269,069, and 8,513,492; a mCry3A toxin disclosed in US Patent Numbers 8,269,069, 7,276,583 and 8,759,620; or a Cry34/35 toxin disclosed in US Patent Numbers 7,309,785, 7,524,810, 7,985,893, 7,939,651 and 6,548,291. In a further embodiment, the DP-023211-2 maize event may be stacked with one or more additional transgenic events containing these *Bt* insecticidal toxins and other *Coleopteran* active *Bt* insecticidal traits for example, event MON863 disclosed in US Patent Number 7,705,216; event MIR604 disclosed in US Patent Number 8,884,102; event 5307 disclosed in US Patent Number 9,133,474; event DAS-59122 disclosed in US Patent Number 7,875,429; event DP-4 114 disclosed in US Patent Number 8,575,434; event MON 87411 disclosed in US Patent Number 9,441,240; and event MON88017 disclosed in US Patent Number 8,686,230 all of which are incorporated herein by reference. In some embodiments, the DP-0232 11-2 maize event may be stacked with MON87427; MON-00603-6 (NK603); MON-87460-4; LY038; DAS-06275-8; BT176; BT11; MIR 162; GA21; MZDT09Y ; SYN-05307-1; and DAS-40278-9.

In some embodiments, a corn plant comprising a DP-0232 11-2 event may be treated with a seed treatment. In some embodiments, the seed treatment may be a fungicide, an insecticide, or a herbicide.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1. Cassette Design for Transgenic Plants Containing Constructs Encoding IPD072 and dsRNA targeting DvSSJl

Cassette designs for IPD072 and DvSSJl expression used in the molecular stacks to generate commercial track events were chosen based upon their efficacy and expression in gene testing transformation experiments. A large number of different regulatory

(promoters, introns) and other elements (terminators, RNAi hairpin designs) were evaluated in gene testing experiments. The large number of different regulatory elements were used to evaluate expression patterns for yield and trait efficacy.

Three gene testing experiments were carried out to evaluate about 40 different IPD072 single cassettes. These experiments involved a gene design screen and two construct matrices in which multiple promoters, terminators and subcellular targeting strategies were evaluated. Four IPD072 cassette designs were chosen from these experiments for inclusion in molecular stacks with DvSSJl.

A similar, but more extensive approach was taken to choose three cassette designs for DvSSJl. About 100 single DvSSJl cassettes were evaluated in multiple TO experiments. These included experiments designed to choose *dvssjl* fragments for hairpin stem design, the loop region of the hairpin, directionality of the hairpin stem and the promoters driving hairpin expression.

In all cases the DvSSJ1 hairpin was cloned upstream of the IPD072 gene. The cassettes were separated by a stack of three terminators. These combinations had not been validated in prior transformations. The genetic elements contained in the T-DNA Region of the selected event construct, Plasmid PHP74643, are described in Table 1.

Table 1: Description of Genetic Elements in the T-DNA Region of Plasmid PHP74643

	Location on T-DNA (Base Pair Position)	Genetic Element	Size (bp)	Description
	20,691 – 20,777	Intervening Sequence	87	DNA sequence used for cloning
DvSSJ1 fragment cassette	20,778 – 21,677	*ubi*ZM1 Promoter	900	Promoter region from the *Zea mays* ubiquitin gene 1
	21,678 – 21,760	*ubi*ZM1 5' UTR	83	5' untranslated region from the *Zea mays* ubiquitin gene 1
	21,761 – 22,773	*ubi*ZM1 Intron	1,013	Intron region from the *Zea mays* ubiquitin gene 1
	22,774 – 22,812	Intervening Sequence	39	DNA sequence used for cloning
	22,813 – 23,022	DvSSJ1 Fragment	210	Fragment of the smooth septate junction protein 1 gene from *Diabrotica virgifera* (Western corn rootworm)
	23,023 – 23,043	Intervening Sequence	21	DNA sequence used for cloning
	23,044 – 23,143	*zm-Adh1* Intron Connector	100	Connector sequence derived from the Intron 1 region of the *Zea mays* alcohol dehydrogenase gene, containing the first 50

				base pairs of the 5' end and the last 50 base pairs of the 3' end
	23,144 – 23,164	Intervening Sequence	21	DNA sequence used for cloning
	23,165 – 23,374 (complementary)	DvSSJ1 Fragment	210	Fragment of the smooth septate junction protein 1 gene from *Diabrotica virgifera* (Western corn rootworm;)
	23,375 – 23,408	Intervening Sequence	34	DNA sequence used for cloning
	23,409 – 23,888	Z27G Terminator	480	Terminator region from the *Zea mays* W64 line 27-kDa gamma zein gene
	23,889 – 23,894	Intervening Sequence	6	DNA sequence used for cloning
	23,895 – 24,796	*UBQ14* Terminator	902	Terminator region from the *Arabidopsis thaliana* ubiquitin 14 gene
	24,797 – 24,802	Intervening Sequence	6	DNA sequence used for cloning
	24,803 – 25,296	*In2-1* Terminator	494	Terminator region from the *Zea mays In2-1* gene
	25,297 – 25,353	Intervening Sequence	57	DNA sequence used for cloning
	25,354 – 25,377	*att*B2	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning System.
	25,378 – 25,414	Intervening Sequence	37	DNA sequence used for cloning
ipd072Aa gene cassette	25,415 – 25,828	BSV(AY) Promoter	414	Promoter region from the banana streak virus (acuminata Yunnan strain) genome
	25,829 – 25,847	Intervening Sequence	19	DNA sequence used for cloning
	25,848 – 26,703	*zm*-HPLV9 Intron	856	Intron region from the *Zea mays* ortholog of an *Oryza sativa* (rice) hypothetical protein
	26,704 – 26,712	Intervening Sequence	9	DNA sequence used for cloning
	26,713 – 26,973	*ipd072Aa*	261	Insecticidal protein *ipd072Aa* gene from *Pseudomonas chlororaphis*
	26,974 – 26,979	Intervening Sequence	6	DNA sequence used for cloning
	26,980 – 27,552	*at*-T9 Terminator	573	Terminator region from an *Arabidopsis thaliana* putative gene of the mannose-binding protein superfamily
	27,553 – 27,591	Intervening Sequence	39	DNA sequence used for cloning
	27,592 – 27,612 (complementary)	*att*B3	21	Bacteriophage lambda integrase recombination site
	27,613 – 27,733	Intervening Sequence	121	DNA sequence used for cloning

	27,734 – 27,781	FRT87	48	Modified Flippase recombination target site derived from *Sacchammyces cenvistae*

Example 2. Transformation of Maize by *Agrobacterium* transformation and Regeneration of Transgenic Plants Containing the IPD072, DvSSJl, PAT, and PMI Genes

DP-02321 1-2 maize event was produced by *Agrobacterium*- mediated SSI transformation with plasmid PHP74643. *Agrobacterium*- mediated SSI was essentially performed as described in U.S. patent application publication number 2017/0240911, herein incorporated by reference (See, for example, Example 3).

Over 2700 immature embryos were infected with PHP74643. After the 105-day selection and regeneration process, a total of 46 TO plantlets were regenerated. Samples were taken from all TO plantlets for PCR analysis to verify the presence and copy number of the inserted IPD072, PMI, mo-PAT and DvSSIl genes. In addition to this analysis, the TO plantlets were analyzed by PCR for the presence of certain *Agrobacterium* binary vector backbone sequences and for the developmental genes, *zm-odp2* and *zm-wus2* disclosed in U.S. Patents 7,579,529 and 7,256,322, herein incorporated by reference in their entireties. Plants that were determined to contain single copy of the inserted genes, no *Agrobacterium* backbone sequences, and no developmental genes were selected for further greenhouse propagation. Samples from those PCR selected TO quality events were collected for further analysis using Southem-by-Sequencing to confirm that the inserted genes were in the correct target locus (also referred to herein as the "landing pad") without any gene disruptions. Maize events DP-02321 1-2 were confirmed to contain a single copy of the T-DNA (See Examples 3 and 4). These selected TO plants were assayed for trait efficacy and protein expression. TO plants meeting all criteria were advanced and crossed to inbred lines to produce seed for further testing. A schematic overview of the transformation and event development is presented in FIG. 4.

Example 3. Identification of Maize Events DP-023211-2

Genomic DNA from leaf tissue representing multiple generations of maize event DP-023211-2, known copy number calibrator controls, a negative control source (DNA from a non-genetically modified maize) and no template controls (NTC) were isolated and subjected to quantitative real-time PCR (qPCR) amplification using event-specific and constmct-specific primer and probes. Real-time PCR analyses of DP-02321 1-2 maize DNA

using event-specific and construct-specific assays confirm the stable integration and segregation of a single copy of the T-DNA of plasmid PHP74643 in leaf samples tested, as demonstrated by the quantified detection of event DP-023211-2, and IPD072, PMI, DvSSJl and mo-PAT transgenes in DP-0232 11-2 maize. The reliability of each event-specific and construct-specific PCR method was assessed by repeating the experiment in quadruplicate. The sensitivity, or Limit of Detection (LOD) of the PCR amplification was evaluated by various dilutions of the genomic DNA from DP-023211-2.

Two generations of maize containing event DP-02321 1-2 were grown in cell-divided flats under typical greenhouse production conditions. Approximately 165 seed were planted for each generation.

Leaf samples were collected from each healthy plant, when plants were between the V5 and V9 growth stages. The samples were taken from the youngest leaf that was emerged from the whorl of each plant. Three leaf punches per plant were analyzed for the copy number of each event's genomic junction and the PHP74643 T-DNA through copy number PCR (qPCR) for the DP- 023211-2 event as well as IPD072, PMI, DvSSJl and mo-PAT transgenes from seed grown at Pioneer Hi-Bred International, Inc. (Johnston, IA). Genomic DNA extractions from the leaf samples were performed using a high alkaline extraction protocol. Validated laboratory controls (copy number calibrators and negative) were prepared from leaf tissue using a standard cetyl trimethylammonium bromide (CTAB) extraction protocol.

Genomic DNA supporting laboratory controls were quantified using Quant-iT PicoGreen® reagent (Invitrogen, Carlsbad, CA). Quantification of genomic test and control samples were estimated using the NanoDrop 2000c Spectrophotometer using NanoDrop 2000/2000c VI. 6.198 Software (ThermoScientific, Wilmington, DE).

Genomic DNA samples isolated from leaf tissue of DP-0232 11-2 as well as control samples were subjected to real-time PCR amplification utilizing event-specific and construct specific primers and probes which span specific regions of the PHP74643 T-DNA as well as the genomic junctions that span each insertion site for events DP-0232 11-2. An endogenous reference gene, High Mobility Group A (*hmg-A*) (Krech, et al. (1999). *Gene* 234: (1) 45-50) was used in duplex with each assay for both qualitative and quantitative assessment of each assay and to demonstrate the presence of sufficient quality and quantity of DNA within the PCR reaction. The PCR target sites and size of expected PCR products for each primer/probe set are shown in Table 3. Primer and probe sequence information supporting each targeted region are shown in Table 4. PCR reagents and reaction

conditions are shown in Table 5. In this study approximately 3-ng of maize genomic DNA was used for all PCR reactions.

Table 3: PCR Genomic DNA Target Site and Expected Size of PCR Products

Primer and Probe Set	Targeted Regions	Expected Size of PCR Product (bp)	Amplicon SEQ ID NO:
SEQ ID NOs: 7-9	DP-023211-2 insertion	75	25
SEQ ID NOs: 10-12	IPD072	72	26
SEQ ID NOs: 13-15	DvSSJ1	77	27
SEQ ID NOs: 16-18	PMI	113	28
SEQ ID NOs: 19-21	mo-PAT	76	29
SEQ ID NOs: 22-24	hmg-A	79	30

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Table 4: Primers and Probe Sequence and Amplicon for PCR Genomic DNA Targeted Regions

Reagent	Sequence (5' to 3')	Length (base)
SEQ ID NO: 7 forward primer	TTACGGCATCTAGGACCGACTAG	23
SEQ ID NO: 8 reverse primer	GAAGCACTTGTTTTTCAATTCCAA	24
SEQ ID NO: 9 probe	6-FAM-CTAGTACGTAGTGAATCTG-MGB	19
SEQ ID NO: 10 forward primer	ACAACAACGCCGTGAAGGA	19
SEQ ID NO: 11 reverse primer	CCAGATTGGTTTCACATACGTATCA	25
SEQ ID NO: 12 probe	6-FAM-AGGGTCGGCTGATC-MGB	14

SEQ ID NO: 13 forward primer	CGTATTCGTAGGTAATTGAGAATTCG	26
SEQ ID NO: 14 reverse primer	CCAAGATTAGTCAGATCAAGAGACAGA	27
SEQ ID NO: 15 probe	6-FAM-TATCAGGTCCGCCTTGT-MGB	17
SEQ ID NO: 16 forward primer	TGACTGTCAAAGGCCACGG	19
SEQ ID NO: 17 reverse primer	AGATGGACAAGTCTAGGTTCCACC	24
SEQ ID NO: 18 probe	6-FAM-CCGTTTAGCGCGTGTTTACAACAAGCTG-BHQ	28
SEQ ID NO: 19 forward primer	CATCGTGAACCACTACATCGAGAC	24
SEQ ID NO: 20 reverse primer	GTCGATCCACTCCTGCGG	18
SEQ ID NO: 21 probe	6'FAM-ACCGTGAACTTCCGCACCGAGC-BHQ1	22
SEQ ID NO: 22 forward primer	TTGGACTAGAAATCTCGTGCTGA	23
SEQ ID NO: 23 reverse primer	GCTACATAGGGAGCCTTGTCCT	22
SEQ ID NO: 24 probe	VIC-GCGTTTGTGTGGATTG-MGB	16
SEQ ID NO: 25: DP-023211-2 assay amplicon sequence (75-bp; primer and probe binding sites are in bold and underlined) **TTACGGCATCTAGGACCGACTAG**CTAACTAA**CTAGTACGTAGTGAATCTG**T**TTG GAATTGAAAAACAAGTGCTTC**		

SEQ ID NO: 26: IPD072 assay amplicon sequence (72-bp; primer and probe binding sites are in bold and underlined) **ACAACAACGCCGTGAAGGA**CCAGGGTCGGCTGATCGAGCCGCTCTCG**TGATACG TATGTGAAACCAATCTGG**
SEQ ID NO: 27: DvSSJ1 assay amplicon sequence (77-bp; primer and probe binding sites are in bold and underlined) **CGTATTCGTAGGTAATTGAGAATTCG**ATATCAGGTCCGCCTTGTTTCTCC**TCTGT CTCTTGATCTGACTAATCTTGG**
SEQ ID NO: 28: PMI assay amplicon sequence (113-bp; primer and probe binding sites are in bold and underlined **TGACTGTCAAAGGCCACGGCCGTTTAGCGCGTGTTTACAACAAGCTG**TAAGAGC TTACTGAAAAAATTAACATCTCTTGCTAAGCTGGG**GGTGGAACCTAGACTTGTCCA TCT**
SEQ ID NO: 29: Mo-PAT assay amplicon sequence (76-bp; primer and probe binding sites are in bold and underlined **CATCGTGAACCACTACATCGAGAC**CTCC**ACCGTGAACTTCCGCACCGAGC**CGCA GACCCCGCAGGAGTGGATCGAC**
SEQ ID NO: 30: hmg-A assay amplicon sequence (79-bp; primer and probe binding sites are in bold and underlined) **TTGGACTAGAAATCTCGTGCTGA**TTAATTGTTTTACGCGT**GCGTTTGTGTGGATT GTAGGACAAGGCTCCCTATGTAGC**

Table 5: PCR Reagents and Reaction Conditions

Step	Description	Temperature (°C)	Time (seconds)	Cycles

1	Initial Denaturation		95	120	1
2a	Amplification	Denaturation	95	1	40[a]
2b		Anneal/Extend	60	20	

[a] If thermal cycling is completed using a Roche LightCycler ® 480, 45 cycles for steps 2a and 2b are performed.

PCR products ranging in size from 72-bp to 1l3-bp, representing the insertion sites for event DP-0232 11-2 as well as the transgenes within the T-DNA from plasmid PHP74643, were amplified and observed in 100 individual leaf samples from event DP-0232! 1-2 as well as eight copy number calibrator genomic controls, but were absent in each of the eight negative genomic controls and eight NTC controls. Each assay was performed a total of four times with the same results observed. C_T values were calculated for each sample and all positive controls.

Using the maize endogenous reference gene *hmg-A*, a PCR product of 79-bp was amplified and observed in 100 individual leaf samples each from event DP-02321 1-2 as well as eight copy number calibrator and eight negative genomic controls. Amplification of the endogenous gene was not observed in the eight No Template (NTC) controls tested with no generation of C_T values. For each sample, each assay was performed in duplex with both insertion sites and all transgenes a total of four times with the same results observed each time. C_T values were calculated for each sample and all positive and negative controls.

To assess the sensitivity of the construct-specific PCR assays, DP-023211-2 maize DNA was diluted in control maize genomic DNA, resulting in test samples containing various amounts of event DP-023211-2 DNA (5-ng, l-ng, lOO-pg, 50-pg, 20-pg, lO-pg, 5-pg, l-pg, 0.5-pg, O.l-pg) in a total of 5-ng maize DNA. These various amounts of DP-0232! 1-2 maize DNA correspond to 100%, 20%, 2%, 1%, 0.4%, 0.2%, 0.1%, 0.01%, and 0.002% of DP-232 11-2 maize maize DNA in total maize genomic DNA, respectively. For the transgene PMI, additional concentrations of 750-pg, 500-pg and 250-pg, or 15%, 10% and 5% of DP-0232 11-2 DNA in total maize genomic DNA were tested. The various amounts of DP-0232 11-2 DNA were subjected to real-time PCR amplification for transgenes IPD072, PMI, DvSSJl and mo-PAT. Based on these analyses, the limit of detection (FOD) in 5-ng of total DNA for event DP-02321 1-2 was determined to be approximately 20-pg for IPD072, or 0.4%, 500-pg for PMI, or 10% (DP-02321 1-2). The determined sensitivity of each assay described is sufficient for many screening applications.

Each concentration was tested a total of four times with the same results observed each time.

Real-time PCR analyses of event DP-023211-2 utilizing event-specific and construct-specific primer/probe sets for event DP-0232 11-2 confirm the stable integration and segregation of a single copy of the T-DNA of plasmid PHP74643 of the event in leaf samples tested, as demonstrated by the quantified detection of IPD072, PMI, DvSSJl and mo-PAT transgenes in DP-0232 11-2 maize. These results were reproducible among all the replicate qPCR analyses conducted. The maize endogenous reference gene assay for detection of hmg-A amplified as expected in all the test samples, negative controls and was not detected in the NTC samples. The sensitivity of each assay under the conditions described ranges from 5-pg to 500-pg DNA, all sufficient for many screening applications by PCR.

Example 4. Southern-by-Sequencing (SbS) Analysis of DP-023211-2 maize for Integrity and Copy Number

Southem-by-Sequencing (SbS) utilizes probe-based sequence capture, Next Generation Sequencing (NGS) techniques, and bioinformatics procedures to isolate, sequence, and identify inserted DNA within the maize genome. By compiling a large number of unique sequencing reads and comparing them to the transformation plasmid, unique junctions due to inserted DNA are identified in the bioinformatics analysis and can be used to determine the number of insertions within the plant genome. One TO plant each of DP-0232 11-2 maize was analyzed by SbS to determine the insertion copy number. In addition, samples of the control maize line were analyzed.

Genomic DNA was extracted from the TO generation of DP-0232 11-2 maize and control plants.

Capture probes used to select PHP74643 plasmid sequences were designed and synthesized by Roche NimbleGen, Inc. (Madison, WI). A series of unique sequences encompassing the plasmid sequence was used to design overlapping biotinylated oligonucleotides as capture probes. The probe set was designed to target most sequences within the PHP74643 transformation plasmid during the enrichment process. The probes were compared to the maize genome to determine the level of maize genomic sequence that would be captured and sequenced simultaneously with the PHP74643 plasmid sequence.

Next-generation sequencing libraries were constructed for the DP-023211-2 maize plants and the control maize lines. SbS was performed as described by Zastrow-Hayes, et

al. *Plant Genome* (2015). The sequencing libraries were hybridized to the capture probes through two rounds of hybridization to enrich the targeted sequences. Following NGS on a HiSeq 2500 (Illumina, San Diego, CA), the sequencing reads entered the bioinformatics pipeline for trimming and quality assurance. Reads were aligned against the maize genome and the transformation construct, and reads that contain both genomic and plasmid sequence were identified as junction reads. Alignment of the junction reads to the transformation construct shows borders of the inserted DNA relative to the expected insertion.

To identify junctions that included endogenous maize sequences, control maize genomic DNA libraries were captured and sequenced in the same manner as the DP-0232 11-2 maize plants. These libraries were sequenced to an average depth approximately five times that of the depth for the DP-02321 1-2 maize plant samples. This increased the probability that the endogenous junctions captured by the PHP74643 probes would be detected in the control samples, so that they could be identified and removed in the DP-02321 1-2 maize samples.

Integration and copy number of the insertion were determined in DP-0232 11-2 maize derived from construct PHP74643. Schematic maps of the PHP74643 plasmid and the T-DNA from PHP74643 used in transformation are provided in FIGs. 1 and 2.

SbS was conducted on the T0 plants of DP-0232 11-2 maize to determine the insertion copy number in the genome. Unique junctions between the genomic flanking sequence and the landing pad were detected. The FRT1 and FRT87 sites are the two junctions where the target trait of PHP74643 T-DNA was integrated into the site-specific integration line. The unique reads at the FRT1 and FRT87 junctions for the plant are shown in FIG. 3. There were no other junctions between the PHP74643 sequences and the maize genome detected in the plant, indicating that there are no additional plasmid-derived insertions present in DP-0232! 1-2 maize. Additionally, there were no junctions between non-contiguous regions of the PHP74643 T-DNA identified, indicating that there are no detectable rearrangements or truncations in the inserted DNA. Furthermore, there were no junctions between maize genome sequences and the backbone sequence of PHP74643 in the plant analyzed, demonstrating that no plasmid backbone sequences were incorporated into DP-0232 11-2 maize.

SbS analysis of the T0 plants of DP-0232 11-2 maize demonstrated that there is a single insertion containing the desired genes from the PHP74643 T-DNA in DP-02321 1-2 maize and that no additional insertions are present in the respective genomes.

Southem-by- Sequencing (SbS) analysis was conducted on the T0 plants of DP-02321 1-2 maize to confirm insertion copy number. The results indicate a single PHP74643 T-DNA

insertion in the plant. No junctions between the PHP74643 T-DNA sequences and the maize genome were detected in control plants, indicating that, as expected, these plants did not contain any insertions derived from PHP74643. Furthermore, no plasmid backbone sequences were detected in the plant analyzed. SbS analysis of the T0 plants of DP-0232! 1-2 maize demonstrated that there is a single insertion of the PHP74643 T-DNA in each of DP-0232 11-2 maize and that no additional insertions are present in the respective genomes.

Example 5. Insect efficacy of maize events DP-023211-2

Efficacy data was generated for five construct designs. Each construct design consisted of three genetic backgrounds with several events (Table 6) within each background. A 42 kernel sample of each entry was characterized prior to planting to confirm the presence of the event by event-specific PCR. Four entries required tissue sampling in the field and all off-type plants were culled from the experiment. Efficacy testing included: WCRW root damage at eight locations. At each location, single-row plots were planted in an incomplete block design with two replications per location.

Plants at approximately the V2 growth stage were manually infested with approximately 375-750 (varied by location) WCRW eggs applied into the soil on each side of the plant (-750-1,500 eggs/plant total). Additionally, plots were planted in fields that had a high probability of containing a natural infestation of WCRW. Plant roots were evaluated at approximately the R2 growth stage. Two plants per plot were tagged with unique identifiers and removed from the plot and washed with pressurized water. The root damage was rated using the 0-3 node injury scale (CRWNIS) (Oleson, *et al.* (2005) *J. Econ. Entomol.* 98(1): 1-8).

For the single location analysis of construct designs (Table 7), a linear mixed model was applied to model node-injury scores for each location separately. Construct design was treated as fixed effect. Effects for replication, replication by incomplete block, background, construct, background by construct, event, field range, field row, plot and residual were treated as independent normally distributed random variables with means of zero. *T*-tests were conducted to compare treatment effects. A difference was considered statistically significant if the *P*-value of the difference was less than 0.05. All data analysis and comparisons were made in ASReml 3.0 (VSN International, Hemel Hempstead, UK, 2009).

For the across locations analysis of events (Table 8), construct design was treated as fixed effect. Effects for location, location by replication, location by replication by

incomplete block, background, concept, background by concept, event, location by background, location by concept, location by background by concept, location by event, field range within each location, field row within each location, plot within each location and residual within each location were treated as independent normally distributed random variables with means of zero. ′/-tests were conducted to compare treatment effects. A difference was considered statistically significant if the /J-value of the difference was less than 0.05. All data analysis and comparisons were made in ASReml 3.0 (VSN International, Hemel Hempstead, UK, 2009). Estimated root damage ratings from WCRW feeding are shown in Tables 6 and 7 showing some constructs performing better than others.

Table 6. Number of Genetic Backgrounds and Events Evaluated for Efficacy in each Construct Design

Construct Design	Number Backgrounds	Number of Events
SSJ72_UBI;BSV(AY)[a]	3	22
SSJ72_UBI;A	3	24
SSJ72_BSV(AY);A	3	15
SSJ72_3XUBI;A	3	7
SSJ72_UBI;B	3	25

[a] Event **DP-023211-2** included in this construct design
*A and B are each different promoters

Table 7. Efficacy of Construct Designs Against mixed populations of northern corn rootworm (NCR) and western corn rootworm (WCR) Larvae in Field Testing.

Location	Construct Design*	Number of Plots	Number of Plants	Estimated CRWNIS[a]	Standard Error	Lower 95% CL	Upper 95% CL	Letter Group [b]
Brookings, SD	SSJ72_UBI;BSV(AY)[a]	46	92	0.08	0.05	-0.03	0.19	A
	SSJ72_UBI;A	48	96	0.08	0.05	-0.02	0.19	A
	SSJ72_BSV(AY);A	36	72	0.11	0.06	-0.01	0.22	A
	SSJ72_3XUBI;A	30	59	0.11	0.06	-0.01	0.23	A
	Positive Control	44	87	0.16	0.05	0.05	0.27	A
	SSJ72_UBI;B	50	99	0.17	0.05	0.07	0.28	A
	Negative Control	50	97	1.42	0.05	1.31	1.52	B
Geneva, NE	SSJ72_UBI;A	48	94	0.16	0.10	-0.05	0.37	A
	SSJ72_3XUBI;A	27	54	0.22	0.11	-0.01	0.45	A B
	SSJ72_UBI;BSV(AY	45	89	0.22	0.10	0.01	0.43	A B
	SSJ72_BSV(AY);A	36	72	0.30	0.11	0.08	0.52	A B C
	Positive Control	42	84	0.35	0.11	0.14	0.57	B C
	SSJ72_UBI;B	50	97	0.43	0.10	0.22	0.64	C
	Negative Control	51	99	2.31	0.10	2.10	2.52	D

Location	Construct Design*	Number of Plots	Number of Plants	Estimated CRWNIS[a]	Standard Error	Lower 95% CL	Upper 95% CL	Letter Group [b]
Mankato, MN	SSJ72_BSV(AY);A	35	70	0.09	0.10	-0.11	0.30	A
	SSJ72_UBI;BSV(AY)	46	91	0.10	0.09	-0.10	0.29	A
	Positive Control	44	88	0.11	0.09	-0.09	0.30	A
	SSJ72_UBI;A	48	94	0.11	0.09	-0.08	0.31	A
	SSJ72_3XUBI;A	30	60	0.17	0.10	-0.04	0.38	A
	SSJ72_UBI;B	50	99	0.21	0.09	0.02	0.40	A
	Negative Control	51	101	2.31	0.09	2.12	2.50	B
Rochelle, IL	SSJ72_UBI;A	48	96	0.10	0.15	-0.21	0.42	A
	SSJ72_UBI;BSV(AY)	46	91	0.11	0.16	-0.21	0.42	A
	SSJ72_BSV(AY);A	36	72	0.39	0.16	0.07	0.71	B
	Positive Control	44	87	0.49	0.16	0.17	0.81	B
	SSJ72_3XUBI;A	28	56	0.55	0.16	0.22	0.88	BC
	SSJ72_UBI;B	50	100	0.65	0.15	0.33	0.96	C
	Negative Control	51	102	1.69	0.15	1.37	2.00	D
Alleman, IA	SSJ72_UBI;A	48	96	0.22	0.09	0.04	0.39	A
	SSJ72_UBI;BSV(AY)	46	92	0.27	0.09	0.10	0.45	A
	SSJ72_BSV(AY);A	36	71	0.51	0.09	0.32	0.69	B
	SSJ72_3XUBI;A	28	55	0.53	0.09	0.33	0.72	B
	Positive Control	44	88	0.56	0.09	0.39	0.74	B
	SSJ72_UBI;B	50	99	0.58	0.09	0.40	0.75	B
	Negative Control	52	104	1.51	0.09	1.34	1.68	C
Mansfield, IL	SSJ72_UBI;A	40	76	0.29	0.15	-0.01	0.60	A
	SSJ72_UBI;BSV(AY)	37	65	0.61	0.15	0.29	0.92	B
	SSJ72_BSV(AY);A	33	64	0.76	0.16	0.44	1.07	B
	SSJ72_3XUBI;A	23	44	0.82	0.17	0.48	1.17	B
	Positive Control	33	59	0.89	0.16	0.57	1.21	B
	SSJ72_UBI;B	39	70	1.45	0.15	1.14	1.76	C
	Negative Control	44	76	2.39	0.15	2.09	2.70	D
Johnston, IA	SSJ72_UBI;A	48	95	0.31	0.08	0.15	0.47	A
	SSJ72_UBI;BSV(AY)	46	91	0.34	0.08	0.18	0.50	A
	SSJ72_BSV(AY);A	36	71	0.81	0.09	0.64	0.98	B
	Positive Control	44	87	0.81	0.08	0.65	0.98	B
	SSJ72_3XUBI;A	30	60	0.83	0.09	0.65	1.01	B
	SSJ72_UBI;B	50	100	0.97	0.08	0.81	1.12	B
	Negative Control	52	104	2.04	0.08	1.88	2.19	C
Fowler, IN	SSJ72_UBI;A	48	95	0.35	0.15	0.04	0.66	A
	SSJ72_UBI;BSV(AY)	45	101	0.55	0.15	0.24	0.86	A
	SSJ72_BSV(AY);A	36	84	0.96	0.16	0.64	1.28	B
	Positive Control	44	87	1.02	0.15	0.71	1.33	B
	SSJ72_3XUBI;A	26	52	1.06	0.16	0.73	1.40	B
	SSJ72_UBI;B	50	112	1.44	0.15	1.13	1.75	C
	Negative Control	51	99	2.22	0.15	1.92	2.53	D

"Damage ratings on individual plant root masses were determined using 0-3 Node Injury Scale (Oleson *et al.* 2005, supra).

"Within a location, estimates with the same letter are not significantly different (*T*-test, $P > 0.05$).
*A and B are each different promoters

Table 8. Efficacy of Events Against mixed populations of NCR and WCR Larvae Across Eight Field Testing Locations.

Event	Number of Plots	Number of Plants	Estimated CRWNIS[a]	Standard Error	Lower 95% CL	Upper 95% CL	Letter Group[b]
DP-023211-2	15	29	0.26	0.15	-0.04	0.56	A
Positive Control	126	248	0.52	0.14	0.23	0.82	B
Negative Control	137	265	1.96	0.14	1.67	2.25	C

[a] "Damage ratings on individual plant root masses were determined using 0-3 Node Injury Scale (Oleson *et al.* 2005, supra).
[b] "Within a location, estimates with the same letter are not significantly different (*T*-test, $P > 0.05$).

Further field testing of DP-02321 1-2 was conducted in year 3 in 14 locations located in commercial maize-growing regions of North America: Benson, MN (MK_BE); Brookings, SD (BR); Fowler, IN (WN_FO); Goodland, IN (WN_GL); Janesville, WI (JV); Johnston, IA (JH and JH_D3); Mankato, MN (MK); Mansfield, IL (CI_MF); Marion, IA (MR); Readlyn, IA (MR); Seymour, IL (CI_SE); and York, NE (YK and YK_LI). No efficacy data were collected at CI_SE, JV, WN_FO, WN_GL, and YK due to a low nodal injury score (CRWNIS) below 0.75 on negative control roots.

Single-row plots (10 feet in length) were planted in an alpha experimental design with two replications. Prior to planting, 42 kernels from each seed lot were characterized to confirm the presence of the traits by PCR. Five consecutive plants were manually infested utilizing a tractor-mounted CRW egg infester at a targeted infestation rate of approximately 750 eggs/plant or 1500 eggs/plant, depending on the location, when plants reached growth stages V2-V4. Eggs were injected into the soil approximately 4 inches deep and approximately 2-3 inches on both sides of each plant. Injury from larval feeding on roots was evaluated between 56 and 78 days after planting. Two com roots were tagged, manually dug from the ground, washed clean of soil with pressurized water, and evaluated for the amount of larval feeding at approximately the R2 growth stage. Root injury was evaluated by visually rating and recording the amount of larval feeding contained on each root using the Iowa State 0-3 node-injury scale (Oleson *et al,* 2005).

The mean node-injury root rating results from CRW for DP-0232 11-2 maize and control maize are provided in Table 9. These results indicate that maize lines containing the insect-active protein IPD072Aa and RNAi trait DvSSJl are efficacious against CRW.

Table 9. Efficacy Results Against Corn Rootworm

Maize Line	Number of Plots	Mean Node-Injury Root Rating ± SD	Range	P-Value
DP-023211-2	27	0.13 + 0.08	0.02 – 0.70	<0.0001[a]
Control	27	1.79 + 0.74	0.50 – 3.00	

[a] Statistically significant difference; (P-value < 0.05)

Example 6. Agronomic and yield field evaluations of maize events DP-023211-2

Agronomic field trials, containing the five molecular stack construct designs as used in Example 5 containing both DvSSJl and IPD072, were executed in the summer of 2016 to generate yield data and to evaluate other agronomic characteristics. Multiple events were tested for each construct design (Table 10). All inbred and hybrid materials tested for an event were generated from a single T0 plant.

Hybrid Trials

Hybrid trials were planted at 16 locations with a single replicate of the entry list at each location. Grain was harvested from 10 of the 16 locations. Each entry in a common background was crossed to three testers to generate hybrid seed for testing. Experiments were nested by testers, with the entries randomized within each nest. Various observations and data were collected at each planted location throughout the growing season. The following agronomic characteristics were analyzed for comparison to a wild type entry (WT), or an entry with the same genetics but without the molecular stacks of DvSSJl and IPD072, also referred to as base comparator (Tables 11-12 and FIGs. 5-6):

1.) Growing degree units to silk (GDUSLK): Measurement records the total accumulated growing degree units when 50% of the plants in the plot have fully emerged silks. A single day equivalent is approximately 2.5 growing degrees units for this data set.

2.) Growing degree units to shed (GDUSHD): Measurement records the total accumulated growing degree units when 50% of the plants in the plot have tassels

that are shedding pollen. A single day equivalent is approximately 2.5 growing degrees units for this data set.

3.) Ear height (EARHT): Measurement from the ground to the attachment point of the highest developed ear on the plant. Ear height is measured in inches.

4.) Plant height (PLTHT): Measurement from the ground to the base of the flag leaf. Plant height is measured in inches.

5.) Moisture (MST): Measurement of the percent grain moisture at harvest.

6.) Yield: Recorded weight of grain harvested from each plot. Calculations of reported bu/acre yields were made by adjusting to measured moisture of each plot.

Inbred Trials

Inbred trials were planted at eight locations with two replicates of the entry list at each location. One replicate at each location was nested by construct design; the other replicate was planted as a randomized complete block. Agronomic data and observations were collected for the inbred trials and analyzed for comparison to a wild type entry (WT), or untraited version of the same genotype. Data generated for the inbred trials included the following agronomic traits (FIGs. 7 and 8):

1.) Growing degree units to silk (GDUSLK): Measurement records the total accumulated growing degree units when 50% of the plants in the plot have fully emerged silks. A single day equivalent is approximately 2.5 growing degrees units for this data set.

2.) Growing degree units to shed (GDUSHD): Measurement records the total accumulated growing degree units when 50% of the plants in the plot have tassels that are shedding pollen. A single day equivalent is approximately 2.5 growing degrees units for this data set.

3.) Ear height (EARHT): Measurement from the ground to the attachment point of the highest developed ear on the plant. Ear height is measured in inches.

4.) Plant height (PLTHT): Measurement from the ground to the base of the flag leaf. Plant height is measured in inches.

5.) Ear photometry yield (PHTYLD): Calculated yield estimates from images of harvested ears from each plot. Units for the values shown are bu/acre.

Trial Results

To evaluate the hybrid data, a mixed model framework was used to perform multi location analysis. In the multi-location analysis, main effect construct design is considered as fixed effect. Factors for location, background, tester, event, background by construct design, tester by construct design, tester by event, location by background, location by

construct design, location by tester, location by background by construct design, location by tester by construct design, location by event, location by tester by event are considered as random effects. The spatial effects including range and plot within locations were considered as random effects to remove the extraneous spatial noise. The heterogeneous residual was assumed with autoregressive correlation as ARl*ARl for each location. The estimate of construct design and prediction of event for each background were generated. The *T*-tests were conducted to compare construct design/event with WT. A difference was considered statistically significant if the *P*-value of the difference was less than 0.05. Yield analysis was by ASREML (VSN International Ltd; Best Linear Unbiased Prediction; Cullis, B. Ret al (1998) *Biometrics* 54: 1-18, Gilmour, A. R. et al (2009); ASReml User Guide 3.0, Gilmour, A.R., et al (1995) *Biometrics* 51: 1440-50).

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Similar experiments were conducted in Year 2 and the results confirmed the performance data from Year 1; two events were selected from the SSJ72_UBI;BSV(AY)_NONE construct design.

Table 10. Number events evaluated for each construct design

Construct Design	Number of Events
SSJ72_UBI;BSV(AY)[a]	14
SSJ72_UBI;A	9
SSJ72_BSV(AY);A	7

Construct Design	Number of Events
SSJ72_3XUBI;A	14
SSJ72_UBI;B	10

[a] Event **DP-023211-2** included in this construct design
*A and B are each different promoters

Table 11. Hybrid performance of construct designs compared to base entry—yield

Construct Design	Number of plots with trait data	Predicted value (bu/acre)	Standard Error
WT (base comparator)	445	202.56	7.00
SSJ72_UBI;BSV(AY)[a]	124	202.26	7.17
SSJ72_UBI;A	83	204.34	7.23
SSJ72_BSV(AY);A	88	204.12	7.23
SSJ72_3XUBI;A	12	199.82	7.69
SSJ72_UBI;B	171	203.49	7.14

[a] Event **DP-023211-2** included in this construct design
*A and B are each different promoters

Table 12. Hybrid performance of events DP-023211-2 compared to base entry—yield

Construct Design	Number of plots with yield data	Predicted value (bu/acre)	Standard Error	Predicted lower 95% CL	Predicted upper 95% CL
WT (base comparator)	445	202.56	7.00	188.31	216.81
DP-023211-2	9	202.79	7.64	187.25	218.33

Example 7. Protein Expression and Concentration

Protein Extraction

Aliquots of processed leaf or root tissue samples were weighed into l.2-ml tubes at the target weight of 10 mg for leaf tissue and 20 mg for root tissue. Samples analyzed for PAT and PMI protein concentrations were extracted in 0.6 ml of chilled PBST and samples analyzed for IPD072Aa protein were extracted in 0.6 ml of chilled PBST with 25% Stabilzyme Select. Following centrifugation, supernatants were removed, diluted in PBST (PAT and PMI) or PBST with 25% Stabilzyme Select (IPD072Aa), and analyzed.

Determination of IPD072Aa Protein Concentration

The IPD072Aa ELISA method utilized a kit developed by produced by Pioneer Hi-Bred International, Inc. to determine the concentration of the IPD072Aa protein in samples. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre-coated with a IPD072Aa-specific antibody. Following incubation, unbound substances were washed from the plate. A different IPD072Aa-specific antibody, conjugated to the enzyme horseradish peroxidase (HRP), was added to the plate and incubated. Unbound substances were washed from the plate. Detection of the bound IPD072Aa-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader.

Determination of PAT Protein Concentration

The PAT ELISA method utilized an ELISA kit produced by EnviroLogix™ Inc. to determine the concentration of PAT protein in samples. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were co-incubated with a PAT-specific antibody conjugated to the enzyme HRP in a plate pre-coated with a different PAT-specific antibody. Following incubation, unbound substances were washed from the plate. Detection of the bound PAT-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the OD of each well was determined using a plate reader.

Determination of PMI Protein Concentration

The PMI ELISA method utilized a kit developed by produced by Pioneer Hi-Bred International, Inc. to determine the concentration of the PMI protein in samples. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre-coated with a PMI-specific antibody. Following incubation, unbound substances were washed from the plate. A different PMI-specific antibody, conjugated to the enzyme HRP, was added to the plate and incubated. Unbound substances were washed from the plate. Detection of the bound PMI-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the OD of each well was determined using a plate reader.

Calculations for Determining Protein Concentrations

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the OD values obtained for each set of sample wells to a protein concentration value.

A standard curve was included on each ELISA plate. The equation for the standard curve was derived by the software, which used a quadratic fit to relate the OD values obtained for each set of standard wells to the respective standard concentration (ng/ml). The quadratic regression equation was applied as follows:

$$y = Cx^2 + Bx + A$$

where x = known standard concentration and y = respective absorbance value (OD)

Interpolation of the sample concentration (ng/ml) was performed by solving for x in the above equation using the values for A, B, and C that were determined for the standard curve.

$$\text{Sample Concentration (ng/ml)} = \frac{-B + \sqrt{B^2 - 4C(A - \text{sampleOD})}}{2C}$$

For example, given curve parameters of A = 0.0476, B = 0.4556, C= -0.01910, and a sample OD = 1.438

$$\text{Sample Concentration} = \frac{-0.4556 + \sqrt{0.4556^2 - 4(-0.01910)(0.0476 - 1.438)}}{2(-0.01910)} = 3.6 \text{ ng/ml}$$

The sample concentration values were adjusted for a dilution factor expressed as l:N by multiplying the interpolated concentration by N.

Adjusted Concentration = Interpolated Sample Concentration x Dilution Factor

For example, given an interpolated concentration of 3.6 ng/ml and a dilution factor of 1:20

Adjusted Concentration = 3.6 ng/ml x 20 = 72 ng/ml

Adjusted sample concentration values obtained from SoftMax Pro GxP software were converted from ng/ml to ng/mg sample weight as follows:

$$\text{Sample Concentration (ng protein/mg sample weight)} = \text{Sample} \times \frac{\text{Extraction Buffer Volume (ml)}}{}$$

$$\frac{\text{Concentration (ng/ml)}}{\text{Sample Target Weight (mg)}}$$

For example, sample concentration = 72 ng/ml, extraction buffer volume = 0.60 ml, and sample target weight = 10 mg

$$\text{Sample Concentration (ng protein/mg sample weight)} = 72 \text{ ng/ml} \times \frac{0.60 \text{ ml}}{10 \text{ mg}} = 4.3 \text{ ng/mg}$$

The reportable assay lower limit of quantification (LLOQ) in ng/ml was calculated as follows:

Reportable Assay LLOQ (ng/ml) = (lowest standard concentration - 10%) x minimum dilution

For example, lowest standard concentration = 0.50 ng/ml and minimum dilution = 10

Reportable Assay LLOQ (ng/ml) = (0.50 ng/ml - (0.50 x 0.10)) x 10 = 4.5 ng/ml

The LLOQ, in ng/mg sample weight, was calculated as follows:

$$\text{LLOQ} = \text{Reportable Assay LLOQ (ng/ml)} \times \frac{\text{Extraction Buffer Volume (ml)}}{\text{Sample Target Weight (mg)}}$$

For example, reportable assay LLOQ = 4.5 ng/ml, extraction buffer volume = 0.60 ml, and sample target weight = 10 mg

$$\text{LLOQ} = 4.5 \text{ ng/ml} \times \frac{0.60 \text{ ml}}{10 \text{ mg}} = 0.27 \text{ ng/mg sample weight}$$

Results

Means, standard deviations, and ranges for IPD072Aa protein in V9 root tissue in two generations of DP-023211-2 maize are provided in Table 13 and means, standard deviations, and ranges for PAT and PMI proteins in V9 leaf tissue in two generations of DP-02321 1-2 maize are provided in Table 14.

Table 13: Expressed IPD072Aa Protein Concentrations in V9 Root Samples of DP-023211-2 maize

Tissue (Growth Stage)	Generation	ng IPD072Aa/mg Tissue Dry Weight			Number of Samples <LLOQ/ Total Number of Samples Reported
		Mean ± SD	Range	Sample LLOQ	
Root (V9)	BC1F1	68 ± 14	51 - 90	0.11	0/5
Root (V9)	BC2F1	81 ± 18	57 - 99	0.11	0/5

Table 14. Expressed PAT and PMI Protein Concentrations in V9 Leaf Samples of DP-023211-2 maize

Tissue (Growth Stage)	Generation	Mean ± SD	Range	Sample LLOQ	Number of Samples <LLOQ/ Total Number of Samples Reported
ng PAT/mg Tissue Dry Weight					
Leaf (V9)	BC1F1	3.7 ± 0.48	3.3 - 4.4	0.11	0/5
Leaf (V9)	BC2F1	4.1 ± 0.23	3.7 - 4.3	0.11	0/5
ng PMI/mg Tissue Dry Weight					
Leaf (V9)	BC1F1	18 ± 4.0	14 - 23	0.54	0/5
Leaf (V9)	BC2F1	20 ± 2.5	17 - 22	0.54	0/5

Example 8. DvSSJl dsRNA Expression

Separate generations (BC1F1 and BC2F1) of DP-023211-2 maize were grown in 4-inch pots, organized in flats containing 15 pots, using typical greenhouse production conditions in 2017 in Johnston, Iowa, USA.

Root samples were collected from 10 plants at approximately the V9 growth stages (*i.e*. when the collar of the ninth leaf becomes visible) and were analyzed using endpoint real-time PCR analysis for the presence or absence of the DP-0232 11-2 maize events and the *ipd072Aa, mo-pat, pmi,* and *DvSSJl* genes. Five plants which tested positive via PCR analysis were selected for further analysis.

Each root sample was obtained by removing the roots from the soil and shaken to remove excess soil. Roots were then thoroughly cleaned with water and then removed from the plant. No above ground brace roots were included in the sample. The root tissue was cut into sections of 1 in. (2.5 cm) or smaller in length and part of the sample was collected into a pre-chilled vial for QuantiGene analysis and the remaining sample was collected into a vial for moisture analysis. All samples were kept on dry ice until transferred to a -80 °C freezer.

Approximately 1.2 g of frozen V9 root tissue sample from DP-02321 1-2 maize plants was weighed, mixed with lysis buffer, and ground. Total RNA from 800 µï of the ground tissue and lysis buffer mix was extracted using mirVana Total RNA Isolation Kit (ThermoFisher Scientific, Carlsbad, CA) based on the manufacturer's instmctions and

eluted in 75 µl of molecular-grade water. The extracted RNAs were quantified using a NanoDrop-8000 and stored in a -80 °C freezer.

The reference standard of DvSSJl hairpin RNA (hpRNA) was produced by *in vitro* transcription. To generate a construct containing the DvSSJl sequence used for *in vitro* transcription, the total RNA was extracted from the transgenic plants and used to synthesize the cDNA of the full-length DvSSJl by reverse transcription using 5' and 3' rapid amplification of cDNA ends (RACE). The resulting cDNA was cloned into a pUC57 vector under the T7 promoter. Plasmid DNA of the DvSSJl full-length construct was isolated from a bacterial culture and used for *in vitro* transcription of DvSSJl hpRNA by SunScript RT RNaseH- kit (Sygnis, Heidelberg, Germany). The working concentration of DvSSJl hpRNA was 10 ng/pl. Nine-point concentrations ranging from 0.0105 to 16 pg per 40 µl were used for generating the standard curve. The measurements of each point of the standard curve were generated and averaged.

250 ng of total RNA per well was analyzed with a standard curve created by nine-point concentrations (at range of 0.0105 to 16 pg per 40 µl reaction volume) of DvSSJ 1 hpRNA reference standard using a validated QuantiGene Plex 2.0 Assay (Affymetrix Inc., Santa Clara, CA). The probe set used in the assay was designed to specifically detect DvSSJl RNA transcripts. Total RNA from non-GM HC69 maize plants was used as negative control.

The QuantiGene assay was conducted according to the manufacturer's instructions with a modification. The test samples, negative control samples, and DvSSJl hpRNA reference standards were assayed in triplicated wells in a volume of 100 µl in a 96-well hybridization plate. In each test sample well, 250 ng of total RNA was mixed with a quarter strength of the probe set and heated at 95 °C. After heating for 3 minutes, the samples were cooled and maintained at 54 °C until use. A mixture of 40 µl of the RNA sample and 5 µl of probe set was transferred to a hybridization plate containing 55 µl of bead mix for overnight hybridization. After signal amplification and washes, the assay plates were read for florescence intensity and by a MagPix analyzer (Luminex. Corp., Austin, TX) according to the manufacturer's instructions. The net median florescence intensity (MFI) from each assay well was reported.

Root tissue sample from five plants per generation was collected to obtain the fresh-weight to dry-weight ratios. Fresh weights were recorded for each sample. Samples were then placed on dry ice, lyophilized, and the dry weights were recorded.

The mean, standard deviation, and coefficient of variation were calculated for each set of triplicate samples using the net MFI value. Standard curves were generated on the QuantiGene Assay plates and used to interpolate DvSSJl dsRNA concentrations based on the net MFI values. The concentration of DvSSJ 1 RNA from each test sample was further converted to a pg/mg fresh weight (fw) value. All fresh weight values were further converted to pg/mg of dry weight (dw) values. The mean, standard deviation, and range of the DvSSJl RNA levels were determined on both fw and dw basis for each of 5 plants in 2 generations.

The reportable assay lower limit of quantification (LLOQ) in pg/ml was calculated as follows:

Reportable Assay LLOQ (pg/ml) = lowest standard concentration x 90% x minimum dilution

The lowest standard concentration was 0.0105 pg/rxn, and the minimum dilution used was 0.574 rxn/mg.

Thus, the LLOQ = 0.0105 pg/rxn x 0.9 x 0.574 rxn/mg = 0.0054 pg/mg

The DvSSJ 1 dsRNA expression results for root samples of DP-02321 1-2 maize were averaged from the five plants analyzed per generation, and the means, standard deviations, and ranges are summarized in Table 15.

Table 15: Summary of DvSSJl RNA Expression Levels in V9 Root Tissue of DP-023211-2 maize

Tissue (Growth Stage)	Generation	pg/mg Fresh Weight			pg/mg Dry Weight	
		Mean ± SD	Range	Sample LLOQ	Mean ± SD	Range
Root (V9)	BC1F1	0.92 ± 0.20	0.62 - 1.16	0.0054	15.42 ± 3.38	10.42 - 19.35
	BC2F1	0.92 ± 0.46	0.55 - 1.69	0.0054	15.34 ± 7.77	9.21 - 28.33

Example 9. LCso and Spectrum Analysis

IPD072Aa and DvSSJl are both effective at controlling Diabrotica virgifera virgifera (Western Corn Rootworm (WCR)), which is an insect pest of corn that feeds on com plant root tissue and reduces yield. Species selected for testing with IPD072Aa and

DvSSJl were based on several criteria: organism relatedness to WCR, established laboratory bioassay methodologies, availability of laboratory reared insects, availability of a suitable diet, and laboratory performance and reproducibility of the response variables for each organism. Method development included on establishing a suitable diet and environmental conditions that enabled robust bioassay performance and establishment of acceptability criteria generally less than 20% control mortality over at least 7 days for IPD072Aa and 14 days for DvSSJl. When possible, other sub-lethal endpoints such as growth and development time were also observed.

In ah cases, fresh diets, with appropriate concentrations of IPD072Aa and DvSSJl, were provided to the organisms as frequently as the organism would allow without exceeding acceptable levels of control mortality, or as test substance stability under bioassay conditions declined. In most cases, fresh diets were provided at least every 3 or 4 days and in some cases daily. Generally, acceptability criteria included $\leq$ 20% mortality in the bioassay controls with $\geq$ 80% mortality observed with various positive controls associated with each bioassay, though $\leq$ 30% control mortality was considered acceptable with WCR given the relatively more variable performance of this organism in laboratory bioassays with artificial diet.

The LC50 for IPD072Aa is 15.9 ppm (with 95% confidence intervals of 12.6-20.6 ppm) generated using a bioassay with a 7-day duration. The 14 day LC50 for DvSSJl is 0.036 ppm (with 95% confidence intervals of 0.0066-0.065 ppm). A longer duration study was conducted with DvSSJl as the RNAi mode of action as DvSSJl requires longer than IPD072Aa to take effect and kill the target pest.

Activity of IPD072Aa and DvSSJl was assessed via laboratory studies with organisms that are related to WCR or species that were available for laboratory studies. Table 16 shows the array of species used in these additional bioassays, some of which represent pests of various grains (com, wheat, soy, etc.) and some species are non-target organisms that provide a beneficial ecosystem service within agricultural fields. Special focus was applied to testing organisms in the Order Coleoptera since WCR is in this Order. The additional organisms selected represent three additional families within the Order Coleoptera. Additionally, four different families in the Order Lepidoptera were tested.

No observed effect concentrations (NOEC) for survival with IPD072Aa ranged between 100 and greater than 1000 ppm (Table 16). No activity was observed outside of the Order Coleoptera at the concentrations tested. NOECs for survival with DvSSJl exceeded 1 ppm for ah organisms tested except *Diabrotica undecimpunctata* (Southern Corn

Rootworm (SCR)) which is a close relative of WCR and is also a pest of corn (Table 16). No activity has been observed with DvSSJl on any organism tested other than western (WCR) and southern corn rootworm (SCR).

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Table 16. NOEC[a] Values for Spectrum of Activity Characterization with IPD072Aa and DvSSJl

Order	Family	Species	Feeding Guild	IPD072Aa[b] Survival NOEC (ppm)	DvSSJ1[c] Survival NOEC (ppm)	Percent (%) Identity to DvSSJ1 dsRNA[d]	Number of SNPs[e]	Number of 21 nt matches (or longest nt sequence)[f]
Coleoptera	Chrysomelidae	*Diabrotica undecimpunctata*	Corn pest	500	0.01	92.9	15	79
Coleoptera	Chrysomelidae	*Leptinotarsa decemlineata*	Potato pest	> 1000	> 1	73.3	56	0 (12 nt)
Coleoptera	Tenebrionidae	*Tenebrio molitor*	Grain pest	100	> 1	NA	NA	NA
Coleoptera	Tenebrionidae	*Zophobas morio*	Grain pest	> 1000	> 1	69.0	65	0 (10 nt)
Coleoptera	Tenebrionidae	*Tribolium castaneum*	Grain pest	> 1000	> 1	69.5	64	0 (11 nt)
Coleoptera	Coccinellidae	*Epilachna varivestis*	Soybean pest	100	> 1	67.6	68	0 (12 nt)
Coleoptera	Coccinellidae	*Coleomegilla maculata*	Predator; Non-target organism	100	> 1	61.9	80	0 (13 nt)
Coleoptera	Coccinellidae	*Hippodamia convergens*	Predator; Non-target organism	500	> 1	NA	NA	NA
Coleoptera	Coccinellidae	*Cryptolaemus montrouzieri*	Predator; Non-target organism	> 1000	NA	63.3	77	0 (8 nt)
Coleoptera	Staphylinidae	*Dalotia coriaria (Atheta coriaria)*	Predator; Non-target organism	> 1000	> 1	64.3	75	0 (8 nt)
Lepidoptera	Crambidae	*Ostrinia nubilalis*	Pest of corn	> 1000	> 1	64.2	79	0 (9 nt)
Lepidoptera	Noctuidae	*Helicoverpa zea*	Pest of corn	> 1000	> 1	60.0	84	0 (8 nt)
Lepidoptera	Nymphalidae	*Vanessa cardui*	Soybean pest	> 1000	> 1	64.8	74	0 (8 nt)
Lepidoptera	Tortricidae	*Cydia pomonella*	Apple pest	> 1000	> 1	60.5	83	0 (8 nt)

Note: Not available (NA)

[a] No Observed Effect Concentration; the greatest concentration at which no biologically relevant adverse effect was observed on survival.

[b] IPD072Aa bioassays were of 7-day duration, except *Tenebrio molitor* and *Zophobas morio* which were 14-day and *Cokomegilla maculata* and *Hippodamia convergent* which were 28-day durations.

[c] Given the different mode of action of DvSSJl and the relatively longer time needed for activity, bioassays conducted were 14-day durations except for *Cokomegilla maculata* and *Hippodamia convergent* which were 28-day durations.

[d] Sequence comparison of DvSSJl dsRNA to species tested for spectrum of activity.

[e] Number of single nucleotide polymorphisms identified during DvSSJl sequence comparison.

[f] Number of 21 nucleotide matches or longest nucleotide matches identified during DvSSJl sequence comparison.

The above description of various illustrated embodiments of the disclosure is not intended to be exhaustive or to limit the scope to the precise form disclosed. While specific embodiments of and examples are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. The teachings provided herein can be applied to other purposes, other than the examples described above. Numerous modifications and variations are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

These and other changes may be made in light of the above detailed description. In general, in the following claims, the terms used should not be construed to limit the scope to the specific embodiments disclosed in the specification and the claims.

The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, manuals, books or other disclosures) in the Background, Detailed Description, and Examples is herein incorporated by reference in their entireties.

Efforts have been made to ensure accuracy with respect to the numbers used (e.g. amounts, temperature, concentrations, etc.) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight; temperature is in degrees Celsius; and pressure is at or near atmospheric.

WHAT IS CLAIMED IS:

1. A corn plant comprising the genotype of the com event DP-023211-2, wherein said genotype comprises a nucleotide sequence as set forth in SEQ ID NO: 31 and SEQ ID NO: 35.

2. The com plant of claim 5, wherein said genotype comprises the nucleotide sequence set forth in SEQ ID NO: 32 and SEQ ID NO: 36.

3. The com plant of claim 5, wherein said genotype comprises the nucleotide sequence set forth in SEQ ID NO: 33 and SEQ ID NO: 37.

4. A DNA construct comprising an operably linked first and second expression cassette, wherein said first expression cassette comprises:

a) An *ubiZMl* Promoter;

b) an *ubiZMl* 5' UTR;

c) an *ubiZMl* Intron;

d) a DvSSJl Fragment;

e) a *zm-Adhl* Intron Connector;

f) a DvSSJl Fragment;

g) a Z27G Terminator

h) a *UBQ14* Terminator; and

i) a maize In2-l terminator;

wherein said second expression cassette comprises:

1) a BSV(AY) Promoter;

2) a zm-HPLV9 Intron;

3) an i*pd072Aa;* and

4) an *at-T9* Terminator.

5. A plant comprising the DNA construct of claim 4.

6. The plant of claim 5, wherein said plant is a com plant.

7. A plant comprising the sequence set forth in SEQ ID NO: 25.

8. A corn event DP-0232 11-2, wherein a representative sample of seed of said com event has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-124722.

9. Plant parts of the corn event of claim 8.

10. Seed comprising com event DP-023211-2, wherein said seed comprises a DNA molecule chosen from SEQ ID NO: 31 and SEQ ID NO: 35, wherein a representative sample of the corn event DP-023211-2 seed of has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-124722.

11. A corn plant, or part thereof, grown from the seed of claim 10.

12. A transgenic seed produced from the com plant of claim 8.

13. A transgenic corn plant, or part thereof, grown from the seed of claim 12.

14. An isolated nucleic acid molecule comprising a nucleotide sequence chosen from SEQ ID NOs: 25, and 31-38, and full length complements thereof.

15. An amplicon comprising the nucleic acid sequence chosen from SEQ ID NOs: 25-30 and full length complements thereof.

16. A biological sample derived from com event DP-02321 1-2 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence which is or is complementary to a sequence chosen from SEQ ID NO: 31 and SEQ ID NO: 35, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-0232 11-2 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-124722.

17. The biological sample of claim 16, wherein said biological sample comprises plant, plant tissue, or seed of transgenic com event DP-02321 1-2.

18. The biological sample of claim 17, wherein said biological sample is a DNA sample extracted from the transgenic corn plant event DP-0232 11-2, and wherein said DNA sample comprises one or more of the nucleotide sequences chosen from SEQ ID NOs: 25-38, and the complement thereof

19. The biological sample of claim 16, wherein said biological sample is chosen from com flour, corn meal, corn syrup, corn oil, corn starch, and cereals manufactured in whole or in part to contain corn by-products.

20. An extract derived from corn event DP-02321 1-2 plant, tissue, or seed and comprising a nucleotide sequence which is or is complementary to a sequence chosen from SEQ ID NO: 31 and SEQ ID NO: 35, wherein a representative sample of said com event DP-02321 1-2 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-124722.

21. The extract of claim 20, wherein said nucleotide sequence is detectable in said extract using a nucleic acid amplification or nucleic acid hybridization method.

22. The extract of claim 21, wherein said extract comprises plant, plant tissue, or seed of transgenic com plant event DP-0232 11-2.

23. The extract of claim 22, wherein the extract is a composition chosen from corn flour, com meal, corn symp, com oil, com starch, and cereals manufactured in whole or in part to contain com by-products, wherein said composition comprises a detectable amount of said nucleotide sequence.

24. A method of producing hybrid corn seeds comprising:

a) sexually crossing a first inbred com line comprising a nucleotide chosen from SEQ ID NOs: 25-38 and a second inbred line having a different genotype;

b) growing progeny from said crossing; and

c) harvesting the hybrid seed produced thereby.

25. The method according to claim 24, wherein the first inbred com line is a female parent.

26. The method according to claim 24, wherein the first inbred com line is a male parent.

27. A method for producing a corn plant resistant to coleopteran pests comprising:

a) sexually crossing a first parent corn plant with a second parent com plant, wherein said first or second parent corn plant comprises event DP-0232 11-2 thereby producing a plurality of first generation progeny plants;

b) selfing the first generation progeny plant, thereby producing a plurality of second generation progeny plants; and

c) selecting from the second generation progeny plants that comprise the event DP-0232 11-2 and are resistant to a coleopteran pest.

28. A method of producing hybrid corn seeds comprising:

a) sexually crossing a first inbred com line comprising the DNA construct of claim 1 with a second inbred line not comprising the DNA construct of claim 1; and

b) harvesting the hybrid seed produced thereby.

29. The method of claim 28, further comprising the step of backcrossing a second generation progeny plant that comprises com event DP-0232 11-2 to the parent plant that lacks the com event DP-02321 1-2 DNA, thereby producing a backcross progeny plant that is resistant to a coleopteran pest.

30. A method for producing a corn plant resistant to a corn rootworm, said method comprising:

a) crossing a first parent corn plant with a second parent com plant, wherein said first or second parent com plant comprises event DP-0232 11-2, thereby producing a plurality of first generation progeny plants;

b) selecting a first generation progeny plant that comprises the event DP-0232! 1-2;

c) backcrossing the first generation progeny plant of step (b) with a parent plant that lacks the com event DP-0232 11-2 DNA, thereby producing a plurality of backcross progeny plants; and

d) selecting from the backcross progeny plants, a plant that comprises the event DP-0232 11-2;

wherein the selected backcross progeny plant of step (d) comprises SEQ ID NO: 25, 31, or 35.

31. The method according to claim 30, wherein the plants of the first parent com line are the female parents or male parents.

32. Hybrid seed produced by the method of claim 30.

33. A method of determining zygosity of a com plant comprising event DP-02321 1-2 in a biological sample comprising:

a) contacting said sample with a first pair of DNA molecules and a second distinct pair of DNA molecules such that:

1) when used in a nucleic acid amplification reaction comprising com event DP-02321 1-2 DNA, produces a first amplicon that is diagnostic for event DP-0232 11-2, and

2) when used in a nucleic acid amplification reaction comprising com genomic DNA other than DP-0232 11-2 DNA, produces a second amplicon that is diagnostic for com genomic DNA other than DP-0232! 1-2 DNA;

b) performing a nucleic acid amplification reaction; and

c) detecting the amplicons so produced, wherein detection of the presence of both amplicons indicates that said sample is heterozygous for com event DP-0232! 1-2 DNA, wherein detection of only the first amplicon indicates that said sample is homozygous for corn event DP-0232 11-2 DNA.

34. The method of claim 33, wherein the first pair of DNA molecules comprises primer pair SEQ ID NOs: 7 and 8.

35. The method of claim 33, wherein the first and second pair of DNA molecules comprise a detectable label.

36. The method of claim 35, wherein the detectable label is a fluorescent label.

37. The method of claim 35, wherein the detectable label is covalently associated with one or more of the primer molecules.

38. A method of detecting the presence of a nucleic acid molecule that is unique to event DP-02321 1-2 in a sample comprising com nucleic acids, the method comprising:

a) contacting the sample with a pair of primers that, when used in a nucleic-acid amplification reaction with genomic DNA from event DP-0232 11-2 produces an amplicon that is diagnostic for event DP-0232 11-2;

b) performing a nucleic acid amplification reaction, thereby producing the amplicon that is diagnostic for event DP-0232 11-2; and

c) detecting the amplicon that is diagnostic for event DP-0232 11-2.

39. The method of claim 38, wherein the nucleic acid molecule that is diagnostic for event DP-0232 11-2 is an amplicon produced by the nucleic acid amplification chain reaction.

40. The method of claim 38, wherein the probe comprises a detectable label.

41. The method of claim 40, wherein the detectable label is a fluorescent label.

42. The method of claim 40, wherein the detectable label is covalently associated with the probe.

43. A plurality of polynucleotide primers comprising one or more polynucleotides which target event DP-0232 11-2 DNA template in a sample to produce an amplicon diagnostic for event DP-0232 11-2 as a result of a polymerase chain reaction method.

44. The pair of polynucleotide primers according to claim 43, wherein

a) the first polynucleotide primer comprises at least 10 contiguous nucleotides of a nucleotide sequence chosen from nucleotides 1-503 of SEQ ID NO: 3, nucleotides 16687-17568 of SEQ ID NO: 3, and the complements thereof; and

b) the second polynucleotide primer comprises at least 10 contiguous nucleotides from nucleotides 1-503 of SEQ ID NO: 3, nucleotides 16687-17568 of SEQ ID NO: 3, and the complements thereof.

45. The pair of polynucleotide primers according to claim 43, wherein

a) the first polynucleotide primer comprises a nucleotide sequence as set forth in SEQ ID NO: 7, and the complements thereof; and

b) the second polynucleotide primer comprises a nucleotide sequence as set forth in SEQ ID NO: 8, and the complements thereof.

46. The primer pair of claim 43, wherein said first primer and said second primer are at least 18 nucleotides.

47. A method of detecting the presence of DNA corresponding to the DP-0232 11-2 event in a sample, the method comprising:

a) contacting the sample comprising maize DNA with a polynucleotide probe that hybridizes under stringent hybridization conditions with DNA from

maize event DP-0232 11-2 and does not hybridize under said stringent hybridization conditions with a non-DP-0232 11-2 maize plant DNA;

b) subjecting the sample and probe to stringent hybridization conditions; and

c) detecting hybridization of the probe to the DNA;

wherein detection of hybridization indicates the presence of the DP-0232 11-2 event.

48. A kit for detecting nucleic acids that are unique to event DP-0232 11-2 comprising at least one nucleic acid molecule of sufficient length of contiguous polynucleotides to function as a primer or probe in a nucleic acid detection method, and which upon amplification of or hybridization to a target nucleic acid sequence in a sample followed by detection of the amplicon or hybridization to the target sequence, are diagnostic for the presence of nucleic acid sequences unique to event DP-0232 11-2 in the sample.

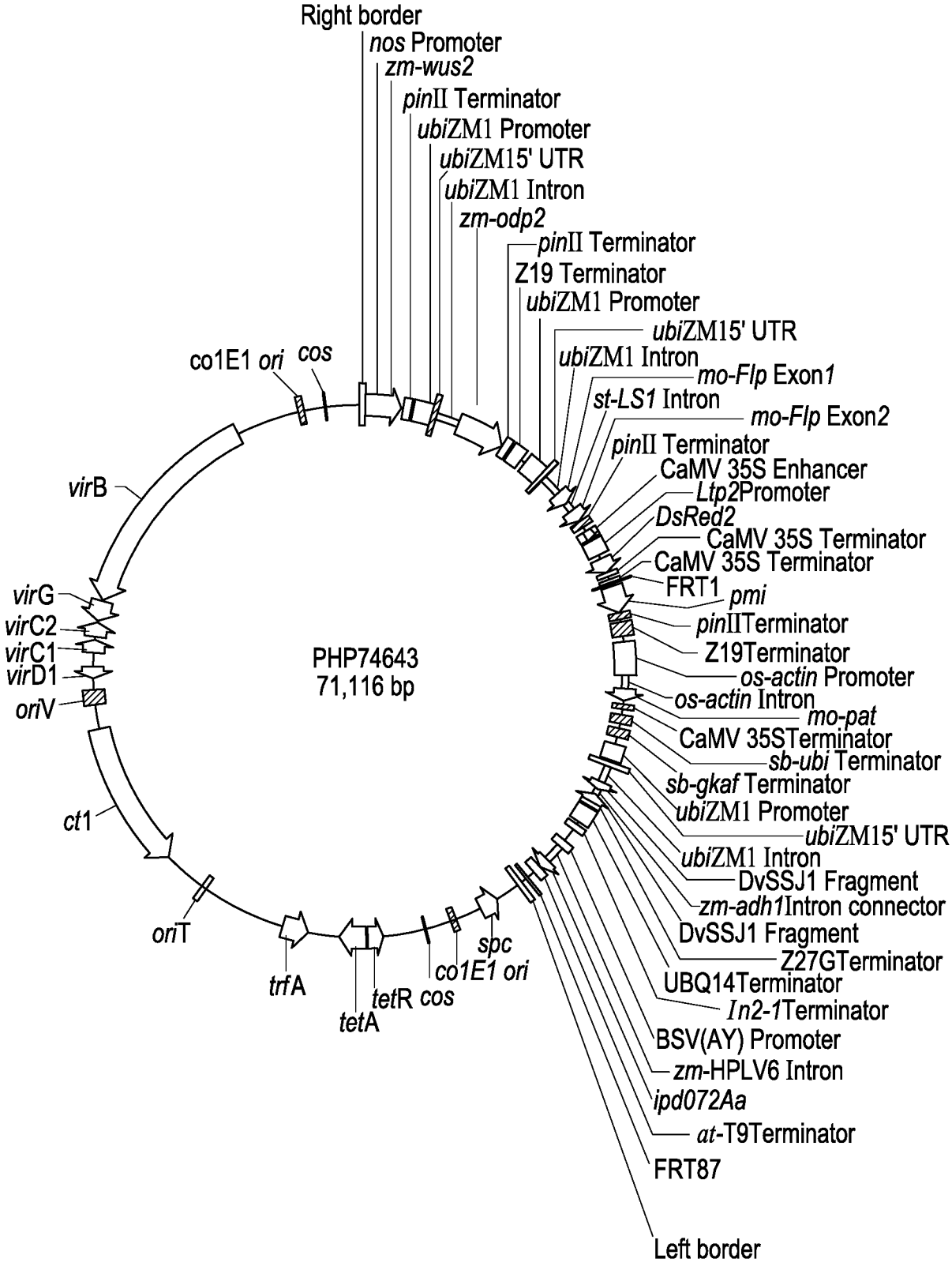
49. The kit according to claim 48, wherein the nucleic acid molecule comprises a nucleotide sequence from SEQ ID NO: 7-38.

50. The kit according to claim 48, wherein the nucleic acid molecule is a primer chosen from SEQ ID NOs: 7-38, and the complements thereof.

51. A corn plant comprising the genotype of the com event DP-0232 11-2, wherein said genotype comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 31 and SEQ ID NO: 35.

52. The com plant of claim 5, wherein said genotype comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 32 and SEQ ID NO: 36.

53. The com plant of claim 5, wherein said genotype comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 33 and SEQ ID NO: 37.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pinII Terminator
ubiZM1 Promoter
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mo-Flp Exon2
pinII Terminator
CaMV 35S Enhancer
Ltp2Promoter
DsRed2
CaMV 35S Terminator
CaMV 35S Terminator
FRT1
pmi
pinIITerminator
Z19Terminator
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os-actin Intron
mo-pat
CaMV 35STerminator
sb-ubi Terminator
sb-gkaf Terminator
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zm-adh1Intron connector
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Z27GTerminator
UBQ14Terminator
In2-1Terminator
BSV(AY) Promoter
zm-HPLV6 Intron
ipd072Aa
at-T9Terminator
FRT87
Left border
co1E1 ori
cos
virB
virG
virC2
virC1
virD1
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oriT
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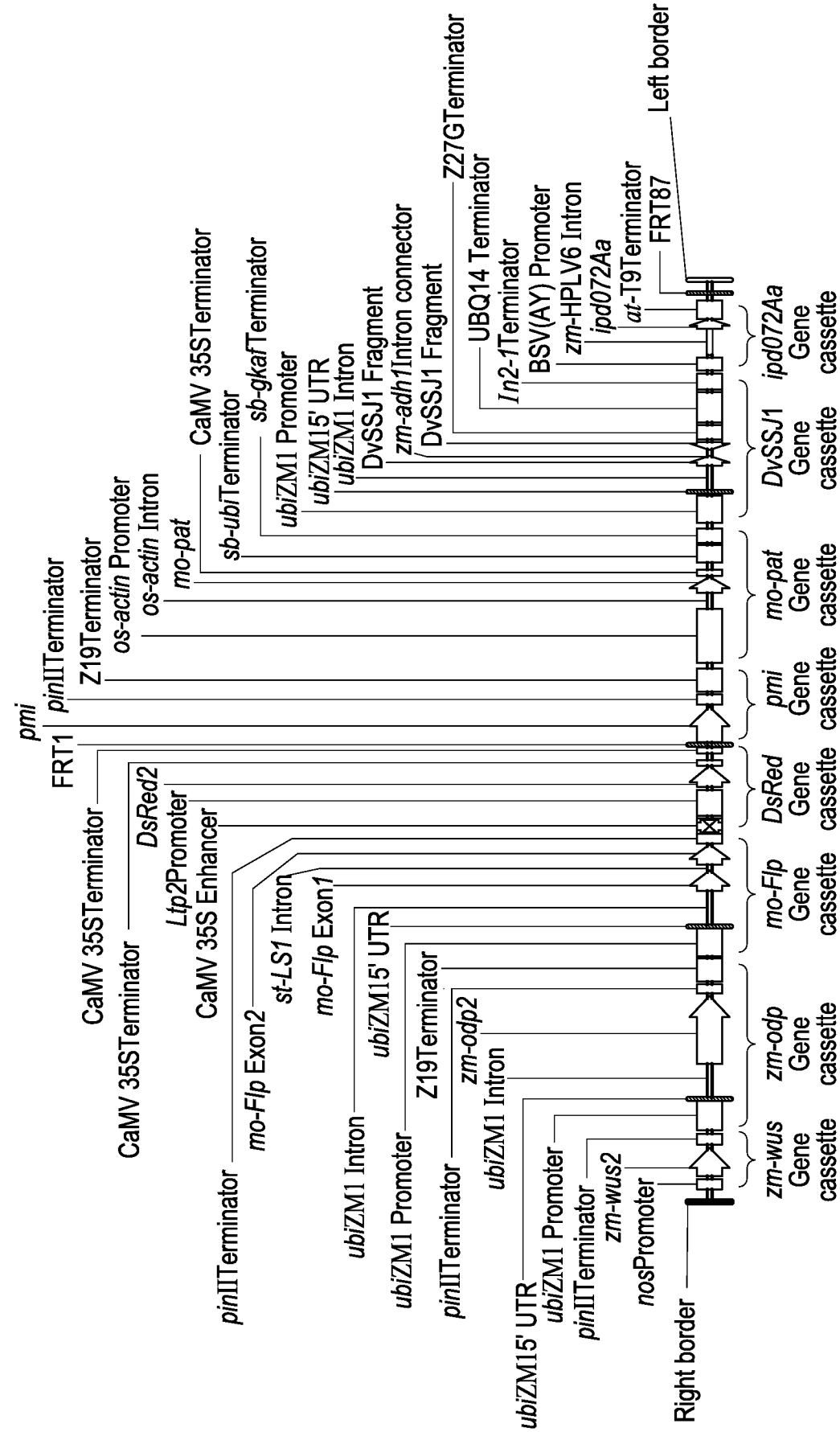
tetR
cos
co1E1 ori
spc
PHP74643
71,116 bp

FIG. 1



PHP74643T-DNA
28,187 bp

FIG. 2

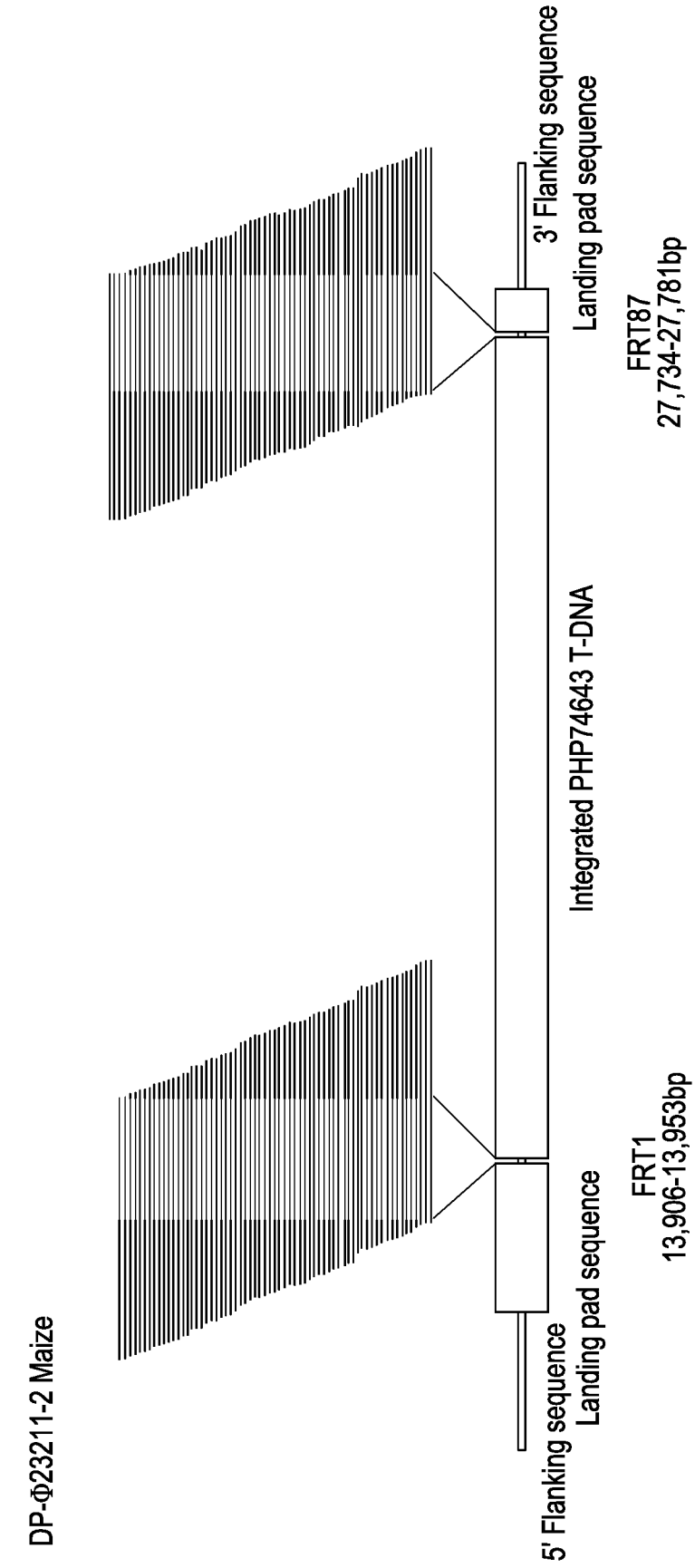

DP-Φ23211-2 Maize
5' Flanking sequence
Landing pad sequence
FRT1
13,906-13,953bp
Integrated PHP74643 T-DNA
FRT87
27,734-27,781bp
Landing pad sequence
3' Flanking sequence

FIG. 3

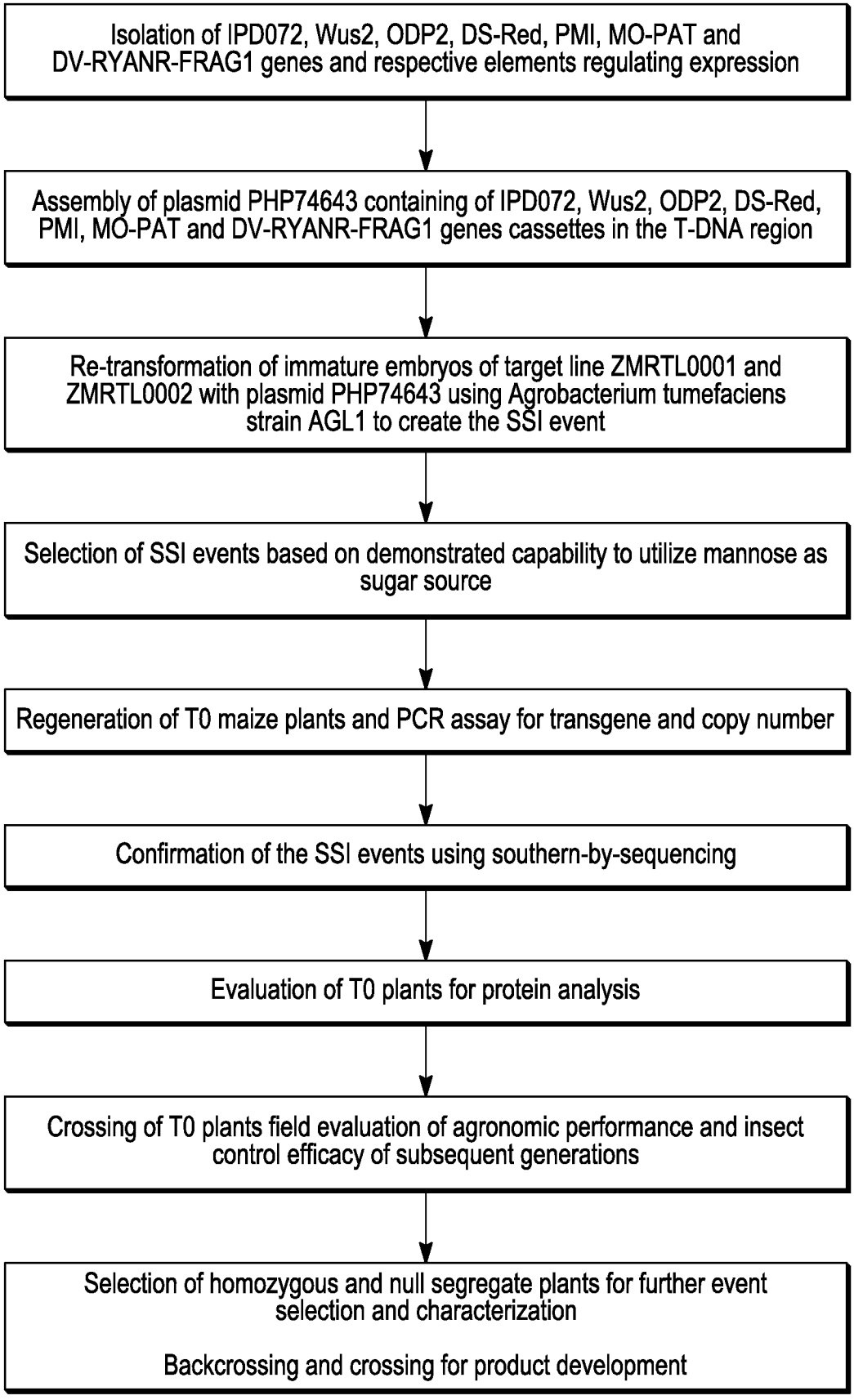

Isolation of IPD072, Wus2, ODP2, DS-Red, PMI, MO-PAT and DV-RYANR-FRAG1 genes and respective elements regulating expression
Assembly of plasmid PHP74643 containing of IPD072, Wus2, ODP2, DS-Red, PMI, MO-PAT and DV-RYANR-FRAG1 genes cassettes in the T-DNA region
Re-transformation of immature embryos of target line ZMRTL0001 and ZMRTL0002 with plasmid PHP74643 using Agrobacterium tumefaciens strain AGL1 to create the SSI event
Selection of SSI events based on demonstrated capability to utilize mannose as sugar source
Regeneration of T0 maize plants and PCR assay for transgene and copy number
Confirmation of the SSI events using southern-by-sequencing
Evaluation of T0 plants for protein analysis
Crossing of T0 plants field evaluation of agronomic performance and insect control efficacy of subsequent generations
Selection of homozygous and null segregate plants for further event selection and characterization
Backcrossing and crossing for product development

FIG. 4

Construct Design	Trait: GDUSLK			Trait: GDUSHD			Trait: EARHT		
	Number of plots with trait data	Predicted value (bu/acre)	Standard Error	Number of plots with trait data	Predicted value (bu/acre)	Standard Error	Number of plots with trait data	Predicted value (bu/acre)	Standard Error
WT (base comparator)	572	140.83	1.62	568	139.95	1.77	378	52.24	1.94
SSJ72_UBI;BSV(AY)[a]	166	140.38	1.63	165	139.67	1.77	104	52.09	1.95
SSJ72_UBI;A	111	140.64	1.63	111	139.68	1.77	71	52.39	1.95
SSJ72_BSV(AY);A	115	140.79	1.63	113	139.91	1.77	75	52.02	1.96
SSJ72_3XUBI;A	17	140.8	1.64	17	139.67	1.78	11	52.02	1.97
SSJ72_UBI;B	223	140.48	1.62	218	139.75	1.77	144	52.20	1.95

Trait: PLTHT			Trait: MST		
Number of plots with trait data	Predicted value (bu/acre)	Standard Error	Number of plots with trait data	Predicted value (bu/acre)	Standard Error
377	106.70	2.77	445	18.72	0.76
106	106.58	2.79	124	18.54	0.76
73	106.91	2.80	83	18.65	0.76
76	106.36	2.80	88	18.75	0.76
10	106.34	2.82	12	18.54	0.77
148	106.40	2.80	171	18.66	0.76

[a]Event DP-023211-2 included in this construct design

FIG. 5

Construct Design	Trait: GDUSLK			Trait: GDUSHD			Trait: EARHT		
	Number of plots with trait data	Predicted value (bu/acre)	Standard Error	Number of plots with trait data	Predicted value (bu/acre)	Standard Error	Number of plots with trait data	Predicted value (bu/acre)	Standard Error
WT (base comparator)	572	140.83	1.62	568	139.95	1.77	378	52.24	1.94
DP-023211-2	13	140.39	1.65	13	139.72	1.79	7	52.52	2.01

Trait: PLTHT			Trait: MST		
Number of plots with trait data	Predicted value (bu/acre)	Standard Error	Number of plots with trait data	Predicted value (bu/acre)	Standard Error
377	106.70	2.77	445	18.72	0.76
7	107.24	2.82	9	18.53	0.77

FIG. 6

Construct Design	Trait: GDUSLK			Trait: GDUSHD			Trait: EARHT		
	Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error	Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error	Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error
WT (base comparator)	199	157.69	2.64	197	153.28	2.50	160	45.12	2.72
SSJ72_UBI;BSV(AY)[a]	88	156.72	2.70	89	152.66	2.54	72	44.09	2.77
SSJ72_UBI;A	59	157.27	2.74	57	152.49	2.56	47	44.37	2.79
SSJ72_BSV(AY);A	58	158.43	2.73	56	154.08	2.56	44	44.59	2.79
SSJ72_3XUBI;A	12	161.05	2.99	11	154.58	2.73	9	42.58	2.98
SSJ72_UBI;B	105	157.58	2.64	106	152.99	2.54	80	43.29	2.76

Trait: PLTHT			Trait: PHTYLD		
Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error	Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error
161	86.45	2.12	213	119.35	10.79
72	86.22	2.22	92	114.53	11.18
47	86.24	2.28	62	112.86	11.39
44	86.95	2.26	63	111.95	11.38
8	86.56	2.56	11	58.40	13.13
80	84.96	2.23	109	112.98	11.13

[a]Event DP-023211-2 included in this construct design

FIG. 7

Construct Design	Trait: GDUSLK			Trait: GDUSHD			Trait: EARHT		
	Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error	Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error	Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error
WT (base comparator)	199	157.69	2.64	197	153.28	2.50	160	86.45	2.72
DP-023211-2	11	158.88	2.70	11	155.05	2.55	8	88.94	2.79

Trait: PLTHT			Trait: PHTYLD		
Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error	Number of plots with analyzed trait data	Predicted value (bu/acre)	Standard Error
161	86.45	2.12	213	119.35	10.79
8	88.94	2.21	10	110.46	11.45

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/US20 19/028485

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N 15/82
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, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2016/144688 A1 (PIONEER HI BRED INT [US]; DU PONT [US]) 15 September 2016 (2016-09-15) claims 1-15; examples 1-4; sequences 981, 850	1-53
Y	US 2015/257389 A1 (HU XU [US] ET AL) 17 September 2015 (2015-09-17) claims 1-39; sequence 729	1-53
Y	WO 2015/038734 A2 (PIONEER HI BRED INT [US]) 19 March 2015 (2015-03-19) claims 1-52; example 7	1-53

-/--

[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) orwhich 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 July 2019	24/07/2019

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	Marchesini, Patrizia

2

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

International application No
PCT/US2019/028485

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	XU HU ET AL: "Discovery of midgut genes for the RNA interference control of corn rootworm", SCIENTIFIC REPORTS, vol. 6, no. 1, 28 July 2016 (2016-07-28), XP055394042, DOI: 10.1038/srep30542 abstract -----	1-53
Y	U. SCHELLENBERG ET AL: "A selective insecticidal protein from Pseudomonas for controlling corn rootworms", SCIENCE, vol. 354, no. 6312, 3 November 2016 (2016-11-03), pages 634-^637, XP055342083, US ISSN: 0036-8075, DOI: 10.1126/science.aaf6056 abstract -----	1-53

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2019/028485

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