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Construction of plant expression vectors harboring synthetic *cry2AX1* gene and its expression in tobacco (*Nicotiana tabacum*)

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Abstract

The *cry2AX1* is a potential synthetic *Bt* gene for developing transgenic crops resistant to lepidopteran insect pests. By using this gene, two new plant expression vectors were constructed in pCAMBIA2300 backbone. In the construct p2300:CaMV:2AX1, the *cry2AX1* gene was driven by CaMV35S promoter and in p2300:CaMV:ctp-2AX1, the chloroplast transit peptide (ctp) sequence of cotton *rbcS1b* was N-terminally fused in frame with synthetic *cry2AX1* coding sequence driven by CaMV35S promoter. These constructs were confirmed by PCR, restriction analysis and nucleotide sequencing. Further the plant transformation vectors were mobilized to *Agrobacterium tumefaciens* LBA4404. Leaf discs of tobacco (*Nicotiana tabacum* L. cv Petite Havana) were used as explants for transformation with the two constructs through *Agrobacterium* mediated transformation. Plantlets were generated *in vitro* on medium containing kanamycin as selection agent. Screening by PCR revealed presence of *cry2AX1* gene in all twelve putative transformants generated from p2300:CaMV:2AX1 and eighteen out of nineteen putative transformants generated from p2300:CaMV:ctp-2AX1 construct. The expression of Cry2AX1 protein in PCR positive T_0 tobacco plants ranged from 3 to 42 ng/g and 1.6 to 28 ng/g of fresh leaf tissue in p2300: CaMV:2AX1 and p2300:CaMV:ctp-2AX1 constructs, respectively. The two new plant transformation vectors were validated for expression of synthetic *cry2AX1* gene in tobacco as model plant.

Keywords: *Bacillus thuringiensis*, *cry2AX1*, tobacco transformation, chloroplast transit peptide, ELISA

Introduction

Losses due to pests and diseases have been estimated at 37% of the agricultural production worldwide, with 13% due to insects (Gatehouse *et al*., 1992). For a long time, they are mainly controlled by chemical insecticides, which are not effective enough besides causing environmental pollution. Therefore, alternative approaches of pest control are needed. One of the choices is *Bacillus thuringiensis* (*Bt),* which is an ubiquitous gram-positive, spore-forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle and its insecticidal activity was attributed largely or completely (depending on the insect) to the parasporal crystals (Schnepf *et al*., 1998). After ingestion of crystal protein by susceptible insects, toxins bind to specific receptors in the midgut and are solubilized and activated by proteinases in the insect midgut epithelium. The activated toxins induce the formation of a lytic pore in the midgut epithelial membrane that results in cell lysis, cessation of feeding, and death of the larva (Daniel *et al*., 2000). The application of Bt-toxins for insect pest resistance has emerged as a powerful tool, being chemically free, eco-friendly and highly specific against target insects (Pigott and Ellar 2007; Bravo *et al*., 2011).

The expression of Cry proteins in transgenic crops has provided a very effective way to control economically important insect pests. A major concern in *Bt*-mediated insect resistance is the continued use of similar Bt proteins against target insect pests leading to the development of resistance to Bt protein in insects. Insects that develop resistance against one protein (Cry1A) are not cross-resistant to another (Cry2A) protein (Tabashnik *et al*., 2000). Stacking multiple toxins to target the same target insect species allows the use of proteins with different modes of action and significantly delays the development of resistance. Another strategy to avoid development of insect resistance is the generation of transgenic plant with high level of toxin expression. Hence it is necessary to increase expression of *Bt* genes to a desirable level without affecting normal physiological processes of

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plants. It has been found that targeting Bt proteins to chloroplast with the help of signal peptide could increase the stability and protein accumulation (Wong *et al*., 1992; Kim *et al*., 2009., and Wu *et al*, 2011).

Tobacco (*Nicotiana tabacum* cv petite Havana) was chosen for genetic transformation as a model plant, because as it is amenable for tissue culture. Besides this, previous research reports have shown that it is a simple and robust method, successfully utilized in confirming gene function and validation of expression in various studies (Oven and Luthar, 2013; Singh *et al*., 2014; Jayaprakash *et al*., 2014 and Karimi *et al*., 2013).

The *cry2AX1* (Accession No.GQ332539.1) is a novel synthetic codon optimised gene constructed in Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India for expression in plants. It is a potential insecticidal gene for developing transgenic crops resistant to lepidopteran insect pests (Jayaprakash *et al*., 2014). The aim of the present study was to develop new construct(s) with *cry2AX1* gene driven by CaMV35S promoter with and without chloroplast transit peptide and validation of its expression in a model plant system, *N. tabacum*.

Material and Methods

Escherichia coli DH5α, *Agrobacterium tumefaciens* LBA4404, pCAMBIA2300 vector, pRT103-cry2AX1, pGEM-ctp vector, restriction endonucleases (*Hind*III, *Eco*RI, *Sac*I , *Nco*I, *Sal*I, *Pst*I, *Nco* and *Bam*HI), 1kb and 100 bp DNA Marker, λ *Hind*III marker, T4 DNA ligase, *Taq* DNA polymerase and tobacco cv Petite Havana.

Construction of plant expression vector(s)

Cotton *rbcS1b* transit peptide (ctp) 186 bp (Paritosh *et al*., 2013) was excised by *Nco*I digestion from its source plasmid (pGEMT Easy vector). Excised product was electrophoresed in 1.5% agarose gel, eluted using gel elution kit (Sigma-Aldrich Company, USA). Simultaneously pRT103-*cry2AX1* vector (harbouring *cry2AX1* gene flanked CaMV35S promoter and CaMV polyA sequence at 5' and 3' ends respectively) was also linearised by *Nco*I enzyme and the released cotton transit peptide sequence was N- terminally fused in frame with *cry2AX1* gene for generating CaMV35S:ctp-*cry2AX1* in pRT103 (Fig.1).

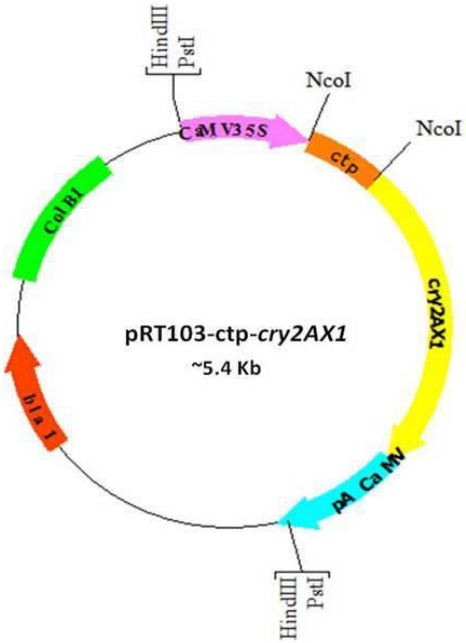

Figure 1. Schematic representation of plasmid pRT103-ctp-cry2AX1

The cassette, CaMV35S:ctp-*cry2AX1-polyA* and CaMV35S:*cry2AX-polyA* were released by *Hin*dIII digestion from pRT103 and sub-cloned into plant transformation vector pCAMBIA2300. Orientation of cloned cassette was confirmed by restriction digestion analysis and sequencing.

Identification of *Agrobacterium* transconjugants by PCR

These two plant expression vectors were mobilized in to *Agrobacterium tumefaciens* strain, LBA4404 by triparental mating method. The *Agrobacterium* transconjugants (positive clones), were identified by colony PCR using *cry2AX1 gene* specific primers.

***Agrobacterium* mediated transformation of tobacco**

Fully expanded leaves were excised from *in vitro* grown wild-type tobacco plants and cut into leaf bits of 5 mm^2 with a sterile scalpel blade. The leaf bits were pre-cultured on regeneration medium (MS Media Powder 4.2 g/L + calcium chloride 0.44g/L + BAP 1 mg/L + NAA 0.1 mg/L + 3% sucrose and 0.8% agar and pH 5.8) in petri dishes incubated at 25◦ C for 2 days. The precultured explants were infected with *A. tumefaciens* carrying the plasmid of interest for 15 min with 100 µM of acetosyringone in MS broth and then placed on plant regeneration medium and incubated for 2 days at 25 °C in dark. After 2 days, the leaf bits were washed with cefotaxime (250 mg/L) and transferred to selection medium (MS media powder 4.2g/L, calcium chloride 0.44g/L, BAP 1mg/L, NAA 1mg /L, kanamycin 100 mg/L and cefotaxime 250 mg/L, 3% sucrose and 0.8% agar) for the selection of transgenic shoot growth. Subsequent sub-culturing was done at two week intervals and actively growing shoots on selection plates were transferred to half strength MS medium with 1.0 mg/L IBA and 100 mg/L kanamycin. Plants which produced profuse roots were transferred to small plastic cups with pot mixture containing soil + sand + vermicompost at the ratio of 1:1:1. Gradually plants were acclimatized to greenhouse condition and then transferred to 7" diameter pots containing above mentioned pot mixture.

PCR analysis

DNA was isolated from putative transgenic tobacco plants using CTAB method (Murray and Thompson, 1980). PCR screening of putative tobacco transformants generated with p2300-CaMV-2AX1 construct was performed using *cry2AX1* gene specific primers (Forward 5'CCTAACATTGGTGGACTTCCAG 3' and Reverse 5' GAGAAACGAGCTCCGTTATCGT 3') and putative tobacco transformants generated with p2300-CaMV-ctp-2AX1 construct using another pair of forward primer 5'-AGCTTAAACCATGGCCTCCT and reverse primer 5'- GAAGAAGCTGGTAACCCTGA specific for *ctp-cry2AX1.* The PCR for the both sets of samples was carried out using a Thermal Cycler (Eppendorf, Germany) in 25 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 (Banglore Genei, India) and kept in a thermo cycler which was programmed for 5 min preheat at 94°C and then 30 cycles of denaturation at 94°C for 1 min, annealing temperature of 60°C for 45 s and extension time of 45 s at 72°C, with a final extension at 72°C for 7 min. The plasmid DNA was used as positive control and wild type non transformed tobacco plant genomic DNA sample was used as negative control for the PCR reaction. The PCR products were run on 1.0 % agarose gel and analyzed on Syngene (G:BOX F3) gel documentation system.

ELISA analysis

A double-antibody sandwich enzyme linked immunosorbant assay (ELISA) was used to detect the presence of the Cry2AX1 protein expressed in the leaves of transgenic tobacco plants. Experiments were performed with double sandwich quantitative Cry2A ELISA kit (Envirologix Inc., Portland, USA). Proteins from leaf samples of transformed and untransformed tobacco plants were extracted using the protein extraction buffer provided in the kit. Each sample was replicated twice. Leaf extract was diluted to fit in the linear range of the provided Cry2A standards and steps were performed according to manufacturer's instructions. Optical density (O.D.) was read at 450 nanometer in an ELISA plate reader (Biotek, USA). The quantity of Cry2AX1 protein present in the sample was calculated by referring to standard graph generated with Cry2A calibration standards provided along with the kit and represented in ng/g fresh weight of leaves.

Results

Development of *cry2AX1* constructs

CaMV35S:cry2AX1-polyA cassette (2589 bp) was released from pRT103 using *Hin*dIII restriction enzyme and cloned in plant transformation vector pCAMBIA2300 (Fig. 2) and confirmed by restriction digestion analysis with *Hin*dIII, *Bam*HI and *Nco*I enzymes which released expected size fragments of 2584, 2343 and 1309 bp, respectively (Fig. 3). Another construct was developed by in frame N-terminal fusion of ctp sequence (186 bp) to *cry2AX1* gene in pRT103-*cry2AX1* using *Nco*I enzyme. Subsequently CaMV35S:ctp-cry2AX1-polyA cassette (~2770 bp) was released by *Hin*dIII digestion and sub-cloned to plant transformation vector pCAMBIA2300 and the construct was named as p2300:CaMV-ctp-2AX1 (Fig. 4). This construct was confirmed by restriction digestion with different restriction enzymes which released expected size fragments (*Hin*dIII-2770 bp, *Eco*RI-11512 bp, *Sac*I-2381 bp, *Nco*I-1309 bp and 186 bp, *Pst*I- ~2770 bp and *Sal*I-11512 bp) as shown in (Fig. 5).

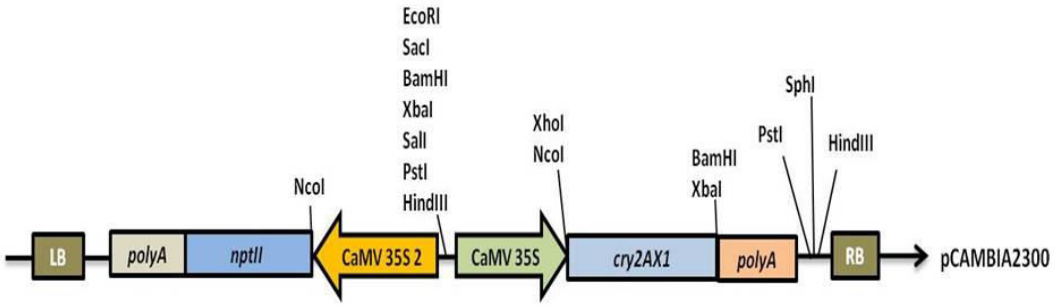

Figure 2. Schematic representation of T-DNA region of the plant transformation construct p2300-CaMV-cry2AX1

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

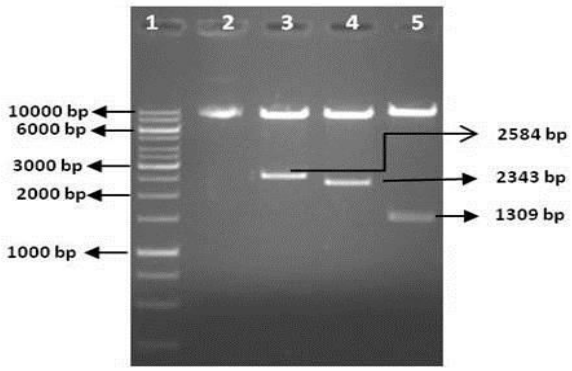

Fig. 3 Restriction analysis of plant transformation vector p2300:CaMV-cry2AX1 with *Hin*dIII, *Bam*HI, *Nco*I enzymes. Lane 1: 1 Kb Marker. Lane 2: uncut plasmid, Lane 3: 2584 bp fragment with *Hin*dIII. Lane 4: 2343 bp fragment with *Bam*HI, Lane 5: *Nco*I digested fragment 1309 bp

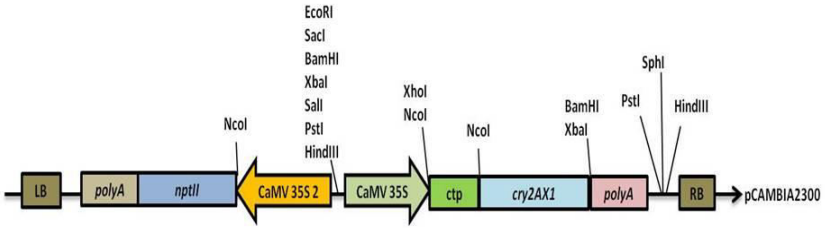

Figure 4. Schematic representation of T-DNA region of the plant transformation constructs p2300:CaMV-ctp-cry2AX1

LB: left border, poly A: CaMV35S terminator, *nptII*: neomycin phosphotransferase gene, CaMV35S2: Cauliflower mosaic virus 35S duplicated promoter, CaMV35S: Cauliflower mosaic virus 35S promoter, ctp: chloroplast transit peptide, *cry2AX1*: gene of interest, RB: right border

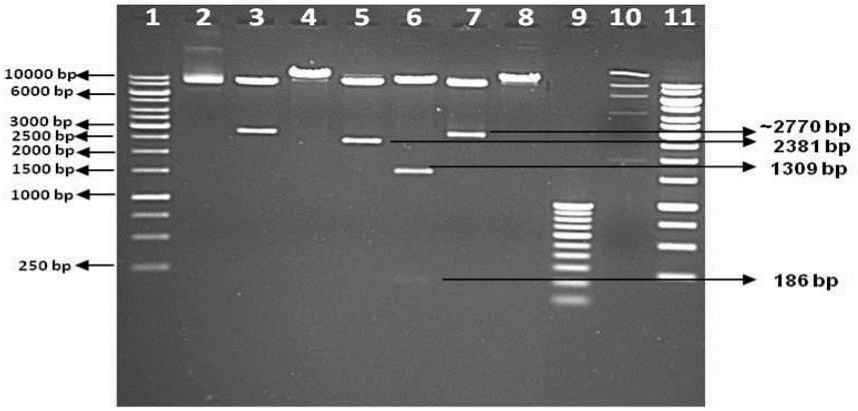

Figure. 5 Restriction analysis of Plant Expression Vector p2300:ctp-CaMV-cry2AX1 with *Hin*dIII, *Eco*RI, *Sac*I, *Nco*I, *Pst*I and *Sal*I enzymes. Lane 1: 1 Kb Marker. Lane 2: uncut plasmid, Lane 3: 2770 bp fragment with *Hin*dIII. Lane 4: 11512 bp fragment with *Eco*RI, Lane 5: *Sac*I digested fragment 2381 bp; Lane 6: *Nco*I digested fragments 1309 bp and 186 bp Lane7: *Pst*I digested fragment ~2770 bp Lane 8: *Sal*I digested linearised fragment (11512 bp), Lane 9: 100 bp ladder lane: 10: *Hin*dIII marker Lane 11: 1kb marker

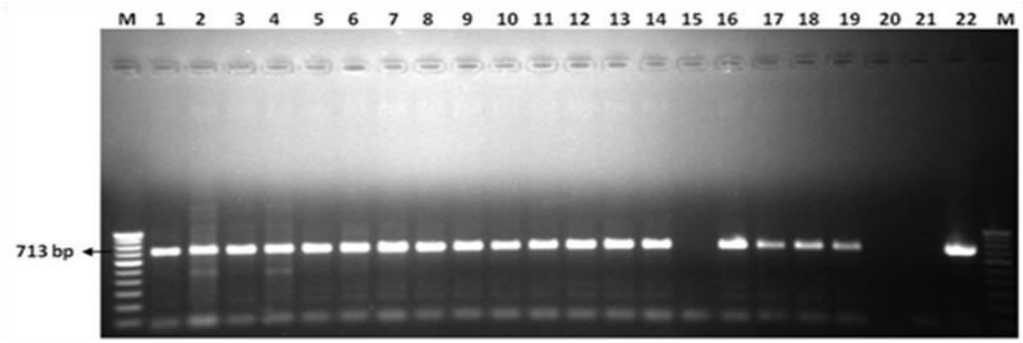

Fig. 6a Agarose gel electrophoresis of PCR amplified products from genomic DNA isolated from nineteen different putative transgenic tobacco plants, showing amplification of correct size (713 bp) fragment of the N- terminal region of *cry2AX1* gene. Lane M 100 bp DNA ladder, Lanes 1–19: amplified products from nineteen putative different transgenic plants lane 20 and 21: blank and negative control and lane 22: positive control.

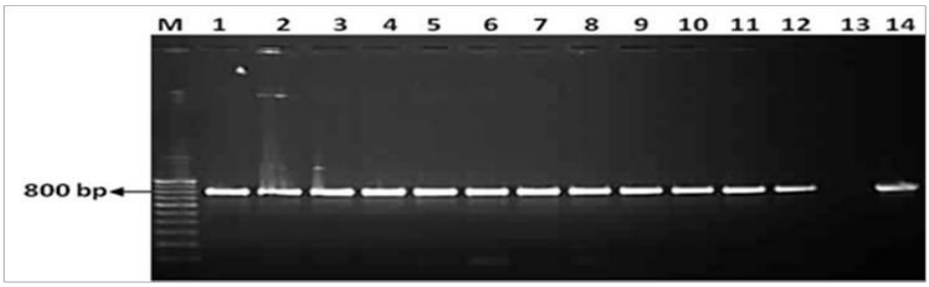

Fig.6b Agarose gel electrophoresis of PCR amplified products from genomic DNA isolated from twelve different putative transgenic tobacco plants, showing amplification of correct size (800 bp) fragment of the C-terminal region of cry2AX1 gene. Lane M: 100 bp DNA ladder, Lanes 1–12: amplified products from putative different transgenic plants, lane 13: negative control and lane 14: positive control.

Molecular analysis of putative *cry2AX1* transgenic tobacco plants

PCR

To confirm the presence of *cry2AX1* gene, DNA from putative transgenic plants was extracted and then subjected to PCR analysis. Out of 19 putative transformants generated with construct p2300:CaMV-ctp-2AX1, 18 plants were positive for ctp-*cry2AX1* gene and all of 12 plants generated with construct p2300:CaMV-2AX1, found positive for *cry2AX1* gene specific primers and showed amplicon size of 713 and 800 bp respectively (Fig. 6a and Fig. 6b), whereas, no amplification was observed in non-transformed control plants.

ELISA

Quantitative ELISA was performed to determine the expression level of Cry2AX1 protein in transgenic plants developed with both constructs. The Cry2AX1 protein detected by ELISA confirmed the expression of the *cry2AX1* gene in the transgenic tobacco plants. The level of Cry2AX1 protein (45 days after transplanting) in transgenic tobacco plants transformed with p2300: CaMV-ctp-2AX1 and p2300:CaMV-2AX1 was in the range of 1.6 to 28 ng/g and 3 to 42 ng/g of fresh weight of leaf tissue, respectively (Table 1. and Table. 2; Fig.7a and Fig. 7b).

Table. 1 Expression of Cry2AX1 protein in T_0 transformants of tobacco generated by construct p2300: CaMV-ctp-2AX1

S. No.	Tobacco events	Cry2AX1 conc. in fresh leaf tissue (ng/g at 45 DAT)
1.	35 –TPX 3	1.60 ± 0.000
2.	35 –TPX 8	6.00 ± 0.000
3.	35 –TPX 10	17.5 ± 0.001
4.	35 –TPX 12	7.00 ± 0.001
5.	35 –TPX 17	28.0± 0.000
6.	Control	0.00

Table. 2 Expression of Cry2AX1 protein in T_0 transformants of tobacco generated by construct p2300: CaMV-2AX1

S. No.	Tobacco events	Cry2AX1 conc. in fresh leaf tissue (ng/g at 45 DAT)
1.	35 –2AX 1	6.00 ± 0.00
2.	35 –2AX4	22.00 ± 0.001
3.	35 –2AX6	8.00 ± 0.000
4.	35 –2AX7	3.00 ± 0.000
5.	35 –2AX8	42.00 ± 0.000
6.	35 –2AX10	24.00 ± 0.000
7.	Control	0.00

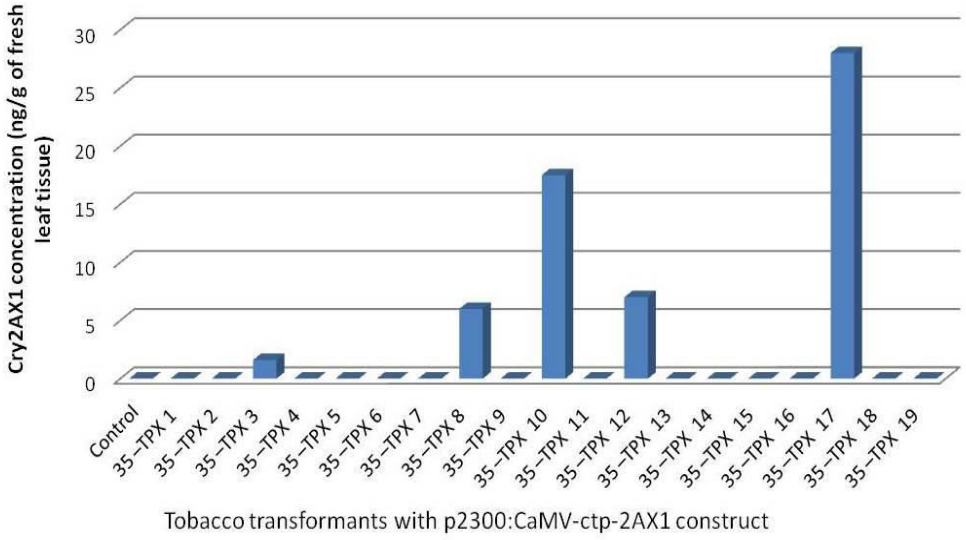

Fig.7a Quantitative estimation of Cry2AX1 protein in different tobacco transgenic lines (45 Days after transplanting)

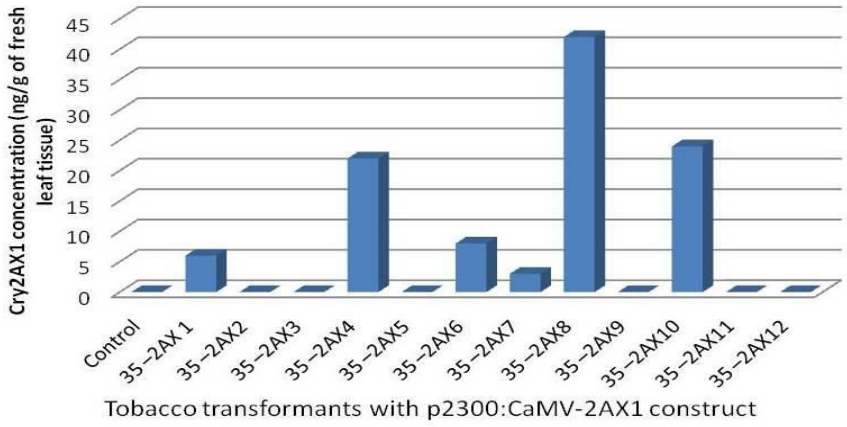

Fig. 7b Quantitative estimation of Cry2AX1 protein in different tobacco transgenic lines (45 Days after transplanting)

Discussion

Tobacco was used as a model plant system for genetic transformation and expression validation of new constructs with *cry2AX1* gene. Molecular and biochemical analysis (PCR and quantitative ELISA) of transgenic plants revealed expression of *cry2AX1* gene in transgenic tobacco plants. However, the level of expression Expression of Cry2AX1 protein in transgenic tobacco plants generated with these new constructs was low compared to earlier studies (Jayaprakash *et al*., 2014) wherein enhanced version of CaMV35S promoter was used to drive *cry2AX1* gene expression. Although most of the T_0 transgenic tobacco plants were PCR positive for *cry2AX1* but only a few of them were positive for ELISA and the rest of them showed no expression at all. This could be due to the complete inactivation of the gene because of its integration into highly repetitive DNA region of the plant's genome (Prols and Meyer, 1992). Integration site of transgene in the genome may have a detrimental effect on its expression. The variable level of gene expression observed among different transgenic lines could be due to integration of genes at different positions (position effect) or methylation, the direct influence of genomic DNA sequences or chromosome structure near the site of transgene integration (Fobert *et al.* 1991; Goldsbrough and Bevan 1991, Matzke and Matzke, 1998). This suggests that screening of a larger population of primary tobacco transformants is necessary to know the full expression potential of both the new constructs harbouring synthetic *cry2AX1* gene.

Conclusion

In this study, we have successfully developed plant expression constructs that could express Cry2AX1 protein and accumulate in cytosol or chloroplasts. Using these constructs we generated transgenic tobacco plants with *cry2AX1* gene. Expression level of Cry2AX1 protein was low and it varied in the limited number of plants tested.

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References

1. **Bravo, A., Likitvivatanavong S., Gill S.S., and Soberon, M.** 2011. *Bacillus thuringiensis*: A story of a successful bio insecticide. *Insect Biochemistry and Molecular Biology*, 4:423–431.

2. **Daniel, J.L., David, J.E., and Paul, J.** 2000. Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac-endotoxin. *Applied and Environmental Microbiology*, 66: 5174–5181.

3. **Fobert, P.R., Miki, B.L. and Iyer, V.N.** 1991. Detection of gene regulatory signals in plants revealed by T-DNA-mediated fusions. *Plant Molecular Biology*, 17: 837–851.

4. **Gatehouse, A.M.R., Hilder, V.A and Boulter, D.** 1992. Potential of plant-derived genes in the genetic manipulation of the crops for insect resistance, In: Biotechnology in Agriculture: Plant genetic manipulation for crop protection, CAB International, pp.155-181.

5. **Goldsbrough, A., and Bevan, M. 1991.** New patterns of gene activity in plants detected using an *Agrobacterium* vector. *Plant Molecular Biology*, 16**:** 263–269.

6. **Jayaprakash, S.P., Nandeesha, P., Sozhavendan, A.E., Naveenkumar, A., Illakiya Priya A., Balakrishnan, N., Balasubramani, V., Singh, P.K., Sudhakar, D., Balasubramanian, P. and Udayasuriyan, V.** 2014. Expression of a novel synthetic *cry* gene of *Bacillus thuringiensis* in transgenic tobacco confers resistance to *Helicoverpa armigera* and *Spodoptera litura. Journal of Pure and Applied Microbiology*, 8: 4687-4692.

7. **Karimi S.M, Sohail, M.N., Amin, I., Mansoor, S. and Mukhtar, Z.** 2013. Molecular characterization of a new synthetic *cry2Ab* gene in *Nicotiana tabacum*. *Biotechnology Letter*, 35: 969-974.

8. **Kim, E.H., Suh, S.C., Park, B.S., Shin, K.S., Kweon, S.J., Park, S.H., Kim, Y.S. and Kim, J.K.** 2009. Chloroplast-targeted expression of synthetic *cry1Ac* in transgenic rice as an alternative strategy for increased pest protection. *Planta*, 230**:** 397-405.

9. **Matzke, A.J. and Matzke, M.A.** 1998. Position effects and epigenetic silencing of plant transgenes. *Current Opinion in Plant Biology*, 1:142–148.

10. **Murray, M.G. and Thompson, W.F.** 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research,* 8: 4321-4325.

11. **Oven, K., Luthar, Z.** 2013. Expression and molecular analysis of *DsRed* and *gfp* fluorescent genes in tobacco (*Nicotiana tabacum* L.) *Acta Agriculturae Slovenica*, 101: 5 – 14.

12. **Paritosh, K., Pental, D. and Burma, P.K.** 2013. Structural and transcriptional characterization of *rbcS* genes of cotton (*Gossypium hirsutum*). *Plant Molecular Biology Reporter*, 31: 1176-1183.

13. **Pigott, C.R., Ellar, D.J.** 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Review*, 71:255–281

14. **Prols, F. and Meyer, P.** 1992. The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in *Petunia hybrid. The Plant Journal*, 2:465-475.

15. **Schnepf, E., Crickmore, N. and Rie, J.V.** 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Review*, 62: 705-806.

16. **Singh, N., Mishra, A., Jha, B.** 2014. Over-expression of the peroxisomal ascorbate peroxidase (*SbpAPX*) gene cloned from halophyte *Salicornia brachiata* confers salt and drought stress tolerance in transgenic tobacco. *Marine Biotechnology*, 16:321-332.

17. **Tabashnik, B.E., Liu, Y.B., De Maagd, R.A. and Dennehy, T.J.** 2000. Cross-resistance of pink bollworm (*P. gossypiella*) to B. thuringiensis toxins. Applied and Environmental Microbiology, 66: 4582 – 4584.

18. **Wong E.Y., Hironaka, C.M. and Fischhoff, D.A.** 1992. *Arabidopsis thaliana* small subunit leader and transit peptide enhance the expression of *Bacillus thuringiensis* proteins in transgenic plants. *Plant Molecular Biology*, 20: 81–93.

19. **Wu, J., Luo, X., Zhang, X., Shi, Y. and Tian, Y.** 2011. Development of insect-resistant transgenic cotton with chimeric TVip3A* accumulating in chloroplasts. *Transgenic Research*, 20:963–97.