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ORIGINAL PAPER

Development of leaffolder resistant transgenic rice expressing *cry2AX1* gene driven by green tissue-specific *rbcS* promoter

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Abstract The insecticidal *cry* genes of *Bacillus thuringiensis* (Bt) have been successfully used for development of insect resistant transgenic rice plants. In this study, a novel *cry2AX1* gene consisting a sequence of *cry2Aa* and *cry2Ac* gene driven by rice *rbcS* promoter was introduced into a rice cultivar, ASD16. Among 27 putative rice transformants, 20 plants were found to be positive for *cry2AX1* gene. The expression of Cry2AX1 protein in transgenic rice plants ranged from 5.95 to 122.40 ng/g of fresh leaf tissue. Stable integration of the transgene was confirmed in putative transformants of rice by Southern blot hybridization analysis. Insect bioassay on T_0 transgenic rice plants against rice leaffolder (*Cnaphalocrosis medinalis*) recorded larval mortality up to 83.33 %. Stable inheritance and expression of *cry2AX1* gene in T_1 progenies was demonstrated using Southern and ELISA. The detached leaf bit bioassay with selected T_1 plants showed 83.33–90.00 % mortality against *C. medinalis*. The whole plant bioassay for T_1 plants with rice leaffolder showed significant level of resistance even at a lower level of Cry2AX1 expression varying from 131 to 158 ng/g fresh leaf tissue during tillering stage.

Keywords Cry2AX1 · Transgenic rice · *C. medinalis* · *rbcS* promoter

Abbreviation

rbcS	*Ribulose biphosphate carboxylase*
SSC	Saline-sodium citrate
SDS	Sodium dodecyl sulphate
dCTP	Deoxycytidine triphosphate

Introduction

Rice (*Oryza sativa* L.,) is one of the most important food crops in the world with more than half of the world population (nearly 3 billion people) depending on it as the primary staple food. Rice cultivation and consumption is mainly concentrated in the developing Asian countries, contributing more than 90 % of the world rice cultivation area. With ever increasing population and decreasing area of cultivable land the demand for rice continues to increase without any sustainable increment in its production. Rice yield has been immensely improved after the green revolution but the yield potential of improved varieties is not actually achieved in farmers' field conditions. This is due to the fact that the productivity is adversely impacted by numerous biotic and abiotic factors. Approximately 52 % of the global production of rice is lost annually owing to the damage caused by biotic factors, of which almost 21 % is attributed to the attack of insect pests (Brookes and Barfoot 2003).

The major constraints in rice production, throughout the rice growing countries of the world, are the insect pests among which the most serious ones are lepidopterans viz. yellow stemborer (YSB) (*Scirpophaga incertulas* Walker), striped stemborer (SSB) (*Chilo supressalis* Walker) and rice leaffolder (RLF) (*Cnaphalocrosis medinalis* Guenee). Rice

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stem borers and leaffolder are potentially the most destructive insect pests, causing up to 30 and 10 % on an average respectively each year (Krishnaiah and Varma 2012). The use of chemical insecticides against these insects has not been found much effective mainly because of their nature of life cycle. Also chemical insecticide increases the cost of production, environmental pollution, and damage to the ecosystem. Moreover the harmful effects caused by the reckless application of the pesticides to control these pests pose threat to human health and biodiversity (Pingali and Roger 1995). Perhaps the most economic and efficient measure to protect rice from insect pests is the production of insect resistant rice varieties.

However the development of insect resistant lines in rice through conventional breeding has been a challenge as the existing rice varieties could not provide sufficient levels of insect resistance, which is mainly due to the scarcity in the diversity of germplasm. So, an alternative and the most attractive strategy is to employ the tools of genetic engineering to produce insecticidal proteins through the introduction of corresponding insecticidal genes into rice. Genetic engineering overcomes incompatibility barriers between different crop species (Muthukrishnan et al. 2001). Several crops such as tomato, cotton, maize and rice have been successfully transformed with different versions of crystal protein gene of *B. thuringiensis* (Christou et al. 2006).

Recent progress in rice transformation technologies has made it possible to produce genetically engineered (GE) rice cultivars with improved resistance to insect pests. The first transgenic rice plant with insect resistant Bt protein was reported by Fujimoto et al. (1993). Thereafter, many rice varieties have been transformed with genes encoding various Bt crystal proteins and have been shown to be resistant to one or more lepidopteran insect pests of rice, the most important of which are YSB, RLF and SSB (Nayak et al. 1997; Tu et al. 2000; Ye et al. 2003; Ramesh et al. 2004; Bashir et al. 2005; Kim et al. 2008; Xia et al. 2011; Yang et al. 2014; Wang et al. 2014). First field trial of Bt rice started in China in 1998 (Tu et al. 2000; Shu et al. 2000) and in 2009 China's Ministry of Agriculture issued biosafety certificates for limited commercialization trial in Hubei Province in China for a 5-year period, 2009–2014 (Chen et al. 2011).

A major concern of Bt mediated insect resistant plants is the continuous use of similar Bt toxins against a target insect pest leading to breakdown of resistance. However, insects that develop resistance against one protein (Cry1A) are not cross-resistant to another (Cry2A) protein (Tabashnik et al. 2000). So, pyramiding of two or more Bt genes with different modes of action, is one of the strategies to delay the resistance development in insects. Commercial Bt crops expressing Cry1Ab, Cry1Ac, Cry1F, Cry2Ab and Cry3Bb proteins, either single or in combination, with different modes of action are now being grown worldwide for protection against a variety of insect pests. Similarly, hybrid Bt toxins produced through inclusion of a domain from another toxin result in increased potency of the fused protein (Bosch et al. 1994). A novel chimeric Bt gene *cry2AX1* was constructed by using the sequences of *cry2Aa* and *cry2Ac* cloned from indigenous isolates of Bt (Udayasuriyan et al. 2010).

Another concern is the expression of recombinant protein in unwanted tissue (e.g. grains). 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. Many experiments demonstrated that *rbcS* is a tissue-specific expression gene, especially present in green tissue and its expression pattern is regulated by light (Kyozuka et al. 1993; Huang et al. 1999; Nomura et al. 2000). Several earlier studies also showed that the Bt gene driven by green tissue specific *rbcS* promoter was successfully expressed in leaves and stems, but not in the seeds (Kim et al. 2009; Ye et al. 2009; Rawat et al. 2011).

In this background, attempts were made in the present study to generate transgenic *indica* rice cultivar, ASD16 with *cry2AX1* gene driven by green tissue specific promoter with chloroplast transit peptide sequence for targeting Cry2AX1 protein in chloroplast and testing the efficacy against rice leaffolder.

Materials and methods

Construction of expression vectors and rice transformation

Codon optimized synthetic *cry2AX1* gene (Accession No. GQ332539.1) under the control of green tissue specific *rbcS-tp* promoter (Jang et al. 1999) and *nos* terminator (3.46 kb), was cloned into the *Hin*dIII and *Sal*I sites of the pCAMBIA1300 vector containing *hpt* (coding for hygromycin phosphotransferase) as a plant selectable marker gene, and the resultant plasmid was designated as *pC1300-rbcS-tp-2AX1* (Fig. 1). This construct was mobilized, into the *Agrobacterium* strain LBA4404 for rice transformation.

Agrobacterium-mediated rice transformation procedure suggested by Hiei and Komari (2008) was followed. The immature embryos of ASD16 were placed on cocultivation medium containing acetosyringone 100 mM and 5 µl of the *Agrobacterium* suspension was dropped on each embryo. The infected immature embryos were incubated at 25 °C in the dark for 7 days. Well developed embryogenic calli from co-cultivated immature embryos were sub-cultured twice on resting medium containing cefotaxime

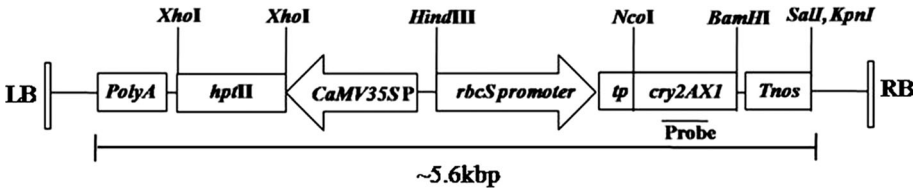

Fig. 1 T-DNA region of plant transformation construct *pC1300-rbcS-tp-2AX1*. The synthetic *cry2AX1* gene is driven by a rice *rbcS* promoter and its transit peptide (tp) and terminated by the *nopaline synthase* (*nos*) terminator. The plant selectable marker gene, *hptII* is under the control of the CaMV35S promoter and tailed by the CaMV35S polyA. *LB* left border of T-DNA region, *RB* right border of T-DNA region

250 mg l^{-1} for 15 days at 30 °C under continuous illumination. The proliferated embryogenic calli were subcultured twice on selection medium containing hygromycin 50 mg l^{-1} and cefotaxime 250 mg l^{-1} for 17 days. The hygromycin resistant calli were transferred to pre-regeneration medium containing hygromycin 40 mg l^{-1} for 7 days. The proliferated calli with green spots were cultured on regeneration medium containing 30 mg l^{-1} hygromycin. The regenerated plantlets were transferred to half strength MS medium containing hygromycin 30 mg l^{-1} for rooting. Finally, the well developed rooted plants were transferred to soil in pots and grown to maturity in a transgenic greenhouse (Fig. 2).

PCR and Southern blot analyses

PCR analysis were performed to demonstrate the presence of *cry2AX1* and *hptII* genes in putative transgenic lines of ASD16 using gene specific primer (2AX1FP, 5′-CCTAAC ATTGGTGGACTTCCAG-3′ and 2AX1RP, 5′-GAGAAA CGAGCTCCGTTATCGT-3′); (HPTFP, 5′-GCTGTTATG CGGCCATTGGTC-3′; HPTRP, 5′ GACGTCTGTCGAGA AGTTTG-3′) (Jayaprakash et al. 2014). These primers amplify 800 and 630 bp internal fragments from *cry2AX1* and *hptII* genes, respectively. The plasmid DNA (*pC1300-rbcS-tp-2AX1*) was used as positive control and DNA isolated from non-transformed control plants was used as negative control. The amplified PCR products were resolved on 1.2 % agarose gel, visualized on UV transilluminator upon ethidium bromide staining.

Total genomic DNA was extracted from leaf tissue of transgenic and control plants using the method described by Dellaporta et al. (1983). For Southern blot analysis, 5 µg of genomic DNA was digested with *Kpn*I, which has a recognition sequence at one end of the T-DNA. The digested products were separated on a 0.8 % agarose gel, and then transferred to a nylon membrane using 20X SSC following standard capillary transfer protocol. The transferred DNA was cross-linked by a UV crosslinker at 1200 µJ min^{-1} for 1 min. The prehybridisation was carried out for 1 h and hybridization for 18 h at 60 °C. For hybridization, PCR amplified 800 bp internal region of *cry2AX1* gene was used as a probe. The probe DNA was labelled with α^{32}P dCTP using Decalabel DNA labelling kit (Thermo Fischer Scientific Inc.) and added to hybridization solution. After hybridization, the blot was washed with 3X SSC + 0.1 % SDS and 2X SSC + 0.1 % SDS for 15 min each, followed by 10 min in 0.5X SSC + 0.1 % SDS. All washings were carried out at 60 °C and the blot was exposed to X-ray film.

Detection of Cry2AX1 protein in transgenic rice plants

Fresh leaf tissue (30 mg) from transgenic and wild type ASD16 plants were collected separately, homogenized in 500 µl of extraction buffer, spun at 3000 g at 4 °C for 10 min and 100 µl of the supernatant was immediately used for assay. Each sample was replicated twice. Cry2AX1 protein expression in transgenic rice plants was determined by ELISA kit (Envirologix, USA) following standard procedures. The protein concentration was calculated on a linear standard curve, using the standards provided in the kit.

Detached leaf bit and whole plant bioassay

Adults of rice leaffolder were collected from the rice field at Paddy Breeding Station, TNAU, Coimbatore and they were released on TN1 rice plants maintained in insect cages (65 cm × 65 cm × 75 cm) for culturing. The adult moths were provided 10 % honey solution to enhance the egg laying capacity. After two generations, the neonates of *C. medinalis* larvae were used for the bioassay. Leaves of transgenic plants were cut into pieces (about 3 cm length) and three leaf bits were placed on a moist filter paper in a plastic petriplate. Ten neonate larvae of rice leaffolder were released in each petriplate. A control was maintained using leaf bits collected from non-transgenic rice ASD16. Three replications were maintained and the experiment was carried out at 25 °C ± 1, 60 % relative humidity. Larval growth and mortality was recorded every day up to 5 days.

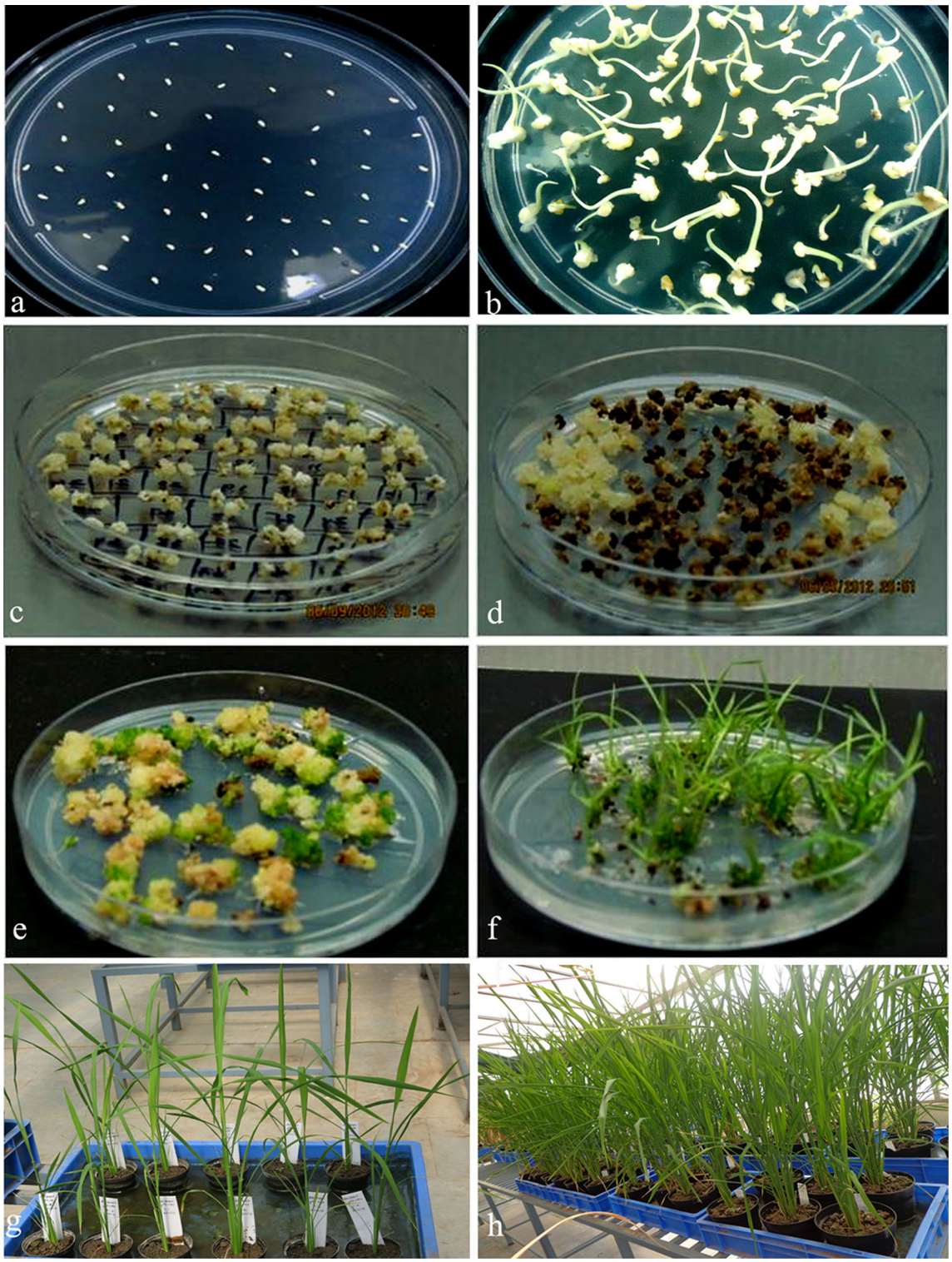

Fig. 2 *Agrobacterium*-mediated transformation of rice (*Oriza sativa* L. cv. ASD16). **a** Pre-treated immature embryos infected with *Agrobacterium* on cocultivation medium. **b** Immature embryos after cocultivation. **c** Subcultured calli on resting medium. **d** Callus proliferation on selection medium. **e** Embryogenic calli on pre-regeneration medium. **f** Regenerated transgenic rice plants. **g** Pre-hardening of transgenic rice plants in greenhouse. **h** Established transgenic rice plants in greenhouse

After 5 days, leaf area damage and surviving larval characteristics were evaluated in transgenic as well as control plants. Percentage of leaf area damage was calculated using the formula:

$$\text{Leaf area damage } (\%) = \frac{\text{Consumed leaf area}}{\text{Total leaf area}} \times 100$$

The whole plant bioassay was carried out with neonates and second instar larvae of *C. medinalis.* The 60 day old transgenic and control plants were kept in separate bioassay cages (75 cm × 75 cm × 100 cm) and 20 numbers of neonate or second instar larvae of *C. medinalis* were released on each treatment. The transgenic plants were evaluated 17 days after infestation. For transgenic and control plants, the level of insect mortality was recorded. The number of damaged leaves along with undamaged ones was recorded and % infestation was determined using the following formula.

$$\text{Leaf infestation } (\%) = \frac{\text{Number of damaged leaves}}{\text{Total number of leaves}} \times 100$$

Statistical analysis

The larval mortality and concentration of Cry2AX1 protein data were subjected to arc sine and square root transformations respectively, before analysis. Data analysis was done by analysis of variance (ANOVA) following the AGRES statistical package. Values for concentration of Cry2AX1 protein and mortality of rice leaffolder are reported as mean ± SD. The association between protein expression and insect mortality was studied by correlation analysis using the Statistical Package for Social Studies (SPSS) software version 16.

Results

Agrobacterium mediated transformation

The *cry2AX1* gene was introduced into immature embryo of the ASD16 cultivar by *Agrobacterium* mediated method. A total 42 embryogenic calli were selected after two rounds of hygromycin selection, the selected embryogenic calli were transferred to pre-regeneration medium followed by regeneration medium for shoot induction. Finally, 27 putative transgenic rice lines were obtained and transferred to greenhouse for molecular analysis.

Molecular analysis

Total genomic DNA from putative rice transformants was subjected to PCR analysis with *hptII* and *cry2AX1* gene specific primers. Out of 27 plants regenerated, 20 were found to be positive for the amplification of ~800 and ~630 bp internal sequences of *cry2AX1* and *hpt* genes, respectively (Fig. 3). A similar band was also observed in positive control (*pC1300-rbcS-tp-2AX1*), whereas untransformed control plant did not show any amplification.

Southern hybridization analysis was performed to confirm T-DNA integration. Genomic DNA from ELISA positive T_0 transgenic rice lines was digested by *Kpn*I enzyme and subjected to Southern blot hybridization using [α-^{32}P]- dCTP labelled 800 bp of *cry2AX1* probe. The results showed a single hybridization signal in seven of the 19 plants analyzed (Rb 2, Rb 4, Rb 5, Rb 6, Rb 7, Rb 8 and Rb 20) whereas the remaining 12 plants had two to five signals. In addition to expected bands, a few unexpected hybridization signals, which were lesser than the expected size were observed (Fig. 4).

Expression analysis

Among the 20 PCR positive rice lines screened by Cry2A quantitative ELISA kit, 19 were found positive for the expression of Cry2AX1 protein in young rice leaf tissue. The expression of Cry2AX1 protein in these transgenic rice lines ranged from 5.95 ± 0.15 to 122.40 ± 2.40 ng/g fresh leaf tissue indicating expression of *cry2AX1* gene in transgenic rice lines (Table 1).

Insect bioassay

In order to determine the insecticidal activity of Cry2AX1 protein in transgenic rice plants, detached leaf bit bioassay carried out using neonate larvae of rice leaffolder on the ELISA positive plants showed larval mortality ranging from 20.00 ± 0.00 to 83.33 ± 5.77 % (Table 1). A significant, positive correlation was found between the concentration of Cry2AX1 protein and insect mortality in T_0 transgenic plants ($r = 0.942$, $p = 0.01$). There was no larval mortality on control plants and the major portion of the leaf tissue was consumed by the surviving larvae over a period of 5 days (Fig. 5).

Analysis of the T_1 generation plants

Thirty plants of T_1 progenies derived from Rb16 (selected based on relatively higher level expression of Cry2AX1 protein in T_0 generation) were established in the greenhouse for further analyses of gene inheritance, expression and efficacy. An amplicon of ~800 bp was found in all the T_1 progenies for the presence of *cry2AX1* gene, whereas no amplification was found in non-transgenic control plant. Among the 30 PCR positive T_1 plants of Rb16, five were subjected to analysis of Cry2AX1 expression at three

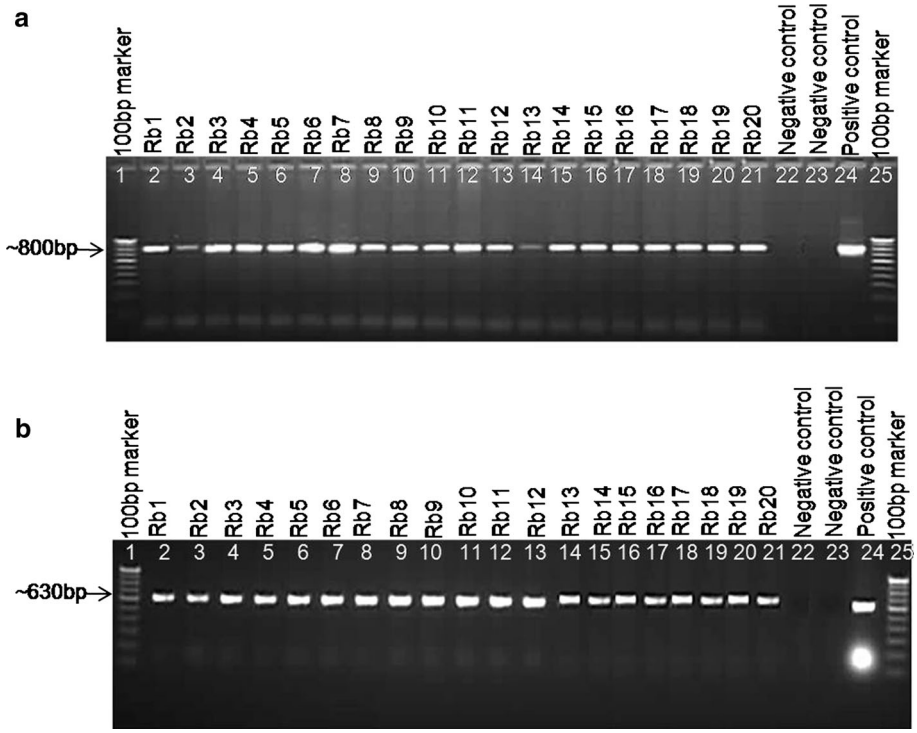

Fig. 3 PCR analysis of *cry2AX1* transgenic rice plants. **a** A 800 bp internal sequence of *cry2AX1* gene was amplified by PCR from the DNA isolated from putative transgenic plants. *Lanes 1* and *25* 100 bp marker, *Lanes 2–21* Putative transgenic plants of ASD16, *Lane 22* Non transformed control plant, *Lane 23* Negative control (water), *Lane 24 pC1300-rbcS-tp-2AX1* plasmid as a positive control. **b** A 630 bp internal sequence of *hptII* gene was amplified by PCR from the DNA isolated from putative transgenic plants. *Lane 1* and *25* 100 bp marker, *Lanes 2–21* Putative transgenic plants of ASD16, *Lane 22* Non transformed control plant, *Lane 23* Negative control (water), *Lane 24*, *pC1300-rbcS-tp-2AX1* plasmid as a positive control

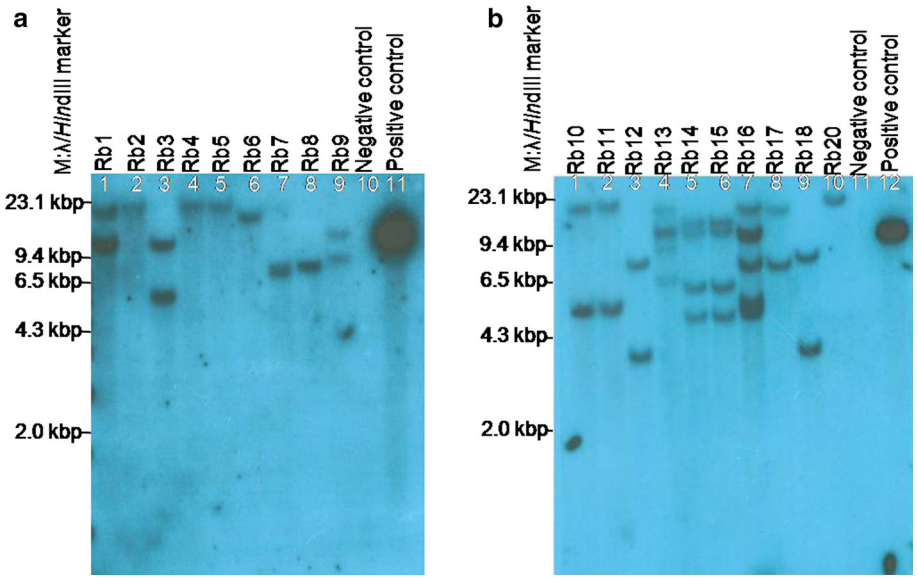

Fig. 4 Southern blot analysis of T_0 transgenic rice plants expressing *cry2AX1* gene. DNA sample isolated from transgenic and non-transgenic plants digested with *Kpn*I restriction enzyme, fractionized by electrophoresis, transferred to nylon membrane and allowed to hybridize with a radioactively labelled 800 bp internal sequence of *cry2AX1* gene. **a** *M* λ/*Hin*dIII marker, *Lanes 1–10* Genomic DNA from transgenic rice plants Rb1, Rb2, Rb3, Rb4, Rb5, Rb6, Rb7, Rb8 and Rb9, respectively, *Lane 10* Non-transformed control plant, *Lane 11* Positive control *pC1300-rbcS-tp-2AX1*. **b** *M* λ/*Hin*dIII marker, *Lanes 1–10* Genomic DNA from transgenic rice plants Rb10, Rb11, Rb12, Rb13, Rb14, Rb15, Rb16, Rb17, Rb18 and Rb20, respectively, *Lane 11* Non-transformed control plant, *Lane 12* Positive control *pC1300-rbcS-tp-2AX1*

phenological stages viz, vegetative, tillering and reproductive and the expression varied from 131.00 ± 5.00 to 236.00 ± 0.00 ng/g fresh leaf tissue, 47.20 ± 0.80 to 158.40 ± 0.00 and 36.08 ± 0.00 to 124.00 ± 0.80 ng/g, respectively (Table 2). The Cry2AX1 protein was not detected in seeds of transgenic plants. Southern blot

Table 1 Quantity ELISA and rice leaffolder bioassay on T_0 rice plants expressing *cry2AX1* gene

S. No	Rice line	Cry2AX1 concentration in fresh leaf tissue Mean ± SD[a]	*C. medinalis* mortality (%)[b]
1	Rb 1	28.10 ± 0.10	43.33 ± 5.77
2	Rb 2	15.50 ± 0.50	30.00 ± 0.00
3	Rb 3	14.50 ± 0.50	23.33 ± 5.77
4	Rb 4	13.50 ± 0.50	30.00 ± 0.00
5	Rb 5	8.30 ± 0.00	NT
6	Rb 6	7.70 ± 0.70	NT
7	Rb 7	23.30 ± 0.10	33.33 ± 5.77
8	Rb 8	15.00 ± 1.00	30.00 ± 0.00
9	Rb 9	14.40 ± 0.00	23.33 ± 5.77
10	Rb 10	5.95 ± 0.15	NT
11	Rb 11	6.00 ± 0.00	NT
12	Rb 12	13.30 ± 0.10	30.00 ± 0.00
13	Rb 13	60.00 ± 0.00	70.00 ± 0.00
14	Rb 14	38.00 ± 0.00	43.33 ± 5.77
15	Rb 15	25.00 ± 0.00	30.00 ± 0.00
16	Rb 16	122.40 ± 2.40	83.33 ± 5.77
17	Rb 17	20.50 ± 0.50	NT
18	Rb 18	33.00 ± 0.00	53.33 ± 5.77
19	Rb 19	0.0	NT
20	Rb 20	11.00 ± 0.00	20.00 ± 0.00
21	Control (ASD16)	0.0	0.0
SED		0.0006	2.08
CD ($P = 0.05$)		0.001	4.24

NT not tested, *SD* standard deviation

[a] Mean of two replications

[b] Mean of three replications

hybridization analysis of selected T_1 progenies (Rb 16-8 and Rb 16-9) showed four and five hybridization signals of ~5.6, ~6.0, ~7.5, ~10.0 and ~14.0 kbp size (Fig. 6).

Detached leaf bit bioassay for two T_1 plants (which expressed relatively higher levels of Cry2AX1 protein) were tested for efficacy of Cry2AX1 protein against the neonates of rice leaffolder. Mortality in rice leaffolder varied from 83.33 ± 2.35 to 90.00 ± 0.00 % in the transgenic rice plants (Table 3). The percentage of leaf area damaged by rice leaffolder larvae varied from 10.68 ± 0.43 to 17.51 ± 0.85 in the transgenic lines compared to 92.73 ± 0.43 % in control plants (Supplementary Figure 1).

Whole plant bioassay was performed on two T_1 plants Rb 16-9 and Rb 16-8 against the neonates and second instar larvae of rice leaffolder, respectively (Tables 4, 5). Mortality was recorded after 17 days. The transgenic rice lines showed 60 and 40 % mortality against neonate and second instar larvae of rice leaffolder whereas no mortality was recorded in the non-transgenic control. In case of neonates, the leaf infestation was 22.22 and 76.47 % in transgenic line and non-transgenic control, respectively. In second instar larvae, leaf infestation were 29.82 and 80.76 % in transgenic and control plants, respectively. Inhibition of growth was observed in larval survivors which fed on transgenic lines compared to normal growth in non-transgenic control (Fig. 7).

Discussion

One of the major concerns in using constitutive promoter is that the expression is throughout the plant which includes tissues which are not fed by insects. Compared with the temporal or spatial specific expression, the constitutive expression of foreign proteins in transgenic plants may cause adverse effects, such as the metabolic burden imposed on plants for constant synthesis of foreign gene products and may increase the potential risk of resistance of the target insects to Bt. There is also undue concern

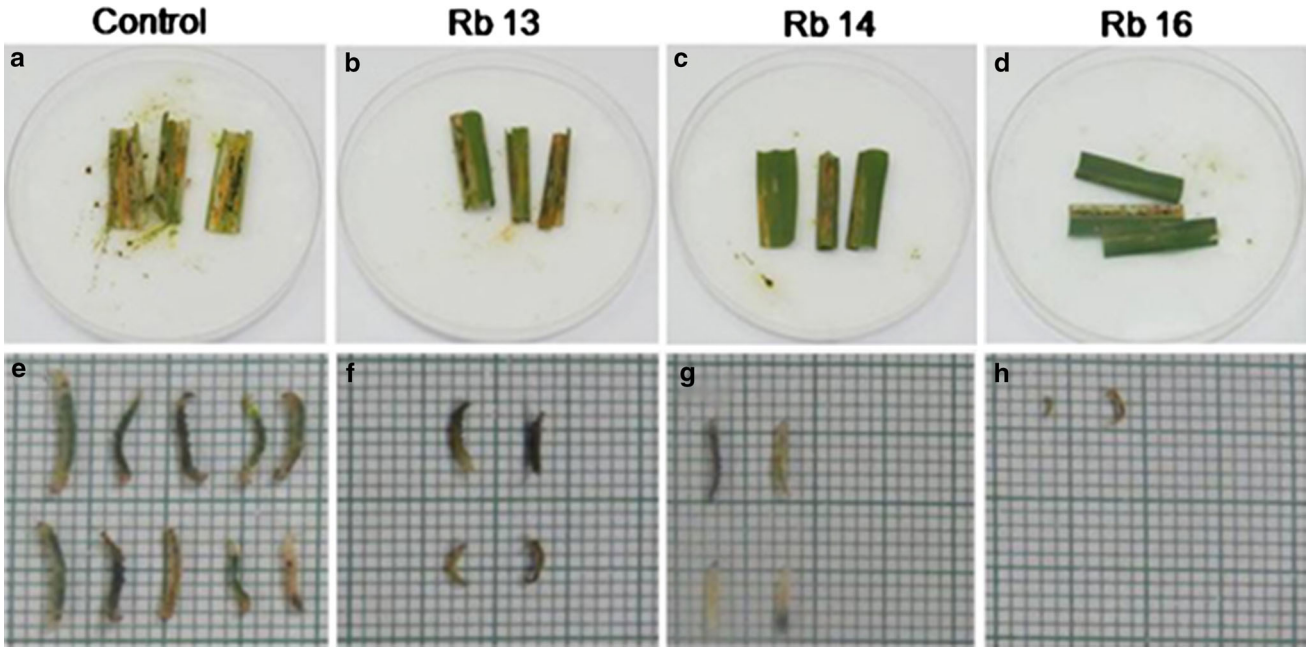

Fig. 5 Detached leaf bit bioassay against rice leaffolder (*C. medinalis*) in T_0 transgenic rice plants expressing Cry2AX1 protein. **a** Non transformed control plant (ASD16). **b–d** Transformed rice plant (Rb13, Rb14, Rb16). **e–h** Size of survivor from non transformed and transformed rice plants

Table 2 Expression of Cry2AX1 protein in T_1 progenies of rice

S. No	Event	T_1 progeny	Cry2AX1 concentration (ng/g)[a] Mean ± SD				
			Vegetative stage (25 DAS)	Tillering stage (55 DAS)	Reproductive stage (85 DAS)	Mean	Seeds
1	Rb 16	Rb 16-4	140.50 ± 0.50	47.20 ± 0.80	36.08 ± 0.00	74.59	0.00
2		Rb 16-5	139.50 ± 1.50	71.20 ± 0.80	58.40 ± 0.80	89.70	0.00
3		Rb 16-8	236.00 ± 0.00	158.40 ± 0.00	124.00 ± 0.80	172.80	0.00
4		Rb 16-9	216.30 ± 3.70	131.20 ± 0.00	100.80 ± 1.60	149.43	0.00
5		Rb 16-11	131.00 ± 5.00	92.80 ± 0.00	69.60 ± 2.40	97.80	0.00
6	Control		0.0	0.0	0.0	0.0	0.00
Mean			143.88	83.47	64.81		
			SED		CD ($P = 0.05$)		
Progeny			0.001		0.002		
DAS			0.0008		0.002		
Progeny X DAS			0.002		0.004		

[a] Mean of two replicates

about the food safety of genetically modified plants. Therefore, in certain circumstances, it is desirable to use tissue-specific promoters which drive the expression of foreign gene in specific plant tissues or organs. The *rbcS* gene, which encodes the small subunit of *ribulose-bisphosphate carboxylase* (Rubisco), is expressed only in leaf mesophyll cells. Indeed, the expression of the Bt gene by tissue-specific promoters enhanced the rice resistance to insects. Kim et al. (2009) reported that use of *rbcS* promoter-transit peptide sequence in transgenic rice increased the *cry1Ac* transcript and protein level by 25- and 100-fold, respectively. Similarly, Ye et al. (2009) reported that the insect resistant gene, Cry1C under rice *rbcS* promoter was transformed into Zhonghua 11 (*Oryza sativa* L. ssp. *japonica*) and transgenic plants were resistant to yellow stem borer, striped stem borer and leaffolder. But the levels

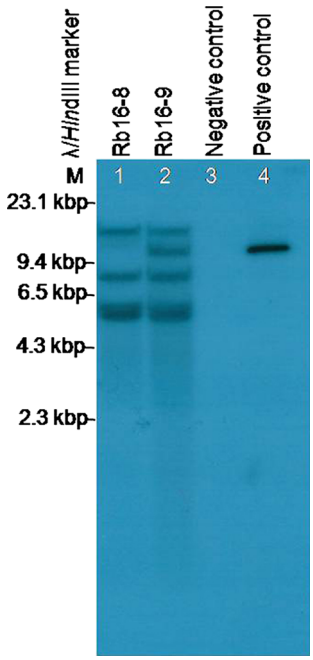

Fig. 6 Southern blot analysis of T_1 transgenic rice plants expressing *cry2AX1* gene. DNA digested with *Kpn*I and probed with a radioactively labelled 800 bp internal sequence of *cry2AX1* gene. *M* λ/*Hin*dIII marker, *Lane 1* Rb 16-8, *Lane 2* Rb 16-9, *Lane 3* Control plant, *Lane 4*, Positive control *pC1300-rbcS-tp-2AX1*

of Cry1C were undetectable in endosperm. In this direction an attempt was made to express the *cry2AX1* gene by green tissue specific rice *rbcS* promoter and target the expressed Cry2AX1 protein to chloroplast using their own transit peptide and to study its efficacy in transgenic rice plants.

Agrobacterium mediated transformation was used for introducing the synthetic *cry2AX1* gene in rice. Out of the twenty-seven putative transformants of rice generated under hygromycin selection, 20 plants were found to be positive for the *cry2AX1* gene in PCR analysis and nineteen of them were found positive in ELISA for expression of Cry2AX1 protein in leaf tissue. The concentration of Cry2AX1 protein varied among the transformants from 5.95 to 122.40 ng/g fresh leaf tissue. Bioassay of selected T_0 transgenic plants showed significant level of mortality in neonates of rice leaffolder ranging from 20.00 to 83.33 %. The bioassay results indicated considerable variation in the level of insect resistance among the independent T_0 transgenic rice lines. The highest larval mortality of 83.33 % was observed on transgenic plant Rb16. The differences in the level of toxicity (morality) observed among the different transgenic lines could be attributed to differences in the level of Bt gene expression whereas non transformed control plants did not show any mortality and major portion of leaf tissue was consumed by surviving larvae. The surviving larvae fed on transgenic leaf bits were severely stunted in growth. Correlation between the Cry2AX1 protein expression and larval mortality showed a highly significant positive relationship ($r = 0.942$ at 1 percent level) indicating that higher the concentration of Cry2AX1 protein has resulted in higher mortality of the larvae. Transformants expressing higher levels of Cry2AX1 protein invariably induced higher larval mortality. The result of bioassay suggests that the Cry2AX1 protein produced in T_0 transgenic rice plants is functionally active.

The ELISA positive T_0 plants were further analyzed by Southern using the coding sequences of *cry2AX1* gene as a probe for T-DNA integration analysis. Southern results indicated that the transgene was inserted in three to five locations of the rice genome in most of the analyzed plants, although in a few cases only one insertion was found. However, smaller in size of hybridizing signal (~3.5 kb) was also observed in few cases of transgenic lines. The

Table 3 Detached leaf bit bioassay on T_1 progenies of transgenic rice plants expressing *cry2AX1* gene

S. No.	Progeny No.	Mortality (%)[a] (56 DAS) Mean ± SD	Leaf area damage (%)[a]
1	Rb 16-8	90.00 ± 0.00	10.68 ± 0.43
2	Rb 16-9	83.33 ± 2.35	17.51 ± 0.85
3	Control	0.0	92.73 ± 0.43

[a] Mean of three replicates

Table 4 Whole plant bioassay with neonate larvae of rice leaffolder on T_1 transgenic rice plants expressing *cry2AX1* gene

S. No	Events	Number of tillers/plant	Number of larvae released	Number of larvae alive	Number of larvae dead	Rice leaffolder mortality (%)	Leaf infestations (%)	Overall larval growth
1	Rb16-9	19	20	8	12	60.00	22.22	Inhibited
2	Control	20	20	20	0	0.00	76.47	Normal

Table 5 Whole plant bioassay with second instar larvae of rice leaffolder on T_1 transgenic rice plants expressing *cry2AX1* gene

S. No	Events	Number of tillers/plant	Number of larvae released	Number of larvae alive	Number of larvae dead	Rice leaffolder mortality (%)	Leaf infestations (%)	Overall larval growth
1	Rb16-8	19	20	12	8	40.00	29.82	Inhibited
2	Control	18	20	20	0	0.00	80.76	Normal

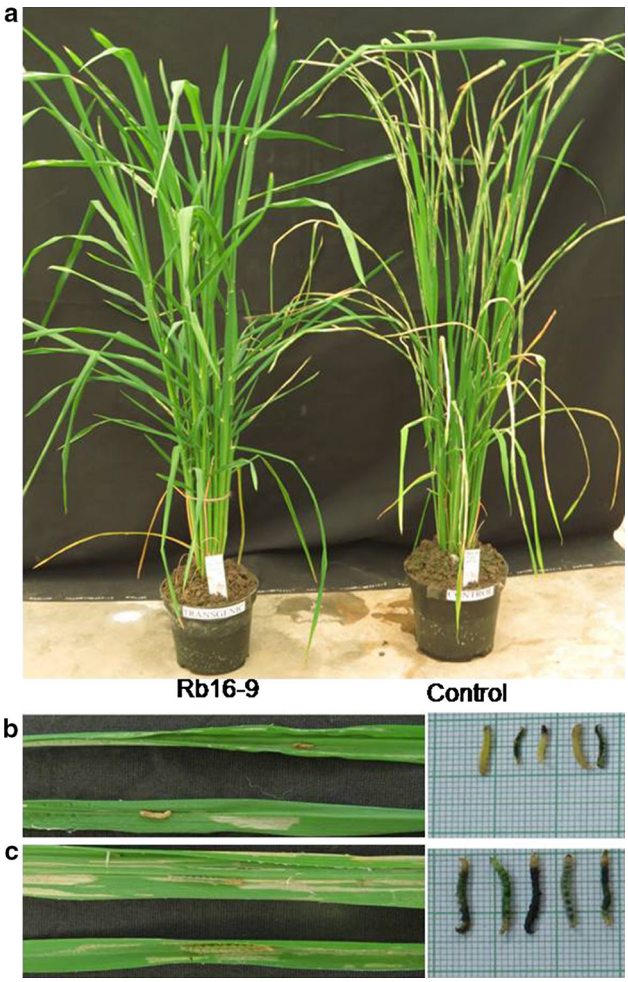

Fig. 7 Whole plant bioassay with neonate larvae of rice leaffolder on T_1 transgenic rice plant expressing *cry2AX1* gene. **a** Comparison of leaf damage from transgenic (Rb16-9) and control plants (ASD16), **b**, **c** close view of leaf area damage and survivors from transformed and control plants

smaller fragment in Rb12 and Rb18 might be a result of the transfer of a truncated T-DNA. The difference in size and banding pattern of T-DNA integration into the rice genome indicated that they were derived from different independent transformants and not from entophytic *Agrobacterium* contamination. It was also observed that the expression of Cry2AX1 transgenic plants appeared to be dependent on the number of *cry2AX1* gene integration into the plant genome. The transgenic plant Rb16 with five insertion of *cry2AX1* gene, expressed relatively higher amounts of Cry2AX1 protein (122.40 ng/g) compared to the other plants with one to four insertion of the transgene. Further studies on the regions flanking the transgene insertion sites in the plant genome might be useful in assessing any relationship between the integration number and the transgene expression levels. Zaidi et al. (2009) also reported that the level of Cry1C expression was higher in transgenic rice plant with three copies of *cry1C* gene when compared to single copy plants. In general, multiple integration of gene might cause unstable inheritance or transgene silence. Therefore, transgenic plants with the single insertion are more important for stable resistance to pest (Ye et al. 2009).

The stable inheritance of *cry2AX1* gene was demonstrated by progenies analysis through PCR. The Mendelian segregation ratio could not be determined due to smaller size (30) of the T_1 progeny of the plants and also due to multiple integration of *cry2AX1* gene. Southern hybridization analysis revealed four and five integrations of *cry2AX1* gene in two of the T_1 progenies tested (event Rb 16).

The expression of Cry2AX1 protein in transgenic rice plants was analyzed at different phenological stages to measure the temporal variation of Cry protein. The expression of Cry2AX1 protein was relatively higher in vegetative stage followed by tillering and reproductive stage. The expression was drastically decreased in tillering stage when compared to vegetative stage. One of the progenies Rb16-8 recorded maximum level of expression of Cry2AX1 protein i.e., 236.00, 158.40 and 124.00 ng/g fresh leaf tissue during vegetative, tillering and reproductive stage, respectively. The ELISA results indicated that the expression of Cry2AX1 protein in leaves decreased towards the end of growing season. Similar results were also reported in previous researches on Bt rice (Wu et al. 2001; Zhao et al. 2004; Han et al. 2009). Wan et al. (2005) reported that expression pattern of Bt toxin in progenies of cotton varies within the season with higher concentration at the beginning and lower at the latter stages. The mechanisms of variation in Cry protein concentration in plants are rather complicated. The possible reason for reduction of Bt

protein expression is that the levels of *cry* gene transcripts decreased in plant tissues as they mature and is likely because of decreased activity of the promoters, which is probably hindered by adverse physiological conditions in the rice plants (Christensen et al. 1992; Han et al. 2009). Bakhsh et al. (2012) reported that the reduction of Bt protein content in later season of cotton tissue could be attributed to the over expression of the Bt gene at earlier stage, which leads to gene regulation at post-transcriptional level and consequently results in gene silencing at a later stage.

In this study the expression of Cry2AX1 protein was detected only in the leaves of the transgenic plants and not in the seeds. Therefore the expression *cry2AX1* gene driven by the *rbcS* promoter and transit peptide could help to reduce the release of Bt toxin into the environment through the seeds and pollen of transgenic plants. Similar results were also reported by earlier workers, when the *cry1C* and *cry1Ac* genes driven by the *rbcS* promoter were transformed into rice (Ye et al. 2009; Kim et al. 2009; Qi et al. 2012; Yang et al. 2014).

The detached leaf bioassay against neonate larvae of rice leaffolder on selected T_1 transgenic rice plants expressing the Cry2AX1 protein indicated that Rb 16-8 recorded the highest mortality of 90 % followed by Rb 16-9 (83.33 %). The mortality data correlated well with data recorded for leaf area damage. The transgenic plants showed low level of leaf damage which ranged from 10.68 to 17.51 % whereas in non-transformed control plant the level of leaf damage was significantly higher (92.73 %). The present study indicated that neonate larvae were highly susceptible to Cry2AX1 protein even at low concentration. Earlier workers reported that Bt crops (Davidson et al. 2002; Kranthi et al. 2005, Meiyalaghan et al. 2006; Katageri et al. 2007; Zaidi et al. 2009; Jafari et al. 2009) offered significant level of protection against neonate larvae. Tang et al. (2006) also reported that transgenic *indica* rice plants expressing *cry1C* gene were highly resistant to *C. medinalis* and *S. incertulas* under laboratory conditions.

In whole plant bioassay, the larval mortality recorded on T_1 plants showed 60 and 40 % against neonate and second instar larvae of rice leaffolder, respectively. Significant difference in damage symptoms like scrapping and folding was noticed in the transgenic lines and non-transgenic control. Transgenic lines showed less damage symptoms compared to non-transgenic control. The surviving larval growth and development were severely affected when fed on transgenic leaf tissues whereas survivors from non transformed control plants grew normally and consumed major portion of leaf tissue. The results suggested that the level of *cry2AX1* gene expression in the transformants were not high enough to confer complete protection against rice leaffolder. In leaf bit bioassay, the larvae are forced to feed on selected leaf bits (leaves selected from top canopy of the plant). The whole plant bioassay may provide a clear picture as the larvae are not restricted to feed particular leaves and also it imposes a situation like in a field. The difference in mortality of leaffolder between leaf bit bioassay and whole plant bioassay could be due to the settlement of larvae in middle or still lower canopy of the plant where the expression of protein may be comparably low.

In earlier report, use of the *rbcS-tp* sequence for expressing *cry1Ac* accumulated Cry1Ac protein in chloroplasts up to 2 % of the total soluble proteins. In the present study the expression level of Cry2AX1 protein in transgenic rice was very low (varying from 36 to 236 ng/g of fresh leaf tissue in different phonological stages among the T_1 plants) when compared to earlier studies. Therefore it is suggested that further modification of *cry2AX1* gene construct with suitable 5′ UTR (untranslated region) sequence and matrix attachment region to overcome position effect of gene integration (Streatfield 2007), coupled with high frequency generation of large number of *cry2AX1* transformants of rice is necessary to identify an elite event with desirable level of Cry2AX1 expression.

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Compliance with ethical standards

Conflict of interest The authors certify that there is no conflict of interest in our manuscript.

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