International Journal of Agriculture, Environment and Biotechnology
Citation: IJAEB: 8(4): 795-803 December 2015
DOI Number: 10.5958/2230-732X.2015.00088.1

Genetics and Plant Breeding

Transformation of Tomato with *Cry2ax1* by Biolistic Gun Method for Fruit Borer Resistance

Chaithra N*, Ramanjini Gowda P.H. and Neha Guleria

Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bengaluru 560065, India

*Corresponding author: chaithran12@gmail.com

Paper No.: 372 Received: 13 Jan. 2015 Accepted: 15 Nov. 2015

Abstract

Tomato (*Solanum esculentum* L.) is an important vegetable crop cultivated throughout India. The crop is extensively damaged by alepidopteran insect, *Helicoverpa armigera* Hubner (fruit borer). To mitigate this problem, an attempt was made to generate transgenic tomato plants resistant to fruit borer. The regeneration protocol being pre-requisite for any transformation studies, 1.0 mg L^{-1} BAP (Benzyl amino purine) in combination with 0.2 mg L^{-1} IAA (Indole-3-acetic acid) was standardized as shoot regeneration media for tomato cv. Pusa Ruby using leaf explant. The highest callus initiation (100%) and an average number of shoots (3.9) were obtained in the above media. Rooting was observed at 2.0 mg L^{-1} of IBA(Indole-3-butyric acid) with a highest average number of roots (1.06). The transformation system has been established by Biolistic Gun method using plant binary vector pCAMBIA2300 mobilized into the *Agrobacterium* strain LBA4404 carrying *cry2AX1* under the control of CaMV 35S promoter. The callus of tomato was bombarded twice from three different distances between tissue and macro carrier assembly. The highest transformation efficiency (75%) was obtained at distance 9 cm and transformants were screened using 50 mg L^{-1} of kanamycin. Out of 72 explants bombarded, only six explants developed completely but only two putative transformed plants were successfully hardened in the greenhouse. The integration and expression of the *cry2AX1* gene in transformed plants were confirmed by PCR and Quantitative ELISA. The cry2AX1 protein accumulated in leaf quantified by ELISA has recorded 38.9 and 79.9 ng ml^{-1} respectively compared to 129 ng ml^{-1} of protein sample in positive control.

Highlights

- Regeneration protocol was standardized for tomato.
- Transformation studies performed using Biolistic gun.
- Integration of *cry2AX1* gene was confirmed using PCRand quantitative ELISA.

Keywords: *Helicoverpa armigera* hubner (fruitborer), biolistic gun method, *cry2AX1*, quantitative ELISA

Tomato (*Solanum esculentum* L.) is considered as the second greatest important vegetable crop in the world after potato. It is grown in almost every country of the world in the field, greenhouses, and net houses. Tomato by its nature is a perennial plant, but it is commercially cultivated as an annual crop either for fresh fruits or processing. At present, India contributes 11% of world tomato production and it is grown in an area of 8, 70, 000 hectare with the productivity of 18.24 MT/hectare (FAO Statistical Database 2014).

Tomato has also been extensively utilized as a model plant system to understand biological processes and to study functional genomics, proteomics, and metabolomics (Arumuganathan and Earle 1991). *In vitro* regeneration of cultivated tomato has been a topic of research because of the economic worth of the crop and its flexibility for further improvement via genetic manipulation. Consequently, many studies on plant regeneration from a varied range of tissues and organs of wild and cultivated tomato germplasm have been conducted (Ohki *et al.* 1978, Kurtz and Lineberger 1983).

DNA transfer by biolistic gun makes use of physical processes to achieve the transformation of crop plants. There is no dependence on bacteria, so the limitations

inherent in organisms such as *Agrobacterium tumefaciens* do not apply. The absence of biological constraints, at least until DNA has entered the plant cell, means that Biolistic gun is a versatile and useful transformation method, not limited by cell type, species or genotype. There are no constitutional vector requirements so transgenes of any size and arrangement can be introduced, and multiple gene co-transformation is straightforward (Altpeter *et al.* 2005).

Tomato is severely damaged by alepidopteran insect, *Helicoverpa armigera*Hubner (fruit borer). Genetically engineered (GE) crops have revolutionized agriculture, especially in the management of insect-pest, weeds and diseases. Commercial production of GE plants expressing proteins from *Bacillus thuringiensis* (*Bt* plants) is rightly considered another form of host plant resistance, which is a cornerstone in the foundation of integrated pest management (Shelton, 2012). *Bacillus thuringiensis*, a gram-positive soil bacterium, produces crystalline inclusions during sporulation, which contain insecticidal proteins called δ--endotoxins. The expression of δ-endotoxins in transgenic plants has provided a very effective means to control target insect pests to overcome the drawback in the spraying of spores such as instability and degradation of *cry* proteins when exposed to ultraviolet radiation and short persistence on the plant. This method of pest management has brought about a reduction in the use of broad-spectrum chemical pesticides, thereby reducing the ecological damage (Saker *et al.* 2011). There is a possibility of developing stable insect-resistant tomato lines through the expression of the insecticidal gene of *Bt*, as documented successfully in several crop plants like cotton, maize, soybean, rice, canola and potato (Sanahuja *et al.* 2011; Tabashnik *et al.* 2011). 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 due to the presence of specific receptors in the midgut, while being non-toxic to beneficial insects and vertebrates owing to the lack of the receptors for toxin interaction and binding (Bravo *et al.* 2011).

This paper attempts to standardize regeneration protocol for tomato using different concentrations of plant growth regulators. The tomato cv. Pusa Ruby is grown throughout India but in severe cases of fruit borer infestation, more than 80% of tomato fruits get damaged. Hence, the transformation of tomato cv. Pusa Ruby by abiolistic gun method with a novel synthetic *cry2AX1* gene was attempted to impart insect resistance to tomato fruit borer (*H. armigera).*

Material and Methods

Plant material and Regeneration

The leaves from 8 days old tomato seedlings were used for plant regeneration and genetic transformation studies. The explants were kept under running tap water to remove adhering dust particles and surface sterilized with 0.4 % (w/v) bavistin and 0.1 % (w/v) mercuric chloride for 1-2 minutes, followed by 3-4 washing with sterile distilled water, under aseptic conditions. After rinsing, leaves were trimmed into pieces of about 1cm^2-1.25cm^2 and then inoculated onto MS (Murashige and

Table 1. Effect of different concentrations of BAP+IAA on Caulogenesis

MS media with (mg L^{-1})		Frequency of callus regenerated into shoots (%)	Average number of shoots per explant	Shooting observation
BAP	IAA			
1.0	0.2	28.1	3.93	Long, Healthy
2.0	0.2	26.5	1.06	Long, Vitrified leaves
3.0	0.2	4.6	0.18	Vitrified
4.0	0.2	12.5	0.50	Vitrified
5.0	0.2	20.3	0.81	Long, Yellowing

Table 2. Effect of different concentrations of IBA on Rhizogenesis

MS media with IBA (mg L^{-1})	Average number of roots per shoots	Rooting observation
2.0	1.06	Long, Thick, healthy
4.0	0.31	Long, Thin
6.0	0.06	Short, Thin
8.0	0.00	—
10.0	0.18	Short, Thin

Skoog 1962) culture media added with 3% (w/v) sucrose, 0.8% agar and supplemented with different concentration of BAP (1.0 mg L^{-1} to 5.0 mg L^{-1}) along with IAA (0.2 mg L^{-1}). The pH of the media was adjusted to 5.8 with 0.1 N NaOH, autoclaved at 121^{0}C and 15 lbs/inch^2 pressures for 15 min. The cultures were maintained at 25 ± 2 C in the growth chamber under a 16 h photoperiod. Regenerated shoots were subculturedin every 15 days of interval and rhizogenesis was obtained using IBA (2.0 mg L^{-1}, 4.0 mg L^{-1}, 6.0 mg L^{-1}, 8.0 mg L^{-1}, and 10.0 mg L^{-1}). Regenerated plantlets with well-developed shoots and roots were washed under gentle flow of tap water for 3-4 hours to remove adhering agar completely. Plantletswere transferred to plastic pots filled with sand and vermicompost at ratio (1:1) and gradually acclimatized to field conditions. The acclimatized plants were transferred to normal bigger pots containing soil, sand and vermicompost at 1:1:1 ratio in the greenhouse.

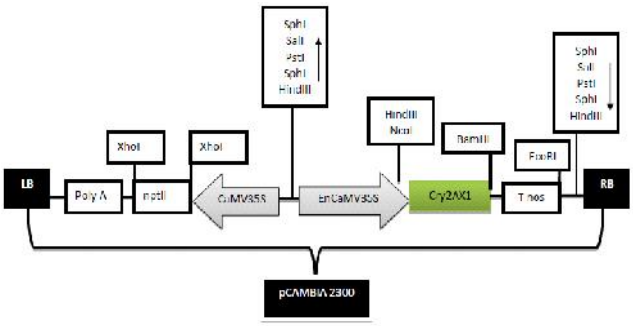

Fig. 1. Vector map of pCAMBIA2300-EnCaMV-*Cry2AX1*-Tnos construct:LB: left border, poly A: CaMV35S terminator, *npt*II: neomycin phosphotransferase gene, CaMV35S: Cauliflower mosaic virus *35S* Promoter, ECaMV35S: Enhanced Cauliflower mosaic virus *35S* promoter, *cry2AX1:* gene of interest, *Tnos*: nopaline synthase terminator, RB: right border

Kanamycin sensitivity test

Yepes-Martinez and Aldwinckle (1994) showed that *npt-II* gene conferring resistance to the amino-glycoside antibiotic, kanamycin was used in many early attempts of plant transformation. The leaf explants were cultured on shoot induction media supplemented with different concentrations of kanamycin (0, 25, 50 and 100 mg L^{-1}) to determine the optimal concentration of kanamycin for callus formation and shoot regeneration. All cultures were maintained at 25 ± 2^{o}C in the growth chamber under a 16 h photoperiod. Each explant was scored for lethality after 20 days.

Agrobacterium strain and plant vector

The plant binary vector pCAMBIA2300 carrying *Cry2AX1* under the control of an EnCaMV 35S (constitutive expression) promoter (Figure 1) was mobilized into disarmed *Agrobacterium tumefaciens* strain LBA4404. The *npt-II* gene is included in the vector as a selectable marker.

Biolistic Gun Transformation

Standardization of Transformation of tomato using *GUS* reporter gene

The tomato leaf callus was transformed with *GUS* reporter gene by biolistic gun method in order to standardize transformation protocol. Callus was maintained overnight on osmotic media [MS + mannitol (30 g L^{-1}) agar petri plates] for four hours. The bombardment was carried out with a Biolistic PDS-100/ He particle delivery system (Bio-Rad). All bombardments were performed at a pressure of 1100psi, twice from 3 different levels of thebiolistic gun (6 cm, 9 cm, and 12 cm away from macro carrier launch assembly) and a vaccum of 25 Hg inch (Guleria and Gowda 2015). Following bombardment, callus was incubated in the dark for two days at 25 ± 2^{o}C. Later, explants were subjected to *GUS* histochemical assay.

Fig. 2. Regeneration and Transformation of tomato leaf explants: (a) Shoot initiation (b) Rooting (c) Tissue culture raised plants (non-transformed) bearing fruits (d) Hardened transgenic tomato plants bearing fruits

GUS Histochemical Assay

The *GUS* gene present in the Plasmid pABC constructs (Fig 1a) was used to transform callus by the biolistic gun method to standardize transformation protocol. The explants were incubated overnight at 37 ^{o}C with 0.1mM 5-bromo-4-chloro-3indolyl β-D-glucuronide (X-gluc) in 50mM sodium phosphate buffer (pH7) supplemented with 0.1 M Potassium Ferrocyanide,0.1M Potassium

Ferricyanide and 10% W/V Triton-X-100 (Jefferson *et al.* 1987). The GUS reaction was stopped by rinsing with 70% ethanol until pigments, such as chlorophyll, cleared completely. The number of individual cells or cell aggregates was observed for blue colour development.

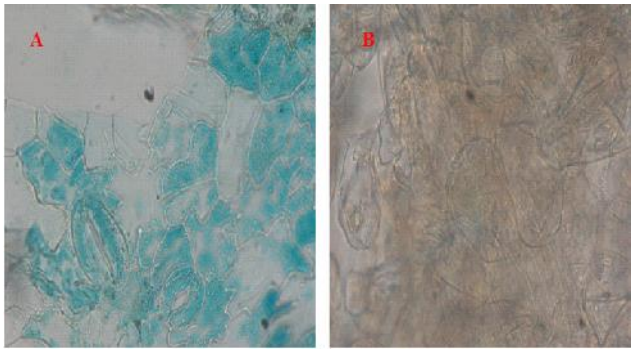

Fig. 3. *GUS* Histochemical Assay A) *GUS* expression in tomato leaf explants transformed by Biolistic gun method B) Control (Non-transformed tomato leaf explant)

Transformation of tomato using *cry2AX1* gene

Callus of tomato explantswas maintained overnight on osmotic media. The bombardmentwas carried out as standardized for *GUS*reporter gene. After bombardment, callus was incubated in the dark for two days at 25 ± 2°C. Initially, callus were placed on shoot induction medium of BAP 1 mg L^{-1} and IAA 0.2 mg L^{-1} without any selection agent i.e. kanamycin. Two weeks later callus were sub-cultured to fresh shoot induction medium containing kanamycin (50 mg L^{-1}) for selection. Putative transgenics were hardened in green house by transferring to pots.

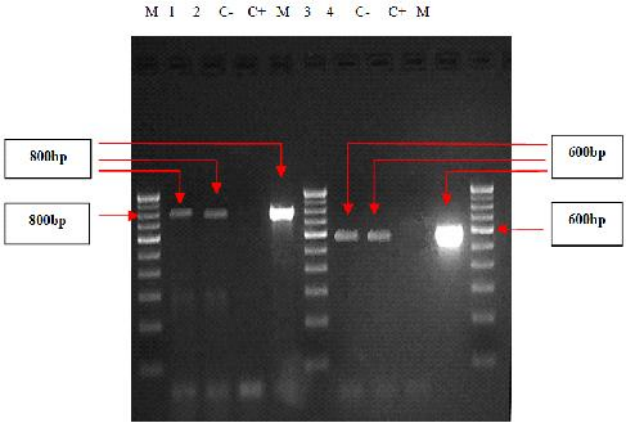

Fig. 4. PCR confirmation of putative transformants using *Cry2AX1*gene specific primers: Lane M: 100bp marker Lane 1, 2: C terminal 800bp Putative transformants with *Cry2AX1* gene; Lane 3, 4: N terminal 600bp Putative transformants with *Cry2AX1* gene;Lane C-: Negative control (Non-transformed plant DNA) Lane C+: Positive control (Plasmid DNA)

Molecular and Biochemical Analysis of Putative transgenic tomato plants

Polymerase Chain Reaction

Genomic DNA was isolated from young leaves of putative transgenic tomato plants using C-TAB method (Sambrook *et al.* 1993). The integration of the *cry2AX1* gene was confirmed by PCR using the primer i) C terminal: (a) Forward primer- 5' CCTAACATTGGTG GACTTCCAG (b) Reverse primer- 5' GAGAAACGA GCTCCGTTATCGT (ii)N terminal: (a) Forward primer- 5' AACGTTCTTAACTCTGGAAGGA (b) Reverse primer- 5' GCAGAAATTCCCCACTCATCAG. PCR was performed initially for 5 min at 95°C followed by 30 cycles of Denaturation at 94°C for 45 sec, annealing at 55°C for 40 sec and Extension at 72°C for 40 sec with a final extension at 72°C for 7 min and a final hold at 4°C (Hanumantappa 2006).

Quantitative ELISA

The ELISA kit used in the study was manufactured by Enviro Logix Co., Portland, USA. The Kit is a "sandwich" Enzyme Linked Immuno Sorbent Assay (ELISA) that uses antibodies and colour change to identify protein. The protocol was followed as mentioned in the Kit.

In the test, sample extracts were added to test wells coated with antibodies. Any residues present in the sample extract bind to the antibodies, and are then detected by addition of enzyme conjugate. The Stop Solution used is 1.0 N Hydrochloric acid that gives bluecolour. After a simple wash step, the results of the assay were visualized with a color development step; colour development is proportional to cry2AX1concentration in the sample extract.

Statistical analysis

Each treatment had four replicates with 16 explants and all the experiments repeated thrice to see the reproducibility of the results. The data were reported as mean standard error and means were analyzed by one-way ANOVA (Gomez and Gomez 1984).

Results

Plant regeneration by indirect organogenesis

Surface sterilized, leaf explants of tomato were cultured on MS medium supplemented with different concentrations of BAP and IAA. After 15 days of culturing, callus initiation and shoot regeneration were

observed from the abaxial side of leaf explants. Repeated experiments revealed that callus induction (100%) and an average number of shoots per explants (3.9) was highest in the combination of 1.0 mg L^{-1} BAP with 0.2 mg L^{-1} IAA (Table1 and Figure 2a, 2b). The rate of shoot multiplication was increased with each subculture and rooting in few explants was obtained when cultures kept for a longer time on same media which can be attributed to the IAA present in media. Butrhizogenesis of most of the explants was obtained on the media supplemented with 2 mg L^{-1} IBA (1.06) which differ significantly over all other treatments (Table 2). After acclimatization rooted shoots were transplanted into pots and maintained in green house (Figure 2c). Percentage survival of hardened plants was found to be 60%.

Effect of Kanamycin concentration

In vitro selection of explants for their intrinsic resistance against kanamycin was carried out at varying concentrations. The frequency of regeneration on antibiotic-free medium (control) reached 100.0%. But regeneration frequency decreased rapidly when kanamycin concentration was increased. Approximately 70% shoots survived at a concentration of 25 mg L^{-1}. Kanamycin at 50 mg L^{-1} completely inhibited cell division from non-transformed explants. At a concentration of 75 and 100 mg L^{-1}, explants remained green for initial days than 90% of explants bleached and died. Taking these results into consideration, to minimize escape and prevent necrosis, 50 mg L^{-1} concentration of kanamycin was used for the selection and regeneration of tomato leaf explants with the *cry2AX1* gene.

Biolistic Gun Transformation

Transformation of tomato with GUS reporter gene (pABC plasmid construct)

GUS assay was performed for the leaf explants bombarded with GUS gene after keeping on regeneration media along with 50 mg L^{-1} of kanamycin (selection agent). The explants with bluecolour development confirmed the presence of thegene of interest (Figure 3). Thus, showing the transformation efficiency in tomato cv. Pusa Ruby plants by biolistic gun method.

Genetic transformation and regeneration of putative transgenics bombarded with *Cry2AX1* gene

To obtain regeneration of transgenic plants, the bombarded calli (Figure 4a) were cultured on selective media allowing shoot initiation, shoot elongation and root formation. In all the 3 levels shooting was obtained but high transformation efficiency (75%) was observed in explants bombarded twice at level 2 (9 cm away from macro carrier launch assembly). Experiment was repeated thrice to check the reproducibility of results. The putative transgenic plantlets regenerated were morphologically identical to non-transgenic plants considered as control, but their development and growth were slower. As a consequence of this delay, which was probably caused by kanamycin, plantlets were obtained only after 8 weeks.

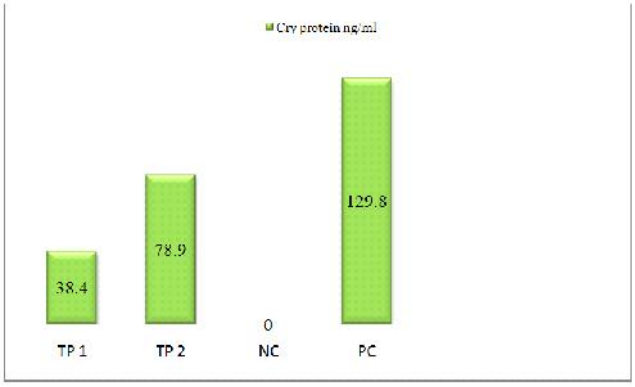

Fig. 5. Quantitative ELISA- TP 1: Protein sample from transgenic plant 1 TP 2: Protein sample from transgenic plant 2 NC: Negative control (protein sample of non-transformed plant) PC: Positive control

The average number of shoots was 3.75 per explant bombarded twice at level 2. The shooted plants were transferred to standardized rooting media for rooting. The average numbers of roots obtained were 4.0 per explants. Finally, 6 completely shooted and rooted plants were transferred to cups filled with potting mixture (soil, sand and vermicompost at 1:1:1 ratio). The cups with plants were finally placed in green house for hardening. Out of 6 putative transformed plants, only two plants were successfully hardened. The growth and development of these two putative transformed tomato plants were normal bearing healthy fruits (Figure 2d).

Molecular characterization

Polymerase Chain Reaction Analysis

To confirm the integration of the *cry2AX1* gene into the genome of two putative transgenic plants, a PCR analysis was carried out. Using the *cry2AX1* gene specific primer set, the expected 800 bp fragment of C terminal and 600 bp fragment of N terminal was found in both putative transformed plants along with positive control (plasmid DNA pCAMBIA2300) but not in control plants (Figure 4). The amplification of DNA samples of *cry2AX1* transformed plants confirms the integration of exogenous DNA into the plant nuclear genome.

Quantitative ELISA

ELISA is a rapid and sensitive technique by which an immunogen can be screened for a large number of samples. The absorbance of transgenic protein samples at 450 nm was 0.038 in transgenic plant 1 and 0.051 in transgenic plant 2. The values of transgenic protein samples were significantly higher than the negative control absorbance value of 0.001. The higher absorbance values of the transgenic plant samples indicated the immunogenic nature of the samples, confirming the presence of the recombinant*cry2AX1* gene. The *cry2AX1* protein concentration (ng mL^{-1}) in transgenic plant 1& 2 was 38.4 and 78.9 respectively; in comparison to positive control (129.8) (Figure 5).

Discussion

In recent years, a mass propagation and reliable regeneration procedure for vegetable crops have been established, because it would be a primary step to facilitate gene introduction and transferring new traits. The *in vitro* morphogenetic response of cultured plant tissues is affected by the different components of culture media, especially by concentration of growth hormones, and it is therefore important to evaluate their effects on plant regeneration (Guleria and Gowda 2015).

The combination of BAP 1.0 mg L^{-1} and IAA 0.2 mg L^{-1} proved to be the best for tomato regeneration using leaf explant. Similarly, the literature shows that the combination of IAA + BAP for shoot regeneration from different explants of tomato was found to be more effective (Chen *et al.* 1999; Chandel and Katiyar 2000). In contrast maximum number of multiple shoots formation was found at 0.2 mg L^{-1} IAA with 1.2 mg L^{-1} Zeatin using tomato cotyledon explant (Vikram *et al.* 2012) and Sidhu *et al.* (2014) observed highest number of buds (21.76) using cotyledon of eggplant at 2.5 mg L^{-1} BAP+ 1.0 mg L^{-1} Kin. The study by Sharma and Srivastava (2014) observed best shoot regeneration at 1.0 mg L^{-1} kin + 1.0 mg L^{-1} IAA. Therefore, optimum ratio of cytokinin to auxins is required for bud formation and shoot regeneration (Kaur *et al.* 2011). However, the reports from Mohamed *et al.* (2010) showed BAP alone can induce shoot organogenesis without auxin. He found media supplemented with 2 mg L^{-1} BAP was the best in the number of shoots (3.43) and shoot length (1.28) in thecase of both hypocotyl and cotyledon explants of tomato. This could be possibly due to the use of different explants in the studies. Therefore, type of explant used also had a significant effect on shoot regeneration studies. The success in regeneration response of tomato to growth regulators has been observedto genotype highlyspecific and as such, the type of explants and concentration of growth hormones suitable for one genotype may not be optimal for others (El-Farash *et al.* 1993). Amongst others, leaf explant is most widely used explant in tomato (Padmanabhan *et al.* 1974 and Kurtz and Lineberger 1983) mainly because of efficient regeneration from this tissue. Even Duzyaman *et al.* (1994) found that the degree of shoot regeneration was in the order of leaves $\geq$ cotyledonse $\geq$ hypocotyls. Thus,the plant regeneration is found to be influenced by the genotype, explant and type of medium as reported by Mamidala *et al.* (2011). Rooting in all the experiments were obtained when cultures kept for a longer time on same media. This may be due to the addition of IAA in regeneration media enhances the root formation. In this connection, Mensuali–Sodi *et al.* (1995) has stated that hormones are not essential for rooting as regenerated tomato contains endogenous phytohormones. In tissue culture, cytokinin interacts with auxin during the formation of plant organs such as shoots and roots (Ying *et al.* 2011). However, Oktem *et al.* (1999) reported that profuse rooting could be induced by using IAA at 0.2 mg L^{-1} and 0.5 mg L^{-1}, respectively. Loc *et al.* (2011) used IBA 0.5 mg L^{-1} for induction of rooting in atransgenic tomato plant.

Optimization of antibiotic concentration is aprior necessity to achieve stringent selection and confirm transgenic plants. Yepes-Martinez and Aldwinckle (1994) showed that *npt-II* gene conferring resistance to the aminoglycosideantibiotic; kanamycin was used in many early attempts of plant transformation and tends to inhibit regeneration strongly even at low doses. In this study, kanamycin concentration optimized at 50 mg L^{-1} for screening transgenic plants is in line with the study conducted in our lab by Guleria and Gowda (2015), Khin *et al.* (2006). Even the study carried out by Ruturaj *et al.* 2014 also used kanamycin at 50 mg L^{-1} to select transformants.

Ramachandra *et al.* (2014) achieved maximum transformation efficiency in rice when tissue was bombarded at 1100psi and target cell distance of 9 cm. These results are in line with the present study. Similarly, cotyledon explant of eggplant showed thebest regeneration when bomabarded at 9 cm target distance and 900 psi (Sidhu *et al.* 2014). Whereas study conducted by Abu El-Heba *et al.* (2008) achieved maximum transformation efficiency (68.9%) when hypocotyls were bombarded twice at 1350psi and target cell distance of 6cm. Even in thecase of *C. forskohlii* transformation by Guleria and Gowda (2015)achieved high transformation efficiency at 6 cm distance (1100psi). The psi, distance between sample and macro carrier and some

bombardments vary depending on type of explants used for the study. While low regeneration efficiency obtained after the attack might be due to harmful effects of this technique on the plant tissues. The work by Sidhu *et al.* (2014) showed thepresence of holes on explants bombarded at 1100 psi causing acute injury and reduced regeneration ability. Bombarding the explants twice increased the number of cells giving positiveevents and this could be because multiple bombardments allow better coverage of the target areas and compensate for misfire from faulty and poorly set rupture disc as was the case in rice (Wang *et al.* 1988). While Sanford *et al.*(1993) found that addition of osmoticum to the bombardment media increase the efficiency of transformation in some tissues, since it provides osmotic support for the tissue and minimizes cell damage.

About present study Arvinth *et al.* (2010) and Khan *et al.* (2013) have observed considerable protection against different lepidopteran insects and significant enhancement in productivity by incorporating cry1Ab insecticidal crystal protein gene in large number of crop plants mainly rice, tomato, maize, sugarcane, and cotton.

The integration and expression of *Cry2AX1* gene in two putative transformants was confirmed by PCR and ELISA. Thetwo putative transformants showed presence of 800bp band of C terminal and 600bp band of N-terminal. Thus, indicating probable insertion of *Cry2AX1* gene into the tomato plants. While the study conducted by Ruturaj *et al.* (2014) also confirms the present investigation.

Several workers have reported a wide range of Bt protein expression in transgenic plants. Independent plants of same genetic background and gene construct show greater differences in the level of expression (Hussain *et al.* 2002; Ramesh *et al.* 2004). To test the expression of Cry2AX1 protein in transgenic plants, quantitative ELISA was performed using antibodies specific to Cry2A protein, and the presence of Cry2AX1 protein was detected in green leaves. This result is similar to the study conducted by Ruturaj *et al.* (2014).

Conclusion

Standardized regeneration protocol was developed for the cv. Pusa Ruby grown throughout India. Biolistic Gun method of tomato transformation with *cry2AX1* gene resulted with transgenics of tomato showing expression of *cry2AX1*gene.

Acknowledgements

Authors are highly thankful to Department of Biotechnology (DBT), Government of India, New Delhi, for giving financial assistance to this project. Authors are also thankful to Dr. V. Udayasuriyan Professor, Department of Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore for providing *cry2AX1* gene construct.

References

Abu-el-heba, G.A., Hussein, G.M. and Abdalla, N.A. 2008. A rapid and efficient tomato regeneration and transformation system. *Agricultural Research* **58**: 103-110

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P.J., Fauquet, C., Huang, N., Kohli, A., Mooibroek, H., Nicholson, L., Nguyen, T.T., Nugent, G., Raemakers, K., Romano, A., Somers, D.A., Stoger, E., Taylor, N. and Visser, R. 2005. Particle bombardment and the genetic enhancement of crops: myths and realities. *Molecular Breeding* **15**: 305- 327.

Arumuganathan, K. and Earle, E. 1991. Estimation of nuclear DNA content of plants by flow cytometry. *Plant Molecular Biology Reporter* **9**: 208-218.

Arvinth, S., Arun, S., Selvakesavan, R.K., Srikanth, J., Mukunthan, N., Kumar, P.A., Premachandran, M.N. and Subramonian, N. 2010. Genetic transformation and pyramiding of aprotinin-expressing sugarcane with *cry1Ab* for shoot borer (*Chiloinfuscatellus*) resistance. *Plant Cell Reports* **29**: 383–395.

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

Chen, H., Zhang, J., Zhuang, T. and Zhou, G. 1999. Studies on optimum hormone levels for tomato plant regeneration from hypocotyls explants cultured *in vitro. Acta Agriculturae Shanghai* **15**: 26-29.

Duzyaman, E., Tanrisever, A. and Gunver, G. 1994. Comparative studies on regeneration of different tissues of tomato *in vitro*. *Acta Horticulturae* **24**: 235-242.

El-farash, E.M., Abdalla, H.I., Taghian, A.S. and Ahmad, M.H. 1993. Genotype, explant age and explant type as effecting callus and shoot regeneration in tomato. *Assuit Journal of Agricultural Sciences* **24**: 3-14.

FAO Statistical Database, 2014. FAOSTAT Agriculture data. http://faostat3.fao.org/ faostat-gateway/ go/to/download/Q/QC/E. Accessed 5 April 2014.

Gomez, K.A. and Gomez, A.A. 1984. Statistical Procedures for Agricultural Research. Intl Rice Res Inst, 2nd edn. Wiley and Sons, New York.

Guleria, N. and Gowda Ramanjini, P.H. 2015. An efficient regeneration and transformation protocol for *Coleus froskohlii* using gene gun. *International Journal of Agriculture, Environment and Biotechnology* **8**(2): 227-235.

Hanumantappa, P. 2006. Standardization of regeneration and transformation protocol for muskmelon with hepatitis B surface antigen. Dissertation, University of Agricultural sciences.

Hussain, T., Asad, J., Maqool, S.B., Datta, S.K. and Riazuddin, S. 2002. Variability in expression of insecticidal *cry1Ab* gene in indica Basmati rice. *Euphytica* **128**: 121-128.

Jefferson, R.A., Kavanagh, and Bevan, M. 1987. GUS fusions: β-glucuronidase as fusion marker in higher plants. *Eur Mol Biol Organ J* **6**: 3901-3907.

Kaur, M.A., Dhatt, A.S., Sandhu, J.S. and Gosal, S.S. 2011. *In vitro* plant regeneration in brinjal from cultured seedling explant. *Indian Journal of Horticulture* **68**(1): 61-65.

Khan, G.A., Baksh, A., Ghazanfar, M., Riazuddin, S. and Husnain, T. 2013. Development of transgenic cotton lines harbouring a pesticidal gene (cry1Ab). *Emirates Journal of Food and Agriculture* **25**(6): 434–442.

Khin, M.L., Gowda, P.H.R., Chandrashekar, S., Gowda, T.K.S., Vasundhara, M., Nagesha, N., Virupakshagouda, U.P., Sreenevasa, S. and Manjunatha, T. 2006. *In vitro* regeneration and transformation of *Coleus forskohlii* with glucanase-chitinase gene. *J Nontimb for Prod* **13**(3): 193-197.

Koul, B., Srivastava, S., Sanyal, I., Tripathi, B., Sharma, V. and Amla, D.V. 2014. Transgenic tomato line expressing modified *Bacillus thuringiensis Cry1Ab* gene showing complete resistance to two lepidopteran pests. *Springer Plus* **3**: 84.

Kurtz, S.M. and Lineberger, R.D. 1983. Genotypic differences in morphogenic capacity of cultured leaf explants of tomato. *Journal of the American Society for Horticultural Science* **108**: 710-714.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Loc, N.H., Thinh, L.T., Yang, M. and Kim, T. 2011. Highly expressed cholera toxin B subunit in fruit of a transgenic tomato. *Biotech Bio Eng* **16**: 576-580.

Mamidala, P. and Nanna, R.S. 2011. Effect of genotype, explants source and medium on *in vitro* regeneration of tomato. *IInternational Journal of Genetics and Molecular Biology* **3**: 45-50.

Mensuali-Sodi, A., Panizza, M. and Tognoni, F. 1995. Endogenous ethylene requirement for adventitious root induction and growth in tomato cotyledons and lavandin microcuttings *in vitro*. *Plant Growth Regulation* **17**: 205-212.

Mohamed, A.N., Ismail, M.R. and Rahman, M.H. 2010. *In vitro* response from cotyledon and hypocotyl explants in tomato by inducing 6-benzylaminopurine. *African Journal of Biotechnology* **9**(30): 4802-4807.

Murashige, J. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Plant Physiol* **15**: 473-497.

Ohki, S., Bigot, C. and Mousseau, J. 1978. Analysis of shoot forming capacity *in vitro* in two lines of tomato (*Lycoperiscon esculentum* Mill.) and their hybrids. *Plant and Cell Physiology* **19**: 27-42.

Oktem, H.A., Bulbul, Y., Oktem, E. and Yucel, M. 1999. Regeneration and *Agrobacterium* – mediated transformation studies in tomato (*Lycopersicon esculentum* Miller). *Turkish Journal of Botany* **23**: 345-348.

Padmanabhan, V., Paddock, E.F. and Sharp, W.R. 1974. Plantlet formation *in vitro* from tomato (*Lycopersicon esculentum)* leaf callus. *Canadian Journal of Botany* **52**: 1429-1432.

Ramachandra, R., Kumar, V., Gowda, P.H.R., Pramila, C.K. and Ganapathy, 2014. Biolistic mediated transformation and transient-1,3 glucuronidase expression in Indica rice variety Mangala. *Bioinfolet* **11**(1A): 109-112.

Ramesh, S., Nagadhara, D., Pasalu, C., Kumari, A.P., Sarma, N.P., Reddy, V.D. and Rao, K.V. 2004. Development of stemborer resistant transgenic parental lines involved in the production of hybrid rice. *Journal of Biotechnology* **111**: 131-141.

Ruturaj, R.B., Naveenkumar, A., Nandeesha, P., Manikandan, R., Balakrishnan, N., Balasubramani, V., Sudhakar, D. and Udayasuriyan, V. 2014. Transformation of tomato with *cry2AX1* gene of *Bacillus thuringiensis*. *International Journal of Therapeutic Applications* **32**: 3-4.

Saker, M.M., Salama, H.S., Salama, M., El-banna, A. and Ghany, A.N.M. 2011. Production of transgenic tomato plants expressing *Cry2Ab* gene for the control of some lepidopteran insects endemic in Egypt. *Journal of Genetic Engineering and Biotechnology* **9**: 149-155.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1993. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T. and Christou, P. 2011. *Bacillus thuringiensis:* a century of research, development and commercial applications. *Plant Biotechnology Journal* **9**: 283–300.

Sanford, J., Smith, F. and Russell, A. 1993. Optimizing the biolistic process for different biological applications. *Methods Enzymol* **217**: 483–509.

Sharma, C. and Srivastava, D.K. 2014. *In vitro* plant regeneration from seedlings-derived explants of tomato (*Lycopersicon esculentum* Mill.*) International Journal of Agriculture, Environment and Biotechnology* **7**: 421-432.

Shelton, A.M. 2012. Genetically engineered vegetables expressing proteins from *Bacillus thuringiensis* for insect resistance. *GM Crops and Food: Biotechnology in Agriculture and the Food Chain* **3**: 175-183.

Sidhu, M.K., Dhatt, A.S., Sandhu, J.S. and Gosal, S.S. 2014. Biolistic transformation of *cry1Ac* gene in eggplant (*Solanum melongena* L.). *International Journal of Agriculture, Environment and Biotechnology* **7**(4): 679-687.

Tabashnik, B.E., Huang, F., Ghimire, M.N., Leonard, B.R., Siegfried, B.D., Rangasamy, M., Yang, Y., Wu, Y., Gahan, L.J., Heckel, D.G., Bravo, A. and Soberón, M. 2011. Efficacy of genetically modified *Bt* toxins against insects with different genetic mechanisms of resistance. *Nature Biotechnol* **29**: 1128–1131.

Vikram, G., Madhusudhan, K., Srikanth, K., Laxminarasu, M. and Swamy, N.R. 2012. Zeatin induced direct multiple shoots development and plant regeneration from cotyledon explants of cultivated tomato (*Solanum lycopersicum* L.). *American Journal of Cultural Sociology* **6**(1): 31-35.

Wang, Y., Klein, T.M., Fromm, M., Cao, J., Sanford, J.C. and Wu, R. 1988. Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. *Plant Molecular Biology* **11**: 433-439.

Yepes, L.M. and Aldwinckle, H.S. 1994. Factors that affect leaf regeneration efficiency in apple, and effect of antibiotics on morphogenesis. *Plant Cell, Tissue and Organ Culture* **37**: 257-269.

Ying-hua, S.U., Yu-bo, L., Zhang and Xian-sheng, 2011. Auxin–Cytokinin Interaction Regulates Meristem Development. *Molecular Plant* **4**: 616-625.