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Agrobacterium Mediated Transformation of Indica Rice with Synthetic *cry2AX1* gene for Resistance against Rice Leaf Folder

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A novel synthetic *cry2AX1* gene consisting sequences of *cry2Aa* and *cry2Ac* genes isolated from Indian strains of *Bacillus thuringiensis* was codon optimized and a sequence encoding cotton *rbcS1b* transit peptide was fused at upstream of coding sequence. The synthetic *cry2AX1* gene, driven by maize ubiquitin1 promoter was cloned in a pCAMBIA- based plant transformation vector. Immature embryos were used as target tissues for *Agrobacterium*-mediated transformation of an elite rice cultivar, ASD16. Among 34 putative rice transformants, 21 plants were found to be positive for *cry2AX1* and *hptII* gene by PCR. Southern blot analysis of transgenic plants confirmed stable T-DNA integration into the genome of transformed plants. The expression of Cry2AX1 protein in transgenic rice plants ranged from 3.3 to 16.0 ng/g of fresh leaf tissue. Insect bioassays of transgenic rice plants against rice leaf folder (*Cnaphalocrosis medinalis Guenee*) recorded larval mortality of upto 40 per cent and significant reduction in leaf feeding. Level of *cry2AX1* expression in transgenic rice plants of the present study is 1000 fold less in comparison to earlier reports on insect resistant Bt-rice plants. These results indicated that the Cry2AX1 protein is effective against rice leaf folder even at a very low concentration in transgenic rice plants. Desirable level of insect resistance could be achieved in rice through *cry2AX1* gene by improving the level of transgene expression.

Key words: Cry2AX1, Rice, *Agrobacterium tumefaciens* and rice leaf folder.

Rice (*Oryza sativa* L.), is one of the predominant staple food crops around the world in general and Asia in particular. The world population is expected to reach 10.5 billion by 2050[30], adding further to global food security concerns. This increase translates into 33% more human mouths to feed, with the greatest demand growth in the poor communities of the world. The worldwide rice yield has increased from 1869 Kg/Ha to 4403 Kg/Ha from 1961 to 2011[29]. However, rice production is challenged by many biotic and abiotic factors. Approximately 52 % of the global production of rice is lost annually due to the damage caused by biotic factors, of which almost 21 % is attributed to insect pests[5]. Among these insects, lepidopteran pests such as yellow stem borer (YSB), striped stem borer (SSB) and rice leaf folder (RLF) are major pests in rice that cause severe yield losses. Once considered as a minor pest, leaf folders have now attained major pest status due to the spread of high-yielding rice varieties, continuous availability of rice crop in double cropping areas and accompanying changes in cultural practices. The RLF larvae damage the plant during vegetative stage of rice by folding the leaves and scraping the green leaf tissues within the folded region, causing significant yield loss by reducing leaf photosynthetic activity. Bautista *et al*.,[3] have clearly reported that the percentage of yield reduction in rice is positively

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correlated with the amount of leaf damaged by RLF. In the past two decades, control of these lepidopteran pests chiefly depended on extensive use of chemical insecticides, leading to considerable environmental pollution and health hazards to farmers, besides increasing the cost of rice production.

Conventional plant breeding of rice enhancing for resistance against YSB, RLF and SSB is less effective because of the complex as well as poor understanding of the genetic mechanism of resistance. Moreover, the genes for resistance against these insects are not available in the related germplasm. With the advent of genetic engineering technology, it is now possible to source genes for resistance from across the kingdoms for use in developing transgenic insect-resistant plants. Transgenic plants expressing insecticidal crystal proteins from the bacterium *Bacillus thuringiensis* (Bt) which are revolutionizing agriculture, are examples. These proteins are known to be toxic towards larvae of different orders of insect pests (Lepidoptera, Diptera, Coleoptera, Hymenoptera and Hemiptera) with different level of efficacies[21].

During the last two decades, considerable efforts have been made to introduce insecticidal crystal protein genes into rice by transgenic approaches. The first transgenic rice plant with insect resistant Bt protein was reported by Fujimoto *et al*[11]. In 1990s, several Bt genes were transferred into higher plants, but the insecticidal activity of the resulting transgenic plants was low, mainly due to mRNA degradation causing low level of protein expression. To further increase the expression of Bt genes in plants, two strategies have been deployed: to target proteins to specific sub cellular sites/compartments of plant cells, such as the chloroplast[24, 25] or to modify the coding sequences of the bacterial gene to plant-preferred coding sequences[18, 22].

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[26]. 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 mode of action are now being grown worldwide for protection against a variety of insect pests. In this direction a potent chimeric Bt gene *cry2AX1*[28] was constructed by using coding sequences of *cry2Aa* and *cry2Ac*, cloned from Indian strains of Bt. In the present investigation, cotton transit peptide was fused in frame at the 5' end of the codon optimised- synthetic *cry2AX1* gene for chloroplast targeted expression. The synthetic *cry2AX1* gene was transformed to rice cultivar ASD16 by *Agrobacterium* mediated transformation to evaluate its insecticidal activity against rice leaf folder.

MATERIALS AND METHODS

Plasmid constructs

The synthetic *cry2AX1* gene is translationally fused at its 5' end to the cotton chloroplast transit peptide (CTP) sequence of *rbcs1b* gene (Source: Dr. P.K. Burma, University of Delhi South Campus, New Delhi, India). The fusion gene driven by maize ubiquitin constitute promoter was cloned in pUH binary vector[1] which harbours *hph* (coding for *hygromycin phosphotransferase*) as a plant selectable marker gene. Transformation vector (Fig.1) was mobilized into disarmed *Agrobacterium* strain, LBA4404 for rice transformation experiments.

Rice transformation

Agrobacterium-mediated rice transformation procedure suggested by Hiei and Komari[12] was followed. The immature embryos of ASD16 were placed on cocultivation medium containing 100 mM acetosyringone and 5 µl of the *Agrobacterium* suspension was dropped on each embryo. The infected immature embryos were incubated at 25 ºC in the dark for 7 days. Well developed embryogenic calli from co-cultivated immature embryos were sub-cultured twice on resting medium containing 250 mg l^{-1} cefotaxime for 15 days at 30 ºC under continuous illumination. The proliferated embryogenic calli were sub-cultured twice on selection medium containing hygromycin 50 mg 1^{-1} and 250 mg l^{-1} cefotaxime for 17 days. The hygromycin resistant calli were transferred to pre-regeneration medium containing

40 mg l^{-1} hygromycin 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 of MS medium containing 30 mg l^{-1} hygromycin for rooting. Finally, the well developed rooted plants were transferred to soil in pots and grown to maturity in a transgenic greenhouse.

PCR and Southern blot analysis

PCR analyses were performed to demonstrate the presence of *cry2AX1* and *hptII* genes in putative transgenic lines of ASD16 using gene specific primer (2AX1FP, 5'-CCTAAC ATTGGT GGACTTCCAG-3' and 2AX1RP, 5'-GA GAAA CGAGCTCCGTTATCGT-3'); (HPTFP, 5'-GCTGTTATGCGGCCATTGGTC-3'; HPTRP, 5'-GACGTCTGTCGAGAAGTTTG-3'). These primers amplify 800 and 640 bp internal fragments from *cry2AX1* and *hptII* genes, respectively. 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*[9]. For Southern blot analysis, 5 µg of genomic DNA was digested with *Bam*HI, 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 ìJ min^{-1} for 1 min. The prehybridisation was carried out for one hour and hybridization for 18 hours at 60 ºC. 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 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 ºC 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 5000 rpm at 4 ºC 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 Envirologix ELISA kit (Envirologix, USA) following standard procedures. The Cry2A protein concentration was calculated on a linear standard curve, using the standards provided in the kit.

Insect culture and detached leaf bioassay

Adults of rice leaf folder were collected from the rice field at Paddy Breeding Station, TNAU, Coimbatore and they were released on the TN1 rice plants maintained in insect cages for culturing. 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 leaf folder 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 ºC ±1, 60 % relative humidity. Larval growth and mortality was recorded every day upto five days.

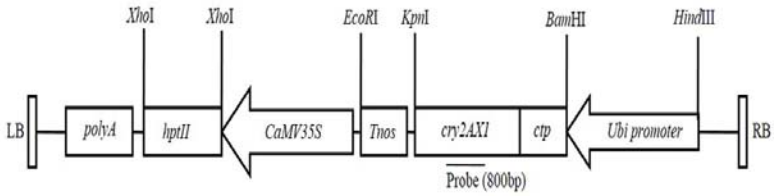
RESULTS

Rice transformation and regeneration

Embryogenic calli developed from co-cultivated immature embryos were subcultured twice on selection medium containing hygromycin. A total of 43 calli lines of ASD16 were recovered after two rounds of hygromycin selection. Transformed tissues grew normally while the non-transformed ones turned brown and dried. The selected embryogenic calli were transferred onto pre-regeneration and regeneration medium for shoot induction. Well developed shoots were separated and transferred onto rooting medium. A total of 34 events were generated.

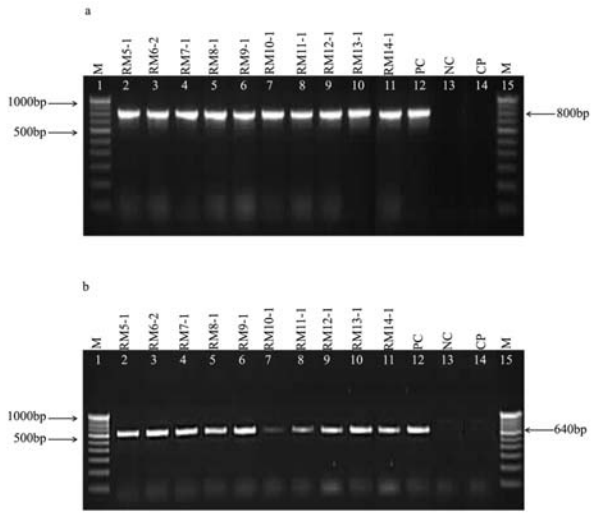
Screening of putative rice transformants by PCR

Total genomic DNA from putative rice transformants was subjected to PCR analysis with *hptII* and *cry2AX1* gene specific primers. Out of 34 plants regenerated, 21 were found to be positive for the amplification of 800 bp (Fig. 2a) and 640 bp (Fig. 2b) internal sequences of *cry2AX1* and *hptII* genes, respectively.



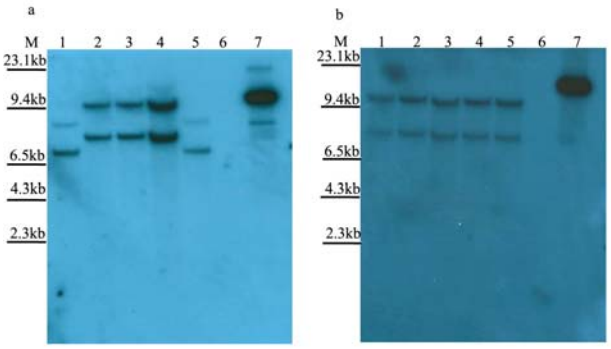
Cotton chloroplast transit peptide (*ctp*) was fused to *cry2AX1* gene. The *ctp-cry2AX1* gene is driven by a maize ubiquitin1 promoter and terminated by the nopaline synthase (*nos*) terminator. The plant selectable marker gene, *hptII* is under the control of the CaMV35S promoter and tailed by the CaMV35S polyA. LB: left border of T-DNA region; RB: right border of T-DNA region.

Fig. 1. T-DNA region of plant transformation construct *pUH-TP2AX1*



a, A 800 bp internal sequence of *cry2AX1* gene was amplified by PCR from the DNA isolated from putative transgenic plants. Lane 1 & 15: 100 bp marker, Lane 2-11: Putative transgenic plants of ASD16, Lane 12: *pUH-TP2AX1* plasmid as a positive control, Lane 13: Non-transformed plant as a negative control, Lanes 14: Water as a negative control.
b, A 640 bp internal sequence of hptII gene was amplified by PCR from the DNA isolated from putative transgenic plants. Lane 1 & 15: 100 bp marker, Lane 2-11: Putative transgenic plants of ASD16, Lane 12: *pUH-TP2AX1* plasmid as a positive control, Lane 13: Non-transformed plant as a negative control, Lanes 14: Water as a negative control.

Fig. 2. PCR analysis of *cry2AX1* transgenic rice plants



DNA digested with *Bam*HI and probed with a radioactively labelled 800 bp internal sequence of *cry2AX1* gene
a. M: λ/HindIII, Lane 1: RM 5-1, Lane 2: RM 6-2, Lane 3: RM 7-1, Lane 4: RM 8-1, Lane 5: RM 9-1, Lane 6: Control plant, Lane 7: PC, *pUH-TP2AX1* plasmid.
b. M: λ/HindIII, Lane 1: RM 10-1, Lane 2: RM 11-1, Lane 3: RM 12-1, Lane 4: RM13-1, Lane 5: RM14-1, Lane 6: Control plant, Lane 7: PC, *pUH-TP2AX1* plasmid

Fig. 3. Southern blot analysis of transgenic rice plants expressing *cry2AX1* gene

ELISA for expression of Cry2AX1 in transgenic rice plants

Expression of Cry2AX1 protein in fresh leaves was analysed at tillering stage from PCR positive rice lines. Among 21 PCR positive plants, 10 were found to be positive in ELISA and the concentration of protein ranged from 3.3 to 16.0 ng/g of fresh leaf tissue (Table 1).

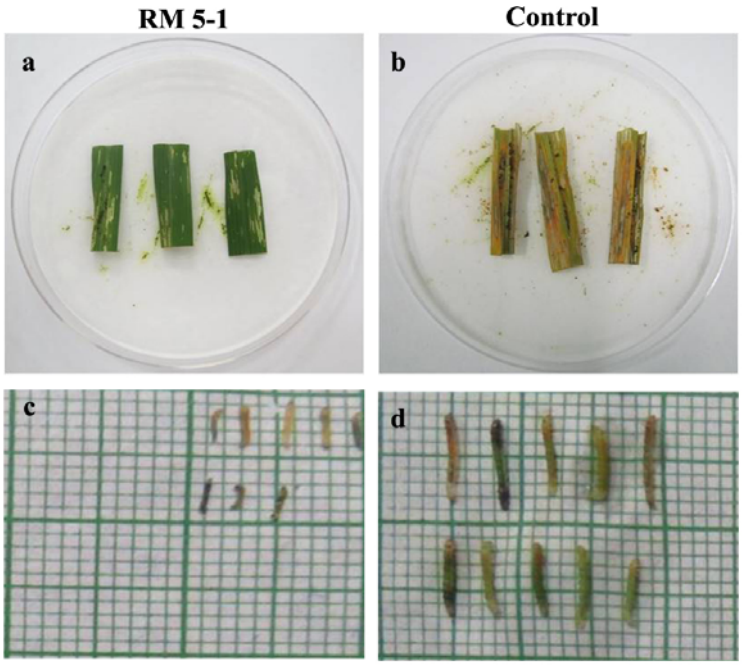
Southern blot hybridization analysis of *cry2AX1* transgenic rice plants

Southern blot hybridization analysis of ten *cry2AX1* transformants indicated the

Table 1. Expression of Cry2AX1 protein and mortality of rice leaf folder neonates in transgenic rice lines

S. No	Transgenic rice lines	Concentration of Cry2AX1 protein (ng/g fresh leaf tissue)[a] Mean ± SD	Mortality of neonates of RLF (%)[b](Mean ± SD)
1	RM 5-1	16.0 ± 0.00	40.00 ± 0.00
2	RM 6-2	14.0 ± 0.10	36.67 ± 4.71
3	RM 7-1	7.50 ± 0.00	16.67 ± 4.71
4	RM 8-1	8.30 ± 0.00	16.67 ± 4.71
5	RM 9-1	4.15 ± 0.00	13.33 ± 4.71
6	RM 10-1	9.15 ± 0.85	20.00 ± 0.00
7	RM 11-1	3.30 ± 0.00	13.33 ± 4.71
8	RM 12-1	12.0 ± 0.10	26.67 ± 4.71
9	RM 13-1	7.75 ± 0.25	16.67 ± 4.71
10	RM14-1	8.30 ± 0.00	16.67 ± 4.71
11	Control	-	-

[a] Mean of two replicates; [b] Mean of three replicates



a. Transformed rice plant (RM 5-1). b. Non transformed control (ASD16). c & d. Size of survivor from transformed and non transformed rice plants

Fig. 4. Detached leaf bioassay against rice leaf folder (*Cnaphalocrocis medinalis*) in transgenic rice plants expressing Cry2AX1 protein

integration of *cry2AX1* gene in all the 10 transgenic rice plants at two loci whereas the untransformed control plant did not show any hybridization signal (Fig.3).

Insect bioassay of *cry2AX1* transgenic rice plants

The detached leaf bioassay against neonate larvae of rice leaf folder on 10 ELISA positive plants showed larval mortality ranging between 13.33 to 40 % (Table 1). The surviving larvae showed severe growth inhibition and significant reduction in leaf feeding. There was no larval mortality on control plants and major portion of the leaf material was consumed by the surviving larvae over a period of five days (Fig. 4).

DISCUSSION

Research and development of transgenic technology in rice have made significant progress in the past decades, particularly in developing countries. 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[2,13,17,19,23,27,31]. We have shown that the expression of a novel synthetic Bt gene encoding the Cry2AX1 protein could be an alternative Bt gene for protection against rice leaf folder.

PCR positive putative rice transformants of *cry2AX1* gene were analysed for the level of expression of Cry2AX1 protein. Ten out of 21 PCR positive plants were found to be positive for the expression of Cry2AX1 protein, ranging from 3.3 to 16.0 ng/g of fresh leaf tissue. A wide range of Bt protein expression in transgenic plants were reported by earlier workers. Genetic background and gene constructs were shown to influence the level of expression[4,14,15,16]. Plant to plant variation in expression is mainly due to integration of transgenic DNA into regions of the genome that are transcriptionally repressed (heterochromatin), which ultimately leads to transgene silencing. Additionally, transgene may be incorporated near endogenous regulatory elements, such as transcriptional enhancers or silencers, which could cause mis-expression[7,10]. These types of position effects often result in the production of transgenic lines exhibiting high and low levels of expression[8].

Insect bioassay on rice transformants expressing Cry2AX1 protein showed mortality of rice leaf folder between 13.33 to 40 % and the amount of Cry protein in these lines ranged from 3.3 to 16.0 ng/g of leaf tissue. In transgenic Bt plants, the protein concentration is directly related to the level of insect-resistance. Chen *et al*.,[6] reported an insect mortality of 100% against rice stem borer in plants with average level of Cry2A protein expression of 10 µg/g fresh weight. Riaz *et al*.,[20] reported a concentration of Cry1Ac protein ranging between 4.6 and 16 µg/g and Cry2A at a level of 0.34 to 1.45 µg/g of tissue in leaves of transgenic rice plants. The levels reported by earlier workers indicate that a relatively higher level of *cry2AX1* gene expression may be necessary for a desirable level of insecticidal activity in rice plants. Therefore, the expression of desirable level of Cry2AX1 protein in rice plants can possibly be achieved by generating large number of events followed by selection of a few best events showing significant level of mortality against rice leaf folder.

Southern blot analysis with *BamH*I digested DNA confirmed integration of *cry2AX1* gene at two loci in all the 10 ten transgenic rice lines. Banding pattern in Southern analysis suggested that these might have derived from three independent events.

In the present study, we have introduced a synthetic chimeric gene, *cry2AX1* into rice to study its insecticidal activity of against rice leaf folder. The rice transformants showed a mortality of up to 40 % against neonates of *C. medinalis* even with a very lower level of Cry2AX1 protein (about 16.0 ng/g of leaf tissue). To obtain an event with a desirable level of protein expression and insecticidal activity, more number of plants/events need to be screened.

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