Madras Agric. J., 105 (4-6): 135-140, June 2018

Development of Transgenic Rice by Using *cry2AX1* Gene against Leaffolder

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Rice transformation was carried out using *Agrobacterium* strain C58C1 (pGV2260::pSSJ1A) containing a binary vector, *pC0390-ubi-rtp-cry2AX1* with a view to generate marker-free transgenic rice plants, resistant to lepidopteran insects. Eight putative transgenic rice plants, positive to *gusA* and *hpt* genes, were generated. Four out of the eight putative transgenic plants were positive for presence of *cry2AX1* gene, indicating co-transformation of the selectable marker gene, *hpt* and the gene of interest, *cry2AX1* in these plants. Cry2AX1 protein concentration in PCR positive T_0 Plants ranged from 0.010 to 0.022 µg/g of fresh leaf tissue. The T_2 generation plants were subjected to ELISA, and Cry2AX1 protein concentration ranged from 0.016 to 0.057 µg/g of fresh leaf tissue. Insect bioassay studies on ELISA positive T_0 and T_2 plants against neonates of leaffolder resulted in larval mortality ranging from 15 to 30 %

Key words : *Cry2AX1*, Transgenic rice, Insect resistance, Rice leaffolder

Rice is one of the most important staple food crops in the world. Yellow stemborer (YSB) (*Tryporyza incertulas*, Walker), striped stem borer (*Chilo suppressalis,* Walker) and leaf folder (*Cnaphalocrocis medinalis,* Guenec) cause severe yield losses (Ye *et al*, 2009). Globally, YSB alone causes yield losses of 10 MT and accounts for 50 % of all insecticides used in rice field (Huesing and English, 2004). For a long time, control of these pests has chiefly depended on the use of large amounts of chemical insecticides, leading to considerable environmental pollution and representing a health hazard to farmers, besides significantly increasing the costs of rice production (Tang *et al*., 2006).

An alternative to overcome the hazards of chemical pesticides is the development of inherently insect resistant varieties which can withstand the attack of such pests. This has been made possible by the use of *Bacillus thuringiensis* (*Bt*), a gram-positive, soil-dwelling bacterium, commonly used as a biological pesticide. They produce crystal proteins called delta endotoxins which are encoded by *cry* genes and have insecticidal action (Dean, 1984). The utility of this bacterium has been made possible in the plant kingdom with the help of transgenic approach which circumvents species and barrier genetic separation amongst organism (Ye *et al*, 2009).

In earlier studies, *cry* genes were introduced into crops such as tobacco (Barton *et al*. 1987; Vaeck *et al*. 1987) and tomato (Fischhoff *et al*, 1987) and the resultant transgenic plants exhibited a certain level of insect resistance. Since then, insect resistant crops harbouring *Bt* genes have been developed at a very fast pace. These crops benefitted the growers and environment by greatly reducing the use of chemicals insecticides (Ferre and Van Rie, 2002).

Plants encoding Bt toxins need no protection against the target pest with insecticides, limited use of pesticides causes less damage to the environment and prevents other negative effects of insecticide application. Well-documented and very important fact is that Bt toxins have no or adverse effects on mammals (including humans) and birds (Goldberg and Tjaden, 1990).

The first step towards the development of insect resistant rice began with the introduction of a truncated delta endotoxin gene, *cry1Ab* of *Bacillus thuringiensis* which has specific biological activity against lepidopteran insects into japonica rice (Fujimoto *et al,* 1993). Subsequent studies and analysis of results in both laboratory and field conditions have shown that *Bt* is highly effective against lepidopteran pests in rice (Wunn *et al*, 1996, Chen *et al*, 2005). The most commonly used *Bt* genes in transgenic crops including rice, are *cry1Ab, cry1Ac* and fusion gene *cry1Ac/cry1Ab* (Fujimoto *et al*. 1993; Nayak *et al*. 1997; Wunn *et al*. 1996; Ghareyazie *et al*. 1997; Wu *et al*. 1997; Cheng *et al*. 1998; Datta *et al*. 1998; Alam *et al*. 1999; Tu *et al*. 2000; Wang *et al*. 2002). The results of binding tests of midgut brush border membrane vesicles indicated that the cry1Aa, cry1Aband cry1Ac toxins share a common binding site (Escriche *et al*. 1997; Ballester *et al*. 1999; Karim and Dean 2000).

Chen *et al.* (2008) developed ten transgenic *Bt* rice lines with different *Bt* genes (two *cry1AC* lines,

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three *cry2A* lines, and five *cry9C* lines) derived from the variety Minghui 63 against YSB and ASIATIC rice borer. All transgenic lines exhibited high toxicity to these two rice borers. Transgenic rice harboring the *cry2A* gene showed a strong field resistance to natural infestation of leaf folders and stemborers (Chen *et al*. 2005). Truncated *cry1Ab* gene has been introduced into several cultivars of rice (*indica* and *japonica*) by microprojectile bombardment and protoplast systems (Datta *et al*. 1998). The effectiveness of this toxin was comparable to *cry1Ab/cry1Ac* genes.

Maqbool *et al*. (1998) transformed the rice cultivars Basmati 370 and Ms7 by using *cry2A* insecticidal gene against the yellow rice stem borer and the rice leaf folder. Nayak *et al*. (1997) reported that two rice lines transformed with synthetic *cry1Ac* were highly toxic to yellow stem borer larvae and reduced the insect feeding. Rice plants expressing *cry1Ab* and *cry1Ac* genes are highly toxic to striped stem borer (*C. suppressalis*) and YSB (*T. incertulas*), with mortalities of 97 to 100 % within 5 days after infestation.

One of the primary concerns in the development of transgenic plants is related to the presence of selectable marker genes. These genes are primarily to confer antibiotic/herbicide resistance which enables selection of the transformed calli from the untransformed calli through the transformation process. The presence of these selectable marker genes has however, aroused safety concerns amongst the public (Daniell *et al.*, 2001). Therefore, removal of marker genes from transgenic plants would likely hasten the public acceptance of transgenic crops (Qiu *et al.*, 2010). There are several strategies to exclude selectable marker gene in transgenic generations, among them co-transformation has been widely practiced (De Block and Debrouwer, 1991; Depicker *et al.*, 1985; Mcknight *et al.*, 1987).

In the present study, we developed transgenic rice plants with codon optimized synthetic *cry2AX1* gene (fused with rice chloroplast transit peptide sequence) using *Agrobacterium* mediated transformation. The insect bioassay results indicated that the transgenic plants expressing synthetic *cry2AX1* gene were resistant to rice leaffolder.

Material and Methods

Agrobacterium-mediated transformation

Rice transformation experiment was carried out using a construct, pC0390-*ubi-rtp-cry2AX1* based on the binary vector pCAMBIA0390 harbouring a synthetic *cry2AX1* gene flanked by maize *ubiquitin* promoter and *nos* terminator (Fig.1) and a co-integrate vector consisting of *gus* reporter gene and hygromycin resistance gene (*hpt:* driven by *CaMV*35S Promoter) as plant selectable marker (Fig.2). The synthetic *cry2AX1* is a codon modified gene consisting sequence of *cry2Aa* and *cry2Ac* genes, developed by Dr. V. Udayasuriyan and his group in CPMB & B, TNAU. The former vector was maintained in *E.coli* DH5α and later one was maintained in *Agrobacterium* strain, C58C1**.** Transformation of rice using immature embryos of elite rice variety ASD16 was carried out following the protocol of Hiei and Komari (2008). A local elite rice cultivar ASD16 was obtained from Department of Rice, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, and used as source of explant for transformation.

Molecular and biochemical analyses of putative transgenic rice plants

The putative T_0 transgenic rice plants just before planting in the transgenic greenhouse were subjected to GUS assay. For this, a small portion of the soft root tissue (about 3-5 mm) was trimmed off and dipped in 5 µl of GUS solution (Jefferson *et al*, 1994). PCR was carried out for the DNA samples isolated from the both T_0 and T_2 putative transgenic rice plants in order to confirm the presence of *cry2AX1*gene using gene specific S2XSF2 (5'CCTAACA TTGGTGGACTTCCAG3') and S2XR2 (5'GAGAAACGAGCTCCGTTATCGT3') Primers. The amplified PCR products were analysed on 0.8 % agarose gel.

The expression of Cry2AX1 protein in the fresh leaves of the PCR positive transgenic lines was analysed at 40-45 day after transferring to pots in the transgenic greenhouse by using the Envirologix *cry2AX1* Quantiplate kit.

Insect bioassay

Transgenic rice plants which showed positive for protein expression were used for insect bioassay with detached leaf bits. About 5 cm length of leaf bit was placed in plastic petriplate containing moist filter paper. Five neonates of rice leaffolder were released in each leaf bit, while thirty larvae were tested per treatment. The experiment was carried out with four replications and maintained at 25 ±1 °C with 60 % relative humidity. Non-transformed ASD16 plants used as a control. The larval mortality was recorded six days of experiment with daily interval. On 6th day, larval mortality, leaf area damage and surviving larval characteristics were recorded in transgenic as well as control plants.

Results and Discussion

Transgenic technology provide opportunity to improve agricultural crops by incorporating genes

Table 1. Expression of Cry2AX1 protein in T_0 transgenic events

Sample ID	Concentration of *cry2AX1* protein in (µg/g of leaf tissue) Mean ±SD
CONTROL	0 .00
SM-ASD16-1	0.010±0.00
SM-ASD16-1	0.022±0.04
SM-ASD16-2	0.017±0.00
SM-ASD16-2	0.015±0.00
SM-ASD16-5	0.010±0.001

from different sources to impart resistance against insect pests. Among the insect resistant genes, *cry* genes of *B. thuringiensis* are the most widely used in plant genetic engineering.

Table 2. Expression and toxicity of cry2AX1 protein in transgenic plants against leaffolder

Transgenic lines	Generation	Concentration of *cry2AX1* protein in (µg/g of leaf tissue) Mean ±SD	Mean larval mortality (%)
SM-ASD16-1	T_0	0.022±0.04	25.00 [ab] (29.72)
SM-ASD16-2	T_0	0.017±0.00	20.00 [ab] (33.20)
SM-ASD16-5	T_0	0.010±0.001	15.00 [b] (20.24)
GR-ASD16-13-3	T_2	0.057±0.04	30.00 [a] (32.89)
CONTROL		0 .00	0.00 [c] (1.28)
SEd		-	5.0341
C.D (0.05)			10.7300

Figures in parentheses are arc sine transformed values.

The present study was taken to develop marker-free transgenic rice events resistant to lepidopteran insects. Co-transformation strategy using *Agrobacterium* strain C58C1 which harbours a co-integrate vector with *gus* and *hpt* genes and a binary vector harbouring a codon optimized synthetic *cry2AX1* gene was used.

Table 3. Expression of Cry2AX1 protein in T_2 transgenic rice plants

Sample ID	Concentration of *cry2AX1* protein in (µg/g of leaf tissue) Mean ±SD
CONTROL	0.00
GR-ASD16-13-3	0.045±0.03
GR-ASD16-13-3	0.057±0.04
GR-ASD16-8-5	0.016±0.01
GR-ASD16-8-5	0.018±0.01
GR-ASD16-17-12	0.032±0.03
GR-ASD16-17-12	0.038±0.01

The *cry2AX1* gene was developed by containing a part of sequences from *cry2Aa* and *cry2Ac* gene (Udayasuriyan *et al.* 2010). The chimeric Cry2AX1 protein exhibited higher level of toxicity than their parental proteins, Cry2Aa and Cry2AC (Udayasuriyan *et al.* 2010). In the present study, a chimeric *Bt*

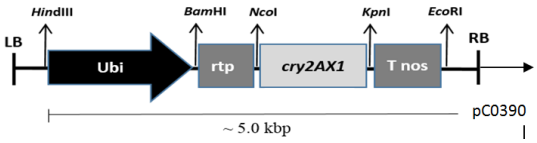


Fig. 1. T-DNA region of binary vector pC0390-ubi-rtp-cry2AX1

gene, *cry2AX1* used for genetic transformation of rice cultivar ASD16 was successfully introduced into rice ASD16 to develop insect resistant transgenic rice against leaffolder.

Molecular analysis of putative T_0 transgenic plants

A total of 39 transgenic plants were derived from eight events and transferred to pots and maintained in transgenic greenhouse (Fig.3). Total genomic DNA was extracted from putative rice transformants was subjected to PCR analysis with *cry2AX1* gene specific primer as described earlier. Out of 8 events regenerated, 4 were found to be positive for the presence of *cry2AX1* gene (Fig.4) indicating 50 per cent efficiency of co-transformation of the gene of interest and marker gene which is on par with the reports already available (Daley *et al*., 1998; De Block and Debrouwer, 1991; Komari *et al*., 1996). The PCR positive transgenic rice events were further screened by quantitative ELISA kit. All the 4 PCR positive events were found positive for the expression of Cry2AX1 protein. The expression of Cry2AX1 protein in these transgenic events ranged 0.010 to 0.022 µg/g of fresh leaf tissue (Table 1). Three ELISA positive T_0

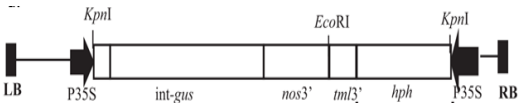


Fig. 2. T-DNA region of co-integrate vector pGV2260::pSSJ1A (Sripriya *et al*., 2011)

plants were considered for insect bioassay studies. The detached leaf bit bioassay using neonate larvae of rice leaffolder showed larval mortality ranging from 15 to 25 per cent (Table 2) (Fig.5).

Inheritance studies

Twenty seven transgenic plants of T_2 progenies from three transgenic events GR-ASD16-8-5, GR-ASD16-13-3, GR-ASD16-17-12 developed in an earlier study were established in greenhouse for further studies on gene inheritance and expression. An amplicon of 800 bp was found in all the 27 T_2 progenies indicating the presence of *cry2AX1* gene. The three PCR positive transgenic events were further screened by quantitative ELISA kit. The expression of Cry2AX1 protein in these transgenic events ranged from 0.016 to 0.05 µg/g of fresh leaf tissue (Table 3).

Insect bioassay

Among the three events in T_2 progeny, GR-ASD16-13-3-4 event showed highest expression level (0.057±0.04) of Cry protein. The detached leaf bit bioassay was carried out for one of the T_2 plants (GR-ASD16-13-3-4) using neonate larvae of rice leaffolder. The larval mortality of 30 per cent was recorded (Table 2). Molecular analysis was done for the confirmation of the transgenes in both T_0 and T_2 plants by PCR. ELISA positive T_0 and T_2 plants were subjected to insect bioassay. The T_0 and T_2 transgenic rice plants expressing Cry2AX1 protein showed mortality ranging from 15 to 25 per cent and upto 30 per cent respectively. It has been demonstrated by earlier researchers that the Cry protein concentration

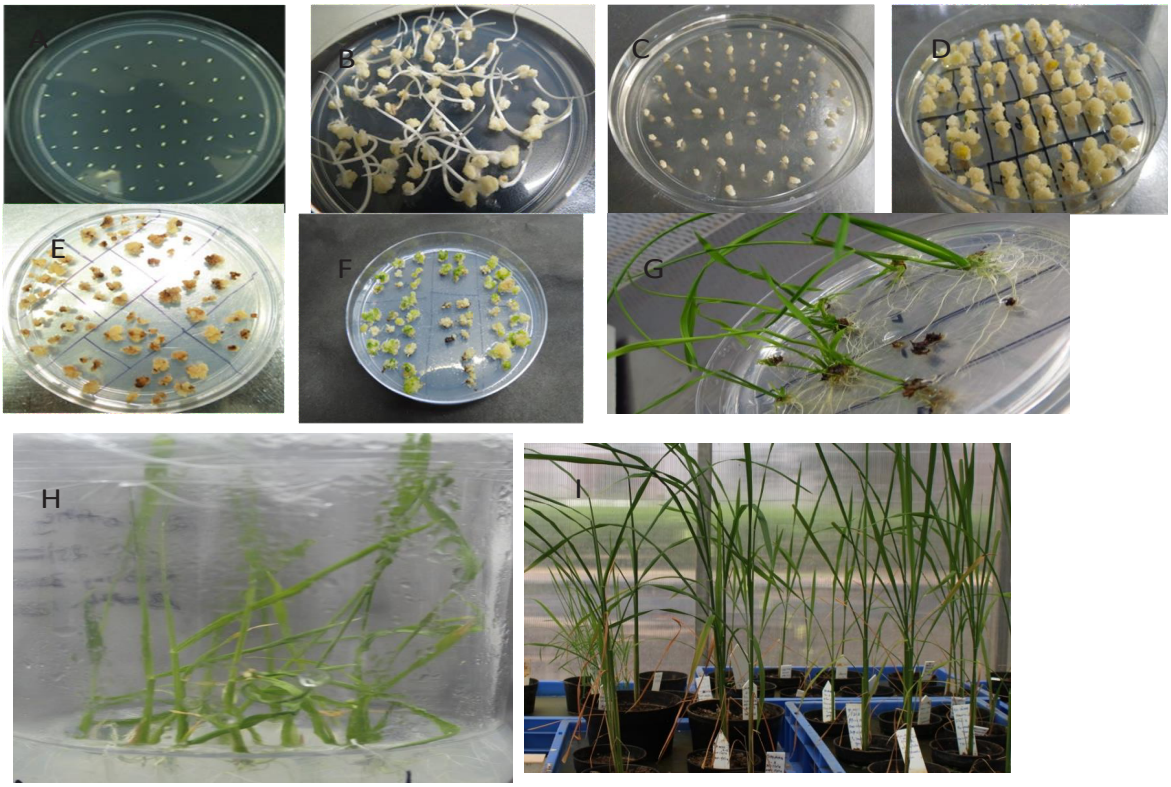


Fig. 3. Agrobacterium mediated transformation of rice (Oryza sativa L, cv, ASD16), (a) immature seeds collected from rice ; (b) pretreated immature embryos infected with Agrobacterium on cocultivation medium; (c) callus initiation (and shoot tips) from cocultivated embryo; (d) subcultured calli on resting medium; (e) callus proliferation on selection medium; (f) embryogenic calli on preregeneration medium; (g) regenerated transgenic rice plants; (h) transgenic plants in rooting medium; (i) established transgenic rice plants in transgenic greenhouse.

is directly proportional to the mortality of insects (Chen *et al.*, 2005; Riaz *et al.*, 2006). Lower level of mortality in the present study may be attributed to low level of expression of Cry2AX1 protein in plant tissues. Though the protein possesses insecticidal activity, the transgenic events did not exhibit a higher

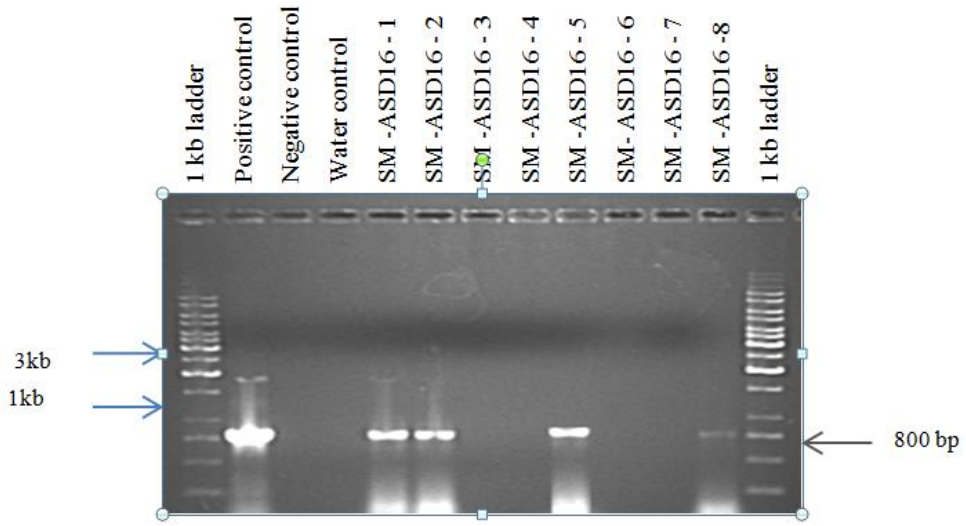
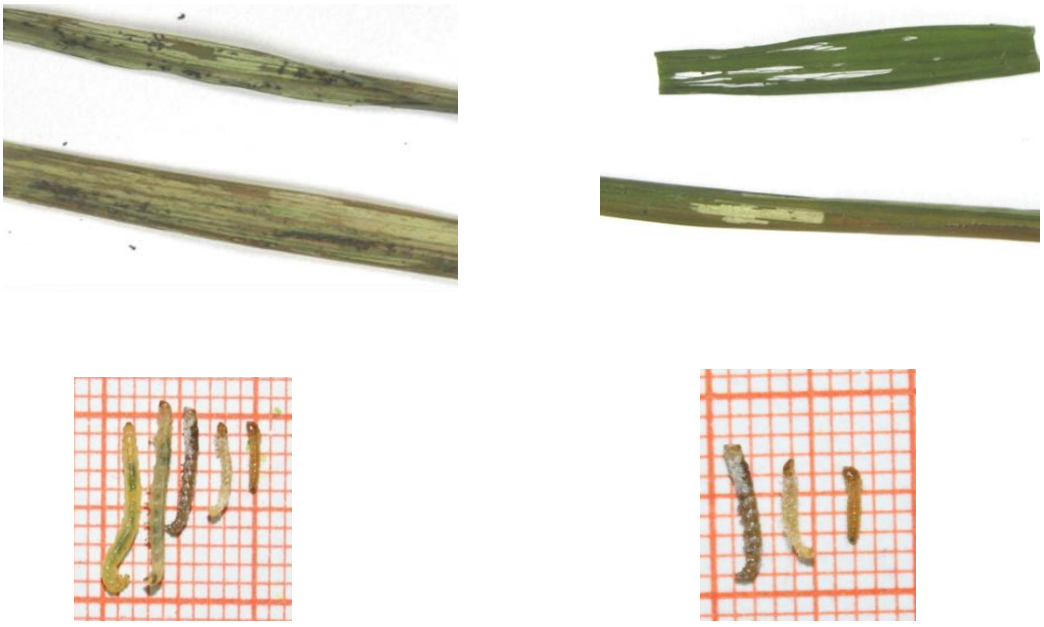


Fig. 4. PCR analysis of T0 Transgenic plants for the presence of cry2AX1 gene

level of mortality due to low level of expression of the insecticidal transgene. Transgenic rice plants having single copy gene may be developed to get higher level expression of the insecticidal protein.



a. Control ASD16 b. Transgenic ASD16

Fig. 5. Insect bioassay on T0 transgenic event, SM-ASD16-1

Conclusion

Rice transformation by *Agrobacterium* containing binary vector *pC0390-ubi-rtp-cry2AX1* harbouring the gene of interest, *cry2AX1* was carried out following the protocol suggested by Hiei and Komari (2008), and a total of eight putative transgenic rice events were generated. All of them were positive for GUS assay. Four out of eight GUS positive rice events were positive for *cry2AX1* gene in PCR analysis. Cry2AX1 protein content in the ELISA positive T_0 transgenic plants ranged from 0.010 to 0.022 µg/g of fresh leaf tissue. In T_2 generation, three PCR positive events (which were developed in an earlier investigation) were subjected to ELISA. Cry2AX1 protein content in the ELISA positive plants ranged from 0.016 to 0.057 µg/g of fresh leaf tissue. The toxicity of Cry2AX1 protein in transgenic plants ranged from 15 to 30 per cent against rice leaffolder.

Acknowledgement

Dr. K. Kumar, Dr. L. Arul of Rice transformation laboratory CPMB & B for providing valuable suggestions and information related to my research work. My sincere, gratitude goes to the member of Rice Transformation Laboratory, Rajadurai. G for guiding me in learning the basics of rice transformation.

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Received : May 07, 2018; Revised : May 14, 2018; Accepted : May 28, 2018