Creation of Borer Pests Resistance Genetically Engineering Peach (Prunus Persica L.) Plants by Cloning cry1Ab Gene

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Abstract

The plasmid pBI121 cry1Ab was used to transform peach explants to produce insect resistant plants. The plasmid was constructed from cloning the synthetic *cryIAb* gene with intron of castor bean catalase-1 gene into the pBI121 binary vector under the control of CaMV35S promoter. Leaf discs of peach (*Prunus Persica* L.) were co-cultivated for two days with *A. tumefaciens* strain LBA 4404. Explants were plated on WPM medium supplemented with 125 mg/l kanamycin, 3% sucrose, 2.5 mg L^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid) and 0.5 mg/l BA (6-benzyladenine) in darkness for callus formation. The calli were then selected on WPM medium supplemented with 125 mg L^{-1} kanamycin, 3% sucrose, 3.00 mg L-1 TDZ (Thidiazuron), 1.0 mg L-1 Kn (kinetin) and 0.5 mg L-1 IAA (Indoleacetic acid) in the light for at least five subcultures (with a regeneration efficiency of 91.8%). The integration of cryIAb gene into the peach genome was confirmed by PCR (polymerase chain reaction) and northern blot analysis. The transformation efficiency (28%) was obtained when leaves were incubated for 15 min. with *A. tumefaciens*. The *cry*IAb gene expression was confirmed using RT-PCR, northern blot hybridization, immune-strip test and insect bioassays, respectively. For insect bioassay, it was evident from data the toxin CryIAb protein expressed in transformed peach plants showed 100% mortality at 1000 ppm against *Synanthedon exitiosa* larvae's after 96 hr. These results obtained improved significantly of CryIAb toxin protein against lepidopteron larvae of peach.

Key Message

In vitro peach regeneration from *in vitro* leaves is difficult, as well as regeneration using the method of genetic transfer such as agrobacterium to impart the desired characteristics of the plants with the aim of improvement is very difficult. This paper is the first to transgenic peach plants carrying cry1Ab gene for insect resistance particularly the peach tree borer.

Introduction

Peach (*Prunus persica*) is a deciduous tree or shrub reaching up to 8-9 meters in height, have the ability to produce fruit for 20 years, believed to have originated in China and considered the eighth most main commercial fruit crop worldwide (Souza et al. 2011). The peach belong to the Rosaceae family, which has many other species of commercial importance, contains many fruit, nut, and ornamental species (Wang et al. 2002; Bodh et al. 2019). The Mediterranean region is the center of world production, especially in Italy, Spain, France and Turkey (Declerck and Korban 1996**;** Gentile et al. 2002). The percentage of areas planted with peaches in terms of the size is distributed as follows: 71.8% Asia, 16.4% Europe, 15% China, 7.4% Americas and 4.1% Africa, but in the last 4 years, the area planted with peaches decreased from 1600 k ha in a year 2015 to 1500 K ha in a year 2019 (FAO 2020) http://www.fao.org/faostat/en/#data/QC/visualize.

Peach trees are exposed to many different pests and diseases such as peach tree borer (*Synanthedon exitiosa*), the yellow peach moth (*Conogethes punctiferalis*), the *cutworm* moths (*Abagrotis*

orbis), *Lyonetia prunifoliella*, the fruit tree borer (*Maroga melanostigma*), which causes strike serious damage of trees and fruits which reducing production and quality (Zehr et al. 1995; Cottrel et al. 2006; CABI 2014). Insect herbivores cause huge losses in crop production, reducing yields in major crops by up to 20% (Ferry et al. 2004). The peach tree borer, *Synanthedon exitiosa* (Say) (Lepidoptera: Sesiidae), is a very dangerous agriculture pest that infects a variety of stone–fruit trees such Prunus spp. including peach (*Prunus persica* L.), is responsible for more damage to peach trees than all other insect pests (Johnson et al. 2005). *S exitiosa* do not attack the fruit, but rather the tree itself, hiding in its trunk near ground level or blowing into it and devouring the live cambium layer of the trunk and large roots that form arcades located around the surface of the soil to a depth of approximately 30 cm. (Shapiro-Ilan et al. 2015). Also, Reilly et al. (1987) mention to the peach tree borer can damage the tree by inducing plant pathogens to invade the weakened tree. Countries around the world spend more than $3 billion each year to reduce insect pests (Wilcox et al. 1986). Sales microbial insecticide are estimated to be less than 1% of this total but are expected to increase by up to 70% by year 2020 (Tabashnik et al. 1990). Numerous studies have indicated the effectiveness of using microbiological control agents as a biological therapeutic method to manage insect pests in agriculture ecosystems (Shapiro-Ilan et al. 2009; El-Gaied et al. 2020). One of these biological insecticides organisms is *B. thuringiensis (Bt)* which used to control Spodoptera exigua (Hübner; Lepidoptera: Noctuidae)–a worldwide, polyphagous pest species (Baranek et al. 2020). *Bt* is notable for its production of a variety of insecticidal crystalline proteins that are encoded by *cry* genes (Höfte and Whiteley 1989; Atia 2020). Cry proteins is safe alternative to chemical pesticides, not toxic to humans, and it does not cause the serious environmental (Balaraman 2005). With this base of knowledge on *Bt* and cry protein the possibility are open to apply several strategies such as using cry protein by genetic engineering to improve pest control and reduce pesticides (Jenkins and Dean 2000). The use of protein engineering has become an interesting topic, due to the significant public debate that exists regarding its potential benefits or adverse effects (Kranthi and Stone 2020).

Modified crops containing cry genes that express sufficient levels of insecticidal proteins have been developed to protect them from attacks insect pests in many plants (Betz et al. 2000; Ranjekar et al. 2003). As a result of the expansion in the cultivation of these GMC on a large commercial scale, this can generate mutation in target insect pests, as has happened with chemical insecticides, Consequently, the potential development of insect resistance is the greatest threat to breeding programs for the sustainable use of modified plants (Shelton et al. 2002; Butko 2013). Accordingly, working on developing genetic engineering techniques will remain the most important duty of plant breeders to sustain the fight against insect diseases. Several reports are available on dicot and monocot species that have been transformed using these genes and become resistant to insect pests using biotechnology tools (Hinchee et al. 1988; Perlak et al. 1993; Nayak et al. 1997; Gomez et al. 2014). There are several genetically modified crops with the *Bt cry1Ab* gene that have been to be highly effective against larval insect pests (Ye et al. 2001; Dutton et al. 2005; Pardo et al. 2013; Hemaid et al. 2017; Xu et al. 2018).

Prunus species is one of the most difficult species of perennial trees to produce *in vitro* regeneration shoots, although reports have obtained of successful in vitro regeneration and transformation species recently (Gentile et al. 2003; Song and Sink 2005; Petri et al. 2008; Zhou et al. 2010; Sidorova et al.

2017and 2019; Sabbadini et al. 2019; Ricci et al. 2020). *In vitro* shoot regeneration of peach from juvenile or seedling explants is achieved, but is difficult to accomplish from mature plant (San et al. 2014). Nevertheless, adding more desirable characteristics while preserving valuable traits of mature genotypes requires reliable and good tools for regeneration and genetic transformation (Zhou et al. 2010).

This work describes the transformation of peach (*Prunus persica* L.) by using *Agrobacterium tumefaciens* having *cry*1*Ab* gene to improve of insect resistant peach and evaluation of *cry*1*Ab* gene expression in peach plant against *Synanthedon exitiosa*. This is the first report of the synthesis and expression of *Cry1Ab* gene in putative transgenic peach plants.

Materials And Methods

Construction of the synthetic *cryIAb* gene and bacterial strain.

The cry1Ab gene under the control of the CaMV35S promoter and kanamycin resistance gene (npt-II) as a plant selectable marker under the NOS promoter were sub-cloned in the binary plasmid vector pBI121, designated as pBI121 cry1Ab has a 1845 bp fragment of the cry1Ab gene containing the intron of castor bean catalase-1 gene (190 bp) with a pair of synthetic linkers of BamHI site and the terminator of nopaline synthase *gene* (Fig.1). The cry1Ab gene has been cloned and their nucleotide sequence was determined from *Bacillus thuringiensis*. The plasmid pBI121 cry1Ab was constructed by Prof. Dr. Hemaid I.A. Soliman from cloning the synthetic cry1Ab gene. The plasmid pBI121 cry1Ab were transformed into the *A. tumefaciens* strain LBA4404 and used for peach (*Prunus Persica* L.) transformation experiments.

In vitro regeneration of peach (*Prunus Persica* L.)

Leaves collected after on month from *in vitro* proliferating microshoots of peach (*Prunus Persica* L.) cv. Balady grown in Taif governorate, Saudi Arabia previously propagated by stem nodal sections on MS medium containing 2 mg L-1 Benzyle adenine (BA), 0.2 mg L-1 indole3 butyric acid (IBA), 30 g l-1sucrose and 2.5 g l-1phytagel as described by Soliman (2013). The MS medium was adjusted for pH 5.8 and was autoclaved for 20 min at 121°C. All samples were incubated at 25 ± 2 ° C with a light period of 16 hrs (3240µmol m^2s^{-1} intensity). The *in vitro* leaves were cut (5 mm length) and planted on two different media: MS (Murashig and Skoog 1962) and WPM (Lloyd and McCown 1980) containing different concentrations of 0.5 - 4.0 mg L-1 2,4-D in combination with 0.25 - 3.0 mg L-1 BAP or kinetin. The samples were incubated in growth room at 25±2°C in the dark for callus induction. For *in vitro* adventitious shoot regeneration, the calli were transferred to two media (MS and WPM) containing 5.0 mg L-1 TDZ alone or in combination with 1.0-3.0 mg L-1 kinetin, 0.5-2.0 mg L-1 BAP and 0.25-1.0 mg L-1 IAA at 4 weeks under the light intensity of 60-70 µmol m^2s^{-1} for shoot induction.

For shoot elongation, adventitious microshoots of regenerated peach from previously step were cultured on MS medium supplemented with 0.5 mg L-1 2iP for 4 weeks and then elongated shoots were transferred to MS liquid medium supplemented with 2.0 mg L-1 IBA , 0.5 mg L-1 NAA and 162 mg L-1 phloroglucinol. The shoots were incubated at 26±2°C in complete dark for one week and then transferred into light condition for three weeks for rooting formation as described by Soliman (2013) and Ricci et al. (2020). For acclimatization, the next applied process was taken according to Metwali et al. (2015); the plants were acclimatized by transferring them to pots containing a mixture of autoclaved peatmoss and sand (3:1), then the pots covered with plastic bags and placed in the growth chamber at 28±2°C and 16-hour light period. After 10 days the plants were hardened by plastic cover removed gradually over a 7–10 day-period.

Kanamycin sensitivity test

Determining the appropriate kanamycin concentration is very important in the selection of transformed plants by inhibiting non-transformed plants growth (Colby and Meredith 1990). Kanamycin was prepared by filtration through filters (0.22 µn) and placed into medium when in pre-cooled (45-50°C). The leaf segments were planted on callus WPM medium supplemented with 25- 150 mg L-1 Kanamycin. Subculture was applied every two weeks onto fresh callus WPM medium under the same composition. The survival explants (kanamycin resistant) percentage was tested after four weeks.

Agrobacterium Transformation

To prepare the bacteria for use in the process of transformation , Agrobactrium t*umefaciens* strain (LBA4404) harboring the binary vector plasmid pBI121 cry1Ab containing the synthetic cryIAb gene and npt-II gene was cultured in LB liquid medium containing both rifampicllin and kanamycin at a concentration of 50 mg L-1 mg L-1 at 28°C for overnight (Sambrook et al**.** 1989) and then *Agrobacterium* suspension culture was mixed to 50 ml WPM medium free hormones and incubated at 28°C until optical density at 600 nm (OD600 nm) – using *spectrophotometers (Unicam5625 Uv/Vis Spectrophotometer, United Kingdom)* - reaches the limit between 0.5-1.0 as an indication of Log phase. Leaf explants were cut into the bacterial solution and left for 15 min. Excess bacterial solution was eliminated by placing the leaf segments on the sterile filter papers, then the inoculated were transferred to solidified WPM medium containing 2,4-D at 2.5 mg L-1 and BA at 0.5 mg L-1 and incubated for 2 days in the dark at 28±2°C. After co-cultivation, the leaf segments were cultured on callus WPM medium supplemented 250 mg L-1 cefotaxime and 125 mg L-1 knamycin for one month at 26±2°C in the dark and then transferred to regenerated WPM medium supplemented with the same antibiotics and incubated at 26±2°C under 16 h light period (60-70µmol m2s1).

DNA isolation and molecular analyses

DNA transgenic leaf peach was isolated and the presence of the *cry1Ab* gene was determined with gene specific primers using PCR (Applied Biosystems™ Cat. No; A24811; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For *cry1Ab*, two primers, F: 5'- GTTACCCTGATTGATAGGC-3' *cry1Ab* R: 5'-ACAGAAGACCTTTCAATATC-3' was made to amplify the 2035 bp fragment of the *cry*IAb gene by PCR using a volume of 50 µl for a final concentration containing 2 ng of plant DNA (as template), 1 *p*moles of each of the primers used, 200 µM of dNTPs , 0.04 U *Taq* DNA polymerase, 2.5 mM MgCl2, 1X PCR buffer and complete the volume dH2O according to manufacturer's instructions (Sigma, USA). Single – step PCR is performed at 95°C/5min. The PCR reaction consisted of a single step of 95°C for 5 min., followed by 35 cycles at 94°C/1 min., 57°C /1 min., 72°C /2 min., and a final extension at 72°C /8 min. 15 µl of PCR reaction mixture with 3 µl loading buffer was loaded onto a 1% agarose gel and run at 80 Volt.

RT-PCR and Northern blot hybridization

Total RNA from peach leaves by phenol/chloro-form procedure (Sambrook et al. **1989**) was separated on 1.2 % on formaldehyde gel and then transferred to a nylon N+ membrane (Amersham) according to the manufacturer's instructions *QIAGEN*. Membranes were hybridized with 32P dCTP-labelled BamHI (2035bp) fragment of the *cryIA(b)* gene as probe overnight at 42°C. The same *cry1Ab* primer set for PCR reactions was also used for the RT-PCR. The RT-PCR reaction was set according to technical bulletin provided with "Access RT-PCR" kit Agdia protocol (Agdia Inc., Elkhart, IN, USA). The amplification conditions protocol are 30 cycles of 94°C/2 min., 65°C/ 30s, 72°C/ 2 min., and the final reaction at 72°C/ 5 min. RT-PCR products were shown in a 1% agarose gel by electrophoresis.

Survey with Trip Tests for the Cry1Ab protein

The leaves of transgenic peach plants which positive cry1Ab gene confirmed by PCR analysis were assayed to detect the expression of Cry 1Ab protein using Immuno Strip® (Immuno Strip® has two test lines; one for the Bt-Cry2A and one for the Bt-Cry1Ab/Ac protein, Agdia Inc.,USA) from Agdia following the procedure given by (Agdia **2003**) and the according to the manufacturer's instruction Agdia protocol (Agdia Inc., Elkhart, IN, USA). The leaves were ground with a rotary pestle and added 0.5ml extract buffer was mixed with the leaf powder. QuickStix strips were incubated with leaf extraction buffer. After ten minutes the reaction results were checked and were examined the bands on the strip.

Bioassay for insect toxicity

Bioassay of transformed peach (*Prunus persica* L.) plants with the *Synanthedon exitiosa* (peach tree borer) larvae's was used to confirm the Cry1ab protein expression in positive transformed peach plants. *Synanthedon exitiosa* (peach tree borer) larvae's that had been collected from infested peach orchard during July 2020 according to procedures described by Cottrell and Shapiro-Ilan (**2006**). When dissolving 0.1 gm of transformed dried peach leaves in 100 ml of water gives 1000 ppm, and then different concentrations from stock concentration (200-1000 ppm) were prepared. To each cup containing artificial media with 500 µl of each dilution was added and three replicates were prepared from each experiment. 10 neonate larvae were incubated on the surface of the medium after the surface

of the solution was completely dry and cups were covered with aluminum foil and left at 26°C (± 2°C). On the other hand, negative control medium was prepared exactly as mentioned above but the toxin replace with an addition 500 µl H2O. Data were taken and mortality was calculated every day for five days.

Statistical analysis

Statistical analysis of all experiments data was performed as a completely randomized design with three replicates. The recorded data were analyzed statistically using the analysis of variance technique (ANOVA) and by multiple range tests (Steel et al**. 1997**). The means significance was compared by applying the (L.S.D.) test at 5% level of probability.

Results And Discussion

Callus induction and plantlet regeneration of peach (*Prunus Persica* L.)

In the current study leaf explants of peach (*Prunus persica* L.) induced callus on both of two different media: MS and WPM supplemented with different concentrations of 0.5-4.0 mg L-1 2,4-D in combination with 0.25-3.0 mg L-1 BAP or kinetin as shown in Table (1). The results showed that, the highest % of explants formed callus (89.5 %; 87.8 %) and the highest callus fresh weight was (3.78 g; 3.68 g) was recorded on 2.5 mg L-1 2,4-D with 0.5 mg L-1 BAP followed by 3.0 mg L-1 2,4-D with 1.0 mg L-1 BAP, respectively on WPM medium compared to MS medium at the same concentration of PGR. Although 2.5 mg L-1 2,4-D with 0.5 mg L-1 BAP on WPM medium recorded the best treatment of % explants formed callus and callus fresh weight, but on MS medium the best values for the both previous traits (84.9% ; 3.55 g) were recorded at concentration 3.0 mg L-1 2,4-D with 1.0 mg L-1 BAP. This result is in agreement with Declerck and Korban (1996) and Pérez-Jiménez et al. (2013). Compared to other auxins, hypotheses developed to explain the positive action of 2, 4-D that could be due to inhibition of the DRT102 protein, which is responsible for stimulating cell division, DNA replication and lead to decrease in the number of cells (Pasternak et al. 2002). Moreover, in previous study of (Zuo et al. 2002), he explained the effect of the hormone 2,4-D as a signal trigged to alert the beginning of hyperpolarization process of membrane polypeptides in in the cell .. Reis et al. (2021) recently mentioned that the growth of callus effectively in the artificial media is linked to 2,4-D hormone homeostasis , which directly effects on the efficient accumulation of storage reserves of callus. On the other hand, the effect of the exogenously applied of 2,4-D and BA may be due to its stimulus to increase the endogenous levels of IAA, which is known to be an activator of cell division and differentiation, especially under dark incubation or low light intensity Migual et al. (1996).

The callus obtained from *in vitro* leaf was transferred to the regeneration media containing 1.0-5.0 mg L-1 TDZ alone or in combination with 1.0-3.0 mg L-1 kinetin, 0.5-2.0 mg L-1 BAP and 0.25-1.0 mg L-1 IAA. Observation of regeneration efficiency was recorded from callus as shown in Table (2). The result showed that, the highest % of callus formed shoots (91.8% ; 88.5%), number of adventitious buds/callus (8.75 ; 7.25) and mean shoot length (3.87 cm ; 3.70 cm) was showed on WPM medium MS medium respectively

supplemented with 3.00 mg L-1 TDZ, 1.0 mg L-1 kinetin and 0.5 mg L-1 IAA . The results indicated that the WPM medium was better than MS medium and the TDZ succeeded in being effective in the case of using with IAA. The results of the present study are in agreement with Pérez-Jiménez et al. (2013) who reported that the best results for regeneration percentages and proliferation of the shoot were obtained with thidiazuron (TDZ) instead of 6-benzylamino-purine (BAP) and TDZ is considered one of the best growth regulators to obtain large number of plantlets and shoot induction of callus comparing with other growth regulators such as BA. Also, previous study indicated the efficiency of TDZ to enhance Agrobacterium-mediated transformation in *Prunus Persica* (Soliman 2013). This availability of the efficient plant regeneration from different meristem tissues is a prerequest to improve peach species via gene transfer technology (Ricci et al. 2020).

Effect of kanamycin on explant regeneration

Kanamycin is the most suitable selective antibiotic for transformed tissues in most of the plant species (Oropeza-Aburto et al. 2020). To determine the optimum concentration of the lethal dose of kanamycin in peach, different concentrations of kanamycin ranged from 25 up to 150 mg L-1 were applied plus the control. The high rate of survival (100 %) for leaf segment explants was obtained under kanamycin free medium. On the contrary, under the medium treated with kanamycin, the survival rate was lower as the concentration of the kanamycin increased in the medium. Leaf segments rate of survival has reached to 18% under of 100 mg L-1 kanamycin, while the lethal kanamycin dose for the survival was assessed at 125 mg L-1 kanamycin (Fig. 2). The concentration of 125 mg L^{-1} kanamycin was then chosen as a selection marker for transformed tissues in peach, where Cosson et al. (2015) indicated that the selection agent should be able to completely inhibit the growth of non-transgenic plant cells, and it is desirable that the lowest concentration that can achieve this must determined. But in different species such as grapevine, vitis and cotton the optimum concentration of kanamycin for selection was 50, 70 and 75 mg L^{-1}, respectively (Dhekney et al. 2006; Sabbadini et al. 2019; Zhang et al. 2001). This variation in kanamycin concentration may be due to the difference in genotypes as well as the type of explant (Abbosi et al. 2020). Also, previous reports demonstrated that development stage of explant may be an influencing factor in determining the concentration of kanamycin and accordingly the selection concentration should be changed along with development of culture (Zhang et al. 2001).

Transformation of **peach (*Prunus Persica* L.)**

Leaf section explants were collected from one month-old *in vitro* peach (*Prunus persica* L.) plants and used as explants (Fig. 3a). Explants were suspended in *A. tumefaciens* LBA4404 for 15 minutes as described in material and methods section. Results showed that kanamycin resistant callus formation (21%) was obtained on WPM medium containing 30 g L^{-1} sucrose, 2.5 mg L-1 2,4-D and 0.5 mg L-1 BAP, 250 mg L-1 cefotaxime and 125 mg L-1 kanamycin compared to the control (89.5%) after one month at 26±2°C in the dark (Fig. 3b–c). Also Kanamycin resistant calluses cultured onto regenerated solidified WPM medium supplemented with 30 g L-1 sucrose, 3.0 mg L-1 TDZ, 1.0 mg L-1 kinetin, 0.5 mg L-1 IAA and 125 mg L-1 Kanamycin succeeded to obtained shoot regeneration (79%) after incubated for six

weeks at 26±2°C under an 8/16 h (light/dark) photoperiod (60-70µmol m^2s^1) as shown in the Fig. (3 d-e-f). Putative transformed adventitious microshoots was elongated on medium containing 0.5 mg L-1 2iP after one month (Fig. 4a) and started to forming root using MS liquid medium supplemented with 100 mg L-1 myo-inositol, 30 g L-1 sucrose, 2.0 mg L-1 IBA and 0.5 mg L-1 NAA and 162 mg L^{-1} phloroglucinol at 26±2°C in complete dark for one week and then transferred into light condition for three weeks (Fig. 4 b-C). Acclimation protocol was successfully achieved highest percentage of survival transgenic peach plants (91%) as described by Soliman (2013) (Fig. 4d). Recently study proposed that the ability of explants from woody fruit species to regenerate shoots and efficiency protocols for *Agrobacterium*-mediated transformation remains a major impediment to genetic modification and gene editing technologies (Song et al. 2019; Orbović 2019).

PCR detection of transformed peach plants

Transformed peach plants were screened for the presence of the *cry1Ab* gene using PCR analysis with *cry1Ab* gene specific primers. The expected 2035 bp *cry1Ab* fragment was amplified only from genomic DNA isolated from the kanamycin resistant peach plants compared to non-transformed peach plants. The PCR has the potential to become a widespread tool to confirm the presence of a foreign gene such as Vis1 in tomato (Metwali et al. 2015), GmbZIP2 in soybean (Yang et al. 2020) and PpMYB10.1 in peach (Xu et al. 2020) in transgenic plant tissues. In the current study out of 60 plants examined from kanamycin resistant peach regenerated plants only 17 gave positive results. This indicated the success of the process of transferring the *cry1Ab* gene through Agrobacterium-mediated transformation method with the rate of 28% as putative transgenic peach plants (Fig. 5). That rate of obtaining genetically modified plants is not constant and varies, and that may be due to a difference in type of explants, culture condition, PGR, genotypes, agrobacterium strain and plasmid type (Aisley et al. 2001; Wang et al. 2015). For peach, internodes (56.8%), cotyledons (52.7%), and embryonic axes (46.7%) had the highest transformation rates where hypocotyl was less competent for transformation (Padilla et al. 2006). Also, previous study indicated the efficiency of TDZ comparing to other PGR to enhance agrobacterium-mediated transformation in *Petunia hybrid,* it shortened the duration of intermodal bud regeneration, in addition to reducing the somaclonal variations in most of the regenerated buds (Thirukkumaran et al. 2009).

RT-PCR and Northern blot analysis

Seven randomly selected putative transgenic peach plants were used for RT-PCR analysis to confirm *cry1Ab* gene expression at transcript level. All seven transgenic plants showed the expected 1845 bp amplified fragment in RT-PCR with *cry1Ab* gene specific primers compared to non-transgenic peach plant (Fig. 6 a). Also, the results were showed that 190bp intron is removed by RNA splicing and gave 1845 bp amplified fragment in RT-PCR. Also, accumulation of mRNA for the introduced *cry1Ab* was determined in putative transgenic of peach plants using Northern blot analysis. The presence of a single 1845 bp band obtained after splicing of a 190 bp intron from the cry1Ab gene showed higher level of gene expression at the transcriptional level compared to non transgenic peach plants (Fig. 6 b).

Cry1Ab protein detection in transgenic peach plants

The expression of Cry1Ab protein was assayed in the peach (*Prunus persica* L.) plants of PCR-positive transgenic plants via immune-strip test and insect bioassays.

Immune-Strips assay

Immune-Strips showed the band related to Cry1Ab protein in the peach transgenic plants. The control band appeared in transgenic and nontransgenic plants, but Cry1Ab protein bands just appeared in transgenic peach plant, but not appeared in the of non-transgenic peach plants (Fig. 7). The results indicated that high gene expression of Cry1Ab protein in transgenic peach plants.

Insect bioassay

Insect bioassay of transformed peach (*Prunus persica* L.) plants with the *Synanthedon exitiosa* (peachtree borer) larvae's was performed and the mortality was recorded every 24 hours for four days. Table **3** shows the mortality percentage of Cry1Ab toxin expressed in transgenic plants against *Synanthedon exitiosa*. The results indicated that the LC50 value from Cry1Ab toxin protein from transformed peach plants were 300 ppm against the *Synanthedon exitiosa* larvae's. The mortality percentage of Cry1Ab toxin expressed in transgenic peach plants against *Synanthedon exitiosa* was 100% with 1000 ppm compared to the other treatments; as they reached 30, 50, 60 and 80 % with 200, 400, 600 and 800 ppm from Cry1Ab toxin protein, respectively (Fig. 8). This data showed the high expression of *cry1Ab* gene in the transformed peach plant.

In constructing plant expression vector pBI121-cry1Ab, the coding sequence of synthetic cry1Ab was fused to CaMV35S promoter and the polyadenylation signal of the octopine synthase gene as described by Soliman et al. (2017). The combination of 35S promoter and the castor bean catalase-1 intron has previously been demonstrated to confer high-level expression of foreign genes in transgenic plants (Tanaka et al., 1990). The stable integration of the cry1Ab gene, presence or absence of specific transcript and accumulation of mRNA was assured in plants using the RT-PCR and Southern blot analysis (Salehian et al. 2021). The copy number determination of transgenes in transgenic plants is important due to the effect of the copy number on the gene expression level and genetic stability. In our study highest gene expression of Cry1Ab protein in transgenic peach plants level was detected, however, this high rate is not necessarily expected in other experiments, as Ramirez-Romero et al. (2008) indicated that the Variability and significant differences of Cry1Ab levels seems to occur related with the type of plant tissue, development stage and physiological conditions of transgenic plants. Our results in this study indicated the importance of Northern plot and Immune-Strips as of molecular analyzes to confirm the stability of crystal protein gene into transgenic peach genome. This result is in agreement with (Chen and Wu 2012). In our experiment a positive correlation between pest resistance and the mortality percentage of Cry1Ab toxin was detected. Kumar et al. (2010) showed that bioassay method and mortality were effective in identifying lines with intermediate to significant resistance against pest attack used. The interest in producing GMO from peaches cloning with Cry1Ab gene is an important scientific

development, where Rahnama et al. (2017) pointed out that the consumption of GMP food carrying Cry1Ab had no effect on the growth rate, and general health status of the rats.

Conclusion

Peach (*Prunus Persica* L.) is an important deciduous fruit tree that is widely cultivated in the world. Peach is susceptible to many pests such as insects, virus, mites and borers which limit peach production. Modern methods of tissue culture and regeneration from *in vitro* leaves via indirect somatic embryogenesis using the method of genetic transfer are useful technique to impart the desired characteristics of peach plants. In this study, genetically peach modified with cry1Ab gene that has inserted into peach genome by Agrobacterium, showed results are resistance to *Synanthedon exitiosa* larvae's. Various molecular analyzes confirmed that CryIAb toxin protein is stable integrated into peach genome. This paper is the first to have successfully used the Bt-cry1Ab gene to produce genetically modified peach plants targeted to improve agricultural efficiency and product quality by acquisition of insect borer's resistance particularly the peach tree borer. These transgenic peach plants could be produced commercially in the future and cultivated to increase the productivity of peach fruits.

Abbreviations

BA: 6-Benzyladenine; **bp**: Base pairs; **Cry**: Crystal protein; **IAA**: Indole-3-acetic acid; **IBA**: Indole-3-butyric acid; **2iP:** N-6-(Δ2-isopentenyl) adenine**, Kn:** kinetin, **TDZ**: Thiadiazuron (N-phenyl-N-1,2,3,-thiadiasol-5-ylurea); 2,4-D: 2,4-Dichlorophenoxyacetic acid; WPM: Woody plant medium; **MS**: Murashige and Skoog's medium; ***npt*II**: Neomycin phosphotransferase; **PCR**: Polymerase chain reaction; RT-PCR: Reverse transcriptase polymerase chain reaction.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The authors have no have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1 The best of different concentrations of plant growth regulators and various culture media on callus induction of peach (*Prunus Persica* L.) after four weeks.

Culture medium and concentration of plant growth regulators			% of explants formed callus	Callus fresh weight (g)
MS (Murashige and Skoog 1962) medium				
2,4-D	**Kinetin**	**BAP**		
2.0	0.0	0.5	33.8±0.27i	2.25±0.18i
2.5	0.0	0.5	49.5±0.38h	2.48±0.23h
3.0	**0.0**	**1.0**	84.9±0.57c	3.55±0.44c
3.5	0.0	1.0	e±0.2476.5	±0.67d3.47
4.0	0.0	1.5	55.2±0.72g	±0.38e3.25
2.0	1.0	0.0	±0.19j27.0	1.85±0.25j
2.5	1.0	0.0	±0.34i38..3	2.05±0.82i
3.0	1.5	0.0	58.4±0.65g	2.65±0.77g
3.5	1.5	0.0	52.7±0.39g	±0.62h2.55
4.0	2.0	0.0	41.8±0.52h	2.29±0.38i
WPM (Lloyd and McCown 1980) medium				
2,4-D	**Kinetin**	**BAP**		
2.0	0.0	.50	85.2±0.88c	2.68±0.79g
2.5	**0.0**	**0.5**	89.5±0.93a	3.78±0.65a
3.0	0.0	1.0	87.8±0.74b	3.68±0.52b
3.5	0.0	1.0	81.4±0.68d	±0.48c3.52
4.0	0.0	1.5	66.5±0.54f	±0.62d3.40
2.0	1.0	0.0	39.8±0.26i	2.05±0.39i
2.5	1.0	0.0	h±0.3744.5	2.35±0.42i
3.0	1.5	0.0	f±0.5165.2	2.85±0.60f
3.5	1.5	0.0	f±0.4262.5	g±0.782.67
4.0	2.0	0.0	±0.38g55.3	2.49±0.82h

Means followed by the same letters in the column do not differ by the Duncan test ($p \leq 0.05$). Value represents the mean ± standard error (S.E.) of ten replicates per treatment in three repeated experiments

Table 2 The best plant **growth regulators concentrations and various culture media on adventitious bud regeneration efficiency of peach (*Prunus Persica* L.) after six weeks.**

Culture medium and concentration of plant growth regulators				% of callus formed shoots	NO. of adventitious buds/callus	Mean shoot length (cm)
MS (Murashige and Skoog medium1962)						
TDZ	IAA	BAP	Kinetin			
1.0	0.0	2.0	0.0	±0.42j38.5	3.55±0.22i	±0.42j1.29
2.0	0.0	1.0	1.0	±0.55i43.9	h±0.394.05	±0.59j1.37
2.0	0.5	2.0	0.0	g±0.7165.8	e±0.575.32	±0.66i1.89
3.0	0.0	0.5	1.0	±0.68h53.4	g±0.394.35	±0.54i1.78
3.0	0.5	0.0	0.0	±0.83d79.5	d±0.655.90	±0.65g2.95
3.0	**0.5**	**0.0**	**1.0**	±0.92b88.5	b±0.837.25	±0.81b3.70
4.0	0.5	0.5	1.0	±0.79c82.4	c±0.696.15	±0.79d3.48
4.0	0.5	0.0	0.0	±0.77f68.3	d±0.785.70	±0.68f3.05
5.0	0.0	0.0	1.0	66.4±0.59g	h±0.364.00	±0.77g2.84
WPM (Lloyd and McCown 1980) medium						
TDZ	IAA	BAP	Kinetin			
1.0	0.0	2.0	0.0	±0.61i49.7	g±0.314.28	±0.29h2.28
2.0	0.0	1.0	1.0	±0.73h55.5	f±0.594.65	±0.46h2.42
2.0	0.5	2.0	0.0	±0.84e70.2	d±0.685.78	±0.61f2.98
3.0	0.0	0.5	1.0	g±0.7366.5	f±0.444.89	2.85±0.59g
3.0	0.5	0.0	0.0	±0.39c82.3	c±0.786.25	±0.72d3.40
3.0	**0.5**	**0.0**	**1.0**	±0.55a91.8	8.75±0.89a	3.87±0.94a
4.0	0.5	1.0	1.0	±0.65b89.2	b±0.757.39	±0.86c3.65
4.0	0.5	0.0	0.0	78.5±0.47d	c±0.696.00	±0.65e3.25
5.0	0.0	0.0	1.0	71.9±0.81e	f±0.624.95	3.00±0.59f

Means followed by the same letters in the column do not differ by the Duncan test ($p \leq 0.05$). Value represents the mean ± standard error (S.E.) of ten replicates per treatment in three repeated experiments

Table 3 Insect bioassay of peach (*Prunus Persica* L.) transformed plants against *Synanthedon exitiosa* (peach tree borer) larvae's of expressing the cry1ab gene after four days of Bioassay on Semi-Artificial diet.

Lethal concentration 50 (LC50)	larvae's)peach tree borer(*Synanthedon exitiosa*					
	Mortality percentage/concentration (ppm)					Peach plants
	200	400	600	800	1000	
—	0%	0%	0%	0%	0%	Nontransformed plants
300 ppm	30	50	60	80	100	Transformed plants

Figures

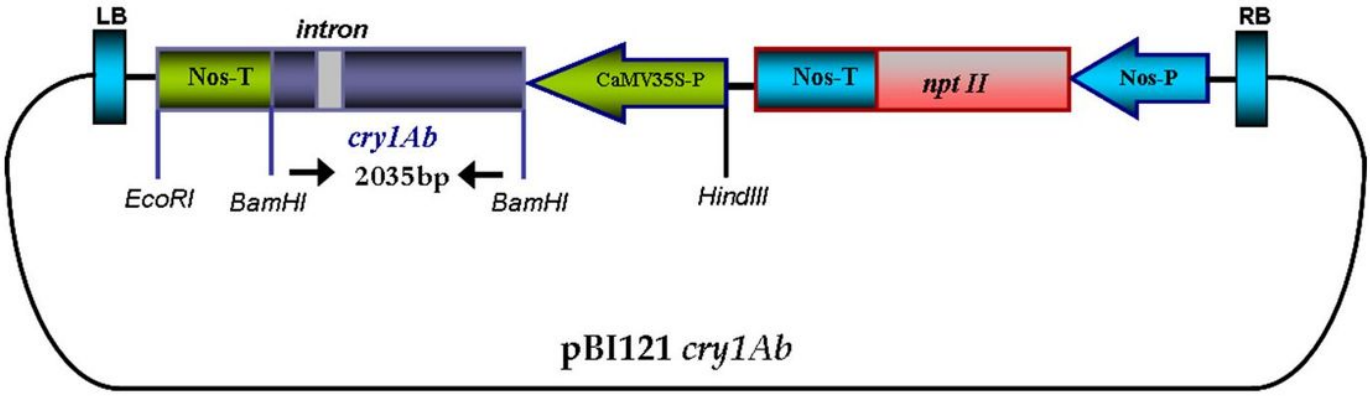

Figure 1

Restriction map of the binary vector pBI121cry1Ab carrying the synthetic cry1Ab gene containing the first intron of castor bean catalase-1 gene was driven by the CaMV35S promoter and nopaline synthase terminator with Kamamycin-resistance gene (npt II) was driven by the Nos promoter and Nos terminator. LB, left border; RB, right border; nptII, neomycin phosphotransferase;CaMV35S-P,cauliflower mosaic virus promoter Nos-P, nopaline synthase promoter; Nos-T, nopaline synthase terminator.

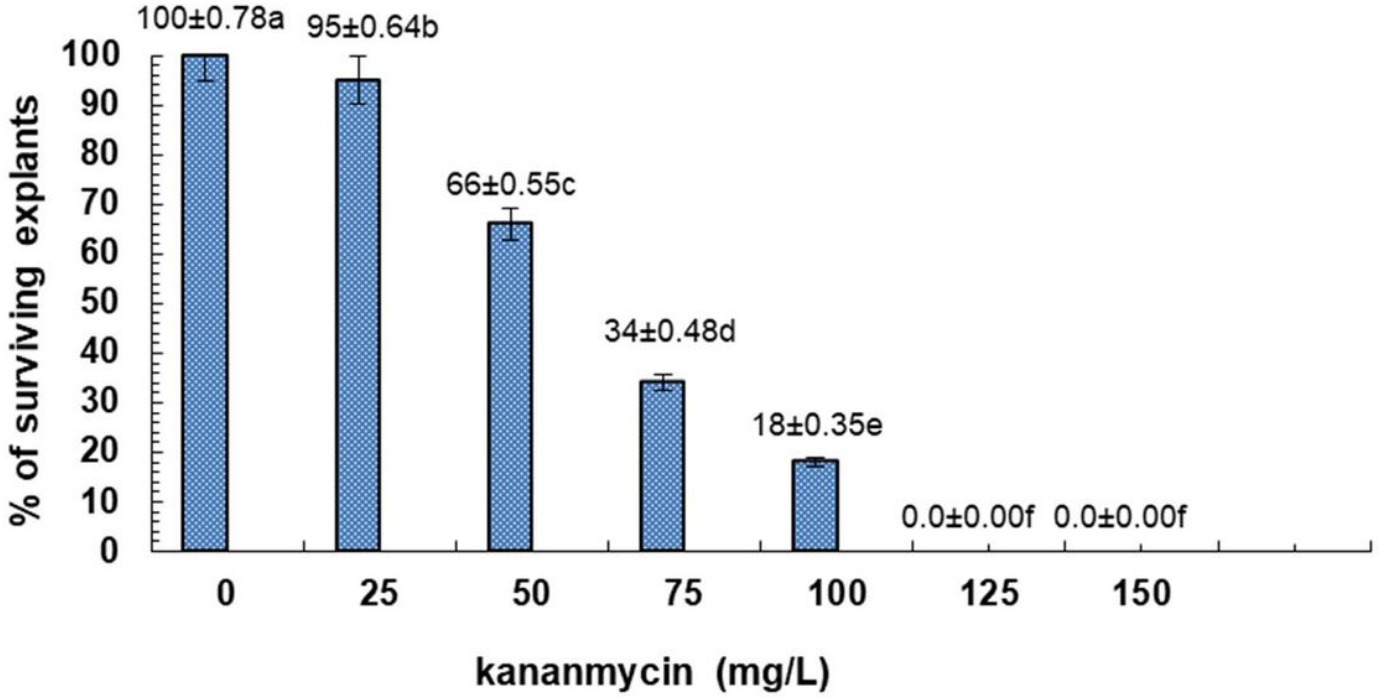

Figure 2

Effect of kanamycin concentrations on leaf segments of peach (Prunus persica L.) plants.

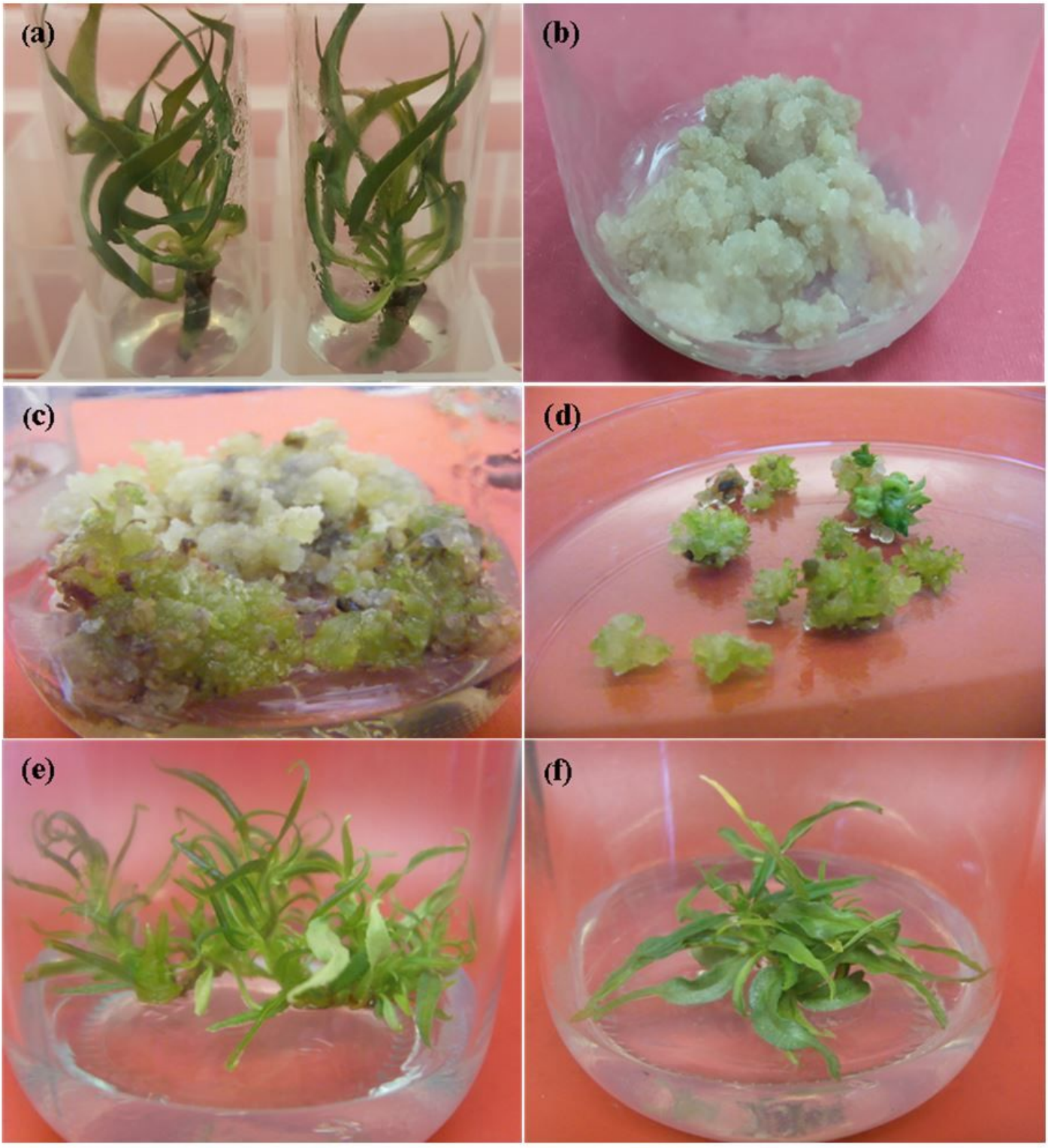

Figure 3

Adventitious shoot regeneration from in vitro leaves of peach. a Leaf explants from in vitro shoots that were previously propagated by stem node sections on MS medium 2.0 mg L-1 BA and 0.2 mg L-1IBA; b Callus formation from leaf explants on WPM medium supplemented with 2.5 mg L-12,4-D and 0.5 mg L-1BAP; c Nodular type callus conducive to adventitious bud formation and shoot differentiation; d

Adventitious shoots induction from callus on WPM medium supplemented with 3.0 mg L-1,1.0 mg L-1kinetin and 0.5 mg L-1 IAA after 35 days; e and f. Developed shoot after 45 and 60 days.

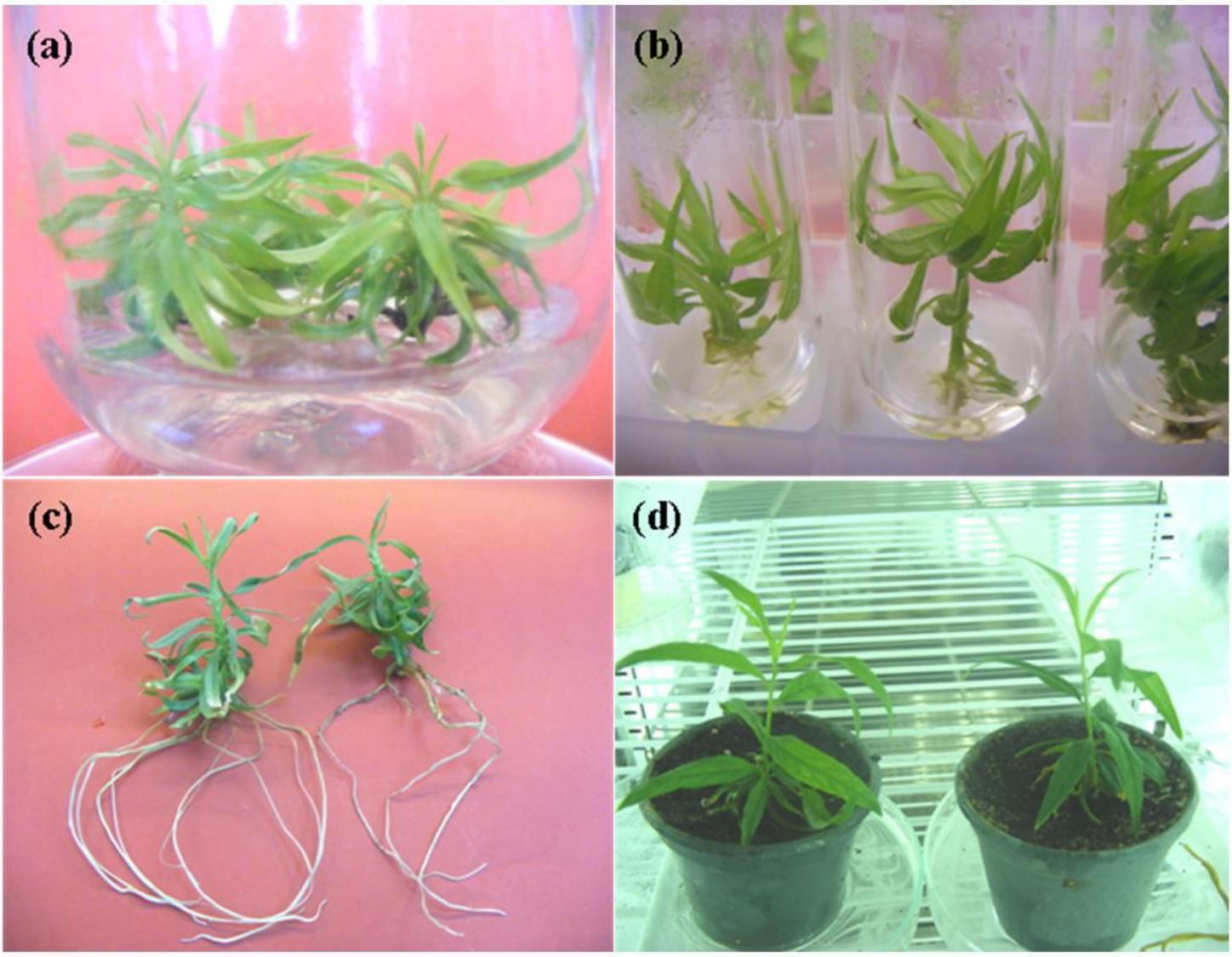

Figure 4

In vitro propagation stages of regenerated transformed peach plants. a elongated shoots were cultured on MS medium containing 0.5 2iP after one month; b Root formation on liquid MS medium supplemented with 2.0 mg L-1 IBA and 0.5 mg L-1 NAA and 162 mg L-1 phloroglucinol after 21 days; c after 45 days; d Acclimatization of transformed peach plants.

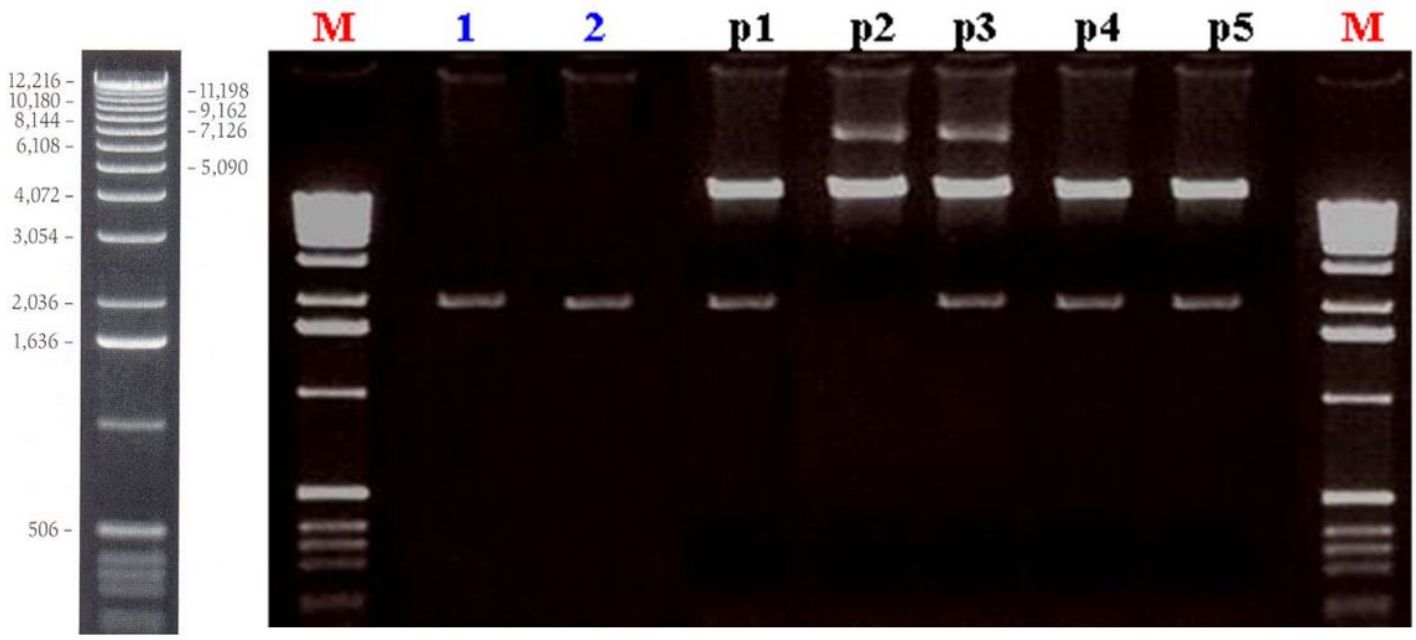

Figure 5

Molecular analysis of the cry1Ab gene in peach (Prunus persica L.). PCR detection of cry1Ab gene with intron in putative transgenic peach plants, amplifying about 2.035 kb with transgenic plants (Lanes 1-2). pBI121cry1Ab digested with BamHI to release the binary plasmid vector pBI121 (upper band) along with the insert cry1Ab gene with intron (2.035kb lower band) (Lanes p1, p2, p4 and p5) compared with the binary plasmid vector pBI121 without cry1Ab gene (Lane p2). Lane M: TrackIt™1 Kb DNA Ladder.

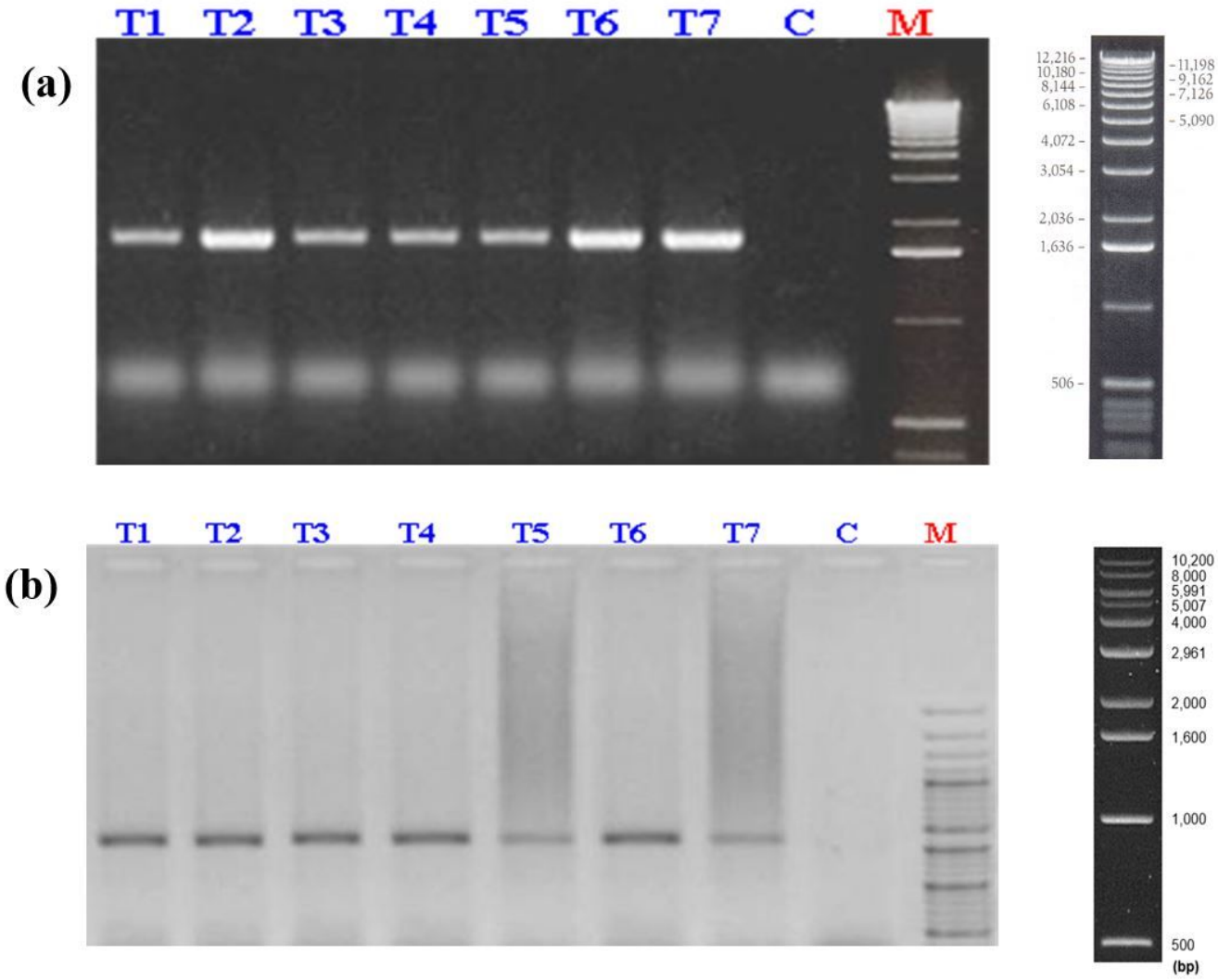

Figure 6

RT-PCR and Northern blot analysis of transgenic peach (Prunus persica L.) plants. a RT-PCR analysis of seven randomly selected transgenic peach plants of cry1Ab showing 1845 bp cry1Ab gene transcripts with specific primer. b Northern blot analysis of transgenic peach plants showing RNA blot with 32P dCTP-labelled cryIAb probe. Lanes (T1-T7) transgenic peach plants. Lane (C) control (non-transgenic peach plants. Lane (M) TrackIt™ and AccuLadder™ 1 Kb DNA Ladder).

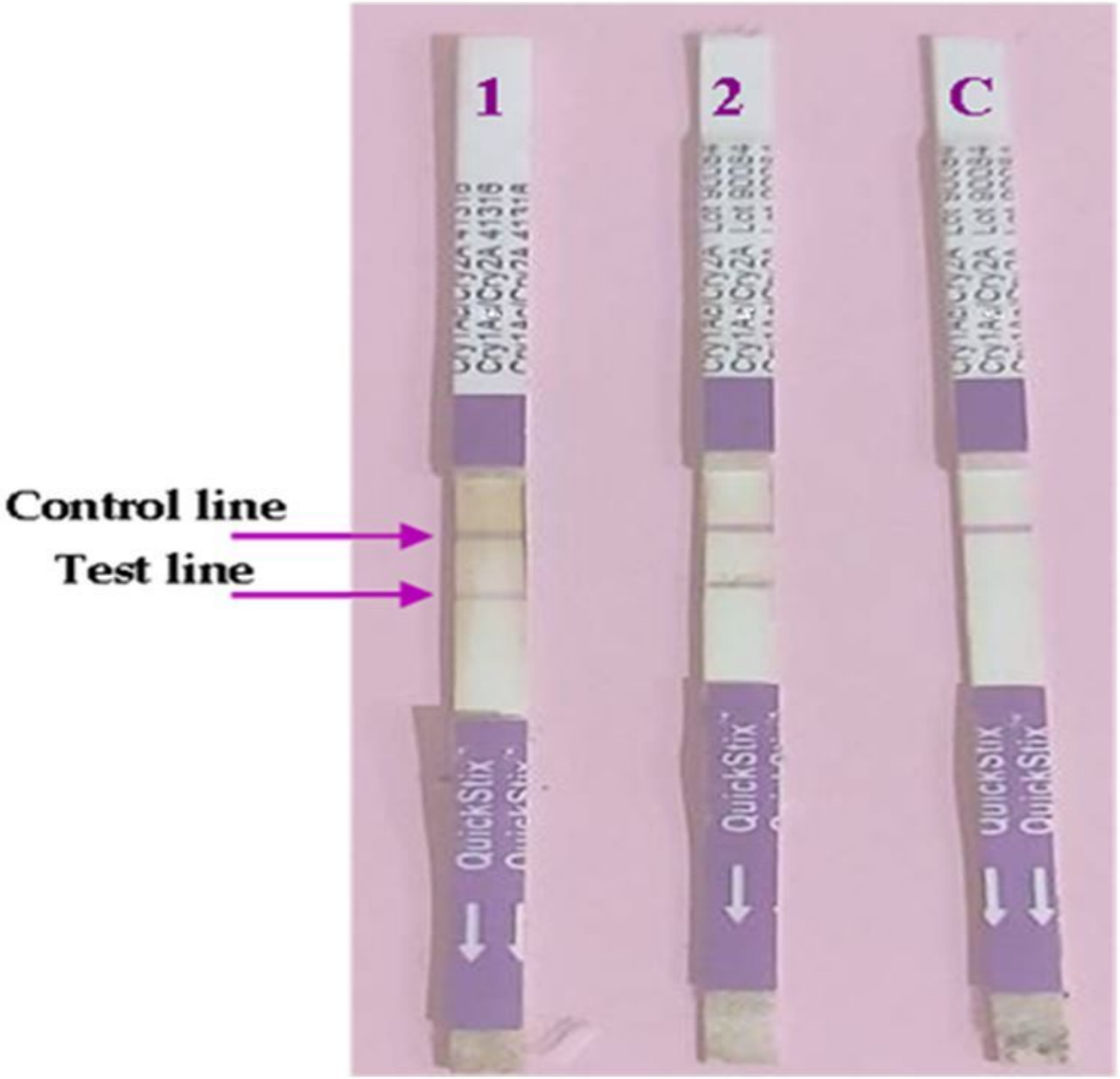

Figure 7

Immunostrip detection of Cry1Ab protein in PCR-positive transgenic peach (Prunus persica L.) plants. Lanes 1 and 2: Transgenic peach plant expressing Cry1Ab protein; Lane c: nontransgenic peach plant.

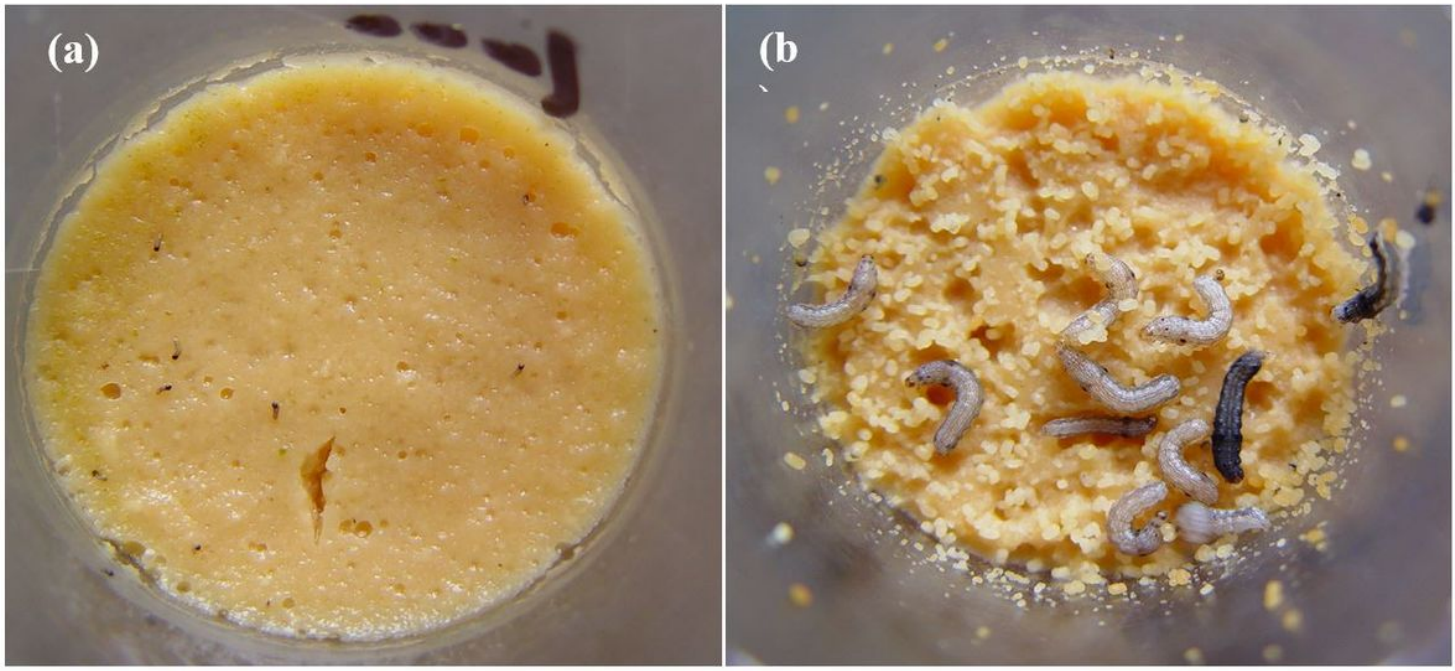

Figure 8

Effect of toxicity of the transformed peach with cryIAb on Synanthedon exitiosa. a mortality reached 100% at 1000 ppm of dried transformed peach leaf; b control.