FORM 2

THE PATENTS ACT 1970
(39 of 1970)
&
The Patent Rules 2005

COMPLETE SPECIFICATION
(see sections 10 & rule 13)

1. TITLE OF THE INVENTION

"TRANSGENIC RICE (*ORYZA SATIVA*) COMPRISING PE-2 EVENT"

2. APPLICANT (S)

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3. PREAMBLE TO THE DESCRIPTION

COMPLETE SPECIFICATION

The following specification particularly describes the invention and the manner in which it is to be performed.

FIELD OF INVENTION

The present invention relates to an insect resistant transgenic rice plant designated as PE-2 event comprising a polynucleotide sequence encoding a Cry1Ac protein.

BACKGROUND OF INVENTION

Rice has been cultivated in the country for at least 5,000 years, and India is considered to be one of the centres of origin. The area under rice cultivation is estimated at 43.4 million ha. with a total production of 130,513,000 Mt (FAO data, 2005, http://faostat.fao.org/). Since 1965, hundreds of high-yielding rice varieties have been released by the national rice research programs, adapted to different agro-climatic regions of the country.

In recent years, however, the production of rice in the Indian sub-continent has been affected by a steady increase in insect pest infestation, especially the yellow stem borer (*Scirpophaga incertulas*). The adult females lay eggs on leaves, and upon hatching, the young larvae bore into the culms (stems) of the rice plant and feed on the soft tissues within. This leads to the formation of 'dead hearts'. Infestations at later times in the crop cycle results in the formation of panicles with the absence of grain-filling, called chaffy ears. In Asia, there is a significant reduction in yield due to stem borers, of up to 10% overall, but local severe infestations can lead to crop losses of up to 70%.

Farmers use large quantities of chemical insecticides singly or in combination to control stem borers. This practice of indiscriminate use of insecticides leads to build up of pesticide residues on the grain, destruction of natural enemies, pest resurgence and environmental pollution.

To reduce pest-linked damage in rice crops as well as to protect the environment from adverse effects of pesticides, deploying the Lepidopteran specific *cry1Ac* gene under the control of a suitable promoter for high level expression in rice would provide an effective built-in control for yellow stem borer. This would result in bringing down the cultivation costs of rice, as contribution of chemical pesticides to rice cultivation is significant.

A number of groups have carried out transformation of rice using different methods. The most successful of the methods are *Agrobacterium*-mediated methods for transformation (Azhakanandam *et al.*, 2000).

The source organism for *cry1Ac* gene is *Bacillus thuringensis* (Bt), which is a gram positive bacterium synthesizing insecticidal crystalline (Cry) inclusions during sporulation. The *cry1Ac* gene encodes the Cry1Ac protein (δ-endotoxins) of 130 kDa and is highly specific to Lepidpoteran larvae. Cry1Ac protein must be ingested by the insect to exhibit insecticidal activity. The protein in its crystalline form is insoluble in aqueous solution at neutral or acidic pH, however the pH of the larval insect gut is alkaline which favours solubilisation of the protein crystal. The solubilised protein is subsequently activated by the proteases in the insect gut. These proteases cleave the carboxy terminal domain from the rest of the protein as well as approximately 28 amino acids from the amino terminal end of the protein. The activated protein, which consists of approximately 600 amino acids, diffuses through the peritrophic membrane of the insect to the midgut epithelium. Here it binds to specific high affinity receptors on the surface of the midgut epithelium of target insects. Pores are formed in the membrane leading to leakage of intracellular content (eg. K^+) into the gut lumen and water into the epithelial gut cells. The larval gut epithelial cells swell due to osmotic pressure and lyse. The gut becomes paralyzed as a consequence of changes in electrolytes and pH in the gut causing the larval insect to stop eating and die.

The expression of a foreign gene in plants is known to be influenced by the location of the transgene in the genome of the plant. Variations in transgene expression occur due to insertion into chromatin regions which may be more transcriptionally active (euchromatin) or less active (heterochromatin). Examples of these are methylated regions in which gene expression is suppressed, or in the proximity of transcriptional regulation elements like enhancers and suppressor, which increase or decrease gene expression respectively. Therefore it is necessary to screen a large number of independent transformation event for the expression of the transgene and to identify the event showing desired expression of the heterologous inserted gene.

SUMMARY OF INVENTION

The present invention relates to an insect resistant transgenic rice plant comprising a specific event PE-2. The transgenic plant is characterized by harbouring the *cry1Ac* gene under the control of CaMV 35S promoter at a specific locus in the rice genome. Further, the invention discloses a method for detection of the specific event PE-2 in transgenic rice. The invention further provides a kit for identifying the transgenic plants comprising the specific event PE-2.

The present invention relates to the transformation of the rice plant with *cry1Ac* gene for conferring insect resistance. The invention pertains to transforming rice plants with plant expression vectors pMH0102 and pCAMBIA1201 by *Agrobacterium*-mediated methods. Further it also relates to the production of more than 55 independent transformation events comprising the *cry1Ac* gene. All the independent events were screened and characterized for the expression of the Cry1Ac protein. Based on the level of expression of the Cry1Ac protein and insect bioassays, three events were selected for further characterization. The present invention also relates to a process of identification of transformation events. One specific event designated PE-2 was identified and characterized in detail.

The specific location of the insertion of the heterologous gene was analyzed by molecular methods. This involves cloning of the genomic region flanking the left border of the T-DNA into suitable vectors. The present invention also relates to the analysis of this flanking region by sequencing. The invention relates to designing primers from this region of the DNA sequence for PCR amplification of the genomic DNA of rice PE-2 event.

The present invention further provides a diagnostic tool to distinguish the rice PE-2 event from other rice transformation events and non-transgenic rice plants.

BRIEF DISCRIPTION OF ACCOMPANYING DRAWINGS

Figure 1 shows a map of the construct pMH0102

Figure 2 shows gel image of the PE-2 event using the event specific primers

DETAILED DISCRIPTION OF THE INVENTION

The present invention relates to an insect resistant transgenic rice plant comprising a specific event PE-2. The transgenic plant is characterized by harbouring the *cry1Ac* gene under the control of CaMV e35S promoter at a specific locus in the rice genome. Further, the invention discloses a method for detection of the specific event PE-2 in transgenic rice plants, plant cells, tissues or seeds. The invention further provides a kit for identifying the transgenic plants comprising the specific event PE-2.

The term "amplicon" or "amplified DNA" "amplified fragment" refers to the product of nucleic acid amplification of a target nucleic acid sequence that is part of nucleic acid template.

The term "Heterologous Gene/DNA" refers to DNA sequence of foreign origin inserted in the plant genome.

The term "event" refers to the original transformant and any progeny produced by a sexual outcross between the original transformant or its descendants bearing the heterologous gene, and another rice variety.

The term "probe" refers to a DNA sequence identical to the gene of foreign origin inserted in the plant genome.

The present invention provides efficient method for transforming plant, plant cells and tissues of Rice (*Oryza sativa*) using *Agrobacterium*-mediated transformation method for conferring resistance to insect pests.

One embodiment of the present invention provides the explants for transformation are selected from a group consisting of embryogenic calli derived from scutelar part of mature seed, embryo, immature embryo, leaf lamina, shoot tip, anther and root or any other suitable explant.

Another embodiment of the present invention is to provide a method of identification of the flanking sequence around the transgenic insertion site for the event PE-2 by PCR amplification. 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).

Transgenic insertion and neighbouring flanking rice DNA were purified by agarose gel electrophoresis and cloned. The cloned fragment was sequenced by methods known in the art.

Another embodiment of the present invention is to provide diagnostic methods for identification of the PE-2 event in transgenic rice plants.

Another embodiment of the present invention is to provide a method of introduction of PE-2 event in other background or cultivars

Another embodiment of the present invention is to provide a method of production of hybrids using the transgenic rice having PE-2 event

Yet another embodiment of the present invention provides a novel DNA molecule having polynucleotide sequence as set forth in the SEQ ID NO: 7 and the complement thereof.

The present invention further provides a method of producing the transgenic rice plant resistant to insect pests comprising transforming a rice cell with the DNA construct pMH0102. The fertile rice plant obtained from the said rice cell can be self pollinated or crossed with compatible rice varieties to produce insect resistant rice plant.

Insecticidal *cry1Ac* gene from *Bacillus thuringiensis* has been transferred into rice line MPH-1 developed by MAHYCO. The present invention provides an efficient method for transforming plant, plant cells and tissues of rice (*Oryza sativa*) plant using *Agrobacterium*-mediated transformation method for conferring resistance to insect pests.

The explants for *Agrobacterium* mediated transformation of rice plant were selected from a group consisting of embryogenic calli derived from scutelar part of mature seed, embryo, immature embryo, leaf lamina, shoot tip, anther and root or any other suitable explant.

The vector pMH0102 (Figure 1) containing the *cry1Ac* gene under the control of a CaMV e35S promoter and Ve10518 polyA terminator; and the vector pCAMBIA1201 containing *hpt* gene under the control of a CaMV 35S promoter as a plant selectable marker gene and a *GUS* gene under the control of a CaMV 35S promoter were transformed into *Agrobacterium tumefaciens*. The recombinant *A. tumefaciens* was inoculated into a suitable medium for the growth of *Agrobacterium*. *Agrobacterium* cells were inoculated into 25 ml of sterile LB medium (pH 7) in a flask. LB medium contains 1% Tryptone; 0.5% Yeast extract and 1% NaCl with pH 7.0. Suitable antibiotics were added to this medium before inoculating bacteria for the selective growth of *Agrobacterium* with the plasmids pMH0102 and pCAMBIA1201. The bacteria were inoculated in LB medium and grown with shaking until the culture reached an optical density (600nm) in the range of 1.5 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 (about 3 days of co-cultivation), these explants were transferred on postculture medium, LS-Cef containing Cefotaxime.

After about 10 days of postculture, the explants were transferred on to selection medium LS-H50 with Hygromycin and Cefatoxime for a period of about 2 weeks. Putative transformants were maintained on fresh selection medium LS-H50 again for about two weeks.

Putative transformants were transferred on to regeneration I medium, RI- MSB2.5 with Hygromycin and Cefotaxime for a period of about 10 days. Theses transformants were regenerated and elongated on regeneration II medium, RII- MSB2.5. Elongated transformant were transferred to rooting medium and the rooted plants were hardened and established in the greenhouse.

A detailed procedure of co-transformation of rice plant with the pMH0102 and pCAMBIA1201 constructs is provided in Example 1.

In the present invention, the *cry1Ac* gene from *Bacillus thuringiensis* has been transferred into rice line MPH-1 developed by MAHYCO.

More than 55 independent transformation events were screened to isolate rice plant PE-2 event. All events underwent transgene segregation analysis and protein expression evaluation to determine the optimum event for commercialization. Details are provided in Example 2.

Molecular characterization of the rice plant PE-2 event was carried out. Details are provided in the Example 3. Further diagnostic methods for identification of rice plants carrying the PE-2 event was carried out. the methods are described in detail in Example 4. Example 5 describes the zygosity assay developed for rice plant PE-2 event.

The rice plant PE-2 event was chosen on the basis of a number of criteria. Segregation analysis over three generations indicated that there is a single locus of insertion of the *cry1Ac* gene in this line. This was confirmed by DNA blot analysis. Protein quantification studies using quantitative ELISA were performed on a number of rice single insertion events. These studies indicated that the PE-2 line was among the highest Cry1Ac protein expressing lines, and that the expression of the inserted gene was stable in a number of different genetic backgrounds, over multiple generations. Phenotypic analysis of the PE-2 event bearing line showed that it was morphologically indistinguishable from the non-transformed parent line from which it was derived, and therefore most suitable for further backcross breeding.

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. Transgenic insertion and neighbouring flanking rice DNA were purified by agarose gel electrophoresis and cloned. The cloned fragment was sequenced by methods known in the art.

While the invention is broadly as defined above, it will be appreciated by those persons skilled in the art that it is not limited thereto and that it also includes embodiments of which the following description gives examples.

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

Transformation of Rice

Sterilization and inoculation of seeds

Rice seeds from line MPH-1 were surface sterilised in a 250 ml conical flask with 1.2 % NaOCl for 20 min. with vigorous shaking (30 ml NaOCl for 500 seeds). After 20 min the solution was decanted and the seeds washed 5 times with sterile distilled water. The seeds were blotted dry on sterile filter paper for 1 hr and inoculated on LS medium (Table 1) in plastic plates at 10 seeds/plate. The seeds were maintained at 28°C in dark for about 14 days to produce calli.

Agrobacterium cultures

A day before co-cultivation was to be done, a culture of the *Agrobacterium* strain harbouring the transformation vectors pMH0102 and pCAMBIA1201 was grown overnight in 25 ml liquid LB medium (Table 1) at 28°C with shaking at 175 rpm with the respective

antibiotics. This overnight culture was started with a loopful of bacterial cells taken from a freshly-streaked solid medium plate containing the same antibiotics.

Calli isolation and preculture

About three days before co-cultivation, the calli of about 14 days were isolated from the seeds and cut into pieces of size 3-4 mm^2 and precultured on LS (Table 1) solid media for co-cultivation. The cultures were maintained at 28°C in dark for about 3 days.

Co-cultivation

On the morning of co-cultivation, the optical density (O.D.) of the overnight grown bacterial culture was measured at 600 nm. The optical density of the overnight grown bacterial culture was adjusted to 1.00 using liquid LS medium. The precultured calli (explants) were incubated in the bacterial culture in a petridish or a glass beaker (50 ml beaker) for 15 min with slow intermittent stirring. The calli were then blotted on a sterile filter paper to remove excess bacteria and cultured on co-cultivation medium LS-As (Table 1) for about three days for co-cultivation (20 explants per plate). The cultures were incubated in dark at 28°C for about three days.

Positive and negative controls were also maintained in each experiment. Positive controls were explants cultured on medium without antibiotics to check the tissue culture regeneration, whereas negative controls were explants maintained on antibiotic-containing media to make sure the antibiotic is checking the growth.

Postculture (LS-Cef medium)

After about 3 days of co-cultivation, the calli were transferred on to postculture medium, LS-Cef (Table 1). The cultures were incubated in dark at 28°C for a period of about 10 days.

Selection (LS-H50 medium)

After about 10 days of postculture, the calli were transferred on to selection medium LS-H50 (Table 1) with Hygromycin 50 mg/l and Cefatoxime 250 mg/l for a period of about 2 weeks. Putative transformed calli were grown and produced protuberances, and non-transformed calli became dark brown, black. Putative transformed calli were maintained on fresh selection medium LS-H50 again for about two weeks. Cultures were incubated in dark at 28°C.

Regeneration I (RI- MSB2.5 medium)

Golden white calli were transferred on to regeneration I medium, RI- MSB2.5 (Table 1), with Hygromycin 40 mg/l and Cefotaxime 250 mg/l for a period of about 10 days. Cultures were incubated in dark at 28°C. At this stage, putative transgenic calli started producing bright protuberances that will turn into small shoot buds while non-transgenic calli turned completely black and did not produce protuberances or shoot buds.

Regeneration II (RII- MSB2.5 medium)

After about 10 days on regeneration I medium, the calli were transferred on to regeneration II medium, RII- MSB2.5 (Table 1), with Hygromycin 30 mg/l and Cefotaxime 250 mg/l, for shoot regeneration. Cultures were incubated at 28°C with a photoperiod regime of 16 hrs light + 8 hrs darkness for a period of about 10 days. At this stage small green shoot buds were visible from putative transgenic calli. These shoot buds were grown on this medium.

Elongation (RII- MSB2.5 medium)

Small shoots were transferred on to fresh RII- MSB2.5 with Hygromycin 30 mg/lit and Cefotaxime 250 mg/lit for a period of about 2 weeks, for shoot multiplication and elongation. Cultures were incubated at 28°C with a photoperiod regime of 16 hrs light + 8 hrs darkness.

Rooting (MSN1.5 medium)

The individual shoots were transferred on to rooting medium MSN1.5 (Table 1) with Hygromycin 20 mg/l and Cefotaxime 250 mg/l for a period of about 2 weeks. The shoots were subcultured for about every 2 weeks till healthy rooting.

Hardening

The rooted plants were washed with sterile distilled water thoroughly to remove the gelling agent (phytagel). Plants were then transferred to cups containing mixture of promix (60%) and soil (40%). The plants were covered with polythene bags for about 7 days. After about 7 days, the polythene bags were cut from the corners to allow the hardening process to begin, which was completed in about 2 weeks.

Table 1: Composition of media used in rice transformation

Sr. No.	Medium	Composition	Purpose
1	LB Solid	Tryptone 10 gm/l; Yeast extract 5gm/l; NaCl 10 gm/l; pH 7.0; Agar agar 15gm/l	Bacterial culture
2	LB liquid	Tryptone 10 gm/l; Yeast extract 5gm/l; NaCl 10 gm/l; pH 7.0	Bacteria inoculation.
3	LS liquid	MS major 100 ml/l; MS minor 10 ml/l; MS $CaCl_2$ 10 ml/l; MS Iron 10 ml/l; LS Vitamins Thimine HCL1mg/l; Myoinositol 100 mg/l; 2,4-D 2.5 mg/l; Maltose 3%; pH 5.8	Bacteria re-suspension
4	LS	MS major 100 ml/l; MS minor 10 ml/l; MS $CaCl_2$ 10 ml/l; MS Iron 10 ml/l; LS Vitamines, Thimine HCL1mg/l; Myoinositol 100 mg/l; 2,4-D 2.5mg/l; Maltose 3%; pH 5.8; Phytagel 3.0 gm/l	Seed inoculation
5	LS-As	MS major 100 ml/l; MS minor 10 ml/l; MS $CaCl_2$ 10 ml/l; MS Iron 10 ml/l; LS Vitamines, Thimine HCL1mg/l; Myoinositol 100 mg/l; 2,4-D 2.5mg/l; Maltose 3%; pH 5.8; Phytagel 3.0 gm/l; Acetosyringone 100 uM	Co-cultivation
6	LS-H	MS major 100 ml/l; MS minor 10 ml/l; MS $CaCl_2$ 10 ml/l; MS Iron 10 ml/l; LS Vitamines, Thimine HCL1mg/l; Myoinositol 100mg/l; 2,4-D 2.5 mg/l; Maltose 3%; pH 5.8; Phytagel 3.0 gm/l; Hygromycin 50 mg/l; Cefotaxime 250 mg/l	Selection.
7	RI- MSB2.5	MS major 100 ml/l; MS minor 10 ml/l; MS $CaCl_2$ 10 ml/l; MS Iron 10 ml/l; MS Vitamins 10 ml/l; , BAP 2.5 mg/l; , Sucrose 5%; Hygromycin 40 mg/l; Cefotaxime 250 mg/l; pH 5.8; Phytagel 0.6%	Regeneration I
8	RII- MSB2.5	MS major 100 ml/l; MS minor 10 ml/l; MS $CaCl_2$ 10 ml/l; MS Iron 10 ml/l; MS Vitamins 10 ml/l; , BAP 2.5mg/l; Sucrose 5%; Hygromycin 30 mg/l; Cefotaxime 250 mg/l; pH 5.8; Phytagel 0.3%	Regeneration II and Elongation
9	MSN1.5	MS major 100 ml/l; MS minor 10 ml/l; MS $CaCl_2$ 10 ml/l; MS Iron 10 ml/l; MS Vitamins 10 ml/l; NAA 1.5mg/l; Sucrose 3%; Hygromycin 20 mg/l; Cefotaxime 250 mg/l; pH 5.8; Phytagel 0.3%	Rooting

Example 2

Identification of rice plant PE-2 event

A large number (>55) of independent transformation events were generated in order to maximize the chance of a high-transgene-expressing, genetically stable event for

production of commercial transgenic rice lines. All rice plants coming out of the transformation experiments were analyzed for presence of the *cry1Ac* gene by PCR, and the positive plants subjected to ELISA for determining the expressivity of the transgene. The initial transformants (T_0) were advanced to the next generation by selfing, and the T_1 progeny plants were checked by PCR to determine the segregation of the transgene. The expected T_1 segregation ratio for the transgene in a line with a single *cry1Ac* gene insertion is 3:1 based on Mendelian genetics. Further, as *cry1Ac* acts as a dominant gene when introduced as a transgene, the expression of the gene was monitored by ELISA in the T_1 generation. Again, in a single insertion event, the expected ratio of Cry1Ac expressing plants to non-expressing plants is 3:1. Insect bioassays were carried out on tissue from selected lines in order to determine which lines would have better efficacy against the yellow stem borer pest. Southern blot analysis of selected individual transformation events was carried out to confirm the number of loci (insert number) at which the transgene integrated in the rice genome.

Based on the above criteria, transformed lines were selected which displayed segregation characteristics of single locus insertion events and showed effective tolerance to yellow stem borer. Conversely, those lines that were found to have abnormal segregation ratios and/or low efficacy against the pest were not taken further. The lines selected for advancement were grown in the greenhouse and Cry1Ac protein was estimated through the life of the crop by quantitative ELISA, which enables determination of the highest protein expressing lines. The tissues analyzed were leaf, culm, panicle and root. After a careful analysis of the above parameters, event PE-2 was found to be one of the best available events, in terms of Cry1Ac expression, efficacy against the pest and genetic stability over three plant generations. The PE-2 event was used for further breeding for developing yellow stem borer-tolerant rice.

Example 3

Molecular characterization of the transgenic rice event PE-2

The Rice transgenic PE-2 event was analyzed to identify rice genomic DNA sequences flanking the *cry1Ac* gene expression cassette using the method of Cottage et al., 2001. Plant genomic DNA was extracted from fresh young leaves of PE-2 event bearing plants, (Dellaporta et al., 1983). Genomic DNA (2µg) was digested with *Dra*I enzyme in 20 µl of reaction volume using standard buffers. The digestion reaction was incubated at 37°C overnight. The digestion product was then incubated at 65°C for enzyme inactivation and was precipitated with 3M sodium acetate and ethanol. DNA was air dried and dissolved in 12 µl sterile distilled water. Digested DNA was ligated to the annealed adapter in ligase buffer supplied by the manufacturer. The sequences of the adapters are as below

ADAP 1: 5'-CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT-3' SEQ ID. NO: 1

ADAP 2: 5'-P-ACC TGC CC-H_2N -3' SEQ ID NO: 2

Both the adapters were at first annealed to each other and then ligated to the digested genomic DNA of PE-2 event.

The ligation mixture was incubated at 15-16^{0}C overnight for ligation of digested genomic DNA to the annealed adapters. The ligation mixture was diluted to 100 µl for obtaining adapter library, and first round amplification was carried out using the following primer combination: Forward primer complementary to the adapter DNA sequence SEQ ID NO: 3 and reverse primer complementary to the inserted heterologous DNA SEQ ID NO: 4.

AP: 5'- GGA TCC TAA TAC GAC TCA CTA TAG GGC-3' SEQ ID NO: 3

MHIP-5: 5'- GAC CTG CAG CCA AGC TTC G -3' SEQ ID NO: 4

Restriction digestion:

Genomic DNA	12.0 µl (2 µg)
10X Reaction buffer	2.0 µl (final concentration 1x)
DraI 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 AP (100 ng/µl)	1.0 µl
Primer MHIP-5 (100ng/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	30 seconds	
58^0c	30 seconds	40
68^0c	4 minutes	
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:

NAP: 5'- TAT AGG GCT CGA GCG GC-3' SEQ ID NO: 5

MHIP-6: 5'- CAA GCT TCG AAT TAA TTC AGT AC -3' 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 NAP (100ng/µl)	1.0 µl
Primer MHIP-6 (100ng/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	30 seconds	
58^0c	30 seconds	40
68^0c	4 minutes	
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 800 bp was amplified from the left border region of the T-DNA after two rounds of PCR (using primers NAP SEQ ID NO:5 and MHIP-6 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 PE-2-*Dra I* -

10. Plasmid DNA from the clone PE-2-*Dra I* -10 was isolated using standard methods known in the art. The cloned fragment (amplicon) was sequenced. The sequence in SEQ ID NO: 7 contains the adapter sequence, PE-2 T-DNA flanking rice genomic DNA sequence, a part of left border, e 35 S promoter and a part of *cry1Ac* gene coding sequence.

SEQ ID NO: 7

TATAGGGCTCGAGCGGCCGCCCGGGCGGGTAAAGCCCCTGGGAGACTCGTCTGAAGGCGTT
AATGTTCTTGATCTCAGTGAGCCTATATAGATTTTTGGCCCATCATCATGAAGATTTGAAG
AATTAGGCCCAGATAGATACAGAGATTAAAAAGCGGAAAGGAATAAGTCTACTTCACCTCC
CTCCACTCATGCGTCTGGGTGAATCACACCCCTCAACCGCAAAACCGGATATAACTTGTCC
CTTAACTTCAAAAACCGTTGCAAATCGACTCCCTCGACGGTTTCGGAGGCGGTTTTTCCCA
CGTGGCACTTACGTGGCGGGTTTATCTGGTCTTCATCCCATGTGACATCGACGTGGCGCTT
ACGTGGCAGTAAAAAACAAACCTAAATAAAAATAAAAAGAAGTTTTGGGCACACGTGTCAG
TTACTCGTCTCCTCACTCGACGGCGAGCGAGATGGAGAGAGCGGCGGNGCAGACGGCGGGA
GCTTGGCAGAGCGGTGAGAGCAGCAAGACGTTGGAAGCGGTGGGACGGCGAGAGCGGTTGA
GGGATACGATTAACCAGGTACATGAGTTGAGGGACGCAATGTAAACTTATTCTTAAAAAAG
CCATGGAGGCCCATTTATCAGGTTTTCCAGATGGTACTAATTGAGAAGCAGAATTGTGTTG
AACCACGCAACATCAGAATGGTCAATGCTTCTTAATTAGCCGCCTGCCGCCTTCATACATC
CAATCCACACGATGCCATAACACATTGCGGGCGTTTTTAATGTACTGAATTAATTCGAAGC
TTGGCTGCAGGTCTGTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTC
CATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACA
AATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCC
CAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCT
TCAAAGCAAGTGGATTGATGTGATGGTCCGATTGAGACTTTTCAACAAAGGGTAATATCCG
GAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAA
GGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCC
TCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAG
ACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGA
TGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCAT
TTGGAGAGGACACATGGACAACAACCCAAACATCAACGAATGCATTCCATACAACTGCTTG
AGTAACCCAGAAGTTGAAGTACTTGGTGGAGAACGCATTGAAACCGGTTACACTCCCATCG
ACATCTCCTTGTCCTTGACACAGTTTCTGCTCAGCGAGTTCGTGCCAGGTGCTGGGTTCGT
TCTCGGACTAGTTGACATCATCTGGGGTAT

SEQ ID NO: 7 consists a part of SEQ ID NO 1 (base 1 to 30), PE-2 T-DNA flanking rice genomic DNA sequence (base 31 to 751), a part of left border (base 752 to 789), e35 S promoter (base 790 to 1416) and a part of *cry1Ac* gene coding sequence (base 1417 to 1616).

Example 4

Diagnostic methods for identification of the transgenic rice PE-2 event:

To detect the presence or absence of the rice PE-2 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 MHTPH-1 (SEQ ID NO: 8) and the second primer is MHIP-5 (SEQ ID NO: 4) to amplify the transgenic insertion locus from PE-2 genomic DNA.

MHTPH-1: 5'- ACT CGT CTC CTC ACT CGA CGG -3' SEQ ID NO: 8

These primer pairs include, but are not limited to, SEQ ID NO: 8 and SEQ ID NO: 4. For the amplification of the 5' region, any primer pair derived from SEQ ID NO: 7 that when used in DNA amplification reaction produces a DNA amplicon diagnostic for PE-2 event is an aspect of the present invention.

However, any modification of these methods that use DNA molecules or complements thereof to produce an amplicon DNA molecule diagnostic for PE-2 is within the ordinary skill of the art. For example if SEQ ID: 8 primer if used in combination with primer1 (SEQ ID NO: 9) will produce an amplicon of 1017 base pair, or in combination with primer 2 (SEQ ID NO: 10) will amplify 1066 base pair from PE-2 event. The sequences of primer 1 and 2 are as below.

Primer 1: 5'- GCA TTC GTT GAT GTT TGG GTT G-3' SEQ ID NO: 9

Primer 2: 5'- CTC CAC CAA GTA CTT CAA CTT C– 3' SEQ ID NO: 10

For the analysis it is important to have positive and negative controls. The PCR method was designed in order to distinguish the PE-2 event from the other rice transgenic events and non-transgenic lines. Genomic DNA from rice PE-2 event was isolated from leaves using the method described by Dellaporta *et al.* (1983). Genomic DNA was also isolated

from other rice transgenic events and non-transgenic rice 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: 8 and SEQ ID NO: 4, 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 MHTPH-1 (100 ng/µl)	1.0 µl
Primer MHIP-5 (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	30 seconds	
58^0c	30 seconds	35
72^0c	1minute	
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 molecular weight marker. Lane 2, the no DNA control sample. Lane 3 represents a non-transgenic rice control. The sample in lane 7 contains DNA from the PE-2 event, while samples in lane 4, 5 and 6 contain DNA from other transgenic rice plants that do not contain the PE-2 event. From the figure it is evident

that the 377 bp fragment is amplified from the rice PE-2 event but not from other transgenic events and non-transgenic rice plants.

Lane 1. Molecular weight marker

Lane 2. No DNA (water) – negative control

Lane 3. DNA template from a non-transgenic rice plant

Lane 4, 5 and 6. DNA from transgenic rice plants that do not contain the PE-2 event

Lane 7. PE-2

Example 5

Zygosity assay for rice PE-2 event

The PE-2 flanking genomic DNA sequence described in Example 3 was used to obtain sequence information adjacent to the T-DNA. A primer was designed having nucleotide sequence as shown in SEQ ID NO: 11.

MHTPH-2: 5`- GGG AGA GAA GGT ACT CTG TGC C –`3 SEQ ID NO: 11

This primer when used in combination with the primer having nucleotide sequence as set forth in SEQ ID NO: 8 and SEQ ID NO: 4 results in the amplification of the following fragments:.

1) 377 base pairs (transgene specific band from SEQ ID NO: 8 and SEQ ID NO: 4

2) 933 base pairs (non-transgenic allele band from SEQ ID NO: 8 and SEQ ID NO: 11

The PCR conditions for conducting zygosity PCR for rice PE-2 event is given below.

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 MHTPH-1 (100 ng/µl)	1.0 µl
Primer MHIP-5 (100 ng/µl)	1.0 µl
Primer MHTPH-2 (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	40 seconds	
58^0c	40 seconds	35
72^0c	1: 40minute	
72^0c	5 minutes	1
4^0c	Hold	---

References

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Dellaporta S, Wood J, and Hicks JB. (1983) A plant DNA minipreparation: version II. *Plant Molecular Biology Reporter* 1:19-21.