Genetic Transformation of Tomato Using Bt Cry2A Gene and Characterization in Indian Cultivar Arka Vikas

V. S. Hanur, B. Reddy*, V. V. Arya, and P. V. Rami Reddy

ABSTRACT

Transgenic tomato plants of south Indian cultivar Arka Vikas were developed using Agrobacterium strain EHA 105, harbouring Bt Cry2A gene with a construct containing 3SS CaMV promoter, OCS terminator and nptII selectable marker, through Agrobacterium-mediated transformation. This study was conducted to improve the regeneration and transformation protocol for south Indian cultivar Arka Vikas. Hypocotyl was used as explant source for transformation due to high regeneration efficiency, molecular analysis through PCR for putative transformants in T₀ generation and qualitative ELISA method was performed for Bt protein expression followed by insect bioassays. Insect bioassay studies was conducted using neonate larva of Helicoverpa armigera to screen the plants and the plants expressing good resistance with molecular and phenotypic characters were carried further for successive generations. The experimental results concluded that Bt gene was deployed in tomato cultivar successfully and had developed resistance to neonate larva of Helicoverpa armigera at laboratory conditions. These results signified that transgenic lines expressed substantial quantity of Bt Cry2A protein efficient in management of Helicoverpa armigera. Precise screening of transgenic T₁ lines is highly important to obtain single copy number plants since the expression of Bt protein in successive generations promotes effective management of this pest in the future.

Keywords: Agrobacterium, Bt Cry2A, Helicoverpa armigera, Transformation.

INTRODUCTION

Tomato (Solanum lycopersicum) is a major vegetable crop cultivated in India for its nutritional and commercial values (Mueller et al., 2005). It has been proved that lycopene, a major constituent in tomato, is known for its medicinal value in reducing the risk of cancer (Giovannucci, 1999), lowers the cholesterol levels (Sesso et al., 2004) and improving human diet. The major limiting factor in tomato crop productivity is due to lepidopteron pest Helicoverpa armigera (Hubner) (Atwal, 1986). This polyphagous pest is abundant in nature affecting many agricultural and horticultural crops (Zalcucki et al., 2002) across the world, causing huge economic failure in tomato production. The loss incurred due to this pest alone in various other crops like cotton, okra, pigeon pea including tomato is estimated to be 10,000 million rupees (Raheja, 1996). Control of this pest is becoming difficult even after spending nearly $600 million worth chemical pesticides (Ghosh, 2001). In India, chemical control of this pest is not always effective due to resistance development to all major pesticides. Biotechnology has become a powerful tool in developing transgenic crops (Chandrashekara et al., 2012) by introducing

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specific genes to combat against the lepidopteron pests. Bt transgenic technology, a suitable alternative option for efficient control of this pest, was proved earlier in case of Bt cotton (Gurinder and Chhabra, 2013). The techniques in genetic engineering highly promotes to alter various crops with biotechnology approach (Sidhu et al., 2010) with additional benefits.. The transformation studies in tomato were done earlier by many researchers for lycopene (Fraser et al., 2002), β-caratone (Romer et al., 2000) and other cry genes (Paramesh et al., 2010). Tomato plant is normally chosen among dicotyledonous for gene introduction (Wing et al., 1994) since in vitro plant regeneration protocol has been standardized with Agrobacterium-mediated transformation (McCormick, 1991). The hypocotyl explants for regeneration and transformation of Cry2A gene in tomato through Agrobacterium-mediated transformation were attempted (Vageeshbabu et al., 2011) for obtaining successful transformants. Regenerating explants on specific media (Madhulatha et al., 2007) is the routine procedure used in obtaining transformants in many crops. Tomato cultivar Arka Vikas is a major hybrid variety developed by IIHR Bangalore and extensively studied in transformation studies due to its adaptability to various stress conditions. The Agrobacterium-mediated transformation is preferred due to its success rate and simplicity in dicot species (Atkinson, 2002) and was standardized in south Indian tomato cultivar Arka vikas (Vageeshbabu et al., 2009). Insect resistant transgenic tomato plants with other cry genes have been reported earlier (Mandaokar et al., 2000) and the present activity is involved in development of Bt tomato plants containing Cry2A gene being effective against lepidopteron pests and resistance phenotype screening of transformed plants were done respectively. Molecular analysis including PCR (using nptII and Cry2A gene specific primers), bt protein expression analysis confirmation using dipstick ELISA method and insect bioassays were carried out for phenotypic resistant characters. Screening was also done for successive generations and the data obtained was recorded. Data on individual plant was recorded regarding Bt protein expression and phenotypic resistance (Vageeshbabu et al., 2012) towards challenge inoculation with first instar neonate larvae and later instar larvae on leaves of tomato fruit borer H. armigera were performed for further progress in development of insect resistant plants. Bioassays conducted on Bt lines showed moderate to high levels of resistance to tomato fruit borers.

MATERIALS AND METHODS

Seed Sterilization

The genuine breeder seeds obtained from Division of Vegetable Crops, Indian Institute of Horticultural Research (IIHR), Bangalore, and were pre-soaked in Captoff, a commercially available fungicide (CAP-50), at a concentration of 0.2% for ten minutes. The seeds were dried and then treated with GA3 (250 ppm) concentration for ten minutes, then used for surface sterilization, seeds were treated with absolute ethanol for 30-45 sec followed by double distilled water wash twice, a drop of tween 20 was added along with NaClO (Sodium hypochlorite) and was let for 6-8 minutes followed by doubled distilled water wash twice. The seeds were blot dried on autoclaved sterile tissue paper and placed in ½MS medium (Shadang et al., 2007) for seed germination.

Agrobacterium Strains

The Agrobacterium strain EHA 105 used was obtained from Dr. P. Ananda Kumar, Principal Scientist, NRCPB, New Delhi. This strain harboring binary vector pBinBt Cry2A gene along with 35S CaMV promoter, OCS terminator sequences and
Neomycin phosphotransferase (nptII) gene resistant to kanamycin was used as selection marker.

Agrobacterium Culture and Plant Transformation

A single colony of Agrobacterium containing Cry2A gene was taken for inoculation with autoclaved 5 mL YEP medium (Yeast extract, Peptone and NaCl) containing 50 mg L\(^{-1}\) kanamycin and was allowed to grow overnight in an incubator shaker at 28°C. The culture was diluted to obtain optimization for co-cultivation and hypocotyls from PreConditioning (PC) media selected were suspended in YEP medium containing Agrobacterium and left for 10-12 minutes. The hypocotyls were then blot dried on autoclaved sterile tissue paper and were placed back into PC media.

Regeneration of the Transformed Explants

The co-cultivated explants after agrobacterium mediated infection were transferred to SIM (Shoot Initiation Medium), subcultured twice every 2 weeks continuously onto fresh medium for shoot bud initiation, and later transferred to SEM (Shoot Elongation Medium) for shoot elongation. These explants were further subjected to RIM (Root Induction Medium) for inducing roots and were maintained for 2 weeks. Then, the plants were subjected to hardening by placing in autoclaved soilrite mixture and were transferred to greenhouse for acclimatization (Figure 1).

Molecular Characterization of Transformants

Molecular characterization was performed for putative transformants and its subsequent generations (Rashid et al., 1996). Genomic DNA was isolated using C-TAB method (Sambrook et al., 1989). Fresh leaf sample from T\(_1\) plants grown in greenhouse was taken for DNA isolation and the same DNA isolated from leaf samples was used for PCR analysis with nptII and gene specific primers.

Enzyme Linked Immunosorbent Assay

Qualitative dot ELISA (Lateral flow immuno diagnostic assay) was used for testing the presence of Cry protein in transgenic plants. Qualitative ELISA on

![Figure 1. Callus initiation from hypocotyls after transformation (a), Multiple shoots from transformed Calli (b).](image-url)
different samples were conducted at laboratory conditions from plants growing in insect proof transgenic nethouse, for determining the Bt protein expression. Cry2A Bt Xpresstrips™ (Desigen, Jalna) were used for accessing the Bt protein expression by following manufacturer’s instructions. The sample extraction was individually carried out for one single plant in each line, and leaf discs were obtained using the same micro centrifuge tube cap and discs were dropped directly into the tubes. Around 500 µL sample extraction buffer was added to each tube and using autoclaved separate micropestles, the leaf tissue was thoroughly crushed for 30 seconds to 1 minute. One Bt Xpresstrip™ was kept in the sample extract for 10 minutes incubation. A purple color band in the of Bt Xpresstrip™ indicates that strip is working, band in bottom line of strip with green color indicates the positive line, the results were recorded for different Bt lines, which varied in the protein expression (Figure 2).

**PCR Analysis of T₁ Transformants**

Individual plant were considered as discrete line during seed collection and maintained separately. The seeds of tomato plants were sown and maintained in greenhouse conditions. The grown plants were labeled individually. Confirmation of insertion of nptII and cry2A genes was performed using PCR specific primers and the results were tabulated. The oligonucleotide primers were obtained from Sigma® and the complete gene sequence for Cry2A gene was provided by Dr. P. Ananda Kumar (Principal Scientist NRCPB), primer sequence is given below.

**Primer Sequence for NPT II Gene**

Forward primer: 5’– AGAAGAACTCGTCAAGAAGGC–3’.
Reverse primer: 5’– GAACAAGATGGATTCACGCA–3’.

**Primer Sequence for Cry2A Gene**

Forward primer: 5’– ATGAACAACGTGCTCAACTCCGGAGGACA–3’.
Reverse primer: 5’– TTAGTAGAGTGCGGAAGGGTTGGTCGCAC–3’.

(See Figure 3.)
Insect Bioassays

Insect bioassays were performed to assess the potential of transformed tomato plants for cry2A gene presence and its gene expression phenotypically. Leaves from the control and transformed plants were collected for insect bioassays. Successful in vitro rearing of H. armigera was done using modified semi synthetic diet (Ahmed and McCaffery, 1991). Screening was also done for successive generations and the data obtained was recorded. Data on individual plant was recorded regarding Bt protein expression and phenotypic resistance (Vageeshbabu et al., 2012) towards challenge inoculation with first instar neonate larvae and later instar larvae on leaves of tomato fruit borer H. armigera, were performed for further progress in development of insect resistant plants. Bioassays conducted on Bt lines showed moderate to high levels of resistance to tomato fruit borers. Neonate larvae of H. armigera were utilized for insect bioassays and for insect cultures a standardized protocol developed in our lab had been selected. The evaluation of resistance phenotype under in vitro conditions is more beneficial in laboratory bioassays (Vageeshbabu et al., 2012) and was attempted during this bioassays. For in vitro bioassay, the challenge inoculation using freshly hatched neonate larva were performed on 45-day old tomato leaves (Figure 4), since the expression of Bt protein in leaves is crucial by contributing more in the control of this pest (Manjunath 2006). Regularly used petri dishes of tissue culture specimen plates (Tarsons) were utilized for detached leaf bioassay. The bottom surface was covered with autoclaved Whatman filter paper presoaked in sterile water was placed to maintain moisture in leaf content. Observations on mortality of dead larva were recorded.

Figure 3. PCR for NPTII (A), and CRY2A (B) genes.

Figure 4. Leaf Bioassay for Cry2A gene, (a), Dead larva from leaf bioassay (b).
Statistical Analysis

Standard deviations along with one way ANOVA for mean values were performed for all the mortality values (Table 1) (Figure 5).

RESULTS AND DISCUSSION

In the present study, synthetic Cry2A gene was introduced to tomato cultivar Arka Vikas and screened initially for molecular and phenotypic traits of resistance. Subsequent generations of transgenic plants were also screened for molecular and phenotypic traits of resistance. In general, transgenic plants are favorable in comparison with orthodox cultivation procedures by reducing the application of chemical pesticides, improving yield and quality of the crop, and reducing risk of environmental pollution caused by application of chemical pesticides. Insect resistant plants are an integral component of integrated pest management and Bt genes are effective sources against tomato fruit borer H. armigera. In developing transgenic plants for insect resistance, the Cry2A gene is expansively considered due to its strict binding action on brush border membrane vesicles of H. armigera (Hernandez-Rodriguez et al., 2008). Introduction of this Cry2A gene to local cultivars requires in-depth study in producing transgenic plants that are effective on lepidopteron and dipteran species (Saleem and Shakoori, 2010). Many researchers have reported transformation in many other diverse tomato cultivars already, but still an attempt made to transform south Indian cultivar Arka Vikas was due to its preference in tomato

Table 1. Percentage mortality of neonate larvae during detached leaf bioassay under lab conditions.

<table>
<thead>
<tr>
<th>Cry2A</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep 1</td>
<td>50</td>
<td>83.3</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Rep 2</td>
<td>50</td>
<td>66.6</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Rep 3</td>
<td>50</td>
<td>83.3</td>
<td>83.3</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Rep 4</td>
<td>33.3</td>
<td>66.6</td>
<td>83.3</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Rep 5</td>
<td>33.3</td>
<td>50</td>
<td>83.3</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Rep 6</td>
<td>33.3</td>
<td>66.6</td>
<td>83.3</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>SEM</td>
<td>41.65±3.7</td>
<td>69.40±5.1</td>
<td>88.87±3.5</td>
<td>100±0.0</td>
<td>100±0.0</td>
<td>0</td>
</tr>
<tr>
<td>CD at 5%</td>
<td>9.991</td>
<td>7.445</td>
<td>5.381</td>
<td>NS</td>
<td>NS</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 5. Daily data on percentage mortality of neonate larva H. armigera (Hubner).
cultivation across India. Various other cultivars like Pusa Ruby the outcome of transformation frequency was about 6% (Vidya et al., 2000) whereas in Micro-Tom cultivar around 40% transformations was reported (Sun et al., 2006) and transformation was studied in Arka vikas cultivars by which 18% transformation rate was attained (Figure 1). Transformation success is heavily dependent on gene integration and its optimum expression in plant and its successive generations. Results obtained from qualitative ELISA proved sufficient protein was expressed in transgenic lines (Figure 2) that showed good mortality against H. armigera. Confirmation of Cry2A gene integration was analyzed using PCR in T₀ and T₁ generations using gene specific primers (Figures 3-A and -B).

Analysis of variance was performed with mean values of mortality data. The larval mortality of challenged neonate larva was 85% to 90% on Bt leaves, except those expressed less protein and, in the control plant leaves, the larva entered into next instar stages. A considerable discrepancy was observed regarding mortality between the 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 in comparison with control plates (Figure 5). The initial larval stages of larva are more susceptible to Bt strains (Sneh et al., 1981) the same protocol repeated with Cry2A gene in neonate larval instars where mortality was 100% and proved Cry2A protein is highly effective at early stages of H. armigera larva. The larva fed on control leaves didn’t show any response and were healthier when compared to larva fed on Bt leaves and successfully completed its life cycle (Figure 4). Phenotypic screening with challenge inoculation of larva on tomato plants initially reduces the efforts for molecular characterization of transformants and its successive generations by reducing time.

CONCLUSION

The wide involvement of Agrobacterium for implementing transgenics through gene insertion has gained importance in agriculture to attain better transgenic plants. This method of gene delivery needs to be tuned highly for achieving success with good transformation rate. Tomato cultivar Arka Vikas was transformed with Cry2A gene through this technique and was standardized for efficient regeneration of transformants. Molecular screening and phenotype screening using H. armigera larva showed significant results in gene expression and insect mortality. The results obtained are a significant step in development of potential insect resistant cultivars in tomato. Several transgenic lines having BT Cry2A gene showed varietal expression. The future prospects of this study, like histopathological effects and protein expression studies on larval feeding, will be investigated in detail and the output will be published elsewhere.

ACKNOWLEDGEMENTS

Authors are highly grateful for ICAR “Network Project on Bt-Transgenic Tomato” for providing financial support to carry this research work.

REFERENCES


کالی‌یوکتار رایج گوشه فرگنگی در جنوب هند به نام Arka Vikas متعلق قرار گرفته است. ترکیب ترمیمی، زیست‌شناسی و شناسایی این کالی‌یوکتار در جنوب هند به نام Vikas به‌همراه یافته‌ی برای این کار از طریق بین‌المللی مطالعه و منابع، نشان می‌دهد که روش ترکیبی که شکلی از این کالی‌یوکتار باعث درد خوندی شده است، ترکیبی از طریق پی سی آر برای ترکیب ترخیص ساز‌های مصرفی Bt در نسل To روش کیفی ال‌ایزآ در بیان بروتنین Bt به دنبال آن زیست آزمون دختره‌ای اجرا شد. helicoverpa armigera (neonate) مطالعه زیست آزمون حشره‌ای با استفاده از لارو پوره‌ای برای غربال کردن گیاهان انجام شده و گیاهانی که مقاومت خوبی با مشخصات ملکولی مورفیکی نشان دادند برای نسل های بعدی ادامه داده شدند. نتایج پژوهش نشان داد که کار برد این Bt در گوشه فرگنگی منطقه بوده و این گیاه نسبت به لارو پوره‌ای آزمایشگاهی مقاومت نشان داد. این نتایج مویی آن است که لایه‌های ترکیب، بین مقدار قابل توجهی از بروتین Bt Cry2A و دفع رگه‌ها (لاین های) ترخیص T1 برای به دست آوردن تک نسخه‌ای گیاهان بسیار مهم است. زیرا این بروتنین Bt در نسل های پی در پی، منجر به ارتقای مدیریت موثر این آفت در آینده می‌شود.