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Genetic Manipulation of Tomato (*Solanum lycopersicum*) cv. PKM-1 using *cry2AX1* Gene for Insect Resistance

E. I. Bamishaiye, S. Varanavasiappan, N. Balakrishnan, V. Udayasuriyan and D. Sudhakar*

Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu, India
**Corresponding author*

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A B S T R A C T

Cotyledonary explants of tomato cv. PKM-1 were co-cultivated with *Agrobacterium tumefaciens* strain, LBA4404 harbouring a codon-optimised chimeric *cry2AX1* gene driven by enhanced *CaMV35S* promoter in pCAMBIA2300 vector backbone. Ninety six putative transgenic plants were regenerated, and the presence of the *cry2AX1* gene in fifty eight plants was demonstrated by PCR analysis. ELISA showed that nine out of the fifty eight plants had detectable level of Cry2AX1 protein expression, which ranged from 0.030 to 0.388 µg/g of fresh tissue. Insect bioassay of transgenic T_0 tomato plants using *H. armigera* neonates recorded a mortality of 16.67 to 100 per cent and showed significant reduction in leaf feeding and inhibition of growth in surviving larvae. The results demonstrated the potential of the chimeric *cry2AX1* gene in developing *H. armigera* resistant transgenic tomato varieties.

Introduction

Tomato (*Solanum lycopersicum*) is one of the world's most preferred vegetable crops. The growth and productivity of tomato crop is often hampered by various biotic as well as abiotic stress factors which results in yield reduction and poor quality of fruits. One of the major limiting factors, which affect tomato production worldwide, is the incidence of pests, and they often infest at different stages of growth and development (Kumar *et al.*, 2017). Some of the invading insect pests include tomato fruit borer (*Helicoverpa armigera*), jassid (*Amrasca biguttula biguttula*), whitefly (*Bemisia tabaci*), mite (*Tetranychus urticae*), aphid (*Myzus persicae*) and leaf miner (*Liriomyza trifolic*). Among all, tomato fruit borer, *Helicoverpa armigera* (Hub.) is an important lepidopteran pest responsible for severe yield losses as it infests fruits (Tewari and Moorthy, 1984;, Kumar and Kumar, 2004) and makes them unfit for consumption thereby leading to massive loss (Sharma *et al.*, 2013). Application of pesticides is an option and indiscriminate use of pesticides results in adverse effects on the beneficial organisms, leading to resurgence and secondary outbreak of pest besides leaving

residue in the edible fruit. Fast changes in climate and consumers' needs as well as the emergence of new plant pests and diseases require continuous development of improved genotypes that can withstand the changes (Anderson *et al.*, 2004, Cardi *et al.*, 2017). In recent years, biotechnological applications have been integrated into crop breeding systems, and it offers many opportunities in developing transgenic plants with improved agronomic traits.

Genetic engineering is one of the biotechnological techniques that is commonly adapted for development of insect resistant crops by the insertion of a gene from *Bacillus thuringiensis* (*Bt*) that produces Cry proteins which are toxic to certain insect (*Lepidoptera*, *Coleoptera* and *Diptera*) families. These insecticidal crystal proteins selectively bind to insect midgut receptors and insert into the gut cell membrane, thereby causing the formation of pores in membrane leading to cell lysis and insect death (Knowles and Dow, 1993). *Agrobacterium*-mediated gene transfer method has been used successfully for transformation of numerous dicot species (Jadav *et al.*, 2015).

Tomato engineered with different insecticidal protein gene(s) from *Bt* has been reported to provide adequate protection to plants against different lepidopteran insect pests (Selale *et al.*, 2017; Koul *et al.*, 2014). Due to the difference in structure and insecticidal activities, *cry2A* genes are suitable candidates for the management of insects in crop plants (Jain *et al.*, 2006). The chimeric Cry2AX1 protein was observed to be more lethal than its parental proteins (Cry2Aa and Cry2Ac) (Udayasuriyan *et al.*, 2010). Considering these facts, the present study was conducted to develop transgenic tomato plants expressing *cry2AX1* gene, using *Agrobacterium* mediated transformation method and to evaluate the efficacy of *cry2AX1* expressed in tomato events against *H. armigera.*

Materials and Methods

Preparation of explants

Genetically pure seeds of tomato cv. PKM1 were obtained from Horticultural College and Research Institute, Tamil Nadu Agricultural University, Periyakulam, Tamil Nadu. Tomato seeds were pre-soaked in water containing a few drops of tween 20 for five min and subjected to vigorous shaking for another 5 min. Seeds were treated with seventy per cent ethanol for 4-5 min, followed by washing with sterile water thrice. The seeds were then treated with 4 per cent sodium hypochlorite for 7 min with occasional swirling followed by doubled distilled water wash thrice. The seeds were blot dried on a sterile tissue paper and placed on half strength MS medium (Murashige and Skoog, 1962) for germination under 72 hours dark period followed by a cycle of 16 hours photoperiod using cool white fluorescent tube light (110-130 $nM/m^2/s$ intensity) and eight hours of darkness at 26˚C in a plant growth chamber (Panasonic, Japan). Cotyledonary explants were collected from 7 day-old *in vitro* grown seedlings (Figure 3A). The distal and proximal ends (1-2 mm) were cut off and the explants were cut into two pieces (Figure 3B) before placing them on the pre-culture medium (MS medium modified with Gamborg B_5 vitamins containing 1 mg/L zeatin) (Ruturaj *et al.*, 2014). They were handled gently with flat forceps to avoid any injury. They were pre-cultured for a day under light prior to co-cultivation.

Binary vectors and strain

The pC2300-*2AX1* construct containing *cry2AX1* gene (Jayaprakash, 2011; Figure 1a) and pC2300- *ctp2AX1* containing *cry2AX1* in

fusion with chloroplast transit peptide sequence (Nandeesha, 2012; Figure 1b) were used in transformation experiments. The gene of interest was placed under the control of *EnCaMV35S* promoter and *nos-polyA* terminator in binary vector, pCAMBIA 2300 backbone. Both vectors harbored the neomycin phosphotransferase (*nptII*) gene driven by CaMV35S promoter, conferring resistance to kanamycin which was used for plant selection. The constructs, independently, were mobilized into *Agrobacterium* strain LBA4404 by triparental mating and the *Agrobacterium* transconjugants were grown on LB (1% Yeast extract, 1% Peptone and 0.5% NaCl pH 7.2) medium containing kanamycin 100 mg/L, tetracycline 5 mg/L and 10 mg/L rifampicin in a 28°C incubator shaker at 200 rpm for 48-72 hours and used for tomato transformation.

Agrobacterium-mediated transformation of tomato

A single colony of *Agrobacterium*, containing LBA4404 (pC2300-*2AX1)* or LBA4404 (pC2300-*ctp2AX1*) gene was inoculated in sterile 3 mL LB medium (Yeast extract, Peptone and NaCl) containing kanamycin 100 mg/L, tetracycline 5 mg/L, and rifampicin 10 mg/ml, was allowed to grow overnight in an incubator shaker at 28°C and 180 rpm. From the overnight culture, an aliquot of 500 µl was inoculated into 30 ml LB with same antibiotics and conditions and grown for 6-8 hours. The bacterial pellets were harvested by centrifugation at 4,000 rpm for 10 min. The pellet was re-suspended in 30 ml of infection medium (modified MS containing 100 µM acetosyringone). *Agrobacterium* density in the suspension was maintained at 0.4 OD at 600 nm. The pre-cultured explants were carefully submerged in the suspension in a sterile petri plate for an infection time of 30 min with gentle agitation. Post infection, explants were blotted on sterile tissue paper and transferred onto co-cultivation medium (MS medium modified with Gamborg vitamins containing zeatin 1 mg/L and 100 µM acetosyringone). Plates were kept under dark condition in a growth chamber for 48 hours.

Regeneration of the transformed explants

After the co-cultivation period, the explants were washed in a washing medium (modified MS containing 200 mg/L timentin) to kill the *Agrobacterium*, blot dried and sub-cultured on selection medium (MS modified with Gamborg vitamins containing kanamycin 100 mg/L, zeatin 1 mg/L and 200 mg/L timentin) for shoot bud initiation and were maintained under 8/16 dark light cycle. The explants that responded well (Figure 3C) were sub-cultured continuously onto fresh medium, and later transferred to shoot elongation medium (MS modified with Gamborg vitamins containing kanamycin 100 mg/L, zeatin 0.5 mg/L and 200 mg/L timentin) for elongation. Elongated shoots (2-3 cm) (Figure 3D) were transferred to the half MS basic medium, supplemented with IBA (1 mg/L) for rooting and maintained under 16 hours light and 8 hours dark. Shoots with no sign of rooting after 21 days were discarded. Well rooted plants (Figure 3E) were hardened in transgenic greenhouse in small cups containing autoclaved coconut peat mixture covered with a polythene cover to maintain humidity. Well-established plants were transplanted into bigger pots and maintained in transgenic greenhouse (Figure 3F).

Molecular characterization of transformants

DNA isolation and PCR analysis

Genomic DNA was isolated from leaves of putative transgenic and non-transgenic tomato plants using CTAB (cetyltrimethyl ammonium bromide) protocol, which is a

modification of the method of Doyle and Doyle (1987). Presence of transgene was confirmed by using *cry2AX1* gene specific primers (Forward 5'CCTAACATTGGTG GA CTTCCAG 3' and Reverse 5' GAGAAA CGAGCTCCGTTATCGT 3') and *nptII* gene specific primers (forward primer 5'-AGAACTCGTCAAGAAGGCGA and reverse primer 5'-CAGACAATCG GCTGCTCTGA). The plasmid DNA was used as positive control. The PCR for both sets of samples was carried out in 50 µl reaction volume containing, 2.5 µl of 10X *Taq* buffer, 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 1.5U of *Taq* DNA polymerase and incubated in a thermal cycler which was programmed for 5 min preheat at 94°C and then 35 cycles of denaturation at 94°C for 1 min, annealing temperature of 58°C for 45 sec and extension time of 45 sec at 72°C, with a final extension at 72°C for 7 min. The PCR products were run on 0.8 % agarose gel, visualized and documented in gel documentation system.

ELISA Analysis

A double-antibody sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) was used to detect and quantify the Cry2AX1 protein expressed in the leaves of transgenic tomato plants. Cry2A quanti-plate (Envirologix, USA) ELISA kit was used for the experiment. Proteins from fresh leaf samples of transformed and untransformed tomato plants were extracted and used for detection of Cry2AX1 protein as per the manufacturer's protocol.

The OD was measured at 450 nm using an ELISA reader (Biotek, USA). The quantity of Cry2AX1 protein present in the sample was calculated by referring to standard graph generated with Cry2A calibration standards and represented in µg/g fresh weight of leaves.

Insect Bioassay

Detached leaf bit bioassay was carried out to determine the level of insect resistance in ELISA positive T_0 transgenic tomato plants with *H. armigera* neonates under laboratory condition.

Leaf bits (1.4 cm diameter) from both the transgenic and 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 in each line.

The experiment was carried out at 27 ±1 ^{0}C and 65 per cent relative humidity. Larval mortality was recorded after 48 hours at 24 hours interval for six days.

Statistical analysis

The experimental data values of Cry2AX1 protein concentration and mortality of *H. armigera* were mean values from three replicates. 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

Plant regeneration

A total of ninety six putative transgenic tomato plants were regenerated out of eight hundred and fifty six cotyledons co-cultivated. Fifty four plants were generated with pC2300-*ctp-2AX1* construct and forty two plants with pC2300-*2AX1* construct. They are considered independent as they were derived from different co-cultivated explants.

PCR analysis

The putative T_0 transgenic tomato plants generated were screened for the presence of *cry2AX1* gene and *nptII* gene with gene specific primers targeting the coding region of *cry2AX1* and *nptII* gene, respectively. Thirty six out of the fifty four plants generated with pC2300-*2AX1* construct and twenty two out of forty two with pC2300-*2AX1* construct were found positive for PCR showing an amplification at the expected size of 430 bp fragment for *nptII* specific primers and 800 bp fragments for *cry2AX1* gene specific primers respectively (Figure 2a and 2b). No amplification was observed in non-transformed control plants.

ELISA Analysis

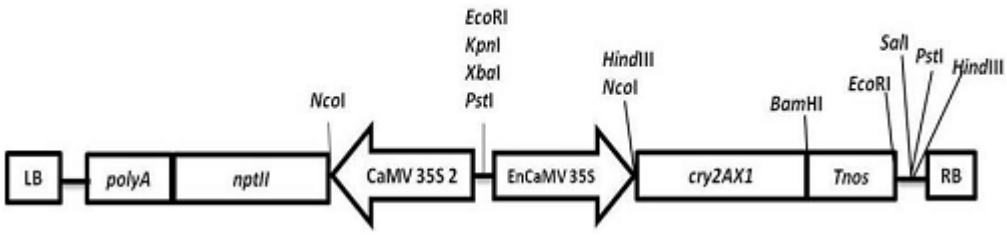
PCR positive plants were further analyzed for the quantification of the insecticidal Cry2AX1 protein by using quantitative ELISA Kit. Out of fifty eight plants tested, nine were found to be positive for the expression of *cry2AX1* and the concentration of Cry2AX1 protein ranged from 0.030 ± 0.01 to 0.388 ± 0.01 µg/g of fresh leaf tissue (Table 1).

Table.1 Quantitative ELISA and *H. armigera* bioassay on T_0 transformants of tomato

Lines	Concentration of Cry2AX1 protein (µg/g fresh leaf tissue) Mean ± SD	Larval mortality (%)
PKM 10	0.327 ± 0.02	90.00 (74.70)
PKM 20	0.058 ± 0.00	33.33 (35.21)
PKM 25	0.388 ± 0.01	100.00 (89.10)
PKM 26	0.071 ± 0.00	43.33 (41.15)
PKM 27	0.379 ± 0.01	100.00 (89.10)
PKM 32	0.231 ± 0.00	66.67 (54.99)
PKM 34	0.030 ± 0.01	16.67 (23.86)
PKM 35	0.048 ± 0.01	36.67 (37.22)
PKM 38	0.267 ± 0.03	40.00 (39.23)
Control		0.00 (0.90)
SEd		5.90
C.D (0.05)		12.18

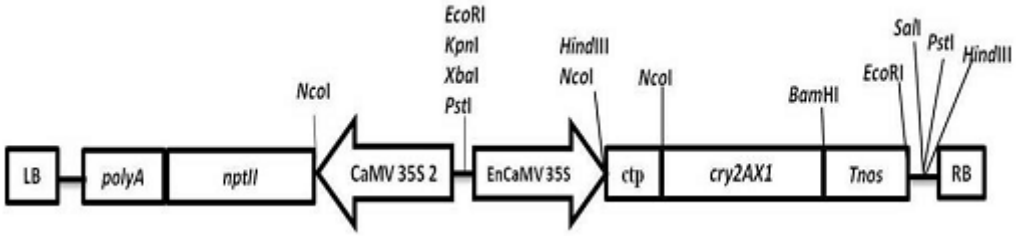
Figures in parentheses are arc sin transformed values.

Fig.1a Schematic representation of T-DNA region of the plant transformation construct pC2300-*2AX1*



LB: left border, poly A: CaMV35S terminator, *nptII*: neomycin phosphotransferase gene, CaMV35S2: Cauliflower mosaic virus 35S duplicated promoter, EnCaMV35S: Enhanced cauliflower mosaic virus 35S promoter, *cry2AX1*: gene of interest, *Tnos*: *nopaline synthase* terminator, RB: right border

Fig.1b Schematic representation of T-DNA region of the plant transformation construct pC2300-*ctp2AX1*



LB: left border, polyA: CaMV35S, *nptII*: neomycin phosphotransferase gene, CaMV35S2: Cauliflower mosaic virus 35S duplicated promoter, EnCaMV35S: Enhanced cauliflower mosaic virus 35S promoter, *ctp*: chloroplast transit peptide, *cry2AX1*: gene of interest, *Tnos*: *nopaline synthase* terminator; RB: right border

Fig.2a Screening of putative transformants of tomato for the presence of *nptII* gene. L: 1000 bp DNA ladder, Lane PC: positive control (pC2300- *En35S-2AX1*), Lane NC: negative control, Lane WC: water control, Lanes 4–13: putative transgenic plants

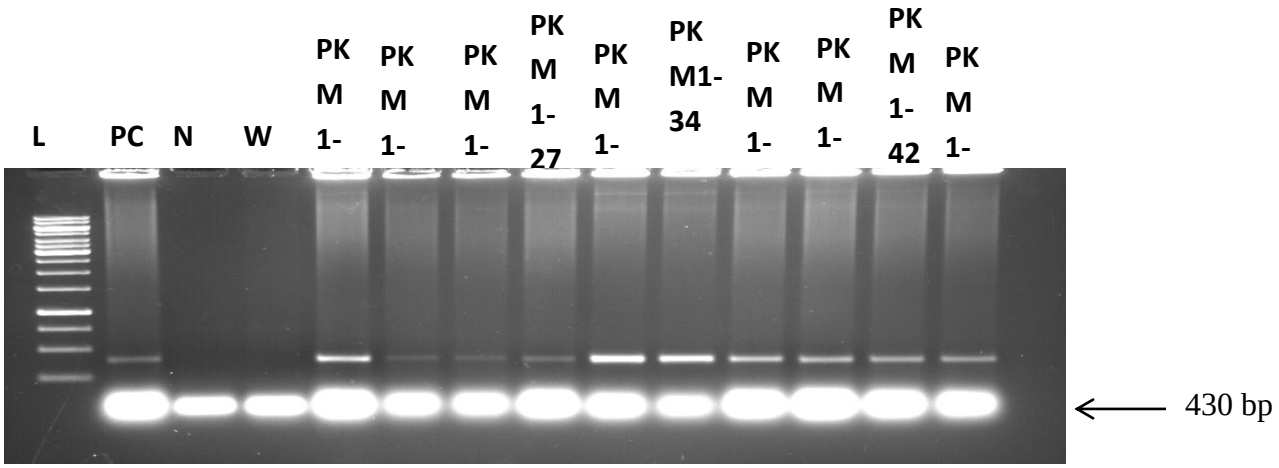


Fig.2b Screening of putative transformants of tomato for the presence of *cry2AX1* gene. L: 1000 bp DNA ladder, Lane PC: positive control (pC2300- *En35S-2AX1*), Lane NC: Negative control, Lane WC: water control, Lanes 4–13: putative transgenic plant

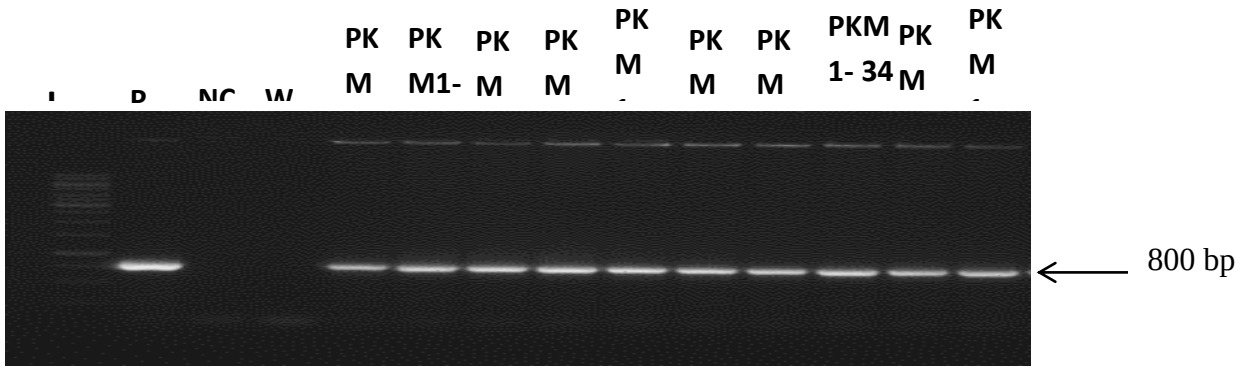


Fig.3 *Agrobacterium*-mediated transformation of tomato cv PKM-1 (A) *In vivo* grown seedlings on germination medium (B) Explants on pre-culture medium (C) Co-cultivated explants on selection medium at 3rd selection (D) Explants on selection medium at 4th selection (E) Elongated shoot on rooting medium (H) Well established transformant in transgenic greenhouse

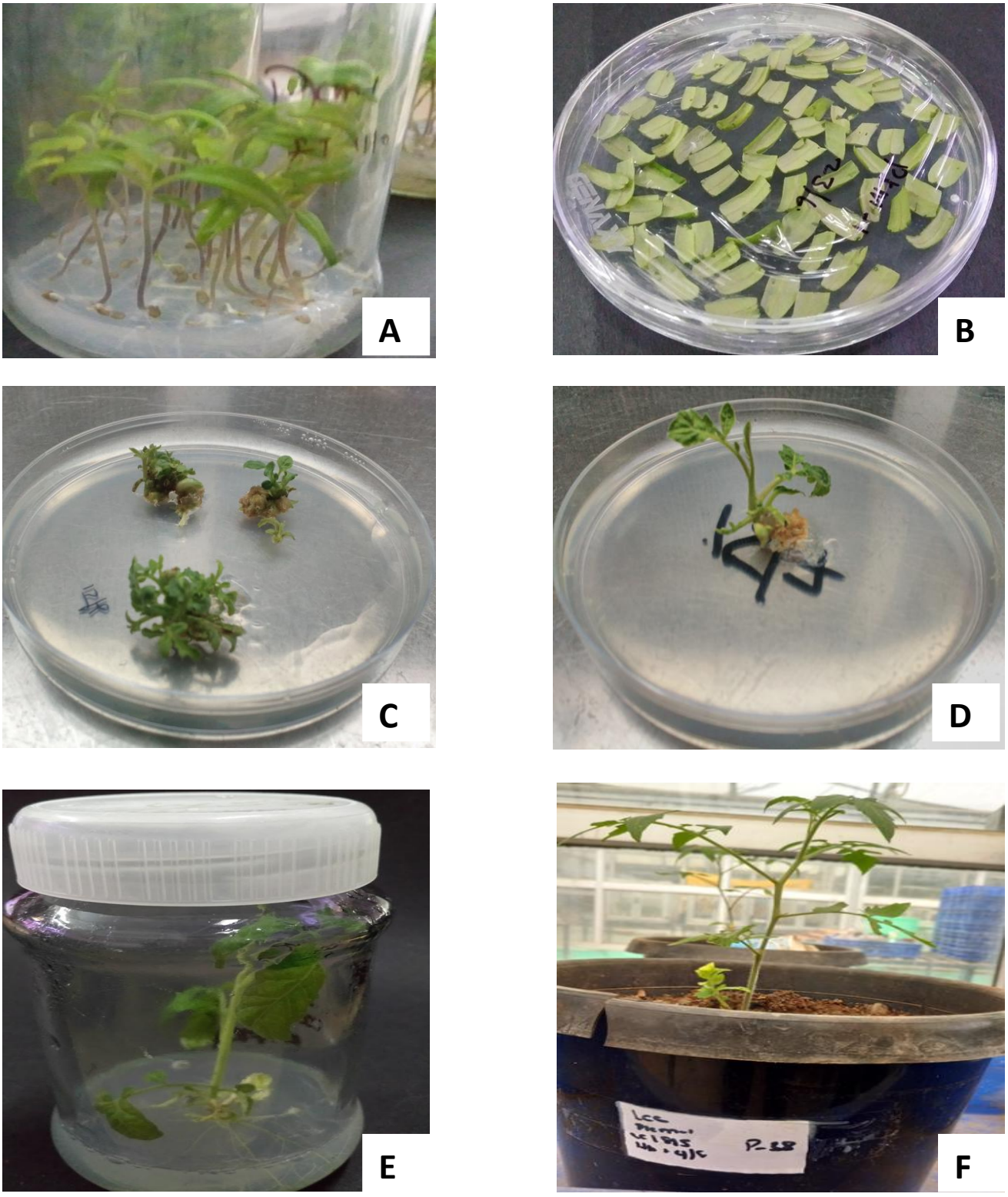
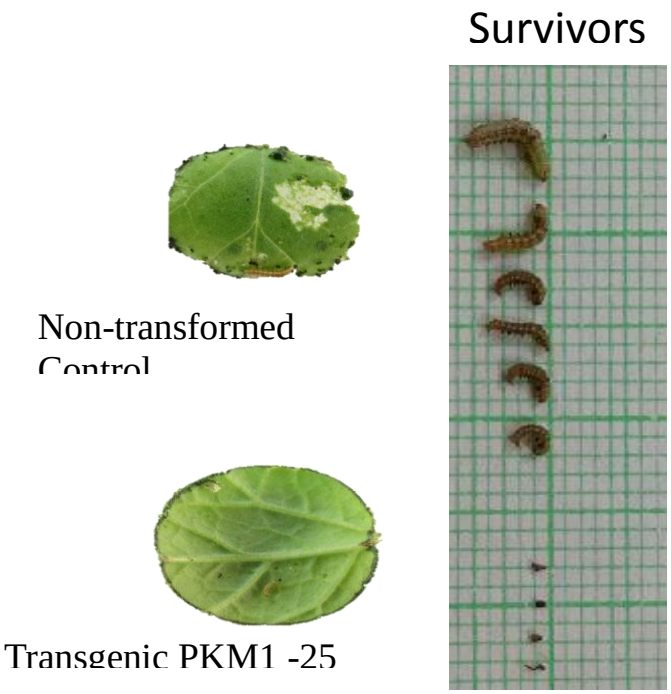


Fig.4 Detached leaf disc bioassay against *Helicoverpa armigera* in transgenic tomato plants expressing the Cry2AX1 protein



Insect Bioassay

The mortality of neonates on T_0 plants ranged from 16.67 to 100 per cent, whereas the control plants showed no mortality (Table 1). There was growth inhibition in surviving larvae and a high reduction in leaf area feeding was observed in the ELISA positive plants whereas larvae released on control plants were alive with normal growth (Figure 4).

The insecticidal crystal proteins genes of Bt have been successfully engineered into many crop plants to yield resistance against lepidopteran insects (Kumar and Sharma, 1994). The levels of toxin expression in plants, however, have been insufficient when the native genes were used, necessitating the use of a truncated version of the genes, modification of the coding sequence, and optimization of the codon usage to achieve higher expression of the gene in plants (Perlak *et al.*, 1991). Significant protection against insects have been reported in many crops such as cotton, maize, rice, potato, eggplant etc. when these modified genes are expressed in them (Mandaokar *et al.*, 2000). Tomato is an excellent plant for stably expressing transgenes, indicating a good target for genetic engineering (Kobayashi *et al.*, 2003; Omura *et al.*, 2007; Sun *et al.*, 2007). These characteristics make it an attractive research target for practical application in food and agricultural industries.

The present investigation was carried out to demonstrate the effect of chimeric *Cry2AX1* on a major target pest of tomato, *H. armigera*. *Agrobacterium*-mediated transformation was used for introducing the synthetic *cry2AX1* gene in tomato. Regenerated transgenic *cry2AX1* tomato events were verified for gene integration through PCR analysis. Out of the ninety six putative transformants of tomato generated under kanamycin selection, fifty

eight plants were found to be positive for both genes. There was no observed phenotypic difference between the transgenic and control plants (data not shown). Transformants expressed Cry2AX1 at levels ranging from 0.030 to 0.388 µg/g of leaf tissue (fresh weight basis). Mandaokar *et al.*, 2000 reported Cry1Ac protein expression level of 0.04 to 0.41 % of total soluble protein while Koul *et al.,* 2014 reported Cry1Ab toxin expression of 0.47 ± 0.01 % in tomato.

A wide range of Bt protein expression in different transgenic plants has been reported previously (Maqbool *et al.*, 2001; Ramesh *et al.*, 2004; Meiyalaghan *et al.*, 2006). Such disparity in expression is usually attributed to unpredictable levels of transgene expression due to position effect, truncation of T-DNA during integration, the chromatin structure, post transcriptional gene silencing and differences in T-DNA copy number (Conner and Christey 1994, Francis and Spiker 2005). The transgenic lines which were positive for PCR but did not express at detectable level of Cry2AX1 protein could be due to the inactivation of the gene because of its integration into highly repetitive DNA region of the plant's genome (Prols and Meyer, 1992) or the site of integration of transgene in the genome may have a detrimental and negative effect on its expression.

The tomato transformants showed mortality ranging from 16.67 to 100 % against the neonates of *H. armigera* even with moderate level of Cry2AX1 protein. A positive correlation was established between the level of Cry2AX1 expression and mortality and reduction in feeding against *H. armigera* larvae. Such positive correlation between expression level of Cry protein and insect mortality have been reported in many studies (Bhattachary *et al.*, 2002; Manikandan *et al.*, 2014). In the present study, the level of expression of Cry protein also showed a positive relationship with insect mortality. Differences in the level of mortality observed among the different transgenic lines could be ascribed to variation in the level of *Bt* gene expression. The surviving larvae on transgenic lines showed severe growth inhibition (Figure 4) and significant differences were observed in the mortality percentage of neonate larvae between the transgenic and non-transformed tomato leaf bits.

In our present investigation, a chimeric Bt gene, *cry2AX1*, was introduced into tomato through *Agrobacterium* - mediated transformation method and tomato plants expressing the gene was generated. Significant mortality of *H. armigera* neonates was observed when fed on transgenic tomato leaves. This demonstrates that Cry2AX1 could be one of the most effective *Bt* genes against lepidopteran insects pests in tomatoes.

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