**Abstract**

Tomato (*Solanum lycopersicon*) is the most imperative vegetable crop cultivated across the world. In India, the tomato productivity is stumpy and one of the major limiting factors is the damage caused by tomato fruit borer *Helicoverpa armigera* (Lepidoptera: Noctuidae). At present there is no source of genetic resistance existing in tomato germplasm against this pest. The conventional methods for management of this particular pest are futile. Bt transgenic technology provides a secure and consistent means for management of this pest. With this analysis, the *cry2A* Bt transgenic tomato cv. Arka Vikas was developed for resistance to fruit borer through *Agrobacterium* mediated transformation. The *Agrobacterium* strain EHA 105 harboring pBINBT *cry2A* containing CaMV35S promoter, OCS terminator, the synthetic *cry2A* gene coding region and neomycin phosphotransferase II (*nptII*) selectable cassette was used for transformation. Transfomrants were obtained through efficient regeneration and transformation protocol developed in our lab. Using PCR and qualitative ELISA, confirmation of transgene integration and expression respectively were respectively examined. The detached leaf insect bioassays were performed with *in vitro* reared freshly hatched first instar neonate of *Helicoverpa armigera* larvae. Transgenic plants showed extensive resistance to *Helicoverpa armigera* and we found a significant mortality (95%) of *H. armigera* within 24 hr of bioassays and outcome of this examination on Bt tomato lines revealed that these transgenic lines were effective in management of *H. armigera*.

**Keywords:** *cry2A, Helicoverpa armigera, Agrobacterium, Tomato.*

**Introduction**

A new epoch has been reputable in area of plant biotechnology addressing the major problems faced in related area making beneficial progress in agriculture to increase the productivity by minimizing the losses occurred. Tomato is second most (*FAOSTAT Database 2010*) important solanaceous vegetable crop cultivated in Indian sub continent for human consumption. In view of economy, it is a short duration high yielding dietary vegetable crop consumed for nutrition. The major limiting factor in tomato production is *Helicoverpa armigera*, commonly called as tomato fruit borer (1) a polyphagous pes (2) affecting many crops and is largely distributed in its population. Application of chemical insecticides is the common practice followed for control of insect pests. Insect pests have almost developed resistance to all major chemical insecticides and its repeated application led to perilous effect on humans (3). An alternative strategy is required for the control of these insect pests. Bt transgenic technology provides a safer
and reliable means for the control of insect pests and reported earlier in tomato (4).

Bt proteins are very much transient in nature, as the toxicity of Bt proteins is specific to insect pests (5). Cry toxins significantly proved active against lepidopteran (6) and other insect pests in case of transgenic crops (7). Bt crops shows a promising way for insect control that are highly insect specific (8). All Bt crops are environmentally safe and globally 58 million hectares Bt crops were cultivated (9) and can majorly reduce amounts of chemical pesticides entering the environment. However, in few cases there is report of resistance developed to this Bt crops which demands finding of other alternative cry genes. In this regard, cry2A gene was opted since earlier studies on cotton plants harboring this gene showed increased resistance to Helicoverpa armigera (10, 11). Plant tissue culture is being the major catalyst in genetic transformation and using Agrobacterium tumefaciens (12) the transformation and regeneration of tomato using cry2A gene was introduced into local cultivar Arka Vikas (13) using MS media along with other growth regulators (BAP, IAA). Agrobacterium mediated gene transformation was performed by co cultivation for production of Bt transgenic tomato plants, since Agrobacterium mediated gene transfer have an added advantage over other biolistic gene transfer methods being genotype independent (14). In vitro regeneration of cultivated tomato has been a subject of research (15) and Bt transgenic tomato against Tomato fruit borer Helicoverpa armigera is being done due the commercial value of tomato crop and its amenability for further improvement via genetic manipulation.

**Materials and Methods**

The experiment was conducted at the Biopesticides laboratory, Division of Biotechnology, Indian Institute of Horticultural Research Bangalore. H. armigera larva were reared in a controlled environment (25 ±2°C, 65±5% R.H.) and freshly hatched neonate larvae were used for challenging and observation. The transgenic plants raised by the means of Agrobacterium mediated transformation. This transformation was mainly done using hypocotyls part as explants. Shoot regeneration were achieved in the transformed explants by different hormonal combination like (BAP, IAA), then the root were induced by the hormone IBA followed by hardening in glasshouse. The transformed explants were isolated from the non-transformants in the kanamycin selection media. The presence of transgene was confirmed by polymerase chain reaction using nptII (for kanamycin resistance) and cry2A gene specific primers. The protein expression was confirmed qualitatively by lateral flow immunodiagnostic assay method (Bt strip method, Desigen™ Jalna). The molecularly confirmed transformants were challenged with in vitro reared freshly hatched neonate larvae of Helicoverpa armigera Hubner. The bioassays performed in vitro and results of this testing were discussed.

**Plasmid and bacterial strains used:** Plasmids pBin cry2A were transformed into Agrobacterium strain EHA-105 along with the gene cry2A, selectable marker nptII – (neomycin phosphotransferase II) which was used for selection of transformed plants (Fig.1).

**Plant transformation:** Tomato seedlings were grown aseptically on half-strength Murashige and Skoog (MS) medium. Hypocotyls from 8-10 day old seedlings were used for co-cultivation with A. tumefaciens (16). Overnight grown A. tumefaciens containing gene in modified M9 medium for 24 h at 28°C and diluted 20-fold before use. Explants were co-cultivated with diluted A. tumefaciens containing cry2A gene. The explants were incubated in Petri dishes on regeneration/selection medium containing MS salts, 3% sucrose, 100 mg/l kanamycin, 250 mg/l Cefotaxime and 0.25% Gelrite (pH 5.7-5.8). The culture conditions were maintained at 25°C, 16 h photoperiod. The explants were regularly sub-cultured every two weeks. The regenerated shoots were grown on shoot elongation media and rooting were done on root induction medium containing MS salts, 3% sucrose, 50 mg/l
kanamycin. The rooted plants were transplanted into soilrite for hardening. After establishment, the plants were shifted to pots into the net house.

**Conformation of cry2A transgene by Polymerase chain reaction (PCR):** The presence of the transgene was investigated in the T₁ generation obtained from the selfing of the T₀ plants, by analyzing for the presence of cry2A transgene by PCR. Genomic DNA was isolated from the leaves of all putative transformants. PCR analysis was carried out by using the primers specific to nptII gene.

Forward primer: 5'-AGAAGAACTCGTCAAGAGGCGG-3'.
Reverse primer: 5'-GAACAAGATGGATTGCA CGCA-3'.

PCR was carried out using standard reaction mixture and thermal cycling was performed for 30 cycles using following parameters: 94°C-10 min; 54°C -90 sec and 72°C, 45sec.

**Insect bioassays:** *Helicoverpa armigera* was artificially reared in the laboratory on modified semi-synthetic diet(17) with slight modifications. The leaves of transformed plants hardened in glasshouse were collected and placed on moisture white filter paper (Fig. 3). The freshly hatched neonate larvae starved for 2-3 hr and released on leaves of control and transgenic leaves. Insect mortality data was recorded with 24 hr intervals. The experiment was repeated thrice for each plant and observation was taken along with confirmation of alive and dead larva after treatment under stereo microscope. All laboratory experiments conducted for bioassay were kept at 25±2°C, 65±5% RH.

Assessment of transgenic plants in the glasshouse for insect-resistance was also performed with challenge inoculation of tomato fruit borer *H. armigera* at glasshouse conditions and this test includes cry2A transgenic lines (T₁ generation), the cultivar cv. Arka Vikas as susceptible control for comparison. Using fine camel hairbrush two larvae were released on both fruit in plant and detached fruit simultaneously for bioassay. In detached leaf bioassay three larvae were released individually and observations were recorded.

**Results and Discussion**

The development of familiar execution procedure is necessary and reports in case of cotton indicate that Bt genes are tremendous against lepidopteran insects ensuing in elevated yields (18) and hence cry genes in case of tomato to local cultivars was employed. Earlier many researchers proved cry1Ab protein was efficient in control of tobacco hornworm but higher proteins was essential for tomato fruit worm, *Helicoverpa* species (19) and lack of resistant varieties in germplasm strongly motivates for new approach of control measures like adaptation of synthetic genes like cry2A that are highly specific against lepidopteran pests. This study includes Agrobacterium mediated transformation in tomato and molecular characterization of transgene and insect phenotyping in putative transformants. Diverse essential crops like cotton, brinjal, maize are transformed with synthetic Bt genes under the control of constitutive promoters such as CaMV 35S exhibited a wide range of levels of foreign protein expression in whole plant benefiting the farmers with less application of chemical pesticides. Many researchers in developing transgenic plants relay heavily on molecular characterization of transfectants and concentrate finally on phenotypic characterization like bioassay protocols. It becomes essential that phenotypic screening should be done first so that molecular characterization can be cross checked in bioassay positive plants reducing the input in molecular screening.

In the present study, Agrobacterium transformation in tomato and phenotypic assessment of CRY2A protein on *Helicoverpa armigera* was performed by insect bioassays for selection of high insect resistant lines. Four transgenic lines were developed and characterized(20) and PCR was performed for both gene specific and npt II (Fig. 2) primers and
positive lines were selected. The insect bioassay results indicate that CRY2A protein confers superior levels of resistance towards neonate larva of Helicoverpa armigera on expression of high levels of Bt toxin (Fig. 3). The concentrations of Bt protein expression varies from crop to crop (21) and among individual lines with similar Bt construct(22) and for stable expression throughout the plant 35SCaMV promoter was used for enhancing the production of Bt protein in transgenic plants. Previous reports have shown that use of 35SCaMV promoter resulted in a constitutive and enhanced production of transgene derived protein (23). From in vitro larval insect bioassays and resistance phenotyping (24) using 1st instar larvae of H. armigera on the transgenic plants (T1 generation) proved that Bt protein expression is lethal to neonate larva. In this study, we have maintained two groups of larva which among one group is allowed to feed on control tomato leaves, whereas other group of larva were allowed to feed on Bt cry2A transgenic plant leaves. Here we observed mortality only in larva group which fed on transgenic leaves (Fig.4). This indicates that the observed mortality perhaps due to consumption of cry protein. Over expression of CRY2A Bt protein may have a negative impact on growth, development of H. armigera (25). Larval mortality in challenged neonate larva was 90 to 95% on Bt plants except those expressed less protein and in control plants the larva entered into next instar stages. A significant difference was observed regarding mortality between control and treated larval groups and a critical difference (CD) at 5% showed a range from 5-9% during 1st day to 3rd day of post-treatment in case of transgenic leaves fed larval groups compared to control (Table 1). Plants which showed relatively high levels of CRY2A protein expression gave the better response for causing larval mortality. This study is a preliminary analysis in order to understand whether these Bt transgenics were having any effect on larvae or not and we found that these transgenic are effective in inducing mortality of H. armigera. Further studies are required in order to fully characterize a transgenic plant which is essential in releasing these transgenic plants for commercialization.

Conclusions

Tomato (Solanum lycopersicum) being an agronomically significant solanaceae crop grown primarily for its nutritional benefits and commercial values. Tomato is prone to many diseases and pests causing serious crop loss economically. Numerous chemical insecticides have been applied for controlling the propagation of harmful insects affecting food chains. The insect populations are resistant to many chemical insecticides leading to opt the most efficiently preferred toxins released by Bacillus thuringiensis. However analysis showed consistently high expression of cry2A gene in the transgenic plants exhibited total protection against the target pest in neonatal stages. Insect bioassay clearly indicates, cry2A transgenic tomato gave total 95% mortality of neonate larva of Helicoverpa armigera. Some lines transgenic tomato having cry2A gene developed during this experiment showed high mortality rates of H. armigera larvae proving their potential in insect controlling strategies and this also may help in delaying the resistance development to Bt toxins. This phenotyping screening based challenge inoculation in other crop species can be utilizes for genetic improvement as well as for diverse investigations in the field of transgenic screening. This study is an example in demonstrating the effectiveness of cry2A gene in controlling of H. armigera and this demonstration could be precedent for Bt transgenic mediated management of other pests in other crops.

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References


Phenotypic assessment of Transgenic tomato


