



Efficacy of *cry2AX1* Gene in Transgenic Tomato Plants against *Helicoverpa armigera* and *Spodoptera litura*

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Tomato crop is infested by large number of insect pests, causing significant yield loss. Fruit borer (*Helicoverpa armigera*) feeds on fruits which leads to quality deterioration and economic loss. Genetic engineering is recognised as a viable tool to engineer resistance in crops against insect pests. Transgenic tomato plants over-expressing *cry2AX1* gene under the control of *CaMV35S* promoter were developed. The T₀ and T₁ plants were characterized by PCR for the presence and segregation of transgenes. Segregation of *cry2AX1* gene in T₁ progeny plants followed the Mendelian pattern of monogenic inheritance of 3:1 ratio. The expression of *Cry2AX1* at 55 days after sowing ranged from 0.034 to 0.219 µg/g of fresh leaf tissue and 0.030 to 0.249 µg/g of fresh leaf tissue at 80 days after sowing. Insect bioassay of detached leaf bits performed on the ELISA positive transgenic tomato plants against *Helicoverpa armigera* and *Spodoptera litura* resulted in mortality of neonates upto 100 per cent in *H. armigera* and 53 per cent in *S. litura*.

Key words: *cry2AX1* - Transgenic tomato - Insect resistance - Fruit borer

Tomato (*Lycopersicon esculentum* L.) is an economically important vegetable crop next to potato (Mamidala and Nanna, 2011). Tomato is attacked by a host of pathogens namely, viruses, viroids, fungi, bacteria, nematodes, parasite weeds, and insect pests (Jones *et al.*, 1997). Among the insects, fruit borer (*Helicoverpa armigera* Hubner), leaf eating worm (*Spodoptera litura*), and leaf miner (*Tuta absoluta*) cause severe damage leading to yield loss in tomato (Fitt 1989). *H. armigera* larvae attacks tomato from transplanting stage to fruit maturity. The pest is prevalent practically throughout the year and has developed resistance against chemical pesticides (Kranthi *et al.*, 2001; Tabashnik and Carriere, 2010).

Bt genes such as *cry1Ac*, *cry2Ab* and *cry1F* derived from *Bacillus thuringiensis*, a soil bacterium, has been known to offer protection against lepidopteran insect pests. Transgene plants were successfully developed introducing *cry* genes into corn, cotton, soybean, rice, potato and canola (Rahman *et al.*, 2015). Insecticidal *cry1Ab* gene expressing tomato was found to confer protection against tomato fruit worm (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987). Saker *et al.* (2011) generated transgenic tomato plants expressing *cry2Ab* gene conferring resistance against lepidopteron insects *viz.*, *Spodoptera littoralis*, *Helicoverpa armigera*, *Phthorimaea operculella*. A chimeric gene, *cry2AX1* was constructed in Tamil Nadu Agricultural University, Coimbatore, India, using the sequences of *cry2Aa* and *cry2Ac*, cloned from Indigenous strains of *Bt*. The *Cry2AX1* protein isolated from recombinant bacterium was found to be more effective against *H. armigera* than

Cry2Aa, *Cry2Ab* and *Cry2Ac* proteins (Udayasuriyan *et al.*, 2010). Raturaj *et al.* (2014) and Bamishaiye *et al.* (2017b) generated transgenic tomato plants expressing *cry2AX1* in tomato cv. PKM1 resistant to fruit borer.

In this present study, we developed transgenic tomato plants over expressing *cry2AX1* gene through *Agrobacterium*-mediated transformation. Insect bioassay on transgenic tomato expressing *cry2AX1* gene showed resistance against two major pest *viz.*, *Helicoverpa armigera* and *Spodoptera litura*.

Material and Methods

Agrobacterium-mediated transformation

Tomato transformation was carried out with a binary vector (pC2300-*tp2AX1*) harbouring a codon optimized 1902 bp long synthetic *cry2AX1* gene (consisting sequences from *cry2Aa* and *cry2Ac*; Acc. No. GQ332539.1) fused with a cotton chloroplast transit peptide sequence (186 bp; Acc. No. JN608790.1) under the control of double enhancer version of *CaMV35S* promoter and *nos* terminator. The construct was maintained in *E.coli* and also in *Agrobacterium* strain LBA4404. Genetically pure seeds of tomato cv. PKM1 were obtained from Horticultural College and Research Institute, Tamil Nadu Agricultural University, Periyakulam, Tamil Nadu. Tomato cultivar PKM1 was aseptically grown and cotyledons were used as explants for transformation (Bamishaiye *et al.*, 2017a). T₀ and T₁ progenies of two events, PKM1-25 and PKM1-26, expressing *Cry2AX1* were characterized by PCR and ELISA.

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Molecular characterization of tomato transgenic events

Plant genomic DNA was isolated from transgenic plants and wild type control plants using a modified CTAB method (Doyle and Doyle, 1987). The presence of transgene was confirmed by *cry2AX1* specific primers (Forward primer 5'-CCTAACATTGGTGGACTTCCAG-3' and Reverse primer 5'-GAGAAACGAGCTCCGTTATCGT-3') and *nptII* specific gene primers (Forward primer 5'-AGAACTCGTCAAGAAGGCGA-3' and Reverse primer 5'-CAGACAATCGGCTGCTCTGA-3'). The PCR products were subjected to electrophoresis in 0.8 % agarose gel, visualized and documented in gel documentation system.

ELISA

A double-antibody sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) was used to detect the level of Cry2AX1 protein expression in the leaves of transgenic tomato plants. Cry2A quantiplate (Envirologix, USA) ELISA kit was used for this experiment. T₁ progenies which showed the presence of *cry2AX1* and *nptII* were subjected to ELISA. ELISA was performed in two stages of plant growth (i.e) on 55 days after sowing (DAS) and 80 DAS.

Insect bioassay

Leaf disc bioassay was carried out to determine the efficacy of insect resistance in ELISA positive T₁

transgenic tomato plants with *H. armigera* and *S. litura* neonates under laboratory condition. Leaf bits of 1.4 cm diameter and 2.0 cm diameter for *H. armigera* and *S. litura*, respectively, from both the transgenic and non-transgenic control plants were placed in a damp filter paper on Petri plates. Ten neonate larvae of *H. armigera* were released per replication and three replications were maintained for each line. In case of *H. armigera* bioassay, only one larva was released per leaf disc in a Petri plate whereas in *S. litura*, five larvae per leaf disc per plate were released. New leaf disc was placed in Petri plate, when the leaf disc was fully consumed by the larva(e). The experiment was carried out at 24-26°C and 60 % RH. Larval mortality was recorded after 48 hours at 24 hours interval for six days. Data on Cry2AX1 expression and mortality rate of *H. armigera* from three replicates were recorded. The data were subjected to arcsine transformations before analysis, followed by analysis of variance (ANOVA). Mean values were separated by Duncan's multiple range test (DMRT) at 5 % probability level (Duncan, 1955).

Results and Discussion

Two T₀ transformants viz., T₀-PKM1-25 and T₀-PKM1-26 showed the presence of *cry2AX1* transgene through PCR. An expected amplicon size of 800 bp fragments for *cry2AX1* gene and 430 bp fragments for *nptII* gene were observed (data not shown). Subsequent PCR analysis of T₁ progeny of the above events is presented in the Table 1.

Table 1. Segregation analysis of *cry2AX1/nptII* gene in T₁ transgenic plants

Transgenic (T ₁) line	No. of T ₁ plants analysed	PCR		Calculated Chi-square value
		Positive	Negative	
T ₁ -PKM1-25	30	20	10	1.13
T ₁ -PKM1-26	30	25	5	1.103

Critical value of chi-square for p=0.01 is 2.706. The calculated value is less than 2.706 and therefore fit to the expected ratio 3:1

PCR positive T₁ progenies of PKM1-25 and PKM1-26 were analysed for the expression of Cry2AX1 protein. At 55 DAS, the Cry2AX1 protein concentration ranged from 0.034 to 0.219 µg/g of fresh leaf tissue where as at 80 DAS, the expression ranged from 0.030 to 0.249 µg/g of fresh leaf tissue.

Insect bioassay was carried out on ELISA positive T₁ progenies of PKM1-25 and PKM1-26. The mortality of neonates on T₁ plants ranged from 16.66 to 93.33 and 16.66 to 100 per cent at 58 and 84 DAS, respectively against *H. armigera*. Whereas, in *S. litura*, mortality of 16.66 to 50.00 and 10.00 to 53.33 per cent at 58 and 84 DAS, respectively was observed (Table 2). The feeding was higher in control plant leaf discs (5 larvae fed 2 leaf discs i.e., 580 mm²). Control plants showed no mortality. Growth inhibition was observed in surviving larvae as well as reduced feeding in the ELISA positive plants. However, larvae released on control plants were alive with normal growth (Figure 1 and 2).

Prior studies from genetic engineering of tomato with *cry1Ab* or *cry1Ac* genes reported significant level

of protection against tomato fruit worm (*Heliothis zea*), tobacco hornworm (*Manduca sexta*) and tomato fruit borer (*Helicoverpa armigera*) (Fischhoff *et al.*, 1987; Delannay *et al.*, 1989; Mandaokar *et al.*, 2000; Kumar and Kumar, 2004). The present study was carried out to demonstrate the expression of Cry2AX1 protein in T₁ progenies. In PCR analysis, out of thirty T₁ progeny plants analyzed in each of the two events, PKM1-25 and PKM1-26, twenty and twenty five plants, respectively were positive for the presence of *cry2AX1* and *nptII*. The segregation of *cry2AX1* among the T₁ progenies followed Mendelian the 3:1 monogenic inheritance ratio in both the events, suggesting a single copy integration of *cry2AX1* in the genome.

Among the PCR characterized T₁ progenies, wide variation was observed in Cry2AX1 expression. The following are the high Cry2AX1 expressing progenies viz., T₁-PKM1-25-1, T₁-PKM1-25-2, T₁-PKM1-25-11, and T₁-PKM1-25-13 at 80 DAS. Of all, T₁-PKM1-25-2 showed significantly higher level of Cry2AX1 expression at 0.219 and 0.249 µg/g of leaf tissue at 55 DAS and 80 DAS, respectively.

Table 2. Expression of Cry2AX1 protein and larval mortality of *Helicoverpa armigera* and *Spodoptera litura* in selected T₁ progenies

Transgenic plants	Cry2AX1 protein concentration (µg/g fresh leaf tissue) Mean ± SD 55 DAS	Larval mortality (%) of <i>H. armigera</i> Mean ± SD 58 DAS	Larval mortality (%) of <i>S. litura</i> Mean ± SD 58 DAS	Cry2AX1 protein concentration (µg/g fresh leaf tissue) Mean ± SD 80 DAS	Larval mortality (%) of <i>H. armigera</i> Mean ± SD 84 DAS	Larval mortality (%) of <i>S. litura</i> Mean ± SD 84 DAS
High expression lines						
T ₁ -PKM1-25-1	0.136 ± 0.01	73.33 ± 5.77 (59.00) ^c	43.33 ± 5.77 (41.15) ^c	0.195 ± 0.01	83.33 ± 5.77 (66.14) ^c	46.66 ± 5.77 (43.07) ^c
T ₁ -PKM1-25-2	0.219 ± 0.09	93.33 ± 5.77 (77.51) ^a	50.00 ± 5.77 (45.00) ^a	0.249 ± 0.22	100 ± 0.00 (89.41) ^a	53.33 ± 5.77 (46.92) ^a
T ₁ -PKM1-25-11	0.176 ± 0.07	83.33 ± 5.77 (66.14) ^b	46.66 ± 5.77 (43.07) ^b	0.168 ± 0.07	73.33 ± 5.77 (63.93) ^d	43.33 ± 0.00 (41.15) ^d
T ₁ -PKM1-25-13	0.115 ± 0.06	63.33 ± 5.77 (52.77) ^d	40.00 ± 0.00 (39.23) ^d	0.202 ± 0.05	83.33 ± 5.77 (68.85) ^b	50.00 ± 5.77 (45.00) ^b
Low expression lines						
T ₁ -PKM1-25-6	0.053 ± 0.01	30.00 ± 0.00 (33.21) ^e	16.67 ± 0.00 (26.56) ^e	0.062 ± 0.03	43.33 ± 5.77 (41.15) ^e	16.67 ± 5.77 (26.56) ^e
T ₁ -PKM1-26-4	0.034 ± 0.04	16.66 ± 5.77 (23.85) ^g	16.66 ± 5.77 (23.85) ^g	0.030 ± 0.00	16.66 ± 5.77 (23.85) ^g	10.00 ± 0.00 (18.43) ^g
T ₁ -PKM1-26-5	0.044 ± 0.02	20.00 ± 5.77 (23.85) ^f	26.66 ± 5.77 (26.96) ^f	0.034 ± 0.00	16.66 ± 5.77 (23.85) ^f	20.00 ± 0.00 (26.56) ^f
PKM1control	0.005 ± 0.00	0.00 ± 0.00 (0.58) ^h	0.00 ± 0.00 (0.55) ^h	0.00 ± 0.00	0.00 ± 0.00 (0.58) ^h	0.00 ± 0.00 (0.55) ^h
SEd		4.07	1.80		3.58	2.41
CD (0.05)		8.63	3.80		7.59	5.08

Figures in parentheses are arc sine transformed values.

The bioassay data presented in the above table was restricted to a few selected high and low expressing T₁ progenies of T₁-PKM1-25 and T₁-PKM1-26.

Towards studying the efficacy of Cry2AX1 toxin, detached leaf bioassay against *H. armigera* neonate larvae and *S. litura* at 58 DAS and 84 DAS showed

effective resistance in some of the high Cry2AX1 expression lines. The larval mortality was observed from 16.66 to 93.33 per cent at 58 DAS and 16.66

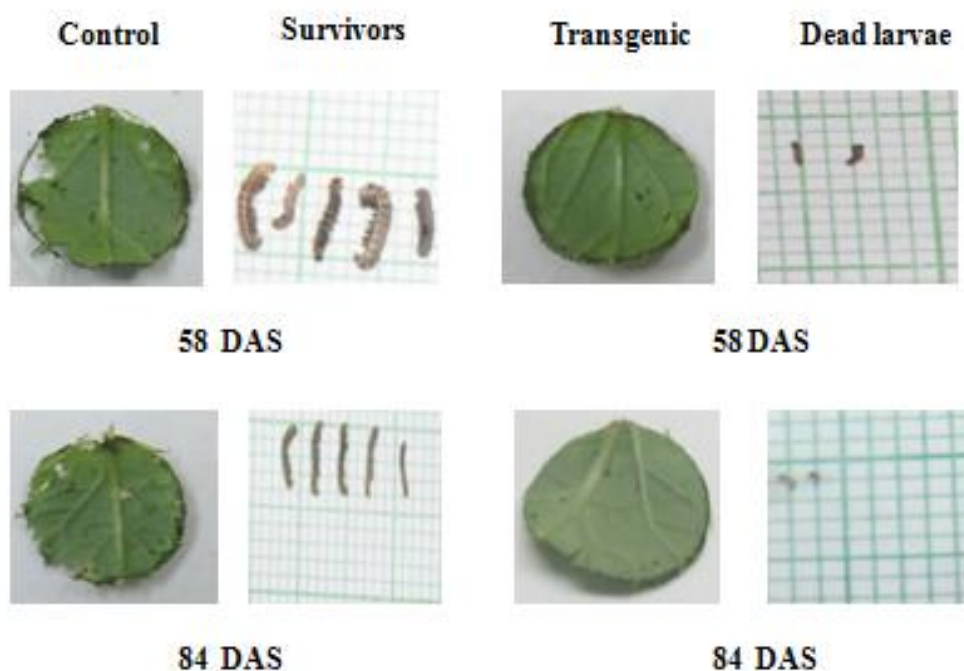


Fig. 1. Insect bioassay on transgenic tomato plant (T₁-PKM1-25-2) against *Helicoverpa armigera*

to 100 per cent at 84 DAS against *H. armigera*. Similarly, *S. litura* mortality ranged from 16.66 to 50.00 per cent at 58 DAS and 10.00 to 53.33 percent at 84 DAS. Inhibition in growth of the surviving larvae

and a larger damaged leaf area were observed in selected T₁ progeny plants with higher levels of Cry2AX1 expression as per ELISA. Thus, a positive correlation was observed between the level of

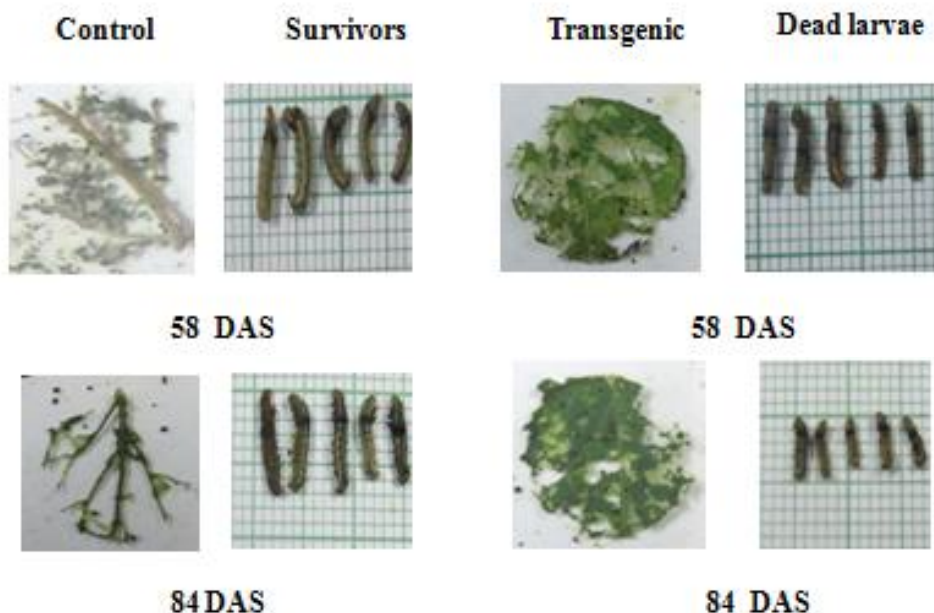


Fig 2. Insect bioassay on transgenic tomato plant (T1-PKM1-25-2) against *Spodoptera litura*

Cry2AX1 expression and mortality of *H. armigera* and *S. litura*. Similar correlation between the expression level of Cry protein and insect mortality was earlier reported (Bhattachary *et al.*, 2002; Manikandan *et al.*, 2014). The progeny plant, T₁-PKM1-25-2 had shown a relatively higher level of Cry2AX1 expression as well as an increased percentage of mortality against the two major pests subjected to bioassay. Still, there is a necessity to generate and evaluate additional transformants expressing this gene so as to identify a promising tomato transformant in future.

Conclusion

Transgenic tomato plants were developed with *cry2AX1* gene to impart insect resistance. T₁ progenies of PKM1-25 and PKM1-26 were subjected to segregation analysis and presence of *cry2AX1* and *nptII* was confirmed by PCR using gene specific primers. Segregation pattern followed the Mendelian ratio of 3:1 with monogenic inheritance of *cry2AX1* gene. The expression of the Cry2AX1 protein were analysed by ELISA at two different stages of plant growth (55 DAS and 80 DAS). Insect bioassay was performed and results indicated that the transgenic tomato plants were resistant against *Helicoverpa armigera* and *Spodoptera litura*.

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