International Journal of Forestry and Crop Improvement
Volume 7 | Issue 1 | June, 2016 | 79-85 | Visit us : www.researchjournal.co.in
e ISSN-2230-9411

RESEARCH ARTICLE

DOI: 10.15740/HAS/IJFCI/7.1/79-85

Transformation of tobacco (*Nicotiana tabaccum*) with cry2AX1 gene and analysis of transgenic plants

R. MANIKANDAN, P. KANIMOZHI, N. BALAKRISHNAN, V. BALASUBRAMANI, D. SUDHAKAR AND V. UDAYASURIYAN

ABSTRACT : A novel synthetic cry2AX1 gene was codon optimized and a sequence encoding cotton rbcS1b transit peptide was fused upstream of coding sequence. The fusion cry2AX1 gene, driven by maize ubiquitin1 promoter was cloned in a pUH plant transformation vector. *Agrobacterium* mediated transformation was carried out with pUH-ctp-2AX1 construct using leaf discs of tobacco as model plant. Screening by PCR revealed presence of cry2AX1 gene in all nine putative transformants and expression of cry2AX1 protein in PCR positive T_0 tobacco plants ranged from 1.5 to 10.0 ng/g. The detached leaf bit bioassay of tobacco transformants with *Helicoverpa armigera* showed 30 per cent mortality even at lower level of cry2AX1 expression. The results indicated a newly developed construct was functionally expressed in tobacco plant.

KEY WORDS : Tobacco, Transformation, Insect resistance, *Helicoverpa armigera*

HOW TO CITE THIS ARTICLE : Manikandan, R., Kanimozhi, P., Balakrishnan, N., Balasubramani, V., Sudhakar, D. and Udayasuriyan, V. (2016). Transformation of tobacco (*Nicotiana tabaccum*) with *cry2AX1* gene and analysis of transgenic plants. *Internat. J. Forestry & Crop Improv.*, **7** (1) : 79-85, **DOI: 10.15740/HAS/IJFCI/7.1/79-85.**

ARTICLE CHRONICAL : Received : *05.02.2016;* Revised : *18.04.2016;* Accepted : *20.05.2016*

INTRODUCTION

The choice of an appropriate promoter is a very important factor in crop transformation experiments. The constitute cauliflower mosaic virus promoter (CaMV35S) has been most widely used for dicot transformation, especially for the development of *cry* gene containing transgenic crop plants (Perlak *et al*., 1990). The maize derived ubiqutine (Ubi) promoter is now being used extensively for the production of Bt gene transformed monocot plants such as maize and rice. Before transformation into rice, newly developed gene construct driven by maize Ubi promoter was validated for the expression of an insecticidal Bt gene in a model dicot plant (tobacco). Tobacco (*Nicotiana tabacum* L. 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;

MEMBERS OF RESEARCH FORUM

Address of the Correspondence : **R. MANIKANDAN,** Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, COIMBATORE (T. N.) INDIA Email: bioinba@gmail.com

Address of the Coopted Authors : P. KANIMOZHI, N. BALAKRISHNAN, V. BALASUBRAMANI, D. SUDHAKAR AND V. UDAYASURIYAN, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, COIMBATORE (T. N.) INDIA

Singh *et al*., 2014; Jayaprakash *et al*., 2014 and Karimi *et al*., 2013).

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. In this direction, the chimeric cry2AX1 (Accession No.GQ332539.1) is a novel synthetic codon optimized 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).

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 plants. It has been found that targeting Bt proteins to sub cellular organs such as chloroplast with the help of signal peptide could increase the stability and protein accumulation (Wong *et al*., 1992; Kim *et al*., 2009, Wu *et al*., 2011 and Manikandan *et al*., 2015). The aim of the present study was to develop new construct(s) with cry2AX1 gene driven by Ubiquitin promoter with chloroplast transit peptide and validation of its expression in a model plant system, *N. tabacum*.

EXPERIMENTAL METHODS

Plasmid construct :

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 (Agarwal *et al*., 2002) which harbours *hph* (coding for *hygromycin phosphotransferase*) as a plant selectable marker gene. Transformation vector (Fig.A) was mobilized into disarmed *Agrobacterium* strain, LBA4404 for tobacco transformation experiments.

The synthetic *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 and RB: indicate left border and right border of the T-DNA region.

Tobacco transformation :

Leaf explants were collected from tobacco plants which were grown on half strength MS medium with 1.5 per cent sucrose (w/v) and 0.8 per cent agar at pH 5.8. The leaf bits were precultured on plant regeneration medium (MS + 1.0 mg BAP/l + 0.1 mg NAA/l + 3 % sucrose (w/v) and 0.8 % agar, pH 5.8) for two days and the pre-cultured explants were infected with *Agrobacterium*. The infected leaf bits were co-cultivated for two days at 25°C on plant regeneration medium with 100 µM acetosyringone to allow efficient T-DNA transfer.

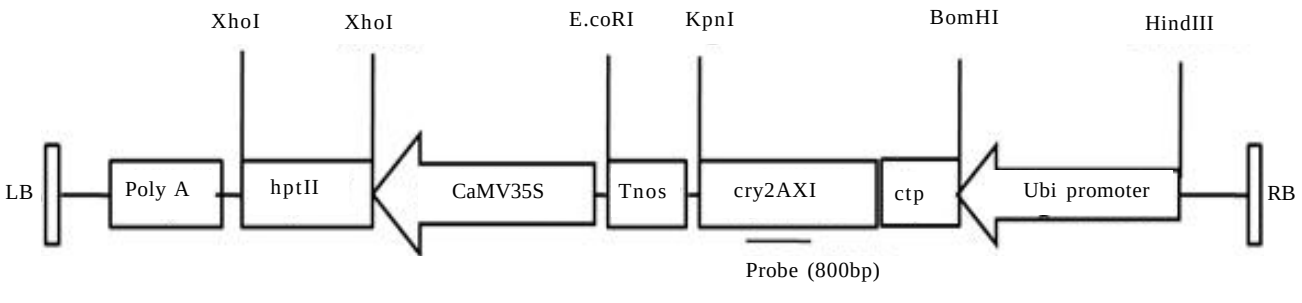

Fig. A : T-DNA region of plant transformation construct *pUH-TP2AXI*

The leaf bits were washed with cefotaxime (250 mg/l) and placed on plant regeneration medium containing 20 mg hygromycin/l. Subsequent sub-culturing was done at every two weeks intervals and individual shoots which were actively growing on selection plates were transferred to ½ strength MS medium which contain 1.0 mg IBA/l and 20 mg hygromycin/l. The plants which produced profuse roots were transferred to small paper 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 in transgenic greenhouse (Fig. B).

Sensitivity test of tobacco to hygromycin :

To identify the lethal concentration of hygromycin for effective selection of transgenic tobacco plants, sensitivity test was carried out using 20, 30 and 40 mg/l concentrations of hygromycin. The leaf discs on shoot regeneration media without hygromycin serve as control.

Detection of transgene by PCR :

PCR analyses were performed to demonstrate the presence of cry2AX1 and *hptII* genes in putative transgenic lines of tobacco using gene specific primer (2AX1FP, 5'-CCTAACATTGGTGGACTTCCAG-3'and 2AX1RP, 5'-GAGAAACGAGCTCCGTTATCGT-3'); (HPTFP, 5'-GCTGTTATGCGGCCATTGGTC-3'; HPTRP, 5'-GAC GTCTGTCGAGAAGTTTG-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 per cent agarose gel, visualized on UV transilluminator upon ethidium bromide staining.

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

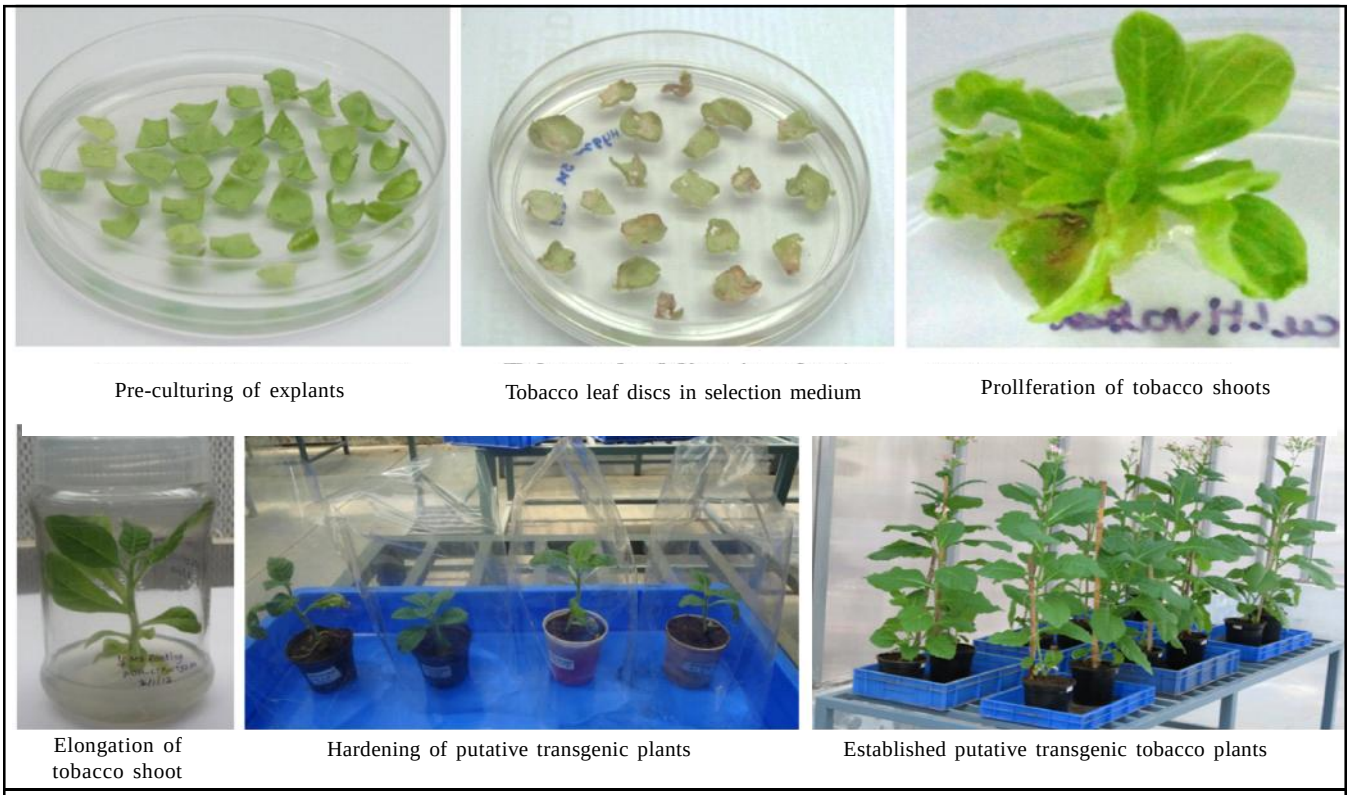

Fig. B : ***Agrobacterium*** **mediated transformation of tobacco**

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.

Insect bioassay of transgenic plants :

Detached leaf disc bioassay was carried out on the ELISA positive tobacco plants with neonate larvae of *H. armigera*. The neonate larvae were released on to fresh leaf discs (in *H. armigera* 1 larvae per disc) placed in 90 mm petriplates lined with wet Whatmann number 1 filter paper. Ten replications were maintained. A control was also maintained by using leaf discs from non-transformed tobacco plants of same age compared to transgenic plants. Final mortality data were recorded on five days of the experiment.

EXPERIMENTAL RESULTS AND ANALYSIS

Tobacco is widely used as a model plant system in transgenic research for several reasons: its molecular genetics is well understood, its genomic mapping is almost complete, genetic transformation can be readily achieved, tobacco plants survive well *in vitro* and under greenhouse conditions.

Agrobacterium-mediated transformation is an effective and widely used approach to introduce foreign DNA into dicotyledonous plants.*Agrobacterium*-mediated transformation of tobacco using different useful genes were reported by several workers (Haughn *et al*., 1988; Svab and Maliga, 1993 and Pugalendhi *et al*., 2008). Several cry genes have been introduced into tobacco plants through genetic transformation, they include cryIIIA (Sutton *et al*., 1992); cryIA (*b*) (Salm *et al*., 1994); cryIC (Strizhov *et al*., 1996); cry1Ia5 (Selvapandiyan *et al*., 1998) and cry9Aa2 (Chakrabarti *et al*., 2006). Success in genetic transformation and regeneration depends on the combination of plant growth regulators used and selection strategy adopted. The tobacco plants established under *in vitro* conditions were used as source for explants as *in vitro* grown plants obviates the need for sterilization of explants.

In this study, tobacco was used as a model plants system to validate the transformation construct for the potential use of Ubi promoter for the expression of Bt gene in dicot model tobacco plants. This evaluation helps to understand the ability of the promoter to express Bt gene at the levels, that is high enough to render the transformed plant, resistant to the target insects, as it does in monocots. The binary construct pUH-ctp-2AX1 harbouring synthetic cry2AX1 gene is driven by ubi promoter and it was used for tobacco transformation.

Generation and molecular analysis of tobacco transformants :

To test the lethal concentration of hygromycin was tested for the selection of transgenic tobacco plants. The sensitivity test was carried out using 20, 30 and 40 mg/lit concentrations of hygromycin with tobacco. Based on observation, hygromycin concentration of 20mg/lit was used for selecting transformed cells/tissues. Shoots of putative transformants were normal and green when cultured in selection medium containing hygromycin (20

Table 1: Expression of cry2AX1 protein and mortality of *H. armigera* neonates in transgenic tobacco lines

Sr. No.	Tobacco events	Cry2AX1 conc. in fresh leaf tissue (ng/g at 40 DAT)* (Mean ± SD)	Detached leaf bit bioassay with *H. armigera* (%)**(Mean ± SD)
1.	TP2A-1	3.0± 0.00	16.66667± 4.71
2.	TP2A-2	2.0± 0.00	NT
3.	TP2A-3	1.5± 0.00	NT
4.	TP2A-4	2.7± 0.01	10.00± 0.00
5.	TP2A-5	4.5± 0.00	NT
6	TP2A-6	7.0± 0.00	20.00± 0.00
7.	TP2A-7	10.0± 0.00	30.00± 0.00
8.	TP2A-8	8.5± 0.01	23.33± 4.71
9	TP2A-9	2.0± 0.00	NT
10.	Control	0.0	0.0

(**Mean of three replications, *Mean of two replications, NT- Not tested)

mg/lit), while the untransformed shoots died, indicating the effective selection of transformed tissues on hygromycin. Transformed cells expressing *hptII* are insensitive to kanamycin and using appropriate cell culture media can regenerate complete plants.

A total of nine individual putative transgenic tobacco shoots were resulted from six experiments. All twenty shoots were regenerated and transferred to rooting medium containing 20mg/lit of hygromycin. The rooted plants were transferred to green house for hardening. The established tobacco plants were used for further analysis by PCR. PCR provides a quick and convenient mechanism for confirming the presence of *cry* gene using specific primers (Dung *et al*., 2006). About 200ng of genomic DNA from the putative transformants were used for DNA template for of cry2AX1 and hptII gene.

A 800 bp internal sequence of cry2AX1 gene was amplified by PCR from the DNA isolated from putative transgenic plants. Lane 1: 100 bp marker, Lane 2-11: Putative transgenic plants of tobacco. Lane 12: pUH-TP2AX1 plasmid as a positive control, Lane 13: Non-transformed plant as a negative control.

A 642 bp internal sequence of hptII gene was amplified by PCR from the DNA isolated from putative transgenic plants. Lane 1: 100 bp marker, Lane 2-11: Putative transgenic plants of tobacco. Lane 12: pUH-TP2AX1 plasmid as a positive control, Lane 13: Non-transformed plant as a negative control.

Total nine tobacco plants were screened by PCR for cry2A and *hpt* gene, all the nine plants were found to be positive for the amplification of 800 bp (Fig. 1a) and 640 bp (Fig. 1b) internal sequences of cry2AX1 and hptII genes, respectively.

Expression and insect bioassay of putative tobacco transformants :

Quantitative ELISA was performed to determine the expression level of cry2AX1 protein in transgenic

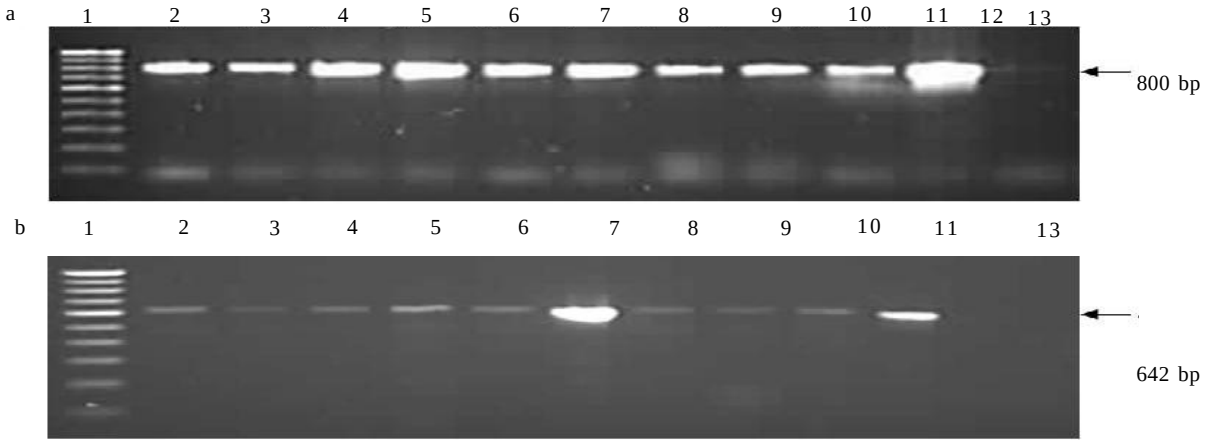

Fig. 1 : PCR analysis of cry2AXI tobacco transformants

Control Transgenic (TP2A-7)

Fig. 2 : Detached leaf bit bioassay against *H. armigera* in T_0 transgenic tobacco plants

plants. The cry2AX1 protein detected by ELISA confirmed the expression of the cry2AX1 gene in the transgenic tobacco plants. The level of cry2AX1 protein in the range of 1.5 to 10 ng/g of fresh weight of leaf tissue (Table 1).

The detached leaf bioassay against neonate larvae of *H. armigera* on ELISA positive plants showed larval mortality ranging between 10.00 to 30 per cent (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 larva over a period of five days (Fig. 1).

Conclusion :

In the present study, the level of cry2AX1 protein expression in tobacco transformants generated with Ubiquitin promoter was very low when compared to transgenic Bt plants reported by earlier workers. Though a few lines generated in the study expressed a detectable level of cry proteins, these lines may not be the best possible lines.All nine events generated by *Agrobacterium* mediated transformation was found to be ELISA positive and showed the larval mortality of maximum of 30 per cent. The study indicated the newly developed transformation vector functionally expressed and produced the protein in transgenic pants. Further studies were not carried out on these plants due to the very low level of expression of cry2AX1 protein.

REFERENCES

Agarwal, K.S., Kapoor, A. and Grover, A. (2002). Binary cloning vectors for efficient genetic transformation of rice. *Curr. Sci.*, **82** : 873-876.

Chakrabarti, S.K., Lutz, K. A., Lertwiriyawong, B., Svab, Z. and Maliga, P. (2006). Expression of the *cry9Aa2* Bt gene in tobacco chloroplasts confers resistance to potato tuber moth. *Trans. Res*., **15** : 481-488.

Dung, T.T. Tan, D., Huu, H.N. and Van, U.N. (2006). *Agrobacterium*-mediated transformation of *cry1Ac* gene to tobacco (*Nicotiana tabaccum*) and evaluation of *Heliothis armigera* resistance. *J. Agric*., **22**: 161-169.

Haughn, G.W., Smith, J., Mazur, B. and Somerville, C. (1988). Transformation with a mutant Arabidopsis *acetolactate synthase* gene renders tobacco resistant to sulfonylurea herbicides. *Mol. Gen. Genet*., **211**: 266-271.

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*. *J. Pure Appl. Microbiol*., **8** : 4687-4692.

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*. *Biotechnol. Lett*., **35** : 969-974.

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.

Manikandan, R., Balakrishnan, N., Balasubramani, V., Sudhakar, D. and Udayasuriyan, V. (2015). Transgenic rice plants expressing synthetic *cry2AX1* gene exhibits resistance to rice leaffolder (*Cnaphalocrosis medinalis*). 3 *Biotech.*, **6** (10): 1-10.

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

Perlak, F.J, Deaton, R.W., Armstrong, T. A., Fuchs, R.L., Sims, S.R., Green Plate, J.T. and Fischhoff, D.A. (1990). Insect resistant cotton plants. *Biotechnol*., **8** : 939-943.

Pugalendhi, T., Premalatha, T. and Bhat, S.G. (2008). *Agrobacterium*-mediated transformation of annexin gene in tobacco (*Nicotiana tabacum*). *Afr. J. Biotechnol*., **8** : 5149-5154.

Salm, T., Bosch, D., Honee, G., Feng, L., Munsterman, E., Bakker, P., Stiekema, W. J. and Visser, B. (1994). Insect resistance of transgenic plants that express modified *Bacillus thuringiensis cry1A(b)* and *cry1C* genes: a resistance management strategy. *Plant Mol. Biol*., **26**: 51-59.

Selvapandiyan, A., Reddy, N.S., Anandakumar, P., Tewari, K.K. and Bhatnagar, R.K. (1998). Transformation of *Nicotiana tabacum* with a native *cry11a5* gene confers complete protection against *Heliothis armigera*. *Mol. Breed*., **4** : 473-478.

Singh, N., Mishra, A. and Jha, J. (2014). Overexpression of the peroxisomal *ascorbate peroxidase* (*SbpAPX*) gene cloned from halophyte *Salicornia brachiataconfers* salt and drought stress tolerance in transgenic tobacco. *Marine Biotechnol*., **16** : 321-332.

Strizhov, N., Keller, M. and Mathur, J. (1996). A synthetic *cryIC* gene, encoding a *B. thuringiensis* δ-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco. *Proc. Natl. Acad. Sci. USA.*, **93**: 15012-15017.

Sutton, D.W., Havastad, P.K. and Kemp, J.D. (1992). Synthetic *cryIIIA* gene from *Bacillus thuringiensis* improved for high expression in plants. *Trans. Res.*, **1**: 228-236.

Svab, Z. and Maliga, P. (1993). High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc. Natl. Acad. Sci. USA.*, **90** : 913-917.

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. *Appl. Environ. Microbiol.*, **66**: 4582-4584.

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 Mole. Biol.*, **20** : 81-93.

Wu, J., Luo, X., Zhang, X., Shi, Y. and Tian, Y. (2011). Development of insect-resistant transgenic cotton with chimeric TVip3A* accumulating in chloroplasts. *Trans. Res.*, **20** : 963-997.