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

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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 35S 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 T_0 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 T_1 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 polyphagus 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 GA_3 (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

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Neomycin phosphotransferase (nptII) gene resistant to kanamycin was used as selection marker.

*Agrobacterium C*ulture 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

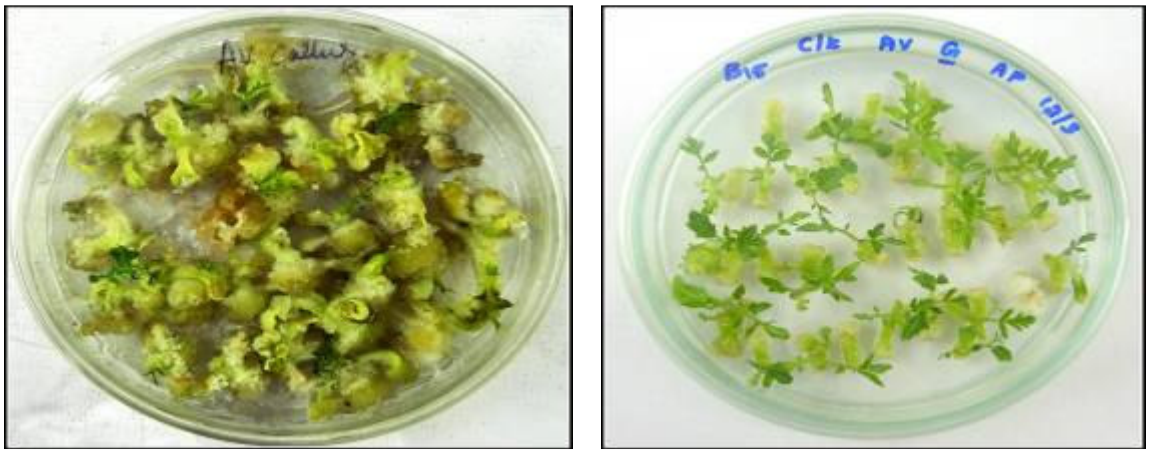
The co-cultivated explants after *agrobacterium* mediated infection were transferred to SIM (Shoot Initiation Medium), subcultured twice every 2 weeks continuously onto fresh medium for shoot bud initiation, and later transferred to SEM (Shoot Elongation Medium) for shoot elongation. These explants were 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 (Figure1).

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



(a) (b)

Figure 1. Callus initiation from hypocotyls after transformation (a), Multiple shoots from transformed Calli (b).

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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 (Figure2).

PCR Analysis of T_1 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'–*GAACAAGATGGATTGCACGCA*–3'.

Primer Sequence for Cry2A Gene

Forward primer: 5'–ATGAACAACGTGCTCAACTCCGGGAGGACA–3'.
Reverse primer: 5'–TTAGTAGAGTGCGGAAGGGTTGGTCGGCAC–3'
(See Figure3.)

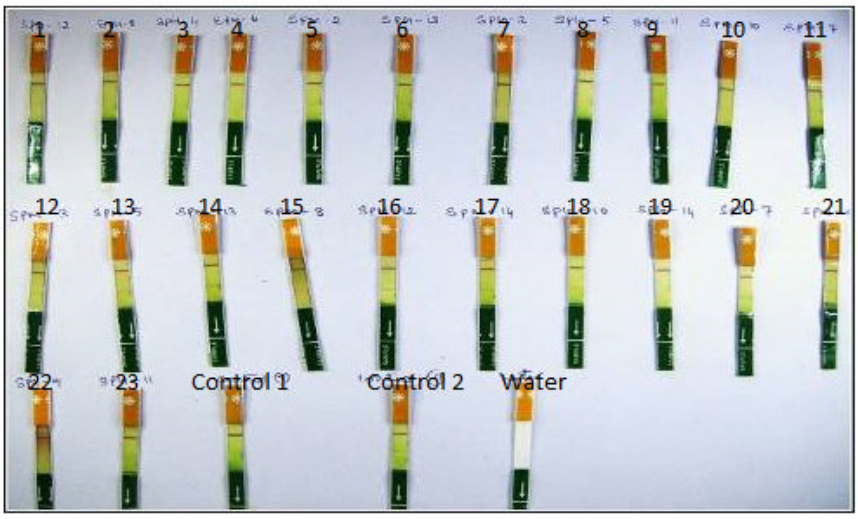

Figure 2. Qualitative dot ELISA for samples 1-23, two controls and water as negative samples.

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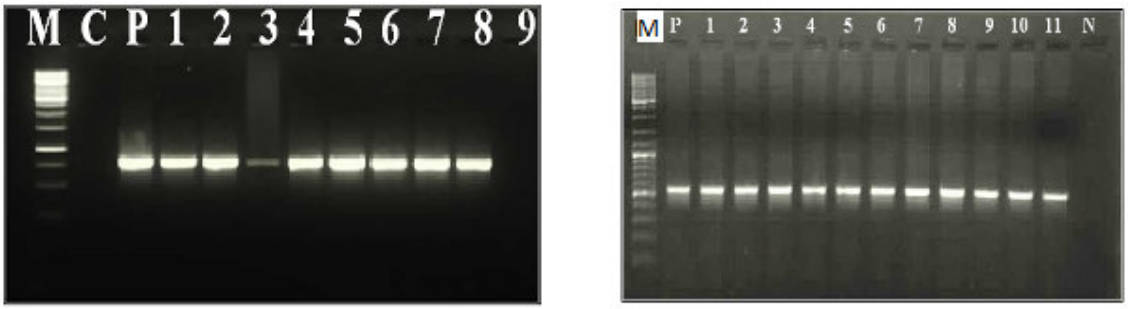
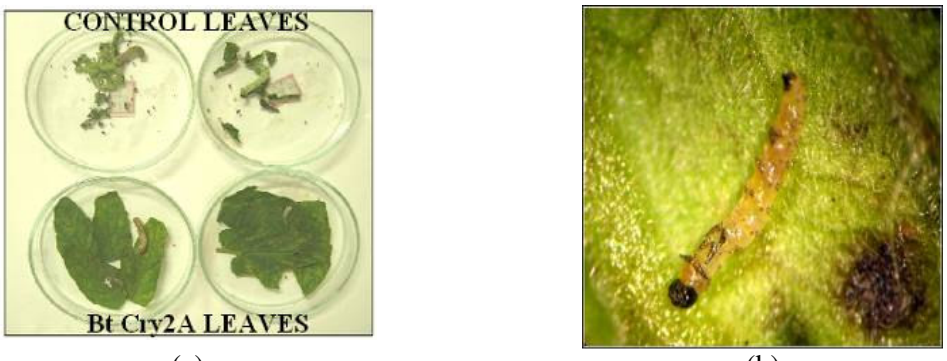


(a) (b)

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

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 larvas 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.



(a) (b)

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

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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.

Cry2A	Day 1	Day 2	Day 3	Day 4	Day 5	Control
Rep 1	50	83.3	100	100	100	0
Rep 2	50	66.6	100	100	100	0
Rep 3	50	83.3	83.3	100	100	0
Rep 4	33.3	66.6	83.3	100	100	0
Rep 5	33.3	50	83.3	100	100	0
Rep 6	33.3	66.6	83.3	100	100	0
SEM	41.65±3.7	69.40±5.1	88.87±3.5	100±0.0	100±0.0	0
CD at 5%	9.991	7.445	5.381	NS	NS	0

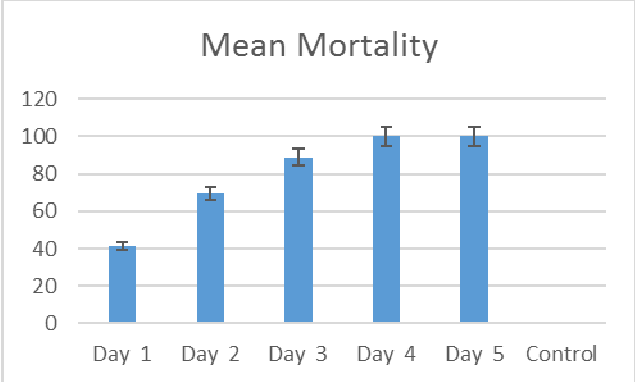

Figure 5. Daily data on percentage mortality of neonate larva *H. armigera* (Hubner).

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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 T_0 and T_1 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.

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تراریخت ژنتیکی گوجه فرنگی با کار برد ژن Bt *Cry2A* و مشخص کردن ویژگیهای آن در کولتیوار Arka Vikas

و. س. هانور، ب. ردی، و. و. آری، و پ. و. رامی ردی

چکیده

در این پژوهش، با استفاده از ریسه EHA 105 اگروباکتریوم که حامل ژن Bt *Cry2A* با سازواره (construct) حاوی آغازگر 35S CaMV و پایان دهنده OCS و مارکر انتخابی *nptII* بود،

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کالتیوار رایج گوجه فرنگی در جنوب هند به نام Arka Vikas تراریخته شد. پژوهش حاضر به این منظور انجام شد که روش باززایی و تراریختی کالتیوار رایج گوجه فرنگی در جنوب هند به نام Arka Vikas بهبود یابد. برای این کار ازهیپوکوتیل که کارآیی باززایی بالایی دارد به عنوان منبع ریز نمونه برای عمل تراریخت استفاده شد و تجزیه ملکولی از طریق پی سی آر برای تراریخت ساز های مفروض در نسل To و روش کیفی الایزا برای بیان پروتئین Btو به دنبال آن زیست آزمون حشره ای اجرا شد. مطالعه زیست آزمون حشره ای با استفاده از لارو پوره ای (neonate) *helicoverpa armigera* برای غربال کردن گیاهان انجام شد و گیاهانی که مقاومت خوبی با مشخصات ملکولی و فنوتیپیکی نشان دادند برای نسل های بعدی ادامه داده شدند. نتیجه پژوهش نشان داد که کار برد ژنBt در گوجه فرنگی موفق بود و این گیاه نسبت به لارو پوره ای (neonate) *helicoverpa armigera* در شرایط آزمایشگاهی مقاومت نشان داد. این نتایج موید آن است که لاین های تراریخت بیان مقدار قابل توجهی از پروتئین Bt *Cry2A*را داشتند که در مدیریت *Helicoverpa armigera* موثر بود.غربال کردن دقیق رگه های (لاین های) تراریخت T_1 برای به دست آوردن تک نسخه ای گیاهان بسیار مهم است زیرا بیان پروتئین Bt در نسل های پی در پی، منجر به ارتقای مدیریت موثر این آفت در آینده می شود.

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