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Genetic engineering of cotton with a novel *cry2AX1* gene to impart insect resistance against *Helicoverpa armigera*

Karunamurthy Dhivya[1], Sundararajan Sathish[1], Natarajan Balakrishnan[1], Varatharajalu Udayasuriyan[1] and Duraialagaraja Sudhakar[1*]

Abstract: *Embryogenic calli of cotton (Coker310) were cocultivated with the Agrobacterium tumefaciens strain LBA4404 harbouring the codon-optimised, chimeric cry2AX1 gene consisting of sequences from cry2Aa and cry2Ac genes isolated from Indian strains of Bacillus thuringiensis. Forty-eight putative transgenic plants were regenerated, and PCR analysis of these plants revealed the presence of the cry2AX1 gene in 40 plants. Southern blot hybridisation analysis of selected transgenic plants confirmed stable T-DNA integration in the genome of transformed plants. The level of Cry2AX1 protein expression in PCR positive plants ranged from 4.9 to 187.5 ng g^{-1} of fresh tissue. A transgenic cotton event, TP31, expressing the cry2AX1 gene showed insecticidal activity of 56.66 per cent mortality against Helicoverpa armigera in detached leaf disc bioassay. These results indicate that the chimeric cry2AX1 gene expressed in transgenic cotton has insecticidal activity against H. armigera.*

Keywords*: cry2AX1, Agrobacterium tumefaciens, somatic embryogenesis.*

INTRODUCTION

Cotton is one of the most important crop species, valued around the world by the textile industry. Besides being the backbone of the textile industry, cotton and its by-products are also part of livestock feed, seed oil, fertilizers, paper, and other consumer products (Wilkins et al. 2000). Since cotton is highly susceptible to biotic and abiotic stresses, it requires intensive crop management. In cotton, bollworms, namely *Helicoverpa armigera* (Hubner), the American bollworm, *Earias vittella* (Fabricius), the spotted bollworm, and *Pectinophora gossypiella* (Saunders), the pink bollworm, pose serious threats to cotton production (Agarwal et al. 1984), causing yield loss of more than 50%. Conventional plant breeding methods have been extensively applied to improve these traits. However, these approaches have been limited by the lack of sufficient genetic variability in the existing germplasm pool (Wu et al. 2004). The control of insect pests has been accomplished primarily through the application of chemical pesticides, which lead to severe environmental problems. Moreover, many insects have developed resistance to different chemical pesticides, resulting in inefficient insect control programs. Considering the problems related to the action of these insecticides on non-target/beneficial organisms, environment and human health, it has become necessary to find alternative methods of control, which can be a part

***Corresponding author:**
E-mail: dsudhakar@hotmail.com

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[1] Tamil Nadu Agricultural University, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Coimbatore, India 641 003

of Integrated Pest Management (IPM).

In this context, genetic engineering provides an enormous scope for widening the genetic diversity of crop plants through stable expression of foreign genes from divergent sources, including bacteria. *Bacillus thuringiensis* (Bt) is perhaps the most important source of insect resistance genes. Resistance to insects through deployment of Bt genes is one of the most successful strategies in modern agriculture. Genetically engineered (GE) crops were grown on 175.2 million hectares globally in 2013 (James 2013). Among them, transgenic cotton expressing insecticidal proteins from Bt has been one of the most rapidly adopted GE crops in the world (James 2012, Lu et al. 2012). Cultivation of Bt transgenic cotton has allowed a significant decrease in the use of chemical insecticides and, consequently, in environmental pollution and human exposure to toxins (Bennett et al. 2004). Bt toxins are also highly specific against insect pests, without affecting predators and other beneficial insects (Christou 2005).Due to these advantages, Bt cotton varieties or hybrids are recognized as a valuable component of an integrated pest management system.

Integration of genes encoding proteins from Bt has made it possible to obtain cotton lines that are resistant to several polyphagous insects (Perlak et al. 1990, Perlak et al. 1991). The ultimate goal is to obtain durable protection, which specifically requires stability of gene expression during the course of selfing or backcrossing, and also requires reducing the probability of development of resistant insects. Transgenic Bt cotton expressing *cry1Ac* has been registered for commercial cultivation in India since 2002, and it primarily targets *Helicoverpa armigera*.

A major concern of using transgenics with a single Bt toxin is the possibility of breakdown of insect resistance in plants. However, Tabashnik et al. (2003) have shown that insects that developed resistance against Cry1A are still susceptible to the Cry2A protein. Therefore, pyramiding two or more *Bt* genes with different modes of action is one of the strategies to delay development of resistance in insects. The combination of *cry1Ac* and *cry2Ab* in the second version of Bt cotton (BGII) exhibited superior control of lepidopteran pests and delayed development of resistance in insects (Perlak et al. 2001). Commercial Bt crops expressing Cry1Ab, Cry1Ac, Cry1F, Cry2Ab, and Cry3Bb proteins, with different modes of action, either individually or in combination, are now being grown worldwide with protection against a variety of insect pests.

With a view toward developing an alternative gene belonging to the *cry2* group, a novel chimeric *cry2AX1* gene was constructed, consisting of sequences from *cry2Aa* and *cry2Ac* (Udayasuriyan et al. 2010). Due to differences in structural and insecticidal mechanisms, *cry2A* genes are potential candidates for management of resistance in insects when deployed in combination with *cry1*. In this study, the codon-optimised synthetic *cry2AX1* gene was used in cotton transformation to develop cotton plants exhibiting insecticidal activity against *H. armigera*.

MATERIAL AND METHODS

Plasmid constructs and binary vector

The codon-optimised 1902 bp synthetic *cry2AX1* gene (Acc. No. GQ332539.1) fused downstream of the cotton transit peptide sequence (186 bp) from the Ribulose bisphosphate carboxylase small subunit (*rbcS1b*) gene family (Acc. No. JN608790.1) was cloned under the control of a double enhancer version of *CaMV35S* promoter and *nos* termination signal in pCAMBIA 2300 backbone (Ruturaj et al. 2014). The *Agrobacterium* strain LBA4404 harbouring the above construct (p2300-tp*2AX1*) (Figure 1) was used for cotton transformation.

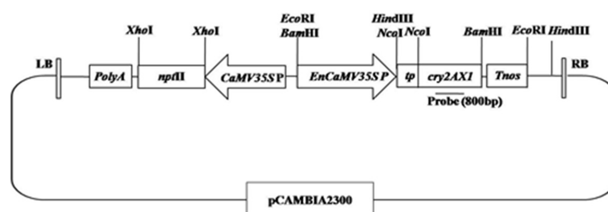

Figure 1. T-DNA region of plant transformation construct p2300-tp*2AX1.* Cotton chloroplast transit peptide (tp) was fused to the *cry2AX1* gene. The tp-*cry2AX1* gene is driven by a double enhancer *CaMV35S* promoter and terminated by the nopaline synthase (*nos*) terminator. The plant selectable marker gene *npt*II is under the control of the duplicated *CaMV35S* promoter and tailed by the *CaMV35S* polyA. LB: left border of T-DNA region; RB: right border of T-DNA region.

Plant material

Seeds of *Gossypium hirsutum cv.* Coker310 were surface sterilized and germinated in half-strength MS medium. Cotyledons and hypocotyls derived from one week old seedlings were used as explants for callus induction.

Callus induction and somatic embryogenesis

Initiation and maintenance of embryogenic calli

Cotyledonary (10-16 mm^2) and hypocotyl (3-5 mm) segments were used as explants for callus induction. Calli were cultured on MS medium supplemented with 0.5 mgL^{-1} kinetin and 0.1 mgL^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid) (Trolinder and Goodin 1988a) and 3% maltose. The proliferated calli were cultured on MS medium containing 1.9 gL^{-1} KNO_3 for initiation of somatic embryogenesis (Trolinder and Goodin 1988b) (Figure 2).

Genetic transformation and plant regeneration

Friable embryogenic calli were cultured with *Agrobacterium* strain LBA4404 (p2300-tp*2AX1*) (Leelavathi et al. 2004). After cocultivation, the calli were subcultured on MS medium supplemented with 1.9 gL^{-1} KNO_3, 25 mgL^{-1} kanamycin, and 250 mgL^{-1} cefotaxime until initiation and maturation of somatic embryos. The mature somatic embryos with true cotyledons were germinated on MS medium containing 0.1 mgL^{-1} GA3 (Giberellic acid), 1.0 mgL^{-1} IAA(Indole-3-acetic acid), and 3% sucrose. The regenerated plantlets were transferred to soil (soil:sand:peat mixture 1:1:1) for hardening (Figure 2).

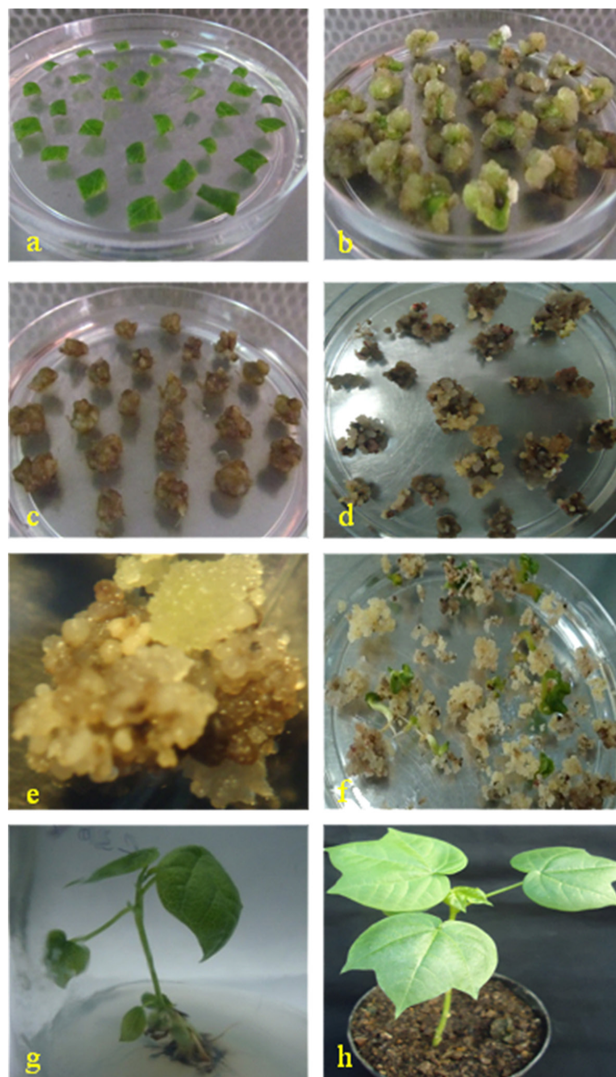

Figure 2. *Agrobacterium*-mediated transformation of cotton. a. Cotyledon explant inoculation (Explant from 7 day old seedlings). b. Callus induction on cotyledon explants (30 days). c. Pre-embryogenic calli for cocultivation (180 day old calli). d. Proliferation of embryogenic calli (30-45 days). e. Proliferated somatic embryos (45-60 days). f. Friable embryogenic cultures with cotyledonary embryos (30 days). g. Regeneration of complete plant (30-45 days). h. Plant with well-developed roots after transfer to soil (15-20 days).

Molecular analysis of putative transgenic plants

Genomic DNA was isolated from the leaves of putative transgenic and non-transgenic control plants (Stewart and Via 1993). PCR analysis was performed to analyse the presence of *cry2AX1* and *npt*II (*Neomycin phosphotransferase*) genes in the putative transgenic lines of Coker310 using gene specific primers, *cry2AX1*: FP 5'-AACGTTCTTAACTCTGGAAGGA-3'; RP 5'-GCAGAAATTCCCCACTCATCAG-3' and *npt*II:FP 5'-CTGATGCTCTTCGTCCAGAT-3'; RP 5'-AGAGGCTATTCGGCTATGACT-3'. The presence of the actin gene was checked as an internal control.

Southern blot hybridisation analysis was done to confirm the integration of the transgene in putative T_0 transgenic plants. For Southern blot hybridisation analysis, 10 µg of genomic DNA was digested with *Eco*RI, which releases the *cry2AX1* gene (~3.1 kb size). The digested products were gel electrophoresed on 0.8% agarose gel and blotted onto a positively charged nylon membrane. For hybridisation, the 800 bp internal region of the *cry2AX1* gene was used as a probe. The probe DNA was radio-labelled with α^{32}-P dCTP (Deoxycytidine triphosphate) by random priming using the Decalabel DNA labelling kit (Thermo Scientific Inc.). The blot was washed with 3X SSC (Saline-sodium citrate) + 0.1% SDS (*Sodium dodecyl sulphate*) and 2X SSC + 0.1% SDS for 15 min each, followed by 10 min in 0.5X SSC + 0.1% SDS at 60°C after hybridisation. The membrane was exposed to X-ray film for a week.

Estimation of Cry2AX1 protein by ELISA

The Cry2AX1 protein levels in the putative transgenic plants were analysed using the Enzyme Linked Immunosorbent Assay quantitative (ELISA) kit (Envirologix, USA) as per manufacturer's instructions. Protein extracts were made by grinding 30 mg of leaf tissues (fully expanded terminal leaf) in 500 mL of extraction buffer (provided in the kit) and centrifuged at 10,000 rpm for 10 min at 4 °C. An aliquot of 100 µL of leaf extracts was loaded into the ELISA plate. Colour intensity was observed at 450 nm. Proper negative and positive controls (standards provided in the kit) were included in the experiment. The Cry2AX1 protoxin was quantified based on standards provided with the kit.

Detached leaf disc insect bioassay

Putative T_0 transgenic plants were subjected to insect bioassay to assess insecticidal activity of the Cry2AX1 protein against cotton bollworm, *H. armigera*. Leaf discs (3 cm diameter) of putative transgenic (T_0) and non-transformed (control) plants were placed on a wet filter paper placed inside a sterile petriplate. Ten first instar larvae of *H. armigera* were released per replication. Three replications were maintained in each line, and the bioassay was carried out at 26-28°C with 60% relative humidity. Larval mortality and larval growth were recorded for five days.

Statistical analysis

The experimental data values of Cry2AX1 protein concentration and mortality of *H. armigera* were mean values from three replicates, and the results were presented as mean ± SD. All mortality data were subjected to arcsine transformations before analysis. Data analysis was done by analysis of variance (ANOVA) following the AGRES statistical package. Mean values were separated by Duncan's multiple range test (DMRT) at a 5 per cent probability level (Duncan 1955).

RESULTS AND DISCUSSION

Generation and evaluation of transgenic plants

The cultivation of transgenic cotton (*G. hirsutum* L.) rapidly gained a great deal of ground in the late 1990s and now accounts for most cotton production in the US and many other countries, including India. The transformation and regeneration of cotton *via* somatic embryogenesis is a long process, and cotton remains one of the recalcitrant species to be manipulated in culture (Wilkins et al. 2000). Somatic embryogenesis in cotton is hampered by its extended culture period, low frequency of embryos, and high incidence of abnormal embryos (Kumria et al. 2003). Low conversion rates of somatic embryos into complete plantlets in cotton

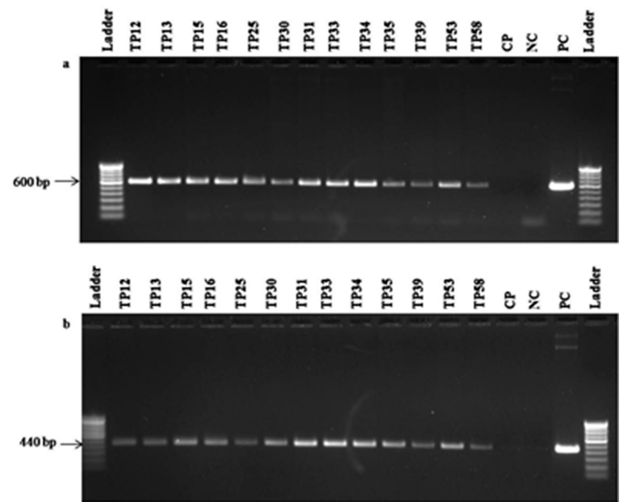

Figure 3. PCR analysis of *cry2AX1* transgenic cotton plants. a. A 600 bp internal sequence of the *cry2AX1* gene amplified by PCR from the DNA isolated from putative transgenic plants. b. A 440 bp internal sequence of the *npt*II gene amplified by PCR from the DNA isolated from putative transgenic plants. Ladder: 100 bp ladder, Lane 2-14: Putative cotton transgenic plants, CP: non-transformed plant, NC: negative control, PC: positive control (p2300-tp*2AX1* plasmid).

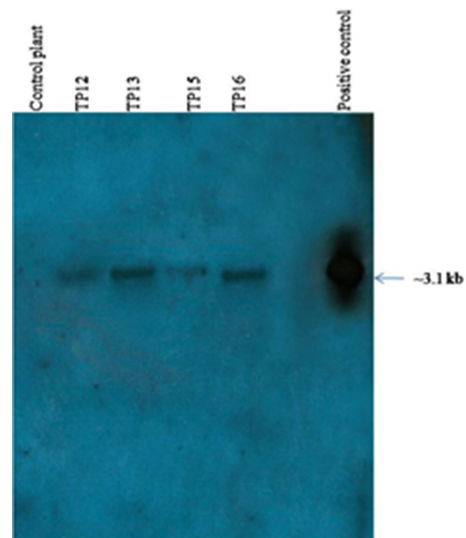

Figure 4. Southern blot analysis of putative T_0 transgenic plants**.** DNA digested with *Eco*RI and probed with a radioactively labelled 800 bp internal sequence of the *cry2AX1* gene. Lane 1: Control plant, Lane 2: TP12, Lane 3: TP13, Lane 4: TP15, Lane 5: TP16, Lane 6: blank, Lane 7: positive control (p2300-tp*2AX1*plasmid).

tissue culture have been reported (Voo et al. 1991, Zhang et al. 1993). In germinating somatic embryos, rooting is an inefficient process and one that has received scant attention. In the Coker genotypes, only ~5-6% of somatic embryos root sufficiently to allow recovery of complete plants (Wilkins et al. 2000).

We introduced a synthetic *cry2AX1* gene into cotton plants (Coker310) through *Agrobacterium* mediated transformation to evaluate its efficacy against cotton bollworm, *H. armigera*. Six month old friable embryogenic calli that were cocultivated with the *Agrobacterium* strain LBA4404 (p2300-tp*2AX1*) construct proliferated into somatic embryos, whereas no proliferation was observed in non-transformed calli. Transformation of cotton with the synthetic *cry2AX1* gene (consisting of sequences of *cry2Aa* and *cry2Ac* genes) resulted in generation of 48 putative transgenic plants.

In PCR analysis carried out with gene specific primers to confirm the presence of transgenes in putative transgenic plants, 40 plants were found to possess *cry2AX1* and *npt*IIgenes (Figure 3a, b). No amplification was observed in non-transformed control plants. Southern blot hybridisation analysis of *cry2AX1* transformants indicated stable integration of the *cry2AX1* gene in the genome of transgenic cotton plants, whereas the untransformed control plant did not show any sign of hybridisation (Figure 4).

The selected PCR positive transgenic plants were subjected to ELISA for quantification of the insecticidal Cry2AX1 protein. Thirteen out of 40 PCR positive plants were found to be positive for expression of the Cry2AX1 protein. However, we observed a wide variation of expression in the transgenic lines developed, ranging from 4.9 to 187.5 ng g^{-1} of fresh leaf tissue (Table 1). A wide range of Bt protein expression in transgenic plants with the same genetic background and gene construct were reported by several earlier studies (Maqbool et al. 2001, Breitler et al. 2000, Ramesh et al. 2004,

Table 1. Bioassay on putative T_0 transgenic cotton lines against *Helicoverpa armigera*

S. No	Lines	**Concentration of Cry2AX1 protein** (ng $g^{-1}$of fresh leaf tissue)* (Mean ± SD)	**Larval mortality** (%)# (Mean ± SD)
1	TP12	6.60 ± 0.0	NT
2	TP13	9.90 ± 0.0	NT
3	TP15	49.0 ± 0.0	33.33±4.71 $(35.22)^{ef}$
4	TP16	16.6 ± 0.0	23.33±4.71 $(28.78)^{g}$
5	TP25	94.9 ± 0.0	46.66±4.71 $(43.08)^{c}$
6	TP30	23.0 ± 0.0	26.66±4.71 $(30.99)^{fg}$
7	TP31	187.5 ± 0.0	56.66±4.71 $(48.85)^{b}$
8	TP33	54.0 ± 0.0	43.33±4.71 $(41.15)^{cd}$
9	TP34	4.90 ± 0.0	NT
10	TP35	17.0 ± 1.41	26.66± 4.71 $(30.99)^{fg}$
11	TP39	61.0 ± 11.31	43.33±4.71 $(41.15)^{cd}$
12	TP53	49.0 ± 0.0	36.66±4.71 $(37.22)^{de}$
13	TP58	32.0 ± 1.41	30.0± 0.0 $(33.21)^{efg}$
14	Control (Non-transformed plant)	0.0 ± 0.0	0.0 ± 0.0 $(0.91)^{h}$
15	BGII (Positive control)	27000.0 ± 0.0	100.0 ± 0.0 $(89.09)^{a}$

* Mean of two replicates; # Mean of three replicates; NT – Not tested; Figures in parentheses are arcsine transformed values; Standard Error of Deviation SEd = 2.50; Critical Difference CD (0.05) = 5.16; Coefficient of Variation CV% = 7.98; Means followed by the same small letters within a column are not significantly different at the 5% level in Duncan's multiple range test (DMRT)

Meiyalaghan et al. 2006). We reason that the variation in the level of transgene expression or lack of expression may be due to mutations in the transgene, truncation of T-DNA during integration, post transcriptional gene silencing, or transcriptional gene silencing (integration of T-DNA into genomic regions such as the heterochromatin that repress transgene expression) (Francis and Spiker 2005). Inactivation of the transgene is often shown to be accompanied by an increase in DNA methylation (Amasino et al. 1984). Inactivation also very frequently correlates with the number of copies of integrated transgenes (Jones et al. 1987).

Insect bioassay of *cry2AX1* transgenic cotton plants

Laboratory biotoxicity assays with the first instar *H. armigera* larvae were conducted to analyse the efficacy of Cry2AX1 protein in cotton transformants. The results of leaf disc bioassay studies showed larval mortality ranging from 23.33 to 56.66 % in the selected ELISA positive plants (Table 1). The surviving larvae on transgenic plants showed severe growth inhibition and significant reduction in leaf feeding, whereas larvae released on control plants were alive with normal growth (Figure 5). Earlier reports suggest that the protein concentration is directly related to the level of insect resistance in Bt transgenic plants (Chen et al. 2005, Rashid et al. 2008, Mehrotra et al. 2011). Differences in the level of toxicity (mortality) observed among the different transgenic lines could be attributed to differences in the level of Bt gene expression. Variation of a single amino acid can also significantly influence the level of toxicity in Cry proteins (Udayasuriyan et al. 1994, Rajamohan et al. 1996).

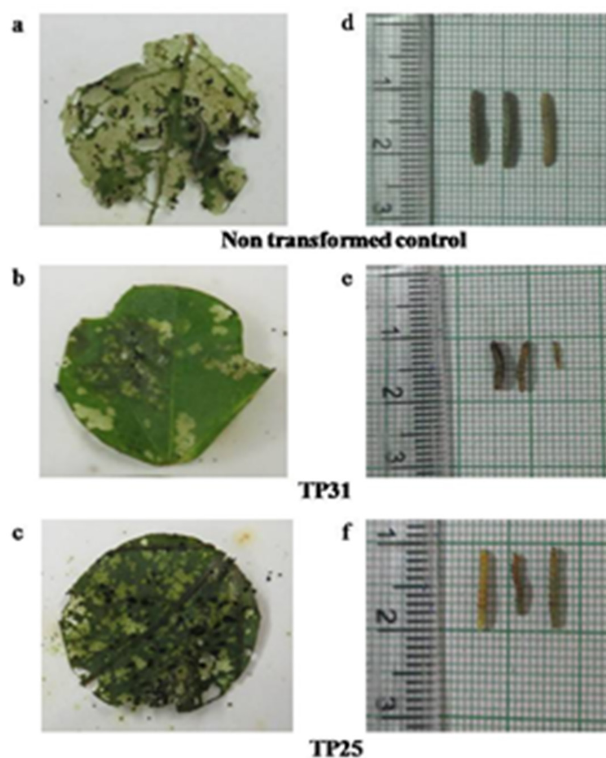

Figure 5. Detached leaf disc bioassay against cotton boll worm (*Helicoverpa armigera*) in transgenic cotton plants expressing the Cry2AX1 protein. a. Non-transformed control (Coker310), b. Transformed cotton plant TP31, c. Transformed cotton plant TP25; d, e, f. Surviving larvae on control and transformed plants.

The Cry2Aa protein is known to be toxic to prominent lepidopteran insect pests like rice stem borers and brinjal fruit and shoot borer (Maqbool et al. 1998, Rao et al. 1999). Pesticidal activity of *B. thuringiensis* δ-endotoxins, Cry1Aa, Cry1Ab, Cry1Ac, and Cry2A against *Helicoverpa zea* was determined by Karim et al. (2000). *H. zea* was susceptible to Bt toxins in the order Cry1Ac, Cry1Ab, Cry1Aa, and Cry2A with 63.60, 89.04, 159.65, and 375.78 ng/larvae, respectively. Indian populations of *H. armigera* were 5- to 30-fold less susceptible to *cry2Aa* than *cry1Ac* (Chakrabarti et al. 1998, Babu et al. 2002, Misra et al. 2002). The expression level of Cry2Ab in commercialized Bt cotton ranges from 16.8 to 22.7 μg g^{-1} in fresh leaf tissue (Li et al. 2011). These findings indicated that a greater amount of Cry2AX1 protein expression may be needed to achieve the desirable level of insecticidal activity in plants.

In the current investigation, an attempt was made to study the insecticidal activity of the *cry2AX1* gene against *H. armigera* in cotton. The *H. armigera* neonates showed mortality up to 56.66% in the transgenic plants, even with a relatively lower level of Cry2AX1 protein in the plant (about 187.5 ng g^{-1} of leaf tissue). The expression of a higher level of Cry2AX1 protein and high insecticidal activity can possibly be achieved by generating or screening more transgenic plants. A transgenic plant carrying the *cry2AX1* gene with higher insecticidal activity could be an alternative management strategy for *H. armigera* in cotton.

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