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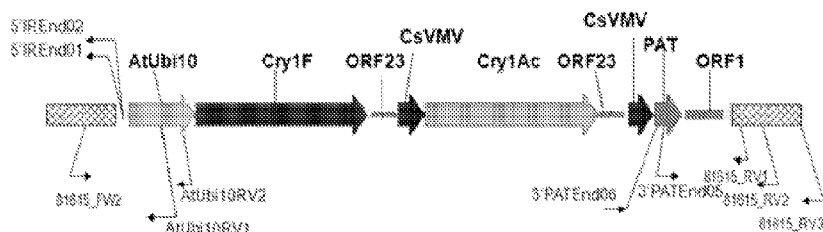
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- (54) **Title:** SOYBEAN EVENT PDAB9582.816.15.1 DETECTION METHOD

Figure 2

The diagram depicts the primer locations for confirming the 5' and 3' border sequence of the soybean event pDAB9582.816.15.1.

- (57) **Abstract:** Soybean Event pDAB9582.816.15.1 comprises gene expression cassettes which contain genes encoding Cry1F, Cry1Ac (synpro), and PAT, affording insect resistance and herbicide tolerance to soybean crops containing the event, and enabling methods for crop protection and protection of stored products. The disclosure provides polynucleotide-related event detection methods. The present disclosure relates to a method for detecting a new insect resistant and herbicide tolerant transgenic soybean transformation event, designated Soybean Event pDAB9582.816.15.1. The DNA of soybean plants containing this event includes the junction/flanking sequences described herein that characterize the location of the inserted DNA within the soybean genome. SEQ ID NO: 1 and SEQ ID NO: 2 are diagnostic for Soybean Event pDAB9582.816.15.1.



SOYBEAN EVENT pDAB9582.816.15.1 DETECTION METHOD

Priority Claims: This disclosure claims the priority of US provisional application 61/663,687, filed on June 25, 2012 and the information therein is incorporated entirely.

Background of Invention

[0001] The genes encoding Cry1F and Cry1Ac synpro (Cry1Ac) are capable of imparting insect resistance, e.g. resistance to lepidopteran insects, to transgenic plants; and the gene encoding PAT (phosphinotricin acetyltransferase) is capable of imparting tolerance to the herbicide phosphinotricin (glufosinate) to transgenic plants. PAT has been successfully expressed in soybean for use both as a selectable marker in producing insect resistant transgenic crops, and to impart commercial levels of tolerance to the herbicide glufosinate in transgenic crops.

[0002] The expression of transgenes 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.*, *Ann. Rev. Genet* 22:421-477, 1988). The presence of the transgene at different locations in the genome will influence the overall phenotype of the plant in different ways. 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. For this reason, 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. As such, 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. 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.

[0003] It is desirable to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene or group of transgenes 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, for example, 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.

[0004] It is possible to detect the presence of a transgenic event by any nucleic acid detection method known in the art including, but not limited to, the polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc., because for many DNA constructs, the coding region is interchangeable. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known. For example, an event-specific PCR assay is described in United States Patent Application 2006/0070139 for maize event DAS-59122-7. It would be desirable to have a simple and discriminative method for the identification of Soybean Event pDAB9582.816.15.1.

Brief Summary of the Invention

[0005] The present disclosure relates to a method for detecting a new insect resistant and herbicide tolerant transgenic soybean transformation event, designated Soybean Event pDAB9582.816.15.1. Representative soybean seed has been deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA, 20110. The deposit, designated as ATCC Deposit No. PTA-12588, was made on behalf of Dow AgroSciences LLC on 23/February/2012. This deposit was made and will be

maintained in accordance with and under the terms of the Budapest Treaty with respect to seed deposits for the purposes of patent procedure.

[0006] The DNA of soybean plants containing this event includes the junction/flanking sequences described herein that characterize the location of the inserted DNA within the soybean genome. SEQ ID NO:1 and SEQ ID NO:2 are diagnostic for Soybean Event pDAB9582.816.15.1. More particularly, sequences surrounding the junctions at bp 1273/1274 of SEQ ID NO:1, and bp 175/176 and bp 316/317 of SEQ ID NO:2 are diagnostic for Soybean Event pDAB9582.816.15.1. Paragraph [0008] below describes examples of sequences comprising these junctions that are characteristic of DNA of soybeans containing Soybean Event pDAB9582.816.15.1.

[0007] An embodiment of the subject disclosure provides a method of detecting Soybean Event pDAB9582.816.15.1 in a sample comprising soybean DNA, said method comprising:

- (a) contacting said sample with a first primer at least 10 bp in length that selectively binds to a flanking sequence within bp 1-1273 of SEQ ID NO:1 or the complement thereof, and a second primer at least 10 bp in length that selectively binds to an insert sequence within bp 1274-1577 of SEQ ID NO:1 or the complement thereof; and
- (b) assaying for an amplicon generated between said primers; or contacting said sample with a first primer at least 10 bp in length that selectively binds to an insert sequence within bp 1-175 of SEQ ID NO:2 or the complement thereof, and a second primer at least 10 bp in length that selectively binds to flanking sequence within bp 176-1687 of SEQ ID NO:2 or the complement thereof; and
- (c) assaying for an amplicon generated between said primers..

In another embodiment, the disclosure provides a method of detecting Soybean Event pDAB9582.816.15.1 comprising:

- a) contacting said sample with a first primer that selectively binds to a flanking sequence selected from the group consisting of bp 1-1273 of SEQ ID NO:1 and bp 176-1687 of SEQ ID NO:2, and compliments thereof; and a second primer that selectively binds to SEQ ID NO:3, or the compliment thereof;

- b) subjecting said sample to polymerase chain reaction; and
- c) assaying for an amplicon generated between said primers.

[0008] In another embodiment the subject disclosure provides an isolated DNA molecule that is diagnostic for Soybean Event pDAB9582.816.15.1. Such molecules include, in addition to SEQ ID NOS: 1 and 2, molecules at least 25 bp in length comprising bp 1273-1274 of SEQ ID NO:1 and at least 10 bp of SEQ ID NO:1 in each direction from the bp 1273/1274 junction; amplicons at least 25 bp in length comprising 175-176 of SEQ ID NO:2 and at least 10 bp of SEQ ID NO:2 in each direction from the bp 175/176 junction. Examples are bp 1258-1288 of SEQ ID NO:1; bp 1223-1323 of SEQ ID NO:1; bp 1173-1373 of SEQ ID NO:1; bp 1073-1473 of SEQ ID NO:1; bp 160-190 of SEQ ID NO:2; bp 125-225 of SEQ ID NO:2; and bp 75-275 of SEQ ID NO:2, and compliments thereof.

[0009] Additionally, the subject disclosure provides assays for detecting the presence of the subject event in a sample (of soybeans, for example). The assays can be based on the DNA sequence of the recombinant construct, inserted into the soybean genome, and on the genomic sequences flanking the insertion site. Kits and conditions useful in conducting the assays are also provided.

[0010] An embodiment of the subject disclosure relates in part to the cloning and analysis of the DNA sequences of the border regions resulting from insertion of T-DNA from pDAB9582 in transgenic soybean lines. These sequences are unique. Based on the insert and junction sequences, event-specific primers can be and were generated. PCR analysis demonstrated that these events can be identified by analysis of the PCR amplicons generated with these event-specific primer sets. Thus, these and other related procedures can be used to uniquely identify soybean lines comprising the event of the subject disclosure.

Brief Description of the Sequences

[0011] **SEQ ID NO:1** is the 5' DNA flanking border sequence for soybean event 9582.816.15.1. Nucleotides 1-1273 are genomic sequence. Nucleotides 1274-1577 are insert sequence.

- [0012]** SEQ ID NO:2 is the 3' DNA flanking border sequence for soybean event 9582.816.15.1. Nucleotides 1-175 are insert sequence. Nucleotides 176-316 are a rearranged sequence from pDAB9582. Nucleotides 317-1687 are genomic sequence.
- [0013]** SEQ ID NO:3 is the DNA sequence of pDAB9582, which is annotated below in Table 1.
- [0014]** SEQ ID NO:4 is oligonucleotide primer 81615_FW2 for confirmation of 5' border genomic DNA.
- [0015]** SEQ ID NO:5 is oligonucleotide primer 81615_RV1 for confirmation of 3' border genomic DNA.
- [0016]** SEQ ID NO:6 is oligonucleotide primer 81615_RV2 for confirmation of 3' border genomic DNA.
- [0017]** SEQ ID NO:7 is oligonucleotide 81615_RV3 for confirmation of 3' border genomic DNA.
- [0018]** SEQ ID NO:8 is oligonucleotide primer 5'IREnd-01 for confirmation of 5' border genomic DNA.
- [0019]** SEQ ID NO:9 is oligonucleotide primer 5'IREnd-02 for confirmation of 5' border genomic DNA.
- [0020]** SEQ ID NO:10 is oligonucleotide primer AtUbi10RV1 for confirmation of 5' border genomic DNA.
- [0021]** SEQ ID NO:11 is oligonucleotide primer AtUbi10RV2 for confirmation of 5' border genomic DNA.
- [0022]** SEQ ID NO:12 is oligonucleotide primer 3'PATEnd05 for confirmation of 3' border genomic DNA.
- [0023]** SEQ ID NO:13 is oligonucleotide primer 3'PATEnd06 for confirmation of 3' border genomic DNA.
- [0024]** SEQ ID NO:14 is the expected sequence of soybean event 9582.816.15.1. Including the 5' genomic flanking sequence, pDAB9582 T-strand insert, and 3' genomic flanking sequence.
- [0025]** SEQ ID NO:15 is oligonucleotide primer 81615_3'F which was used for the TAQMAN assay to detect the 3' border of soybean event 9582.816.15.1.

[0026] **SEQ ID NO:16** is oligonucleotide primer 81615_3'R which was used for the TAQMAN assay to detect the 3' border soybean event 9582.816.19.1.

[0027] **SEQ ID NO:17** is oligonucleotide probe 81615_3'P which was used for the TAQMAN assay to detect the 3' border soybean event 9582.816.19.1. This probe had a FAM fluorescent moiety added to the 5' end and an MGB quencher added to the 3' end.

[0028] **SEQ ID NO:18** is oligonucleotide primer GMS116 F which was used for the TAQMAN assay to detect the endogenous reference gene, GMFL01-25-J19 (GenBank: AK286292.1).

[0029] **SEQ ID NO:19** is oligonucleotide primer GMS116 R which was used for the TAQMAN assay to detect the endogenous reference gene, GMFL01-25-J19 (GenBank: AK286292.1).

[0030] **SEQ ID NO:20** is oligonucleotide probe GMS116 which was used for the TAQMAN assay to detect the endogenous reference gene, GMFL01-25-J19 (GenBank: AK286292.1). This probe had a HEX fluorescent moiety added to the 5' end and an BHQ quencher added to the 3' end.

Brief Description of the Figures

[0031] **Figure 1** is a plasmid map of pDAB9582 containing the *cryIF*, *cryIAC* and *pat* expression cassette.

[0032] **Figure 2** depicts the primer locations for confirming the 5' and 3' border sequence of the soybean event pDAB9582.816.15.1.

[0033] **Figure 3** depicts the genomic sequence arrangement in soybean event pDAB9582.816.15.1.

[0034] **Figure 4.** depicts the primer and probe locations for the TAQMAN assay of the soybean event pDAB9582.816.15.1.

Detailed Description of the Invention

[0035] Both ends of event Soybean Event 9582.816.19.1 insertion have been sequenced and characterized. Event specific assays were developed. The event has also been mapped onto chromosome 03 of the soybean genome. The event can be introgressed into further elite lines.

[0036] As alluded to above in the Background section, the introduction and integration of a transgene into a plant genome involves some random events (hence the name “event” for a given insertion that is expressed). That is, with many transformation techniques such as *Agrobacterium* transformation, the biolistic transformation (i.e. gene gun), and silicon carbide mediated transformation (i.e. WHISKERS), it is unpredictable where in the genome a transgene will become inserted. Thus, identifying the flanking plant genomic DNA on both sides of the insert can be important for identifying a plant that has a given insertion event. For example, PCR primers can be designed that generate a PCR amplicon across the junction region of the insert and the host genome. This PCR amplicon can be used to identify a unique or distinct type of insertion event.

[0037] Definitions and examples are provided herein to help describe the present disclosure and to guide those of ordinary skill in the art to practice the invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. The nomenclature for DNA bases as set forth at 37 CFR §1.822 is used.

[0038] As used herein, the term “progeny” denotes the offspring of any generation of a parent plant which comprises Soybean Event pDAB9582.816.15.1.

[0039] A transgenic “event” is produced by transformation of plant cells with heterologous DNA, *i.e.*, a nucleic acid construct that includes the transgenes of interest, 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. The term “event” refers to the original transformant and progeny of the transformant that include the heterologous DNA. The term “event” also refers to progeny produced by a sexual outcross between the transformant and another variety that includes the genomic/transgene DNA. Even after repeated back-crossing to a recurrent parent, the inserted transgene DNA and flanking genomic DNA (genomic/transgene DNA) from the transformed parent is present in the progeny of the cross at the same chromosomal location. The term “event” also refers to DNA from the original transformant and progeny thereof comprising the inserted DNA and flanking genomic sequence immediately adjacent to the inserted DNA which 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.

[0040] A “junction sequence” or “border sequence” spans the point at which DNA inserted into the genome is linked to DNA from the soybean native genome flanking the insertion point, the identification or detection of one or the other junction sequences in a plant's genetic material being sufficient to be diagnostic for the event. Included are the DNA sequences that span the insertions in herein-described soybean events and similar lengths of flanking DNA. Specific examples of such diagnostic sequences are provided herein; however, other sequences that overlap the junctions of the insertions, or the junctions of the insertions and the genomic sequence, are also diagnostic and could be used according to the subject disclosure.

[0041] The subject disclosure relates in part to event identification using such flanking, junction, and insert sequences. Related PCR primers and amplicons are included in the disclosure. In accordance with embodiments of the subject disclosure, PCR analysis methods using amplicons that span across inserted DNA and its borders can be used to detect or identify commercialized transgenic soybean varieties or lines derived from the subject proprietary transgenic soybean lines.

[0042] The flanking/junction sequences are diagnostic for Soybean Event pDAB9582.816.15.1. Based on these sequences, event-specific primers were generated. PCR analysis demonstrated that these soybean lines can be identified in different soybean genotypes by analysis of the PCR amplicons generated with these event-specific primer sets. Thus, these and other related procedures can be used to uniquely identify these soybean lines. The sequences identified herein are unique.

[0043] Detection techniques of the subject disclosure are especially useful in conjunction with plant breeding, to determine which progeny plants comprise a given event, after a parent plant comprising an event of interest is crossed with another plant line in an effort to impart one or more additional traits of interest in the progeny. These PCR analysis methods benefit soybean breeding programs as well as quality control, especially for commercialized transgenic soybean seeds. PCR detection kits for these

transgenic soybean lines can also now be made and used. This is beneficial for product registration and product stewardship.

[0044] Furthermore, flanking soybean/genomic sequences can be used to specifically identify the genomic location of each insert. This information can be used to make molecular marker systems specific to each event. These can be used for accelerated breeding strategies and to establish linkage data.

[0045] Still further, the flanking sequence information can be used to study and characterize transgene integration processes, genomic integration site characteristics, event sorting, stability of transgenes and their flanking sequences, and gene expression (especially related to gene silencing, transgene methylation patterns, position effects, and potential expression-related elements such as MARS [matrix attachment regions], and the like).

[0046] In light of all the subject disclosure, it should be clear that the subject disclosure includes seeds available under the ATCC Deposit No. identified in paragraph [0005]. The subject disclosure also includes a herbicide-tolerant soybean plant grown from a seed deposited with the ATCC Deposit No. identified in paragraph [0005]. The subject disclosure further includes parts of said plant, such as leaves, tissue samples, seeds produced by said plant, pollen, and the like (wherein they comprise *cryIF*, *cryIAC*, *pat*, and SEQ ID NOS: 1 and 2).

[0047] As used herein, the term “soybean” means *Glycine max* and includes all varieties thereof that can be bred with a soybean plant.

[0048] The DNA molecules of the present disclosure can be used as molecular markers in a marker assisted breeding (MAB) method. DNA molecules of the present disclosure can be used in methods (such as, AFLP markers, RFLP markers, RAPD markers, SNPs, and SSRs) that identify genetically linked agronomically useful traits, as is known in the art. The insect resistance and herbicide-tolerance traits can be tracked in the progeny of a cross with a soybean plant of the subject disclosure (or progeny thereof and any other soybean cultivar or variety) using the MAB methods. The DNA molecules are markers for this trait, and MAB methods that are well known in the art can be used to track the herbicide-resistance trait(s) in soybean plants where at least one soybean line of

the subject disclosure, or progeny thereof, was a parent or ancestor. The methods of the present disclosure can be used to identify any soybean variety having the subject event.

[0049] As used herein, a “line” is a group of plants that display little or no genetic variation between individuals for at least one trait. Such lines may be created by several generations of self-pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques.

[0050] As used herein, the terms “cultivar” and “variety” are synonymous and refer to a line which is used for commercial production.

[0051] “Stability” or “stable” means that with respect to the given component, the component is maintained from generation to generation and, preferably, at least three generations.

[0052] “Commercial Utility” is defined as having good plant vigor and high fertility, such that the crop can be produced by farmers using conventional farming equipment, and the oil with the described components can be extracted from the seed using conventional crushing and extraction equipment

[0053] “Agronomically elite” means that a line has desirable agronomic characteristics such as yield, maturity, disease resistance, and the like, in addition to the insect resistance and herbicide tolerance due to the subject event(s). Any and all of these agronomic characteristics and data points can be used to identify such plants, either as a point or at either end or both ends of a range of characteristics used to define such plants.

[0054] As one skilled in the art will recognize in light of this disclosure, preferred embodiments of detection kits, for example, can include probes and/or primers directed to and/or comprising “junction sequences” or “transition sequences” (where the soybean genomic flanking sequence meets the insert sequence). For example, this includes a polynucleotide probes, primers, and/or amplicons designed to identify one or both junction sequences (where the insert meets the flanking sequence). One common design is to have one primer that hybridizes in the flanking region, and one primer that hybridizes in the insert. Such primers are often each about at least ~15 residues in length. With this arrangement, the primers can be used to generate/amplify a detectable amplicon that indicates the presence of an event of the subject disclosure. These primers can be

used to generate an amplicon that spans (and includes) a junction sequence as indicated above.

[0055] The primer(s) "touching down" in the flanking sequence is typically not designed to hybridize beyond about 1200 bases or so beyond the junction. Thus, typical flanking primers would be designed to comprise at least 15 residues of either strand within 1200 bases into the flanking sequences from the beginning of the insert. That is, primers comprising a sequence of an appropriate size from (or hybridizing to) base pairs 451 to 1331 of SEQ ID NO:1 and/or base pairs 212 to 350 of SEQ ID NO:2 are within the scope of the subject disclosure. Insert primers can likewise be designed anywhere on the insert, but base pairs 451 to 1331 and 13695 to 13833 of SEQ ID NO:3, can be used, for example, non-exclusively for such primer design.

[0056] One skilled in the art will also recognize that primers and probes can be designed to hybridize, under a range of standard hybridization and/or PCR conditions wherein the primer or probe is not perfectly complementary to the exemplified sequence. That is, some degree of mismatch can be tolerated. For an approximately 20 nucleotide primer, for example, typically one or two or so nucleotides do not need to bind with the opposite strand if the mismatched base is internal or on the end of the primer that is opposite the amplicon. Various appropriate hybridization conditions are provided below. Synthetic nucleotide analogs, such as inosine, can also be used in probes. Peptide nucleic acid (PNA) probes, as well as DNA and RNA probes, can also be used. What is important is that such probes and primers are diagnostic for (able to uniquely identify and distinguish) the presence of an event of the subject disclosure.

[0057] It should be noted that errors in PCR amplification can occur which might result in minor sequencing errors, for example. That is, unless otherwise indicated, the sequences listed herein were determined by generating long amplicons from soybean genomic DNAs, and then cloning and sequencing the amplicons. It is not unusual to find slight differences and minor discrepancies in sequences generated and determined in this manner, given the many rounds of amplification that are necessary to generate enough amplicon for sequencing from genomic DNAs. One skilled in the art should recognize and be put on notice that any adjustments needed due to these types of common sequencing errors or discrepancies are within the scope of the subject disclosure.

[0058] It should also be noted that it is not uncommon for some genomic sequence to be deleted, for example, when a sequence is inserted during the creation of an event. Thus, some differences can also appear between the subject flanking sequences and genomic sequences listed in GENBANK, for example.

[0059] Components of the DNA sequence “insert” are illustrated in the Figures and are discussed in more detail below in the Examples. The DNA polynucleotide sequences of these components, or fragments thereof, can be used as DNA primers or probes in the methods of the present disclosure.

[0060] In some embodiments of the disclosure, compositions and methods are provided for detecting the presence of the transgene/genomic insertion region, in plants and seeds and the like, from a soybean plant. DNA sequences are provided that comprise the subject 5' transgene/genomic insertion region junction sequence provided herein (between base pairs 451 -- 1331 of SEQ ID NO:1 and 451 -- 1331 of SEQ ID NO:3), segments thereof, and complements of the exemplified sequences and any segments thereof. DNA sequences are provided that comprise the subject 3' transgene/genomic insertion region junction sequence provided herein (between base pairs 212 -- 350 of SEQ ID NO:2 and 13695 -- 13833 of SEQ ID NO:3), segments thereof, and complements of the exemplified sequences and any segments thereof. The insertion region junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the soybean cell flanking the insertion site. Such sequences can be diagnostic for the given event.

[0061] Based on these insert and border sequences, event-specific primers can be generated. PCR analysis demonstrated that soybean lines of the subject disclosure can be identified in different soybean genotypes by analysis of the PCR amplicons generated with these event-specific primer sets. These and other related procedures can be used to uniquely identify these soybean lines. Thus, PCR amplicons derived from such primer pairs are unique and can be used to identify these soybean lines.

[0062] In some embodiments, DNA sequences that comprise a contiguous fragment of the novel transgene/genomic insertion region are an aspect of this disclosure. Included are DNA sequences that comprise a sufficient length of polynucleotides of transgene insert sequence and a sufficient length of polynucleotides of soybean genomic sequence

from one or more of the three aforementioned soybean plants and/or sequences that are useful as primer sequences for the production of an amplicon product diagnostic for one or more of these soybean plants.

[0063] Related embodiments pertain to DNA sequences that comprise at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more contiguous nucleotides of a transgene portion of a DNA sequence identified herein (such as SEQ ID NO:1 and segments thereof), or complements thereof, and a similar length of flanking soybean DNA sequence from these sequences, or complements thereof. Such sequences are useful as DNA primers in DNA amplification methods. The amplicons produced using these primers are diagnostic for any of the soybean events referred to herein. Therefore, the disclosure also includes the amplicons produced by such DNA primers.

[0064] This disclosure also includes methods of detecting the presence of DNA, in a sample, that corresponds to the soybean event referred to herein. Such methods can comprise: (a) contacting the sample comprising DNA with a primer set that, when used in a nucleic acid amplification reaction with DNA from at least one of these soybean events, produces an amplicon that is diagnostic for said event(s); (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon.

[0065] Further detection methods of the subject disclosure include a method of detecting the presence of a DNA, in a sample, corresponding to said event, wherein said method comprises: (a) contacting the sample comprising DNA with a probe that hybridizes under stringent hybridization conditions with DNA from at least one of said soybean events and which does not hybridize under the stringent hybridization conditions with a control soybean plant (non-event-of-interest DNA); (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.

[0066] DNA detection kits can be developed using the compositions disclosed herein and methods well known in the art of DNA detection. The kits are useful for identification of the subject soybean event DNA in a sample and can be applied to methods for breeding soybean plants containing this DNA. The kits contain DNA sequences homologous or complementary to the amplicons, for example, disclosed herein, or to DNA sequences homologous or complementary to DNA contained in the

transgene genetic elements of the subject events. These DNA sequences can be used in DNA amplification reactions or as probes in a DNA hybridization method. The kits may also contain the reagents and materials necessary for the performance of the detection method.

[0067] A “probe” is an isolated nucleic acid molecule to which is attached a conventional detectable label or reporter molecule (such as a radioactive isotope, ligand, chemiluminescent agent, or enzyme). Such a probe is complementary to a strand of a target nucleic acid, in the case of the present disclosure, to a strand of genomic DNA from one of said soybean events, whether from a soybean plant or from a sample that includes DNA from the event. Probes in accordance with embodiments of the disclosure 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.

[0068] “Primers” are isolated/synthesized nucleic acids 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 of the present disclosure refer to their use for amplification of a target nucleic acid sequence, *e.g.*, by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

[0100] Probes and primers are generally 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217,

218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, or 500, or 1000, or 2000, or 5000 polynucleotides or more in length. Such probes and primers hybridize specifically to a target sequence under stringent hybridization conditions. Preferably, probes and primers in accordance with embodiments of the disclosure have complete sequence similarity with the target sequence, although probes differing from the target sequence and that retain the ability to hybridize to target sequences may be designed by conventional methods.

[0101] 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. PCR-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose.

[0102] 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.

[0103] The nucleic acid probes and primers of the present disclosure 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. A nucleic acid molecule is said to be the “complement” of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit “complete complementarity” when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Molecules that exhibit complete complementarity will generally hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional “high-stringency” conditions. Conventional high-stringency conditions are described by Sambrook et al., 1989.

[0104] Two molecules are said exhibit “minimal 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. Conventional low stringency conditions are described by Sambrook *et al.*, 1989. In order for a nucleic acid molecule to serve as a primer or probe it need only exhibit minimal complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

[0105] The term “stringent condition” or “stringency conditions” is functionally defined with regard to the hybridization of a nucleic-acid probe to a target nucleic acid (*i.e.*, to a particular nucleic-acid sequence of interest) by the specific hybridization procedure discussed in Sambrook *et al.*, 1989, at 9.52-9.55. *See also*, Sambrook *et al.*, 1989 at 9.47-9.52 and 9.56-9.58.

[0106] Depending on the application envisioned, one can use varying conditions of stringent conditions or polynucleotide sequence degeneracy of a probe or primer to achieve varying degrees of selectivity of hybridization towards the target sequence. For applications requiring high selectivity, one will typically employ relatively stringent

conditions for hybridization of one polynucleotide sequence with a second polynucleotide sequence, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50° C to about 70° C. Stringent conditions, for example, could involve washing the hybridization filter at least twice with high-stringency wash buffer (0.2X SSC, 0.1% SDS, 65° C). Appropriate stringency conditions which promote DNA hybridization, for example, 6.0X sodium chloride/sodium citrate (SSC) at about 45° C, followed by a wash of 2.0X SSC at 50° C are known to those skilled in the art. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0X SSC at 50° C to a high stringency of about 0.2X SSC at 50° C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22° C, to high stringency conditions at about 65° C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand. Detection of DNA sequences via hybridization is well-known to those of skill in the art, and the teachings of U.S. Patent Nos. 4,965,188 and 5,176,995 are exemplary of the methods of hybridization analyses.

[0107] A nucleic acid of an embodiment present disclosure will specifically hybridize to one or more of the primers (or amplicons or other sequences) exemplified or suggested herein, including complements and fragments thereof, under high stringency conditions. In one aspect of the present disclosure, a marker nucleic acid molecule of the present disclosure has the nucleic acid sequence as set forth herein in one of the exemplified sequences, or complements and/or fragments thereof.

[0108] In another aspect of the present disclosure, a marker nucleic acid molecule of the present disclosure shares between 80% and 100% or 90% and 100% sequence identity with such nucleic acid sequences. In a further aspect of the present disclosure, a marker nucleic acid molecule of the present disclosure shares between 95% and 100% sequence identity with such sequence. Such sequences may be used as markers in plant breeding methods to identify the progeny of genetic crosses. The hybridization of the probe to the target DNA molecule can be detected by any number of methods known to

those skilled in the art, these can include, but are not limited to, fluorescent tags, radioactive tags, antibody based tags, and chemiluminescent tags.

[0109] Regarding the amplification of a target nucleic acid sequence (*e.g.*, by PCR) using a particular amplification primer pair, “stringent conditions” are conditions that permit the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product, the amplicon.

[0110] The term “specific for (a target sequence)” indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

[0111] As used herein, “amplified DNA” or “amplicon” refers to the product of nucleic-acid amplification of a target nucleic acid sequence that is part of a nucleic acid template. For example, to determine whether the soybean plant resulting from a sexual cross contains transgenic event genomic DNA from the soybean plant of the present disclosure, DNA extracted from a soybean plant tissue sample may be subjected to nucleic acid amplification method using a primer pair that includes a primer derived from flanking sequence in the genome of the plant adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA. The amplicon is of a length and has a sequence that is also diagnostic for the event. The amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair, and/or the combined length of the primer pairs plus about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194,

195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, or 500, 750, 1000, 1250, 1500, 1750, 2000, or more nucleotide base pairs (plus or minus any of the increments listed above). Alternatively, a primer pair can be derived from flanking sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence. A member of a primer pair derived from the plant genomic sequence may be located a distance from the inserted DNA sequence. This distance can range from one nucleotide base pair up to 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.

[0112] 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 U.S. Patent No. 4,683,195 and U.S. Patent No. 4,683,202. PCR amplification methods have been developed to amplify up to 22 kb of genomic DNA. These methods as well as other methods known in the art of DNA amplification may be used in the

practice of the present disclosure. The sequence of the heterologous transgene DNA insert or flanking genomic sequence from a subject soybean event can be verified (and corrected if necessary) by amplifying such sequences from the event using primers derived from the sequences provided herein followed by standard DNA sequencing of the PCR amplicon or of the cloned DNA.

[0113] The amplicon produced by these methods may be detected by a plurality of techniques. Agarose gel electrophoresis and staining with ethidium bromide is a common well known method of detecting DNA amplicons. Another such method is Genetic Bit Analysis where an DNA oligonucleotide is designed which overlaps both the adjacent flanking genomic 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 genomic 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. Analysis of a bound product can be completed via quantitating the amount of fluorescent signal. A fluorescent signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

[0114] Another 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 genomic DNA and insert DNA junction. The oligonucleotide is designed to hybridize to single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking genomic 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 that 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.

[0115] Fluorescence Polarization is another method that can be used to detect an amplicon of the present disclosure. Following this method, an oligonucleotide is designed which overlaps the genomic flanking and inserted DNA junction. The oligonucleotide is

hybridized to single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking genomic 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 of the fluorescently labeled ddNTP 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.

[0116] TAQMAN® (PE Applied Biosystems, Foster City, Calif.) is a method of detecting and quantifying the presence of a DNA sequence. Briefly, a FRET oligonucleotide probe is designed that overlaps the genomic 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. During specific amplification, the Taq DNA polymerase proofreading mechanism releases 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.

[0117] Molecular Beacons have been described for use in polynucleotide sequence detection. Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking genomic 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 genomic 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 genomic/transgene insert sequence due to successful amplification and hybridization.

[0118] Having disclosed a location in the soybean genome that is excellent for an insertion, the subject disclosure also comprises a soybean seed and/or a soybean plant comprising at least one non-Soybean Event 9582.816.15.1 insert in the general vicinity of this genomic location. One option is to substitute a different insert in place of the one

from pDAB9582.816.15.1 exemplified herein. In these general regards, targeted homologous recombination, for example, can be used according to the subject disclosure. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (US 20030232410). Thus, the subject disclosure includes plants and plant cells comprising a heterologous insert (in place of or with multi-copies of the *cryIF*, *cryIAC*, or *pat* genes), flanked by all or a recognizable part of the flanking sequences identified herein (bp 1-1273 of SEQ ID NO:1 and bp 176-1287 of SEQ ID NO:2). An additional copy (or additional copies) of a *cryIF*, *cryIAC*, or *pat* could also be targeted for insertion in this / these manner(s).

[0119] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[0120] The following examples are included to illustrate procedures for practicing the disclosure and to demonstrate certain preferred embodiments of the disclosure. These examples should not be construed as limiting. It should be appreciated by those of skill in the art that the techniques disclosed in the following examples represent specific approaches used to illustrate preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in these specific embodiments while still obtaining like or similar results without departing from the spirit and scope of the disclosure. Unless otherwise indicated, all percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

[0121] The following abbreviations are used unless otherwise indicated.

bp	base pair
°C	degrees Celsius
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
kb	kilobase
µg	microgram
µL	microliter
mL	milliliter
M	molar mass
PCR	polymerase chain reaction
PTU	plant transcription unit or expression cassette

SDS sodium dodecyl sulfate
 SSC a buffer solution containing a mixture of sodium chloride
 and sodium citrate, pH 7.0
 TBE a buffer solution containing a mixture of Tris base, boric
 acid and EDTA, pH 8.3

EXAMPLES

Example 1: Transformation and Selection of the Cry1F and Cry1Ac Soybean event pDAB9582.816.15.1

[0122] Transgenic soybean (*Glycine max*) containing the soybean event pDAB9582.816.15.1 was generated through *Agrobacterium*-mediated transformation of soybean cotyledonary node explants. The disarmed *Agrobacterium* strain EHA101 (Hood et al., 1993), carrying the binary vector pDAB9582 (**Figure 1**) containing the selectable marker, *pat v6*, and the genes of interest, *cry1F v3* and *cry1 Ac synpro*, within the T-strand DNA region, was used to initiate transformation. The T-strand DNA sequence for pDAB9582 is given in SEQ ID NO:3, which is annotated below in **Table 1**.

Table 1. Gene elements located on pDAB9582.

bp (SEQ ID NO:3)	Construct Element	Reference
272 – 1593	AtUbi10 Promoter	Callis, <i>et al.</i> , (1990) <i>J. Biol. Chem.</i> , 265: 12486-12493
1602 – 5048	Cry1F	Referenced above
5151 – 5607	ORF23 3'UTR	U.S. Pat. No. 5,428,147
5671 – 6187	CsVMV Promoter	Verdaguer <i>et al.</i> , (1996) <i>Plant Mol. Biol.</i> , 31: 1129-1139
6197 – 9667	Cry 1AC	Referenced above
9701 – 10157	ORF23 3'UTR	U.S. Pat. No. 5,428,147
10272 – 10788	CsVMV Promoter	Verdaguer <i>et al.</i> , (1996) <i>Plant Mol. Biol.</i> , 31: 1129-1139
10796 – 11347	PAT	Wohlleben <i>et al.</i> , (1988) <i>Gene</i> 70: 25-37
11450 – 12153	ORF1 3'UTR	Huang <i>et al.</i> , (1990) <i>J. Bacteriol.</i> 172:1814-1822

[0123] *Agrobacterium*-mediated transformation was carried out using a modified procedure of Zeng et al. (2004). Briefly, soybean seeds (cv Maverick) were germinated

on basal media and cotyledonary nodes were isolated and infected with *Agrobacterium*. Shoot initiation, shoot elongation, and rooting media were supplemented with cefotaxime, timentin and vancomycin for removal of *Agrobacterium*. Glufosinate selection was employed to inhibit the growth of non-transformed shoots. Selected shoots were transferred to rooting medium for root development and then transferred to soil mix for acclimatization of plantlets.

[0124] Terminal leaflets of selected plantlets were leaf painted with glufosinate to screen for putative transformants. The screened plantlets were transferred to the greenhouse, allowed to acclimate and then leaf-painted with glufosinate to reconfirm tolerance and deemed to be putative transformants. The screened plants were sampled and molecular analyses for the confirmation of the selectable marker gene and/or the gene of interest were carried out. T₀ plants were allowed to self fertilize in the greenhouse to give rise to T₁ seed.

[0125] This event, soybean event pDAB9582.816.15.1, was generated from an independent transformed isolate. The T₁ plants were backcrossed and introgressed into elite varieties over subsequent generations. The event was selected based on its unique characteristics such as single insertion site, normal Mendelian segregation, stable expression, and a superior combination of efficacy, including insect resistance, herbicide tolerance and agronomic performance. The following examples contain the data which were used to characterize soybean event pDAB9582.816.15.1.

Example 2: Characterization of Protein Expression in Soybean event pDAB9582.816.15.1

[0126] The biochemical properties of the recombinant Cry1F, Cry1Ac, and PAT proteins expressed in soybean event pDAB9582.816.15.1 were characterized. Quantitative enzyme-linked immunosorbent assay (ELISA) is a biochemical assay known within the art that can be used to characterize the biochemical properties of the proteins and confirm expression of these proteins in soybean event pDAB9582.816.15.1.

Example 2.1: Expression of the PAT, Cry1F, and Cry1Ac Protein in Plant Tissues

[0127] Samples of soybean tissues were isolated from the test plants and prepared for expression analysis. The PAT protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST) containing 0.5% Bovine Serum Albumin (BSA). The plant tissue was centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer as necessary, and analyzed using an PAT ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Envirologix, Portland, ME). This assay measured the concentrations of expressed PAT protein.

[0128] The Cry1F protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST). The plant tissue was centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer as necessary, and analyzed using an Cry1F ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Strategic Diagnostics Inc., Newark, DE). This assay measured the concentrations of expressed Cry1F protein.

[0129] The Cry1Ac protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST) containing 0.5% Bovine Serum Albumin (BSA). The plant tissue was centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer as necessary, and analyzed using an Cry1Ac ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Strategic Diagnostics Inc., Newark, DE). This assay measured the concentrations of expressed Cry1Ac protein.

[0130] Detection analysis was performed to investigate the expression stability and inheritability both vertically (between generations) and horizontally (between lineages within a generation) in soybean event pDAB9582.816.15.1.

Example 2.2: Expression of the Cry1F, Cry1Ac and PAT Proteins in Plant Tissues

[0131] Levels of Cry1F, Cry1Ac and PAT proteins were determined in Soybean event pDAB9582.816.15.1 using the protocols described above. The soluble, extractable proteins were measured using a quantitative enzyme-linked immunosorbent assay

(ELISA) method from soybean leaf tissue. From T₂ to T₆ generations soybean event pDAB9582.816.15.1, expression was stable (not segregating) and consistent across all lineages. **Table 2** lists the mean expression level of the transgenic proteins in soybean event pDAB9582.816.15.1.

Table 2. Mean expression level of different transgenic proteins in soybean event pDAB9582.816.15.1.

Expression Level of Different Proteins (ng/cm ²)			
Event	Cry1F	Cry1 Ac	PAT
Soybean event pDAB9285.816.15.1	89	18.6	9.9

Example 3: Cloning and Characterization of DNA Sequence in the Insert and the Flanking Border Regions of Soybean Event pDAB9582.816.15.1

[0132] To characterize and describe the genomic insertion site, the sequence of the flanking genomic T-DNA border regions of soybean event pDAB9582.816.15.1 were determined. Genomic sequence of soybean event pDAB9582.816.15.1 was confirmed, comprising 1273 bp of 5' flanking border sequence (SEQ ID NO:1) and 1371 bp of 3' flanking border sequence (SEQ ID NO:2). PCR amplification based on the soybean event pDAB9582.816.15.1 border sequences validated that the border regions were of soybean origin and that the junction regions are unique sequences for soybean event pDAB9582.816.15.1. The junction regions could be used for event-specific identification of soybean event pDAB9582.816.15.1. In addition, the T-strand insertion site was characterized by amplifying a genomic fragment corresponding to the region of the identified flanking border sequences from the genome of untransformed soybean. Comparison of soybean event pDAB9582.816.15.1 with the untransformed genomic sequence revealed that a deletion of about 21 bp from the original locus resulted during the T-strand integration. Overall, the characterization of the insert and border sequence of soybean event pDAB9582.816.15.1 indicated that an intact copy of the T-strand from pDAB9582 was present in the soybean genome.

Table 3. List of primers and their sequences used in the confirmation of soybean genomic DNA in soybean event pDAB9582.816.15.1.

SEQ ID NO:	Primer Name	Size (bp)	Sequence (5'to 3')	Purpose
SEQ ID NO:4	81615_F W2	30	TTACACCCTTAGGATC GAGACACTTAGAGC	confirmation of 5' border genomic DNA, used with AtUbi10RV1 or RV2; with 5'IREnd-01 or 5'IREnd-02
SEQ ID NO:5	81516_R V1	27	GATTCATGTCCTTCCT AATGCGAATTG	confirmation of 3' border genomic DNA, used with 3'PATEnd05 or 3'PATEnd06
SEQ ID NO:6	81516_R V2	25	AATTTACATTTACCC CACTTGCGA	confirmation of 3' border genomic DNA, used with 3'PATEnd05 or 3'PATEnd06
SEQ ID NO:7	81516_R V3	28	GGAGGTGCAGTGAGG AAGGTAATAATGA	confirmation of 3' border genomic DNA, used with 3'PATEnd05 or 3'PATEnd06DNA
SEQ ID NO:8	5'IREnd-01	29	CGAGCTTTCTAATTTC AAACTATTCGGGC	confirmation of 5' border genomic DNA, used with 81615_FW2
SEQ ID NO:9	5'IREnd-02	30	TCCTAGATCATCAGTT CATACAAACCTCCA	confirmation of 5' border genomic DNA, used with 81615_FW2
SEQ ID NO:10	AtUbi10 RV1	29	CGGTCCTAGATCATCA GTTTCATACAAACC	confirmation of 5' border genomic DNA, used with 81615_FW2
SEQ ID NO:11	AtUbi10 RV2	28	CACTCGTGTTTCAGTCC AATGACCAATAA	confirmation of 5' border genomic DNA, used with 81615_FW1, FW2 or FW3
SEQ ID NO:12	3'PATEnd05	20	GCTCCTCCAAGGCCA GTTAG	confirmation of 3' border genomic DNA, used with 81615_RV1, RV2 or RV3
SEQ ID NO:13	3'PATEnd06	20	CCAGTTAGGCCAGTT ACCCA	confirmation of 3' border genomic DNA, used with 81615_RV1, RV2 or RV3

Table 4. Conditions for standard PCR amplification of the border regions and event-specific sequences in soybean event pDAB9582.816.15.1.

Target Sequence	Primer Set	PCR Mixture	Pre-denature (°C/min)	Denature (°C/sec.)	Extension (°C/min:sec)	Final Extension (°C/min)
5' border	81615_FW2/AtUbi10 RV1	D	95/3	98/10	68/4:00	72/10
				32 cycles		
5' border	81615_FW2/5'IREnd-01	D	95/3	98/10	68/4:00	72/10
				32 cycles		
5' border	81615_FW3/5'IREnd-01	D	95/3	98/10	68/4:00	72/10
				32 cycles		
3' border	3'PATEnd05/81615_RV1	D	95/3	98/10	68/4:00	72/10
				32 cycles		
3' border	3'PATEnd05/81615_RV2	D	95/3	98/10	68/4:00	72/10
				35 cycles		
3'border	3'PATEnd06/81615_RV1	D	95/3	98/10	68/4:00	72/10
				32 cycles		
3'border	3'PATEnd06/81615_RV2	D	95/3	98/10	68/4:00	72/10
				32 cycles		
Across the insert locus	81615_FW2/81615_RV3	D	95/3	98/10	68/4:00	72/10
				32 cycles		

Table 5. PCR mixture for standard PCR amplification of the border regions and event specific sequences in soybean event pDAB9582.816.15.1.

PCR Mixture A		PCR Mixture B	
Reagent	1 x reaction (μL)	Reagent	1 x reaction (μL)
H2O	0.8	H2O	14.6
AccuPrime pfx SuperMix	20	10X LA Taq buffer	2
---	---	MgCl ₂ (25mM)	0.6
---	---	dNTP (2.5uM)	1.6
10uM primer	0.2	10uM primer	0.1
gDNA digestion	1	gDNA digestion	1
---	---	LA Taq (5U/uL)	0.1
rxn vol:	22	rxn vol:	20
PCR Mixture C		PCR Mixture D	
Reagent	1 x reaction (μL)	Reagent	1 x reaction (μL)
H2O	28	H2O	11.6
10X PCR buffer II (Mg-plus)	5	10X PCR buffer II (Mg-plus)	2
MgCl ₂ [25mM]	1.5	MgCl ₂ [25mM]	0.6
dNTP[2.5mM]	8	dNTP[2.5mM]	3.2
Adaptor PCR primer (10μM)	1	primer1 (10μM)	0.4
GOI nested primer (10μM)	1	primer2 (10μM)	0.4
DNA binded Beads	5	DNA Template	0.2
LA Taq (5U/uL)	0.5	LA Taq (5U/uL)	1.6
rxn vol:	50	rxn vol:	20

Example 3.1: Confirmation of Soybean Genomic Sequences

[0133] The 5' and 3' flanking borders aligned to a *Glycine max* whole genome shotgun sequence from chromosome 03, indicating that the transgene of soybean event pDAB9582.816.15.1 was inserted in soybean genome chromosome 03. To confirm the

insertion site of soybean event pDAB9582.816.15.1 from the soybean genome, PCR was carried out with different pairs of primers (**Figure 2, Table 3, Table 4, and Table 5**). Genomic DNA from soybean event pDAB9582.816.15.1 and other transgenic or non-transgenic soybean lines was used as a template. To confirm that the 5' border sequences are correct a primer designed to bind to the At Ubi10 promoter gene element, for example AtUbi10RV1, and a primer designed to bind to the cloned 5' end border on soybean genome chromosome 03, designated 81615_FW2, was used for amplifying the DNA segment that spans the At Ubi10 promoter gene element to 5' end border sequence. Similarly, for confirmation of the cloned 3' border sequence a *pat* specific primer, for example 3'PATEnd05, and primers designed according to the cloned 3' end border sequence, designated 81615_RV1 and 81615_RV2, were used for amplifying DNA segments that span the *pat* gene to 3' border sequence. DNA fragments with expected sizes were amplified only from the genomic DNA of soybean event pDAB9582.816.15.1 with each primer pair, but not from DNA samples from other transgenic soybean lines or the non-transgenic control. The results indicate that the cloned 5' and 3' border sequences are the flanking border sequences of the T-strand insert for soybean event pDAB9582.816.15.1.

[0134] To further confirm the DNA insertion in the soybean genome, a PCR amplification spanning the soybean border sequences was completed on genomic DNA which did not contain the T-strand insert for soybean event pDAB9582.816.15.1. Primer 81615_FW2, designed according to the 5' end border sequence, and one primer 81615_RV3, designed for the 3' end border sequence, were used to amplify DNA segments which contained the locus where the pDAB9582 T-strand integrated. As expected, PCR reactions completed with the primer pair of 81615_FW2 and 81615_RV3 produced an approximately a 1.8 kb DNA fragment from all the other soybean control lines but not pDAB9582.816.15.1. Aligning the identified 5' and 3' border sequences of soybean event pDAB9582.816.15.1 with a *Glycine max* whole genome shotgun sequence from chromosome 03 revealed about 21 bp deletion from the original locus. (**Figure 3**). These results demonstrated that the transgene of soybean event pDAB9582.816.15.1 was inserted into the site of soybean genome chromosome 03.

Example 4: Soybean Event pDAB9582.816.15.1 Characterization via Southern Blot

[0135] Southern blot analysis was used to establish the integration pattern of soybean event pDAB9582.816.15.1. These experiments generated data which demonstrated the integration and integrity of the *cryIAc* and *cryIF* transgenes within the soybean genome. Soybean event pDAB9582.816.15.1 was characterized as a full length, simple integration event containing a single copy of the *cryIAc* and *cryIF* PTU from plasmid pDAB9582.

[0136] Southern blot data suggested that a T-strand fragment inserted into the genome of soybean event pDAB9582.816.15.1. Detailed Southern blot analysis was conducted using probes specific to the *cryIAc* and *cryIF* gene, contained in the T-strand integration region of pDAB9582.816.15.1, and descriptive restriction enzymes that have cleavage sites located within the plasmid and produce hybridizing fragments internal to the plasmid or fragments that span the junction of the plasmid with soybean genomic DNA (border fragments). The molecular weights indicated from the Southern hybridization for the combination of the restriction enzyme and the probe were unique for the event, and established its identification patterns. These analyses also showed that the plasmid fragment had been inserted into soybean genomic DNA without rearrangements of the *cryIAc* and *cryIF* PTU.

Example 4.1: Soybean Leaf Sample Collection and Genomic DNA (gDNA) Isolation

[0137] Genomic DNA was extracted from leaf tissue harvested from individual soybean plants containing soybean event pDAB9582.816.15.1. In addition, gDNA was isolated from a conventional soybean plant, Maverick, which contains the genetic background that is representative of the substance line, absent the *cryIAc* and *cryIF* genes. Individual genomic DNA was extracted from lyophilized leaf tissue following the standard CTAB method. Following extraction, the DNA was quantified spectrofluorometrically using Pico Green reagent (Invitrogen, Carlsbad, CA).

Example 4.2: DNA Digestion and Separation

[0138] For Southern blot molecular characterization of soybean event pDAB9582.816.15.1, ten micrograms (10 µg) of genomic DNA was digested. Genomic

DNA from the soybean event pDAB9582.816.15.1 and non-transgenic soybean line Maverick was digested by adding approximately five units of selected restriction enzyme per µg of DNA and the corresponding reaction buffer to each DNA sample. Each sample was incubated at approximately 37°C overnight. The restriction enzymes *AseI*, *HindIII*, *NsiI*, and *NdeI* were used individually for the single digests (New England Biolabs, Ipswich, MA). The restriction enzymes *NotI* and *ApaI* were used together for a double digestion (New England Biolabs, Ipswich, MA). In addition, a positive hybridization control sample was prepared by combining plasmid DNA, pDAB9582 with genomic DNA from the non-transgenic soybean variety, Maverick. The plasmid DNA / genomic DNA cocktail was digested using the same procedures and restriction enzyme as the test samples.

[0139] After the digestions were incubated overnight, 25µL Quick-Precip Plus solution (Edge Biosystems, Gaithersburg, MD) was added and the digested DNA samples were precipitated with isopropanol. The precipitated DNA pellet was resuspended in 15 µL of 1X loading buffer (0.01% bromophenol blue, 10.0 mM EDTA, 10.0% glycerol, 1.0 mM Tris pH 7.5). The DNA samples and molecular size markers were then electrophoresed through 0.85% agarose gels with 0.4X TAE buffer (Fisher Scientific, Pittsburgh, PA) at 35 volts for approximately 18-22 hours to achieve fragment separation. The gels were stained with ethidium bromide (Invitrogen, Carlsbad, CA) and the DNA was visualized under ultraviolet (UV) light.

Example 4.3: Southern Transfer and Membrane Treatment

[0140] Southern blot analysis was performed essentially as described by Memelink, et al. (1994). Briefly, following electrophoretic separation and visualization of the DNA fragments, the gels were depurinated with 0.25M HCl for approximately 20 minutes, and then exposed to a denaturing solution (0.4 M NaOH, 1.5 M NaCl) for approximately 30 minutes followed by neutralizing solution (1.5 M NaCl, 0.5 M Tris pH 7.5) for at least 30 minutes. Southern transfer was performed overnight onto nylon membranes using a wicking system with 10X SSC. After transfer, the DNA was bound to the membrane by UV crosslinking followed by briefly washing membrane with a 2X SSC solution. This process produced Southern blot membranes ready for hybridization.

Example 4.4: DNA Probe Labeling and Hybridization

[0141] The DNA fragments bound to the nylon membrane were detected using a labeled probe (**Table 6**). Probes were generated by a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, into the DNA fragment amplified from plasmid pDAB9582 using primers specific to gene elements. Generation of DNA probes by PCR synthesis was carried out using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's recommended procedures.

[0142] Labeled probes were analyzed by agarose gel electrophoresis to determine their quality and quantity. A desired amount of labeled probe was then used for hybridization 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 Diagnostics, Indianapolis, IN). Briefly, nylon membrane blots containing fixed DNA were briefly washed with 2X SSC and pre-hybridized with 20-25 mL of pre-warmed DIG Easy Hyb solution in hybridization bottles at approximately 45-55°C for about 2 hours in a hybridization oven. The pre-hybridization solution was then decanted and replaced with ~15 mL of pre-warmed DIG Easy Hyb solution containing a desired amount of specific probes denatured by heating in a thermal cycler for approximately five minutes. The hybridization step was then conducted at approximately 45-55°C overnight in the hybridization oven.

[0143] At the end of the probe hybridization, DIG Easy Hyb solutions containing the probes were decanted into clean tubes and stored at approximately -20°C. These probes could be reused for twice according to the manufacturer's recommended procedure. The membrane blots were rinsed briefly and washed twice in clean plastic containers with low stringency wash buffer (2X SSC, 0.1% SDS) for approximately five minutes at room temperature, followed by washing twice with high stringency wash buffer (0.1X SSC, 0.1% SDS) for 15 minutes each at approximately 65°C. The membrane blots briefly washed with 1X Maleic acid buffer from the DIG Wash and Block Buffer Set (Roche Diagnostics, Indianapolis, IN) for approximately 5 minutes. This was followed by blocking in a 1X blocking buffer for 2 hours and an incubation with anti-DIG-AP (alkaline phosphatase) antibody (Roche Diagnostics, Indianapolis, IN) in 1X blocking

buffer also for a minimum of 30 minutes. After 2-3 washes with 1X washing buffer, specific DNA probes remain bound to the membrane blots and DIG-labeled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System (Roche Diagnostics, Indianapolis, IN) following the manufacturer's recommendation. Blots were exposed to chemiluminescent film for one or more time points to detect hybridizing fragments and to visualize molecular size standards. Films were developed with an All-Pro 100 Plus film developer (Konica Minolta, Osaka, Japan) and images were scanned. The number and sizes of detected bands were documented for each probe. DIG-labeled DNA Molecular Weight Marker II (DIG MWM II) and DIG-labeled DNA Molecular Weight Marker VII (DIG MWM VII), visible after DIG detection as described, were used to determine hybridizing fragment size on the Southern blots.

Table 6. Location and length of probes used in Southern analysis.

Probe Name	Genetic Element	Length (bp)
<i>CryIAC</i>	<i>cryIAC</i>	1720
<i>CryIF</i>	<i>cryIF</i>	1746
<i>specR</i>	<i>Spectinomycin resistance gene</i>	750
<i>OriRep</i>	<i>Ori Rep</i>	852
<i>trfA</i>	<i>Replication initiation protein trfA</i>	1119

Example 4.5: Southern Blot Results

[0144] Expected and observed fragment sizes with a particular digest and probe, based on the known restriction enzyme sites of the *cryIAC* and *cryIF* PTU, are given in **Table 7**. Two types of fragments were identified from these digests and hybridizations: internal fragments where known enzyme sites flank the probe region and are completely contained within the insertion region of the *cryIAC* and *cryIF* PTU, and border fragments where a known enzyme site is located at one end of the probe region and a second site is expected in the soybean genome. Border fragment sizes vary by event because, in most cases, DNA fragment integration sites are unique for each event. The border fragments provide a means to locate a restriction enzyme site relative to the integrated DNA and to evaluate the number of DNA insertions. Southern blot analyses completed on multiple generations of soybean containing soybean event pDAB9582.816.15.1 produced data

which suggested that a low copy, intact *cryIAc* and *cryIF* PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.816.15.1.

[0145] Table 7. Predicted and observed hybridizing fragments in Southern blot analysis. 1. Expected fragment sizes are based on the plasmid map of pDAB9582. 2. Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker II and Mark VII fragments.

DNA Probe	Restriction Enzymes	Samples	Expected Fragment Sizes (bp) ¹	Observed Fragment Size (bp) ²
<i>CryIAc</i>	<i>AseI</i>	pDAB9582	13476	>14000
		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	>7286	~8500
	<i>Nsi I</i>	pDAB9582	15326	>15000
		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	>9479	>10000
	<i>Not I+ApaLI</i>	pDAB9582	4550	~4500
		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	4550	~4500
<i>CryIF</i>	<i>NdeI</i>	pDAB9582	8071	~8000
		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	>5569	~7500
	<i>Nsi I</i>	pDAB9582	11044	11000
		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	>9479	>10000
	<i>Hind III</i>	pDAB9582	7732	~7700
		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	7732	~7700
<i>SpecR</i>	<i>NsiI</i>	pDAB9582	15320	~15000
		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	none	none
<i>trfA</i>	<i>NsiI</i>	pDAB9582	15320	~15000

		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	none	none
<i>oriREP</i>	<i>NdeI</i>	pDAB9582	5239	~5000
		Maverick	none	none
		Soybean Event pDAB9582.816.15.1	none	none

[0146] The restriction enzymes *AseI* and *NsiI* bind and cleave unique restriction sites in plasmid pDAB9582. Subsequently, these enzymes were selected to characterize the *cryIAC* gene insert in soybean event pDAB9582.816.15.1 border fragments of >7286 bp or >9479 bp were predicted to hybridize with the probe following *AseI* and *NsiI* digests, respectively (**Table 7**). Single *cryIAC* hybridization bands of about 8500 and >10000 bp were observed when *AseI* and *NsiI* digests were used, respectively. The hybridization of the probe to bands of this size suggests the presence of a single site of insertion for the *cryIAC* gene in the soybean genome of soybean event pDAB9582.816.15.1. Restriction enzymes *NotI* and *ApaLI* were selected to perform a double digestion and to release a fragment which contains the *cryIAC* plant transcription unit (PTU; promoter/gene/terminator) (**Table 7**). The predicted 4550bp fragments were observed with the probe following *NotI* and *ApaLI* double digestion. Results obtained with the enzyme digestion of the pDAB9582.816.15.1 samples followed by probe hybridization indicated that an intact *cryIAC* PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.816.15.1.

[0147] The restriction enzymes *NdeI* and *NsiI* bind and cleave restriction sites in plasmid pDAB9582. Subsequently, these enzymes were selected to characterize the *cryIF* gene insert in soybean event pDAB9582.816.15.1. Border fragments of > 5569 bp and > 9479 were predicted to hybridize with the probe following the *NdeI* and *NsiI* digests, respectively (**Table 7**). Single *cryIF* hybridization bands of ~7500 bp and >10000 bp were observed when *NdeI* and *NsiI* were used, respectively. The hybridization of the probe to bands of this size suggests the presence of a single site of insertion for the *cryIF* gene in the soybean genome of soybean event pDAB9582.816.15.1. Restriction enzyme, *HindIII*, was selected to release a fragment which contains the *cryIF* plant transcription unit (PTU; promoter/gene/terminator) (**Table 7**). The predicted 7732 bp fragment was observed with the probe following the *HindIII* digestions. Results obtained

with the enzyme digestion of the pDAB9582.816.15.1 samples followed by probe hybridization indicated that an intact *cryIF* PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.816.15.1.

Example 4.6: Absence of Backbone Sequences

[0148] Southern blot analysis was also conducted to verify the absence of the spectinomycin resistance gene (*specR*), Ori Rep element and replication initiation protein trfA (trf A element) in soybean event pDAB9582.816.15.1. No specific hybridization to spectinomycin resistance, Ori Rep element or trf A element is expected when appropriate positive (pDAB9582 added to Maverick genomic DNA) and negative (Maverick genomic DNA) controls are included for Southern analysis. Following the *NsiI* digestion and hybridization with the *specR* specific probe, one expected size band of 15320 bp was observed in the positive control sample (pDAB9582 added to Maverick genomic DNA). The *specR* probe did not hybridize to samples of the negative control and soybean event pDAB9582.816.15.1. Similarly, one expected size band of 15320 bp was detected in the positive control sample (pDAB9582 plus maverick) but absent from the samples of the negative control and soybean event pDAB9582.816.15.1 after *NsiI* digestion and hybridization with trfA probe. Another expected size band of 5329 bp was detected in the positive control sample (pDAB9582 added to Maverick genomic DNA) but absent from the samples of the negative control and soybean event pDAB9582.816.15.1 after *NdeI* digestion and hybridization with OriRep specific probe. These data indicate the absence of spectinomycin resistance gene, Ori Rep element and replication initiation protein trfA in soybean event pDAB9582.816.15.1.

Example 5: Agronomic and Yield Field Trial and Herbicide Tolerance

[0149] Replicated agronomic trials were run to compare the agronomic efficacy of soybean event pDAB9582.816.15.1 with the null isolate - Maverick. The majority of the field trials were planted at distinct geographical locations throughout the United States where the soybean variety which contains soybean event pDAB9582.816.15.1 is cultivated. Additional field trials were completed outside of these locations, and were selected to expose the soybean variety which contains soybean event

pDAB9582.816.15.1 to potential stresses that occur from growing in non-preferred locations. Due to environmental variability within a small number of the field trials, some of the sites were discontinued from the study.

[0150] The experiment was set up as a randomized complete block design with two replications per location. There were eight entries including soybean event pDAB9582.816.15.1. Each plot consisted of two rows, 12.5 feet long, planted 30 inches apart. Throughout the season, field plots were maintained under normal agronomic practices and kept free from weeds.

[0151] Seed for the study was produced in winter nursery in Puerto Rico during the. Seed of soybean event pDAB9582.816.15.1 and Maverick were grown in the same nursery and treated in a similar manner in order to minimize any seed source variability. Next, the seed was shipped back to North America where it was packaged and distributed to the various planting locations. Throughout the season a number of agronomic characteristics were measured. These characteristics and the growth stage when the data were collected are listed in **Table 8**.

Table 8. List of agronomic characteristics measured in field trials to compare soybean event pDAB9582.816.15.1 with Maverick.

Characteristic Measured	Growth stage when measurement taken
1. Emergence: Stand count divided by the number of seeds planted in a one meter section multiplied by 100.	Calculated based on early stand count
2. Seedling vigor: Percent vigor with 0% representing a plot with all dead plants and 100% representing plots that look very healthy.	V1 – V3
3. Days to Flowering: Days from planting when 50% of the plants in the plot began to flower.	R1
4. Stand count at R2: Number of plants in a representative one meter section of row at the R2 growth stage.	R2
5. Disease incidence: Severity of disease in the plot rated on a scale of 0-100%.	R6
6. Insect damage: Percentage of plant tissue in the plot damaged by insects.	R6
7. Plant height: Average height in centimeters	R8

of the plants in each plot measured from the soil surface to the tip after leaves have fallen.	
8. Lodging: Percent lodging at harvest time with 0% = no lodging and 100% = all plants in a plot flat on the ground.	R8
9. Days to maturity. Days from planting when 95% of the pods in a plot reached their dry down color.	R8
10. Shattering: Percentage of pods shattered per plot.	R8
11. Yield: Bushels per acre adjusting to 13% moisture.	R8
12. 100 seed weight: For each plot count out 100 seeds and record the weight in grams.	R8

[0152] At the end of the growing season, data from all locations were combined and an across location analysis was performed. Data analysis was carried out using JMP® Pro 9.0.3 (SAS, Cary, NC). A mixed model was used for analysis where entry was considered a fixed effect and location, location by entry, and replication effects were considered random. Least square means from the analysis are reported in **Table 9**. For variables where a significant entry effect was measured a subsequent mean separation was performed using Student's T test to make the comparison between Maverick and soybean event pDAB9582.816.15.1. The probability level for determining significance was set at $p=0.05$.

Table 9. Least square means from the across location analysis comparing soybean event pDAB9582.816.15.1 to Maverick. Levels not connected by the same letter are significantly different at $p=0.05$ according to Student's T test.

Name	Soybean Event pDAB9582.816.15.1	Maverick
Emergence (%)	84 (A)	89 (A)
Vigor (V1-V3)	85 (A)	90 (A)
Days to Flowering (days from planting)	44.9 (A)	43.0 (B)
Stand Count at R2 (plants/m)	24 (A)	25 (A)
Disease Incidence at R6 (%)	2 (A)	2 (A)
Insect Damage R6 (%)	6 (A)	7 (A)
Height (cm)	110 (A)	114 (A)
Maturity (days from planting)	123 (A)	122 (B)
Lodging (%)	24 (A)	21 (A)
Shattering (%)	0 (A)	0 (A)

Yield (bu/ acre)	47.6 (A)	50.5 (A)
100 Seed Weight (g)	12.3 (B)	13.2(A)

[0153] All traits measured with the exception of days to flowering, maturity and 100 seed weight exhibited parity between soybean event pDAB9582.816.15.1 and Maverick. Soybean event pDAB.816.15.1 flowered about 2 days later than Maverick. The two day delay is not a severe delay for producers, and does not impair crop performance. Plots were considered to be flowering when approximately 50% of the plants in a plot exhibited open flowers. Soybean event pDAB9582.816.15 also matured one day later than Maverick at the end of the season, but this delay did not result in a meaningful agronomic difference which would impair crop performance. Likewise, 100 seed weight of soybean event pDAB9582.816.15 was statistically different than Maverick, but this did not result in a significant reduction in yield. The results indicate that soybean event pDAB9582.816.15 may develop differently than Maverick but the difference is minimal and not outside the normal range of commercially grown soybeans.

[0154] To test the herbicide tolerance of soybean event pDAB9582.816.15.1 the event was planted in an efficacy trial Santa Isabel, Puerto Rico. The cultivar Maverick, which was originally transformed to produce soybean event pDAB9582.816.15.1, was planted in each nursery and included as a control in the experiments. Seed for the T₃ nursery was derived from single plant selections at the T₂ stage and seed for the T₄ nursery was derived from single plant selections at the T₃ stage. Four lineages of the event were tested at each generation. Each lineage was planted in a plot which was 4 rows wide and 7.5 feet long. The spacing between rows was 30 inches. Plots were grown under lights for approximately 2.5 weeks to compensate for the short day length in Puerto Rico. Each nursery was sprayed with glufosinate at a rate of 411 g ae/ha. One plot of the control plants, Maverick, was sprayed with the same rate of glufosinate and a second plot was non-sprayed and used as control comparison for the event. Soybean event pDAB9582.816.15.1 showed tolerance to the glufosinate herbicide application. In contrast, none of the Maverick plants were tolerant to the herbicide treatments.

Example 6: Characterization of Insecticidal Activity for Soybean Event pDAB 9582.816.15.1

[0155] Field and greenhouse evaluations were conducted to characterize the level of plant protection provided by the Cry1Ac and Cry1F proteins in soybean event pDAB9582.816.15.1 against soybean pests including the following Lepidopteran insects, including *Anticarsia gemmatalis* (velvetbean caterpillar), *Pseudoplusia includens* (soybean looper), *Spodoptera frugiperda* (fall armyworm) and *Heliothis virescens* (tobacco budworm).

[0156] A greenhouse trial was conducted on approximately four week old plants. Fifteen plants were used to evaluate soybean event pDAB9582.816.15.1 and the Maverick control. For each insect species tested (*A. gemmatalis*, *P. includes*, and *S. frugiperda* neonate larvae), three leaf discs were cut from each plant for a total of 45 leaf discs per plant per insect species. The 1.4 cm leaf punches were placed in a test arena on top of 2% water agar, infested with one neonate larvae and sealed with a perforated plastic lid.

[0157] Mortality and leaf consumption were rated after infestation. Larvae that were not responsive to gentle probing were considered dead. The mortality rates of the insects which were placed on plant materials containing soybean event pDAB9582.816.15.1 were significantly higher (86% mortality for *Spodoptera frugiperda*, 100% mortality for *Anticarsia gemmatalis*, and 100% mortality for *Pseudoplusia includens*) than the insects which were placed on the Maverick controls. **Table 10.** Leaf damage was assessed by visually scoring the percentage of leaf disc consumed by the insect. The results obtained from the greenhouse experiments indicated that soybean event pDAB9582.816.15.1 sustained significantly lower leaf damage and higher insect mortality as compared to Maverick control plants for infestation from *Anticarsia gemmatalis*, *Pseudoplusia includens*, and *Spodoptera frugiperda*.

[0158] An efficacy evaluation of field-grown soybean event pDAB9582.816.15.1 plants was conducted by collecting leaf samples from seed increase nursery plots in Santa Isabel, Puerto Rico and sending these leaves to Indianapolis, IN for bioassay. The nursery plot for the T₃ soybean event pDAB9582.816.15.1 plants consisted of approximately 180 plants arranged in four rows. Each row was 2.3 m long and spaced 76.2 cm apart; individual plants were spaced 5.1 cm apart within each row. The bioassays were performed on one fully-expanded, mainstem trifoliate leaf, located

approximately four nodes below the meristem. The trifoliolate leaf tissue was excised from 10 individual soybean plants bearing event pDAB9582.816.15.1 and 10 individual Maverick plants. The leaves were packed and transferred to the laboratory. In the laboratory, one or two 3.33 cm diameter leaf discs were punched from each trifoliolate leaf to provide a total of 16 leaf discs. Each leaf disc was placed in a test arena on top of 2% agar, infested with one neonate *S. frugiperda* larva, and sealed with a perforated plastic lid. The leaf discs were held in a controlled environment chamber for 7 days, at which time mortality and leaf consumption were rated. Larvae not responsive to gentle probing were considered dead. Leaf damage was assessed by visually scoring the percentage of leaf punch consumed by the insect.

[0159] Mortality and leaf consumption were rated after infestation. Larvae that were not responsive to gentle probing were considered dead. The mortality rates of the insects which were placed on plant materials containing soybean event pDAB9582.816.15.1 were significantly higher (68% mortality for *Spodoptera frugiperda*) than the insects which were placed on the Maverick controls (0% mortality for *Spodoptera frugiperda*).

Table 10. Leaf damage was assessed by visually scoring the percentage of leaf disc consumed by the insect. The results obtained from this leaf bioassay indicated *Spodoptera frugiperda* larvae exposed to soybean event pDAB9582.816.15.1 sustained significantly lower leaf damage and insect survival (also described as higher insect mortality) than *Spodoptera frugiperda* larvae exposed to the Maverick control plants.

[0160] The efficacy of soybean event pDAB9582.816.15.1 was evaluated in a first field trial (First Field Trial in **Table 10**). Soybean seeds from the T₄ generation, containing soybean event pDAB9582.816.15.1 and seeds of the untransformed soybean variety Maverick were planted in a randomized complete block design with 2 replicates. Each replicate plot consisted of 2 rows, 2.3 m in length and spaced 0.76 m apart. There were 40 seeds planted per row, spaced 5.7 cm apart within the row. The trial was planted and one replicate was sprayed with glufosinate herbicide at 411 g ae/ha and the other replicate was not, resulting in only the unsprayed replicate of Maverick plants surviving for bioassay.

[0161] Leaves for the bioassay were collected when the soybean plants were in the R2 growth stage. Several days before leaves were collected for bioassay, leaf punches

were taken from the leaf one node lower (and an older leaf) on the same plants and analyzed for expression of Cry 1Ac and Cry 1F proteins using an ELISA method similar to that described in Example 2 (**Table 12**). Fully-expanded, mainstem trifoliolate leaves, showing no signs of damage or discoloration and located four nodes below the meristem, were excised for bioassay. A single trifoliolate leaf was excised from each of 15 plants per replicate. The leaves were stored at 15° C and bioassayed. The leaflets of each trifoliolate were excised and a single 3.33 cm diameter disc cut from the center of each soybean event pDAB9582.816.15.1 leaflet and two 3.33 cm diameter discs were cut from each Maverick leaflet. These leaf discs were placed individually in separate, labeled wells of 32-well plastic bioassay trays; each well containing a thin layer of agar. A single, neonate *P. includens* larva, neonate *A. gemmatilis* larva, or neonate *S. frugiperda* larva was placed on each leaf disc. The bioassay trays were sealed with adhesive plastic sheets perforated to provide ventilation. For each species, 30 larvae were exposed to leaf tissue from soybean event pDAB9582.816.15.1, and 30 larvae were exposed to leaf tissue from Maverick. The plastic trays holding the infested leaf discs were held at 25° C and 40% relative humidity (RH). After 7 days, larvae were determined to be dead (no movement when stimulated with a sharp probe), stunted (smaller in size than larvae held on Maverick leaves), or alive (normal in size and response to stimulus).

[0162] Mortality was rated after infestation. The mortality rates of the insects which were placed on plant materials containing soybean event pDAB9582.816.15.1 were significantly higher (97% mortality for *Spodoptera frugiperda*, 100% mortality for *Anticarsia gemmatilis*, and 100% mortality for *Pseudoplusia includens*) than the insects which were placed on the Maverick controls. **Table 10**. The results obtained from this leaf bioassay indicated *Spodoptera frugiperda*, *Anticarsia gemmatilis*, and *Pseudoplusia includens* larvae exposed to soybean event pDAB9582.816.15.1 sustained significantly lower insect survival (also described as higher insect mortality) than *Spodoptera frugiperda*, *Anticarsia gemmatilis*, and *Pseudoplusia includens* larvae exposed to the Maverick control plants.

[0163] The efficacy of soybean event pDAB9582.816.15.1 was evaluated in a second separate field trial (Second Field Trial in **Table 10**). Soybean seeds from the T₄ generation, containing soybean event pDAB9582.816.15.1 and seeds of the

untransformed soybean variety Maverick were planted in a randomized complete block design with 4 replicates. Each replicate plot consisted of 4 rows, 6.1 m in length and spaced 1.02 m apart. There were 160 seeds planted per row, spaced 3.8 cm apart within the row. Additional rows of Maverick were planted between and around the trial plots to attract native insect pest populations.

[0164] Leaves for the bioassay were collected when the soybean plants were in the R2 growth stage. Leaves for an additional bioassay were collected from the same plants when the soybean plants were in the R5 growth stage. Several days before leaves were collected for each bioassay, leaf punches were taken from the leaf one node lower on the same plants and analyzed for Cry 1Ac and Cry 1F proteins using an ELISA method similar to that described in Example 2 (**Table 12**). Fully-expanded, mainstem trifoliolate leaves, showing no signs of damage or discoloration and located four nodes below the meristem, were excised for bioassay. A single trifoliolate leaf was excised from each of 15 plants per replicate; 4 trifoliates per replicate were used for the bioassay of *P. includens*, 4 trifoliates per replicate were used for the bioassay of *S. frugiperda*, 4 trifoliates per replicate were used for the bioassay of *H. virescens*, and 3 trifoliates per replicate were used for the bioassay of *A. gemmatilis*. The two side leaflets of each trifoliolate were excised and placed in separate, labeled petri plates containing a thin layer of agar. Two second instar larvae of *P. includens*, *A. gemmatilis*, *S. frugiperda*, or *H. virescens* were placed on each leaflet. For *P. includens*, *S. frugiperda*, and *H. virescens*, 64 larvae were exposed to leaf tissue from soybean event pDAB9582.816.15.1 and Maverick plants. For *A. gemmatilis*, 48 larvae were exposed to leaf tissue from soybean event pDAB9582.816.15.1 and Maverick control plants. The petri plates holding the infested leaflets were covered with lids and held at 25° C and 40% RH. After 4 days, larvae were determined to be dead (no movement when stimulated with a sharp probe), moribund (larva responds to stimulus but unable to right itself if placed on side), stunted (smaller in size than larvae held on Maverick leaves), or alive (normal in size and response to stimulus).

[0165] The bioassay procedure at R5 stage was the same as the procedure used at R2 stage with the exception that for *S. frugiperda* and *H. virescens*, all three leaflets were excised from each trifoliolate and a single, second instar larva was placed on each leaflet.

This resulted in 48 larvae of *S. frugiperda* and *H. virescens* being exposed to leaflets from soybean event pDAB9582.816.15.1 and Maverick.

[0166] Mortality was rated after infestation for both the R2 and R5 leaf bioassays. The mortality rates of the insects which were placed on plant materials containing soybean event pDAB9582.816.15.1 were significantly higher (69% mortality for the R2 leaf bioassay and 54% mortality for the R5 leaf bioassay of *Spodoptera frugiperda*, 100% mortality for both the R2 and R5 leaf bioassays of *Anticarsia gemmatilis*, 95% mortality for the R2 leaf bioassay and 70% mortality for the R5 leaf bioassay of *Heliothis virescens*, and 100% mortality for the R2 leaf bioassay and 98% mortality for the R5 leaf bioassay of *Pseudoplusia includens*) than the insects which were placed on the Maverick controls. **Table 10.** The results obtained from this leaf bioassay indicated *Spodoptera frugiperda*, *Anticarsia gemmatilis*, *Pseudoplusia includens* and *Heliothis virescens* larvae exposed to soybean event pDAB9582.816.15.1 sustained significantly lower insect survival (also described as higher insect mortality) than *Spodoptera frugiperda*, *Anticarsia gemmatilis*, *Pseudoplusia includens* and *Heliothis virescens* larvae exposed to the Maverick control plants.

[0167] Soybean pods were collected from soybean event pDAB9582.816.15.1 and Maverick soybean plants grown at the second field trial, and bioassayed with *H. virescens* larvae. The two uppermost pods on the mainstem were excised from six plants selected at random within each replicate plot. Each set of pods was placed in a plastic petri dish and infested by a single second instar *H. virescens* larva. The experiment was designed so that 24 larvae would be exposed to a set of pods from that had been harvested from soybean event pDAB9582.816.15.1 and Maverick control plants. The petri dishes were held under the same conditions as the excised leaf bioassay described previously. After 2 days, survival of the *H. virescens* larvae was observed using the procedure described for the leaf bioassays.

[0168] Mortality was rated after infestation of the soybean pods. The mortality rates of the insects which were placed on soybean pods containing soybean event pDAB9582.816.15.1 were significantly higher (50% mortality for the soybean pod bioassay of *Heliothis virescens*) than the insects which were placed on the Maverick control pods. **Table 10.** The results obtained from this assay on field-grown soybean

Pods indicated that *Heliothis virescens* larvae exposed to soybean event pDAB9582.816.15.1 sustained significantly lower insect survival (also described as higher insect mortality) than *Heliothis virescens* larvae exposed to the Maverick control plants.

[0169] The terminals (the uppermost section of mainstem bearing two to three expanding trifoliate leaves and a cluster of immature pods) of soybean event pDAB9582.816.15.1 and Maverick control soybean plants were infested in the field with *H. virescens* eggs. Sections of cheese cloth bearing approximately twenty eggs from *H. virescens* were placed on the terminals of five plants selected at random within each replicate plot (20 plants tested in total), and held in place with a plastic-covered paper clip. Cloth mesh bags were placed over the terminals and the open end of the mesh bag was secured around the mainstem with a twist-tie. Egg hatching was monitored daily in a representative set of mesh bags. After all the eggs had hatched, the number of live *H. virescens* larvae in each mesh bag attached to the five plants was counted.

[0170] The average number of live insect larvae which were placed on soybean terminals of soybean event pDAB9582.816.15.1 was significantly lower (0.00 number of insects for the soybean terminal bioassay of *Heliothis virescens*) than the insects which were placed on the terminals of the Maverick controls. **Table 11.** The results obtained from this assay indicated that *Heliothis virescens* larvae exposed to soybean event pDAB9582.816.15.1 sustained significantly lower numbers of insect survival than *Heliothis virescens* larvae exposed to the Maverick control plants.

[0171] Counts of native *P. includens* larvae in the trial plots were made once a week over a four week period. The center two rows of each plot were sampled. A 91 cm × 91 cm white cloth was placed at a randomly selected location between the center two rows. The plants in the section of row adjacent to one edge of the sheet were bent over the cloth and shaken 15 times to dislodge any insects present. This process was repeated for the row on the opposite edge of the cloth. Larvae were counted by species and size: larvae < 6 mm in length were counted as small larvae and larvae ≥ 6 mm in length were counted as large larvae. All insects were removed from the cloth before taking the next measurement. The cloth was moved to a second randomly selected location between the

two center rows and the sampling process was repeated, resulting in two subsamples per plot at each sampling date.

[0172] The average number of insects which were counted over a 1.82 m row of soybean event pDAB9582.816.15.1 was significantly lower (0.00 number of insects for *Pseudoplusia includens*) than the number of insects which were counted over a 1.82 m row of the Maverick controls. **Table 11.** The results obtained from this assay indicated that *Pseudoplusia includens* infestation of soybean event pDAB9582.816.15.1 was significantly lower than *Pseudoplusia includens* infestation exposed to the Maverick control plants.

[0173] The results obtained from these replicated experiments indicated that Lepidoptera larvae exposed to soybean event pDAB9582.816.15.1 sustained significantly lower survival than larvae exposed to the Maverick control plants for all insect species tested. Thus, the soybean event pDAB9582.816.15.1 has insecticidal activity over this broad range of pest insects.

TABLE 10. Insect mortality counts for Lepidopteran insects which were bioassayed on soybean leaf and pod material from soybean event pDAB9582.816.15.1 as compared to control Maverick plants.

Trial	Pest species and stage tested	Test	Soybean event pDAB9582.816.15.1		Maverick	
			Number of dead larvae	Total larvae tested	Number of dead larvae	Total larvae tested
Greenhouse	<i>A. gemmatilis</i> neonate larvae	leaf bioassay	45	45	3	45
Greenhouse	<i>P. includens</i> neonate larvae	leaf bioassay	45	45	3	45
Greenhouse	<i>S. frugiperda</i> neonate larvae	leaf bioassay	39	45	0	45
Santa Isabel, PR	<i>S. frugiperda</i> neonate larvae	leaf bioassay	11	16	0	16
First Field Trial	<i>A. gemmatilis</i> neonate larvae	leaf bioassay	30	30	3	30
	<i>P. includens</i> neonate larvae	leaf bioassay	30	30	6	30

	<i>S. frugiperda</i> neonate larvae	leaf bioassay	29	30	2	30
Second Field Trial	<i>A. gemmatalis</i> 2 nd instar larvae	R2 leaf bioassay	48	48	0	48
	<i>P. includens</i> 2 nd instar larvae	R2 leaf bioassay	64	64	0	64
	<i>S. frugiperda</i> 2 nd instar larvae	R2 leaf bioassay	44	64	0	64
	<i>H. virescens</i> 2 nd instar larvae	R2 leaf bioassay	61	64	0	64
	<i>A. gemmatalis</i> 2 nd instar larvae	R5 leaf bioassay	48	48	4	48
	<i>P. includens</i> 2 nd instar larvae	R5 leaf bioassay	63	64	0	64
	<i>S. frugiperda</i> 2 nd instar larvae	R5 leaf bioassay	26	48	1	48
	<i>H. virescens</i> 2 nd instar larvae	R5 leaf bioassay	34	48	3	48
	<i>H. virescens</i> 2 nd instar larvae	pod bioassay	12	24	4	24

TABLE 11. Average number of live Lepidopteran insects which were present on soybean event pDAB9582.816.15.1 as compared to control Maverick plants.

Location	Pest species and stage counted	Test	Average number of live larvae	
			Soybean Event pDAB9582.816.15.1	Maverick
Second Field Trial	<i>H. virescens</i> larvae	terminal bioassay	0.00 per terminal	3.75 per terminal
	<i>P. includens</i> small+large larvae	First week field count	0.00 per 1.82 m of row	2.25 per 1.82 m of row
	<i>P. includens</i> small+large larvae	Second week field count	0.50 per 1.82 m of row	6.25 per 1.82 m of row
	<i>P. includens</i> small+large larvae	Third week field count	0.00 per 1.82 m of row	22.75 per 1.82 m of row
	<i>P. includens</i> small+large larvae	Fourth week field count	0.00 per 1.82 m of row	4.00 per 1.82 m of row

TABLE 12. Mean expression level of different transgenic proteins isolated from soybean event pDAB9582.816.15.1 plant materials which were used for insect bioassays.

Field Trial	Plant stage tested	Tissue tested	ng/cm ² of protein isolated from leaf tissue			
			Soybean Event pDAB9582.816.15.1		Maverick	
			Cry 1Ac	Cry 1F	Cry 1Ac	Cry 1F
First Field Trial	R2	leaf	6.8	52.9	0.0	0.0
Second Field Trial	R2	leaf	0.79	2.73	0.0	0.0
Second Field Trial	R5	leaf	0.39	2.28	0.0	0.0

Example 7: Expected Sequence of Soybean Event pDAB9582.816.15.1

[0174] SEQ ID NO:14 provides the expected sequence of soybean event pDAB9582.816.15.1. This sequence contains the 5' genomic flanking sequence, the expected T-strand insert of pDAB9582 and 3' genomic flanking sequences. With respect to SEQ ID NO:14, residues 1-1273 are 5' genomic flanking sequence, residues 1274 - 13658 are residues of the pDAB9582 T-strand insert, 13659 – 13821 are residues of a rearrangement from the pDAB9582 plasmid and residues 13822 – 15170 are 3' flanking sequence. The junction sequence or transition with respect to the 5' end of the insert thus occurs at residues 1273 – 1274 of SEQ ID NO:14. The junction sequence or transition with respect to the 3' end of the insert thus occurs at residues 13658 – 13659 of SEQ ID NO:14.

[0175] It should be noted that SEQ ID NO:14 is the expected representation of soybean event pDAB9582.816.15.1 and was assembled from an alignment of SEQ ID NO:1, SEQ ID NO:2, and the t-strand of pDAB9582. The actual sequence of the T-strand insert of soybean event pDAB9582.816.15.1 may slightly deviate from SEQ ID NO:14. During the transformation process of introducing an T-stand insert into the genome of plant cells, it is not uncommon for some deletions or other alterations of the insert to occur. Moreover, errors in PCR amplification can occur which might result in minor sequencing errors. For example, flanking sequences listed herein were determined

by generating amplicons from soybean genomic DNAs, and then cloning and sequencing the amplicons. It is not unusual to find slight differences and minor discrepancies in sequences generated and determined in this manner, given the many rounds of amplification that are necessary to generate enough amplicon for sequencing from genomic DNAs. One skilled in the art should recognize and be put on notice that any adjustments needed due to these types of common sequencing errors or discrepancies are within the scope of the subject disclosure. Thus, the relevant segment of the plasmid sequence provided herein might comprise some minor variations. Thus, a plant comprising a polynucleotide having some range of identity with the subject insert sequence is within the scope of the subject disclosure. Identity to the sequence of SEQ ID NO:14 can be a polynucleotide sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity with a sequence exemplified or described herein. The sequence of the flanking sequences plus insert sequence can be confirmed with reference to the deposited seed. Thus, some differences between SEQ ID NO:14 and the actual T-strand insert of soybean event pDAB9582.816.15.1 may be identified.

Example 8: Event Specific TaqMan® Assay

[0176] An event specific TAQMAN® assay was developed to detect the presence of soybean event pDAB9582.816.15.1 and to determine zygosity status of plants in breeding populations. Soybean event pDAB9582.816.15.1 contains the T-strand of the binary vector pDAB9582 (**Figure 1**). For specific detection of soybean event pDAB9582.816.15.1, specific TAQMAN primers and probes were designed according to the DNA sequences located in the 5' (SEQ ID NO:1) or 3' (SEQ ID NO:2) insert-to-plant junction (**Figure 4**). One event specific assay for soybean event pDAB9582.816.15.1 was designed to specifically detect a 139 bp DNA fragment that spans the 3' integration junction using two primers and a target-specific MGB probe synthesized by Applied Biosystems (ABI) containing the FAM reporter at its 5' end. Specificity of this TAQMAN® detection method for soybean event pDAB9582.816.15.1 was tested against 7 different events which contain the *CryIAC* and *CryIF* PTUs and a control non-transgenic soybean variety (Maverick) in duplex format with the soybean specific

endogenous reference gene, GMFL01-25-J19 (Glycine max cDNA, GenBank: AK286292.1).

Example 8.1: gDNA Isolation

[0177] gDNA samples of 7 different soybean events and non-transgenic soybean varieties were tested in this study. Genomic DNA was extracted using modified QIAGEN MAGATTRACT PLANT DNA KIT® (Qiagen, Valencia, CA). Fresh soybean leaf discs, 8 per sample, were used for gDNA extraction. Samples were diluted with DNase-free water resulting in a concentration of approximately 10 ng/μL for the purpose of this study.

Example 8.2: TaqMan® Assay and Results

[0178] Specific TAQMAN® primers and probe were designed for a soybean event pDAB9582.816.15.1 specific TAQMAN® assay. These reagents can be used with the conditions listed below to detect the transgene within soybean event pDAB9582.816.15.1. **Table 13** lists the primer and probe sequences that were developed specifically for the detection of soybean event pDAB9582.816.15.1.

Table 13. TAQMAN® PCR Primers and Probes.

Event Target Reaction			
	Name	Description	Sequence
SEQ ID NO:15	81615_3'F	Event specific forward Primer	GGCCAAAGGAACCCAAGGT
SEQ ID NO:16	81615_3'R	Event specific reverse Primer	CTAGTGTATTCAAGCTACTGGCAACC
SEQ ID NO:17	81615_3'P	Event specific probe used with 81615_3'F and 81615_3'R	5'FAM/AAGTGTCCGATTGCAAT-MGB

Reference Target Reaction			
	Name	Description	Sequence
SEQ ID NO:18	GMS116 F	Forward Primer	GTAATATGGGCTCAGAGGAATGGT
SEQ ID NO:19	GMS116 R	Reverse Primer	ATGGAGAAGAACATTGGAATTGC
SEQ ID NO:20	GMS116 Probe	Probe	5'HEX/CCATGGCCCGGTACCATCTGGTC/3BHQ_1/3'

[0179] The multiplex PCR conditions for amplification are as follows: 1X Roche PCR Buffer, 0.4 μ M event specific forward primer, 0.4 μ M event specific reverse primer, 0.4 μ M Primer GMS116 F, 0.4 μ M Primer GMS116 R, 0.2 μ M Event specific probe, 0.2 μ M GMS116 Probe, 0.1% PVP, 6-20 ng gDNA in a total reaction of 10 μ L. The cocktail was amplified using the following conditions: i) 95°C for 10 min., ii) 95°C for 10 sec, iii) 60°C for 40 sec, iv) repeat step ii-iii for 40 cycles, v) 40°C hold. The Real time PCR was carried out on the ROCHE LIGHTCYCLER 480®. Data analysis was based on measurement of the crossing point (Cp value) determined by LIGHTCYCLER 480® software, which is the PCR cycle number in which the rate of change in fluorescence reaches its maximum.

[0180] The TAQMAN® detection method for soybean event pDAB9582.816.15.1 was tested against 7 different events which contain the *CryIAc* and *CryIF* PTUs and a non-transgenic soybean variety in duplex format with soybean specific endogenous reference gene, GMFL01-25-J19 (GenBank: AK286292.1). The assay specifically detected the soybean event pDAB9582.816.15.1 and did not produce or amplify any false-positive results from the controls (i.e. the different events which contain the *CryIAc* and *CryIF* PTUs and a non-transgenic soybean variety). The event specific primers and probes can be used for the detection of the soybean event pDAB9582.816.15.1 and these conditions and reagents are applicable for zygosity assays.

[0181] Having illustrated and described the principles of the present disclosure, it should be apparent to persons skilled in the art that the disclosure can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

[0182] All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS

1. A method of detecting Soybean Event pDAB9582.816.15.1 in a sample comprising soybean DNA, said method comprising:
 - (a) contacting said sample with a first primer at least 10 bp in length that selectively binds to a flanking sequence within bp 1-1273 of SEQ ID NO:1 or the complement thereof, and a second primer at least 10 bp in length that selectively binds to an insert sequence within bp 1274-1577 of SEQ ID NO:1 or the complement thereof; and assaying for an amplicon generated between said primers; or
 - (b) contacting said sample with a first primer at least 10 bp in length that selectively binds to an insert sequence within bp 1-175 of SEQ ID NO:2 or the complement thereof, and a second primer at least 10 bp in length that selectively binds to flanking sequence within bp 176-1687 of SEQ ID NO:2 or the complement thereof; and assaying for an amplicon generated between said primers.
2. The method of Claim 1 wherein said sample comprising soybean DNA is obtained from soy plants comprising event pDAB4468.18.07.1 the seeds of which have been deposited under ATCC accession number PTA-12588.

Figure 1. is a plasmid Map of pDAB9582 containing the *cryIF* v3, *cryIAc* and *pat* v6 expression cassette.

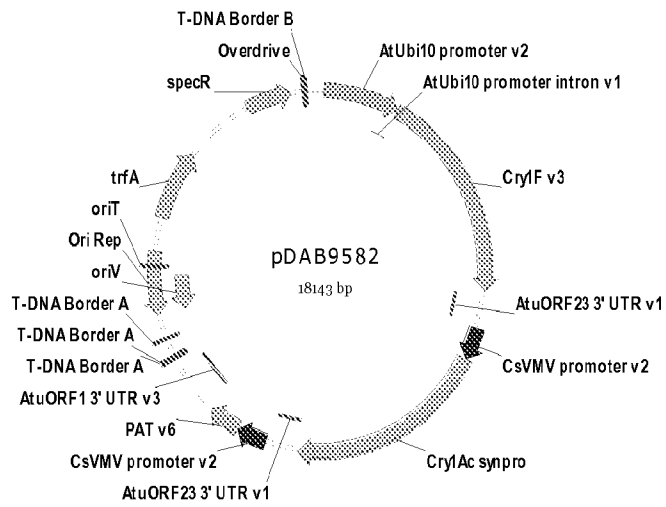


Figure 2. The diagram depicts the primer locations for confirming the 5' and 3' border sequence of the soybean event pDAB9582.816.15.1.

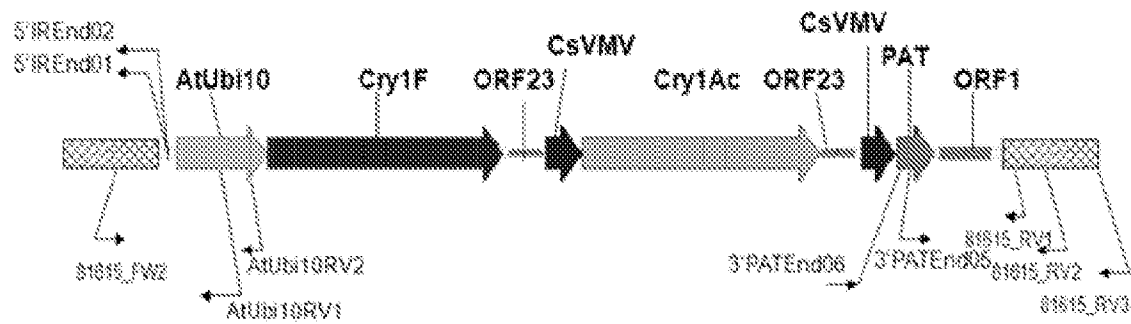


Figure 3. The diagram depicts the genomic sequence arrangement in soybean event pDAB9582.816.15.1

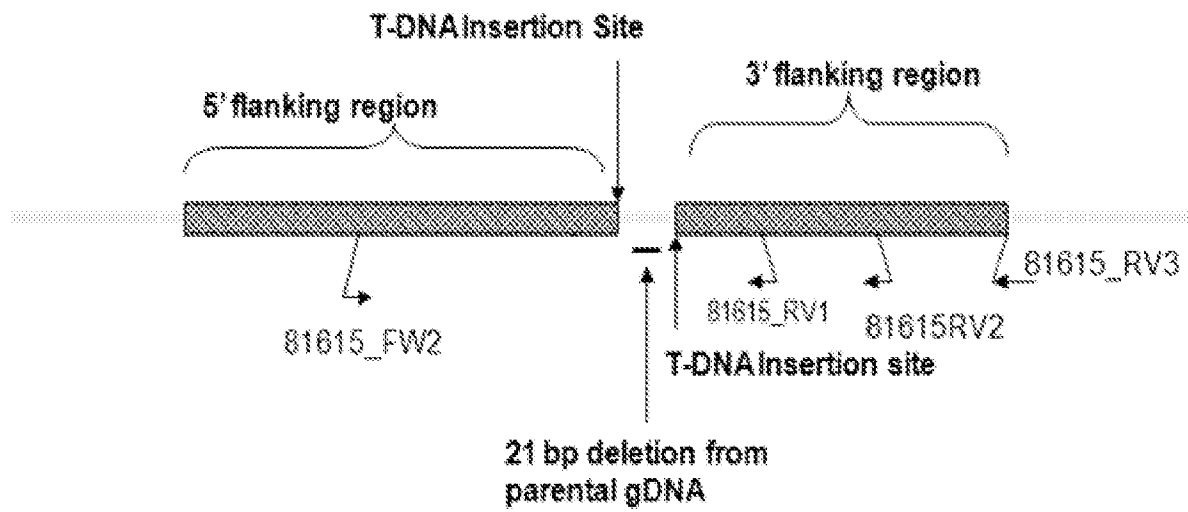
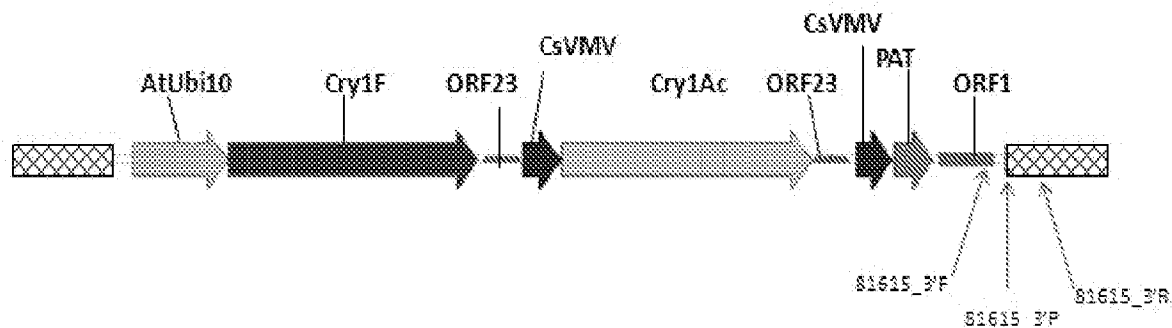


Figure 4. The diagram depicts the primer and probe locations for the TaqMan assay of the soybean event pDAB9582.816.15.1.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/047566

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - C12N 15/11 (2013.01) USPC - 800/265 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC(8) - A01P 13/00, 13/02, 17/00; A23J 1/14; A23K 1/00; C07H 21/04; C07K 14/415; C12N 5/04, 5/10, 5/14, 15/09, 15/11, 15/29, 15/31, 15/52, 15/62, 15/82 (2013.01) USPC - 504/206; 530/370; 536/23.71; 554/1; 800/265, 302 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched CPC - C12N 9/00, 9/0069, 9/0071, 9/1029, 9/1092, 15/00, 15/8274, 15/8275, 15/8277, 15/8286; C12Q 1/6895, 2600/13 (2013.01) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatBase, Google Patents, PubMed		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2006/029076 A2 (ROMMENS et al) 16 March 2006 (16.03.2006) entire document	1, 2
A	WO 2012/075426 A1 (CUI et al) 07 June 2012 (07.06.2012) entire document	1, 2
A	CRESPO et al. "Cross-resistance and mechanism of resistance to Cry1Ab toxin from <i>Bacillus thuringiensis</i> in a field-derived strain of European corn borer, <i>Ostrinia nubilalis</i> ," Journal of Invertebrate Pathology, 29 April 2011 (29.04.2011), Vol. 107, Pgs. 185-192. entire document	1, 2
☐ Further documents are listed in the continuation of Box C. ☐		
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