FIELD OF INVENTION

The present invention relates to a novel insect resistant OE-17A okra event, wherein the presence of the event confers resistant to insect pests in plants.

BACKGROUND OF INVENTION

Abelmoschus esculentus (L.) Moench. commonly known as lady's fingers, okra or bhindi is an important vegetable crop cultivated in different countries of the world. Okra has relatively good nutritional value and is a good complement in developing countries where there is often a great alimentary imbalance. Okra, like other Malvaceae members, is susceptible to a large variety of pests and diseases – insects, fungi, nematodes and viruses. While Yellow Vein Mosaic Virus (YVMV) disease transmitted by the white fly is the most serious disease of okra in most of the okra growing regions, the spotted boll worm - *Earias vittella.* (Fabricius) - is the most serious insect pest of this crop in Asia. Classical and mutation breeding programs aimed at resistance to diseases have rarely succeeded in okra. Genetic improvement by conventional plant breeding is protracted due to the lack of resistance sources to pests and diseases available in okra germplasm. However, with the establishment of genetic engineering methods, a number of genes for resistance to insect pests and diseases, and nutritional enrichment can be incorporated into this crop.

Okra is severely attacked by the spotted bollworm (*E. vittella*). The larvae bore into shoots or fruit, eating on internal contents causing the withering of the plant and reduction in marketable value of the fruit. The severity of damage varies from place to place during different seasons. Farmers often apply pesticides on the crop on alternate days in some localities, where pest pressure is high. The extent of loss

to okra due to infestation of *Earias* spp. has been reported by various researchers to be in the range of 12 to 51%. Even though relative intervarietal variation has been observed in okra against *Earias* sp., none of the varieties showed complete resistance in these studies. The fruit borer (*Helicoverpa armigera*) is also an important insect pest of okra in many parts of India.

The development of gene transfer techniques for plants species is of great interest, importance and value because it can be used for the transfer of beneficial genes of interest into plants.

SUMMARY OF THE INVENTION

The present invention discloses a novel insect resistant OE-17A okra event comprising *cry1Ac* gene under the control of 35S promoter. The present invention further discloses a novel recombinant DNA molecule comprising the okra genomic DNA sequence and *cry1Ac* gene sequence. The invention also discloses a process for detecting the okra plant comprising OE-17A okra event. Further the present invention discloses a process for distinguishing the transgenic plant comprising the event.

In one aspect of the present invention, there is provided a recombinant DNA characteristic of an insect resistant OE-17A okra event, wherein nucleotide sequence of the DNA is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11 and complements thereof.

In another aspect of the present invention, there is provided a primer set comprises a first and a second oligonucleotide useful for detection of presence of an insect resistant OE-17A okra event in a sample, wherein the first oligonucleotide comprises at least 15 nucleotides from position 30 to position 429

of the nucleotide sequence as set forth in SEQ ID NO: 10 and the second oligonucleotide comprises at least 15 nucleotides from position 430 to position 1045 of the nucleotide sequence as set forth in SEQ ID NO: 10.

In yet another aspect of the present invention, there is provided a primer set useful for detection of presence of an insect resistant OE-17A okra event in a sample, wherein the primer set consists of the first oligonucleotide sequence as set forth in SEQ ID NO: 9 and the second oligonucleotide sequence as set forth in SEQ ID NO: 5.

In still yet another aspect of the present invention, there is provided a primer set useful for detection of presence of an insect resistant OE-17A okra event in a sample, wherein the primer set consists of the first oligonucleotide sequence as set forth in SEQ ID NO: 9 and the second oligonucleotide sequence as set forth in SEQ ID NO: 12.

In further aspect of the present invention, there is provided a primer set useful for detection of presence of an insect resistant OE-17A okra event in a sample, wherein the primer set consists of the first oligonucleotide sequence as set forth in SEQ ID NO: 9 and the second oligonucleotide sequence as set forth in SEQ ID NO: 13.

The present invention also provides a process for detecting the presence of an insect resistant OE-17A okra event in a sample, the process comprises a) contacting the sample with a primer set comprising a first and a second oligonucleotide sequence useful for detection of presence of an insect resistant OE-17A okra event in a sample, wherein the first oligonucleotide comprises at least 15 nucleotides from position 30 to position 429 of the oligonucleotide sequence as set forth in SEQ ID NO: 10 and the second oligonucleotide comprises

at least 15 nucleotides from position 430 to position 1045 of the nucleotide sequence as set forth in SEQ ID NO: 10; b) performing amplification reaction; and c) detecting amplification of an amplicon; wherein detection of the amplicon is diagnostic for the presence of the insect resistant OE-17A okra event.

In another aspect, the present invention provides a process for detecting the presence of an insect resistant OE-17A okra event in a sample, the process comprises a) contacting the sample with a probe, wherein said probe is the recombinant DNA characteristic of an insect resistant OE-17A okra event, wherein nucleotide sequence of the DNA is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11 and complements thereof; b) subjecting the sample and the probe to stringent hybridization conditions; and c) detecting hybridization of the probe to the DNA; wherein detection of the hybridization is diagnostic for the presence of the insect resistant OE-17A okra event.

Yet another aspect of the present invention provides a process for producing an insect resistant okra plant comprising OE-17A event, the process comprises (a) sexually crossing a first parental okra line comprising an insect resistant OE-17A okra event, and a second parental okra line that does not comprise the event, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that comprises the event, wherein the event comprises the nucleotide sequence selected from a group consisting of as set forth in SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11 and complements thereof.

In yet another aspect, the present invention provides a kit for detection of presence of OE-17A okra event in a sample, wherein the kit comprises a primer set comprising a first and a second oligonucleotide useful for detection of

presence of OE-17A okra event in a sample, wherein the first oligonucleotide comprises at least 15 nucleotides from position 30 to position 429 of the nucleotide sequence as set forth in SEQ ID NO: 10 and the second oligonucleotide comprises at least 15 nucleotides from position 430 to position 1045 of the nucleotide sequence as set forth in SEQ ID NO: 10.

In still yet another aspect of the present invention there is provided a kit for detection of presence of OE-17A okra in a sample, wherein the kit comprises a probe, wherein the probe is the recombinant DNA characteristic of an insect resistant OE-17A okra event, wherein nucleotide sequence of the DNA is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11 and complements thereof

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1 illustrates a circular map of plasmid pC2300cry1Ac10518ve that was used to generate OE-17A okra event

Figure 2 shows gel image of the amplified product from okra plants using the event specific primers

Lane 1: BT7 DNA digested with *Eco*RI marker.

Lane 2: Negative control without template DNA

Lane 3: Negative control DNA from non-transgenic okra plant

Lane 4: DNA from OE-17A event

Lane 5 and 6: DNA from other transgenic plants that do not contain OE-17A event.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an insect resistant transgenic okra plant comprising a specific OE-17A okra event. The transgenic okra plant is

characterized by harbouring the *cry1Ac* gene under the control of CaMV 35S promoter at a specific locus in the okra genome. Further, the present invention also provides a process for detection of the specific OE-17A okra event in transgenic okra. The invention further provides a kit for identifying the transgenic plants comprising the OE-17A okra event.

A transgenic "event" is produced by transformation of plant cells with heterologous DNA, i.e., a recombinant DNA molecule comprising the gene of interest under the control of a regulatory sequence such as a promoter, enhancer and terminator, wherein the heterologous DNA is inserted into specific locus of the genome of the plant.

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 include the heterologous DNA. Even after repeated back-crossing to a recurrent parent, the inserted DNA and flanking 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 comprising the inserted DNA and flanking genomic sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

As used herein, the term "okra" means *Abelmoschus esculentus* and includes all plant varieties that can be bred with okra, including wild okra species.

As used herein, the term "comprising" means "including but not limited to".

The term "Primer" or "Primers" used in the present invention is referred to synthetic oligonucleotides useful for detection of the event.

The "Primers" are isolated or synthetic nucleotide sequence 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, and then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs of the present invention 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.

Primers and probes based on the flanking DNA and inserted heterologous sequences disclosed herein can be used to confirm the disclosed sequences by conventional methods, e.g., by re-cloning and sequencing such flanking DNA and inserted sequences.

A term "DNA" or "nucleotide" or "oligonucleotide" or "nucleic acid" used herein are interchangeable.

A "junction sequence" is the nucleotide sequence point at which the heterologous DNA inserted into the genome is linked to the genomic DNA of okra plant 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. The present invention provides nucleotide sequences that span the insertions in herein-described okra 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 invention.

A term "expression" used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid of the invention. The term "expression" also refers to translation of mRNA into a polypeptide or protein.

Probes and primers are generally consists of at least about 11 nucleotides, preferably 18 nucleotides or more, more preferably 24 nucleotides or more, and most preferably 30 nucleotides or more. Such probes and primers hybridize specifically to a target sequence under high stringency hybridization conditions. Preferably, probes and primers according to the present invention 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. Primers and probes are often interchangeable, and so primers may be used as probes and probes may be used as primers where effective. One skilled in the art would know how and when to use a probe as a primer and how and when to use a primer as a probe.

Included are DNA sequences that comprise at least 10 or more (e.g., 15, 18, 25, 28, 30 and 50) nucleotides of insert sequence from okra event OE-17A and similar length of flanking DNA from okra event OE-17A. Such DNA sequences are diagnostic for the okra event. Nucleic acid amplification of genomic DNA from the event produces an amplicon comprising such diagnostic DNA sequences.

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

ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, "Ausubel et al., 1992"); and Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. Thermal amplification-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, .COPYRGT. 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.).

Nucleic-acid amplification can be performed by method known in the art such as polymerase chain reaction (PCR). A variety of amplification methods are known in the art and are described in various documents such as PCR Protocols: A Guide to Methods and Applications, ed. Innis et al., Academic Press, San Diego, 1990. Thermal amplification methods have been developed to amplify up to 22 kb of genomic DNA and up to 42 kb of bacteriophage DNA (Cheng et al., Proc. Natl. Acad. Sci. USA 911:5695-5699, 1994). These methods as well as other methods known in the art of DNA amplification may be used in the practice of the present invention.

As used herein, "detection kit" refers to a kit used to detect the presence of OE-17A okra event in a sample, wherein the kit comprises the primers or probe which hybridize under stringent conditions to a target DNA sequence, and other materials necessary to enable nucleic acid hybridization or amplification methods.

An insect resistant okra plant comprising the OE-17A event can be produced by sexually crossing a first parental okra plant comprising the event derived from transformation with the expression cassettes comprising the heterologous DNA of the present invention and a second parental okra plant that lacks the event, thereby producing a plurality of first progeny plants; and then selecting a first progeny

plant that comprises the event and selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants comprising the event.

In accordance with the present invention there is provided a process for producing an insect resistant okra plant comprising OE-17 A event, the process comprising (a) sexually crossing a first parental okra line comprising okra event OE-17A that exhibits a trait which confers resistance to insect pests upon the event, and a second parental okra line that does not comprise the event, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that comprises the event and exhibits resistance to insect pests. The process for producing an insect resistant okra plant comprising OE-17A event is useful for introgressing the insect resistance trait into different genetic backgrounds.

According to one aspect of the invention, DNA sequences are provided that comprise junction sequence of OE-17A okra event having nucleotide sequence as set forth in SEQ ID NO: 11 and a complement thereof, wherein a junction sequence spans the junction between heterologous DNA inserted into the okra genome and DNA from the okra genome flanking the insertion site and is diagnostic for the event.

In accordance with the present invention, one embodiment provides a recombinant DNA characteristic of an insect resistant OE-17A okra event, wherein nucleotide sequence of the DNA is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, , SEQ ID NO: 11 and complements thereof.

Another embodiment of the present invention is to provide a primer set comprising a first and a second oligonucleotide useful for detection of presence of

OE-17A okra event in a sample, wherein the first oligonucleotide comprises at least 15 nucleotides from position 30 to position 429 of the nucleotide sequence as set forth in SEQ ID NO: 10 and the second oligonucleotide comprises at least 15 nucleotides from position 430 to position 1045 of the nucleotide sequence as set forth in SEQ ID NO: 10.

Yet another embodiment of the present invention is to provide the primer set comprising a first and a second oligonucleotide useful for detection of presence of OE-17A okra event in a sample, wherein the first oligonucleotide is as set forth in SEQ ID NO: 9.

Still yet another embodiment of the present invention is to provide the primer set comprising a first and a second oligonucleotide useful for detection of presence of OE-17A okra event in a sample, wherein the second oligonucleotide is selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 12 and SEQ ID NO: 13.

In one of the embodiment, the present invention provides a primer set useful for detection of presence of OE-17A okra event in a sample, wherein the primer set consists of the first oligonucleotide sequence as set forth in SEQ ID NO: 9 and the second oligonucleotide sequence as set forth in SEQ ID NO: 5.

In another embodiment of the present invention, there is provided a primer set useful for detection of presence of OE-17A okra event in a sample, wherein the primer set consists of the first oligonucleotide sequence as set forth in SEQ ID NO: 9 and the second oligonucleotide sequence as set forth in SEQ ID NO: 12.

In yet another embodiment of the present invention, there is provided a primer set useful for detection of presence of an insect resistant OE-17A okra event in a

sample, wherein the primer set consists of the first oligonucleotide sequence as set forth in SEQ ID NO: 9 and the second oligonucleotide sequence as set forth in SEQ ID NO: 13.

Still yet another embodiment of the present invention is to provide a process for detecting the presence of an insect resistant OE-17A okra event in a sample, the process comprises a) contacting the sample with a primer set comprising a first and a second oligonucleotide sequence useful for detection of presence of an insect resistant OE-17A okra event in a sample, wherein the first oligonucleotide comprises at least 15 nucleotides from position 30 to position 429 of the oligonucleotide sequence as set forth in SEQ ID NO: 10 and the second oligonucleotide comprises at least 15 nucleotides from position 430 to position 1045 of the nucleotide sequence as set forth in SEQ ID NO: 10; b) performing amplification reaction; and c) detecting amplification of an amplicon; wherein detection of the amplicon is diagnostic for the presence of the insect resistant OE-17A okra event.

The present invention further provides the process for detecting the presence of an insect resistant OE-17A okra event in a sample, wherein the primer set used comprises first oligonucleotide as set forth in SEQ ID NO: 9 and the second oligonucleotide selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 12 and SEQ ID NO: 13.

The present invention also provides a process of detecting the presence of an insect resistant OE-17A okra event in a sample, the process comprises a) contacting the sample with a probe, wherein the probe is the recombinant DNA characteristic of an insect resistant OE-17A okra event, wherein nucleotide sequence of the DNA is selected from the group consisting of SEQ ID NO: 7,

SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11 and complements thereof; b) subjecting the sample and the probe to stringent hybridization conditions; and c) detecting hybridization of the probe to the DNA.; wherein detection of the hybridization is diagnostic for the presence of the insect resistant OE-17A okra event.

Further, the present invention provides a process for producing an insect resistant okra plant comprising OE-17A event, the process comprises (a) sexually crossing a first parental okra line comprising an insect resistant OE-17A okra event, and a second parental okra line that does not comprise the event, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that comprises the event, wherein the event comprises the nucleotide sequence selected from a group consisting of as set forth in SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11 and complements thereof.

In one embodiment there is provided a transgenic plant, plant cell or a progeny thereof comprising the insect resistant OE-17A okra event, wherein the plant is produced according to the process disclosed in the present invention.

In one of the embodiment of the present invention there is provided a transgenic plant, plant cell or a progeny thereof comprising an insect resistant OE-17A okra event.

The present invention also provides a kit for detection of presence of OE-17A okra event in a sample, wherein the kit comprises a primer set comprising a first and a second oligonucleotide useful for detection of presence of OE-17A okra event in a sample, wherein the first oligonucleotide comprises at least 15 nucleotides from position 30 to position 429 of the nucleotide sequence as set forth in SEQ ID NO: 10 and the second oligonucleotide comprises at least 15

nucleotides from position 430 to position 1045 of the nucleotide sequence as set forth in SEQ ID NO: 10.

The kit for detection of presence of OE-17A okra event in a sample disclosed in the present invention comprises a set of primer comprising a first oligonucleotide and a second oligonucleotide, wherein the first oligonucleotide sequence is as set forth in SEQ ID NO: 9 and the second oligonucleotide sequence is selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 12 and SEQ ID NO: 13.

In one embodiment, there is provided a kit for detection of presence of OE-17A okra event in a sample, wherein the kit comprising a probe, wherein the probe is the recombinant DNA characteristic of an insect resistant OE-17A okra event, wherein nucleotide sequence of the DNA is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 13 and complements thereof.

Molecular characterization of the event OE-17A

Okra has been genetically modified to resist Lepidoptera insect infestation. One means for producing plants, which are resistant to insect infestation comprises the insertion of a DNA cassette that contains a sequence encoding an insecticidal Cry1Ac protein, derived from the bacterium *Bacillus thuringiensis* into the genome of okra plant. Okra plants can be transformed using a variety of methods known in the art, however, in the present invention okra plants were transformed using an *Agrobacterium tumefaciens* mediated transformation system using a DNA fragment derived from plasmid pC2300cry1Ac10518ve.

Insecticidal *cry1Ac* gene from *Bacillus thruingiensis* has been transferred into okra plant developed by the Applicant. The present invention provides an efficient

method for transforming plant, plant cells and tissues of okra (*Abelmoschus esculentus*) plant using *Agrobacterium*- mediated transformation method for conferring resistance to insect pests.

The explants for *Agrobacterium* mediated transformation of okra plant were selected from a group consisting of cotyledon with petiole, hypocotyls, embryo, immature embryo, leaf lamina, cotyledonary axil, shoot tip, anther, root and callus or any other suitable explants.

The vector pC2300cry1Ac10518ve (Figure 1) containing *cry1Ac* gene under the control of CaMV e35S promoter and polyA terminator; *nptII* gene under the control of CaMV 35S promoter as a plant selectable marker gene was transformed in the *Agrobacterium tumefaciens* cells. The recombinant *A. tumefaciens* was inoculated into a suitable medium for the growth of *Agrobacterium*. *Agrobacterium* cells were inoculated into 25 ml of sterile 2YT medium (pH 7) in a flask. 2YT medium contains 1% Yeast extract, 1.6% Tryptone and 0.5% NaCl. Suitable antibiotics were added to this medium before inoculating bacteria for the selective growth of *Agrobacterium* with the plasmid pC2300cry1Ac10518ve. The bacteria were inoculated in 2YT medium in flask and kept on a shaker to get Optical Density (600nm) in the range of 0.01 to 2, preferably 1.8.

Explants were inoculated in recombinant *Agrobacterium* suspension (preferably 15 minutes), blotted dry on sterile filter paper and later transferred to petri plates containing suitable growth medium for co-cultivation.

After the co-cultivation (2 to 5 days preferably 2 days of co-cultivation), these explants were washed in liquid medium with 500mg/l Cefotaxime to inhibit the growth of *Agrobacterium* and were transferred on selection medium.

Transformants regenerated on the selection medium were transferred to rooting medium and the rooted plants were hardened and established in green house.

Detailed procedure of transformation of okra plant with the pC2300cry1Ac10518ve construct is provided in the Example 1.

About 30-50 transgenic okra plant comprising *cry1Ac* gene were obtained. All the transformed okra plants were analysed for expression of Cry1Ac protein. Of these one transformant showing desired expression of Cry1Ac protein was selected and designated as OE-17A okra event.

The OE-17A okra event is an event which is selected from a group of events, obtained by transformation with the same transforming recombinant DNA comprising *cry1Ac* gene or by back-crossing with plants obtained by such transformation, based on the phenotypic expression and stability of the transgene i. e. *cry1Ac* gene and the absence of negative impact on the agronomic characteristics of the plant comprising it.

Thus the criteria for OE-17A okra event selection is that the event is characterized by a well defined molecular configuration which is stably inherited and shows an appropriate and stable spatial and temporal phenotypic expression of Cry1Ac protein in homozygous condition of the event at a commercially acceptable level in a range of environmental conditions in which the plants carrying the event are likely to be exposed in normal agronomic use.

The okra transgenic OE-17A event was analyzed to identify okra genomic DNA sequences flanking the expression cassette comprising *cry1Ac* gene using the method of Cottage et al. (Cottage, A., Yang, A., Maunders, H., de Lacy, R.C. and Ramsay, N.A. (2001) Identification of DNA sequences flanking T-DNA

insertions by PCR-walking. *Plant Molecular Biology Reporter* 19:321-327). Details are provided in Example 2.

Diagnostic methods for identification of the OE-17A event

To detect the presence or absence of the okra OE-17A event, a molecular diagnostic method was developed. The sequence analysis of the fragment (SEQ ID NO: 7) was carried out and the primers were designed to amplify the transgenic insertion locus for use as a diagnostic tool. The two primers designed were forward primer MHTOK-1 (SEQ ID NO: 9) and the second primer is MHIP-19 (SEQ ID NO: 5) to amplify the transgenic insertion locus from OE-17A genomic DNA.

These primer pairs include, but are not limited to, SEQ ID NO: 9 and SEQ ID NO: 5. For the amplification of event specific amplicon, any primer pair derived from SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11 or complements thereof that when used in DNA amplification reaction produces a DNA amplicon diagnostic for OE-17A event is an aspect of the present invention.

Amplicon analyses were performed on genomic DNA extracted from OE-17A okra event and non-transgenic okra plant to verify the DNA sequences flanking of the insert in OE-17A okra event. The control reactions containing no DNA template as well as the reactions containing non-transgenic okra DNA did not generate an amplicon with either primer set described above, as expected. Thermal amplifications with event OE-17A DNA generated products of the expected sizes of 237 bp. Details are provided in Example 2.

The process for identification of OE-17A okra event was carried out by primer walking method. This can also be carried out by the method well known in the art.

The following examples are included to demonstrate examples of certain preferred embodiments of the invention. The person having ordinary skill in the art should, in light of the present invention, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention

EXAMPLES

The examples given are merely illustrative of the uses, processes and products claimed in this invention, and the practice of the invention itself is not restricted to or by the examples described.

Example 1

***Agrobacterium* –Mediated Transformation of Okra**

Preparation of explants

Okra genotypes representing a variety of agronomic varieties or hybrids including Arka Anamika, Parbhani Kranti and proprietary lines of Maharashtra Hybrid Seeds Company Ltd. (MHSCL)] were used in these experiments. The mature seeds were surface sterilized preferably in 0.1% (weight/volume) $HgCl_2$ in distilled water for 1 to 60 minutes (preferably 30 minutes). These seeds were washed many times in sterile distilled water (Preferably, these seeds were imbibed in sterile water).

The embryos were isolated from these seeds in sterile conditions by pressing to remove the seed coat with tweezers or any other means. The imbibed seeds were preferred over non-imbibed. However the non-imbibed seeds also can be used. The cotyledons were separated from embryos, and these embryos measure 1 to 8

mm preferably 5 mm long at the time of isolation. These isolated embryos were washed many times in sterile water blotted dry on filter paper and placed on soaked sterile filter paper. These embryos were wounded at the plumule tip and used for the co-cultivation in *Agrobacterium* suspension.

B. Preparation of recombinant *Agrobacterium tumefaciens*

The recombinant vector pC2300 cry1Ac10518ve carries c*ry1A(c)* gene and *nptII* gene as plant selectable marker in the T-DNA region. The recombinant vector was introduced into the *Agrobacterium tumefaciens* strain EHA 105 using the method known in the art. The recombinant *Agrobacterium tumefaciens* strain EHA 105 carrying pC2300 cry1Ac10518ve plasmid was used for transformation of okra.

The antibiotic 50mg/l kanamycin and 10 mg/l chloramphenicol were added to 2YT medium for the selective growth of the *Agrobacterium* with the plasmid containing either *Cry1A(c)* gene.

The antibiotic kanamycin (preferably 50mg/l) was used to select the transgenic tissue on $MS0Z_2K_{50}C$ medium comprising MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.8, 2mg/l Zeatin supplemented with 25 to 200mg/l, preferably 50mg/l kanamycin and preferably 500mg/l Cefotaxime or any other antibiotic to inhibit the growth of *Agrobacterium.*

Analysis of putative transgenic plants using double antibody sandwich ELISA

The putative transformed plants were tested for the expression of c*ry1A*(c) gene using ELISA assay.

The ELISA plate was coated with monoclonal antibodies specific to Cry1A(c) protein. According to the manufacturer's protocol the assay was carried out as follows. Leaf samples were collected from putative transgenic plants and control plants. Total protein was extracted from these leaf samples were extracted in 500 µl of 1X PBST buffer. Each well in the pre-coated ELISA plate was loaded with 50 µl of protein sample. Polyclonal antibodies (150µl) specific to Cry1A(c) protein in the ratio 1: 20,000 dilution in PBSTO was added to each well. The samples were incubated at 4^0C overnight. The overnight incubated samples were washed thrice with PBST on the next day. Detection antibody labelled with alkaline phosphate (200µl) at 1:6000 dilution in PBSTO, was added to the wells containing the samples. The samples were incubated for 2 hours at room temperature. After the incubation the plate was washed thrice with PBST. Finally 250µl substrate buffer containing 1mg/ml paranitro phenyl phosphate was added to the wells containing sample. Colour development was recorded at 405 nm wavelength using an ELISA reader. The positive samples were selected on the development of yellow colour giving OD value > 0.2 (after 30 minutes incubation) after subtracting the blank value which was compared to the negative (non-transgenic) control (Table 2). Composition of all the buffers used for ELISA is summarized in Table 1.

Table 1: Details for the buffers used for ELISA for screening putative transgenic plants

Buffer	Composition
10X PBST (pH 7.4)	10X PBST buffer was prepared by adding 80 gm/l Sodium chloride, 11.5 gm/l Sodium phosphate dibasic, 2 gm/l potassium chloride and 2 gm/l Potassium dihydrogen phosphate and adjusted the pH to 7.4. After making up the volume to 1 liter, 5 ml of Tween 20 was added.

Buffer	Composition
1X PBST	1X PBST buffer was prepared by diluting 10X PBST buffer using distilled water
1X PBSTO	1X PBST with 0.5% Ovalbumin
Substrate buffer	Ethanolamine = 96 ml HCl + Milli Q water = 52ml + 48 ml = 100 ml Made up the final volume to 1 liter by adding Milli Q water

Table 2: ELISA results of 25 transgenic Okra plants positive for *Bt* with the respective ELISA readings

Sr. No.	Plant ID	ELISA reading
1	TOC2b	0.64
2	TOC3a	0.74
3	TOC4a	0.26
4	TOC5	0.41
5	TOC9a	0.64
6	TOC12	0.66
7	TOC13	0.36
8	TOC29b	0.45
9	TOC32a	0.58
10	TOC41a	0.68
11	TOC42a	0.58
12	TOC43a	0.79
13	TOC44a	0.81
14	TOC45	0.64
15	TOC46a	0.6
16	TOC49a	0.63
17	TOC50a	0.62
18	TOC52a	0.75

Sr. No.	Plant ID	ELISA reading
19	TOC53	0.78
20	TOC54	0.7
21	TOC55a	0.57
22	TOC58	0.64
23	TOC59	0.32
24	TOC60a	0.44
25	TOC62a	0.49
Okra (Non transgenic)	NTO1	0.01

Molecular characterization of the event OE-17A

The okra transgenic OE-17A event was analysed to identify okra genomic DNA sequences flanking the expression cassette comprising *cry1Ac* gene using the method of Cottage et al. (Cottage, A., Yang, A., Maunders, H., de Lacy, R.C. and Ramsay, N.A. (2001) Identification of DNA sequences flanking T-DNA insertions by PCR-walking. *Plant Molecular Biology Reporter* 19:321-327).

Plant genomic DNA was extracted from fresh young leaves of OE-17A event bearing plants, (Dellaporta S., Wood J. and Hicks, J B. (1983) A plant DNA mixipreparation: version II. *Plant Molecular Biology Reporter* 1:19-21).

Fresh, young leaf tissue of okra (2 g) was grinded in fine powder in presence of liquid nitrogen. The ground powder was transferred into a clean autoclaved 50 ml centrifuge tube and 15 ml of extraction buffer (prewarmed at 65°C) was added. The mixture was incubated at 65°C for 30 minutes with occasional inversion. Cold potassium acetate (5ml) (3M potassium 5M acetate; Sambrook et al., Molecular Cloning, vol. 3) was added to it. The mixture was incubated on ice for 30 minutes and centrifuged at 10,000 rpm for 5 min. Supernatant was filtered and collected in a tube and 10 ml of isoporopanol was added to it and incubated on ice for 30 minutes for DNA precipitation. The precipitated DNA was hooked out with

the help of a 1 ml pipette tip, and transferred to 1.5 ml tube. The DNA was pelleted and washed with 70% ethanol to remove residual salt. The pellet was dried and resuspended in 500 ul of sterile water. To this 3 ul of RNase (10mg/ml) was added and the mixture was incubated at 37°C for 60 min. To precipitate the DNA 3M sodium acetate and 2 volumes of 100% ethanol was added and centrifuged at 10,000 rpm for 10 min. Supernatant was discarded and the pellet was washed with 70% ethanol. The DNA was dried and re-suspended the pellet in 300 ul of sterile water.

Extraction Buffer: 100mM Tris-Cl pH 8.0, 50mM EDTA pH 8.0, 500mM NaCl, SDS 1.25%, b-mercaptoethanol 0.4%. Prepare buffer fresh before extraction and prewarm at 65^0C before use.

Genomic DNA (2μg) was digested with *Ssp-I* enzyme in 20 μl of reaction volume using standard buffers. The digestion reaction was incubated at 37°C overnight. The digestion product was incubated at 65°C for enzyme inactivation and was precipitated with 3M sodium acetate and ethanol. The digested DNA was air dried and dissolved in 12 μl of sterile distilled water and was ligated to the annealed adapter (SEQ ID NO: 1 and SEQ ID NO: 2) in ligase buffer. Both the adapters were annealed to each other and then ligated to the digested genomic DNA of OE-17A event.

ADAP 1: SEQ ID. NO: 1

CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT

ADAP 2: P-ACC TGC CC-H_2N SEQ ID NO: 2

The ligation mixture was incubated at 15-16^0C overnight for ligation of digested genomic DNA to the annealed adapters. The ligation mixture was diluted to 100 μl for obtaining adapter library. First round of amplification was carried out using

the following primer combination: Forward primer (SEQ ID NO: 3) is complementary to the inserted heterologous and reverse primer (SEQ ID NO: 4) complementary to the adapter DNA sequence.

MHIP-18: CAG CGC ATC GCC TTC TAT C SEQ ID NO: 3

AP: GGA TCC TAA TAC GAC TCA CTA TAG GGC SEQ ID NO: 4

Restriction digestion:

Genomic DNA	12.0 µl (2 µg)
10X Reaction buffer	2.0 µl (final concentration 1x)
SspI enzyme	1.0 µl (10units/ µl)
Sterile water	Make up the volume to 20.0 µl.

Ligation:

Digested Genomic DNA (heat inactivated)	12.0 µl (2 µg)
Annealed adapters	2.0 µl (100 ng/µl)
10X Reaction buffer	3.0 µl (final concentration 1x)
T4-ligase enzyme	1.0 µl (5units/ µl)
Sterile water	Make up the volume to 30.0 µl

First PCR:

Reagents	**Volume**
Nuclease-free water	Make up to 25 µl
10X reaction buffer (with $MgCl_2$)	2.5 µl
10mM dNTPs	0.5 µl
Primer MHIP-18(100ng/µl)	1.0 µl
Primer AP (100 ng/µl)	1.0 µl
Taq DNA polymerase (5 units/µl)	0.5 µl

DNA template	3.0 µl

Thermal Cycler program:

Temperature	Time	Cycles
95^0c	5 minutes	1
94^0c 58^0c 68^0c	30 seconds 30 seconds 4 minutes	40
68^0c	10 minutes	1
4^0c	Hold	

The second round of PCR was necessary to obtain the specific flanking region adjacent to the inserted heterologous gene. PCR was carried out with forward primer (SEQ ID NO: 5) and a reverse primer (SEQ ID NO: 6). Details are given below:

MHIP-19: GGT TTC GCT CAT GTG TTG AGC SEQ I D NO: 5

NAP: TAT AGG GCT CGA GCG GC SEQ ID NO: 6

Second PCR:

Reagents	Volume
Nuclease-free water	Make up to 25 µl
10X reaction buffer (with $MgCl_2$)	2.5 µl
10mM dNTPs	0.5 µl
Primer MHIP-19(100ng/µl)	1.0 µl
Primer NAP (100 ng/µl)	1.0 µl
Taq DNA polymerase (5 units/µl)	0.5 µl
DNA template	2.0 µl

Thermal Cycler program:

Temperature	Time	Cycles

95^0c	5 minutes	1
94^0c 58^0c 68^0c	30 seconds 30 seconds 4 minutes	40
68^0c	10 minutes	1
4^0c	Hold	

A small amount of PCR product was analyzed on a 1% agarose gel, and the amplified fragment was eluted from the gel by using the method known in the art. A DNA fragment (amplicon) of 588 bp was amplified from the left border region of the T-DNA after two rounds of PCR (using primers MHIP-19 SEQ ID NO: 5 and NAP SEQ ID NO: 6). The amplified fragment (amplicon) was cloned into pGEM-T Easy vector to obtain a recombinant vector. This recombinant vector was transformed in the strain of *E. coli* by using method known in the art. The strains can be DH5α, Top 10 etc. The clone comprising this recombinant vector selected for analyzing the sequence was designated as OE-17A-*Ssp I* -4. Plasmid DNA from the clone OE-17A-*SspI* -4 was isolated using standard methods known in the art. The cloned fragment (amplicon) was sequenced using SP-6 and T7 primers. The polynucleotide sequences obtained are as shown in SEQ ID NO: 7 and SEQ ID NO: 8. The sequence in SEQ ID NO: 7 contains the T-DNA vector sequence consisting of MHIP-19 primer (SEQ ID NO: 5), left border and OE-17A T-DNA flanking okar genomic DNA sequence and two bases of adapter. The sequence in SEQ ID NO: 8 contains the adapter sequence, OE-17A T-DNA flanking okra genomic DNA sequence and T-DNA vector sequence.

SEQ ID NO: 7

GGTTTCGCTCATGTGTTGAGCATATAAGAAACCCNTTAGTATGTATTTG
TATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAA
ATCCAGTCTAAAATCCAGATCCCCCGAATTAATTCGGCGTTAATTCAG
TCATTAAAAACGTCAGAATGACAATCCTAATGTCTGTCTGTCATTCAA

ACACATGCAGAGACAATTAAAAGCTGTTTGTATAGCAGCCTCCAAAGG
AGAAATTCTGACAAGGAAAACGATTGGATATGTCCACCGGTCCACATT
ATTATGAGTTAAGGGTAAGGTTTCTAATCTGTCCCATTCATTGTCCGAA
ACTGTTGGTTTTTTTCACTAGAATCCCCTTTTCAATACGTCAATTTCGC
GAAATTTGTGTATGAATGGAATTTTTTGGTTGAAATAGTTGATGGAAT
GGTCATGAATTGCGTTATATCGACTCGAAGCCAACATTGAAGACTCCG
TCTTAATGCGGTTCACTTGTAATGTGTAATGTTAGGATATACATAGAG
AATAATGATGTATATTTTATTAGATAAATAC

Sequence ID NO: 7 consists of a part of the T-DNA sequence starting with primer MHIP-19 (SEQ ID NO: 5, base pair 1-21) followed by T-DNA sequence (base pairs 22-160) adjacent to which is flanking okra genomic DNA sequence of OE-17A event (base pairs 161 to561) followed by two bases of adapter (base pairs 562 to 563).

SEQ ID NO: 8

TATAGGGCTCGAGCGGCCGCCCGGGCAGGTATTTATCTAATAAAATAT
ACATCATTATTCTCTATGTATATCCTAACATTCACATTCAAGTGTAACC
GCATTAAGACGGAGTCTTAATGTTGGCTTCGAGTCGATATAACGCAAT
TCATGACCATTCCATCAACTATTTCAACCAAAAAATTCCATTCATACAC
AAATTTCGCGTAAATTGACGTATTGAAAAGGGGATTCTAGTGAAAAAA
ACCAACAGTTTCGGACAATGTAATGGGACAGATTAGAAACCTTCCCTT
AACTCATAATAATGTGGACCGGTGGACATATCCAATCGTTTTCCTTGTC
AGAATTTCTCCTTTGGAGGCTGCTATCAAACAGCTTTTAATTGTCTCTG
CATGTGTTTGAATGACAGACAGACATTAGGTATTGTCATTCTGACGTTT
TTAATGTACTGAATTAACGCCGAATTAATTCGGGGGATCTGGATTTTA
GTACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTATT
TTCAAATCAAATACATACTAAGGGTTTCTTATATGCTCAACACATGA

Sequence ID NO: 8 consists a part of adapter sequence (SEQ ID NO: 1, base pair 1-30) adjacent to which is flanking okra genomic DNA sequence of OE-17A event (base pairs 31 to 431) followed by T-DNA sequence (base pairs 432 to 582)

Example 2

Diagnostic methods for identification of the OE-17A event

To detect the presence or absence of the okra OE-17A event, a molecular diagnostic method was developed. The sequence analysis of the fragment shown as SEQ ID NO: 7 was carried out and the primers were designed to amplify the transgenic insertion locus for use as a diagnostic tool. The two primers designed were forward primer MHTOK-1 (SEQ ID NO: 9) and the second primer is MHIP-19 (SEQ ID NO: 5) to amplify the transgenic insertion locus from OE-17A genomic DNA.

MHTOK-1: TGG AGG CTG CTA TCA AAC AGC T SEQ ID NO: 9

These primer pairs include, but are not limited to, SEQ ID NO: 9 and SEQ ID NO: 5. For the amplification of the 5'region, any primer pair derived from SEQ ID NO: 7 or SEQ ID NO: 8 and SEQ ID NO: 10 and SEQ ID NO: 11 that when used in DNA amplification reaction produces a DNA amplicon diagnostic for OE-17A event is an aspect of the present invention.

SEQ ID NO: 10 (1045 nts)

TATAGGGCTCGAGCGGCCGCCCGGGCGGTATTTATCTAATAAAATATA
CATCATTATTCTCTATGTATATCCTAACATTCACATTCAAGTGTAACCG
CATTAAGACGGAGTCTTAATGTTGGCTTCGAGTCGATATAACGCAATT
CATGACCATTCCATCAACTATTTCAACCAAAAAATTCCATTCATACAC
AAATTTCGCGTAAATTGACGTATTGAAAAGGGGATTCTAGTGAAAAAA
ACCAACAGTTTCGGACAATGTAATGGGACAGATTAGAAACCTTCCCTT
AACTCATAATAATGTGGACCGGTGGACATATCCAATCGTTTTCCTTGTC
AGAATTTCTCCTTTGGAGGCTGCTATCAAACAGCTTTTAATTGTCTCTG
CATGTGTTTGAATGACAGACAGACATTAGGTATTGTCATTCTGACGTTT
TTAATGTACTGAATTAACGCCGAATTAATTCGGGGGATCTGGATTTTA
GTACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTATT
TTCAAATCAAATACATACTAAGGGTTTCTTATATGCTCAACACATGAG

CGAAACCCTATAGGAACCCTAATTCCCTTATCTGGGAACTACTCACAC
ATTATTATGGAGAAACTCGAGCTTGTCGATCGACTCTAGCTAGAGGAT
CGATCCGAACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCG
ATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCA
CGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCAC
GGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGC
CACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCG
GCAAGCAGGCATCGCCATGTGTCACGACGAGATCCTCGCCGTCGGGC
ATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGA
TGCTCTTCGTCCAGATCATCCTGATCGACAAGACC

SEQ ID NO: 10 consist of SEQ ID NO: 8 (from base 1 to 580), part of SEQ ID NO: 7 (complementary from base 581 to 588) and T-DNA sequence (from base 589 to1045).

SEQ ID NO: 11:

TTCCTTGTCAGAATTTCTCCTTTGGAGGCTGCTATCAAACAGCTTTTAA
TTGTCTCTGCATGTGTTTGAATGACAGACAGACATTAGGTATTGTCATT
CTGACGTTTTTAATGTACTGAATTAACGCCGAATTAATTCGGGGGATC
TGGATTTTAGTACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGAT
AGAAG

SEQ ID NO: 11 consists of junction sequence comprising the okra genomic FNA and the T-DNA i.e. 200 nts from position 321 to 529 of SEQ ID NO 10)

However, any modification of these methods that use DNA molecules or complements thereof to produce an amplicon DNA molecule diagnostic for OE-17A is within the ordinary skill of the art. For example if primer SEQ ID NO: 9 is used in combination with primer 1 (SEQ ID NO: 12) will produce an amplicon of 490 base pair, or in combination with primer 2 (SEQ ID NO: 13) will amplify 668 base pair from OE-17A event. The sequences of primer 1 and 2 are as below.

Primer 1: - CAGGACATAGCGTTGGCTACC SEQ ID NO: 12

Primer 2: CGAAGAGCATCAGGGGCTC SEQ ID NO: 13

For the analysis it is important to have positive and negative controls. The PCR method was designed in order to distinguish the OE-17A event from the other okra transgenic events and non-transgenic lines. Genomic DNA from okra OE-17A event was isolated from leaves using the method described by Dellaporta et al (Dellaporta S., Wood J. and Hicks, J B. (1983) A plant DNA mixipreparation: version II. Plant Molecular Biology Reporter 1:19-21). Genomic DNA was also isolated from other okra transgenic events and non-transgenic okra lines as controls for the PCR detection method. A control reaction having no DNA in the reaction mixture was also included.

The genomic DNA from different plants was subjected to amplification using two primers namely SEQ ID NO: 9 and SEQ ID NO: 5. The details are as follows:

Reagents	Amount to be added
Nuclease-free water	Make up to 25 µl
10X reaction buffer (with $MgCl_2$)	2.5 µl
10mM dNTPs	0.5 µl
Primer MHIP-19 (100 ng/µl)	1.0 µl
Primer MHTOK-1 (100 ng/µl)	1.0 µl
Taq DNA polymerase (5 units/µl)	0.5 µl
DNA template	2.0 µl

Temperature	Time	Cycles
95^0c	5 minutes	1
94^0c 58^0c 72^0c	30 seconds 30 seconds 1minute	35

72^0c	5 minutes	1
4^0c	Hold	-

The amplified product was analyzed on agarose gel electrophoresis. The results obtained are shown in Figure 2. Lane 1 contains BT7 DNA digested with *Eco*RI marker. The sample in lane 2 contains no DNA sample, samples in lane 3 contain DNA from non-transgenic okra plant, lane4 contains DNA from OE-17A event while lane 5and 6 contains DNA from other transgenic plants that do not contain OE-17A event. From the figure it is evident that the 237 bp fragment is amplified from the okra OE-17A event but not from other transgenic events and non-transgenic okra plants.