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# AGROBACTERIUM-MEDIATED TRANSFORMATION OF TOMATO (LYCOPERSICON ESCULENTUM Mill.) USING A SYNTHETIC Cry1Ab GENE FOR ENHANCED RESISTANCE AGAINST TUTA ABSOLUTA (Meyrick)

*Hemaid I. A. Soliman*[1,2], *Fatma M. Abo-El-Hasan*[2], *Ayman S. El-seedy*[3] *and Yasser M. Mabrouk*[3]

***Address(es):***
[1]Plant Genetic Resources Department, Desert Research Center, El-Matariya 11753, Cairo, Egypt.
[2]Tissue Culture and Biotechnology Labs., Maryout Research Station, Desert Research Center, Alexandria, Egypt.
[3]Plant Genetic Department, Faculty of Agriculture, Alexandria University.

*Corresponding author: **hahemaid@yahoo.com**

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**ABSTRACT**

The effect of different plant hormones on *in vitro* regeneration of tomato (*Lycopersicon esculentum* Mill.) two cultivars Castle Rock and Super strain B, obtained from hypocotyls and cotyledons of cultured *in vitro* seedlings was examined. The optimal concentrations of plant growth regulators were the MS medium containing 3 mg $L^{-1}$ of zeatin and 0.1 mg $L^{-1}$ of NAA of cotyledon and hypocotyl explants of Super strain B cultivar. While, the best regeneration medium was the MS medium containing 1 mg $L^{-1}$ ZEA, 0.5 mg $L^{-1}$ BAP and 0.1 mg $L^{-1}$ IAA of cotyledon and hypocotyl explants of Castle Rock cultivar. *Agrobacterium*-mediated genetic transformation system has been expanded of tomato cultivar Super strain B. Transformation efficiency of hypocotyl explants was studied with *Agrobacterium tumefaciens* strain GV3101 harboring binary vector pICBV19, containing *gus* and *bar* genes. Transformed tomato shoots were obtained from hypocotyls explants on MS medium contained with 3 mg $L^{-1}$ ZEA and 0.1 mg $L^{-1}$ NAA. The gene expression of *gus* and *bar* genes was tested in the putatively transformed tomato plantlets through GUS histochemical and leaf painting assays. The plasmid pBI121 containing an insect resistance gene (*cry1Ab*) and kanamycin gene (*nptII*) as a plant selectable marker were introduced into these explants. Putatively transformed plantlets were examined by PCR and southern blot analysis. The expression of *cry*1Ab gene was tested through insect bioassay. For insect bioassay, experiments were conducted to determine the mortality percentage of Cry1Ab toxin protein against *Tuta absoluta* (Meyrick). The results indicated that Cry1Ab toxin protein expressed in transformed tomato plants showed 100% instars larval mortality was obtained after feeding for 4–5 days.

**Keywords:** Transformation, transgenic, tomato, *Lycopersicon esculentum* Mill, cry1Ab gene, insect bioassay

## INTRODUCTION

Tomatoes *(Lycopersicon esculentum* Mill*)* are an integral part of human diet world wide. It ranks third in the world's vegetable production, next to potato and sweet potato, placing itself first as processing crops among the vegetables (**Bhatia *et al.*, 2004**). There are many factors that affect on *in vitro* plant regeneration of which most important of these factors are medium composition, plant hormones, temperature and light intensity (**Reed, 1999**). Many of the literatures have mentioned about *in vitro* regeneration of tomato using different explant types such as; hypocotyl, cotyledon meristem, stems petioles, leaves, and inflorescences explants (**Moghaleb *et al.*, 1999; Raziuddin *et al.*, 2004; Brichkova *et al.*, 2002**). In addition, Several reports showed of shoots induction of tomato when used cotyledons, hypocotyl and leaf explants and cultured on MS medium with different concentrations of BAP, kinetin, TDZ and zeatin alone and also in combination with different concentrations of IAA (**Zelcer *et al.*, 1984; Ishag *et al.*, 2009**). Egyptian ministry of agriculture report showed (2009), the tomato cultivated area and productivity in Egypt is estimated by 4929 thousand tons from a total area of 265,200 hectares. Tomato farmers endured substantial damages in Egypt because of the very high incidence of some lepidopterous insect pests (**Saker *et al.*, 2011**). Lepidopterous insect limit production and have led farmers to use chemical insecticides deliberately, such as *Tuta absoluta* (**Vallejo, 1999**).The tomato borer *Tuta absoluta* (Meyriek) is a standout amongst the most critical lepidopteran insects connected with the tomato crops. T. absoluta has a high conceptive potential, females lay eggs on airy parts of their host plants and a one female can lay a sum of around 260-300 eggs during their life cycle and there might be 10-12 generations for each year (**EPPO, 2005**). *Tuta absoluta* is considered as a key pest of tomato both in the field and under protected conditions. Fruit production of tomato will decline greatly from direct feeding of pests and optional pathogens which may then enter through the injuries made by the pest. Tomato fruits lose as a result of this insect attack commercial value. It has been reported losses estimated at 60 to 100% of tomato fruits (**Cristina *et al.*, 2008**).

The larvae instar which is the main source of infection was difficult to control because of its presence into the plant. The larvae eat on mesophyell tissues and make unequal mines inside the leaf surface. Larvae can damage reach to 100% which happens all through the whole developing cycle of tomatoes (**Souza *et al.*, 1992; Marques and Alves, 1996**). *Bacillus thuringiensis* has been utilized to control different insects because of its eco-friendly nature, security and target specificity. Delta endotoxin protein produced in the bacteria, which the insect larvae ingests and its are broken up and activated under alkaline state in the insect midgut, thus releasing the active peptides that bind to particular receptors in the midgut epithelial cells and making pores in the epithelial membrane (**Charles *et al.*, 1996**). Transformed plants containing toxic proteins of *Bacillus thuringiensis* is realness offer as supportable and powerful technique to stop crop casualties due to *Helicoverpa armigera* (Hübner) infestation. Therefore, it is important to put a genetic transformation system for development of foreign genes into plants so as to produce resistant varieties (**Rashid and Bal, 2011**).

Crystal proteins from these bacteria are toxic to a large group of insects which are important from the economic point insect of pests. More than hundred cry genes encoding the crystal proteins have been isolated and labeled in 22 groups and diverse subgroups with respect to their amino resemblance (**Crickmore *et al.*, 1998; Schnepf *et al.*, 1998**). Crystal proteins for lepidopteron insects have a place with the cry1and cry2 groups (**Aly, 2007**). Cry1Ab is active against Lepidoptera (**Bradley *et al.*, 1995**).

The main objective of this search is to establish a transformation and regeneration systems of tomato cvs. Castle Rock and Super strain B using selectable marker and reporter genes. *Cry1Ab* gene was used for transforming tomato plants to protect the tomato plant against insects particularly tomato borer *Tuta absoluta* (Meyriek).

## MATERIALS AND METHODS

### Bacterial strains and plasmid vector

Agrobacterium strain GV3101 harbouring the binary vector pICBV19 containing the *gus* and *bar* genes (Figure 1) was used to adapt the transformation process in tomato (**Ehab *et al.*, 2015**).
*Cry1Ab* gene was subcloned into the *Bam*HI site of pBI121. Gene orientation was verified by double restriction digestion (*Eco*RI and *Hin*d III). The *cry1Ab* gene expression was under the control of the CaMV promoter and kanamycin resistance (*npt*-II*)* gene under the NOS promoter and transformed in *Agrobacterium tumefaciens* strain LBA 4404 were grown in LB medium (**Sambrook *et al.*, 1989**) supplemented with 50 mg/l rifampicllin and 50 mg/l kanamycin for two days at 28$^{O}$C on shaker (200 r.p.m.) and then used in tomato transformation methods.

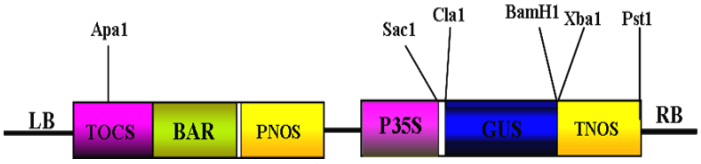


**Figure 1** Schematic presentation of pICBV19 binary vector containing the bar and *gus* genes.

### Tomato regeneration procedure and conditions

Source materials were obtained from tomato seedling cvs. Castle Rock and Super strain B grown for 10 days. Hypo-cotyledon and Cotyledon sections were used as explants because of their high efficiency of regeneration. In addition, tomato seedlings were grown *on* hormone free MS (**Murashige and Skoog 1962**) medium solidified with 0.25 % (w/v) phytagel and incubated at 25°C in the dark for 3 days then they were maintained under photoperiod of 16 h illumination for another 5 days. (Figure 2a). Cotyledons were explanted and passed in sterile medium to prevent desiccation and cut near the inner edge and the middle of the cotyledons (about 0.5 cm in length) to increase the area of infection surface. The explants were cultured under sterile conditions on the tomato regeneration media consisted of MS salts 4.3 g $L^{-1}$, sucrose 30g/L, myo -inositol 100 mg $L^{-1}$, containing different concentrations; 0.5-2.5 g $L^{-1}$ of benzyl adenine (BA), zeatin (Zn) (1.0- 3.0 mg $L^{-1}$) in combination with 0.1 mg $L^{-1}$ IAA or 0.1 mg $L^{-1}$ NAA. The pH of the medium was adjusted to 5.6 by KOH befor adding the phytagel and after that put in an incubation room at a temperature of 25±2°C under 16 hours photoperiod of 3000 Lux. In addition, the MS salts without growth regulators were used as a standard tomato regeneration medium.

### Shoot elongation

To promote the elongation of small shoots, the shoots (between 0.3-0.5 cm in length) were transferred to the elongation medium which contained the (1.5 mg $L^{-1}$) of Gibberellic acid (GA3) and were incubated at the same conditions as the regeneration step-conditions for four weeks.

### Root formation and acclimatization

Shoots about ~4 cm in length obtained from elongation stage were cultured on rooting media consisted of MS salts 4.3 g $L^{-1}$ with vitamins, sucrose 30g $L^{-1}$, myo-inositol 100 mg $L^{-1}$ in addition to different treatments of Indole Butyric Acid (IBA) (0.5-2.0 mg $L^{-1}$) alone or in combination with 0.5 mg $L^{-1}$ Naphthaleneacetic acid (NAA). Cultures were transferred to growth room under the same environmental conditions used for shoot regeneration. Plantlets produced from rooting stage were transferred from the test tubes under tap water to minimize injury and to free the roots from phytagel. The *in vitro* plantlets were transferred to plastic pots containing peatmoss and sand at equal volume (1: 1) in plastic pots and then covered transparent plastic bags and incubated under 3000 Lux light intensity for 16 hours photo period at 26 ±1°C in greenhouse. Three weeks later, plastic pots were opened completely and plantlets were kept up under incubation conditions.

### *Agrobacterium* mediated transformation of tomato plants

### Bialaphos sensitivity test

The herbicide resistance of non-transformed tomato tissues was determined by planting hypocotyl explants on MS salts basal medium with different concentrations, i.e., 0, 1, 2, 3, 4 and 5 mg $L^{-1}$ of Bialaphos phosphinothricin (PPT) using 15 explants for each concentration. Bialaphos was sterilized by filtration through filters (0.22 µn) and incorporated into precooled (45-50°C) autoclaved medium. The percentages of survival explants were recorded after 4 weeks from culturing.

### Transformation with *gus* gene

*Agrobactrium* strain (GV3101) containing the binary plasmid pICBV19 with *bar* as selectable marker and *gus* as reporter gene were used for adapting tomato transformation system. A single clone of bacteria was cultured over night in 5ml LB growth medium contained Streptomycin 50µg/ml, and kanamycin 100 µg/ml at 28°C in the dark on 220 r.p.m., then it was poured into a 50 ml LB medium and grown overnight at 28°C on shaker (220 r.p.m.). Overnight culture growth had an O.D. at 600 nm of between 0.5-1.0 and Cultures were centrifuged at 5000 g for five minutes. The supernatant was discarded and the *Agrobacterium* pellet was resuspended in 50 ml of MS salts medium –free hormones and then was incubated on at 28°C for shaken (175rpm) 2h before used. Hypocotyl explants of tomato *(Lycopersicon esculentum* Mill*)* cultivar Super strain were prepared and dipped in *Agrobacterium* suspension content on pICBV19 containing the *gus* and *bar* genes for 15 min. After incubation, the excess of bacteria were blotted on sterile filter paper and the explants were cultured on co-cultivation medium (without antibiotics) for two days at 28±2°C in the dark and then explants were planted on regeneration medium containing bialaphos compound and 250 mg/L cefotaxime. Transformation efficiency was determined by counting the number of explants expressing GUS.

### Transformation with *cry1Ab* gene

The binary vector pBI121contains the synthetic *cry*1Ab gene (modified for higher expression in plants) under the control of e35S promoter were used for transforming tomato explants using *Agrobacterium*-mediated transformation. The binary vector also contains the kanamycin resistance gene (*nptII*) as a plant selectable marker.
A single clone of bacteria was grown overnight in 5ml Liquid LB medium supplemented with 50µg/ml rifampicin and 50µg/ml kanamycin in 28°C and 180 rpm, then it was subcultured into a 50 ml LB medium and grown 5 h at 28°C with 180 rpm unit the growth reached O.D. 0.5-1.0 at 600 nm. Cells were collected by centrifugation at 5000 g for 5min. The supernatant was discarded and the *Agrobacterium* pellet was resuspended in 50 ml of MS medium. Suspended bacterial cells were shaken (175rpm) at 28°C for 2h before used.

### Detection of the transformed tomato tissues

### GUS histochemical assay

Transformation was confirmed by the GUS assay in different stages of regeneration of transformed explants (**Jefferson, 1987; Hemaid *et al.*, 2010**). Bialaphos resistant-shoots from regeneration medium were incubated in the substrate solution [(50 mM NaPO4 (pH 7.0), 1 mM EDTA, 0.1 % SDS, 100 µM ferrocyanid PO4) with 1 µg/l X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc, Sigma)] overnight at 37°C, then the tissues were cleared with 95% ethanol for easily visualization the blue staining.

### *Bar* gene expression

The *bar* gene which encodes the enzyme phosphinothricin acetyl transferase (PAT) (**Block *et al.*, 1987**), which inactivates phosphinothricin, the active ingredient of the herbicide bialaphos was used a selectable marker gene. The young transgenic plants (around ten weeks after acclimatization) were painted by 1 g $L^{-1}$ of BASTA solution. Resistances of leaflets were scored after 3-7 days.

### Polymerase chain reaction (PCR)

The DNA extracted from putatively transformed and non-transformed control tissues were subjected to PCR using a DNA thermal cycler (**Dellaporta *et al.*, 1983**). The oligonucleotide primers used for PCR amplification of a 1000bp fragment of the *gus* gene were: *GUS F*: 5- CAA CGT CTG CTA TCA GCG CGA AGT, *GUS R*: 5′- TAT CCG GTT CGT TGG CAA TAC TCC -3′ and for *cry1Ab* primer F: 5′-AGG AAG TTC ATT CAT TTG CAG-3′ *cry1Ab* R: 5′-TAA CTT CGG CAG GCA CAAAC-3′ was PCR amplification of a 1800 bp fragment of the *cry*IAb gene. The PCR was conducted in a volume of 50 µl containing a final concentration of each of the followed: 1X PCR buffer, 200 µM of each of dNTPs (dCTP, dTTP, dGTP and dATP), 2.5 mM MgCl2, 1 *p*moles from each of the used primers, 2 ng of plant DNA (as template), 0.04 U *Taq* DNA polymerase and $dH_2O$. The PCR profile consisted of one step of 95°C for five minutes, followed by 35 cycles at 94°C for one minute, 57°C for one minute, 72°C for two minuets and a final extension at 72°C for eight minutes. Applicants was visualized by agarose gel electrophoresis by loading 15 µl of PCR reaction mixture with 3 µl loading buffer on a 1% agarose gel and run at 80 Volt.

**Southern blot hybridization**

Southern blot hybridization of transformed plants was conducted by (**Sambrook *et al*., 1989**) which used the DIG High Prime DNA Labeling of detection transgenic plants. The DNA was extracted from leaves of transgenic and non-transgenic tomato plants. The 50 µg from genomic DNA was digested restriction enzyme with *Bam*HI (Promega) at 37°C over night and then separated on 0.8% agarose gel. DNA from agarose gel was shifted to a positively charged Hybond $N^+$ nylon membrane within of 20 x SSC solutions (NaCl + Na-Acetate, pH 7). Coding sequence of the *cry1Ab* gene (*Bam*HI fragment ~2.0 kb) was labeled with the DIG DNA labeling kit and used as a probe.

**Insect bioassay**

Leaves were derived from tomato transgenic and non-transgenic to be washed by autoclaved distilled water. *Tuta absoluta* larvae were collected from the vials and used in the bioassay, in the entomology testing chamber. The temperature in this chamber was maintained at 25-28°C and 85% humidity. Endotoxin protein produced in the transgenic tomato was observed by feeding the *Tuta absoluta* larvae on transgenic tomato leaves. Each plate contained on two leaves was infected with ten larvae. It has been closed with Parafilm and was incubated in 26-28°C in entomology testing chamber. This experiment was replicated three times. The numbers of live and dead insects were recorded 3 and 5 days after infestation. The mortality and the damage degree of the leaf were measured according to a previously described method (**Gallie *et al*., 1988**).

**Statistical analysis**

Data was analyzed using ANOVA and the differences among means for all treatments were tested for significance at 5 % level using **Duncan (1955)** new multiple range test as described by **Snedecor and Cochran (1967)**. Means followed by the same letter are not significantly different at $p<0.05$.

**RESULTS AND DISCUSSION**

**In vitro regeneration of tomato**

Growth regulators that were able to initiate shoots formation *via* direct somatic embryogenesis are presented in Table 1. The adventitious shoot formation depending on the explant type and plant growth regulators concentrations was added to the regeneration medium. The best results of the shoot formation percentage (96 and 98%) were obtained on MS medium supplemented with 3 mg $L^{-1}$ ZEA and 0.1 mg $L^{-1}$ NAA from cotyledon and hypocotyl explants in Super strain B cultivars, respectively (Table 1**)**. While, the best results of the shoot formation percentage (93 and 95%) were obtained on MS medium supplemented with 1 mg $L^{-1}$ ZEA ,0.5 mg $L^{-1}$ BAP and 0.1 mg $L^{-1}$ NAA from cotyledon and hypocotyl explants cultivar Castle Rock, respectively (Figure 2b). The best results of mean number of shoots formed per explant (2.65) from cotyledons explants and (2.74) from hypocotyl explants were produced on concentration 1 mg $L^{-1}$ ZEA and 0.1 mg $L^{-1}$ NAA of Super strain B cultivar (Figures 2c and 2d). While the best results for mean number of shoots primordial per explants (2.45) from cotyledons explants and (2.62) from hypocotyl explants were obtained on MS medium containing 1 mg $L^{-1}$ ZEA, 0.5 mg $L^{-1}$ BAP and 0.1 mg $L^{-1}$ IAA of Castle Rock cultivar, compared to the other treatments.

In two cultivars studied, the best of *in vitro* regeneration was observed when using hypocotyl explants with concentrations 3 mg $L^{-1}$ ZEA and 0.1 mg $L^{-1}$ NAA of Super strain B or 1 mg $L^{-1}$ ZEA, 0.5 mg $L^{-1}$ BAP and 0.1 mg $L^{-1}$ IAA of Castle Rock (Figure 2e). These outcomes concur with those got by **Habib *et al.* (2009)** reported that the induction of shoots were induced from two types of explants (cotyledons and primary leaves) of tomato cv. Rio Grande were obtained when two explants were grown in media containing 1 mg/L and 0.1 mg/L IAA which gave the high optimal shoot regeneration compared to the medium containing BAP and IAA it was relatively low of in vitro adventitious buds. In addition **Ehab *et al*. (2015)** who reported *in vitro* regeneration from hypocotyls and cotyledon explants of tomato and they fond that the most effective in shoots induction on MS medium containing 1 mg/l ZEA, 0.1 mg $L^{-1}$ IAA and 5 mg $L^{-1}$ sliver nitrate in the cultivar Pusa Ruby. However, in the cultivar Super Strain B with MS medium containing 1 mg $L^{-1}$ ZEA, 0.1 mg $L^{-1}$ NAA and 5 mg $L^{-1}$ sliver nitrate from cotyledons and hypocotyls explants. In our experiments, shoot elongated was observed when cultured shoots on MS medium containing 1.5 mg $L^{-1}$ GA3 of two cultivars Castle Rock and Super strain B (Figure 2f). Research results agreement with those obtained by **Ehab *et al*. (2015)** they observed that elongated shoots were obtained with MS medium containing 1.0 mg $L^{-1}$GA3 and 0.5 mg $L^{-1}$ 2iP of Pusa Ruby and Super strain B cultivars.

**Table 1** Effect of MS medium supplemented with different growth regulators on shoot formation from cotyledon and hypocotyl explants of tomato cvs. Castle Rock and Super strain B

| MS medium with different combination of growth regulators (mg$L^{-1}$) | | | | Percentage of forming shoots primordial/explants | | | | Mean number of shoots primordial/explants | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Castle Rock | | Super Strain B | | Castle Rock | | Super Strain B | |
| ZEA | BAP | NAA | IAA | cotyledon | hypocotyl | cotyledon | hypocotyl | cotyledon | Hypocotyl | cotyledon | hypocotyl |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | $0.00^{g}$ | $0.00^{g}$ | $0.00^{g}$ | $0.00^{g}$ |
| 1.0 | 0.0 | 0.1 | 0.0 | 57 | 60 | 69 | 82 | $1.12^{e}$ | $1.00^{f}$ | $0.98^{f}$ | $1.10^{f}$ |
| 2.0 | 0.0 | 0.1 | 0.0 | 68 | 71 | 85 | 86 | $1.23^{d}$ | $1.25^{e}$ | $1.24^{d}$ | $1.72^{c}$ |
| 3.0 | 0.0 | 0.1 | 0.0 | 62 | 68 | 96 | 98 | $1.35^{d}$ | $1.40^{d}$ | $2.65^{a}$ | $2.74^{a}$ |
| 1.0 | 0.0 | 0.0 | 0.1 | 75 | 78 | 72 | 72 | $1.65^{c}$ | $1.42^{d}$ | $1.05^{e}$ | $1.45^{d}$ |
| 2.0 | 0.0 | 0.0 | 0.1 | 78 | 80 | 76 | 77 | $1.25^{d}$ | $1.37^{d}$ | $1.58^{c}$ | $1.65^{c}$ |
| 1.0 | 0.5 | 0.1 | 0.0 | 72 | 77 | 90 | 91 | $1.35^{d}$ | $1.24^{e}$ | $1.92^{b}$ | $2.20^{b}$ |
| 1.0 | 0.5 | 0.0 | 0.1 | 93 | 95 | 87 | 81 | $2.45^{a}$ | $2.62^{a}$ | $1.23^{d}$ | $1.45^{d}$ |
| 0.5 | 0.5 | 0.1 | 0.0 | 88 | 85 | 80 | 84 | $1.32^{d}$ | $1.28^{e}$ | $1.75^{b}$ | $1.98^{c}$ |
| 0.5 | 0.5 | 0.0 | 0.1 | 90 | 91 | 83 | 79 | $2.00^{b}$ | $1.72^{c}$ | $0.75^{f}$ | $1.25^{e}$ |
| 0.0 | 1.0 | 0.1 | 0.0 | 58 | 62 | 69 | 72 | $1.00^{f}$ | $1.04^{f}$ | $0.48^{f}$ | $1.05^{f}$ |
| 0.0 | 2.0 | 0.1 | 0.0 | 76 | 72 | 71 | 78 | $1.15^{e}$ | $1.29^{e}$ | $1.65^{c}$ | $1.35^{e}$ |
| 0.0 | 1.0 | 0.1 | 0.1 | 79 | 80 | 77 | 89 | $1.25^{d}$ | $1.77^{c}$ | $1.78^{b}$ | $1.95^{c}$ |
| 0.0 | 2.0 | 0.1 | 0.1 | 83 | 85 | 88 | 90 | $1.92^{b}$ | $1.98^{b}$ | $1.72^{b}$ | $2.05^{b}$ |
| 0.0 | 3.0 | 0.1 | 0.1 | 87 | 88 | 55 | 69 | $1.64^{c}$ | $1.69^{c}$ | $0.85^{f}$ | $1.25^{e}$ |
| **LSD (0.05)** | | | | **1.89** | **1.83** | **1.78** | **1.92** | **0.45** | **0.46** | **0.42** | **0.44** |

**Rooting and acclimatization**

Data recorded in Table (2) indicates the percentage of shoots forming roots reached the highest value of (96%) and the best of mean number of roots formed on the explant (4.75) when using MS medium containing 1 mg $L^{-1}$ IBA of Castle Rock cultivar compared to the other treatments (Figure 2g) while, percentage of shoots forming roots (95%) and mean length of roots (5.64) treated with MS containing MS medium with 1.0 mg $L^{-1}$ IBA and 0.5 mg $L^{-1}$ NAA was significantly higher of super strain cultivar compared to the other treatments. Previous work also found that higher rooting responses (97%) for tomato were obtained after 28 - days in light on MS medium containing 1 mg $L^{-1}$ IBA and 0.5 mg $L^{-1}$ NAA (**Indrani *et al*., 2013**). For acclimatization, plants produced from rooting stage reaching 3- 5cm, were rinsed once with water and then transferred into pots containing equal parts of peat and sand, then incubated under transparent plastic bags on 16h photo period at 25°C for 4 weeks before transfer to greenhouse. After 8 weeks from transfer into greenhouse, they were repotted into sterile soil consisting equal parts of peat and sand (v/v) **(**Figure 2h). Some literatures have reported on acclimatization of tomato (**Mahmoud *et al*., 2013; Ehab *et al*., 2015**) they reported that the survival percentage of tomato plantlets produced from acclimatization was 100 %.


**Figure 2** Different stages of tomato (*Lycopersicon esculentum* Mill) cultivars Castle Rock and Super strain B *in vitro* regeneration and transformation. (a); germinated seeds of tomato on MS-based medium free-hormones. (b); induction of adventitious shoots from cotyledon and hypocotyl explants in cultivar Castle Rock on MS medium supplemented with 3 $mgL^{-1}$ ZEA and 0.1 $mgL^{-1}$NAA. (c and d); induction of adventitious shoots from cotyledon and hypocotyl explants in cultivar Super strain B on MS medium supplemented with 1 $mgL^{-1}$ZEA, 0.5 $mgL^{-1}$BA and 0.1 $mgL^{-1}$IAA . (e); the best frequency of regeneration was observed when hypocotyl explants were used. (f); Shoot elongated was observed when cultured shoots on MS medium supplemented with 1.5 mg $L^{-1}$GA3. (g); Young plantlet with well developed roots on selection medium. (h); A soil acclimated plantlet.(i); Mature transgenic tomato plants bearing fruit cultivar super strain B containing the *cryIAb* gene.

**Establishment of tomato (*Lycopersicon esculentum* Mill.) transformation system**

In this study, we have tried to improve the transformation efficiency of the tomato (*Lycopersicon esculentum* Mill.) cv. Super strain B.

***Agrobacterium*- mediated transformation of tomato**

Tomato transformation was carried out for the using *A. tumefaciens* strain GV3101 has the binary plasmid pICBV19 contains *bar* and *gus* reporter genes were used for adapting tomato transformation system. Explants were soaked with overnight *Agrobacterium* culture for 10 min and then explants were transferred to the MS medium –free hormones at 28 ± 2°C in the dark of two days. Subsequently, hypocotyledons explants were transferred to the regeneration medium added to it 2.5 mg $L^{-1}$ bialaphos to select transgenic tomato shoots and 300 mg $L^{-1}$ cefatoxime to avoid bacterial contamination. The cultures were incubated under similar conditions; bialaphos was added to select the transformed tomato cells while the cefatoxime were added to prevent *Agrobacterium* growth. After four weeks of incubation, Shoots were subcultured to fresh medium. Controls included hypo-cotyledons were cultured on regeneration medium without treatment with *Agrobacterium* and antibiotics.
Bialaphos resistant-explants were further evaluated for detecting and studying the expression of the transgenes using GUS assay. The transformation efficiency was calculated on the basis; the number of transgenic shoots per inoculated explants x 100. The shoot induction percentage of (*Lycopersicon esculentum* Mill.) cv. Super strain B decrease after incubated with *Agrobacterium* on the regeneration medium. The results indicated that the regeneration percentage was 42.5% compared to control regeneration percentage (98%) and thus the efficiency of genetic transformation resulting from this experience about 22.8 %. This percentage has agreed with that reported by **Frary and Earle (1996)** reported the *Agrobacterium*-mediated transformation in tomato cultivar Money maker and transformation efficiency of 10.6% was obtained. While, **Ellul *et al*. (2003)** found that transgenic plants ranged from 24.5% to 80% with tomato by *Agrobacterium*-mediated transformation. And **Sun *et al*. (2006)** mentioned that transformed cotyledon explants of tomato, Micro Tom cultivar with *A. tumefaciens* transformation efficiency of 40%. While, **Hasan *et al*. (2008)** reported the genetic transformation of tomato with *Agrobacterium* contains *Arabidopsis* early flowering gene AP1. The results obtained from the resistant plants of kanamycin gene about 87.9% while showed 12.07% with AP1 expression when explants were incubated at 48 hours with *agrobacterium* strains.

**Plant transformation with synthetic *cry1Ab* gene**

The binary vector pBI121 which harbor the insect resistance (*cry1Ab*) was used for transforming tomato (*Lycopersicon esculentum* Mill.) explants using *Agrobacterium*-mediated transformation. The binary vector also contains the kanamycin resistance gene (*nptII*) as a plant selectable marker. Hypocotyl explants of cultivar Super strain were collected in sterile Petri dishes under aseptic conditions. Explants were immersed in resuspended *Agrobacterium* culture for suitable time. After incubation, the excess of bacteria were blotted on sterile filter paper and the explants were cultured on co-cultivation medium (without antibiotics) for two days at 28±2°C in the dark and then leaf explants were planted on regeneration medium containing 125 mg $L^{-1}$ kanamycin and 300 mg $L^{-1}$ cefotaxime. The cultures were kept in the growth chamber at 26±2°C under 16 hours photoperiod of 3000 Lux supplied with cool white fluorescent lamps.

**Table 2** The efficiency of shoots forming roots for tomato cvs. Castle Rock and Super strain B after growing on MS medium supplemented with different auxin concentrations.

| **Auxin concentration** ($mgL^{-1}$) | | **% of shoot forming roots** | | **Mean number of roots/explant** | |
|---|---|---|---|---|---|
| **IBA** | **NAA** | **Castle Rock** | **Super strain B** | **Castle Rock** | **Super strain B** |
| 0.0 | 0.0 | 0.5 | 3.9 | 0.05f | 0.04f |
| 1.0 | 0.0 | 96 | 65 | $4.75^{a}$ | $2.29^{d}$ |
| 1.5 | 0.0 | 93 | 70 | $2.46^{d}$ | 1.69e |
| 2.0 | 0.0 | 90 | 87 | $3.32^{c}$ | $3.58^{c}$ |
| 1.0 | 0.5 | 89 | 95 | $4.25^{b}$ | $5.64^{a}$ |
| 1.5 | 0.5 | 84 | 91 | 1.55e | $4.15^{b}$ |
| 2.0 | 0.5 | 86 | 93 | 1.62e | $4.25^{b}$ |
| **LSD (0.05)** | | **1.64** | **1.52** | **0.48** | **0.43** |

**Gene expression in tomato plants**

Three methods were used in this study to detect the presence of the *gus and bar* genes in the putatively transformed tomato tissues; the histochemical assay, leaf painting assay and PCR analysis.

**Histochemical detection of GUS expression in putative transformants**

GUS enzyme activity was detected in the transformed tomato tissues that were resistance to bialaphos, by histochemical *gus* assay (**Jefferson *et al*., 1987)**. The use of 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as a substrate for the GUS enzyme provide an effective methods for monitoring the expression of GUS gene introduced into plant cells. X-gluc is the best substrate currently available for histochemical localization of β-Glucuronidase activity in tissue and cells **(Martin *et al*., 1992)**. This substrate works very well, giving a blue precipitate at the site of enzyme activity. Our results explained that GUS activity in transformed tomato explants which were co-cultivated with *Agrobacterium* could be detected histochemical for GUS expression after 4 weeks for shoot regeneration as the first evidence of transformation (Figure 3). Most of tissues turned to deep blue color compared with control. Transformed plants were incubated at 37°C in the GUS buffer with X-gluc for 24 hours after that treated with Clorox (commercial bleach) to remove effect of pigments.
Research results agree with the results obtained by **Ehab *et al*. (2015**) as they mentioned that for the results showed that the gus gene were detected in bialaphos resistant plant materials of tomato (*Lycopersicon esculentum* Mill.) cv. Pusa Ruby. Also, they found incubating the bialaphos resistant plant materials with GUS buffer contains X-gluc at 37 °C for 24 h then showed blue color of tested explants. Also, **Manoj *et al*. (2009)** they showed GUS expression level in different tomato explants of cv. Pusa Ruby results was non- uniform of gene expression, they showed blue color when incubated with b-glucorondiase substrate X-Gluc for 10–24 hours, while, showed very high expression of gus gene within one –two hours when incubated with b-glucorondiase substrate X-Gluc . The lack of GUS expression in some of bialaphos resistant shoots and positive PCR analysis which was recorded in some tissues may be due to change or gus gene loss resulting from rearrangement of the coding sequence or gene methylation, as suggested by **Ottaviani *et al*. (1993)**.

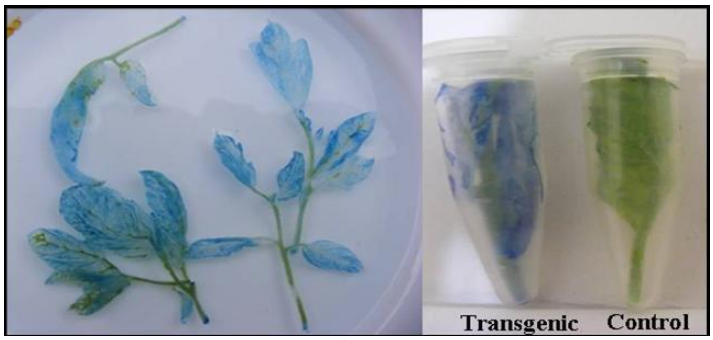


**Figure 3** Histochemical GUS assay for *Agrobacterium*-mediated transformed tomato plants

**Expression of *bar* gene in tomato plants**

The most important standard to evaluate the transgenic plants, which contain the herbicide-resistant *bar* gene, is using leaf painting with 0.2% of the herbicide basta **(Spencer *et al*., 1990)**. The *bar* gene expression of tomato transgenic was tested at bio-containment stage with 1g/L basta through painting the middle green parts from both sides of the plant leaves. Results in Figure 4 showed that the leaves of the herbicide-resistant tomato plants retained green color while in the non-resistant herbicide or control (non-transformed plants), color turned yellow within three days. The use of leaf painting to identify the transgenic tomato plants is a fast method and highly efficient to detect the expression of bar gene in the DNA genomic of tomato. In cereals, similar observation was obtained by **Eisa (2001)**. She used the Basta herbicide to detect the transgenic wheat plants through painting the middle green parts of the plant leaves with 1g $L^{-1}$ basta. Also, **Moemen *et al*. (2005)** reported that *bar* gene expression was tested of putative transformed faba bean plants by painting the leaflets of putative transgenic plants with 1g $L^{-1}$ basta BASTA.

**Figure 4** Herbicide leaf painting test showing the resistance of tomato transgenic leaf to BASTA application (left) and the control of non transgenic leaflet showing necrotic after painting (right).

**PCR detection**

The genomic DNA was isolated from young leaves of bialaphos resistant tomato plants by the method of **Delaporta *et al*. (1983)** and used in PCR analyzes. The gus-specific primer was used to detect the gus gene in putative transformed tomato plants which were treated with *agrobaterium* contains the plasmid pICBV19 and PCR product from these plants were able to amplify a fragment of ~1000 bp (Figure 5) for gus gene. PCR was recorded that only 65 out of 150 (transformed tomato plants with pICBV19) plants certain to be transgenic, representing a percentage of 43.3%. Tomato plants gave an expected PCR amplicon no such amplicon was observed in negative control (nontransformed) plants. Ten transgenic plants from every group (transformed either with pICBV19) which are used to study for *gus* gene expression. Our results are in agreement with **Ehab *et al*. (2015)** they found that transgenic tomato cv. Pusa Ruby plants produced from direct transformation with the plasmid pICBV19 was able to amplify a fragment of ~1000bp for *gus* gene. PCR analysis was used in many studies with transformed tomato plants to reveal the stable integration of the *gus* gene into the tomato genome. **Hasan *et al*. (2008)** used PCR for identify of the transformation and detection of the presence of the NPTII, Gus and API gene into plant genome. While, **El-Siddig *et al*. (2009)** used PCR technique T0 investigate the presence of AFP gene of transgenic tomato cv. Castle Rock. Also, **Chaudhry and Rashid (2010)** used PCR analysis to confirm that hygromycin resistance gene is in transgenic tomato cv. Riogrande.

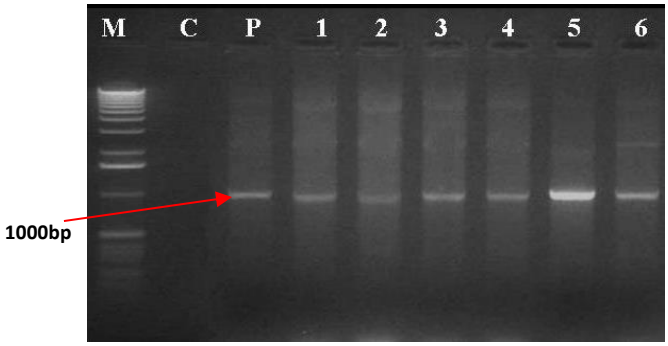


**Figure 5** PCR product of GUS amplifying partial length (~1000bp) in putative tomato tissues transformed *via Agrobacterium*. Lane M= 1kb DNA ladder (Marker) Lane C= negative control and Lane P= cry1Ab plasmid. Lanes 1-6= putative transformed tomato plants.

**Confirmation of transformation with synthetic *Bt* gene**

Three methods were used in this study to confirm that the *cry1Ab* gene in the putatively transformed tomato tissues; the PCR analysis, Southern blot hybridization and insect bioassay.

**PCR reaction**

Genomic DNA was isolated from the kanamycin resistant tomato plants that have been transformed by the *Agrobacterium* mediated gene transfer. The polymerase chain reaction was used to identify the *cry1Ab* gene into the genomic DNA of the putative kanamycin resistant tissues of tomato (*Lycopersicon esculentum* Mill.) cultivar Super strain B as shown in Figure 6. In this study, the *cry1Ab* specific primers were designed to amplify a fragment of 1800 bp of the *cry1Ab* gene. Out of 130 plants examined from kanamycin resistant tissues only 38 gave positive results (29.2%).The PCR is a very powerful technique which is now used in

many areas of biology and it used to identify of foreign gene in genetically modified plants **(Lassner *et al*., 1989)**. While, **Nguyen *et al*. (2002)** they used PCR to detect of *cry1Ac* gene into the genomic DNA of transformed rice tissue.

**Southern blot hybridization**

From transgenic tomato plants were selected based on southern blot analysis. Showed the results of southern blot analysis of transgenic tomato plants developed with the presence of the *cry1Ab* gene and resistance to tomato borer *Tuta absoluta* (Meyriek). Genomic DNA was digested with *Bam*HI and data revealed that only one copy number was obtained fragment of 2.0 kbp that included the whole coding sequence of *cry1Ab* gene and the nos terminator but was absent with the negative control (Figure 7). This result indicates that integrating *cry1Ab* gene into the genomic DNA of the tomato transformed. No hybridization signal could be detected for the DNA extracted from the untransformed plants.

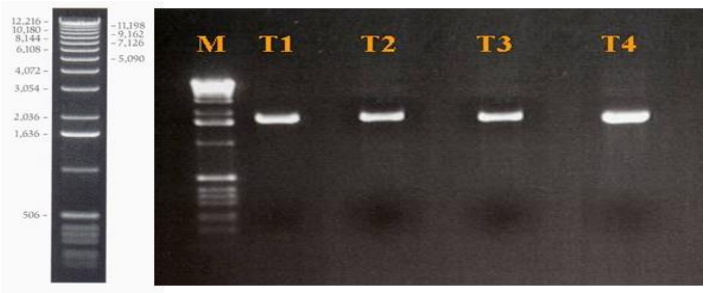


**Figure 6** PCR product of amplified fragment of *cry1Ab* gene with a length of 1800bp in putative tomato tissues transformed *via Agrobacterium*. Lanes (T1-T4) putative transformed tomato plants and Lane (M) 1kb DNA ladder (Marker).

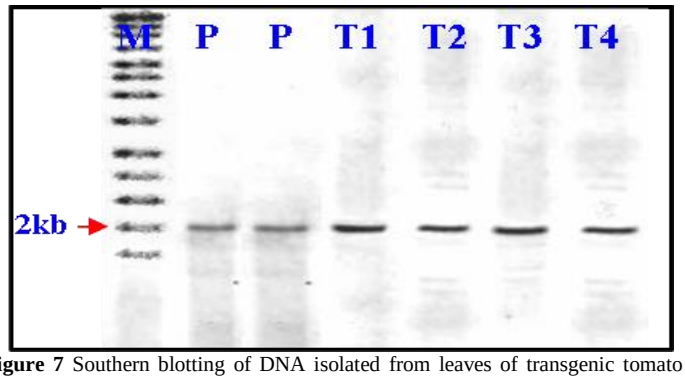


**Figure 7** Southern blotting of DNA isolated from leaves of transgenic tomato (*Lycopersicon esculentum* Mill.) cultivar Super strain B; **M,** 1.0 kb plus DNA ladder; **P,** BamHI digested DNA from plasmid pBI121-*cry1Ab*; **lanes T1-T4**, BamHI digested DNA from transgenic tomato plants.

**Insect bioassay**

Tomato borers, *Tuta absoluta* (Meyrick) were collected from the tomato fields of Alexandria province, Egypt (Figure 8). Laboratory scale bioassays for the transformed tomato plants were carried out.

Bioassays were carried out to evaluate the potential of transformed tomato plants. Leaves from non-transformed and transformed plants were used for insect feeding. The toxicity of introduced insecticidal genes was evaluated by feeding the larvae instars (third and forth larval stages) of *Tuta absoluta* on transgenic tomato leaves. Ten different larvae instars of each of treatment of the target insect were used in the bioassay. Larval mortality of larval instars against the target insect pests was tabulated in Table (3).

In the case of *Tuta absoluta*, it appears that the 4$^{th}$ instars are more rapidly affected than the 3$^{rd}$ instars larvae; where 100% larval mortality was obtained after 4–5 days (Table 3). The tested transgenic tomato plants exhibited 10% larval mortality against larvae of of *Tuta absoluta* after one day and reached 70% larval mortality on the third day. The instars larvae treated with the tested transgenic tomato plants cv. super strain B showed 100% larval mortality after feeding for 4–5 days as the shown Figures 9 and 10. Each PCR- positive tomato plant showing a higher resistance against insect pests with mortality higher than 98% were collected for inheritance analysis and selection of the homozygous positive plants. Our results are in agreement with **Cabello *et al.* (2009a)** they found that the influence of *B. thuringiensis* subsp *kurstaki* on all larval instars have showed the influence of effective against *T. absoluta* larval infestations. Mortality of the infected larvae may be due to the undigestion of the ingested food, or result paralysis and/or the physiological disruption result the toxicity of the haemolymph **(Lotfy, 1988)**.

In the present experiment it was obtained that the mortality was very low after 2 days of feeding while after four days the mortality was very high. This might be due to the fact that *B. thuringiensis* being stomach poison within 24 hours of feeding when Bt protein in to the midgut of insect and its are broken up and activated under alkaline pH in the insect midgut, releasing the active peptides which bind to particular receptors in the midgut epithelial cells and making pores in the epithelial membrane **(Schnepf *et al.*, 1998)**.


**Figure 8** Tomato leafminer, *Tuta absoluta* (larval stages) were collected from planted tomatoes infected with *Tuta absoluta*.

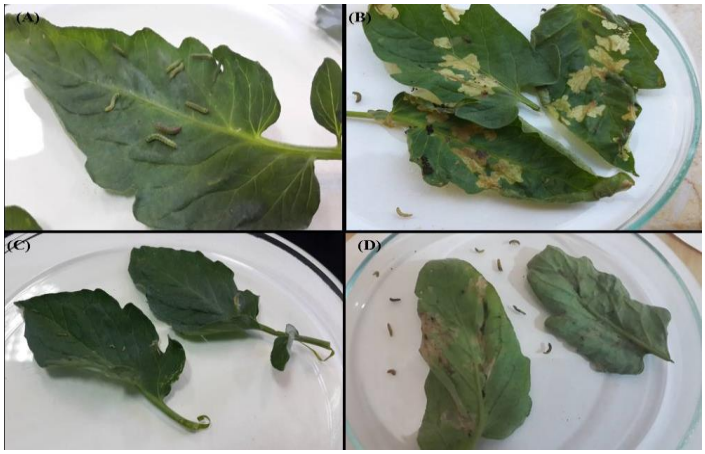


**Figure 9** Bioassay using *Tuta absoluta*. (A) First, *Tuta absoluta* larvae on leaves of non-transformed tomato plants (B) After 5 days on leaves of non-transgenic tomato control plants, larvae were alive and progressed to second instar. Extensive visible damage was observed and no dead larvae were recovered. (C) ) first, *Tuta absoluta* larvae on leaves of transformed tomato plants.(D) After 5 days on leaves of tomato transgenic cv. super strain B, larvae were all dead. No visible damage, no live larvae recovered.

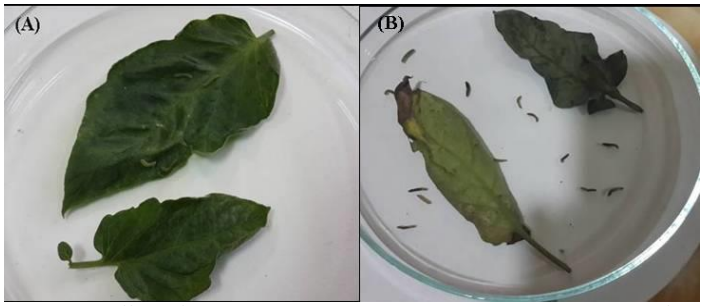


**Figure 10** Effect of Cry1AB expression on the larvae instars (third and forth larval stages) of an insect *Tuta absoluta* (A); After feeding of *Tuta absoluta* larvae on the transformed tomato plants (B); Tomato transformed Cry1Ab toxin protein howed 100% mortality after feeding for four days.

**Table 3** Effect of transgenic tomato leaves on daily larval mortality of *Tuta absoluta* after five days.

| Days | % Mortality after feeding on non-transgenic tomato leaves (Control) | % Mortality after feeding on transgenic tomato leaves | % Leaf damage of nontransgenic plants (Control) | % Leaf damage of transgenic plants |
|---|---|---|---|---|
| **1st day** | $0.0\pm0.00^{i}$ | $0.95^{g}\pm10$ | $15\pm1.08^{f}$ | $0.0\pm0.00^{i}$ |
| **2nd day** | $0.0\pm0.00^{i}$ | $1.25^{e}\pm30$ | $1.38^{d}\pm45$ | $0.5\pm0.15^{d}$ |
| **3rd day** | $0.0\pm0.00^{i}$ | 1.82c±70 | $1.92^{b}\pm87$ | $0.46^{c}\pm1.5$ |
| **4th day** | $0.0\pm0.00^{i}$ | $2.05^{a}\pm 100$ | $2.06^{a}\pm 100$ | $0.52^{b}\pm2.5$ |
| **5th day** | $0.0\pm0.00^{i}$ | $2.04^{a}\pm100$ | $2.05^{a}\pm 100$ | $1.04^{a}\pm3.0$ |

## CONCLUSION

There are hundreds of insects and mites that live in the consumption of this plant, few causing considerable damage, and that in many regions or times make it difficult or almost impossible to culture. Tomato growers suffered heavy losses of about 90-100% in Egypt because of the very high incidence of *Tuta absoluta*. The high frequency *in vitro* regeneration system of tomato was established by using zeatin in the treatments for two cultivars. Shoot regeneration of tomato cv. Super strain B is greater to produce the plants derived from cotyledon and hypocotyl explants on medium that contained zeatin with NAA while, the plants are produced from cotyledon and hypocotyl explants of Castle Rock cultivar increased in all tested on medium that contained zeatin with BAP in the presence of IAA. Success of transformation depends on the integration of the *cry1Ab* gene into the tomato genome and gene expression, and on its inheritance in progeny plants. PCR analysis was used to detect of *cry1Ab* gene into the genomic DNA of transformed tomato plants. The Cry1Ab toxin proved to be expressed by transgenic tomato plant and it remains biologically active when ingested by the target insects. Obvious effects of Cry1Ab were judged by the mortality of *Tuta absoluta* when incubated with leaves from transgenic tomato plants. Our research indicated that 100% larval mortality was obtained when it keep the larvae on the leaves for after 4–5 days this indicates Cry1Ab gene expression of the transgenic plants.

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