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Research Article

Generation of insect resistant marker-free transgenic rice with a novel *cry2AX1* gene

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Abstract
A synthetic *cry2AX1* gene, consisting sequence of *cry2Aa* and *cry2Ac* genes, driven by green tissue specific *rbcS* promoter was used to develop insect resistant marker-free transgenic rice events. The gene of interest *cry2AX1* and the selectable marker gene *hpt* were present in two independent T-DNAs of the *Agrobacterium* strain, C58C1. Seventy seven hygromycin and GUS positive T_0 transgenic plants were recovered. Among them, 48 were found to be co-transformed with *cry2AX1*. The Cry2AX1 protein expression level in T_0 transgenic events ranged from 0.027 to 0.110 µg/g of fresh leaf tissue. The toxicity level of Cry2AX1 protein was assessed through *in vitro* bioassay against target insects. The larval mortality ranged from 46.66 to 66.67 per cent against rice leaffolder (*C. medinalis*) while it ranged from 60 to 100 per cent against rice yellow stem borer (*S. incertulas*) in T_0 transgenic plants. A marker free transgenic event, which expressed 0.043 µg/g of Cry2AX1 in fresh leaf tissue exhibited 45 and 55 per cent larval mortality against rice leaffolder and rice yellow stem borer, respectively in T_2 generation.

Key words
Transgenic plants, *cry2AX1*, insect resistance, rice leaffolder, rice yellow stem borer

Introduction

Rice is a major staple food crop of the world, particularly Asia, Africa, Central America, and the Middle East (Zhang *et al.,* 2011; Durand-Morat *et al.,* 2015). The predicted global demand for rice is gradually increasing by 0.9 and 1.2 per cent every year (Durand-Morat *et al.,* 2015). Insect pests have been identified as the major factor causing yield loss in rice (Waddington *et al.,* 2010). However, yield loss due to lepidopteran insects inflicted damage between 5 to 25 % with occasional localized outbreaks of up to 60 per cent and remains a major challenge (Pathak and Khan, 1994; Bandong and Litsinger, 2005). In India, average yield losses in recent years due to rice stem borers and leaffolders account for 30 and 10 per cent, respectively (Krishnaiah and Varma, 2012), while the co-incidence of yellow stem borer and rice leaffolder cause more damage than when these insects infest the crop separately (Selvaraj *et al.,* 2012).

Chemical pesticides are predominantly used to control rice insect pests. Indiscriminate use of pesticides in rice is becoming a major concern for rice ecosystem, affecting the beneficial insects (Waddington *et al.,* 2010). However, application of chemical pesticides is becoming less effective in controlling yellow stem borer and rice leaffolder due to their feeding nature. They live either inside the stem or inside folded leaf nests or in the soil, avoiding direct contact with the applied pesticides. On the contrary, due to non-availability of rice germplasm that are resistant to these insects, breeding high yielding insect resistant rice cultivars through conventional breeding approaches is as well a challenge.

Genetic engineering technology is considered as an alternative tool to develop insect resistant crop varieties. Crop varieties developed through *Bt* technology represent one of the most commercially successful technologies in modern agriculture. *Bt* crop technology has been demonstrated to be economically viable and environmentally sustainable approach for pest management (Sreevathsa *et al.,* 2015). *Bt* genes are very effective against several important pests, particularly lepidopteran and coleopteran insects. The action of *Bt* relies upon toxic proteins produced during sporulation which are known to form pores in the midgut membrane and possess insecticidal activity when ingested by certain group of insects while being harmless to all vertebrates including humans (Bravo *et al.,* 2007). *Bt*

rice has the potential to eliminate yield losses caused by lepidopteran pest besides substantially reducing the use of broad-spectrum chemical insecticides, which has health and environment benefits (High *et al.,* 2004).

Transgenic rice plants which express the fusion proteins of *Cry1Ac* and *Cry1I* (Yang *et al.,* 2014) and that of *Cry1Ab* and *Cry1Ac* (Cheng *et al.,* 1998) were reported to be effective against rice leaffolder and yellow stem borer. A broad-spectrum of toxicity against lepidopterans was observed in *Cry2A* family of proteins (Mandal *et al.,* 2007). Rice lines expressing *cry1Ac* and *cry2A* genes separately exhibited high level of resistance to yellow stem borer, rice leaffolder and rice skipper (Bashir *et al.,* 2004), while the codon optimized *cry2A* was found to be effective against yellow stem borer and leaffolder (Chen *et al.,* 2005) in laboratory and field studies. Recently, expression of a chimeric *cry2AX1* gene (fusing *cry2Aa* and *cry2Ac* genes) was reported to be toxic against *Helicoverpa armigera* and *Spodoptera litura* in tobacco (Udayasuriyan, 2012; Jayaprakash *et al.,* 2014) as well as tomato (Balakrishnan *et al.,* 2012) and against rice leaffolder in rice (Manikandan *et al.,* 2014a).

Transgene driven by constitutive promoters, *viz*. *CaMV35S* or maize *ubiquitin1* express in all parts of the plants. However, tissue specific promoters drive the expression of transgenes only in desirable tissues and one such promoter is green tissue specific *rbcS* promoter, which is regulated by light (Kyozuka *et al.,* 1993; Huang *et al.,* 1999; Nomura *et al.,* 2000). In the present study, an attempt was made to express the Cry2AX1 protein in greener parts of rice plant which are fed by the larvae of target pests.

Selectable marker genes (SMGs) are essential components for transgenic plant development but their retention in transgenic plants may negatively impact public acceptance, even though the products of SMGs are proved to be safe. In order to improve public acceptance and successful commercial deployment, removal of selectable marker after selection process could lead a safer publically acceptable produce. Hence, in the present study, the marker gene, *hpt* gene was segregated from the gene of interest leading to development of marker free transgenic event.

Materials and Methods

Agrobacterium tumefaciens strain, C58C1 harbouring a co-integrate plasmid pGV2260::pSSJ1 and a binary plasmid pMF-rbcS-tp-*cry2AX1* was used for co-transforming rice cultivar, ASD16. The T-DNA of the binary plasmid pMF-*rbcS-tp-cry2AX1* (Fig. 1A) harbours *cry2AX1* gene driven by green tissue specific promoter, *rbcS* and its chloroplast transit peptide (Jang *et al.,* 1999) and the co-integrate vector pGV2260::pSSJ1 (Jacob and Veluthambi, 2002) carries *hph* and *gus* genes (Fig. 1B). The binary vector was mobilized into *A. tumefaciens* strain, C58C1 by triparental mating.

Agrobacterium-mediated rice transformation was followed as per the protocol of Hiei and Komari (2008). Two days old bacterial culture were suspended in AA infection medium with 50 µM acetosyringone for infecting embryos. Fourteen day old immature seeds were collected from the rice var. ASD16, healthy seeds were dehusked and sterilized. Surface sterilization involves 70% ethanol treatment for 1 min and 0.1% mercuric chloride for 3 min intervened by three washes with sterile distilled water. Isolated healthy immature embryos were transferred into sterile eppendorf tubes containing sterile water and incubated at 43 ^{o}C for 30 min in water-bath followed by one min incubation on ice and centrifuged at 1,100 rpm for 10 min. Cocultivation was carried out with the pretreated immature embryos by placing on NB-As (10X N6 major salts 100ml L^{-1}, 100x FeEDTA 10ml L^{-1}, 100X B5 minor salts 10 ml L^{-1}, 100X B5 vitamins 10 ml L^{-1}, 100 mg L^{-1} 2,4-D, 100 mg L^{-1} NAA, 100 mg L^{-1} 6BA, sucrose 20 g L^{-1}, glucose 10 g L^{-1}, proline 500 mg L^{-1} and vitamin assay casamino acids 500 mg L^{-1}) medium containing 100 mM acetosyringone and 5 µl of the *Agrobacterium* suspension was dropped on each embryo and incubated at 26 ^{o}C in dark for 30 min. Infected embryos were transferred to fresh place on the same plate and incubated at 26 ^{o}C in dark for seven days. Putative embryogenic calli from co-cultivated immature embryos were separated from shoots and cultured for 15 days at 31 ^{o}C under continuous illumination on resting medium (10X CC major salts 100 ml L^{-1}, 100X FeEDTA 10 ml L^{-1}, 100X CC minor salts 10 ml L^{-1}, 100 X CC vitamins 10 ml L^{-1}, 100 mg L^{-1} 2,4-D, 100mg L^{-1} NAA, 100 mg L^{-1} 6BA, maltose 20 g L-1, mannitol 36 g L-1, proline 500 mg L^{-1} and vitamin assay casamino acids 500 mg L^{-1}) containing 250 mg l^{-1} cefotaxime. The proliferated embryogenic calli were subcultured twice on selection medium (10X CC major salts 100 ml L^{-1}, 100X FeEDTA 10 ml L^{-1}, 100X CC minor salts 10 ml L^{-1}, 100 X CC vitamins 10 ml L^{-1}, 100 mg L^{-1} 2,4-D, 100 mg L^{-1} NAA, 100 mg L^{-1} 6BA, maltose 20 g L-1, mannitol 36 g L-1, proline 500 mg L^{-1} and vitamin assay casamino acids 500 mg L^{-1}) containing hygromycin 50 mg 1^{-1} and 250 mg l^{-1} cefotaxime for 17 days and transferred to pre-regeneration medium (10X N6 major salts 100 ml L^{-1}, 100X FeEDTA 10 ml L^{-1}, 100X B5 minor salts 10 ml L^{-1}, 100X vitamins, 100 mg L^{-1} 2,4-D, 100 mg

L^{-1} NAA, 100 mg L^{-1} 6BA, 30 g L^{-1} maltose, 500 mg L^{-1} proline, 500 mg L^{-1} vitamin assay casamino acids and 30 g L^{-1} glutamine) containing hygromycin 40 mg 1^{-1} incubated at 31 ^{o}C for 7 days under continuous illumination. The proliferated calli with green spots were transferred to regeneration medium containing 30 mg l^{-1} hygromycin. The regenerated plantlets were transferred to half strength MS medium containing 30 mg l^{-1} hygromycin for rooting. Well developed putative transformants were transferred to protray filled with soil and maintained in transgenic greenhouse for hardening.

GUS expression in regenerated plantlets were determined using the histochemical GUS assay as described by Wu *et al*. (2003). About 3-5mm of leaf and root bits were incubated overnight at 37 ^{o}C in 10 µl of X-Gluc buffer (pH 7.0) containing 1 mM X-Gluc, 100mM sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% (v/v) Triton X-100. Untransformed ASD 16 plant samples were used as negative control. GUS positive plantlets were tagged and grown in a greenhouse.

Plant genomic DNA were isolated from the putative transformants and wild type control plants by CTAB method (Dellaporta *et. al.,* 1983). Presence of *cry2AX1* gene were analysed with gene specific primers(CRY2FP-5'-CTAACATTGGTGGACTTCCAG - 3' and CRY2RP- 5'-GAGAAACGAGCTCCGTTATCGT-3'). PCR of 20 µl reactions were carried out in Eppendorf thermocycler (Vapo protect). The amplified products were subjected to 0.8 % agarose gel electrophoresis and visualized on UV transilluminator upon ethidium bromide staining.

Thirty milligram of fresh leaves were collected and homogenized with 500 µl of extraction buffer (prepared 1X working extraction buffer from the stock provided with kit), centrifuged at 6000 rpm for 7 min at 4 ^{o}C. Supernatant of 100 µl were used for assay with Envirologix ELISA kit (Envirologix, USA) and experiment was done as per manufacturer's instructions with two replications for each sample. Untransformed ASD16 plants were used as negative control. Cry2AX1 protein levels were expressed as µg/ gram of fresh leaves.

Adult moths of rice leaffolder were collected from rice fields of Paddy Breeding Station, TNAU, Coimbatore and reared on TN1 rice plants maintained in insect cages (65 cm x 65 cm x 75 cm). The egg laying capacity of adult moths was enhanced by supplementation of 10 % honey solution with vitamin E. The neonate stage larvae were used for the bioassay. About 5 cm long leaf bits from transgenic and non-transgenic plants were placed on a moist filter paper in a plastic petriplate. For each treatment, thirty neonate (five larvae/ leaf bit) larvae of rice leaffolder were released. The experiment was done with three replications and maintained at 25 ^{o}C ± 1, 60 % relative humidity for 6 days. Larval mortality and leaf area feeding was recorded daily from the 3rd day. The larval mortality and leaf area damage was expressed in percentage.

Adult moths of rice yellow stem borer collected from rice fields of Paddy Breeding Station, TNAU, Coimbatore were released on TN1 rice plants maintained in insect cages (65 cm x 65 cm x 75 cm). Egg masses were collected and maintained at insect bioassay laboratory with optimum environmental conditions. About 5 cm length of stem bits from transgenic and non-transformed plants were placed on a moist filter paper in a plastic petriplate. Cut end of the stem bits were damaged using knife to ensure that the neonates get soft tissues for feeding. In each stem bit, five active neonates were released and thirty larvae were used per treatment with three replications. Non transgenic ASD16 rice was used as a control. The experimental setup was maintained at 25 ^{o}C ± 1, 60 % relative humidity for 6 days. The larval mortality and feeding damage was recorded on 6th day by split opening of the stem bits.

T_1 seeds collected form the T_0 generation were used for segregation analysis in order to identify marker-free transgenic plants. After 10 days of sowing, the seedlings were subjected to GUS histochemical analysis. The plants which showed negative for GUS expression were subjected to PCR analysis to check the presence of *cry2AX1* gene with gene specific primers (CRY2FP– 5'-CCTAACATTGGTGGACTTCCAG – 3' and CRY2RP- 5'-GAGAAACGAGCTCCGTTATCGT-3'). Plants which were negative for GUS expression and PCR positive for *cry2AX1* were considered marker-free plants.

Results and Discussion

The elite *indica* rice cultivar, ASD16 was transformed with *cry2AX1* gene through *Agrobacterium* mediated transformation. A total of 77 putative transgenic ASD16 lines were generated under hygromycin selection and transferred to transgenic greenhouse for further analysis. Putative hygromycin resistant transgenic plants were subjected to GUS histochemical analysis to confirm the presence of *gus* gene. Blue colour was observed in all the samples, which indicated the presence and expression of *gus* gene in all the transgenic plants while control ASD16 samples did not show blue colour development.

Genomic DNA from the transgenic plants was subjected to PCR analysis to confirm the presence of *hpt* and *cry2AX1* genes with gene specific primers. All the transgenic plants were positive for *hpt* (data not shown). However, out of 77 plants generated, 48 were found to be positive for *cry2AX1* (Fig. 2). The co-transformation frequency was 62 per cent.

PCR positive transgenic plants were subjected to expression assay for *cry2AX1* using Cry2A quantitative ELISA kit. Out of 48 PCR positive plants, 9 plants showed detectable level of Cry2AX1 protein in fresh leaf tissues (Table 1). The expression level ranged from 0.027±0.02 to 0.110±0.03 µg/gram of fresh leaf tissues, while non-transformed ASD16 did not show detectable level of expression. Among the transgenic events tested, the maximum expression level (0.110 µg/g) of Cry protein was observed in GR-ASD16-21.

The larval mortality ranged from 46.66 to 66.67 per cent and the transgenic event, GR-ASD16-21 showed highest larval mortality of 66.67 per cent, while no mortality was observed in ASD 16 control plant. Similarly, 5 positive events were assayed against neonates of yellow stem borer and larval mortality ranged from 60 to 100 per cent while non-transformed ASD16 did not show any larval mortality. Among the transgenic events tested, the transgenic event, GR-ASD16-21 showed maximum larval mortality of 100 % (Table 1).

Eight transgenic events selected based on relative level of Cry2AX1 expression in T_0 generation were established in transgenic greenhouse in order to identify marker-free transgenic plants. Out of eight transgenic events tested, a progeny of the event, GR-ASD16-17 was found to be free of selectable marker gene with presence of *cry* gene (Table 2).

The marker-free transgenic plants (event GR-ASD16-17) were forwarded to T_2 generation to check their efficacy against target insects. The protein expression level in transgenic plants was 0.043 µg/gram of fresh leaf tissues, while non-transformed ASD16 did not show any detectable level of expression. When subjected to insect bioassay, marker-free plants of event GR-ASD16-17 showed a mortality of 45 per cent for leaffolder and 55 per cent for yellow stem borer (Table 3). Moreover, the surviving larvae on transgenic palnts were found to be stunted and deformed (Fig. 3).

Transgenic plants expressing insecticidal crystal proteins are known to impart resistance against lepidopteran insect pests and could potentially reduce the use of broad-spectrum insecticides. However, there is a risk that the target insects, on continuous exposure, could develop resistance to the *Bt* toxins. To overcome this risk, gene pyramiding for simultaneous expression of two or more *cry* genes in transgenic plants is advocated and routinely followed (Datta *et al.*, 2002). In our centre, a chimeric *Bt* gene (*cry2AX1*) was developed with *cry2Aa* and *cry2Ac* sequences from indigenous isolates of *Bt* with a view to enhance the insecticidal property of the Cry2 toxin. Our earlier reports showed that the chimeric Cry2AX1 protein expressed in transgenic rice was toxic to lepidopteran insects (Manikandan *et al.*, 2014a; Chakraborty *et al.*, 2016; Manikandan *et al.*, 2016a).

Out of 77 events generated in the study, 48 events found to be positive for gene of interest (*cry2AX1* gene) with a co-transformation efficiency of 62.34 per cent. Among the PCR positive transformants 9 putative events showed detectable level of Cry2AX1 protein with varying levels by expression. Variation in levels of expression in *Bt* rice was reported by earlier researchers (Wu *et al.*, 2001; Yang *et al.*, 2014; Chakraborty *et al.*, 2016; Manikandan *et al.*, 2016 a and b). However, the level of expression observed in the present study was low. The expression could provide a moderate level of protection (47 % - 67 % larval mortality) against rice leaffolder. Several earlier workers have demonstrated that the expression of Bt protein in transgenic plants had offered significant level of protection against neonate larvae of which insect? (Zaidi *et al.*, 2009; Manikandan *et al.*, 2014b; Chakraborty *et al.*, 2016; Manikandan *et al.*, 2016 a) in rice.

However, the same level toxin could achieve upto 100 per cent of larval mortality in yellow stem borer. This result indicates that the neonate larvae of yellow stem borer is more susceptible to Cry2AX1 protein even at low concentration. Earlier studies have reported 100 per cent mortality of yellow stem borer in transgenic rice plants expressing *cry1Ac* gene (Tu *et al.*, 2000; Li *et al.*, 2005), *cry2A* gene (Chen *et al.*, 2005; Chakraborty *et al.*, 2016) and *cry1C* gene (Tang *et al.*, 2006).

Out of eight transgenic events tested in T_1 generation, an event GR-ASD16-17 found to be free of selectable marker gene with 0.037 µg/gram of Cry2AX1 protein expression level. The low level of protein expression (0.043 µg/gram) in marker-free T_2 transgenic plants could provide a mortality of 45 per cent in rice leaffolder and 55 per cent in yellow stem borer. Moreover, the surviving larvae on transgenic palnts were found to be stunted with abnormal development.

The presence of selectable marker genes in transgenic plants brings about safety concerns, particularly in food crops, which are directly consumed by humans. Majority of marker genes are purposeless after the generation of transgenic plants. A number of marker-

free plant transformation strategies have been reported and they include *Agrobacterium*-mediated co-transformation. In present study, the strategy of 'two T-DNAs in separate vectors' in a single *Agrobacterium* strain, C58C1 was used for genetic transformation and marker- free transgenic rice was identified in T_1 generation. Single *Agrobacterium* strain method is found to improve co-transformation frequency. Two compatible binary vectors, first with the gene of interest and the second with the selectable marker gene were successfully used for marker elimination which yielded a 50 % co-transformation efficiency and a 50 % segregation frequency (Daley *et al.*, 1998; Parkhi *et al.*, 2005). The co-transformation frequency was improved by placing the gene of interest (GOI) on a multicopy binary plasmid and the selectable marker gene on a low copy number co-integrate Ti plasmid (Jacob and Veluthambi, 2002).

Co-transformation of two T-DNAs from a single strain of *Agrobacterium* is more efficient than two *Agrobacterium* strains with independent binary vectors (Komari *et al.*, 1996; Miller *et al.*, 2002; Poirier *et al.*, 2000). In the single-strain method involving a cointegrate vector and a binary vector, the co-transformation efficiency of the non-selected T-DNA (carrying *nptII*) was high (56-74%) when the non-selected GOI was placed in a multi-copy binary plasmid (Jacob and Veluthambi, 2002). The marker-free *cry1Ab*/Ac transgenic *indica* rice TT51 has been issued with security certificate by Chinese government in 2014, indicating its commercialization potential (Ling *et al.*, 2016). In the present study, a broad host-range SMG-free binary vector pCAMBIA0390 was constructed and used for co-transformation experiments. By placing the GOI (*cry2AX1*) in the T-DNA of pCAMBIA0390 and the SMG in a single-copy cointegrate vector, a co-transformation efficiency of 62 per cent was obtained.

The expression of recombinant protein in unwanted tissue (*e.g.* grains) is the major concern when constitutive promoters are used in transformation. Transgene driven by tissue specific promoter will be expressed only in tissues where the transgene product is desired, leaving the rest of the tissues unmodified by transgene expression. The *rbcS* promoter is a tissue-specific expression gene, especially present in green tissue and its expression pattern is regulated by light. Earlier workers demonstrated that the protein expression was detected only in green tissues and not in seeds when the *cry* genes driven by the *rbcS* promoter were used to transform rice (Ye *et al*., 2009; Kim *et al.*, 2009; Qi *et al.*, 2012; Yang *et al.*, 2014). The expression level of *cry2AX1* gene driven by the *rbcS* promoter and transit peptide could possibly reduce the spread of *Bt* toxin into the environment through seeds and pollen of transgenic plants.

The local rice cultivar ASD16 was transformed with *cry2AX1* gene driven by green tissue specific promoter *rbcS* through *Agrobacterium* mediated transformation to generate the marker-free transgenic plants. In this study, co-transformation of transgenic rice harbouring SMG-free *cry2AX1* gene with *A. tumefaciens* C58C1 (pG2260::pSSJ1) were used to generate marker-free transgenic plants and 48 of the 77 transgenic plants (62.34%) were found to be co-transformed. The marker-free transgenic plants identified had moderate level of resistance to rice leaffolder and yellow stem borer.

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Table 1. Protein expression and insect bioassay on T_0 transgenic rice events expressing *cry2AX1* gene
Values are arc sine transformed, mean of original value given in the table. NT - Not tested

Sl. No.	Rice line	Cry2AX1 expression (µg/g)	Larval mortality (%)	
			Yellow stem borer	Leaffolder
1.	GR-ASD16-6	0.027±0.15	NT	NT
2.	GR-ASD16-9	0.051±0.01	60.00 (50.77)[b]	NT
3.	GR-ASD16-12	0.029±0.04	NT	NT
4.	GR-ASD16-17	0.079±0.03	95.00 (77.08)[a]	46.66 (43.09)
5.	GR-ASD16-20	0.075±0.04	90.00 (71.57)[a]	NT
6.	GR-ASD16-21	0.110±0.01	100.00 (90.00)[a]	66.67 (54.73)
7.	GR-ASD16-22	0.046±0.02	NT	NT
8.	GR-ASD16-33	0.034±0.00	60.00 (50.77)[b]	NT
9.	ASD 16 (Control)	0.00	0.00[c]	0.00
SEd			**6.8066**	-
CD (0.05)			**14.3003**	-

Table 2. Identification of marker-free transgenic plants in T_1 generation

Sl. No.	Rice line	Number of plants assayed	Positive for *hpt* and *cry* gene (+/+) (nos)	Positive for *hpt* gene and negative for *cry* gene (+/-)	Negative for *hpt* and positive for *cry* gene (-/+)	Negative for hpt and *cry* gene (-/-)
1.	GR-ASD16-6	25	11	9	0	5
2.	GR-ASD16-9	30	16	6	0	8
3.	GR-ASD16-12	28	17	7	0	6
4.	GR-ASD16-17	30	13	6	5	6
5.	GR-ASD16-20	30	17	6	0	7
6.	GR-ASD16-21	17	13	0	0	4
7.	GR-ASD16-22	16	12	3	0	1
8.	GR-ASD16-33	35	19	8	0	8

Table 3. Cry2AX1 expression and efficacy on target insects in T_2 transgenic progenies

Sl. No.	Rice line	Protein concentration (µg/g)	Larval mortality (%)	
			Leaffolder	Yellow stem borer
1.	GR-ASD16-17-66	0.043±0.01	45.00 (42.13)	55.00 (47.87)
2.	ASD16 control	0.00	0.00	0.00

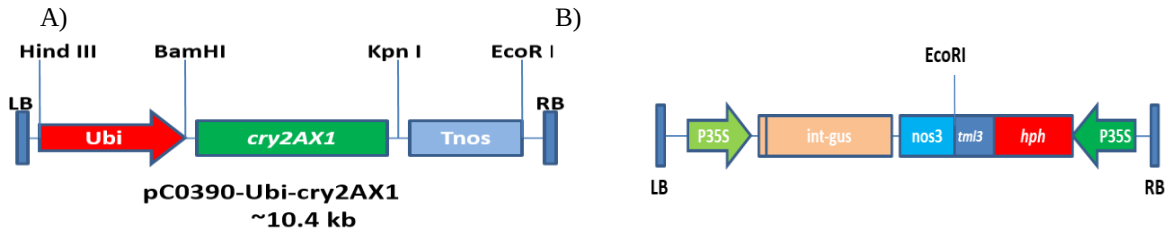

Fig. 1. A) T-DNA region of plant transformation construct pMF-ubi-*cry2AX1*. The synthetic *cry2AX1* gene is driven by a maize ubiquitin promoter and terminated by the nopaline synthase (nos) terminator**. B)** T-DNA region of the cointegrate vector pGV2260::pSSJ1 which harbours p35S-hph and p35S-int-gus genes p35S, cauliflower mosaic virus 35S promoter; int-gus, β-glucuronidase gene with catalase intron; nos, nopaline synthase gene polyadenylation signal; LB, left T-DNA border; RB, right T-DNA border; tml, tumor morphology large gene polyadenylation signal.

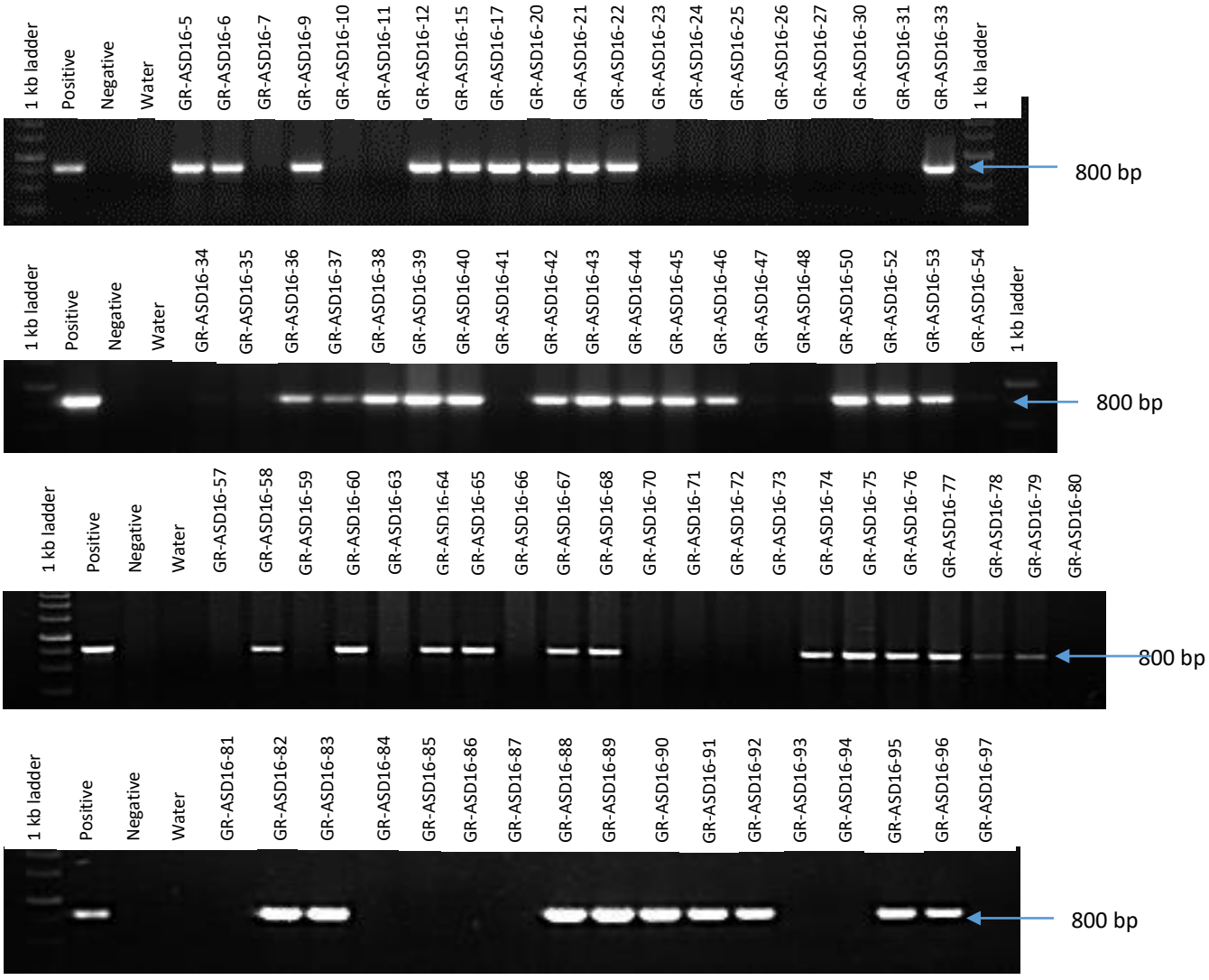

Fig. 2. PCR analysis of transgenic T_0 plants for presence of *cry2AX1*
A 800 bp internal sequence of *cry2AX1* gene was amplified by PCR from the DNA isolated from putative transgenic plants, Lane 2 pMF-rbcS-tp-2AX1 plasmid as a positive control, Lane 3 Negative - Non transformed control plant and Lane 4 Negative control (water).

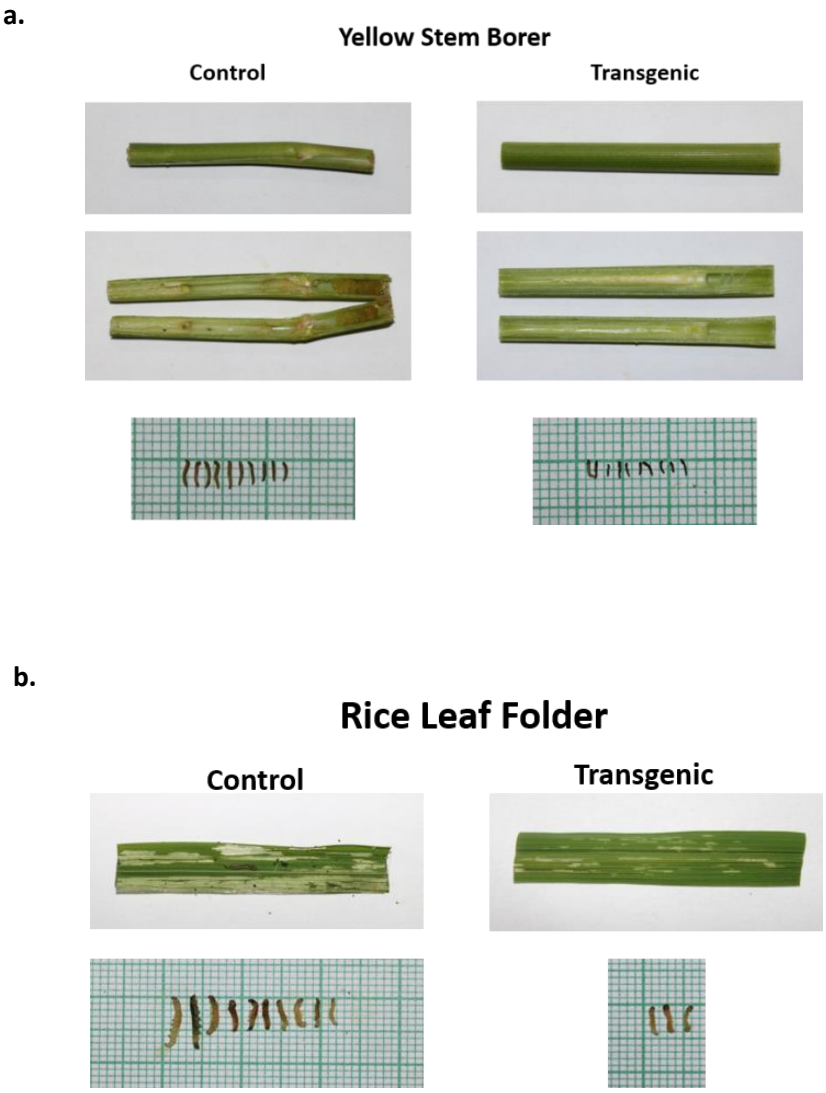

Fig. 3. *In vitro* insect bioassay on transgenic rice

a). Insect bioassay on transgenic and control plants against neonates of yellow stem borer. b). Insect bioassay on transgenic and control plants against neonates of rice leaffolder.