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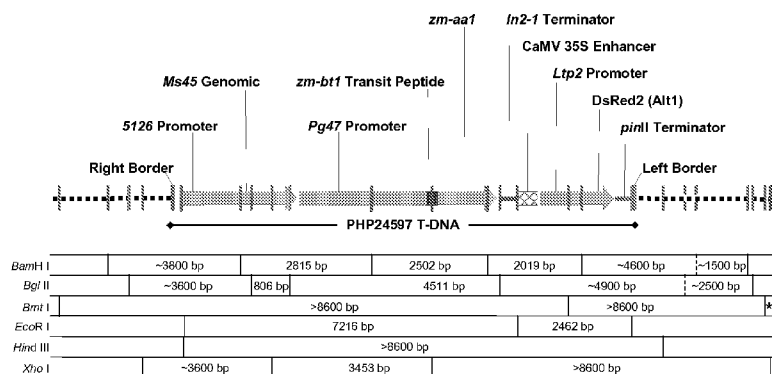
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[Continued on next page]

(54) Title: PLANT GENOMIC DNA FLANKING SPT EVENT AND METHODS FOR IDENTIFYING SPT EVENT

FIGURE 3



(57) Abstract: Compositions and methods related to transgenic plants comprising seed production technology are provided. Specifically, maize plants having a E6611.32.1.38 event which confers seed production technology are provided. The plant harboring the E6611.32.1.38 event at the recited chromosomal location comprises the genomic/transgene junctions described. The plant genomic DNA flanking the integrated E6611.32.1.38 event can be used to design assays that will be specific for the E6611.32.1.38 event. The characterization of the genomic insertion site of the E6611.32.1.38 event provides for an enhanced breeding efficiency and enables the use of molecular markers to track the transgene insert in the breeding populations and progeny thereof. Various methods and compositions for the identification, detection, and use of the maize E6611.32.1.38 event are provided.



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PLANT GENOMIC DNA FLANKING SPT EVENT AND METHODS FOR IDENTIFYING SPT EVENT

5 FIELD OF THE INVENTION

Embodiments of the present invention relate to the field of plant molecular biology. More specifically, embodiments of this invention relate to transgenic maize plants containing a seed production technology (SPT) event and plant genomic DNA flanking the transgenic sequence.

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BACKGROUND OF THE INVENTION

The expression of foreign genes in plants is known to be influenced by their 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). At the same time the presence of the transgene at different locations in the genome influences the overall phenotype of the plant in different ways. For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of an introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant tissues, that may not correspond to the patterns expected from transcriptional regulatory elements present in the introduced gene construct. It is also observed that the transgene insertion can affect the endogenous gene expression. For these reasons, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual outcrossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure reliable gene expression in a number of varieties that are well adapted to local growing conditions.

It would be advantageous to be able to detect the presence or absence of a particular event in a plant or seed, or progeny of such plants or seeds, not only with respect to the transgene itself, but also with respect to its location in the genome of a host plant or seed. It would be particularly advantageous to be able to detect the presence of an event in order to determine whether progeny of a sexual cross contain a transgene of interest. In addition, a

method for detecting a particular event would be helpful for complying with regulations requiring the pre-market approval and labeling of foods derived from recombinant crop plants, or for use in environmental monitoring, monitoring traits in crops in the field, or monitoring products derived from a crop harvest, as well as, for use in ensuring compliance of parties subject to regulatory or contractual terms. Event-specific detection methods can identify a unique junction between the inserted DNA and the recipient genome.

BRIEF SUMMARY OF THE INVENTION

Maize has been modified by the insertion of the SPT event as described herein. Relevant descriptions were also provided in US Patent Application Publication Number US2009/0038026; and in US Patent Application Publication Number 2006/0288440. The genetic elements in the T-DNA of PHP24597, used to create Event E6611.32.1.38, are summarized in Table 12.

Provided are compositions and methods related to transgenic maize plants containing the specific exogenous DNA that was introduced via standard maize transformation, referred to herein as "Seed Production Technology (SPT) event" or "Event E6611.32.1.38" or "E6611.32.1.38" or "Event DP-32138-1" or "DP-32138-1" or "Event 32138" or "32138". The transformed plants or seeds may also be referred to as "32138" or "32138 maize". Also provided are constructs useful for generating transgenic events, and materials and methods useful for identifying particular transgenic events that result in transgenic plants that comprise the SPT event.

Further provided are the seeds deposited as ATCC Patent Deposit Number PTA-9158 and plants, plant cells, plant parts, grain and plant products derived therefrom. Applicant(s) have made a deposit of at least 2500 seeds of maize event 32138 (designated as E6611.32.1.38) with the American Type Culture Collection (ATCC®), Manassas, VA 20110-2209 USA, on April 15, 2008, and the deposits were assigned ATCC Deposit Number PTA-9158. 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. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The seeds deposited with the ATCC on April 15, 2008, were taken from the deposit maintained by Pioneer Hi-Bred International, Inc., 7250 NW 62nd Avenue, Johnston, IA 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 made. This deposit of seed of

maize event 32138 will be maintained in the ATCC depository, which is a public depository, for a period of 30 years, or 5 years following 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 32138 under the Plant Variety Protection Act (7 USC 2321, et seq.). Unauthorized seed multiplication prohibited. The seed may be regulated.

An additional embodiment of the invention relates to the specific flanking sequences described herein, which can be used to develop specific identification methods for E6611.32.1.38 in biological samples. Further embodiments relate to the left border and/or right border flanking regions of E6611.32.1.38, which can be used for the development of specific primers and probes. A further embodiment of the invention relates to identification methods for the presence of E6611.32.1.38 in biological samples based on the use of such specific primers or probes.

According to another embodiment of the invention, methods of detecting the presence of DNA corresponding to Event E6611.32.1.38 in a sample are provided. In a particular embodiment, 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 a plant comprising event E6611.32.1.38, produces an amplicon that is diagnostic for event E6611.32.1.38; (b) performing a nucleic acid amplification reaction, thereby producing the amplicon and (c) detecting the amplicon.

A DNA molecule comprising the novel transgene/flanking insertion region, for example the sequence indicated in Figure 11, or a molecule homologous or complementary thereto, is an embodiment of this invention.

Additional embodiments comprise DNA sequences that comprise the novel flanking and transgene flanking/insertion regions of SEQ ID NO: 1 or Figure 11. DNA sequences that comprise a sufficient length of polynucleotides of transgene insert sequence and a sufficient length of polynucleotides of maize genomic and/or flanking sequence of SEQ ID NO: 1 or Figure 11 that are useful as primer sequences for the production of an amplicon product diagnostic for plants comprising Event E6611.32.1.38 are additional embodiments of the invention.

Further embodiments of the invention include the DNA sequences that comprise at least 11 or more nucleotides of the transgene portion of the DNA sequence of the T-DNA region of 32138 maize, or complements thereof, and a similar length of flanking maize DNA

sequence indicated in SEQ ID NO: 1 or Figure 11, or complements thereof. These DNA sequences may be useful as DNA primers in DNA amplification methods. The amplicons produced using these primers are diagnostic for event E6611.32.1.38. Therefore, embodiments of the invention also include the amplicons produced by DNA primers homologous or complementary to the transferred T-DNA region of 32138 maize alone or in combination with the identified flanking sequences.

According to another embodiment of the invention, methods of detecting the presence of a DNA molecule corresponding to event E6611.32.1.38 in a sample are provided, such methods comprising: (a) contacting the sample comprising DNA extracted from a plant with a DNA probe, said probe comprising a molecule that hybridizes under stringent hybridization conditions with DNA extracted from event E6611.32.1.38 and does not hybridize under the stringent hybridization conditions with a control plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions and (c) detecting hybridization of the probe to the DNA. More specifically, another embodiment of the invention comprises methods for detecting the presence of a DNA molecule corresponding to the event E6611.32.1.38 event in a sample, such methods consisting of (a) contacting the sample, which comprises DNA extracted from a maize plant, with a DNA probe molecule that consists 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 a plant comprising maize event E6611.32.1.38 and does not hybridize under the stringent hybridization conditions with a control maize plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions and (c) detecting hybridization of the probe to the DNA.

Another embodiment of the invention further relates to a DNA detection kit for identifying Event E6611.32.1.38 in biological samples. The kit comprises a first primer which specifically recognizes the left or right border flanking region of E6611.32.1.38, and a second primer which specifically recognizes a sequence within the foreign DNA of E6611.32.1.38, for use in a PCR identification protocol. A further embodiment of the invention relates to a kit for identifying event E6611.32.1.38 in biological samples, which kit comprises a specific probe having a sequence which corresponds or is complementary to, a sequence having between 80% and 100% sequence identity with a specific region of event E6611.32.1.38. The sequence of the probe corresponds to a specific region comprising part of the 5' or 3' flanking region of event E6611.32.1.38.

Further embodiments of the invention relate to using the methods and kits encompassed by the embodiments of the present invention for different purposes such as, but not limited to, the following: to identify event E6611.32.1.38 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; identifying transgenic plant material for purposes of segregation between transgenic and non-transgenic material; and determining the quality of plant material comprising maize event E6611.32.1.38. The kits may also contain the reagents and materials necessary for the performance of the detection method.

In another aspect, the present invention provides a method for producing progeny plants comprising Event E6611.32.1.38. The progeny plants may be inbred or hybrid plants. In a further application, the present invention provides a method for performing marker-assisted breeding for Event E6611.32.1.38. According to another aspect of the present invention, a stably transformed maize plant comprising Event E6611.32.1.38 is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a plasmid map of PHP24597. Plasmid size is 52869 bp.

Figure 2 provides a map of the T-DNA region from plasmid PHP24597. The locations of the probes used are shown as boxes in the lower part of the map and are identified below:

Number	Probe Name
1	<i>5126</i> promoter
2	<i>Ms45</i>
3	<i>Pg47</i> promoter
4	<i>zm-bt1</i>
5	<i>zm-aa1</i>
6	<i>In2-1</i> terminator
7	35S enhancer
8	<i>Ltp2</i> promoter
9	DsRed2
10	<i>pinII</i> terminator

Figure 3 provides a map of the insertion in DP-32138-1 maize and a schematic of restriction digests of the insertion based on Southern blot analysis data from this study. *Bam*H I, *Bgl* II, *Bmt* I, *Eco*R I, *Hind* III, and *Xho* I restriction enzyme sites are indicated. Southern blot analysis indicated a single copy of the PHP24597 T-DNA had inserted within the maize genome. Locations of enzyme sites outside the T-DNA region are not to scale. An asterisk (*) indicates that the relative locations of these enzyme sites are uncertain due to the large size of the fragments generated from these sites. Dashed vertical lines indicate the *Bam*H I and *Bgl* II sites located outside the Left Border junction that demonstrated blocked digestion on the Southern blots.

Figure 4 provides a schematic diagram of PHP24597 without restriction sites.

Figure 5 provides a schematic diagram of the T-DNA region of PHP24597, indicating the Ms45 genomic region, zm-aa1 gene and the DsRed2(Alt1) gene along with their respective regulatory elements. The size of the T-DNA is 9950 bp.

5 **Figure 6** provides a schematic map of T-DNA from plasmid PHP24597 with location of primers (08-0-2544/08-0-2582) used for construct-specific PCR. The figure is not drawn to scale. T-DNA size is 9950 bp.

10 **Figure 7** shows results of construct-specific PCR analysis of leaf DNA from 32138 maize and non-genetically-modified control maize. PCR amplification was conducted with primer set 08-0-2544/08-0-2582 targeting the unique 5126 promoter/Ms45 gene junction of PHP24597 T-DNA present in 32138 maize. Expected amplicon size is 233 bp. Samples were loaded as follows:

Lane Assignments:

Lane	Sample	Lane	Sample
1	Low DNA Mass Ladder	11	32138 maize plant 3
2	Blank	12	32138 maize plant 4
3	Control plant 1	13	32138 maize plant 5
4	Control plant 2	14	Blank
5	Control plant 3	15	No-template control
6	Control plant 4	16	Blank
7	Control plant 5	17	PHP24597
8	Blank	18	Blank
9	32138 maize plant 1	19	Low DNA Mass Ladder
10	32138 maize plant 2		

15 **Figure 8** shows results of maize invertase gene PCR analysis of leaf DNA from 32138 maize and non-genetically-modified control maize. PCR amplification of endogenous maize invertase gene with primer set 02-O-197/02-O-198, targeting the maize invertase gene, was used as a positive control for PCR amplification. Expected amplicon size is 225 bp. Samples were loaded as follows:

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Lane Assignments:

Lane	Sample	Lane	Sample
1	Low DNA Mass Ladder	11	32138 maize plant 3
2	Blank	12	32138 maize plant 4

3	Control plant 1	13	32138 maize plant 5
4	Control plant 2	14	Blank
5	Control plant 3	15	No-template control
6	Control plant 4	16	Blank
7	Control plant 5	17	PHP24597
8	Blank	18	Blank
9	32138 maize plant 1	19	Low DNA Mass Ladder
10	32138 maize plant 2		

Figure 9 shows the sensitivity of PCR analysis for 32138 maize for leaf DNA samples. PCR amplification was conducted with primer set 08-0-2544/08-0-2582 targeting the unique 5126 promoter/Ms45 gene junction of PHP24597 T-DNA present in 32138 maize. Expected amplicon size is 233 bp. Samples were loaded as follows, with decreasing amount of 32138 maize DNA (Lanes 3-10), and non-genetically modified control DNA (Lane 12):

Lane Assignments:

Lane	Sample	Lane	Sample
1	Low DNA Mass Marker	8	10 pg of 32138 maize
2	Blank	9	5 pg of 32138 maize
3	50 ng of 32138 maize	10	500 fg of 32138 maize
4	5 ng of 32138 maize	11	No-template control
5	500 pg of 32138 maize	12	50 ng of Control DNA
6	100 pg of 32138 maize	13	Blank
7	50 pg of 32138 maize	14	Low DNA Mass Marker

Figure 10 is a schematic representation of insert and genomic border regions sequenced in 32138 maize. The diagram indicates the PCR fragments generated from 32138 maize genomic DNA that were cloned and sequenced: Fragment A (07-0-2286/07-0-2277), Fragment B (08-0-2329/08-0-2398), Fragment C (08-0-2402/07-0-2024), Fragment D (08-0-2505/08-0-2504) and Fragment E (08-0-2408/08-0-2526). The vertical dashed line represents the genomic border/insert junction. Fragment F (08-0-2528/08-0-2538) and G (08-0-2539/08-0-2530) represent the 5' and 3' genomic border regions, respectively. Figure 10 is not drawn to scale.

Figure 11 provides the sequence of T-DNA insert and genomic border regions in 32138 maize. The 5' and 3' genomic border regions are underlined: bp1 to 2114 and bp 119997 to 13998, respectively. Directly upstream of the complete insert at positions 2115-11996, within the "genomic border region," is a partial insert of T-DNA, as shown in Figure 15.

Figure 12 shows results of PCR analysis of the 5' and 3' genomic border regions in 32138 maize and control maize plants.

12A: Primer pair 08-0-2528/08-0-2538 amplifies a PCR product (294 bp) from within the 5' flanking genomic DNA. Samples were loaded as follows:

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Lane	Sample
1	Low DNA Mass Marker
2	Blank
3	Control Maize Plant C-1
4	Control Maize Plant C-2
5	Blank
6	32138 Maize Plant T-1
7	32138 Maize Plant T-2
8	32138 Maize Plant T-3
9	32138 Maize Plant T-4
10	No-template Control
11	Blank
12	Low DNA Mass Marker

12B: Primer pair 08-0-2539/08-0-2530 amplifies a PCR product (297 bp) from within the 3' genomic border region. Samples were loaded as follows:

Figure 13 is a schematic map of plasmid PHP24597 indicating *EcoR* I and *BamH* I

Lane	Sample
1	Low DNA Mass Marker
2	Blank
3	Control Maize Plant C-1
4	Control Maize Plant C-2
5	Blank
6	32138 Maize Plant T-1
7	32138 Maize Plant T-2
8	32138 Maize Plant T-3
9	32138 Maize Plant T-4
10	No-template Control
11	Blank
12	Low DNA Mass Marker

restriction enzyme sites with base pair positions. The Right border and Left border regions flank the T-DNA (Figure 2) that is expected to be transferred during *Agrobacterium*-mediated transformation. The location of the probes used for Southern analysis is indicated as lettered boxes inside the plasmid map. **A:** RB probe; **B:** LB probe; **C:** *spc* probe; **D:** *virG* probe; **E:** *tet* probe. The locations of additional enzymes used for analysis are as follows:

Enzyme	Locations (bp)
<i>Bmt</i> I	8503, 36160, 38359, 39502, 45917
<i>Hind</i> III	179, 34837, 45900, 46809, 47930, 49445

Figure 14 is a schematic map of T-DNA from PHP24597 indicating restriction enzyme sites for *BamH* I, *Bmt* I, *EcoR* I, and *Hind* III and the *Ms45*, *zm-aa1* and *DsRed2(Alt1)* coding and regulatory regions. T-DNA size is 9950 bp. The locations of the probes used are shown as numbered boxes below the map and are identified further as follows:

Number	Probe Name
1	5126 promoter

2	<i>Ms45</i>
3	<i>Pg47</i> promoter
4	<i>zm-bt1</i>
5	<i>zm-aa1</i>
6	<i>ln2-1</i> terminator
7	35S enhancer
8	<i>Ltp2</i> promoter
9	<i>DsRed2(Alt1)</i>
10	<i>pinII</i> terminator

Figure 15 is a schematic showing detail of the 5' flanking sequence and insertion site. Alternative characterizations of the 23-bp or 27-bp partial insert are provided.

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BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 is the 13,998-base pair region of 32138 maize which comprises 2114 bp of the 5' genomic border sequence, 2002 bp of the 3' genomic border sequence, and 9882 bp of inserted T-DNA from PHP24597.

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SEQ ID NOS: 2-5 are primer sequences used in PCR reactions; see also, Table 17.

SEQ ID NOS: 6-19 are primer sequences used in PCR reactions; see also, Table 16.

SEQ ID NO: 20 is the deduced amino acid sequence from translation of the spliced exons from the *Ms45* gene from plasmid PHP24597. The full-length MS45 protein is 412 amino acids in length and weighs approximately 47kDa.

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SEQ ID NO: 21 is the deduced amino acid sequence from translation of the *zm-bt1* transit peptide + *zm-aa1* region from plasmid PHP24597. The complete translation product, including transit peptide (amino acids 1-75) is 495 amino acids in length and weighs approximately 54 kDa. The processed ZM-AA1 protein, with the transit peptide removed, is 420 amino acids in length and weighs approximately 46 kDa.

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SEQ ID NOS: 22 and 23 are forward and reverse primers, respectively, for a qPCR method for detecting the 32138 event.

SEQ ID NO: 24 is the sequence for a fluorescent-labeled probe for a qPCR method for detecting the 32138 event.

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SEQ ID NO: 25 is an amplicon raised using SEQ ID NOS: 22-24 in the qPCR method for detecting the 32138 event. The amplicon spans the 3' junction.

SEQ ID NOS: 26 and 27 are forward and reverse primers, respectively for a gel-based method for detecting the 32138 event.

SEQ ID NO: 28 is an amplicon raised using SEQ ID NOS: 26 and 27 in the gel-based method for detecting the 32138 event. The amplicon spans the 3' junction.

SEQ ID NOS 29 and 30 are forward and reverse primers, respectively, for detection of the 32138 event.

5 SEQ ID NO: 31 is the sequence for a fluorescent-labeled probe for detection of the 32138 event.

SEQ ID NO: 32 is an amplicon raised using SEQ ID NOS: 29-31 in detecting the 32138 event.

10 SEQ ID NO: 33 is the deduced amino acid sequence from translation of the *DsRed2(Alt1)* gene from plasmid PHP24597. The DsRED2 protein is 225 amino acids in length and weighs approximately 26 kDa.

SEQ ID NO: 34 represents T-DNA of PHP24597 with right and left border repeats indicated. Description and position of genetic elements is provided in Table 12.

SEQ ID NO: 35 provides the DsRed2(Alt1) coding sequence as indicated.

15 SEQ ID NO: 36 provides a partial insert and left border flanking sequence of event 32138.

SEQ ID NO: 37 provides a partial insert and right border flanking sequence of event 32138.

ABBREVIATIONS

Certain abbreviations are used herein, including the following:

5126	Anther-specific gene from maize
Bp	Base pair
DIG	Digoxigenin
ID	Identifier
<i>In2-1</i>	Benzenesulfonamide-inducible 2-1 gene from maize
Kb	Kilobase or Kilobase pair
LB	Left T-DNA border
<i>Ltp2</i>	Aleurone-specific lipid transfer protein 2 gene from <i>Hordeum vulgare</i>
<i>Ms45</i>	Maize fertility restoration gene
<i>Ms45</i> Genomic	<i>Ms45</i> gene and associated 3' untranslated region
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
<i>pinII</i>	Proteinase inhibitor II gene from <i>Solanum tuberosum</i>
RB	Right T-DNA border
SDS	Sodium dodecyl sulfate
SSC	0.015 M sodium citrate, 0.15 M sodium chloride, pH 7.0
T-DNA	Transferred DNA
<i>zm-aa1</i>	Truncated version of the α -amylase gene from maize
<i>zm-bt1</i>	Transit peptide from the <i>Brittle-1</i> gene from maize

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DETAILED DESCRIPTION OF THE INVENTION

The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art.

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As used herein, the term "event E6611.32.1.38 specific" refers to a polynucleotide sequence which is suitable for discriminatively identifying event E6611.32.1.38 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.

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As used herein, the term "maize" is any maize plant and includes all plant varieties that can be bred with maize. As used herein, the term "plant" includes whole plants, plant cells, plant organs, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, stalks, roots, root tips, anthers and the like. Parts of transgenic plants understood to be within the scope of the invention comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, leaves and roots originating in transgenic plants or their progeny previously

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transformed with a DNA molecule of the invention and therefore consisting at least in part of transgenic cells. Grain is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these plants comprise a E6611.32.1.38 event. The class of plants that can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. "Foreign" refers to material not normally found in the location of interest. Thus "foreign DNA" may comprise recombinant DNA and/or newly introduced, rearranged DNA of the plant. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. The site in the plant genome where a recombinant DNA has been inserted may be referred to as the "insertion site" or "target site".

"Flanking DNA" can comprise either genomic DNA naturally present in an organism such as a plant, or foreign (heterologous) DNA introduced via the transformation process, e.g. fragments associated with the transformation event. Thus, flanking DNA may include a combination of native and foreign DNA. A "flanking region" or "flanking sequence" or "genomic border region" or "genomic border sequence" as used herein refers to a sequence of at least 3, 5, 10, 11, 15, 20, 50, 100, 200, 300, 400, 1000, 1500, 2000, 2500 or 5000 base pairs or greater which is located either immediately upstream or downstream of, and contiguous with, the original foreign insert DNA molecule. When this flanking region is located downstream it may also be referred to as "left border flank" or "3' flank" or "3' genomic border region" or "genomic 3' border sequence", and the like. When this flanking region is located upstream it may also be referred to as the "right border flank" or "5' flank" or "5' genomic border region" or "genomic 5' border sequence", and the like. Non-limiting examples of the flanking regions of the E6611.32.1.38 event are set forth in SEQ ID NO: 1 (bp 1-2114 and bp 11,997-13,998) and Figure 11 (see, underlined regions).

Transformation procedures leading to random integration of the foreign DNA will result in transformants containing different flanking regions characteristic of and unique for each transformant. When recombinant DNA is introduced into a plant through traditional crossing, its flanking regions will generally not be changed. Transformants will also contain unique
5 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"
10 refers to DNA that comprises a junction point. Junction points for the E32138 event are apparent from Figure 11 and Figure 15.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign 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
15 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 or analogous 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 or analogous species, is substantially modified from its original form.

"Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences,
20 introns and polyadenylation recognition sequences.

"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 can comprise proximal and more distal upstream elements. The more distal upstream elements are often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence that can stimulate promoter activity and
25 may be an innate element of the promoter or a heterologous element inserted to enhance the activity 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 promoters may direct the expression of a gene in different tissues or cell
30 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
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most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg, (1989) *Biochemistry of Plants* 15:1-82. 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. Examples of translation leader sequences have been described (Turner and Foster, (1995) *Mol. Biotechnol.* 3:225-236).

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 effecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht, *et al.*, (1989) *Plant Cell* 1:671-680.

A "protein" or "polypeptide" is a chain of amino acids arranged in a specific order determined by the coding sequence in a polynucleotide encoding the polypeptide.

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, enhancers, 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 prokaryote cells or eukaryotic cells. Expression cassettes of the embodiments of the present invention are designed to express in plant cells.

The DNA molecules of embodiments of the invention may be provided in expression cassettes for expression in an organism of interest. The cassette may include 5' and 3' regulatory sequences operably linked to a coding sequence. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a polynucleotide of interest and a regulatory sequence (i.e., a promoter) is a functional

link that allows for the expression of the polynucleotide of interest. The operably linked regulatory sequence may initiate and mediate transcription of the polynucleotide of interest. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, operably linked is intended to mean that the coding regions are in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes or multiple DNA constructs. Such an expression cassette may be provided with a plurality of restriction sites and/or recombination sites for insertion of the polynucleotide to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette may include in the 5' to 3' direction of transcription, a transcriptional and translational regulatory region (i.e. a promoter), a coding region, and a transcriptional and translational termination region functional in plants. The transcriptional regulatory 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. The regulatory regions (e.g., promoters, transcriptional regulatory regions, RNA processing or stability regions, introns, polyadenylation signals and translational termination regions) and/or the coding region may be native/analogous or heterologous to the host cell or to each other.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved. The expression cassette can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues.

It is to be understood that as used herein the term "transgenic" 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 transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring

events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition or spontaneous mutation.

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.

Typically, a number of plant cells are transformed, producing a population of plants from which a particular plant is selected. The term "event" refers to the original transformant and progeny of the transformant that include the exogenous DNA inserted into a particular and unique location in the genome, i.e., event DNA. The term "event" also refers to progeny produced by a sexual outcross, a self-pollination, or repeated backcrossing, wherein at least one of the plants used in the breeding is of any generation of the original transformant containing event DNA.

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 include the heterologous DNA. Even after repeated back-crossing to a recurrent parent, the inserted DNA and flanking DNA from the transformed parent are present in the progeny of the cross at the same chromosomal location. 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.

Thus, a transgenic "event" is a plant comprising and defined by an "event DNA." In this way, "Event E6611.32.1.38 " comprises " E6611.32.1.38 Event DNA." A plant may comprise two or more different event DNAs and thus comprise two or more different events. In addition, a plant lacking a given transgene event X does not comprise that event DNA X in question. Event DNA may be transferred from plant to plant, generation to generation, by any breeding scheme, method, or tool known to those of skill in the art of plant breeding.

Transformation of plants typically utilizes a selectable marker and selection method to distinguish the transformed cells of the culture from the non-transformed cells. In some instances the selectable marker gene remains in the transgenic plant; in other instances it is desirable to remove the selectable marker gene or other sequences introduced in the exogenous DNA. Homologous recombination is one method useful for the deletion of marker

genes residing within a transgenic plant (US Patent Number 6,580,019, incorporated herein by reference in its entirety). Another useful tool for removing sequences from a plant involves the use of site-specific recombinase enzymes and their respective site-specific target sites.

A number of different site-specific recombinase systems could be employed in accordance with the instant invention, including, but not limited to, the Cre/lox system of bacteriophage P1 and the FLP/FRT system of yeast. The bacteriophage P1 Cre/lox and the yeast FLP/FRT systems constitute two particularly useful systems for site-specific integration or excision of transgenes. In these systems, a recombinase (Cre or FLP) will interact specifically with its respective site-specific recombination sequence (lox or FRT, respectively) to invert or excise the intervening sequences. The sequence for each of these two systems is relatively short (34 bp for lox and 47 bp for FRT) and therefore, convenient for use with transformation vectors. The FLP/FRT and Cre/lox recombinase systems have been demonstrated to function efficiently in plant cells. In a particular embodiment, a Cre/lox recombinase system is employed to remove selectable marker sequences, particularly an NPT II marker gene flanked by lox P recombination sites. (Russell, *et al.*, (1992) *Mol. Gen. Genet.* 234:45 59).

DNA molecules are provided that comprise at least a portion of the heterologous insert DNA and plant genomic flanking DNA (referred to herein as a "junction sequence" or "junction").

The term "Event E6611.32.1.38 DNA" refers to a DNA segment comprising an insertion of the T-DNA of Figure 2 (see also, bp 2115-11,996 of SEQ ID NO: 1) into a particular location in the genome and adjacent flanking DNA that would be expected to be transferred to a progeny plant from a parent plant containing the inserted DNA. More specifically, Event E6611.32.1.38 DNA also refers to each of the DNA regions that include an interface of the plant native genomic DNA and the integrated transgenic DNA in the genome of the plant, e.g., a region around one interface where the 5' end is in plant native genomic DNA and the 3' end is in integrated transgenic DNA. In addition, the sequence of the exogenous DNA may be altered while resident in its particular location in a host genome, e.g., a portion of the sequence may be changed, deleted or amplified and still constitute said event DNA, providing said exogenous DNA continues to reside in the same location in the genome.

A E6611.32.1.38 plant can be bred by first sexually crossing first and second parental plants. The first parental plant is a plant grown from the transgenic E6611.32.1.38 plant or progeny thereof, i.e., derived from transformation with the expression cassettes of the embodiments of the present invention that confer seed production technology. The first parental plant is pollinated by a second parental plant, thereby producing a plurality of first progeny plants, and a first progeny plant is selected that comprises the SPT event. The first progeny plant may be selfed, thereby producing a plurality of second progeny plants; and from

the second progeny plants is selected a plant comprising the SPT event. These steps can further include the back-crossing of a progeny plant comprising the SPT event to the second parental plant or a third parental plant, thereby producing a plant comprising the SPT event. The SPT event is transmitted through female gametes and not male gametes.

Two different transgenic plants can also be sexually crossed to produce offspring that contain two independently segregating exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several references, e.g., Fehr, in *Breeding Methods for Cultivar Development*, Wilcox, ed., American Society of Agronomy, Madison Wis. (1987).

The term "germplasm" refers to an individual, a group of individuals, or a clone representing a genotype, variety, species or culture, or the genetic material thereof.

A "line" or "strain" is a group of individuals of identical parentage that are generally inbred to some degree and that are generally isogenic or near isogenic.

Inbred maize lines are typically developed for use in the production of maize hybrids and for use as germplasm in breeding populations for the creation of new and distinct inbred maize lines. Inbred maize lines are often used as targets for the introgression of novel traits through traditional breeding and/or molecular introgression techniques. Inbred maize lines need to be highly homogeneous, homozygous and reproducible to be useful as parents of commercial hybrids. Many analytical methods are available to determine the homozygosity and phenotypic stability of inbred lines.

The phrase "hybrid plants" refers to plants which result from a cross between genetically different individuals.

As used herein "crossed" or "cross" is intended to mean the fusion of gametes, e.g., via pollination to produce progeny (i.e., cells, seeds or plants) in the case of plants. The term encompasses both sexual crosses (the pollination of one plant by a genetically distinct plant) and selfing (self-pollination, i.e., when the pollen and ovule are from the same plant or from genetically identical plants).

The term "introgression" refers to the transmission of a desired allele of a genetic locus from one genetic background to another. In one method, the desired alleles can be introgressed through a sexual cross between two parents, wherein at least one of the parents has the desired allele in its genome.

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

methods of plant transformation include *Agrobacterium*-mediated transformation (De Blaere, *et al.*, (1987) *Meth. Enzymol.* 143:277) and particle-accelerated or "gene gun" transformation technology (Klein, *et al.*, (1987) *Nature* (London) 327:70-73; US Patent Number 4,945,050, incorporated herein by reference). Additional transformation methods are disclosed below.

Thus, isolated polynucleotides of the invention can be incorporated into recombinant constructs, typically DNA constructs, which are capable of introduction into and replication in a host cell. Such a construct can 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.

A "probe" is an isolated polynucleotide to which is attached a conventional 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 polynucleotide, for example, in some embodiments, to a strand of isolated DNA from event E6611.32.1.38 whether from a plant or from a sample that includes DNA from the event. Probes include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

As used herein, "primers" are isolated polynucleotides that are annealed 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 polynucleotide, e.g., by the polymerase chain reaction (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, US Patent Numbers 4,683,195 and 4,800,159; herein incorporated by reference). Any combination of primers disclosed herein can be used such that the pair allows for the detection of a E6611.32.1.38 event or specific region.

Non-limiting examples of primer pairs include SEQ ID NOS: 6 and 7, 8 and 9, 10 and 11, 12 and 13, 14 and 15, 16 and 17 and 18 and 19, as shown in Table 16.

Probes and primers are of sufficient nucleotide length to bind to the target DNA sequence and specifically detect and/or identify a polynucleotide having a E6611.32.1.38 event. It is recognized that the hybridization conditions or reaction conditions can be determined by the operator to achieve this result. Generally, 5, 8, 11, 14, 16, 18, 20, 22, 24, 26, 28, 30, 40, 50, 75, 100, 200, 300, 400, 500, 600, 700 nucleotides or more or between about 11-20, 20-30, 30-40, 40-50, 50-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800 or more nucleotides in length are used. Such probes and primers can hybridize specifically to a target sequence under high stringency hybridization conditions. Probes and primers may have complete DNA sequence identity of contiguous nucleotides with the target sequence, although probes differing from the target DNA sequence and that retain the ability to specifically detect and/or identify a target DNA sequence may be designed by conventional methods. Accordingly, probes and primers can share about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater sequence identity or complementarity to the target polynucleotide, or can differ from the target sequence by 1, 2, 3, 4, 5, 6 or more contiguous nucleotides. 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 can be used to amplify an integration fragment to produce an amplicon that can be used as a "specific probe" for identifying event E6611.32.1.38 in biological samples. Alternatively, a probe can be used during the PCR reaction to allow for the detection of the amplification event (i.e., a Taqman probe or a MGB probe, so-called real-time PCR). When the probe is hybridized with the polynucleotides 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 E6611.32.1.38 in the biological sample. Such identification of a bound probe has been described in the art. In one embodiment, the specific probe is a sequence which, under optimized 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 at least 80%, between 80 and 85%, between 85 and 90%, between 90 and 95% or between 95 and 100% identical (or complementary) to a specific region of the E6611.32.1.38 event. A quantitative real-time PCR assay specific for the E6611.32.1.38 event may comprise, for example, the primer pair of SEQ ID NOS: 29 and 30 and the fluorescent probe of SEQ ID NO: 31.

Methods for preparing and using probes and primers are described, for example, in Molecular Cloning: A Laboratory Manual, 2nd ed, vol. 1-3, ed. Sambrook, *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989 (hereinafter, "Sambrook, *et al.*,"

1989"); Current Protocols in Molecular Biology, ed. Ausubel, *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, "Ausubel, *et al.*, 1992"); 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, Wis.) and Primer (Version 0.5.COPYRGT., 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.

As used herein, "kit" refers to a set of reagents for the purpose of performing the method embodiments of the inventions, more particularly, the identification and/or the detection of the E6611.32.1.38 event in biological samples. The kit can be used, and its components can be specifically adjusted, for purposes of quality control (e.g., purity of seed lots), detection of event E6611.32.1.38 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.

In specific embodiments, a kit for identifying event E6611.32.1.38 in a biological sample is provided. The kit comprises a first and a second primer, wherein the first and second primer amplify a polynucleotide comprising a E6611.32.1.38 and/or flanking DNA specific region. In further embodiments, the kit also comprises a polynucleotide for the detection of the E6611.32.1.38 and/or flanking DNA specific region. The kit can comprise, for example, a first primer comprising a fragment of a polynucleotide of SEQ ID NO: 1, 2, 3, 4 or 5 wherein the first or the second primer shares sufficient sequence identity or complementarity to the polynucleotide to amplify said E6611.32.1.38 and/or flanking DNA specific region. The primer pair can comprise a fragment of SEQ ID NO: 1 and a fragment of any of SEQ ID NOS: 2-3 and 6-19. The primers can be of any length sufficient to amplify the E6611.32.1.38 region including, for example, at least 6, 7, 8, 9, 10, 15, 20, 15 or 30 or about 7-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45 nucleotides or longer.

Further provided are DNA detection kits comprising at least one polynucleotide that can specifically detect a E6611.32.1.38 and/or flanking DNA specific region, wherein said polynucleotide comprises at least one DNA molecule of a sufficient length of contiguous nucleotides homologous or complementary to SEQ ID NO: 1.

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 of the present invention hybridize under stringent conditions to a target DNA sequence.

Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. Nucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure.

In hybridization techniques, all or part of a polynucleotide that selectively hybridizes to a target polynucleotide having a E6611.32.1.38 specific event is employed. By "stringent conditions" or "stringent hybridization conditions" when referring to a polynucleotide probe, conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background) are intended. Regarding the amplification of a target polynucleotide (e.g., by PCR) using a particular amplification primer pair, "stringent conditions" are conditions that permit the primer pair to hybridize to the target polynucleotide to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce an identifiable amplification product (the amplicon) having a E6611.32.1.38 specific region in a DNA thermal amplification reaction. Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of identity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length or less than 500 nucleotides in length.

As used herein, a substantially complementary or identical sequence is a polynucleotide that will specifically hybridize to the nucleic acid molecule to which it is being compared, or to its complement, respectively, under high stringency conditions. Appropriate stringency conditions which promote DNA hybridization, for example, 6X sodium chloride/sodium citrate (SSC) at about 45°C., followed by a wash of 2XSSC at 50°C., are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Typically, stringent conditions for hybridization and detection will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M

NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours. The duration of the wash time will be at least a length of time sufficient to reach equilibrium.

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. For DNA-DNA hybrids, the T_m (thermal melting point) can be approximated from the equation of Meinkoth and Wahl, (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; 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. The 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. 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 $\geq 90\%$ identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3 or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9 or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15 or 20°C lower than the thermal melting point (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), it is optimal 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, *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See, Sambrook, *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press,

Plainview, New York) and Haymes, *et al.*, (1985) In: *Nucleic Acid Hybridization, a Practical Approach*, IRL Press, Washington, D.C.

As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the polynucleotide 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, *et 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.

Any of the polynucleotides and fragments and variants thereof employed in the methods and compositions can share sequence identity to a region of the transgene insert of the E6611.32.1.38 event, a junction sequence of the E6611.32.1.38 event, or a flanking sequence of the E6611.32.1.38 event. Methods to determine the relationship of various sequences are known. As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two polynucleotides. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100 or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence, a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller, (1988) *CABIOS* 4:11-17; the local alignment algorithm of

Smith, *et al.*, (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch, (1970) *J. Mol. Biol.* 48:443-453; the search-for-local alignment method of Pearson and Lipman, (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul, (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul, (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys Inc., 9685 Scranton Road, San Diego, California, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins, *et al.*, (1988) *Gene* 73:237-244 (1988); Higgins, *et al.*, (1989) *CABIOS* 5:151-153; Corpet, *et al.*, (1988) *Nucleic Acids Res.* 16:10881-90; Huang, *et al.*, (1992) *CABIOS* 8:155-65 and Pearson, *et al.*, (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller, (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul, *et al.*, (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul, (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the inventions. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the inventions. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul, *et al.*, (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See, Altschul, *et al.*, (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See, www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3 and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison

program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch, (1970) *J. Mol. Biol.* 48:443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the GCG Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the Quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the GCG Wisconsin Genetics Software Package is BLOSUM62 (see, Henikoff and Henikoff, (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent

sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

As used herein, "percentage of sequence identity" is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100.

As used herein, "amplified DNA" or "amplicon" refers to the product of polynucleotide amplification of a target polynucleotide that is part of a nucleic acid template. For example, to determine whether a plant resulting from a sexual cross contains the E6611.32.1.38 event, DNA extracted from the plant tissue sample may be subjected to a polynucleotide 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 E6611.32.1.38 event DNA. By "diagnostic" for a E6611.32.1.38 event, the use of any method or assay which discriminates between the presence and the absence of a E6611.32.1.38 event in a biological sample is intended. Alternatively, the second primer may be derived from the junction sequence. In still other embodiments, 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 polynucleotide of the expression construct as well as the sequence flanking the transgenic insert. The amplicon is of a length and has a sequence that is also diagnostic for the event (i.e., has a junction DNA from a E6611.32.1.38 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. 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, or about twenty thousand nucleotide base pairs. 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 the polymerase chain reaction (PCR). A variety of amplification methods are known in the art and are described, inter alia, in US Patent Numbers 4,683,195 and 4,683,202 and in PCR Protocols: A Guide to Methods and Applications, ed. Innis, *et al.*, Academic Press, San Diego, 1990. 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.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:5695-5699). 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 invention. 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.*, (1994) *Nucleic Acid Res.* 22:4167-4175) 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 (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 (*Innov. Pharma. Tech.* 00:18-24, 2000). 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 (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.*, (*Genome Res.* 9:492-498, 1999) is also a method that can be used to detect an amplicon of the invention. 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

(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.

Taqman® (PE Applied Biosystems, Foster City, Calif.) is described as a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by the manufacturer. Briefly, 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.*, (*Nature Biotech.* 14:303-308, 1996). 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 (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.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the present disclosure and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

Compositions and methods related to transgenic plants comprising the SPT event are provided. Specifically, plants, and more particularly, maize plants, having event E6611.32.1.38 are provided. A maize plant having event E6611.32.1.38 has been modified by the insertion of the seed production technology event. The SPT event E6611.32.1.38 comprises the 5126 promoter driving MS45 Genomic DNA, the PG47 promoter driving ZM-BT1 TP encoding Brittle1 transit peptide linked to ZM-AA1 encoding alpha-amylase, and the CaMV35S enhancer combined with the LTP2 promoter to drive DS-RED2 (ALT1) encoding a variant of DISCOSOMA sp. red fluorescent protein. (See, Figure 1; see also, US Patent Application Serial Number 11/833,363 filed August 3, 2007, and published February 5, 2009, as 2009/0038026; and US Patent Application Publication Number 2006/0288440.) The event insertion maps to Chromosome 3 at 84.1 to 84.5, between markers MZA 12392 and MZA 2358. Thus, a plant comprising event E6611.32.1.38 exhibits enhanced seed production technology capability.

The polynucleotides conferring the seed production technology are linked on the same DNA construct and are inserted at a characterized position in the maize genome and thereby produce the E6611.32.1.38 event. The plant harboring the E6611.32.1.38 event at the recited chromosomal location comprises genomic/transgene junctions as indicated in SEQ ID NO: 1 and Figures 11 and 15. The characterization of the genomic insertion site of the E6611.32.1.38 event provides for an enhanced breeding efficiency and enables the use of molecular markers to track the transgene insert in the breeding populations and progeny thereof. Various methods and compositions for the identification, detection, and use of plants, plant parts, seeds and grain products containing the maize E6611.32.1.38 event are provided herein.

In some embodiments, the polynucleotides conferring the maize E6611.32.1.38 event are engineered into a molecular stack. In other embodiments, the molecular stack further comprises at least one additional transgenic polynucleotide. Said polynucleotide may confer additional aspects of seed production technology or may confer an unrelated trait.

In certain embodiments, the plant is stacked with any combination of polynucleotide sequences of interest in order to create plants with a desired combination of traits. A trait, as used herein, refers to the phenotype derived from a particular sequence or groups of sequences. The combinations generated can also include multiple copies of any one or more of the polynucleotides of interest.

In some embodiments, the maize E6611.32.1.38 event can also be combined with at least one other trait to produce plants that further comprise a variety of desired trait combinations including, but not limited to, traits desirable for animal feed such as high oil content (e.g., US Patent Number 6,232,529); balanced amino acid content (e.g., hordothionins

(US Patent Numbers 5,990,389; 5,885,801; 5,885,802 and 5,703,409; US Patent Number 5,850,016); barley high lysine (Williamson, *et al.*, (1987) *Eur. J. Biochem.* 165:99-106; and WO 98/20122) and high methionine proteins (Pedersen, *et al.*, (1986) *J. Biol. Chem.* 261:6279; Kirihaara, *et al.*, (1988) *Gene* 71:359 and Musumura, *et al.*, (1989) *Plant Mol. Biol.* 12:123));
5 increased digestibility e.g., modified storage proteins (US Patent Application Serial Number 10/053,410, filed November 7, 2001); and thioredoxins (US Patent Application Serial Number 10/005,429, filed December 3, 2001)); the disclosures of which are herein incorporated by reference. Desired trait combinations also include low linolenic acid content; see, e.g., Dyer, *et al.*, (2002) *Appl. Microbiol. Biotechnol.* 59:224-230 and high oleic acid content; see, e.g.,
10 Fernandez-Moya, *et al.*, (2005) *J. Agric. Food Chem.* 53:5326-5330.

In still further embodiments, the maize E6611.32.1.38 event can also be combined with other desirable traits such as, for example, fumonisin detoxification genes (US Patent Number 5,792,931), avirulence and disease resistance genes (Jones, *et al.*, (1994) *Science* 266:789; Martin, *et al.*, (1993) *Science* 262:1432; Mindrinos, *et al.*, (1994) *Cell* 78:1089) and traits
15 desirable for processing or process products such as modified oils (e.g., fatty acid desaturase genes (US Patent Number 5,952,544; WO 94/11516)); modified starches (e.g., ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (SDBE)); and polymers or bioplastics (e.g., US Patent Number 5,602,321; beta-ketothiolase, polyhydroxybutyrate synthase and acetoacetyl-CoA reductase
20 (Schubert, *et al.*, (1988) *J. Bacteriol.* 170:5837-5847) which facilitate expression of polyhydroxyalkanoates (PHAs)); the disclosures of which are herein incorporated by reference. One could also combine seed production technology polynucleotides with polynucleotides providing agronomic traits such as male sterility (e.g., see, US Patent Number 5,583,210), improved stalk strength, altered flowering time or transformation technology traits such as cell
25 cycle regulation or gene targeting (e.g., WO 99/61619, WO 00/17364 and WO 99/25821); the disclosures of which are herein incorporated by reference.

In another embodiment, the maize E6611.32.1.38 event can also be combined with the Rcg1 sequence or a biologically active variant or fragment thereof. The Rcg1 sequence is an anthracnose stalk rot resistance gene in corn. See, for example, US Patent Application Serial
30 Numbers 11/397,153, 11/397,275 and 11/397,247, each of which is herein incorporated by reference.

These stacked combinations can be created by any method including, but not limited to, breeding plants by any conventional methodology or genetic transformation. If the sequences are stacked by genetically transforming the plants, the polynucleotide sequences of interest
35 can be combined at any time and in any order. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of

transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transformation cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucleotide of interest. This may be combined with any combination of suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855 and WO99/25853, all of which are herein incorporated by reference.

As used herein, the term "polynucleotide" is not intended to be limited to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides can comprise ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The polynucleotides provided herein also encompass all forms of sequences including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures and the like.

Isolated polynucleotides are provided that can be used in various methods for the detection and/or identification of the maize E6611.32.1.38 event. An "isolated" or "purified" polynucleotide or biologically active portion thereof, is substantially or essentially free from components that normally accompany or interact with the polynucleotide as found in its naturally occurring environment. Thus, an isolated or purified polynucleotide is substantially free of other cellular material or culture medium when produced by recombinant techniques or substantially free of chemical precursors or other chemicals when chemically synthesized.

In specific embodiments, polynucleotides are provided that comprise the junction DNA sequences as indicated in SEQ ID NO: 1 and Figures 11 and 15. Fragments and variants of junction DNA sequences are suitable for discriminatively identifying event E6611.32.1.38. As discussed elsewhere herein, such sequences find use as primers and/or probes.

In other embodiments, polynucleotides that can detect a E6611.32.1.38 event or a E6611.32.1.38-specific region are provided. Such sequences include any polynucleotide set forth in SEQ ID NOS: 2-3, 6-19 and variants and fragments thereof. Fragments and variants of polynucleotides that detect a E6611.32.1.38 event or a E6611.32.1.38 specific region are suitable for discriminatively identifying event E6611.32.1.38. As discussed elsewhere herein, such sequences find use as primers and/or probes. Further provided are isolated DNA nucleotide primer or probe sequences comprising or consisting of a sequence set forth in SEQ

ID NO: 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 24, 26, 27, 29, 30, 31, or a complement thereof.

"Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a polynucleotide having deletions (i.e., truncations) at the 5' and/or 3' end; deletion and/or addition of one or more nucleotides at one or more internal sites in the native polynucleotide and/or substitution of one or more nucleotides at one or more sites in the native polynucleotide.

Various methods and compositions for identifying event E6611.32.1.38 are provided. Such methods find use in identifying and/or detecting a E6611.32.1.38 event in any biological material. Such methods include, for example, methods to confirm seed purity and methods for screening seeds in a seed lot for a E6611.32.1.38 event. In one embodiment, a method for identifying event E6611.32.1.38 in a biological sample is provided and comprises contacting the sample with a first and a second primer and, amplifying a polynucleotide comprising a E6611.32.1.38 specific region.

A biological sample can comprise any sample in which one desires to determine if DNA having event E6611.32.1.38 is present. For example, a biological sample can comprise any plant material or material comprising or derived from a plant material such as, but not limited to, food or feed products. In specific embodiments, the biological sample comprises a maize tissue.

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 polynucleotide probes and primers specifically detect a target DNA sequence. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. By "specifically detect" it is intended that the polynucleotide can be used either as a primer to amplify a E6611.32.1.38 specific region or the polynucleotide can be used as a probe that hybridizes under stringent conditions to a polynucleotide having a E6611.32.1.38 event or a E6611.32.1.38 specific region. The level or degree of hybridization which allows for the specific detection of a E6611.32.1.38 event or a specific region of a E6611.32.1.38 event is sufficient to distinguish the polynucleotide with the E6611.32.1.38 specific region from a polynucleotide lacking this region and thereby allow for discriminately identifying a E6611.32.1.38 event. By "shares sufficient sequence identity or complementarity" to allow for the amplification of a E6611.32.1.38-specific event, is intended that the sequence shares at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity or complementarity to a fragment or across the full length of the polynucleotide having the E6611.32.1.38-specific region.

Regarding the amplification of a target polynucleotide (e.g., by PCR) using a particular amplification primer pair, "stringent conditions" are conditions that permit the primer pair to hybridize to the target polynucleotide to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce an identifiable amplification product (the amplicon) having a E6611.32.1.38 specific region in a DNA thermal amplification reaction. In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify a E6611.32.1.38 specific region. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook, *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also, Innis, *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Methods of amplification are further described in US Patent Numbers 4,683,195, 4,683,202 and Chen, *et al.*, (1994) *PNAS* 91:5695-5699. These methods as well as other methods known in the art of DNA amplification may be used in the practice of the inventions. 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 amplified polynucleotide (amplicon) can be of any length that allows for the detection of the E6611.32.1.38 event or a E6611.32.1.38 specific region. For example, the amplicon can be about 10, 50, 100, 200, 300, 500, 700, 100, 2000, 3000, 4000, 5000 nucleotides in length or longer. In specific embodiments, the specific region of the E6611.32.1.38 event is detected.

Any primer can be employed in the methods provided herein that allows a E6611.32.1.38 specific region to be amplified and/or detected. For example, in specific embodiments, a primer comprises a fragment of a polynucleotide of SEQ ID NO: 1, 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 26, 27, 29, or 30 wherein the primer shares sufficient sequence identity or complementarity to the polynucleotide to amplify said E6611.32.1.38 or flanking DNA specific region. A primer pair can comprise a fragment of SEQ ID NO: 1 and a fragment of SEQ ID NO: 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 26, 27, 29, or 30. The primers can be of any length sufficient to amplify a E6611.32.1.38 region including, for example, at least 6, 7, 8, 9, 10, 15, 20, 15 or 30 or about 7-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45 nucleotides or longer.

As discussed elsewhere herein, any method to PCR amplify the E6611.32.1.38 event or specific region can be employed, including for example, real time PCR. See, for example,

Livak, *et al.*, (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system for detecting PCR product and nucleic acid hybridization. PCR methods and Applications. 4:357-362; US Patent Number 5,538,848; US Patent Number 5,723,591; Applied Biosystems User Bulletin No. 2, "Relative Quantitation of Gene Expression," P/N 4303859; and, Applied Biosystems User Bulletin No. 5, "Multiplex PCR with Taqman VIC probes," P/N 4306236; each of which is herein incorporated by reference.

Thus, in specific embodiments, a method of detecting the presence of event E6611.32.1.38 or progeny thereof in a biological sample is provided. The method comprises (a) extracting a DNA sample from the biological sample; (b) providing a pair of DNA primer molecules; (c) providing DNA amplification reaction conditions; (d) performing the DNA amplification reaction, thereby producing a DNA amplicon molecule and (e) detecting the DNA amplicon molecule, wherein the detection of said DNA amplicon molecule in the DNA amplification reaction indicates the presence of maize event E6611.32.1.38. 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.

Further provided are methods of detecting the presence of DNA corresponding to the E6611.32.1.38 event in a sample. In one embodiment, the method comprises (a) contacting the biological sample with a polynucleotide probe that hybridizes under stringent hybridization conditions with DNA from maize event E6611.32.1.38 and specifically detects the E6611.32.1.38 event; (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 E6611.32.1.38 event.

Embodiments are further defined in the following Examples. It should be understood that these Examples are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention and without departing from the spirit and scope thereof, can make various changes and modifications of the embodiments to adapt it to various usages and conditions. Thus, various modifications of the embodiments, in addition to those shown and described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

EXAMPLES

Example 1: Characterization of Event DP-32138-1

Maize (*Zea mays* L.) event DP-32138-1, also referred to 32138 maize, has been produced by *Agrobacterium*-mediated transformation with the plasmid PHP24597 (Figure 1)

essentially as described in Zhao, *et al.*, (2001) *Molecular Biology* (formerly *Molecular Breeding*) 8:323-333; U.S. Patent No. 5,981,840; and PCT patent publication WO98/32326, all of which are hereby incorporated by reference. The T-DNA region of this plasmid is represented schematically in Figure 2 and its sequence is provided in SEQ ID NO: 34. A summary of the genetic elements and their positions on plasmid PHP24597 and on the T-DNA are described in Tables 11 and 12, respectively. Transformation resulted in the insertion of three gene cassettes containing a fertility restoration genomic sequence, *Ms45*, the α -amylase gene, *Zm-aa1* and the *DsRed2* (*Alt1*) gene as a visible marker. These three gene cassettes are elements of a seed production system to create male-sterile lines for the generation of hybrid seed without the need to mechanically or manually detassel plants.

The first cassette contains the *Ms45* Genomic sequence, which is a region of maize genomic DNA comprising the *Ms45* gene and associated 3' untranslated region (Albertsen, *et al.*, 1993; Albertsen, *et al.*, 1995). The *Ms45* gene includes four exons with three introns that are removed by splicing. Expression of the MS45 protein encoded by the *Ms45* gene in the anther tapetum is required for the production of fertile male pollen by the maize plant (Cigan, *et al.*, 2001). The full-length MS45 protein is comprised of 412 amino acids and has a molecular weight of approximately 47 kDa (SEQ ID NO: 20). The expression of the *Ms45* gene is controlled by the maize anther-specific 5126 promoter (Cigan and Albertsen, 1997). The terminator for the *Ms45* gene is the endogenous *Ms45* 3' untranslated region sequence from the maize genome, which is included in the *Ms45* Genomic sequence (Albertsen, *et al.*, 1993).

The second cassette contains a truncated maize α -amylase (*zm-aa1*) gene that encodes the ZM-AA1 protein. The *zm-aa1* coding region is preceded by the transit peptide from the maize *Brittle-1* (*zm-bt1*) gene (Sullivan, *et al.*, 1991) that targets the ZM-AA1 protein to the amyloplast. The ZM-AA1 protein prevents accumulation of starch in the nascent pollen grain, thus preventing the pollen from developing and germinating normally. The complete translation product, including transit peptide, comprises 495 amino acids and has a molecular weight of approximately 54 kDa (SEQ ID NO: 21). The processed ZM-AA1 protein with the transit peptide removed is 420 amino acid residues in length and has a molecular weight of approximately 46 kDa. The processed ZM-AA1 protein differs from the native protein in that it lacks the 21 N-terminal amino acid residues found in the native protein, including the initial methionine residue. The expression of the *zm-aa1* gene and attached transit peptide is controlled by the *Pg47* promoter, which is the 5' regulatory region from the maize pollen-specific polygalacturonase (*Pg47*) gene (Allen and Lonsdale, 1993). The terminator for the *zm-aa1* gene is the 3' terminator sequence from the maize *ln2-1* gene (Hershey and Stoner, 1991).

The third cassette contains the *DsRed2(Alt1)* gene. The *DsRed2(Alt1)* gene is a modified version of the original *DsRed2* gene (*Clontech*, 2001) in which an internal *BstE*

II restriction site was removed without altering the amino acid sequence of the expressed protein. The *DsRed2(Alt1)* coding sequence is provided at SEQ ID NO: 35. The *DsRed2(Alt1)* gene encodes the DsRed2 protein. Expression of the DsRed2 protein in the aleurone layer of the maize seed produces a red coloration in seeds containing the DNA insertion, allowing for differentiation of seed containing event DP-32138-1 during seed sorting. The full-length DsRed2 protein has a length of 225 amino acids and a molecular weight of approximately 26 kDa (SEQ ID NO: 33). The expression of the *DsRed2(Alt1)* gene is controlled by the barley lipid transfer protein (*Ltp2*) promoter, which provides aleurone-specific transcription of the gene (Kalla, *et al.*, 1994). Located 5' to the *Ltp2* promoter is the enhancer region from the cauliflower mosaic virus (CaMV 35S enhancer) (Franck, *et al.*, 1980; Odell, *et al.*, 1985). The terminator for the *DsRed2(Alt1)* gene is the 3' terminator sequence from the proteinase inhibitor II gene of *Solanum tuberosum* (*pinII* terminator) (Keil, *et al.*, 1986; An, *et al.*, 1989).

Southern blot analysis of Event 32138 - Methods

Southern blot analysis was conducted on DP-32138-1 maize to confirm insertion copy number and integrity and to generate a physical restriction enzyme map of the insertion. Genomic DNA samples from individual plants of DP-32138-1 maize were analyzed by digestion with the restriction enzymes *BamH* I, *Bgl* II, *Bmt* I, *EcoR* I, *Hind* III and *Xho* I, and double digestions with *BamH* I/*Hind* III and *Bgl* II/*Hind* III. These digests were hybridized to probes to the *Ms45* Genomic region and the *zm-aa1* and *DsRed2(Alt1)* genes, along with the remaining genetic elements in the T-DNA including the *5126* promoter, *Pg47* promoter, *zm-bt1* transit peptide, *In2-1* terminator, CaMV 35S enhancer, *Ltp2* promoter, and *pinII* terminator. Analysis with *Bmt* I and *Xho* I, examining sites in the bordering genome, indicated a single copy of the PHP24597 T-DNA is present in DP-32138-1 maize. The *EcoR* I analysis indicated that the PHP24597 T-DNA had inserted intact in the genome, as the internal *EcoR* I restriction sites were present. Analysis with the remaining enzymes was used in conjunction with these data to create a physical restriction map of the T-DNA insertion in DP-32138-1 maize.

Test Substances

The test substances in this study were defined as T1S1 red seed of DP-32138-1 maize, PHI log number T-F-07-132C. All seed were obtained from Pioneer Hi-Bred International, Inc. (Pioneer, Johnston, IA) and pedigree information is on file with staff breeders. The test substance seed used in this study were selected from a segregating population by their red color to ensure that they contained the DP-32138-1 event.

Control Substances

The control substances were defined as seed from maize lines that are not genetically modified. The unmodified lines have genetic backgrounds representative of the test substance background; however, they do not contain the DP-32138-1 insertions. All seed were obtained from Pioneer and pedigree information is on file with staff breeders.

PHI Log #	Description
C-F-07-69C	705
PHIS01-70C	Hi-II
C-F-04-98C	Hi-II
C-F-07-131C	Hi-II(<i>ms45</i>) (Hi-II line homozygous for <i>ms45</i> gene)

Reference Substances

Plasmid PHP24597 was used as a positive control for Southern analysis to verify probe hybridization and to confirm the sizes of fragments internal to the inserted T-DNA. The plasmid DNA used was prepared from plasmid stocks obtained from Pioneer. The plasmid stocks were copies of the plasmid used for transformation to produce DP-32138-1 maize and were digested with restriction enzymes to confirm the plasmid map. The probes used in this study were derived from this plasmid or from a plasmid containing equivalent genetic elements.

DNA molecular weight markers for gel electrophoresis and Southern blot analysis were used to determine approximate molecular weights and amounts of DNA fragments. For Southern analysis, DNA Molecular Weight Marker VII, digoxigenin (DIG) labeled (Roche, Indianapolis, IN), was used as a size standard for hybridizing fragments. Φ X174 RF DNA/*Hae* III Fragments were used to determine sufficient migration on the agarose gel for Southern analysis. Low Mass DNA Ladder (Invitrogen, Carlsbad, CA) was used as an approximate quantitation standard.

Sample Collection

Ten seeds each of T-F-07-132C, C-F-07-131C and C-F-07-69C were planted in the Regulatory Science growth chambers at the DuPont Experimental Station (Wilmington, DE) to produce plant tissue for extraction and analysis. One seed was planted per pot, and the pot was uniquely identified with study number, plant identifier, initials of the person who planted the seeds, and planting date. All plants were grown with light, temperature, and water regulated for healthy plant growth.

The first leaf harvest of the plants occurred when the plants were very small (V2–V3 leaf stage). For each of the control and test substance plants, sufficient leaf material from above the growing point was collected and placed directly into Geno/Grinder™ tubes (SPEX

CertiPrep, Inc., Metuchen, NJ) on dry ice. The samples were then transferred to a freezer and were maintained frozen ($<-50^{\circ}\text{C}$) until processing. The second and third leaf collections occurred after the plants had re-grown sufficiently and prior to the R1 stage; the youngest leaf was collected and placed in a pre-labeled, re-sealable bag. The sampling bags were placed directly on dry ice. The samples were then transferred to a freezer and were maintained frozen ($<-50^{\circ}\text{C}$) until processing. Frozen leaf tissue of Hi-II control maize plants was obtained from previous internal studies. All leaf samples were uniquely labeled with the plant identifier, date of harvest, study number, and initials of the harvester.

Event-specific PCR Analysis

DNA was extracted from each leaf sample using the Extract-N-Amp™ Plant PCR kit using the described procedure (Sigma-Aldrich, St. Louis, MO). Real-time PCR was performed on each DNA sample utilizing an ABI PRISM® 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA). TaqMan® probe and primer sets were designed to detect a target sequence from the DP-32138-1 event. In addition, a second TaqMan® probe and primer set for a reference maize endogenous gene was used to confirm the presence of amplifiable DNA in each reaction. The analysis consisted of quantitative real-time PCR determination of qualitative positive/negative calls. The extracted DNA was assayed using optimized and validated primer and probe concentrations in TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Inc.). Initial incubation was at 95°C for 10 minutes followed by 40 cycles as follows: 95°C for 15 seconds, 60°C for 1 minute.

Positive or negative determination for the event was based on comparison of the C_T (threshold cycle) of the insertion target PCR to that of the maize endogenous reference target. If the event and endogenous PCR targets amplified above C_T threshold, then the plant was scored as positive for that event. If the endogenous target amplified and the event target did not, then the plant was scored as negative. If neither target amplified for a particular sample, then it was determined to be a poor quality sample or failed run and the assay was repeated.

DNA Extraction and Quantitation

Genomic DNA was extracted from leaf tissue of test and control plants. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep, Inc.) instrument and the genomic DNA isolated using a standard Urea Extraction Buffer procedure. Following extraction, the DNA was quantified on a spectrofluorometer using Pico Green® reagent (Molecular Probes, Inc., Eugene, OR) and visualized on an agarose gel to confirm values from Pico Green analysis and to determine the DNA quality.

Digestion of DNA and Electrophoretic Separation

For characterization of the DP-32138-1 insertion, genomic DNA samples extracted from both test and control plants were digested with *Bam*H I, *Bgl* II, *Bmt* I, *Eco*R I, *Hind* III and *Xho* I or a combination of two restriction enzymes: *Bam*H I/*Hind* III and *Bgl* II/*Hind* III (New England Biolabs, Ipswich, MA).

Following digestion with the restriction enzyme, the fragments produced were electrophoretically separated by size through an agarose gel and a molecular weight standard [Φ X174 RF DNA/*Hae* III Fragments (Invitrogen)] was used to determine sufficient migration and separation of the fragments on the gel.

Southern Transfer

Agarose gels containing the separated DNA fragments were depurinated, denatured, and neutralized *in situ*, and transferred to a nylon membrane in 20x SSC buffer using the method as described for the TURBOBLOTTER™ Rapid Downward Transfer System (Schleicher and Schuell, Keene, NH). Following transfer to the membrane, the DNA was bound to the membrane by UV crosslinking (Stratalinker, Stratagene, La Jolla, CA).

Probe Labeling and Southern Blot Hybridization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized to a labeled probe. Probes used for Southern hybridization are provided in Table 1 and their locations shown in Figure 2. Probes for the *Ms45* Genomic region, *zm-aa1* gene, *DsRed2(Alt1)* gene, *5126* promoter, *Pg47* promoter, *Ltp2* promoter, *zm-bt1* transit peptide, *In2-1* terminator, *pinII* terminator and CaMV 35S enhancer elements were used for Southern hybridization. A probe containing the *zm-bt1* transit peptide combined with the *zm-aa1* gene (*zm-bt1-aa1* probe) was used for preliminary Southern blot analysis instead of separate probes for the two elements. Fragments of the elements were generated by PCR from plasmid PHP24597 using specific primers and were gel purified (Qiagen, Valencia, CA). With the exception of the *Pg47* promoter, each probe covered the majority of the respective genetic element, with only small regions left out due to requirements imposed by the PCR labeling procedure. DNA probes were generated from these fragments by PCR that incorporated a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, into the fragment. PCR labeling of isolated fragments was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

Labeled probes were hybridized to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG Easy Hyb solution (Roche). DIG labeled DNA Molecular Weight Marker VII (Roche), visible after

DIG detection as described below, was used to determine hybridizing fragment size on the Southern blots.

Detection of Hybridized Probes

5 DIG-labeled probes, hybridized to DNA bound to the nylon membrane after stringent washes, and DIG-labeled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Blots were exposed to X-ray film for one or more time points to detect hybridizing fragments and to visualize molecular weight standards. Images were digitally captured by detection with the
10 Luminescent Image Analyzer LAS-3000 (Fujifilm Medical Systems, Stamford, CT). Digital images were compared to original X-ray film exposures as verification for use in this report. The sizes of detected bands were documented for each digest and each probe.

Stripping of Probes and Subsequent Hybridizations

15 Following hybridization and detection, membranes were stripped of DIG-labeled probe to prepare the blot for subsequent re-hybridization to a different probe. Membranes were rinsed briefly in distilled, de-ionized water and then stripped in a solution of 0.2 M NaOH and 0.1% SDS at 37°C with constant shaking. The membranes were then rinsed in 2x SSC and either used directly for subsequent hybridizations or stored for later use. The alkali-based
20 stripping procedure effectively removes probes labeled with the alkali-labile DIG used in these experiments.

Southern blot analysis of Event 32138 – Results and Discussion

Genotype Confirmation and Preliminary Southern Blot Analysis of T1S1 Maintainer Plants of 25 DP-32138-1

All individual plants were tested for the presence of the DP-32138-1 insertion by event-specific PCR analysis. All ten DP-32138-1 T1S1 plants were positive for the insertion. The results of the event-specific PCR assays for the DP-32138-1 plants are summarized in Table 2. All control plants tested negative for the DP-32138-1 insertion (data not shown). The results of
30 the event-specific PCR analysis substantiate the visual screen of the seeds. Furthermore, these event-specific PCR results also verify that the Left Border of the PHP24597 T-DNA insertion remains stable in plants containing the DP-32138-1 insertion.

Genomic DNA extracted from ten individual DP-32138-1 plants and 21 control plants (ten Hi-II(*ms45*) plants, ten 705 plants, and one Hi-II plant) was analyzed by preliminary
35 Southern blots for the presence of the *Ms45* Genomic, *zm-bt1-aa1* and *DsRed2(Alt1)* genetic elements. DNA from these plants was digested with *EcoR* I or *BamH* I, blotted, and probed

with the *Ms45*, *zm-bt1-aa1* and *DsRed2* probes. Results from these Southern blots correspond directly with the results of the event-specific PCR assays. All DP-32138-1 plants contained the *Ms45* Genomic, *zm-bt1-aa1*, and *DsRed2(Alt1)* genetic elements (Table 2) and the control plants were negative for the genes (data not shown). This preliminary Southern blot analysis also indicated that the inserted DNA was identical in all DP-32138-1 plants, as the banding pattern with each probe was identical for all plants. Because control plant samples within a particular inbred line had differing hybridization patterns, these blots were also used to select controls for the Southern blot analysis described below and confirmed controls selected would account for all bands due to the endogenous genome. Hybridization of the same blots with a probe for the *zm-bt1-aa1* coding sequence resulted in many overlapping endogenous bands that were also seen in the control plants. This probe was split into separate *zm-bt1* and *zm-aa1* probes for use in the detailed characterization of a subset of DNA samples in this study as described below.

Detailed Southern Blot Analysis of DP-32138-1

A total of six restriction enzymes were selected to use in a detailed Southern blot analysis of DP-32138-1 maize: *Bam*H I, *Bgl* II, *Bmt* I, *Eco*R I, *Hind* III and *Xho* I, which are shown on the PHP24597 T-DNA map (Figure 2). In addition, double digests with *Bam*H I/*Hind* III and *Bgl* II/*Hind* III were utilized for Southern analysis. The locations of the *Bam*H I and *Eco*R I restriction sites are shown on the plasmid map of PHP24597 (Figure 1) and the base pair locations of restriction sites for enzymes used in the analysis are given in Table 1. PHP24597 was added to 705 maize control DNA, digested, and included on the blots to provide both a positive control for probe hybridization and a size reference for DNA fragments that are contained completely within the T-DNA. Table 1 and Table 18 provide further information on the probes used.

The schematic map of plasmid PHP24597 (Figure 1) indicates restriction enzyme sites for *Bam*H I and *Eco*R I and the *Ms45*, *zm-aa1* and *DsRed2* coding regions. Left Border and Right Border flank the T-DNA (Figure 2) that is expected to be inserted during *Agrobacterium*-mediated transformation. Plasmid size is 52869 bp. The locations of additional enzymes used in this study are given below:

Enzyme	Locations (bp)
<i>Bgl</i> II	1713, 2519, 7030, 38258, 41055, 42018, 45737, 46167, 49645
<i>Bmt</i> I	8503, 36160, 38359, 39502, 45917
<i>Hind</i> III	179, 34837, 45900, 46809, 47930, 49445
<i>Xho</i> I	2134, 5587, 34814, 36128, 37028, 37074, 37711, 45532

The schematic map of the T-DNA region of Plasmid PHP24597 (Figure 2) indicates restriction enzyme sites for *Bam*H I, *Bgl* II, *Bmt*I, *Eco*R I, *Hind* III and *Xho* I and the *Ms45*, *zm-aa1* and DsRed2 coding and regulatory regions. The locations of enzymes used in this study are given below:

Enzyme	Locations (bp)
<i>Bam</i> H I	1456, 4271, 6773, 8792
<i>Bgl</i> II	1713, 2519, 7030
<i>Bmt</i> I	8503
<i>Eco</i> R I	191, 7407, 9869
<i>Hind</i> III	179
<i>Xho</i> I	2134, 5587

Each restriction digest was hybridized with ten different probes covering the genetic elements within the PHP24597 T-DNA: *Ms45*, *zm-bt1*, *zm-aa1* and DsRed2, and probes for the regulatory regions 5126 promoter, *Pg47* promoter, *In2-1* terminator, 35S enhancer, *Ltp2* promoter, and *pinII* terminator. Detailed descriptions of all probes are provided in Table 1 and the locations of the probes are shown on the T-DNA map (Figure 2). Each of these probes hybridizes to only one element within the T-DNA. The actual number of hybridizing bands for each probe depends on the location of the specific enzyme restriction sites in relation to the genetic element and ranges from one to three bands for a single insertion of the PHP24597 T-DNA with the enzymes used in this study.

Two types of fragments would be observed from these digests and hybridizations: a) border fragments, where an enzyme site is located within the PHP24597 T-DNA at one end of the hybridizing fragment and a second enzyme site is expected in the maize genome and b) internal fragments where known enzyme sites flank the probe region and the fragments are completely contained within the PHP24597 T-DNA. Border fragment sizes are unique for each event and provide a means to demonstrate the number of copies of a particular element based on the number of bands observed. One hybridizing band produced from an enzyme that cleaves once in the insert, outside of the probe region, indicates the presence of one copy of the inserted T-DNA at a single locus in the genome. Border fragments formed from the insertion of a full-length T-DNA are typically larger than the size predicted from the T-DNA sequence due to the inclusion of genomic DNA in the fragment. The exact size of border fragments cannot be predicted in advance due to the unknown location of the cleavage site in

the maize genome. Internal fragments provide a means to assess the integrity of the inserted T-DNA and that it has not been changed from the intended arrangement.

The Southern blot data were used to generate a restriction map of the DP-32138-1 T-DNA insertion (Figure 3). *Bam*H I, *Bgl* II, *Bmt* I, *Eco*R I, *Hind* III and *Xho* I restriction enzyme sites are indicated. Southern blot analysis indicated a single copy of the PHP24597 T-DNA had inserted within the maize genome. Locations of enzyme sites outside the T-DNA region are not to scale. An asterisk (*) indicates that the relative locations of these enzyme sites are uncertain due to the large size of the fragments generated from these sites. Dashed vertical lines indicate the *Bam*H I and *Bgl* II sites located outside the Left Border junction that demonstrated blocked digestion on the Southern blots.

The *Ms45*, *zm-bt1*, *zm-aa1*, 5126 promoter, *Pg47* promoter, and *In2-1* terminator probes used in this study are derived from endogenous maize genomic DNA sequences. As a result, Southern blots with these probes also exhibited a number of bands that were due to hybridization to the endogenous sequences in addition to the bands due to the DP-32138-1 insertion. These bands were confirmed by their presence in one or more of the control plant lines (705, Hi-II, or Hi-II(*ms45*)). The DP-32138-1 generation utilized in this study (T1S1) was derived from the original transformant of the Hi-II(*ms45*) background (Hi-II with a backcrossed *ms45* allele), crossed to 705, and then self-crossed. Thus, the T1S1 plants exhibited endogenous bands derived from both the Hi-II and 705 control lines, which varied between T1S1 individuals depending on the digest and probe used. In addition, some of the hybridizing bands showed differences between plants within the Hi-II and Hi-II(*ms45*) control lines themselves, thus requiring the inclusion of two plants from each of these control lines on some of the Southern blots in order to account for all of the endogenous bands observed in the DP-32138-1 plants.

With the exception of the *Pg47* promoter, each probe covers the majority of the respective genetic element, with only small regions left out due to requirements imposed by the PCR labeling procedure. In the case of the *Pg47* promoter, test hybridizations with probe fragments encompassing the approximately 2.7 kb element resulted in intense hybridization to all lanes containing genomic DNA. The full-length *Pg47* promoter probe tested but not used in the study, as described herein, is shown as a solid line below the *Pg47* promoter element in the map of Figure 2. Further investigation yielded a 424 bp fragment located at the 3' end of the *Pg47* promoter that hybridized to a reduced number of endogenous bands and thus provided usable information about this element in DP-32138-1 maize.

Copy Number of Inserted DNA in DP-32138-1 Maize

The restriction enzymes *Bmt* I and *Xho* I were selected to confirm the number of copies of the PHP24597 T-DNA inserted in DP-32138-1 maize.

Bmt I has a single restriction site located within the T-DNA and will result in border fragments at the Right and Left Borders for each single insertion of the T-DNA depending on the probe used (Figure 2). The site for *Bmt* I is located at bp 8503 within the T-DNA, and would be expected to yield fragments of greater than about 8500 bp and greater than about 1400 bp for a single inserted T-DNA. The fragment of greater than 8500 bp will hybridize to the 5126 promoter, *Ms45*, *Pg47* promoter, *zm-bt1*, *zm-aa1*, *In2-1* terminator, 35S enhancer, and *Ltp2* promoter probes, while the fragment of greater than 1400 bp will hybridize to the *Ltp2* promoter, DsRed2, and *pinII* terminator probes. Since the *Bmt* I site is located within the *Ltp2* promoter element, hybridizing with this probe would allow both fragments to be visible simultaneously on a Southern blot.

In the *Bmt* I Southern analysis, a band of greater than 8600 bp hybridized in DP-32138-1 maize with the 5126 promoter, *Ms45*, *zm-bt1*, *zm-aa1*, *In2-1* terminator, 35S enhancer, and *Ltp2* promoter probes (Table 3). In addition, endogenous bands were observed in all samples with the 5126 promoter, *Ms45*, *zm-bt1*, *zm-aa1*, and *In2-1* terminator probes. A band of greater than 8600 bp was also observed with the *Ltp2* promoter, DsRed2, and *pinII* terminator probes (Table 3), which can be identified as the larger of the two bands seen with the *Ltp2* promoter probe by comparison with the *pinII* terminator and 5126 promoter hybridizations. No specific band can be identified with the *Pg47* promoter on the *Bmt* I blot due to the intensity of the co-migrating endogenous bands. However, the presence of the greater than 8600 bp band with the probes flanking the *Pg47* promoter element (*Ms45* and *zm-bt1*) indicates the *Pg47* promoter band is likely to be located in the region of the blot obscured by endogenous bands above the 8600 bp marker. Furthermore, based on the *Xho* I analysis discussed below, a single copy of the *Pg47* element was determined to be inserted in DP-32138-1 maize.

Xho I has two restriction sites located within the PHP24597 T-DNA (Figure 2), and would be expected to produce one internal fragment and two border fragments at the Right and Left Borders for each single insertion. The two restriction sites are located at bp positions 2134 and 5587 (Figure 2). Digestion with this enzyme will produce three fragments from a single T-DNA insertion: a border fragment of greater than about 2100 bp that hybridizes to the 5126 promoter and *Ms45* probes, an internal fragment of 3453 bp that hybridizes to the *Ms45*, *Pg47* promoter, and *zm-bt1* probes, and a second border fragment of greater than about 4400 bp that hybridizes to the *zm-bt1*, *zm-aa1*, *In2-1* terminator, 35S enhancer, *Ltp2* promoter, DsRed2 and *pinII* terminator probes.

Hybridization of *Xho* I-digested DNA from DP-32138-1 maize with the 5126 promoter probe resulted in a single band of about 3600 bp from the T-DNA insertion, in addition to two bands resulting from endogenous maize sequences (Table 4). Hybridization to the *Ms45* probe resulted in two bands as expected: the approximately 3600 bp border band also seen with the 5126 promoter probe and an internal band matching the 3453 bp plasmid fragment (Table 4). The same 3453 bp internal fragment was observed with the *Pg47* promoter probe (Table 4). The PHP24597 plasmid band is weak in many of the hybridizations, but can be observed on the films of the Southern blots. The *zm-bt1* probe showed two insert-derived bands as expected from the location of the *Xho* I site within the element: the 3453 internal band seen with the *Ms45* and *Pg47* promoter probes and a border band of greater than 8600 bp (Table 4). Endogenous bands were again observed with the *Ms45*, *Pg47* promoter, and *zm-bt1* probes. The greater than 8600 bp border band was also seen in hybridizations with the *In2-1* terminator (Table 4), 35S enhancer and *Ltp2* promoter (Table 4), and DsRed2, and *pinII* terminator (Table 4) probes. Endogenous bands were observed with the *In2-1* terminator probe. No extra insert-derived bands were seen with any of these probes. Hybridization of the *Xho* I-digested DNA with the *zm-aa1* probe did not provide any information about the DP-32138-1 insertion, as the region in which the expected greater than 8600 bp band is located, as shown by the flanking *zm-bt1* and *In2-1* probes, was obscured by intense endogenous bands. However, since the *zm-bt1* and *In2-1* probes hybridized to the same greater than 8600 bp band that was observed with the probes of the DsRed2 cassette and, as described above in the *Bmt* I analysis, a single band hybridized to the *zm-aa1* probe, a single copy of the *zm-aa1* gene was determined to be inserted in DP-32138-1 maize.

Therefore, Southern analysis of DP-32138-1 with *Bmt* I and *Xho* I digestion demonstrates that there is a single copy of the T-DNA insertion within the maize genome. Except for those elements in which the restriction site is located within the element, there are only single bands for each of the genetic elements, as expected for fragments from a single copy insertion. The elements in the T-DNA that contain the restriction sites also show the expected number of bands, based on the restriction enzyme location, for a single copy insertion. The presence of single border bands, and no extra insert-derived bands, demonstrates that there is only a single copy of the PHP24597 T-DNA within the DP-32138-1 maize genome.

Integrity of Inserted DNA in DP-32138-1 Maize

The restriction enzyme *EcoR* I was used to confirm the integrity of the PHP24597 T-DNA insertion. This enzyme has three restriction sites within the PHP24597 T-DNA: one site located between the Right Border and the 5126 promoter element at bp position 191, one site

between the *pinII* terminator region and the Left Border at bp position 9869, and a third site located between the *In2-1* terminator and the CaMV 35S enhancer element at bp position 7407 (Figure 2). Digestion with *EcoR* I should produce two internal fragments, of 7216 bp and 2462 bp, from both the PHP24597 plasmid control and plants containing an intact T-DNA insertion.

5 The band observed will depend on the probe used on a given Southern blot. Hybridization with the 5126 promoter, *Ms45*, *Pg47* promoter, *zm-bt1*, *zm-aa1*, and *In2-1* terminator probes will result in the 7216 bp band, while the 35S enhancer, *Ltp2* promoter, DsRed2, and *pinII* terminator probes will hybridize to the 2462 bp band. The presence of the appropriate band with a given probe and absence of any other insert-derived band for that probe provides a
10 strong indication that the T-DNA is complete and was not truncated upon insertion.

Hybridization of the *EcoR* I digested DNA with the 5126 promoter and *Ms45*, probes resulted in a single insert-derived band of 7216 bp that matched the internal plasmid band (Table 5). The same band of 7216 bp was observed with the *Pg47* promoter and *zm-bt1* probes (Table 5) and the *zm-aa1* and *In2-1* terminator probes (Table 5). The 7216 bp band
15 was somewhat obscured on the *zm-aa1* blot by the presence of hybridizing bands due to the maize endogenous background and could not be confirmed. This element was determined to be inserted intact based on analysis discussed below with *BamH* I and *Bgl* II, because digestion with these enzymes released an internal fragment from the T-DNA of the appropriate size. Furthermore, each of these analyses showed only the expected internal fragment and no
20 other bands due to the DP-32138-1 insertion. In addition, there were a number of bands due to hybridization of endogenous maize sequences with the 5126 promoter, *Ms45*, *Pg47* promoter, *zm-bt1*, *zm-aa1*, and *In2-1* terminator probes, that are indicated in Table 5 by an asterisk (*) and gray shading. The 7216 bp band was observed to have run on the gel at an apparent molecular size slightly below the expected size when hybridized to these probes in both the
25 plasmid lanes and the DP-32138-1 maize lanes. This anomalous size appears to be specific to this fragment, as the 2462 bp *EcoR* I fragment ran at the expected size in comparison to the molecular weight markers. There may be a shift in electrophoretic mobility of the 7216 bp fragment due to the presence of the control maize DNA sample causing it to exhibit an apparent size on the gel somewhat smaller than the actual size. The insert-derived band of
30 2462 bp was observed with the 35S enhancer and *Ltp2* promoter probes (Table 5) and the DsRed2 and *pinII* terminator probes (Table 5). No other bands specific to the DP-32138-1 plants were observed in these Southern blots. Table 5 summarizes the expected and observed bands on the *EcoR* I blots.

The *EcoR* I blots demonstrate that the PHP24597 T-DNA in DP-32138-1 is intact, as
35 only the expected two bands of 7216 bp and 2462 bp are observed with their respective probes. The two fragments comprise the entire PHP24597 T-DNA with the exception of the

small regions between the *EcoR* I sites near the ends of the T-DNA and the Left and Right Border elements. The presence of the only two expected bands, and the absence of other bands specific to the DP-32138-1 plants, indicates that the PHP24597 T-DNA inserted intact in the maize genome.

5

Physical Map of Inserted DNA in DP-32138-1 Maize

Three additional restriction enzymes were chosen to provide a complete analysis of the DP-32138-1 insertion: *Hind* III, *Bam*H I and *Bgl* II. Digests with each of these enzymes were hybridized to the ten probes used on the preceding blots and the information derived from these hybridizations was combined with the data described above to create a detailed restriction map of the T-DNA insertion and the surrounding maize genomic DNA in DP-32138-1 (Figure 3). Additional double digestions were carried out with *Bam*H I/*Hind* III and *Bgl* II/*Hind* III to clarify parts of the insertion as seen with the *Bam*H I and *Bgl* II single digests.

There is one *Hind* III site in the PHP24597 T-DNA, located between the Right Border and the 5126 promoter at bp 179 (Figure 2). All of the probes used in this study would hybridize to a single border fragment of greater than about 9800 bp (Table 6).

Hybridization of *Hind* III digested DNA from DP-32138-1 with the probes used in this study yielded a single insert-derived band of greater than 8600 bp, with the exception of the *Pg47* promoter probe (Table 6). In addition to the insert-derived bands, endogenous hybridization was observed in the *Hind* III digests with the 5126 promoter, *Ms45*, *Pg47* promoter, *zm-bt1*, *zm-aa1*, and *ln2-1* terminator probes, that are indicated in Table 6 by an asterisk (*) and gray shading. In the case of the *Pg47* promoter probe, the insert-derived band was obscured by a series of very intense endogenous bands above the 8600 bp molecular weight marker. However, as there were no other bands present in the DP-32138-1 plants that could be determined to be from the insert, and the flanking *Ms45* and *zm-bt1* probes both hybridized to the same greater than 8600 bp band, it is likely that the *Pg47* promoter probe band is indeed of the size expected from the other hybridizations but is merely masked by the endogenous bands. As discussed above for the *Xho* I and *EcoR* I analysis with the *Pg47* probe, a single intact copy of this element was determined to be inserted in DP-32138-1 maize. As the upper molecular weight marker is 8600 bp, and the *Hind* III band is located above the marker band, the exact size of the *Hind* III band cannot be determined, although it appears large enough to exceed its minimum expected size of 9800 bp. Additional data, as discussed below in the *Hind* III double digestions, show that the *Hind* III site in the genome is located approximately 1200 to 1400 bp away from the Left Border of the DP-32138-1 insertion providing evidence that the insertion is intact from base pair position 179.

There are four restriction sites for *Bam*H I within the PHP24597 T-DNA, located at bp positions 1456, 4271, 6773 and 8792 (Figure 2). Digestion with this enzyme will result in five expected fragments for a single T-DNA insertion: a border fragment of greater than about 1500 bp that hybridizes to the 5126 promoter and *Ms45* probes, an internal fragment of 2815 bp that hybridizes to the *Ms45* probe, an internal fragment of 2502 bp that hybridizes to the *Pg47* promoter, *zm-bt1* and *zm-aa1* probes, a third internal fragment of 2019 bp that would hybridize to the *zm-aa1*, *In2-1*, 35S enhancer and *Ltp2* promoter probes and a second border fragment of greater than about 1200 bp that hybridizes to the DsRed2 and *pinII* terminator probes (Table 7). Although the *Bam*H I site at bp location 4271 is located within the *Pg47* promoter element, and thus a full *Pg47* promoter probe would hybridize to both the 2815 and 2502 bp fragments, only the 2502 bp fragment would be seen with the 424 bp *Pg47* promoter probe that was used in this study due to its location at the 3' end of the element (Table 7).

A band of about 3800 bp was observed upon hybridization of *Bam*H I-digested DNA from DP-32138-1 with the 5126 promoter and *Ms45* probes (Table 7). The expected 2815 bp internal band was also observed with the *Ms45* probe (Table 7). Hybridization with the *Pg47* promoter, *zm-bt1* and *zm-aa1* probes resulted in a band of 2502 bp that matched the plasmid control band (Table 7). The *zm-aa1* probe also hybridized to an internal band of 2019 bp that matched the plasmid band, as did the *In2-1* terminator, 35S enhancer, and *Ltp2* promoter probes (Table 7). Endogenous bands were observed with all probes in the *Ms45* and *zm-aa1* cassettes. Two border bands were observed with the DsRed2 and *pinII* terminator probes: a strongly hybridizing band of about 4600 bp and a very faint band of about 6100 bp (Table 7). Table 7 gives the expected and observed bands seen with this digest. In order to investigate the possibility that the faint 6100 bp band resulted from blocked digestion of the genomic *Bam*H I site that resulted in the 4600 bp band, a double digestion with *Bam*H I/*Hind* III was performed as described below.

To demonstrate that the faint second band of about 6100 bp seen with the *Bam*H I digest and the DsRed2 and *pinII* terminator probes was due to blocked digestion at the *Bam*H I site in the maize genome, a double digest was performed with *Bam*H I and *Hind* III. *Hind* III was selected due to the location of its restriction site in the maize genome close to the Left Border junction of the DP-32138-1 insertion as described above. Digestion with these two enzymes should yield a border fragment of greater than 1200 bp from the *Bam*H I site located at bp 8792 in the T-DNA and the *Hind* III site in the maize genome. As the *Hind* III site appears to be located closer to the Left Border than the genomic *Bam*H I site, digestion with both enzymes should release a single, unique fragment hybridizing to the DsRed2 and *pinII* terminator probes from a single T-DNA insertion. The presence of only a single band with the double digest would provide evidence that the faint second band of about 6100 bp on the

*Bam*H I Southern blot did result from blocked cleavage at the genomic *Bam*H I site responsible for the approximately 4600 bp band. However, if two bands are again observed with the double digestion and these two probes, it would be evidence that there is more than one copy of the elements.

Hybridization of *Bam*H I/*Hind* III digested maize DNA with the DsRed2 and *pin*II terminator probes resulted in a single band of about 2600 bp in the DP-32138-1 plants and no other bands (Table 8). This 2600 bp fragment is smaller than the approximately 4600 bp fragment observed in the *Bam*H I analysis and, thus, the location of the genomic *Hind* III site can be estimated to be approximately 1400 bp away from the Left Border junction and is located between the Left Border and the genomic *Bam*H I site. The lack of any other bands with the double digestion demonstrates that there is only one copy of this region of the PHP24597 T-DNA in the DP-32138-1 genome. Furthermore, this indicates the faint band of about 6100 bp seen with the *Bam*H I digest is indeed due to blocked cutting by this enzyme at the restriction site located closest to the Left Border junction in the maize genome. One possible explanation for the blocked cleavage at this *Bam*H I site is methylation of the restriction site or adjoining DNA sequence that results in lowered efficiency of digestion by *Bam*H I (Brown, 1998). Hybridizations of the *Bam*H I/*Hind* III digested maize DNA to the remaining probes used to characterize DP-32138-1 maize all showed the expected bands based on the PHP24597 T-DNA map.

Bgl II has three restriction sites within the PHP24597 T-DNA, located at bp positions 1713, 2519 and 7030 (Figure 2). Digestion of the inserted T-DNA with *Bgl* II will yield four fragments: a border fragment of greater than about 1700 bp hybridizing to the 5126 promoter and *Ms45* probes, an internal fragment of 806 bp that hybridizes to the *Ms45* probe, another internal fragment of 4511 bp hybridizing to the *Ms45*, *Pg47* promoter, *zm-bt1* and *zm-aa1* probes and a third border fragment of greater than about 2900 bp that hybridizes to the *In2-1* terminator, 35S enhancer, *Ltp2* promoter, DsRed2 and *pin*II terminator probes (Figure 2).

All of the four bands expected with the various probes and *Bgl* II digested DNA from DP-32138-1 were seen on the Southern blots. The 5126 promoter and *Ms45* probes both hybridized to a border band of about 3600 bp (Table 9). The *Ms45* probe also hybridized to two internal bands of 806 bp and 4511 bp that correspond to the plasmid control bands (Table 9). The 4511 bp band is faint due to the small overlap of about 140 bp with the *Ms45* probe, while the 806 bp band overlaps an endogenous band of about 800 bp, but can be detected by the increased intensity of the band compared to the control plant lanes. The 4511 bp internal band that matched the plasmid band was also detected using the *Pg47* promoter, *zm-bt1* and *zm-aa1* probes (Table 9). The *In2-1* terminator probe detected a strongly hybridizing border band of about 4900 bp (Table 9). As with the other digests, a number of endogenous bands of

varying intensities were detected with all the probes of the *Ms45* and *zm-aa1* cassettes. Hybridization with the 35S enhancer, *Ltp2* promoter, DsRed2, and *pinII* terminator probes resulted in the detection of two bands: a strongly hybridizing border band of about 4900 bp and a much fainter band of about 7400 bp (Table 9). It is likely that the 7400 bp band resulted from blocked digestion of the maize genomic DNA with *Bgl* II at the site that should result in the 4900 bp border band, and a double digestion with *Bgl* II/*Hind* III was performed as described below to test this hypothesis. Based on the map of the PHP24597 T-DNA (Figure 2), it is likely that the 7400 bp band would also be detected with the *In2-1* terminator probe, but it was not observed due to the weak hybridization to this band and the presence of endogenous bands in that region of the blot.

Similar to the *Bam*H I/*Hind* III analysis described above, a double digest was performed with *Bgl* II and *Hind* III to demonstrate that the faint second band of about 7400 bp seen with the *Bgl* II digest and the 35S enhancer, *Ltp2* promoter, DsRed2 and *pinII* terminator probes was due to blocked digestion at the *Bgl* II site in the maize genome. Digestion with these two enzymes and hybridization with the 35S enhancer, *Ltp2* promoter, DsRed2 and *pinII* terminator probes should yield a border fragment of greater than 2900 bp from the *Bgl* II site located at bp 7030 and the *Hind* III site in the maize genome. As the *Hind* III site appears to be located closer to the Left Border than the genomic *Bgl* II site, digestion with both enzymes should release a single, unique fragment hybridizing to these probes from a single T-DNA insertion. The presence of only a single band with the double digest would provide evidence that the faint second band of about 7400 bp on the *Bgl* II Southern blot did result from blocked cleavage at the genomic *Bgl* II site responsible for the approximately 4900 bp band. However, if two bands are again observed with the double digestion and these two probes, it would be evidence that there is more than one copy of the elements.

Hybridization of *Bgl* II/*Hind* III digested maize DNA with the 35S enhancer, *Ltp2* promoter, DsRed2 and *pinII* terminator probes resulted in a single band of about 4100 bp in the DP-32138-1 plants (Table 10). This 4100 bp fragment is smaller than the approximately 4900 bp fragment observed in the *Bgl* II analysis and positions the genomic *Hind* III site between the Left Border junction and the *Bgl* II site, at approximately 1200 bp from the border. Additionally, the lack of any other insertion-related bands with the double digestion demonstrates that there is only one copy of this region of the PHP24597 T-DNA in the DP-32138-1 genome, and that the faint band of about 7400 bp seen with the *Bgl* II digest alone is likely due to blocked cutting by this enzyme at the site in the maize genome located closest to the Left Border junction. One possible explanation for the blocked digestion at this *Bgl* II site is methylation of the restriction site or adjoining DNA sequence that results in lowered efficiency of digestion by *Bgl* II (Brown, 1998). Hybridizations of the *Bgl* II/*Hind* III digested maize DNA to the remaining

probes used to characterize DP-32138-1 maize all showed the expected bands based on the PHP24597 T-DNA map. The difference between the location of the genomic Hind III restriction site determined with the *Bgl* II/*Hind* III Southern blot (1200 bp from the Left Border) and the *Bam*H I/*Hind* III analysis (1400 bp from the Left Border) is likely due to electrophoretic mobility shifts between Southern blots and the inherent inaccuracy of band size approximation based on the DIG VII markers used in this study.

The information obtained from the *Hind* III, *Bam*H I, *Bgl* II, *Bam*H I/*Hind* III, and *Bgl* II/*Hind* III digests was combined with the data derived from the *Bmt* I, *Xho* I, and *Eco*R I blots to generate a restriction map of the T-DNA insertion and the surrounding maize genome (Figure 3). The digests and hybridizations are consistent with the presence of a single, intact PHP24597 T-DNA in the genome of DP-32138-1 maize.

Example 1 Conclusions

Southern blot analysis was conducted on DP-32138-1 maize to confirm insertion copy number and integrity and to generate a physical restriction enzyme map of the insertion. Genomic DNA samples from individual plants of DP-32138-1 maize were analyzed by digestion with the restriction enzymes *Bam*H I, *Bgl* II, *Bmt* I, *Eco*R I, *Hind* III, and *Xho* I and double digestions with *Bam*H I/*Hind* III and *Bgl* II/*Hind* III. These digests were hybridized to probes to the *Ms45* Genomic region and the *zm-aa1* and *DsRed2(Alt1)* genes, along with the remaining genetic elements in the T-DNA including the *5126* promoter, *Pg47* promoter, *zm-bt1* transit peptide, *In2-1* terminator, CaMV 35S enhancer, *Ltp2* promoter, and *pinII* terminator. Analysis with *Bmt* I and *Xho* I, examining sites in the bordering genome, indicated a single intact copy of the PHP24597 T-DNA is present in DP-32138-1 maize. The *Eco*R I analysis indicated that the PHP24597 T-DNA had inserted intact in the genome, as the internal *Eco*R I restriction sites were present. Analysis with the remaining enzymes was used in conjunction with these data to create a physical restriction map of the T-DNA insertion in DP-32138-1 maize.

Table 1: Description of DNA Probes Used for Southern Hybridization

Probe Name	Probe Lot#	Genetic Element	Probe Position on PHP24597 T-DNA (bp to bp) ¹	Probe Position on PHP24597 Plasmid (bp to bp) ²	Probe Length (bp)
5126 promoter	08-DP-5	5126 promoter	304-706	304-706	403
<i>Ms45</i> ³	08-DP-29	<i>Ms45</i> Genomic region	707-1426	707-1426	720
	07-DP-29		1427-2258	1427-2258	832
	08-DP-33		2263-2658	2263-2658	396
<i>Pg47</i> promoter	08-DP-34	<i>Pg47</i> promoter (3' region)	5028-5451	5028-5451	424
<i>zm-bt1</i>	08-DP-12	<i>zm-bt1</i> transit peptide	5469-5693	5469-5693	225
<i>zm-aa1</i> ⁴	08-DP-32	<i>zm-aa1</i> gene	5701-6333	5701-6333	603
	08-DP-7		6334-6935	6334-6935	602
<i>zm-bt1-aa1</i> ⁵	07-DP-34	<i>zm-bt1</i> transit peptide + <i>zm-aa1</i> gene	5469-6333	5469-6333	865
	08-DP-7		6334-6935	6334-6935	602
<i>In2-1</i> terminator	07-DP-33	<i>In2-1</i> terminator	7049-7377	7049-7377	329
35S enhancer	08-DP-8	CaMV 35S enhancer region	7427-7846	7427-7846	420
<i>Ltp2</i> promoter	08-DP-16	<i>Ltp2</i> promoter	7906-8759	7906-8759	854
DsRed2	08-DP-11	<i>DsRed2(Alt1)</i> gene	8810-9487	8810-9487	678
<i>pinII</i> terminator	08-DP-6	<i>pinII</i> terminator	9582-9815	9582-9815	234

1. The probe position is based on the PHP24597 T-DNA map (Figure 2).

2. The probe position is based on the PHP24597 plasmid map (Figure 1).

5 3. The *Ms45* probe is comprised of three non-overlapping labeled fragments that are combined in the hybridization solution.

4. The *zm-aa1* probe is comprised of two non-overlapping labeled fragments that are combined in the hybridization solution.

10 5. The *zm-bt1-aa1* probe is comprised of two non-overlapping labeled fragments that are combined in the hybridization solution. This probe was used for the preliminary Southern blot analysis only.

Table 2: Results of Event-Specific Polymerase Chain Reaction Analysis and Preliminary Southern Blot Analysis of Plants Grown from Test Substance Seed

Plant ID Number	Sample ID (Event/ Sample #)	Polymerase Chain	Preliminary Southern Blot	Preliminary Southern Blot	Preliminary Southern Blot
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		Reaction Assay Results for Event DP-32138-1¹	Results with <i>Ms45</i> Probe²	Results with <i>zm-bt1-aa1</i> Probe²	Results with <i>DsRed2</i> Probe²
T-F-07-132C-1	DP-32138-1/T1	Positive	Positive	Positive	Positive
T-F-07-132C-2	DP-32138-1/T2	Positive	Positive	Positive	Positive
T-F-07-132C-3	DP-32138-1/T3	Positive	Positive	Positive	Positive
T-F-07-132C-4	DP-32138-1/T4	Positive	Positive	Positive	Positive
T-F-07-132C-5	DP-32138-1/T5	Positive	Positive	Positive	Positive
T-F-07-132C-6	DP-32138-1/T6	Positive	Positive	Positive	Positive
T-F-07-132C-7	DP-32138-1/T7	Positive	Positive	Positive	Positive
T-F-07-132C-8	DP-32138-1/T8	Positive	Positive	Positive	Positive
T-F-07-132C-9	DP-32138-1/T9	Positive	Positive	Positive	Positive
T-F-07-132C-10	DP-32138-1/T10	Positive	Positive	Positive	Positive

1. A positive result indicates the amplification above a C_T threshold of the DP-32138-1 target sequence in the plant. A negative result indicates no amplification above the C_T threshold of the DP-32138-1 target sequence. A positive or negative determination indicates the maize endogenous reference in the plant was amplified.
2. A positive result indicates the presence of the band for *Ms45*, *zm-bt1-aa1*, or *DsRed2(Alt1)* on the Southern blot, while a negative result indicates the absence of the appropriate band.

5

Table 3: Predicted and Observed Hybridizing Bands on Southern Blots; *Bmt* I Digest

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences, as can be determined by the presence of the same band in DP-32138-1 and control plants.

Probe	Figure	Predicted Fragment Size from PHP24597 T-DNA ¹ (bp)	Predicted Fragment Size from Plasmid PHP24597 ² (bp)	Observed Fragment Size in DP-32138-1 ³ (bp)
5126 promoter	5	>8500 (border) ⁴	15455	>8600 ~7800* ~2700*
<i>Ms45</i>	5	>8500 (border)	15455	>8600 ~5200*
<i>Pg47</i> promoter	6	>8500 (border)	15455	Obscured by endogenous bands 3 bands >8600* 2 bands 6100 – 8600* 2 bands 4900 – 6100* 2 bands 3600 – 4900*
<i>zm-bt1</i>	6	>8500 (border)	15455	>8600 ~4200*
<i>zm-aa1</i>	7	>8500 (border)	15455	>8600 3 bands >8600* 3 bands 6100 – 8600* 2 bands 4900 – 6100* 2 bands 3600 – 4900* ~3000* ~2700* ~2500* ⁵ ~1500*
<i>In2-1</i> terminator	7	>8500 (border)	15455	>8600 ~8600* ~8000* ~4700* ~3200*
35S enhancer	8	>8500 (border)	15455	>8600
<i>Ltp2</i> promoter	8	>8500 (border) >1400 (border)	15455 27657	>8600 >8600
DsRed2	9	>1400 (border)	27657	>8600
<i>pinII</i> terminator	9	>1400 (border)	27657	>8600

1. Predicted size for probe hybridization to genomic DNA of DP-32138-1 maize containing a complete T-DNA insertion based on the map of the T-DNA from PHP24597 (Figure 2). Border fragment sizes are rounded to the nearest 100 bp.
2. Predicted size for probe hybridization to plasmid PHP24597 based on the plasmid map of PHP24597 (Figure 1).
3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion.
5. The ~2500 bp endogenous band is seen in only one of the DP-32138-1 plants and the Hi-II control plants.

Table 4: Predicted and Observed Hybridizing Bands on Southern Blots; *Xho I* Digest

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences, as can be determined by the presence of the same band in DP-32138-1 and control plants.

Probe	Figure	Predicted Fragment Size from PHP24597 T-DNA ¹ (bp)	Predicted Fragment Size from Plasmid PHP24597 ² (bp)	Observed Fragment Size in DP-32138-1 ³ (bp)
5126 promoter	10	>2100 (border) ⁴	9471	~3600 ~8600* ~3200*
<i>Ms45</i>	10	>2100 3453	9471 3453	~3600 3453 ⁵ ~8600* ~6500* ~600*
<i>Pg47</i> promoter	11	3453	3453	3453 ⁵ 4 bands >8600* 3 bands 6100 – 8600* ~4100*
<i>zm-bt1</i>	11	3453 >4400 (border)	3453 29227	3453 ⁵ >8600 ~8000* ~6700*
<i>zm-aa1</i>	12	>4400 (border)	29227	Obscured by endogenous bands 3 bands >8600* 3 bands 6100 – 8600* 2 bands 4900 – 6100* 3 bands 3600 – 4900* 2 bands 2800 – 3600* 5 bands 1950 – 2800* 4 bands 1200 – 1950* ~800*
<i>In2-1</i> terminator	12	>4400 (border)	29227	>8600 3 bands >8600* 2 bands 6100 – 8600* ~3800* ~3000*
35S enhancer	13	>4400 (border)	29227	>8600
<i>Ltp2</i> promoter	13	>4400 (border)	29227	>8600
DsRed2	14	>4400 (border)	29227	>8600
<i>pinII</i> terminator	14	>4400 (border)	29227	>8600

1. Predicted size for probe hybridization to genomic DNA of DP-32138-1 maize containing a complete T-DNA insertion based on the map of the T-DNA from PHP24597 (Figure 2). Border fragment sizes are rounded to the nearest 100 bp.
2. Predicted size for probe hybridization to plasmid PHP24597 based on the plasmid map of PHP24597 (Figure 1).
3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion.
5. Observed size is same as expected size due to equivalent migration to the plasmid band.

Table 5: Predicted and Observed Hybridizing Bands on Southern Blots; *EcoR* I Digest

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences, as can be determined by the presence of the same band in DP-32138-1 and control plants.

Probe	Figure	Predicted Fragment Size from PHP24597 T-DNA ¹ (bp)	Predicted Fragment Size from Plasmid PHP24597 ² (bp)	Observed Fragment Size in DP-32138-1 ³ (bp)
5126 promoter	15	7216	7216	7216 ⁴ 2 bands >8600*
<i>Ms45</i>	15	7216	7216	7216 ⁴ >8600*
<i>Pg47</i> promoter	16	7216	7216	7216 ⁴ 3 bands >8600* ~7400* ~5600*
<i>zm-bt1</i>	16	7216	7216	7216 ⁴ >8600* ~8600* ~3300*
<i>zm-aa1</i>	17	7216	7216	7216 ⁴ 2 bands >8600* 2 bands 4900 – 8600* 4 bands 3600 – 4900* ~4300* ⁵ 2 bands 2800 – 3600* 4 bands 1950 – 2800* ~1700*
<i>In2-1</i> terminator	17	7216	7216	7216 ⁴ 2 bands >8600* ~8600* ~7400* ~3400*
35S enhancer	18	2462	2462	2462 ⁴
<i>Ltp2</i> promoter	18	2462	2462	2462 ⁴
DsRed2	19	2462	2462	2462 ⁴
<i>pinII</i> terminator	19	2462	2462	2462 ⁴

1. Predicted size for probe hybridization to genomic DNA of DP-32138-1 maize containing a complete T-DNA insertion based on the map of the T-DNA from PHP24597 (Figure 2). Border fragment sizes are rounded to the nearest 100 bp.
2. Predicted size for probe hybridization to plasmid PHP24597 based on the plasmid map of PHP24597 (Figure 1).
3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Observed size is same as expected size due to equivalent migration to the plasmid band.
5. The ~4300 bp endogenous band is seen in only one of the DP-32138-1 plants and one of the Hi-II control plants.

Table 6: Predicted and Observed Hybridizing Bands on Southern Blots; Hind III Digest

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences, as can be determined by the presence of the same band in DP-32138-1 and control plants.

Probe	Figure	Predicted Fragment Size from PHP24597 T-DNA ¹ (bp)	Predicted Fragment Size from Plasmid PHP24597 ² (bp)	Observed Fragment Size in DP-32138-1 ³ (bp)
5126 promoter	20	>9800 (border) ⁴	34658	>8600 ⁵ ~5900* ~4900*
<i>Ms45</i>	20	>9800 (border) ⁴	34658	>8600 ⁵ ~5400*
<i>Pg47</i> promoter	21	>9800 (border) ⁴	34658	Obscured by endogenous bands 4 bands >8600* 2 bands 6100 – 8600* ~4800* ~2100*
<i>zm-bt1</i>	21	>9800 (border) ⁴	34658	>8600 ⁵ ~8300*
<i>zm-aa1</i>	22	>9800 (border) ⁴	34658	>8600 ⁵ >8600* ⁶ >8600* 3 bands 6100 – 8600* ~4200* ~3800* ⁶ ~3100* 4 bands 1950 – 2800* 6 bands 1200 – 1950* ~900*
<i>In2-1</i> terminator	22	>9800 (border) ⁴	34658	>8600 ⁵ 2 bands >8600* 2 bands 6100 – 8600* ~2700*
35S enhancer	23	>9800 (border) ⁴	34658	>8600 ⁵
<i>Ltp2</i> promoter	23	>9800 (border) ⁴	34658	>8600 ⁵
DsRed2	24	>9800 (border) ⁴	34658	>8600 ⁵
<i>pinII</i> terminator	24	>9800 (border) ⁴	34658	>8600 ⁵

1. Predicted size for probe hybridization to genomic DNA of DP-32138-1 maize containing a complete T-DNA insertion based on the map of the T-DNA from PHP24597 (Figure 2). Border fragment sizes are rounded to the nearest 100 bp.
2. Predicted size for probe hybridization to plasmid PHP24597 based on the plasmid map of PHP24597 (Figure 1).
3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion.
5. The expected size of this fragment is >9800 bp based on the PHP24597 T-DNA map (Figure 2). However, as the largest molecular weight marker band on the blot is ~8600 bp, and the band runs above this marker, the observed size of the band is reported as >8600bp.
6. The >8600 bp and ~3800 bp endogenous bands are seen in only one of the DP-32138-1 plants and the Hi-II and Hi-II(*ms45*) control plants.

Table 7: Predicted and Observed Hybridizing Bands on Southern Blots; *Bam*H I Digest

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous

Probe	Figure	Predicted Fragment Size from PHP24597 T-DNA ¹ (bp)	Predicted Fragment Size from Plasmid PHP24597 ² (bp)	Observed Fragment Size in DP-32138-1 ³ (bp)
5126 promoter	25	>1500 (border) ⁴	8826	~3800 2 bands >8600* ~7000*
<i>Ms45</i>	25	>1500 (border) 2815	8826 2815	~3800 2815 ⁵ ~7000* ~2700*
<i>Pg47</i> promoter	26	2502	2502	2502 ⁵ 3 bands >8600* 3 bands 6100 – 8600* 4 bands 4900 – 6100* 2 bands 3600 – 4900*
<i>zm-bt1</i>	26	2502	2502	2502 ⁵ 3 bands >8600*
<i>zm-aa1</i>	27	2502 2019	2502 2019	2502 ⁵ 2019 ⁵ 4 bands >8600* 3 bands 6100 – 8600* 2 bands 4900 – 6100* 5 bands 3600 – 4900* 2 bands 2800 – 3600* 4 bands 1950 – 2800* 4 bands 1200 – 1950*
<i>In2-1</i> terminator	27	2019	2019	2019 ⁵ 2 bands >8600*
35S enhancer	28	2019	2019	2019 ⁵
<i>Ltp2</i> promoter	28	2019	2019	2019 ⁵
DsRed2	29	>1200 (border)	27225	~4600 ~6100 (faint)
<i>pinII</i> terminator	29	>1200 (border)	27225	~4600 ~6100 (faint)

sequences, as can be determined by the presence of the same band in DP-32138-1 and control plants.

- 5 1. Predicted size for probe hybridization to genomic DNA of DP-32138-1 maize containing a complete T-DNA insertion based on the map of the T-DNA from PHP24597 (Figure 2). Border fragment sizes are rounded to the nearest 100 bp.
2. Predicted size for probe hybridization to plasmid PHP24597 based on the plasmid map of PHP24597 (Figure 1).
- 10 3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion.
- 15 5. Observed size is same as expected size due to equivalent migration to the plasmid band.

Table 8: Predicted and Observed Hybridizing Bands on Southern Blots; BamH I/Hind III Digest

Probe	Figure	Predicted Fragment Size from PHP24597 T-DNA ¹ (bp)	Predicted Fragment Size from Plasmid PHP24597 ² (bp)	Observed Fragment Size in DP-32138-1 ³ (bp)
DsRed2	30	>1200 (border) ⁴	26045	~2600
<i>pinII</i> terminator	30	>1200 (border)	26045	~2600

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1. Predicted size for probe hybridization to genomic DNA of DP-32138-1 maize containing a complete T-DNA insertion based on the map of the T-DNA from PHP24597 (Figure 2). Border fragment sizes are rounded to the nearest 100 bp.
2. Predicted size for probe hybridization to plasmid PHP24597 based on the plasmid map of PHP24597 (Figure 1).
3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion.

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Table 9: Predicted and Observed Hybridizing Bands on Southern Blots; Bgl II Digest

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences, as can be determined by the presence of the same band in DP-32138-1 and control plants.

Probe	Figure	Predicted Fragment Size from PHP24597 T-DNA ¹ (bp)	Predicted Fragment Size from Plasmid PHP24597 ² (bp)	Observed Fragment Size in DP-32138-1 ³ (bp)
5126 promoter	31	>1700 (border) ⁴	4937	~3600 2 bands >8600*
Ms45	31	>1700 (border) 806 4511	4937 806 4511	~3600 806 ^{5,6} 4511 ⁵ ~7400* ~5000* ~800*
Pg47 promoter	32	4511	4511	4511 ⁵ 2 bands >8600* 2 bands 6100 – 8600* 2 bands 4900 – 6100* ~4600*
zm-bt1	32	4511	4511	4511 ⁵ >8600* ~8000*
zm-aa1	33	4511	4511	4511 ⁵ overlapping bands >8000* (not resolved separately) 2 bands 6100 – 8000* 2 bands 4900 – 6100* ~4500* ~3000* ~2900* ⁷
In2-1 terminator	33	>2900 (border)	31228	~4900 >8600* 2 bands 6100 – 8600* ~5900* ~2000*
35S enhancer	34	>2900 (border)	31228	~4900 ~7400 (faint)
Ltp2 promoter	34	>2900 (border)	31228	~4900 ~7400 (faint)
DsRed2	35	>2900 (border)	31228	~4900 ~7400 (faint)
pinII terminator	35	>2900 (border)	31228	~4900 ~7400 (faint)

1. Predicted size for probe hybridization to genomic DNA of DP-32138-1 maize containing a complete T-DNA insertion based on the map of the T-DNA from PHP24597 (Figure 2). Border fragment sizes are rounded to the nearest 100 bp.
2. Predicted size for probe hybridization to plasmid PHP24597 based on the plasmid map of PHP24597 (Figure 1).
3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion.
5. Observed size is same as expected size due to equivalent migration to the plasmid band.
6. Band overlaps an endogenous band but can be detected by increased hybridization intensity.
7. The ~2900 bp endogenous band is seen in only one of the DP-32138-1 plants and the 705 control plant.

Table 10: Predicted and Observed Hybridizing Bands on Southern Blots; Bgl III/Hind III Digest

1. Predicted size for probe hybridization to genomic DNA of DP-32138-1 maize containing a complete T-DNA

Probe	Predicted Fragment Size from PHP24597 T-DNA ¹ (bp)	Predicted Fragment Size from Plasmid PHP24597 ² (bp)	Observed Fragment Size in DP-32138-1 ³ (bp)
35S enhancer	>2900 (border) ⁴	27807	~4100
<i>Ltp2</i> promoter	>2900 (border)	27807	~4100
DsRed2	>2900 (border)	27807	~4100
<i>pinII</i> terminator	>2900 (border)	27807	~4100

PHP24597 (Figure 2). Border fragment sizes are rounded to the nearest 100 bp.

- Predicted size for probe hybridization to plasmid PHP24597 based on the plasmid map of PHP24597 (Figure 1).
- Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
- Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion.

Table 11: Description of Genetic Elements in Plasmid PHP24597

Region	Location on plasmid (base pair position)	Known Genetic Element	Size (base pairs)	Description
T-DNA	1 to 9950		9950	see Table 12 for information on the elements in this region
Plasmid Construct	9951 to 34822	includes elements below	24872	DNA from various sources for plasmid construction and plasmid replication
	11126 to 11914	<i>spc</i>	789	Spectinomycin resistance gene from bacteria (Fling <i>et al.</i> , 1985)
	13037 to 13406	colE1 ori	370	Bacterial origin of replication region (<i>E. coli</i>) (Tomizawa <i>et al.</i> , 1977)
	14503 to 14515	<i>cos</i>	13	<i>cos</i> site; cohesive ends from lambda bacteriophage DNA (Komari <i>et al.</i> , 1996)
	16221 to 16871	<i>tetR</i>	651	Tetracycline resistance regulation gene from bacteria (Komari <i>et al.</i> , 1996)
	16977 to 18176	<i>tetA</i>	1200	Tetracycline resistance gene from bacteria (Komari <i>et al.</i> , 1996)
	18807 to 20996	<i>rep</i>	2190	<i>rep</i> operon from bacteria (includes <i>trfA</i> below) (Komari <i>et al.</i> , 1996)
	19449 to 20597	<i>trfA</i>	1149	Trans-acting replication gene from bacteria (Komari <i>et al.</i> , 1996)
	24411 to 24522	oriT	112	oriT origin of transfer region from bacteria (Komari <i>et al.</i> , 1996)
	26362 to 32632	<i>ctl</i>	6271	Central control operon region from bacteria (Komari <i>et al.</i> , 1996)
	33640 to 34350	oriV	711	oriV origin of replication region from bacteria (Komari <i>et al.</i> , 1996)
Ti Plasmid Backbone	34823 to 49637	includes elements below	14815	Virulence (<i>vir</i>) gene region and intergenic regions from Ti plasmid of <i>Agrobacterium tumefaciens</i> . (Komari <i>et al.</i> , 1996)
	35847 to 36541	<i>virC1</i>	695	Virulence gene important for T-DNA insertion into genome
	36544 to 37152	<i>virC2</i>	609	Virulence gene important for T-DNA insertion into genome
	37263 to 38066	<i>virG</i>	804	Virulence gene important for T-DNA insertion into genome
	38198 to 47633	<i>virB</i>	9436	Virulence gene important for T-DNA insertion into genome
	49934 to 50303	colE1 ori	370	Bacterial origin of replication region (<i>E. coli</i>) (Tomizawa <i>et al.</i> , 1977)
	51400 to 51412	<i>cos</i>	13	<i>cos</i> site; cohesive ends from lambda bacteriophage DNA (Komari <i>et al.</i> , 1996)

Table 12: Description of Genetic Elements in the T-DNA Region of Plasmid PHP24597

Location on T-DNA (base pair position)	Genetic Element	Size (base pairs)	Description
1 to 25	Right Border	25	T-DNA Right Border region from Ti plasmid of <i>Agrobacterium tumefaciens</i>
26 to 177	Ti Plasmid Region	152	Non-functional sequence from Ti plasmid of <i>A. tumefaciens</i>
178 to 203	Polylinker Region	26	Region required for cloning genetic elements
204 to 706	5126 Promoter	503	Maize anther-specific 5126 promoter (Cigan and Albertsen, 1997)
707 to 2658	Ms45 Genomic Region	1952	Maize genomic DNA including the Ms45 coding sequence and associated 3' untranslated region as indicated below: Exon 1 bp 708 to 1086; Intron 1 bp 1087 to 1215; Exon 2 bp 1216 to 1499; Intron 2 bp 1500 to 1596; Exon 3 bp 1597 to 1764; Intron 3 bp 1765 to 1850; Exon 4 bp 1851 to 2258; 3' UTR bp 2384 to 2658 (Albertsen <i>et al.</i> , 1993; Albertsen <i>et al.</i> , 1995)
2659 to 2730	Polylinker Region	72	Region required for cloning genetic elements
2731 to 5466	Pg47 Promoter	2736	Promoter from the maize pollen-specific polygalacturonase (Pg47) gene (Allen and Lonsdale, 1993)
5467 to 5468	Polylinker Region	2	Region required for cloning genetic elements
5469 to 5693	zm-bt1 Transit Peptide	225	Amyloplast-targeting transit peptide from the maize <i>Brittle-1</i> gene (Sullivan <i>et al.</i> , 1991)
5694 to 6956	zm-aa1 Gene	1263	Maize α -amylase gene
6957 to 7033	Polylinker Region	77	Region required for cloning genetic elements
7034 to 7377	In2-1 Terminator	344	Terminator sequence from the maize <i>In2-1</i> gene (Hershey and Stoner, 1991)
7378 to 7411	Polylinker Region	34	Region required for cloning genetic elements
7412 to 7849	CaMV 35S Enhancer	438	Enhancer region from the Cauliflower Mosaic Virus genome (Franck <i>et al.</i> , 1980; Odell <i>et al.</i> , 1985).
7850 to 7905	Polylinker Region	56	Region required for cloning genetic elements

Location on T-DNA (base pair position)	Genetic Element	Size (base pairs)	Description
7906 to 8761	<i>Ltp2</i> Promoter	856	Promoter from barley lipid transfer protein (<i>Ltp2</i>) gene (Kalla <i>et al.</i> , 1994)
8762 to 8809	Polylinker Region	48	Region required for cloning genetic elements
8810 to 9487	<i>DsRed2(Alt1)</i> Gene	678	Modified <i>DsRed2</i> gene (Clontech, 2001) with internal <i>BstE</i> II restriction site removed
9488 to 9528	Polylinker Region	41	Region required for cloning genetic elements
9529 to 9839	<i>pinII</i> Terminator	311	Terminator region from <i>Solanum tuberosum</i> proteinase inhibitor II gene (Keil <i>et al.</i> , 1986; An <i>et al.</i> , 1989).
9840 to 9872	Polylinker Region	33	Region required for cloning genetic elements
9873 to 9925	Ti Plasmid Region	53	Non-functional sequence from Ti plasmid of <i>A. tumefaciens</i>
9926 to 9950	Left Border	25	T-DNA Left Border region from Ti plasmid of <i>Agrobacterium tumefaciens</i>

Example 2. Sequencing of T-DNA region of PHP24597

SEQ ID NO: 20 provides the deduced amino acid sequence from translation of the spliced exons from the *Ms45* gene from plasmid PHP24597. The full-length MS45 protein is 412 amino acids in length and weighs approximately 47 kDa.

SEQ ID NO: 21 provides the deduced amino acid sequence from translation of the *zm-bt1* transit peptide + *zm-aa1* region from plasmid PHP24597. The amino acids at positions 1-75 comprise the transit peptide. The complete translation product, including transit peptide is 495 amino acids in length and weighs approximately 54 kDa. The processed ZM-AA1 protein, with the transit peptide removed, is 420 amino acids in length and weighs approximately 46 kDa.

SEQ ID NO: 33 provides the deduced amino acid sequence from translation of the *DsRed2(Alt1)* gene from plasmid PHP24597. The DsRed2 protein is 225 amino acids in length and weighs approximately 26 kDa.

SEQ ID NO: 1 provides the sequence of the T-DNA insert plus genomic border regions in 32138 maize.

Example 3. Polymerase Chain Reaction (PCR) analysis of maize event DP-32138-1

Polymerase chain reaction (PCR) amplification of the unique junctions spanning the introduced genetic elements can distinguish 32138 maize plants from their non-genetically-

modified counterparts and can be used to screen for the presence of the inserted T-DNA. The purpose of this study was to verify the effectiveness and reliability of a construct-specific PCR assay on genomic DNA from leaf tissue of 32138 maize, and to assess the sensitivity of this method.

5

Experimental Design

Genomic DNA from leaf tissue of the test substance (seed from 32138 maize: lot# T-F-07-132C) and the control substance (seed from a non-genetically modified maize with a genetic background representative of the event background: lot# C-F-07-131C) was isolated and subjected to qualitative PCR amplification using a construct-specific primer pair. The PCR products were separated on a 2% agarose gel to confirm the presence of the inserted construct in the genomic DNA isolated from the test substance and the absence of the inserted construct in the genomic DNA isolated from the control substance. A reference standard (Low DNA Mass Ladder; Invitrogen Corporation Catalog # 10380-012) was used to determine the PCR product size. The reliability of the construct-specific PCR method was assessed by repeating the experiment three times. The sensitivity of the PCR amplification was evaluated by various dilutions of the genomic DNA from event DP-32138-1.

DNA Extraction

Test and control leaf samples (V5-V7 leaf stage) were harvested from plants grown at the DuPont Experimental Station (Wilmington, DE) from seed obtained from Pioneer Hi-Bred International, a DuPont Company (Johnston, IA). Genomic DNA extraction from the test and control leaf tissues was performed using a standard urea extraction protocol.

Genomic DNA was quantified using the NanoDrop® 1000 Spectrophotometer using ND-1000 V3.6 Software (ThermoScientific, Wilmington, DE). DNA samples were visualized on an agarose gel to confirm quantitation values and to determine the DNA quality.

Polymerase Chain Reaction

Genomic DNA isolated from leaf of 32138 maize and control samples was subjected to PCR amplification (AccuPrime Taq DNA Polymerase High Fidelity, Invitrogen Corporation Catalog # 12346) utilizing the construct-specific primer pair 08-0-2544/08-0-2582 (SEQ ID NOS: 2 and 3; Table 17) which targets the maize 5126 Promoter and the Ms45 Genomic sequences and allows for the unique identification of 32138 maize. The expected size of PCR product using this primer pair is 233 bp. A second primer set, 02-O-197/02-O-198 (SEQ ID NOS: 4 and 5; Table 17) was used to amplify the endogenous maize invertase gene (GenBank accession number AF171874.1) as a positive control for PCR amplification. The expected size

of PCR product using this primer pair is 225 bp. PCR reagents and reaction conditions are shown in Table 13. In this study, 50 ng of leaf genomic DNA was used in all PCR reactions.

Table 13: PCR Reagents and Reaction Conditions

PCR Reagents		PCR Reaction Conditions			
Reagent	Volume (μL)	Cycle Element	Temp (°C)	Time (sec)	# Cycles
Template DNA (50 ng/μl)	1	Initial Denaturation	94	120	1
Primer 1 (10 μM)	2	Denaturation	94	15	35
Primer 2 (10 μM)	2	Annealing	60	20	
PCR Master Mix*	5	Elongation	68	60	
ddH ₂ O	39.7	Final Elongation	68	420	1
Polymerase**	0.3	Hold Cycle	4	Until analysis	

5 PCR: POLYMERASE CHAIN REACTION

DDH₂O: DOUBLE-DISTILLED WATER

*10X AccuPrime PCR Buffer II

**AccuPrime Taq DNA Polymerase High Fidelity

10 *Construct-Specific PCR Analysis for 32138 Maize*

A PCR product of approximately 230 bp in size amplified by the construct-specific primer set 08-0-2544/08-0-2582 was observed in PCR reactions using plasmid PHP24597 (50 ng) as template and all 32138 maize DNA samples, but absent in all control maize samples and the no-template control. This experiment was repeated three times, and similar results were obtained. Figure 7 represents results observed for DNA extracts from five 32138 maize plants and five control maize plants. These results correspond closely with the expected PCR product size (233 bp) for samples containing 32138 maize genomic DNA. A PCR product approximately 220 bp in size was observed for both 32138 maize and control maize samples following PCR reaction with the primer set 02-O-197/02-O-198 for detection of the endogenous maize invertase gene (Figure 8). These results correspond closely with the expected PCR product size (225 bp) for genomic DNA samples containing the maize endogenous invertase gene. The endogenous target band was not observed in the no-template control.

Sensitivity of Construct-Specific PCR Analysis for 32138 Maize

25 In order to assess the sensitivity of the PCR amplification, various concentrations of a single DNA sample of 32138 maize plant were added to non-genetically modified control DNA, resulting in 32138 maize DNA amounts ranging from 50 ng to 500 fg (the total amount of

genomic DNA in all PCR samples was 50 ng). Each dilution was subjected to PCR amplification as previously conducted. Based on this analysis, the limit of detection (LOD) was determined to be approximately 50 pg of 32138 maize DNA in 50 ng of total DNA, or 0.1% 32138 maize DNA (Figure 9). This is sufficient sensitivity for many screening applications.

5

Example 3 conclusions

Qualitative PCR analysis utilizing a construct-specific primer set for 32138 maize confirmed that the test plants contained the inserted T-DNA from PHP24597, as evidenced by the presence of the construct-specific target band in all test plant samples analyzed, and the
10 absence in the non-genetically modified control plants. This result was reproducible. Test and control plants both contained the endogenous maize invertase gene. The predicted sensitivity of the analysis under the conditions described is 0.1% 32138 maize DNA.

Example 4. Sequencing of the inserted DNA and flanking regions of Event 32138

Southern blot analyses indicated that a single intact T-DNA from plasmid PHP24597 was inserted into the maize genome to produce 32138 maize; see, Example 1. This example provides the sequence of the inserted T-DNA in 32138 maize and further confirms that a single intact T-DNA was inserted in maize genome with a partial T-DNA insertion of 23 bp or 27 bp at
15 5' genomic border region. In addition, this example describes cloning and sequencing of the genomic border regions undertaken to obtain DNA sequence that could be used to uniquely identify 32138 maize.
20

The sequence of the insert and genomic border regions was determined to confirm the integrity of the inserted DNA and to characterize the genomic sequence flanking the insertion site present in 32138 maize. In total, 13998 bp of 32138 maize genomic sequence was confirmed, comprising 2114 bp of the 5' genomic border sequence, 2002 bp of the 3' genomic
25 border sequence, and 9882 bp of inserted T-DNA from PHP24597. The inserted T-DNA in 32138 maize was found to have a 45 bp deletion on the Right Border (RB) end and a 23 bp deletion on the Left Border (LB) end. Also, a partial T-DNA insertion of 23bp is located at positions 2092-2114 of the indicated genomic 5' border sequence. The sequence surrounding
30 this partial insertion comprises additional junction sequences unique to 32138 maize and which may be used to specifically identify the 32138 event. All remaining transgenic sequence indicated at positions 2115 through 11,996 is intact and identical to the T-DNA of plasmid PHP24597.

The 5' and 3' genomic border regions of 32138 maize were verified to be of maize
35 origin by PCR amplification and sequencing of the genomic border regions from both 32138 maize and control plants. Overall, characterization of the insert and genomic border sequence

in 32138 maize confirms that a single intact insertion of the T-DNA from plasmid PHP24597 is present in the maize genome with a partial T-DNA insertion of 23 bp or 27 bp at 5' genomic border region.

5 *Test Substance*

The test substance is defined as maize seed containing event DP-32138-1 obtained from a T1S1 generation of 32138 maize. Only red kernels were selected for use in the study. Further details regarding the source of the seed are provided in Example 1.

10 All seeds were obtained from Pioneer Hi-Bred International, Inc. (Pioneer, Johnston, IA) and pedigree information is on file with staff breeders. The log number is T-F-07-132C.

Control Substance

The control substance is defined as seed from a maize line that does not contain 32138 maize. The unmodified maize line has a genetic background representative of the event
15 background; however, it does not contain the *Ms45*, *zm-aa1* and *DsRed2* (Alt1) gene cassettes. The log number is C-F-07-131C. Further details are provided in Example 1.

The Low DNA Mass Ladder (Invitrogen Corp., Carlsbad, CA), High DNA Mass Ladder (Invitrogen Corp.), and the GeneRuler 1kb DNA Ladder (Fermentas, Glen Burnie, MD) were used for gel electrophoresis to estimate DNA fragment sizes on agarose gels.

20

Plant Growth and Sample Collection

The 32138 maize seed and the control seed were planted in the Regulatory Science growth chambers at the DuPont Experimental Station (Wilmington, DE) to produce plant tissues used for this study. One seed was planted per pot, and the pot was uniquely identified.

25 All plants were grown with light, temperature, and water regulated for healthy plant growth.

Leaf samples were collected from each of the control and 32138 maize plants. For each sample, sufficient leaf material from above the growing point was collected and placed in a pre-labeled sample bag. The samples were placed on dry ice and were transferred to an ultra low freezer (<-55°C) following collection. All samples were maintained frozen until
30 processing. All leaf samples were uniquely labeled with the plant identifier and the date of harvest.

Genotype Confirmation via Event-Specific PCR Analysis

A leaf sample was taken from all test and control plants for event-specific PCR analysis.
35 DNA was extracted from each leaf sample using the Extract-N-Amp™ Plant PCR kit using the described procedure (Sigma-Aldrich, St. Louis, MO).

Real-time PCR was performed on each DNA sample utilizing an ABI PRISM® 7500HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). TaqMan® probe and primer sets were designed to detect a target sequence from the 32138 maize. In addition, a second TaqMan® probe and primer set for a reference maize endogenous gene was used to confirm the presence of amplifiable DNA in each reaction. The analysis consisted of real-time PCR determination of qualitative positive/negative calls. The extracted DNA was assayed using optimized and validated primer and probe concentrations in TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Inc.). Initial incubation was at 95°C for 10 minutes followed by 40 cycles as follows: 95°C for 15 seconds, 60°C for 1 minute.

Positive or negative determination for 32138 maize was based on comparison of the C_T (threshold cycle) of the event-specific target PCR to that of the maize endogenous reference target. If the event and endogenous PCR targets amplified above C_T threshold, then the plant was scored as positive for that event. If the endogenous target amplified and the event target did not, then the plant was scored as negative. If neither target amplified for a particular sample, then it was determined to be a poor quality sample or failed run and the assay was repeated.

DNA Extraction and Quantitation

Frozen leaf samples (1-2 gram quantities) were ground, and the genomic DNA was isolated using a standard Urea Extraction Buffer procedure. Following extraction, the DNA was visualized on an agarose gel to determine the DNA quality, and was quantified using the NanoDrop 1000 Spectrophotometer and ND-1000 V3.6 Software (ThermoScientific, Wilmington, DE).

Polymerase Chain Reaction (PCR)

PCR primers were synthesized by IDT (Coralville, IA) and used at a concentration of 0.2 - 0.4 μ M with 30 - 250 ng genomic DNA as template in a PCR reaction. DNA isolated from five 32138 maize plants was used as template DNA. PCR products were cloned and sequenced from four of the five 32138 maize plants. The following regions were PCR amplified from genomic DNA isolated from 32138 maize: the PHP24597 T-DNA insert, the 5' and 3' insert/genomic border junctions, and the 5' and 3' genomic border regions. PCR systems used were: AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen Corp.), High Fidelity PCR system (Roche, Mannheim, Germany), Expand Long Template PCR System (Roche) and the Advantage®- GC2 genomic PCR mix (Clontech, Palo Alto, CA). The PCR products were visualized under UV light following electrophoresis through 1 - 2.5% agarose gel with 1X TBE (or 1X TAE) stained with ethidium bromide, or by 1.2% agarose E-Gel with SYBER® Green

(Invitrogen Corp.) under a blue light. Products were excised and purified from the gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA).

Cloning of PCR Products

- 5 PCR products were cloned using the TOPO TA Cloning® Kit (pCR2.1-TOPO vector, Invitrogen) or pGem T-Easy Vector System (Promega). Plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen), screened by restriction enzyme digests, and sequenced.

DNA Sequencing

- 10 DNA fragments were cloned and submitted for sequencing at the Pioneer Crop Genetics Research sequencing facility (Wilmington, DE). Sequencher™ software from Gene Codes Corporation (Ann Arbor, Michigan) was used to assemble the sequences. Sequence annotation was performed using Vector NTI 9.1.0 (Invitrogen Corp) by comparing the T-DNA insert sequences generated from 32138 maize with the sequences from the T-DNA region of
15 plasmid PHP24597 (plasmid used for transformation to produce 32138 maize).

Sequencing of the T-DNA from Plasmid PHP24597

The T-DNA region of plasmid PHP24597, used for creating 32138 maize, was sequenced and compared with the inserted sequence generated from 32138 maize.

20

Sequencing of the Inserted T-DNA in 32138 Maize

- For verification of the DNA sequence of the inserted T-DNA from plasmid PHP24597 in 32138 maize, primers were designed based on the sequence information from the T-DNA of plasmid PHP24597. Five overlapping PCR products were generated using genomic DNA from
25 at least four different 32138 maize plants as template in the PCR. These PCR products were cloned and sequenced from 32138 maize plants.

Sequencing of 5' and 3' Flanking Genomic Border Regions

- Initial sequence characterization of the 5' and 3' flanking border region were carried out
30 using several rounds of inverse PCR (Silver and Keerikatte, (1989); Ochman, *et al.*, (1988); Triglia, *et al.*, (1988)), with primers anchored within various regions of the 5' and 3' ends of the inserted T-DNA. Sequence information obtained from inverse PCR was subjected to BLASTn analysis and showed identity to maize genomic DNA sequence (GenBank accession number AC196124) from the NCBI (National Center for Biotechnology Information) GenBank nucleotide
35 dataset. This sequence was then used to design primers that spanned the 5' and 3' insert/genomic junctions of 32138 maize. The PCR products generated from genomic DNA

isolated from four 32138 maize plants were cloned and sequenced to verify the 5' and 3' insert/genomic junctions and the genomic border regions. In order to demonstrate that the identified 5' and 3' genomic border sequences were of maize origin, PCR was performed within this 5' and 3' genomic regions on genomic DNA from 32138 maize and control plants. Cloned
5 PCR products from four 32138 maize plants and two control plants were sequenced.

Genotype Confirmation via Event-Specific PCR Analysis

All individual plants used for this study were tested for the presence of the 32138 insertion by an event-specific PCR analysis. All 32138 maize plants were positive for the
10 insertion, whereas all the control plants were negative for the insertion. The results of the event-specific PCR assays for the 32138 plants and control plants are summarized in Table 13.

Sequence Characterization of Inserted DNA and Genomic Border Regions

The T-DNA sequence information of plasmid PHP24597 was used to design primers to
15 verify the inserted sequence in 32138 maize (Tables 14 and 15). Preliminary sequences obtained by inverse PCR from 32138 maize genomic DNA were subjected to BLASTn analysis and showed identity to maize genomic DNA sequence (GenBank accession number AC196124) from NCBI (National Center for Biotechnology Information) GenBank nucleotide dataset. The sequence information from AC196124 was then used to design primers to extend
20 additional genomic border regions (Tables 14 and 15).

To characterize the inserted T-DNA in 32138 maize, PCR primers were designed to amplify the T-DNA insert in five separate, overlapping PCR products: Fragment A (07-0-2286/07-0-2277; SEQ ID NOS: 6 and 7), Fragment B (08-0-2329/08-0-2398; SEQ ID NOS: 8 and 9), Fragment C (08-0-2402/07-0-2024; SEQ ID NOS: 10 and 11), Fragment D (08-0-2505/08-0-2504; SEQ ID NOS: 12 and 13), Fragment E (08-0-2408/08-0-2526; SEQ ID NOS: 14 and 15) (Figure 10 and Tables 15 and 16). As expected, the predicted PCR products were
25 generated only from 32138 maize genomic DNA samples, and were not present in the control samples. The five PCR products from 32138 maize plants were cloned and sequenced from four 32138 maize plants. When comparing the sequence of the inserted T-DNA in 32138
30 maize to the T-DNA region of plasmid PHP24597 used for creating 32138 maize, it was determined that there was a 45 bp deletion on the RB end, and a 23 bp deletion on the LB end. RB and LB termini deletions often occur in *Agrobacterium*-mediated transformation (Kim, *et al.*, (2007) *Plant J.* 51:779-791). All remaining sequence is intact and identical to that of plasmid PHP24597. The sequence of the insertion is presented in SEQ ID NO: 1 and Figure 11.

To verify the additional 5' genomic border sequence, PCR was performed with a
35 forward primer (07-0-2286; SEQ ID NO: 6) in the 5' genomic border region and a reverse

primer (07-0-2277; SEQ ID NO: 7) within the inserted T-DNA. The resulting 2198 bp PCR fragment A from 32138 maize genomic DNA samples was cloned and sequenced. The 2114 bp of 5' genomic border region sequence is presented as underlined sequence in Figure 11.

To verify the additional 3' genomic border sequence, PCR was performed with a forward primer (08-0-2408; SEQ ID NO: 14) within the inserted T-DNA and a reverse primer (08-0-2526; SEQ ID NO: 15) in the 3' genomic border region. The resulting 2683 bp PCR fragment E from 32138 maize genomic DNA samples was cloned and sequenced. The 2002 bp of 3' genomic border region sequence is presented as underlined sequence in Figure 11.

In total, 13998 bp of sequence from genomic DNA of 32138 maize were confirmed: 2114 bp of the 5' genomic border sequence, 2002 bp of the 3' genomic border sequence, and 9882 bp comprising the inserted T-DNA (Figures 10 and 11).

To demonstrate that the identified 5' and 3' flanking border sequences are of maize origin, PCR was performed within the 5' and 3' genomic border regions (primer pairs 08-0-2528/08-0-2538, SEQ ID NOS: 16 and 17; and 08-0-2539/08-0-2530, SEQ ID NOS: 18 and 19, respectively) on 32138 maize genomic DNA samples and control DNA samples. The expected PCR fragment F (294 bp for 5' genomic region) and PCR fragment G (297 bp for 3' genomic region) were generated from both 32138 maize and control samples. These PCR products were cloned and sequenced, and the corresponding products from the 32138 maize and the control maize were identical, indicating that the sequences were of maize genomic origin (Figures 12A and 12B).

Table 14. Summary of Genotype Confirmation via Event-Specific PCR Analysis of 32138 Maize and Control Maize Plants

Maize Plant ID	Plant ID abbreviation	Event-specific PCR ¹
T-F-07-132C-1	T1	+
T-F-07-132C-2	T2	+
T-F-132C-3	T3	+
T-F-132C-4	T4	+
T-F-132C-5	T5	+
Control Maize Plant ID		
C-F-07-131C-1	C1	-
C-F-07-131C-2	C2	-

- Summary of event-specific real time PCR assay for 32138 maize. Positive (+) indicates the presence of 32138 maize event. Negative (-) indicates the absence of 32138 maize event.

Table 15. PCR Primers Used to Characterize the Genomic Border Regions and Inserted T-DNA in 32138 Maize

PCR Fragment	Primer Pair	Size in bp	<u>Amplified Region</u>
A	07-0-2286/07-0-2277	2198	5' Genomic border region and insert
B	08-0-2329/08-0-2398	3052	5' Genomic border region and Insert
C	08-0-2402/07-0-2024	3550	Insert
D	08-0-2505/08-0-2504	4073	Insert
E	08-0-2408/08-0-2526	2683	3' Genomic border region and insert
F	08-0-2528/08-0-2538	294	5' Genomic border region
G	08-0-2539/08-0-2530	297	3' Genomic border region

Figure 10 indicates the PCR fragments generated from 32138 maize genomic DNA that were cloned and sequenced: Fragment A (07-0-2286/07-0-2277), Fragment B (08-0-2329/08-0-2398), Fragment C (08-0-2402/07-0-2024), Fragment D (08-0-2505/08-0-2504), and Fragment E (08-0-2408/08-0-2526). The vertical dashed line represents the genomic border/insert junction. Fragment F (08-0-2528/08-0-2538) and G (08-0-2539/08-0-2530) represent the 5' and 3' genomic border regions, respectively (red arrows). Figure 10 is not drawn to scale.

In Figure 12A, primer pair 08-0-2528/08-0-2538 amplifies a PCR product (294 bp) from within the 5' flanking genomic DNA. In Figure 12B, primer pair 08-0-2539/08-0-2530 amplifies a PCR product (297 bp) from within the 3' genomic border region.

Example 4 Conclusions

The sequence of the insert and genomic border regions was determined to confirm the integrity of the inserted DNA and to characterize the genomic sequence flanking the insertion site present in 32138 maize. In total, 13998 bp of 32138 maize genomic sequence was confirmed, comprising 2114 bp of the 5' genomic border sequence, 2002 bp of the 3' genomic border sequence, and 9882 bp of inserted T-DNA from PHP24597. The inserted T-DNA in 32138 maize was found to have a 45 bp deletion on the Right Border (RB) end and a 23 bp deletion on the Left Border (LB) end. Also, a partial T-DNA insertion of 23 bp is located at positions 2092-2114 of the indicated genomic 5' border sequence. The sequence surrounding

this partial insertion comprises additional junction sequences unique to 32138 maize and which may be used to specifically identify the 32138 event. All remaining transgenic sequence indicated at positions 2115 through 11,996 is intact and identical to the T-DNA of plasmid PHP24597.

- 5 The 5' and 3' genomic border regions of 32138 maize were verified to be of maize origin by PCR amplification and sequencing of the genomic border regions from both 32138 maize and control plants. Overall, characterization of the insert and genomic border sequence in 32138 maize confirms that a single intact insertion of the T-DNA from plasmid PHP24597 is present in the maize genome with a partial T-DNA insertion of 23 bp or 27 bp at 5' genomic
10 border region.

Table 16. SEQUENCE AND LOCATION OF PRIMERS USED FOR PCR REACTIONS.

PCR Fragment	SEQ ID NO:	Primer Name	Sequence (5' – 3')	Target Sequence Location (bp to bp) ¹
A	6	07-0-2286	CGAGACTTCACTGCCAGTTGATCG	1-24
	7	07-0-2277	ACGGCTTGTCCTCCGCGTCATC	2198-2179
B	8	08-0-2329	GGTGCAGCTGACAATTACCAACCGATCTTGGTG	1766-1797
	9	08-0-2398	CAGACAGTGTCCTCGGTGCAGATCC	4817-4795
C	10	08-0-2402	TCCGCGACCGCGAATTGGGC	4634-4653
	11	07-0-2024	TCCCGTCCGAGTACTGCGTGTC	8183-8162
D	12	08-0-2505	AGCATCGATCACGACACCATGGCG	7520-7543
	13	08-0-2504	CATGTGGTACCTACGCGTTCGAATATCCA	11592-11564
E	14	08-0-2408	CACCGAGCGCCTGTACCCCCCG	11316-11336
	15	08-0-2526	TTATGCGCATGCAGCAGGAAACAGATACACC	13998-13968
F	16	08-0-2528	TTCTTGATGGAGAGATCGCAGCTCTGTTC	1815-1843
	17	08-0-2538	CCGCTCATGATCAGATTTCACTGTGAGC	2108-2081
G	18	08-0-2539	GGAGATCCTGGTACACTATCTGTAGCAGTTTGG	12000-12032
	19	08-0-2530	GGTCATTTCCTAGAGTTTGATATACTTTATCATAG	12296-12261

¹ Location in sequence of 32138 Maize (see SEQ ID NO: 1). Bases 1 - 2114 = 5' genomic border region, bases 2115 – 11996 = insert, bases 11997 – 13998 = 3' genomic border region.

Table 17. List of Primer Sequences Used in PCR Reactions

SEQ ID NO:	Primer Name	Sequence 5' – 3'	Target Sequence
2	08-0-2544	TCAAGCCGTGAGCAGACATGTTGCAG	5126 Promoter
3	08-0-2582	CGAAGAAGAGGTGAGGGTACTGCACG	Ms45 Genomic
4	02-O-197	CCGCTGTATCACAAGGGCTGGTACC	Maize invertase gene
5	02-O-198	GGAGCCCGTGTAGAGCATGACGATC	Maize invertase gene

The articles "a" and "an" are used herein to refer to one or more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing inventions have been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Table 18. Description of DNA Probes Used for Southern Hybridization

Probe Name	Genetic Element	Figure Probe	Position on PHP24597 T-DNA (bp to bp) ¹	Position on PHP24597 Plasmid (bp to bp) ²	Length (bp)
5126 promoter	5126 promoter	Figure 14 probe 1	304 to 706	304 to 706	403
<i>Ms45</i> ³	<i>Ms45</i> Genomic region	Figure 14 probe 2	707 to 1426 1427 to 2258 2263 to 2658	707 to 1426 1427 to 2258 2263 to 2658	720 832 396
<i>Pg47</i> promoter	<i>Pg47</i> promoter (3' region)	Figure 14 probe 3	5028 to 5451	5028 to 5451	424
<i>zm-bt1</i>	<i>zm-bt1</i> transit peptide	Figure 14 probe 4	5469 to 5693	5469 to 5693	225
<i>zm-aa1</i> ⁴	<i>zm-aa1</i> gene	Figure 14 probe 5	5701 to 6333 6334 to 6935	5701 to 6333 6334 to 6935	603 602
<i>ln2-1</i> terminator	<i>ln2-1</i> terminator	Figure 14 probe 6	7049 to 7377	7049 to 7377	329
35S enhancer	CaMV 35S enhancer region	Figure 14 probe 7	7427 to 7846	7427 to 7846	420
<i>Ltp2</i> promoter	<i>Ltp2</i> promoter	Figure 14 probe 8	7906 to 8759	7906 to 8759	854
<i>DsRed2(Alt1)</i>	<i>DsRed2(Alt1)</i> gene	Figure 14 probe 9	8810 to 9487	8810 to 9487	678
<i>pinII</i> terminator	<i>pinII</i> terminator	Figure 14 probe 10	9582 to 9815	9582 to 9815	234
RB	Plasmid backbone adjacent to T-DNA Border Right	Figure 13 probe A	N/A ⁶	52436 to 52825	390
LB	Plasmid backbone adjacent to T-DNA Border Left	Figure 13 probe B	N/A ⁶	9975 to 10320	346
<i>spc</i>	Spectinomycin resistance gene	Figure 13 probe C	N/A ⁶	11130 to 11904	775
<i>virG</i>	<i>virG</i> gene	Figure 13 probe D	N/A ⁶	37294 to 38037	744
<i>tet</i> ⁵	Tetracycline resistance gene	Figure 13 probe E	N/A ⁶	16983 to 17521 17627 to 18084	539 458

1. The probe position is based on the PHP24597 T-DNA map (Figure 14).
2. The probe position is based on the PHP24597 plasmid map (Figure 13).
3. The *Ms45* probe is comprised of three non-overlapping labeled fragments that are combined in the hybridization solution.
4. The *zm-aa1* probe is comprised of two non-overlapping labeled fragments that are combined in the hybridization solution.
5. The *tet* probe is comprised of two labeled fragments that are combined in the respective hybridization solutions.
6. Not Applicable as this element is not located in the PHP24597 T-DNA region.

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- 25

THAT WHICH IS CLAIMED

1. A method for identifying event E6611.32.1.38 in a biological sample, comprising detecting a E6611.32.1.38-specific region with a probe or first primer which specifically recognizes a sequence within the T-DNA insert of SEQ ID NO: 1, and
5 a second probe or primer which specifically recognizes a sequence within either flanking sequence of SEQ ID NO: 1.
2. The method of claim 1, further comprising amplifying a DNA fragment from a nucleic acid present in said biological sample using a polymerase chain reaction with at least two primers, wherein said first primer recognizes a sequence within
10 the T-DNA insert of SEQ ID NO: 1, and a second primer recognizes a sequence within either flanking sequence of SEQ ID NO: 1.
3. The method of claim 2, wherein said first primer is selected from the group consisting of SEQ ID NOS: 7, 9, 10, 11, 12, 13, and 14, and said second primer is selected from the group consisting of SEQ ID NOS: 6, 8, 15, 16, 17, 18 and 19.
- 15 4. A method of detecting the presence of event E6611.32.1.38 or progeny thereof in a biological sample, comprising:
 - (a) extracting a DNA sample from said biological sample;
 - (b) providing a pair of DNA primers, wherein one of said primers recognizes a sequence within either flanking sequence of SEQ ID NO: 1, and the
20 second of said primers recognizes a sequence within the T-DNA insert of SEQ ID NO: 1;
 - (c) providing DNA amplification reaction conditions;
 - (d) performing a DNA amplification reaction, thereby producing a DNA amplicon molecule; and
 - 25 (e) detecting said DNA amplicon molecule, wherein the detection of said DNA amplicon molecule in said DNA amplification reaction indicates the presence of event E6611.32.1.38.
5. An isolated DNA molecule comprising any one of the amplicons produced by the method of claim 4.
- 30 6. A method of detecting the presence of DNA corresponding to the E6611.32.1.38 event in a sample, the method comprising:
 - (a) contacting the sample comprising DNA with a polynucleotide probe that hybridizes under stringent hybridization conditions with DNA from event E6611.32.1.38 and does not hybridize under said stringent hybridization
35 conditions with a non- E6611.32.1.38 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 E6611.32.1.38 event.

7. An isolated DNA nucleotide primer sequence comprising a sequence selected from the group consisting of: SEQ ID NOS: 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,
5 16, 17, 18, 19 or its complement.
8. An isolated DNA molecule comprising a junction sequence amplified by the primer pair of SEQ ID NO: 6 and 7; or SEQ ID NO: 8 and 9; or SEQ ID NO: 14 and 15.
9. A method for screening seeds for the presence of event E6611.32.1.38, comprising detection of a E6611.32.1.38 specific region with a specific primer or
10 probe which specifically recognizes a junction sequence of Event E6611.32.1.38.
10. A pair of isolated DNA sequences, each comprising at least ten nucleotides and which when used together in a DNA amplification procedure will produce an amplicon diagnostic for event E6611.32.1.38.
11. The pair of isolated DNA sequences of claim 10 wherein the first member of the
15 pair is chosen from the group consisting of SEQ ID NOS: 7, 9, 10, 11, 12, 13, and 14, and said second member of the pair is selected from the group consisting of SEQ ID NOS: 6, 8, 15, 16, 17, 18 and 19.
12. The amplicon of Claim 5, wherein said amplicon has the sequence of SEQ ID NO: 25, 28 or 32.
- 20 13. A plant comprising event E6611.32.1.38.
14. Seed produced by a plant of Claim 13.
15. A transgenic plant, cell, tissue, seed, or DNA-containing part thereof, for which representative seeds have been deposited with the American Type Culture Collection under accession number PTA-9158.
- 25 16. A plant, or DNA-containing part thereof, comprising a DNA insert of nucleotides 2115 through 11,996 of SEQ ID NO: 1.
17. A plant, or DNA-containing part thereof, comprising DNA of nucleotides 2071 through 2170 of SEQ ID NO: 1.
18. A plant, or DNA-containing part thereof, comprising DNA of nucleotides 11,951
30 through 12,050 of SEQ ID NO: 1.

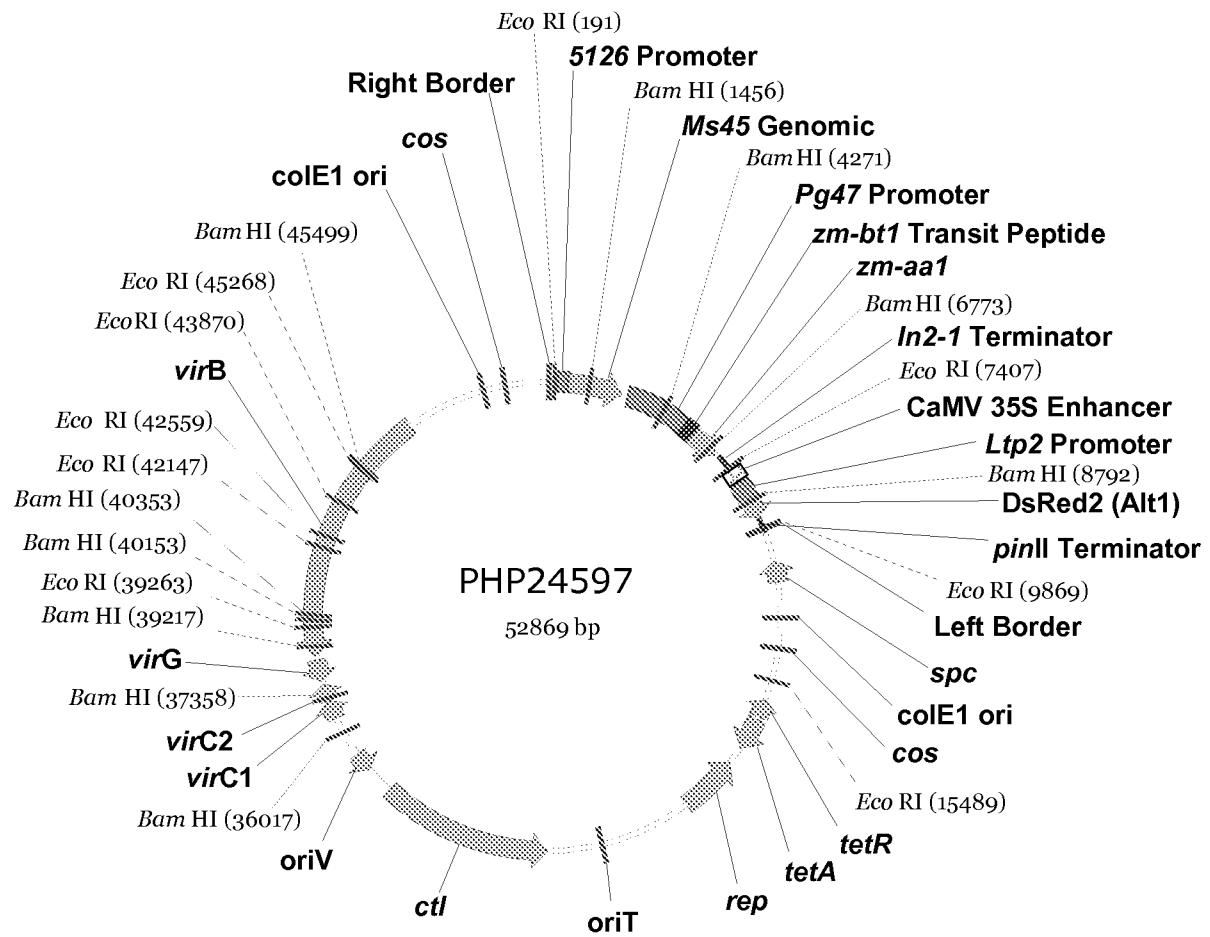
FIGURE 1

FIGURE 2

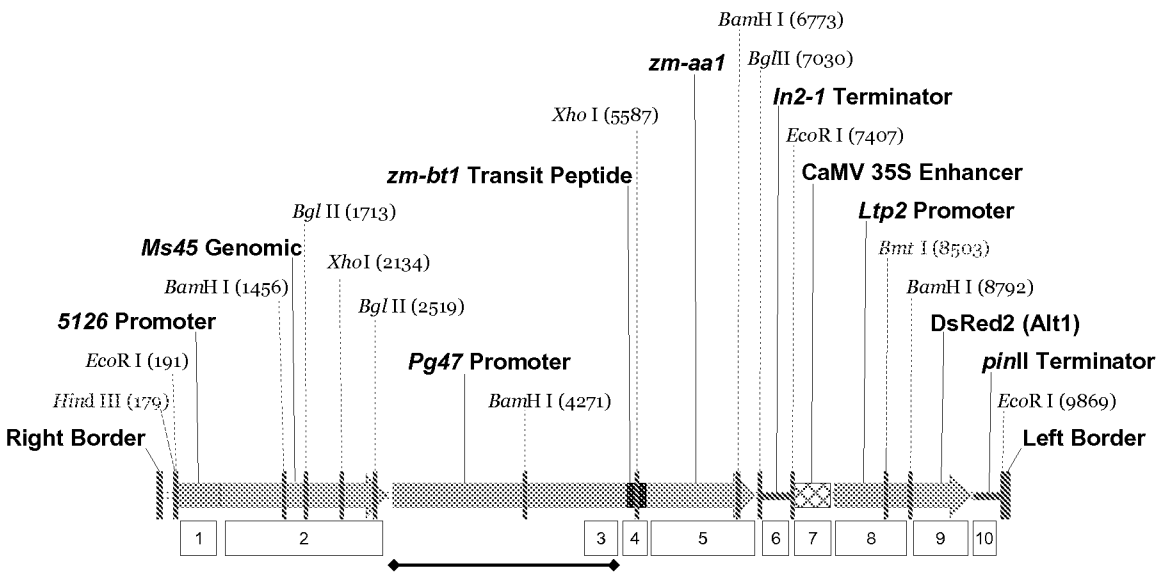


FIGURE 3

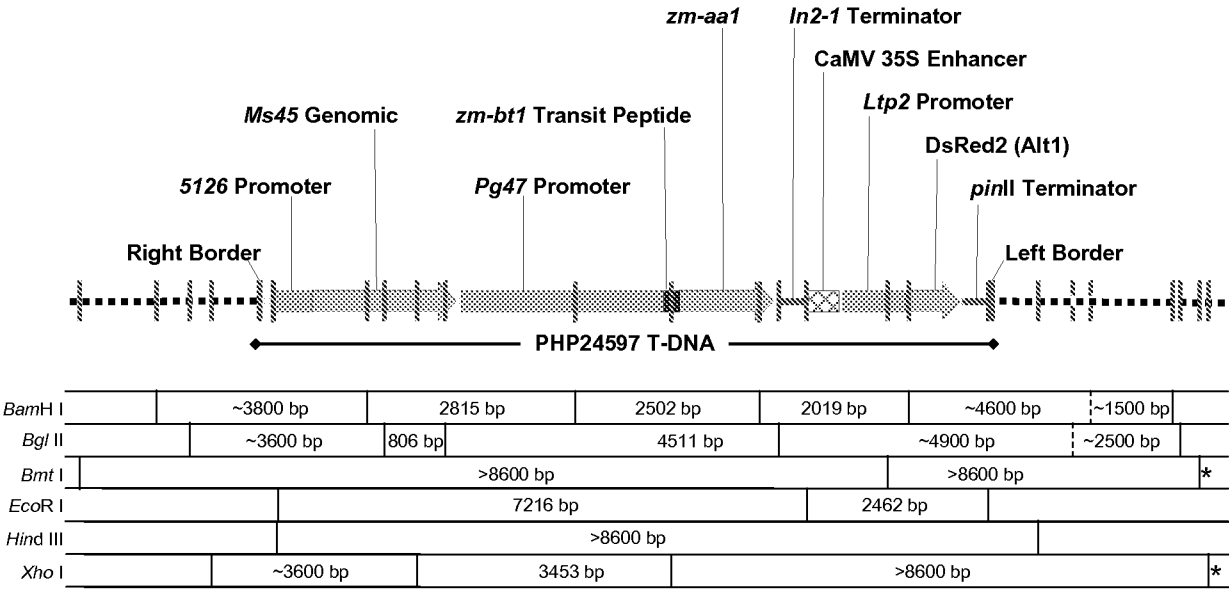


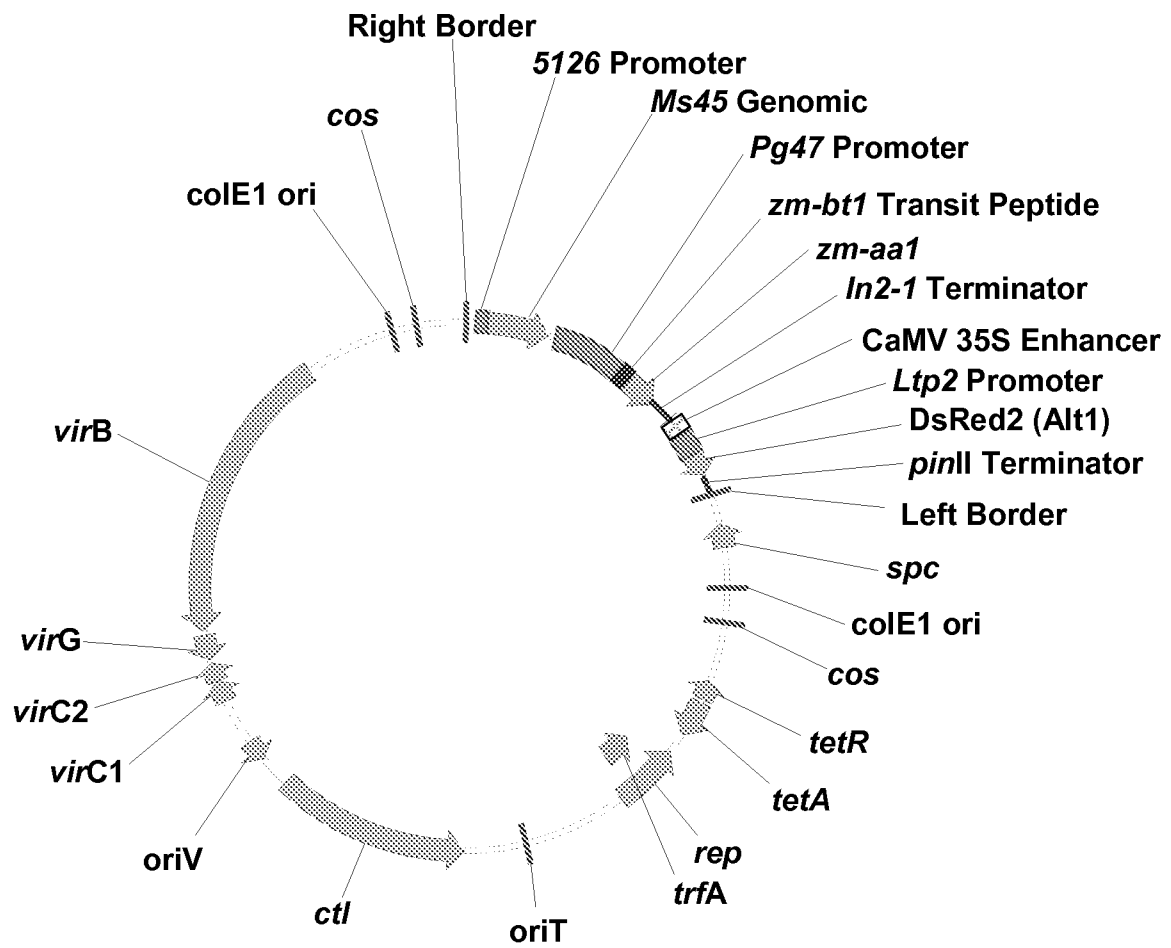
FIGURE 4

FIGURE 5

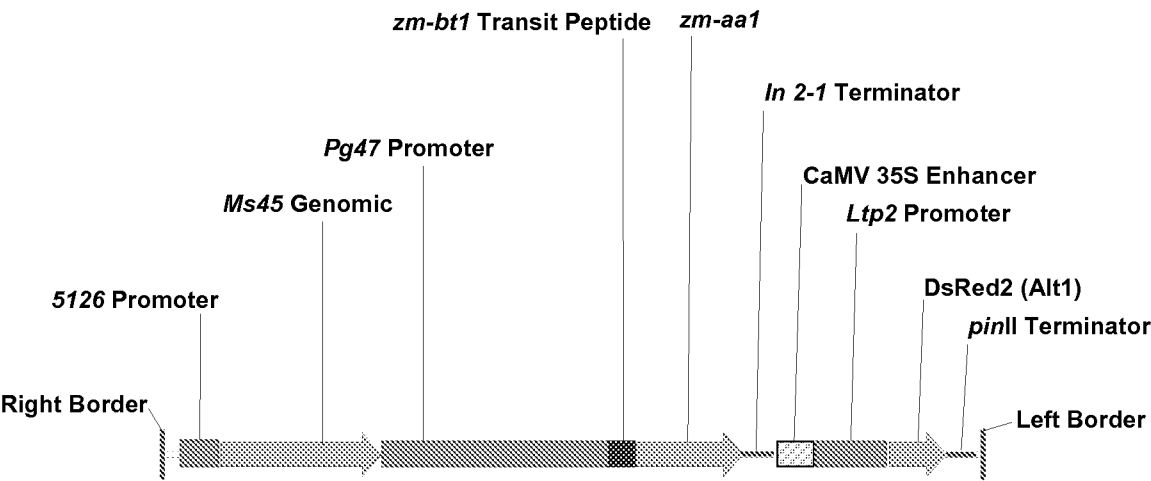


FIGURE 6

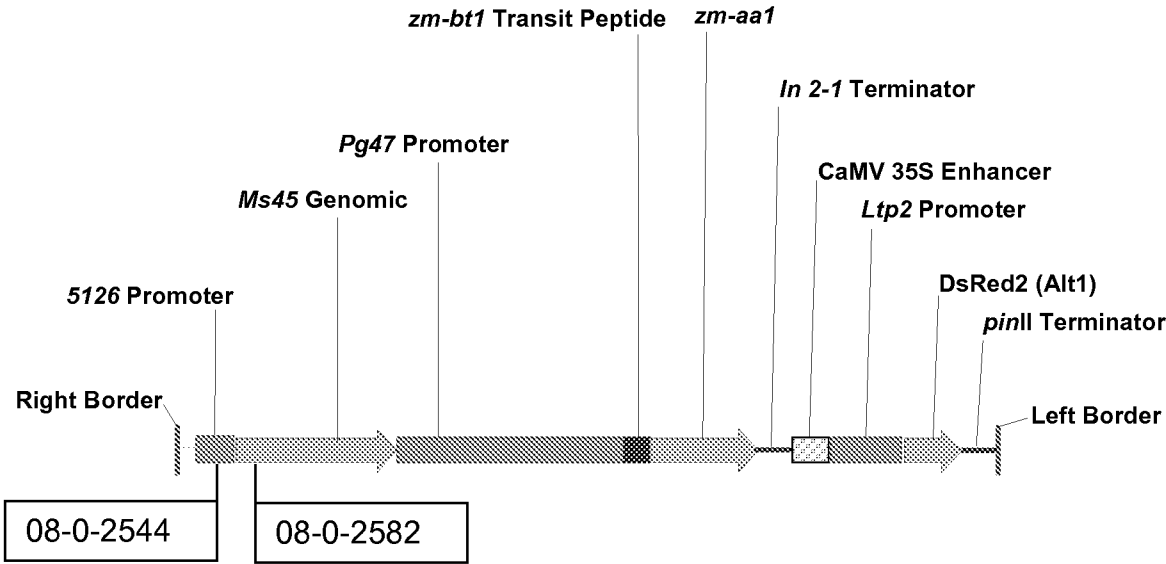


FIGURE 7

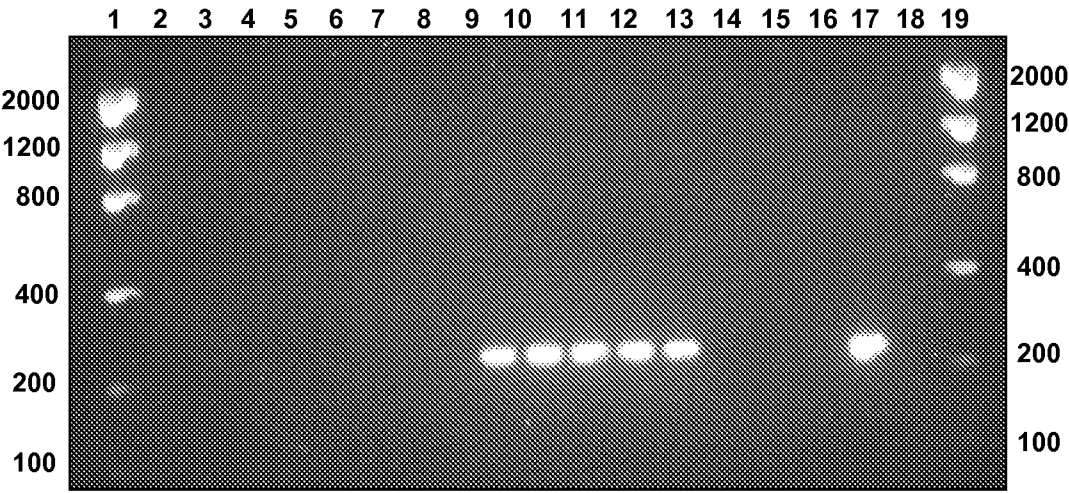


FIGURE 8

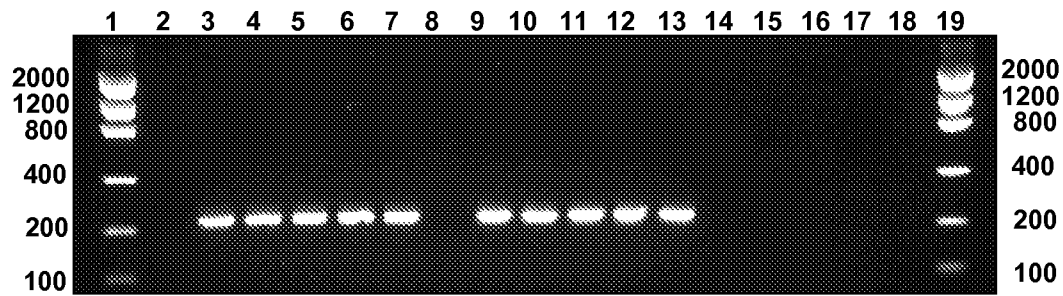


FIGURE 9

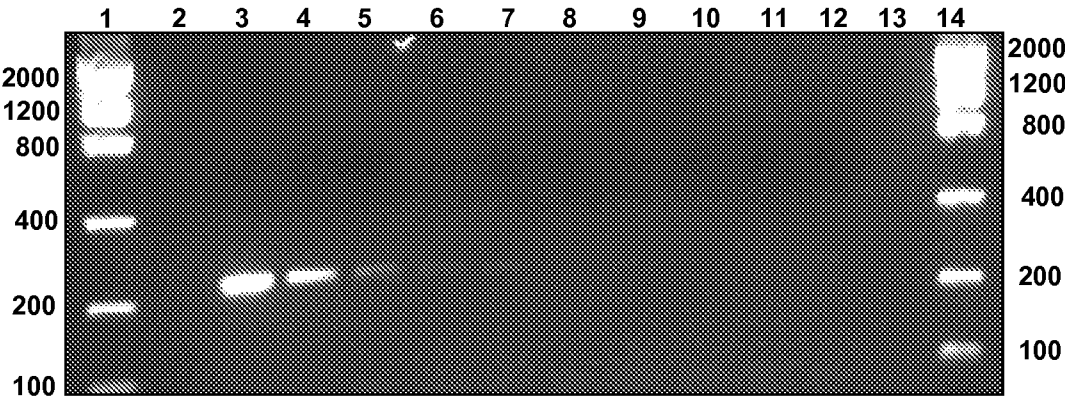


FIGURE 10

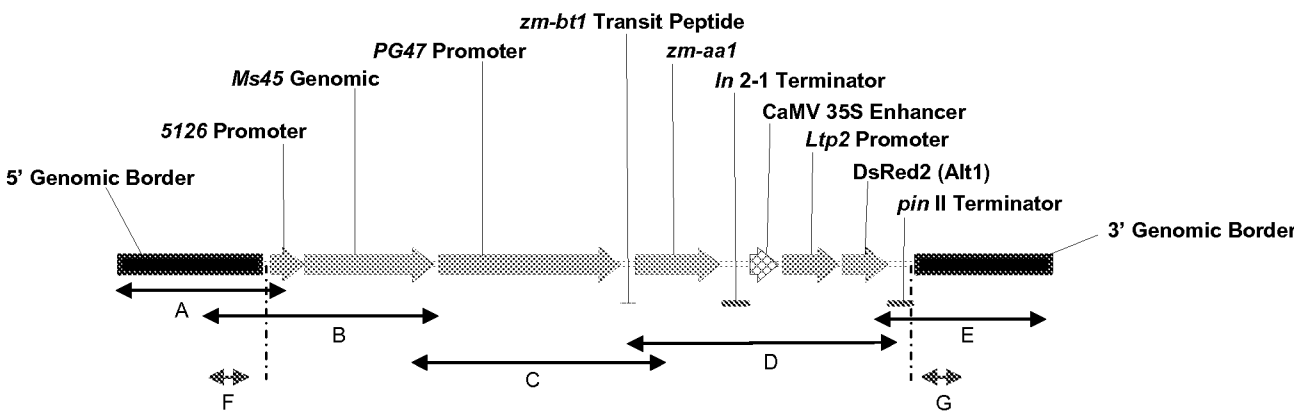


Figure 11.

1	CGAGACTTCA	CTGCCAGTTG	ATCGTCCCAT	GAGGTTGTCA	GAAATTCATG
51	TTGCAGCTGA	GCATGCACTG	AAACAAACGA	TATCAGATCC	TGATTTTATG
101	ACATCACTTT	CATCACTAGA	AGAATTTGAG	GTA GTACATT	CTTGACATC
151	ACAATTTTTT	TTGAAACAAT	GGCAAGAGTT	CTGCCTTTCA	ACCAAGGCGA
201	AATGAATTTT	TTATACAAGA	AGGGGTAGCC	AAACGTGATG	CCTACCACAA
251	GACACTCAAA	TGGCTAACCC	ACCCACGCGA	CGTGGCAGAG	TACAAAAC TA
301	CTATGAGTCA	TTGTTGCTGA	GCATGGTTGC	ATAGCAGCCA	ACATGTCCGG
351	CTTACCATGG	CTGATCCAGA	CGACCCACTC	TAATGATGTT	GGGCACTCGA
401	GCCAGAATTA	AGAGTCCACT	ACAGGTCATC	GTTGCCAATG	TCAAGCGTGC
451	CACATAAGAG	CAGCTTTGAC	TTTTTATTTT	GAACCCTTGG	TGCCGCATAA
501	GAGCAGCCTA	TTGTGGTTCC	TGCTGATGTG	CCATTTTCAGA	TTGCATAATC
551	TTGCTCCTAT	TCCTGGTACT	GAGTCTTTTA	TGGGATGGTG	GGAGAAGATT
601	GACAAAAGTT	CTGGAGATTG	GCTATGAGGG	GTCTCAATTC	TCTTATTGCC
651	TTGGGCGCCT	GGATTCTTTG	GAATCATTGG	AATCGAATTG	TCTTTGATGG
701	ATTATCCCCT	ACCCCTAGTG	TTTCCGCAGC	TCTCTGTCAG	GCAAGGGAGA
751	AACAACAGTT	GTGGGAAATG	GCAGGAGCTA	AGGGTCTCTC	CTTTCTTGCC
801	GCCACCAACA	GTGTCCCAT A	GCTGCTTGTT	G TAGGGTTGT	ATTTGTTTAT
851	CATGGATCGT	TTTCTTTTTT	TTTGTTTTAA	ACTTCCTTAG	GGTTTTTTTT
901	TGTGTGTACT	GGGGTCTATT	TTGGACCTTC	TTTTCCTAAT	ACTTAATATA
951	ATGATGCGTA	GTTCTCCTGC	GCTTTCGAGA	AAAAAAATCA	CCACTATTGT
1001	CATCATTGTC	CTATCACCCCT	G TAGAAGTCT	CTCTAGAGGG	CCATCAACGG
1051	TAGCTGCACA	CAACAAACGC	ATAGCTGCAT	CGGCTGCCAG	CCCCTCCAAT
1101	CGGGCATCAC	ATGGAATCAG	ATTTGATCCT	GCTGCACACG	TACACCACCA
1151	CACCCATAGC	CATTGTCCTC	ACATGTGCGC	CCC GCCACAG	AGGGCACCAC
1201	CCCCTGCCCT	TGTGTCGCCG	GATTTTCCCG	CTAGGTCCTC	GGCCTCGGCG
1251	CCCCGGATCG	ATGCCGCTGC	CATCGCCTGG	GCATGGACAC	CAGTGCGCCG
1301	TGCATCCGGT	CCTGAGACTG	AATTTTGGAC	CCCAGACAAG	GAAGCAGCCA
1351	TGGGCACCGT	CCTGGTGGCG	CTGAGCCGCG	CGCAGCGACA	GCATACGACA
1401	GATCCATACC	AAGGGCCATC	GGACCCACAC	TCAAGGATGG	CAGAATCCAT
1451	TCCGCTGGCG	CTAGAGCCAA	TGATGGCAAT	GTCCCGGCAG	GCCGGCATAG
1501	CTGCGCTTGC	GTGCTCTCAT	CGTGACCCCC	AGCCTTTGGT	GGCATGCTCC
1551	GGCGGTGGCG	AGGGGGAGGG	G TAGGGAGGA	GGCTCAACGG	TGCGTGCGAT
1601	GGGCTTCGCT	CTAACCGTCT	AGGCAGGCAA	CGCAGGGGTT	GGGGGGGGAA
1651	TTGCGATGCT	CATCACAATA	TATACACATT	ATGGACTTTA	ATTTTCGTAA
1701	TAATGCTTCT	GTGTTTCTTT	TGAACTATTT	TTGTGTTACA	GAAAAGATAT
1751	ATGGAGCTTA	CTAAAGGTGC	AGCTGACAAT	TACCACCGAT	CTTGGTGGAA
1801	AAGACATGGA	GTTGTTCTTG	ATGGAGAGAT	CGCAGCTCTG	TTCTTTAAGC
1851	ATGGAAATTA	TGACCTGGCT	GTGAAATCCT	ATGAGAAAGT	TTGTGCTCTC
1901	TATTCTGCAG	AAGGCTGGGA	AGAGCTGTTG	GCAGATGTTT	TTCTTGATCT
1951	TGCAGAAATG	CAGAAGATTG	TTAATGATGA	AGCTGGTTAT	TTGGCTTCTT
2001	GTGTAAAGTT	ACTATCGCTG	GACAGTGGCT	TGTTTTCATC	TAAAGAGCGG
2051	CAAGGTTTCC	AGTCAGAAAT	TGTTCTGACT	GCTCACAGTG	AAATCTGATC
2101	ATGAGCGGAG	AATTAAGGCG	GGAAACGACA	ATCTGATCAT	GAGCGGAGAA
2151	TTAAGGGAGT	CACGTTATGA	CCCCCGCCGA	TGACGCGGGA	CAAGCCGTTT
2201	TACGTTTGGA	ACTGACAGAA	CCGCAACGTT	GAAGGAGCCA	CTCAGCAAGC
2251	TTGATATCGA	ATTCTGTCAG	CCCTATGATT	TAGAATAATA	TACAAATATA
2301	TTACATAAAA	AATATATTAA	TTGAATTAGT	GTTGTCTAAT	TTATAATTAT
2351	TAGAATGTAA	TTCAATTCCA	ACGAAAACAAC	GGGGCCTTAG	GTTTAAATATC
2401	TTCCTTACAC	TGCGAAAATG	TTGTTACACT	TGCCAAAAAA	AATCAATCGC
2451	ATATTTACCT	TACAAGGACA	TATTTTAGCA	AAATGCTATA	GACATGAATC
2501	CAACGTAATC	AATAGAGTGA	GATTTACTGG	TAAACTACCA	ATTGCTCATC
2551	TGCTCGGTAC	CAACCAGCCT	TTCTATTAC	CATGCACATG	TTGCCTCTCA
2601	ACTGCAGCAT	CTTTCAAGCC	GTGAGCAGAC	ATGTTGCAGA	TCGAAGTAAG
2651	GTATATATGT	GCATAGTCTC	CTAATTCTTC	ATCTTCAACC	TCTAGCTGAT
2701	TGATCTCTGG	TATTTACCAC	TCTTTCCTTC	CTTCCTTCCT	TCAATTCTAA

Figure 11, Cont'.

2751	ATACCACAAA	TCAAAGTTGC	TTTGCCATGG	AGAAGAGGAA	CCTGCAGTGG
2801	CGGCGAGGGC	GTGATGGCAT	CGTGCAGTAC	CCTCACCTCT	TCTTCGCGGC
2851	CCTGGCGCTG	GCCCTCCTAG	TCGCGGACCC	GTTTCGGCCTC	AGTCCGCTGG
2901	CCGAGGTCGA	CTACCGGCCG	GTGAAGCACG	AGCTCGCGCC	GTACGGGGAG
2951	GTCATGGGCA	GCTGGCCCAG	AGACAATGCC	AGCCGGCTCA	GGCGCGGGAG
3001	GCTGGAGTTC	GTCGGCGAGG	TGTTTCGGGCC	GGAGTCTATC	GAGTTCGATC
3051	TCCAGGGCCG	CGGGCCGTAC	GCCGGCCTCG	CCGACGGCCG	CGTCGTGCGG
3101	TGGATGGGCG	AGGAGGCCGG	GTGGGAGACG	TTCGCCGTCA	TGAATCCTGA
3151	CTGGTAAGTG	CTCGATATCG	CTCCGGCGTC	CACTCGTTAC	ATGCTATAAT
3201	ATAGTAGTAC	TAAGATATTT	TGATCTGATT	TTTTGCATTTC	TTGGGAGAAA
3251	CGTCATGCAA	AATTTGTTGT	TTCTTGCCAA	AGGTCAGAAG	AAGTCTGTGC
3301	CAATGGAGTG	AACTCAACGA	CGAGGAAGCA	GCACGAGAAG	GAGGAGTTCT
3351	GCGGCCGGCC	GCTCGGCCTG	AGGTTCACG	GGGAGACCGG	CGAGCTCTAC
3401	GTCGCCGACG	CGTACTACGG	TCTCATGGTC	GTTGGCCAGA	GCGGCGGCGT
3451	GGCGTCCTCC	GTCGCGAGGG	AAGCCGACGG	GGACCCCATC	CGGTTTCGCGA
3501	ACGACCTCGA	TGTGCACAGG	AATGGATCCG	TATTCCTTAC	TGACACGAGC
3551	ATGAGATACA	GCAGAAAAGTG	AGCAAAAGCGA	CGTAACAATC	CGGCTTCTCA
3601	TTTTCAAACG	CCTCTGTATT	CTCTGCTGAA	AGAGTAGCTC	ACCAGACAAG
3651	AGCTGAATTT	GCAGGGACCA	TCTGAACATC	CTGTTAGAAG	GAGAAGGCAC
3701	CGGGAGGCTG	CTCAGGTATG	ATCCAGAAAC	AAGCGGTGTC	CATGTCGTGC
3751	TCAAGGGGCT	GGTGTTCCTA	AACGGCGTGC	AGATCTCAGA	GGACCATCAG
3801	TTTCTTCTCT	TCTCCGAGAC	AACAACTGC	AGGTAACAAA	AATACTATCT
3851	GACGATGCTC	ATGATTCTAC	CTATCCATA	GTCAATGAACA	CAAACACAC
3901	GAATCTGGCC	TTGACCAGGA	TAATGAGGTA	CTGGCTGGAA	GGCCCAAGAG
3951	CGGGCGAGGT	AGAGGTGTTT	GCGAACCTGC	CGGGCTTCCC	CGACAACGTG
4001	CGCTCCAACG	GCAGGGGGCCA	GTTCTGGGTG	GCGATCGACT	GCTGCCGGAC
4051	GCCGGCGCAG	GAGGTGTTTC	CCAAGAGGCC	GTGGCTCCGG	ACCTGTACT
4101	TCAAGTTCCC	GCTGTCGCTC	AAGGTGCTCA	CTTGGAAGGC	CGCCAGGAGG
4151	ATGCACACGG	TGCTCGCGCT	CCTCGACGGC	GAAGGGCGCG	TCGTGGAGGT
4201	GCTCGAGGAC	CGGGGCCACG	AGGTGATGAA	GCTGGTGAGC	GAGGTGCGGG
4251	AGGTGGGCCG	CAAGCTGTGG	ATCGGAACCG	TGGCGCACAA	CCACATCGCC
4301	ACCATCCCCCT	ACCCTTTAGA	GGACTAACCA	TGATCTATGC	TGTTTTCAATG
4351	CCTCCTAATC	TGTGTACGTC	TATAAATGTC	TAATGCAGTC	ACTGGTTGTA
4401	ATCTTGTTTG	TGTTTGCCAA	ATTGGCATAA	TAATGGACAG	ATTCAATGGG
4451	CATTGGTGCT	GTAGTCGCAT	CACACTAATT	GAATGGGATC	ATGTTGAGCT
4501	CTCACTTTGC	TACAATTTGC	TCCAGCTTGT	ACGGTTGTAC	CCTCTTGCTC
4551	GTCTATAGTA	AGGGCCATCT	AAAAAAAACT	CAAATTAGAT	CTGCAATACA
4601	AGTATGATTG	GGCCGAATTT	GGATTGTCAC	GGGTCCGCGA	CCGCGAATTG
4651	GGCTCGGTTT	GATTTAGCCG	ACATAGTAGT	GACCGACCCG	AGCCGGCGGC
4701	GAGCAAACCC	GAGCGGACGC	CGCCATGATC	AAGCTATCGG	ACGGCCGCTC
4751	TAGACTAGT	GGATCAGCTT	GCATGCCTGC	AGGTGCACTC	TAGGAGATCT
4801	GCACCGGACA	CTGTCTGGTG	GCATACCAGA	CAGTCCGGTG	TGCCAGATCA
4851	GGGCACCCCT	CGGTTCCCTT	GCTCCTTTGC	TTTTGAACCC	TAACCTTGAT
4901	CGTTTATTGG	TTTGTGTTGA	ACCTTTATGC	ACCTGTGGAA	TATATAATCT
4951	AGAACAAACT	AGTTAGTCCA	ATCATTTGTG	TTGGGCATTTC	AACCACCAAA
5001	ATTATTTATA	GGAAAAGGTT	AAACCTTATT	TCCCTTTCAA	TCTCCCCCTT
5051	TTTGGTGATT	GATGCCAACA	CAAACCAAAG	AAAATATATA	AGTGCAGAAT
5101	TGAACTAGTT	TGCATAAGGT	AAGTGCATAG	GTTACTTAGA	ATTAAATCAA
5151	TTTATACTTT	TACTTGATAT	GCATGGTTGC	TTTCTTTTAT	TTTAACATTT
5201	TGGACCACAT	TTGCACCACT	TGTTTTGTTT	TTTGCAAATC	TTTTTGGAAA
5251	TTCTTTTTTCA	AAGTCTTTTT	CAAAATAGTCA	AAGGTATATG	AATAAGATTG
5301	TAAGAAGCAT	TTTCAAGATT	TGAAATTTCT	CCCCCTGTTT	CAAATGCTTT
5351	TCCTTTGACT	AAACAAAACT	CCCCCTGAAT	AAAATTCTCC	TCTTAGCTTT
5401	CAAGAGGGTT	TTAAATAGAT	ATCAATTGGA	AATATATTTA	GATGCTAATT
5451	TTGAAAATAT	ACCAATTGAA	AATCAACATA	CCAATTTGAA	ATTAAACATA
5501	CCAATTTAAA	AAATTTCAAA	AAGTGGTGGT	GCGGTCCCTT	TGCTTTGGGC
5551	TTAATATTTT	TCCCCCTTTG	GCATTAATCG	CCAAAAACGG	AGACTTTGTG
5601	AGCCATTTAT	ACTTCTCTCC	CATTGGTAAA	TGAAATATGA	GTGAAAGATT

Figure 11, Cont'.

5651	ATACCAAATT	TGGACAGTGA	TGCGGAGTGA	CGGCGAAGGA	TAAACGATAC
5701	CGTTAGAGTG	GAGTGGAAAGC	CTTGCTTCG	CCGAAGACTC	CATTTCCCTT
5751	TCAATCTACG	ACTTAGCATA	GAAATACACT	TGAAAACACA	TTAGTCGTAG
5801	CCACGAAAGA	GATATGATCA	AAGGTATACA	AATGAGCTAT	GTGTGTAATG
5851	TTTCAATCAA	AGTTTCGAGA	ATCAAGAATA	TTTAGCTCAT	TCCTAAGTTT
5901	GCTAAAGGTT	TTATCATCTA	ATGGTTTGGT	AAAGATATCG	ACTAATTGTT
5951	CTTTGGTGCT	AACATAAGCA	ATCTCGATAT	CACCCCTTTG	TTGGTGATCC
6001	CTCAAAAAGT	GATACCGAAT	GTCTATGTGC	TTAGTGCGGC	TGTGTTCAAC
6051	GGGATTATCC	GCCATGCAGA	TAGCACTCTC	ATTGTCACAT	AGGAGAGGGA
6101	CTTTGCTCAA	TTTGTAGCCA	TAGTCCCTAA	GGTTTTGCCT	CATCCAAAGT
6151	AATTGCACAC	AACAATGTCC	TGCGGCAATA	TACTTGGCTT	CGGCGGTAGA
6201	AAGAGCTATT	GAGTTTTGTT	TCTTTGAAGT	CCAAGACACC	AGGGATCTCC
6251	CTAGAACTG	ACAAGTCCCT	GATGTGCTCT	TCCTATCAAT	TTTACACCTT
6301	GCCCAATCGG	CATCTGAATA	TCCTATTAAA	TCAAAGGTGG	ATCCCTTGGG
6351	GTACCAAAGA	CCAAATTTAG	GAGTGTA AAC	TAAATATCTC	ATGATTCTTT
6401	TCACGGCCCT	AAGGTGAACT	TCCTTAGGAT	CGGCTTGGA	TCTTGCACAC
6451	ATGCATATAG	AAAGCATACT	ATCTGGTCGA	GATGCACATA	AATAGAGTAA
6501	AGATCCTATC	ATCGACCGGT	ATACCTTTTG	GTCTACGGAT	TTACCTCCCG
6551	TGTCGAGGTC	GAGATGCCCA	TTAGTTCCCA	TGGGTGTCCT	GATGGGCTTG
6601	GCATCCTTCA	TTCCAAACTT	GTTGAGTATG	TCTTGAATGT	ACTTTGTTTG
6651	GCTGATGAAG	GTGCCATCTT	GGAGTTGCTT	GA CTTGAAAT	CCTAGAAAAT
6701	ATTTCAACTT	CCCCATCATA	GACATCTCGA	ATTTCCGGAAT	CATGATCCTA
6751	CTAAACTCTT	CACAAGTAGA	TTTGTTAGTA	GACCCAAATA	TAATATCATC
6801	AACATAAATT	TGGCATACAA	ACAAAAC TTT	TGAAATGGTT	TGAGTAAAGA
6851	GAGTAGGATC	GGCTTTACTG	ACTCTGAAGC	CATTAGTGAT	AAGAAAATCT
6901	CTTAGGCATT	CATACCATGC	TGTTGGGGCT	TGCTTGAGCC	CATAAAGCGC
6951	CTTTGAGAGT	TTATAAACAT	GGTTAGGGTA	CTCACTATCT	TCAAAGCCGA
7001	GAGGTTGCTC	AACATAGACC	TATTCACCCC	ATTTGATCAC	TTTTTTTGGTC
7051	CTTCAGGATC	TAATAGTTAT	GTATAATTTA	GAGTCTCTTG	TTTAATGGCC
7101	AGATATTTCT	AATTAATCTA	AGAATTTATG	ATATTTTTTA	ATTTTTTTATC
7151	ATGTCTGATG	AGAATTAACA	TAAAGGCTCA	ATTGGGTCCT	GAATTAATAA
7201	TAGAGTGAAA	ATTAATCCAG	AGGCTCTATT	AGAACCTTCA	ATTAGTAATA
7251	CCAAGATATA	TATAAGATAG	TAGAGTATAG	TTTAAATGTT	GGCATTGTTT
7301	ATTCTTTCTT	TTGTTATTTA	ATTTATGCTT	TCCACGGTGG	TTAGTGGTTA
7351	CTTCTGAAGG	GTCCAAAATA	TGCATGAAGA	GTTTGAGGAC	AAGAAGTCTG
7401	CCCTAAAAAT	AGCGATGCAA	AGGCATGGTG	TCCAAGCCAT	ACATATAGCG
7451	CACTAATTTT	ATCAGCAGAA	CAATGGTATT	TATAGGTCCT	AGTGCCCAGG
7501	CAACAAGAGA	CACGAATAAA	GCATCGATCA	CGACACCATG	GCGGCGACAA
7551	TGGCAGTGAC	GACGATGGTG	ACGAGGAGCA	AGGAGAGCTG	GTCTGTCATTG
7601	CAGGTCCCGG	CGGTGGCATT	CCCTTGGAAG	CCACGAGGTG	GCAAGACCGG
7651	CGGCCTCGAG	TTCCCTCGCC	GGGCGATGTT	CGCCAGCGTC	GGCCTCAACG
7701	TGTGCCCGGG	CGTCCCGGCG	GGGCGCGACC	CGCGGGAGCC	CGATCCCAAG
7751	GTCGTCCGGG	CGGCCTGCGG	CCTGGTCCAG	GCACAAGTCC	TCTTCCAGGG
7801	GTTTAACTGG	GAGTCGTGCA	AGCAGCAGGG	AGGCTGGTAC	AACAGGCTCA
7851	AGGCCCAGGT	CGACGACATC	GCCAAGGCCG	GCGTCACGCA	CGTCTGGCTG
7901	CCTCCACCCT	CGCACTCCGT	CTCGCCACAA	GGCTACATGC	CAGGCCGCCCT
7951	ATACGACCTG	GACGCGTCCA	AGTACGGCAC	GGCGGCGGAG	CTCAAGTCCC
8001	TGATAGCGGC	GTTCCACGGC	AGGGGCGTGC	AGTGCGTGCC	GGACATCGTC
8051	ATCAACCACC	GGTGCGCGGA	AAAGAAGGAC	GCGCGCGGCG	TGTACTGCAT
8101	CTTCGAGGGC	GGGACTCCCG	ACGACCGCCT	GGACTGGGGC	CCCGGGATGA
8151	TCTGCAGCGA	CGACACGCAG	TACTCGGACG	GGACGGGGCA	CCGCGACACG
8201	GGCGAGGGGT	TCGCGGCGGC	GCCCAGACATC	GACCACCTCA	ACCCGCGCGT
8251	GCAGCGGGAG	CTCTCCGCCCT	GGCTCAACTG	GCTCAGGTCC	GACGCCGTGG
8301	GGTTCGACGG	CTGGCGCCTC	GACTTCGCCA	AGGGCTACTC	GCCGGCCGTC
8351	GCCAGAATGT	ACGTGGAGAG	CACGGGGCCG	CCGAGCTTCG	TCGTCGCGGA
8401	GATATGGAAC	TCGCTGAGCT	ACAGCGGGGA	CGGCAAGCCG	GCGCCCAACC
8451	AGGACCAGTG	CCGGCAGGAG	CTGCTGGACT	GGACGCGGGC	CGTCGGCGGG
8501	CCCGCCATGG	CGTTCGACTT	CCCCACCAAG	GGCCTGCTGC	AGGCGGGCGT

Figure 11, Cont'.

8551	GCAGGGGGAG	CTGTGGCGGC	TGCGCGACAG	CTCCGGCAAC	GCGGCCGGCC
8601	TGATCGGGTG	GGCGCCCGAG	AAGGCCGTCA	CCTTCGTCGA	CAACCATGAC
8651	ACCGGGTCGA	CGCAGAAGCT	CTGGCCGTTC	CCATCCGACA	AGGTCATGCA
8701	GGGCTACGCC	TACATCCTCA	CCCATCCAGG	AGTCCCCTGC	ATTTTCTACG
8751	ACCACATGTT	CGACTGGAAC	CTGAAGCAGG	AGATATCCAC	GCTGCTCGCC
8801	ATCAGGGCGC	GGAACGGCAT	CCGCGCCGGG	AGCAAGCTGC	GGATCCTCGT
8851	GGCGGACGCG	GACGCGTACG	TGGCCGTTCG	CGACGAGAAG	GTCATGGTGA
8901	AGATCGGGAC	AAGGTACGGC	GTGAGCAGCG	TGGTCCCGTC	GGATTTCCAC
8951	CCGGCGGCGC	ACGGCAAGGA	CTACTGCGTC	TGGGAGAAAG	CGAGCCTCCG
9001	CGTCCCGGCG	GGGCGCCACC	TCTAGCAGCT	CAGATTGCTC	AGTCTTGTGC
9051	TGCATTGCAA	ACACAGCAGC	ACGACACTGC	ATAACGTCTT	TTCCTTGAGA
9101	TCTGACAAAG	CAGCATTAGT	CCGTTGATCG	GTGGAAGACC	ACTCGTCAGT
9151	GTTGAGTTGA	ATGTTTGATC	AATAAAATAC	GGCAATGCTG	TAAGGGTTGT
9201	TTTTTATGCC	ATTGATAATA	CACTGTACTG	TTCAGTTGTT	GAACTCTATT
9251	TCTTAGCCAT	GCCAAGTGCT	TTTCTTATTT	TGAATAACAT	TACAGCAAAA
9301	AGTTGAAAGA	CAAAAAAAAAA	AACCCCCGAA	CAGAGTGCTT	TGGGTCCCAA
9351	GCTACTTTAG	ACTGTGTTTCG	GCGTTCCCCC	TAAATTTCTC	CCCCTATATC
9401	TCACTCACTT	GTCACATCAG	CGTTCTCTTT	CCCCTATATC	TCCACGTCGA
9451	CGCGGCCGAT	CCCCCGGGCT	GCAGGAATTC	CCATGGAGTC	AAAGATTCAA
9501	ATAGAGGACC	TAACAGAACT	CGCCGTAAAG	ACTGGCGAAC	AGTTCATACA
9551	GAGTCTCTTA	CGACTCAATG	ACAAGAAGAA	AATCTTCGTC	AACATGGTGG
9601	AGCACGACAC	GCTTGTCTAC	TCCAAAAATA	TCAAAGATAC	AGTCTCAGAA
9651	GACCAAAAGG	CAATTGAGAC	TTTTCAACAA	AGGGTAATAT	CCGGAAACCT
9701	CCTCGGATTC	CATTGCCAG	CTATCTGTCA	CTTTATTGTG	AAGATAGTTG
9751	AAAAGGAAGG	TGGCTCCTAC	AAATGCCATC	ATTGCGATAA	AGGAAAGGCC
9801	ATCGTTGAAG	ATGCCTCTGC	CGACAGTGGT	CCCAAAGATG	GACCCCCACC
9851	CACGAGGAGC	ATCGTGGAAG	AAGAAGACGT	TCCAACCACG	TCTTCAAAGC
9901	AAGTGGATTG	ATGTGATATC	TCCACTGACG	TAAGGGATGA	CGCACAATCC
9951	CACTAAGCTG	ACCGAAGCTG	GCCGCTCTAG	AACTAGTGGA	TCTCGATGTG
10001	TAGTCTACGA	GAAGGGTTAA	CCGTCTCTTC	GTGAGAATAA	CCGTGGCCTA
10051	AAAATAAGCC	GATGAGGATA	AATAAAATGT	GGTGGTACAG	TACTTCAAGA
10101	GGTTTACTCA	TCAAGAGGAT	GCTTTTCCGA	TGAGCTCTAG	TAGTACATCG
10151	GACCTCACAT	ACCTCCATTG	TGGTGAAATA	TTTTGTGCTC	ATTTAGTGAT
10201	GGGTAAATTT	TGTTTATGTC	ACTCTAGGTT	TTGACATTTT	AGTTTTGCCA
10251	CTCTTAGGTT	TTGACAAATA	ATTTCCATTC	CGCGGCAAAA	GCAAAACAAT
10301	TTTATTTTAC	TTTTACCACT	CTTAGCTTTC	ACAATGTATC	ACAAATGCCA
10351	CTCTAGAAAT	TCTGTTTATG	CCACAGAAATG	TGAAAAAAAAA	CACTCACTTA
10401	TTTGAAGCCA	AGGTGTTTCAT	GGCATGGAAA	TGTGACATAA	AGTAACGTTT
10451	GTGTATAAGA	AAAAATTGTA	CTCCTCGTAA	CAAGAGACGG	AAACATCATG
10501	AGACAATCGC	GTTTGGAAGG	GTTTGCATCA	CCTTTGGATG	ATGCGCATGA
10551	ATGGAAATCGT	CTGCTTGCTA	GCCCTCGCCT	ACCGCCCACT	GAGTCCGGGC
10601	GGCAACTACC	ATCGGCGAAC	GACCCAGCTG	ACCTCTACCG	ACCGGACTTG
10651	AATGCGCTAC	CTTCGTCAGC	GACGATGGCC	GCGTACGCTG	GCGACGTGCC
10701	CCCGCATGCA	TGGCGGCACA	TGGCGAGCTC	AGACCGTGCG	TGGCTGGCTA
10751	CAAATACGTA	CCCCGTGAGT	GCCCTAGCTA	GAAACTTACA	CCTGCAACTG
10801	CGAGAGCGAG	CGTGTGAGTG	TAGCCGAGTA	GATCCCCCGG	GCTGCAGGTC
10851	GACTCTAGAG	GATCCACCGG	TCGCCACCAT	GGCCTCCTCC	GAGAACGTCA
10901	TCACCGAGTT	CATGCGCTTC	AAGGTGCGCA	TGGAGGGCAC	CGTGAACGGC
10951	CACGAGTTTC	AGATCGAGGG	CGAGGGCGAG	GGCCGCCCTT	ACGAGGGCCA
11001	CAACACCGTG	AAGCTGAAGG	TGACGAAGGG	CGGCCCCCTG	CCCTTCGCCT
11051	GGGACATCCT	GTCCCCCCAG	TTCCAGTACG	GCTCCAAGGT	GTACGTGAAG
11101	CACCCCGCCG	ACATCCCCGA	CTACAAGAAG	CTGTCCTTTC	CCGAGGGCTT
11151	CAAGTGGGAG	CGCGTGATGA	ACTTCGAGGA	CGGCGGCGTG	GCGACCGTGA
11201	CCCAGGACTC	CTCCCTGCAG	GACGGCTGCT	TCATCTACAA	GGTGAAGTTC
11251	ATCGGCGTGA	ACTTCCCCTC	CGACGGCCCC	GTGATGCAGA	AGAAGACCAT
11301	GGGCTGGGAG	GCCTCCACCG	AGCGCCTGTA	CCCCCGCGAC	GGCGTGCTGA
11351	AGGGCGAGAC	CCACAAGGCC	CTGAAGCTGA	AGGACGGCGG	CCACTACCTG
11401	GTGGAGTTCA	AGTCCATCTA	CATGGCCAAG	AAGCCCGTGC	AGCTGCCCCG

Figure 11, Cont'.

11451	CTACTACTAC	GTGGACGCCA	AGCTGGACAT	CACCTCCCAC	AACGAGGACT
11501	ACACCATCGT	GGAGCAGTAC	GAGCGCACCG	AGGGCCGCCA	CCACCTGTTT
11551	CTGTAGCGGC	CCATGGATAT	TCGAACGCGT	AGGTACCACA	TGGTTAACCT
11601	AGACTTGTC	ATCTTCTGGA	TTGGCCAACT	TAATTAATGT	ATGAAAATAA
11651	AGGATGCACA	CATAGTGACA	TGCTAATCAC	TATAATGTGG	GCATCAAAGT
11701	TGTGTGTTAT	GTGTAATTAC	TAGTTATCTG	AATAAAAAGAG	AAAGAGATCA
11751	TCCATATTTT	TTATCCTAAA	TGAATGTCAC	GTGTCTTTAT	AATTCTTTGA
11801	TGAACCAGAT	GCATTTTCATT	AACCAAATCC	ATATACATAT	AAATATTAAT
11851	CATATATAAT	TAATATCAAT	TGGGTTAGCA	AAACAAATCT	AGTCTAGGTG
11901	TGTTTTGCGA	ATGCGGCCGC	CACCGCGGTG	GAGCTCGAAT	TCAGTACATT
11951	AAAAACGTCC	GCAATGTGTT	ATTAAGTTGT	CTAAGCGTCA	ATTTGTGATG
12001	GAGATCCTGG	TACACTATCT	GTAGCAGTTT	GGAATGGCTT	CCCAGATGAC
12051	ATCACACTGG	AGTCTCTCAG	TTTAAGGTTG	TCAGCTTCTT	CTAGTGCAGA
12101	<u>TGAAGGTATC</u>	<u>AAGGTACAAG</u>	<u>TACTGTGTTA</u>	<u>GCATTACTGA</u>	<u>TTGCTACCCT</u>
12151	<u>TTTCCTCTGT</u>	<u>GGCATGTCTT</u>	<u>ATTCTGTTAT</u>	<u>TCATGGTTTG</u>	<u>GATGCACTAA</u>
12201	<u>TATGTTTTCA</u>	<u>AGTAAAATTC</u>	<u>AGTTCCATCC</u>	<u>TTGCAGAACA</u>	<u>GATTTTCATAA</u>
12251	<u>ATCAAAACTA</u>	<u>CTATGATAAA</u>	<u>GTATATCAAA</u>	<u>CTCTAGAGAA</u>	<u>ATGACCGCAC</u>
12301	<u>ATAACATATT</u>	<u>CTTCAGTCAC</u>	<u>CTAATGGTTT</u>	<u>AATGTTGTCA</u>	<u>TCCATGCAGG</u>
12351	<u>CAATTAAAAG</u>	<u>TTCAGATTCT</u>	<u>CATGTTCTAG</u>	<u>TACCAGGTAG</u>	<u>AAATATCATC</u>
12401	<u>TCTTTTGACA</u>	<u>TTCTCGTCA</u>	<u>AAAGCCTGGC</u>	<u>TCCTATGTGT</u>	<u>TGGGTGCTCT</u>
12451	<u>CACTGGACAG</u>	<u>ATTGGCAAGC</u>	<u>TGTCATTGAG</u>	<u>ATCACATGGA</u>	<u>TTTTCCCAAG</u>
12501	<u>ATGGTCCAGT</u>	<u>TGAAACTGAT</u>	<u>GAATTTATGA</u>	<u>GCTTTGAGAA</u>	<u>ACCGACAAGA</u>
12551	<u>CCTGTTTGA</u>	<u>AGGTAATTGC</u>	<u>TAAAATGAGC</u>	<u>TGAAGATTAC</u>	<u>TTAGATAGTTT</u>
12601	<u>TTCTGTGGGC</u>	<u>ATGACTGGAG</u>	<u>CTAGATCCCT</u>	<u>TAGAAGTTTC</u>	<u>TGGCCTTAAG</u>
12651	<u>TTTTTTGGGG</u>	<u>TTTCCCTTA</u>	<u>AGATTTTTTT</u>	<u>TTTCTTTTTT</u>	<u>CCATTTTTTC</u>
12701	<u>CATAACTGGC</u>	<u>ACCTTGATAT</u>	<u>GGGTCTATTT</u>	<u>TAGGCCTTTC</u>	<u>TTTCTCTTCT</u>
12751	<u>TAATATATTG</u>	<u>ATGTACAGTT</u>	<u>CTCCTACAGT</u>	<u>TCGAGAAAAA</u>	<u>AAGAGCTGAC</u>
12801	<u>GATCTGAACT</u>	<u>GTTTTATAAA</u>	<u>ATCGTAGCCC</u>	<u>TACCCCATGT</u>	<u>CACTAAACTG</u>
12851	<u>CCATAAGAGA</u>	<u>TACTATCCAT</u>	<u>ACTTTCAAAA</u>	<u>CAAAACCGGA</u>	<u>GCATAGTTTC</u>
12901	<u>CTCTAATTAG</u>	<u>TGAACATGGT</u>	<u>CAGTTATATT</u>	<u>GTTTAAGACA</u>	<u>GATATGTTCT</u>
12951	<u>CATTGCATTT</u>	<u>GTTTCTCTGT</u>	<u>TGTAGGTGAG</u>	<u>AAAACCAAGG</u>	<u>GCTTTAGTTG</u>
13001	<u>ATATTACACC</u>	<u>TGCTGTGTCC</u>	<u>TCTGCTTTGC</u>	<u>TTATGAATGA</u>	<u>GCTCCAATGG</u>
13051	<u>ATTGGATTAA</u>	<u>TCGTAAAGCC</u>	<u>TATAGACTAT</u>	<u>TCTTTAAAAG</u>	<u>GTGGAATATT</u>
13101	<u>GCATATCGAT</u>	<u>GCTGGCGCTG</u>	<u>AACTGAAAAAT</u>	<u>TGAGGAGTCT</u>	<u>CAGATGATTG</u>
13151	<u>AAATAGAAAAT</u>	<u>TTACGTAAAGT</u>	<u>GATATGGAGT</u>	<u>GTGCTAATTC</u>	<u>TGCCAACAGC</u>
13201	<u>TCCATCAAAG</u>	<u>CTGGAAAGGT</u>	<u>TGAAAAGGTA</u>	<u>CCTATTGAAA</u>	<u>ATGGAAAGAT</u>
13251	<u>AGAACTTCCT</u>	<u>GATTGGGCTA</u>	<u>GTGATGTGAC</u>	<u>TACTCTTGTT</u>	<u>TGGTTTCCTG</u>
13301	<u>TTCTGTGCTAT</u>	<u>TGATGACACA</u>	<u>ATTGCAAGAG</u>	<u>GAGAATCCCT</u>	<u>AGGTCTGTAT</u>
13351	<u>GTAACAAGTG</u>	<u>ATAAATTCAC</u>	<u>TATGTTATGT</u>	<u>TTCAATCAGT</u>	<u>TAGTTGACCT</u>
13401	<u>TTCTTTGTCC</u>	<u>TTGTTTCAGT</u>	<u>GTCTCAGAAA</u>	<u>CAGAGCGTTG</u>	<u>TAGATGGGAT</u>
13451	<u>GAGAATGATT</u>	<u>GCTCTCAAGC</u>	<u>TTGAGTTTGG</u>	<u>AGTTTTCCTG</u>	<u>AACCAAGTGT</u>
13501	<u>TTGAAAGGTA</u>	<u>TCTCTCTGTT</u>	<u>TTTCCCTCA</u>	<u>AACATGCAGA</u>	<u>AGAGCTGTGT</u>
13551	<u>CATGTCTGCC</u>	<u>ATTGTATAAG</u>	<u>AAAAGATGAG</u>	<u>ATACTTCGAT</u>	<u>ACATTGAACT</u>
13601	<u>TTAGATATAG</u>	<u>ATACTCAACA</u>	<u>AATTTATTAT</u>	<u>TATGATTTTC</u>	<u>AGGACCATTG</u>
13651	<u>CAGTTCATTT</u>	<u>TACTAACCCA</u>	<u>TTCCATGTAA</u>	<u>GCACACGCGT</u>	<u>CGTGGACAAG</u>
13701	<u>TGCAATGATG</u>	<u>GAGCTCTACT</u>	<u>TCTACAGGTA</u>	<u>AAGTTCTTTC</u>	<u>TTGTCTGGCA</u>
13751	<u>CATGATTGTC</u>	<u>ATTGTTGTAT</u>	<u>TCCTAGATTT</u>	<u>AAAACACACA</u>	<u>CACACACACA</u>
13801	<u>CATATATATA</u>	<u>CAGGGATATT</u>	<u>GGGAGCCCTG</u>	<u>CTCTTCCAAA</u>	<u>CATATATACT</u>
13851	<u>AGAAGCCCCA</u>	<u>GCCGTGCCCT</u>	<u>GCACCACCCG</u>	<u>ACCGGCTTGG</u>	<u>CCACCTAGAT</u>
13901	<u>CCCGTGACAC</u>	<u>CGTCCATCCC</u>	<u>CTGCATGCGG</u>	<u>ATATTTGTTT</u>	<u>CCATTTGCAT</u>
13951	<u>GTGCGAAAAA</u>	<u>TTTGGAAGGT</u>	<u>GTATCTGTTT</u>	<u>CCTGCTGCAT</u>	<u>GCGCATAA</u>

FIGURE 12

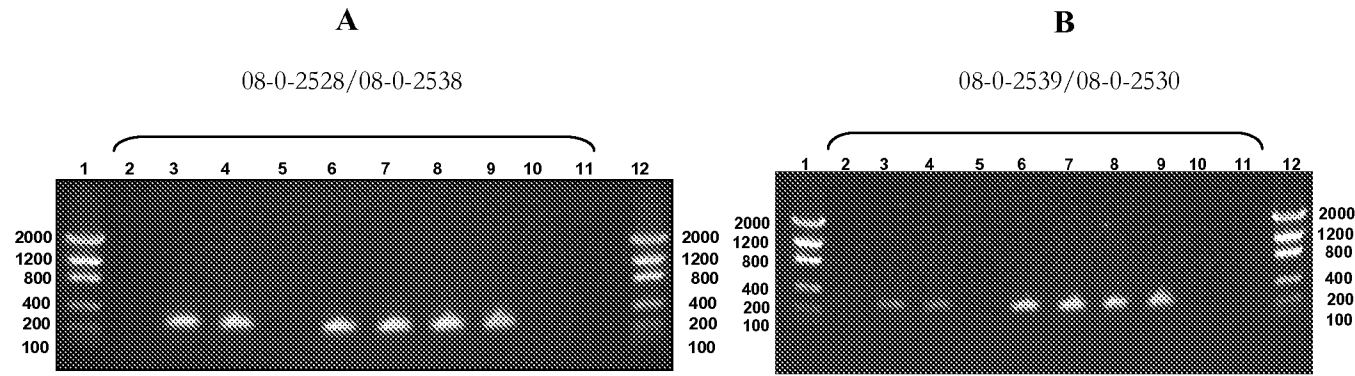


FIGURE 13

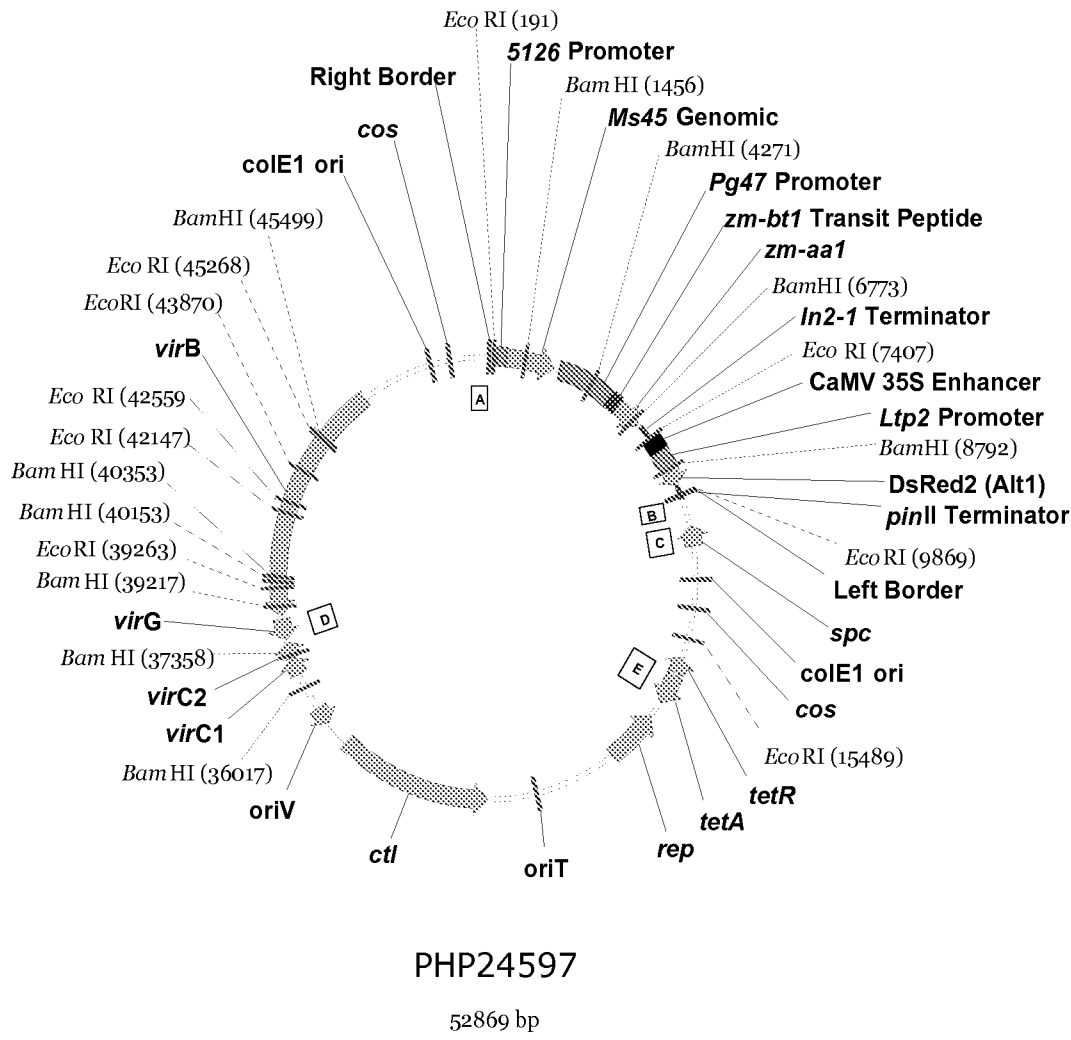
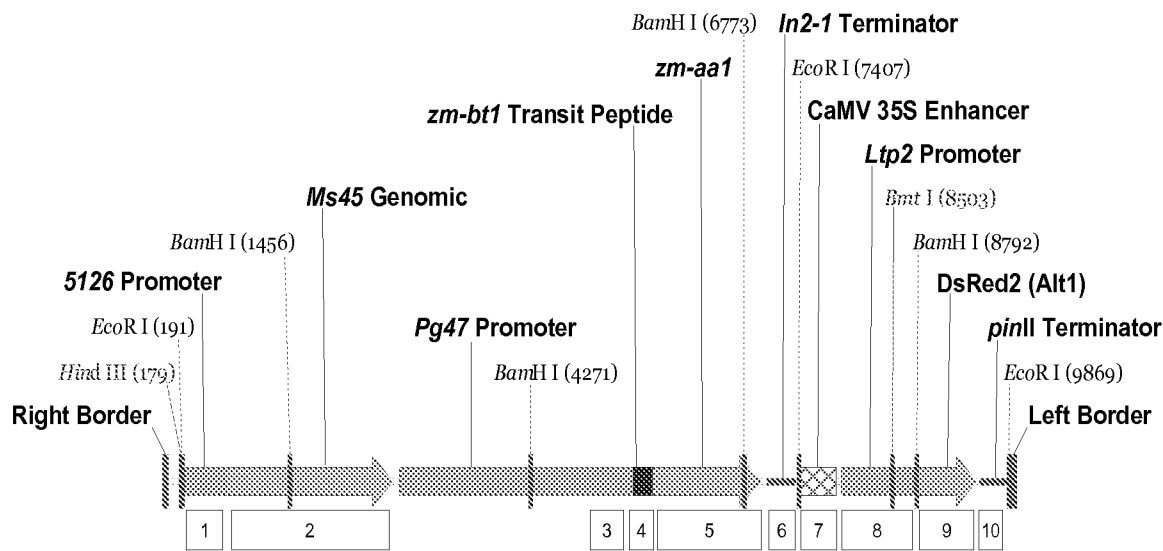


FIGURE 14



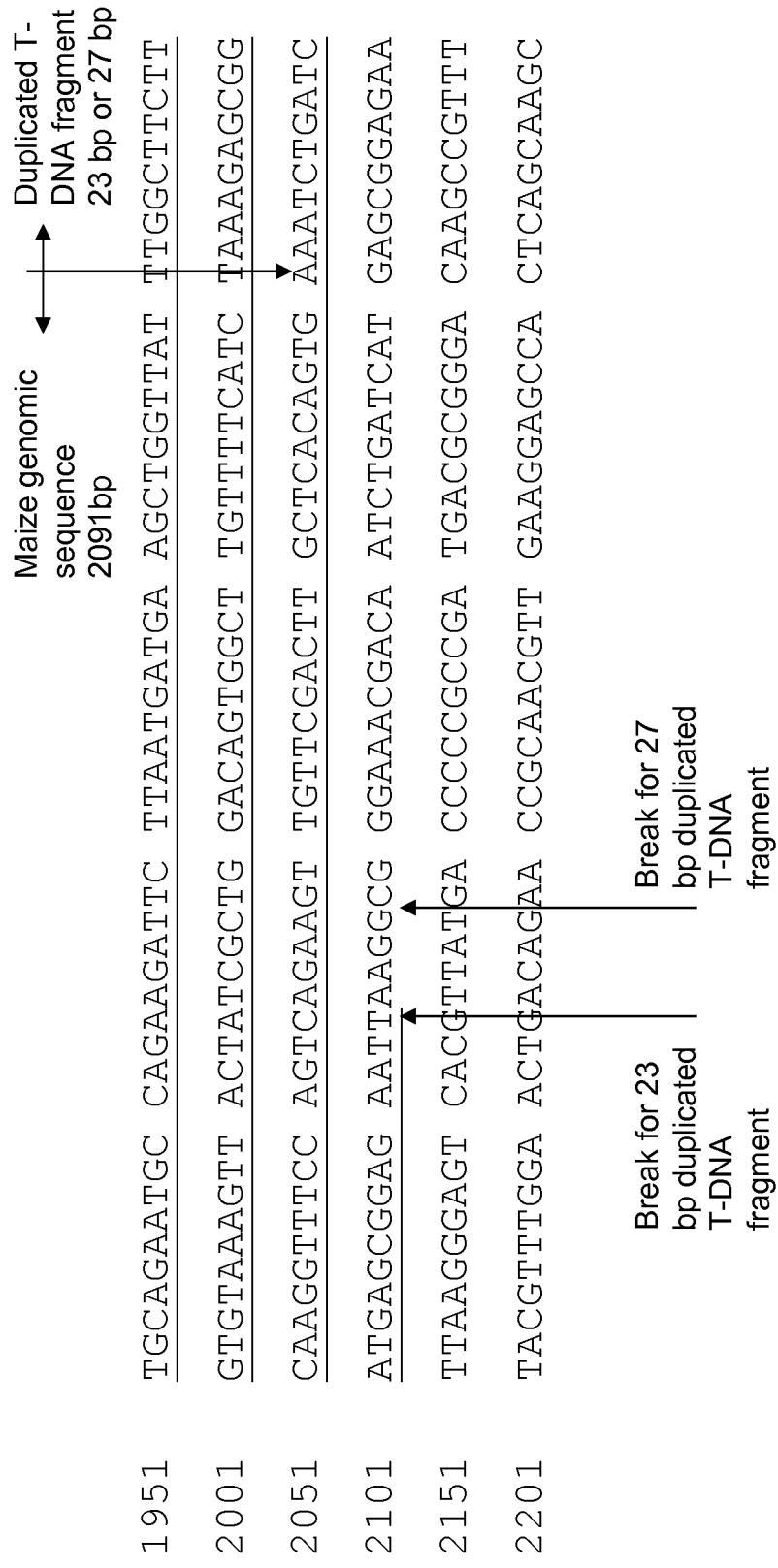


Figure 15.