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Transformation of Citrus with Coleopteran Specific δ-Endotoxin Gene from *Bacillus thuringiensis ssp. tenebrionis*

Seong Lyul Rhim[1*], Il Gi Kim[1], Tae Eun Jin[1], Jin Hyoung Lee[1], Ching I Kuo[2], Suk Chul Suh[3], Li Chun Huang[2]

[1]Department of Genetic Engineering, Hallym University, Chuncheon, Gangwon 200-702, Korea; [2]Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, 11529 Republic of China; [3]National Institute of Agricultural Biotechnology, RDA, Suwon 441-707, Korea

Abstract

A modified δ-endotoxin gene of *Bacillus thuringiensis* ssp. *tenebrionis (B.t.t.),* encoding a coleoptera-specific toxin, was utilized to transform citrus plants, *Citrus reticulata* Blanco 'Ponkan' mandarian. By co-culturing the nucelli with *Agrobacterium tumefaciens* harboring the modified gene in the binary vector pBinAR-Btt, the chimeric toxin gene was transferred into citrus plants. The transgenic plants were selected on modified Murashige and Skoog medium containing kanamycin. Hybridization experiments demonstrated that the transgenic plants contained and expressed the toxin protein gene.

Key words: *Bacillus thuringiensis*, δ-endotoxin gene, transgenic citrus plants

Introduction

The genus *Bacillus thuringiensis* (*B.t.*) is comprised by several subspecies of entomocidal, spore forming bacteria which confer specific toxicity to insects of the orders Lepidoptera, Diptera and Coleoptera (Höoefte and Whiteley 1989). The *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) was first described by Krieg et al. (1983) and shown to produce insecticidal proteins directed against coleopteran larvae. The insecticidal crystal of *B.t.t.* differs from those of other *B.t.*-strains in shape and size of protein subunits. *B.t.t.* produces very characteristic flat, plate-like crystals that are quadrangular to rhomboidal (Krieg et al. 1987). In contrast, lepidopteran-active *B.t.* strains such as *B.t.* subsp. *kurstaki* (*B.t.k.*) produce bipyramidal crystals (Aronson et al. 1986). In *B.t.t.* and in *E. coli*, the toxin gene produces two proteins of 74 kDa and 68 kDa (McPherson et al. 1988; Rhim et al. 1990; Sekar et al. 1987). This characteristic has been attributed to two translation initiation sites in the gene (McPherson et al. 1988; Rhim et al. 1990). The *B.t.k.* strains typically produce a large precursor protein of 130 kDa to 140 kDa (Höefte and Whiteley 1989). The *B.t.t.* insecticidal crystal proteins are not immunologically cross reactive with several *B.t.k.* crystal proteins (McPherson et al. 1988; Sekar et al. 1987). This provides further evidence of significant differences between the DNA sequences of genes encoding lepidopteran-specific and coleopteran-specific insecticidal proteins. A region of DNA approximately 70 bp upstream of the translational initiation site, which is highly conserved among lepidopteran-active genes and found in a dipteran-active gene (Thorne et al. 1986), is absent in the *B.t.t.*-gene. Previously, we constructed a binary vector pBinAR-Btt containing the chimeric gene of the *B.t.t.*-toxin gene, mutagenized at the 5'-end region, and introduced it into tomato plant cells (Rhim et al. 1990, 1995). The transgenic tomato plants produced significant quantities of *B.t.t.*-toxin and showed strong insecticidal activity against Coloraro potato beetle.

In this report, we describe the transformation of citrus plants, *Citrus reticulata* Blanco 'Ponkan' mandarian, with the vector pBinAR-Btt. The citrus stem boring longicorn or longhorn beetle (*Anoplophora maculata* Thompson) damages significantly the fruit trees in South East Asia, especially in Taiwan. Thus, the intend of this investigation was to produce the transgenic citrus plants that might be able to resist the citrus longicorn beetles.

* Corresponding author, E-mail: slrhim@hallym.ac.kr
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Materials and Methods

Reagents and enzymes

Restriction endonucleases, T4-DNA ligase, Taq DNA polymerase, and Dioxigenin (DIG) for labelling of DNA fragments were purchased from Roche (Mannheim, Germany). Nitrocellulose paper membranes were obtained from Schleicher & Schuell, and the other biochemicals from Sigma Chemical Co.

Transformation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens LBA4404 was cultured at 28℃ over night. The binary vector plasmid pBinAR-Btt was then transferred into *Agrobacterium* by electroporation (Easy Jet System, Belgium) using 2 mm cuvettes and conditions of 25 μF and 2.5 kV in 500 μL of 10% glycerin solution. The transformants were selected on LB agar medium containing 50 mg/L of kanamycin.

Transformation of citrus plants

Transformation of citrus, *Citrus reticulata* Blanco 'Ponkan' mandarian, was attempted by co-culturing 30 nucellus explants, freshly excised from immature fruits, for 48 h with *Agrobacterium tumefaciens* carrying the binary vector. The co-culture medium contained MS salts (Murashige and Skoog 1962), 3% sucrose, MT vitamins and glycine (Murashige and Tucker 1969), 100 mg/L i-inositol, 0.3 mg/L BA (N_6-benzyladenine), 10 mg/L adenine sulfate dihydrate, 500 mg/L Difco Bacto malt extract, and 0.2% Gelrite™. The co-culturing was followed by alternately sub-culturing all tissues at weekly intervals between a medium supplemented with 500 mg/L carbenicillin, 250 mg/L cefotaxime and 20 mg/L kanamycin sulfate, and another containing no antibiotics. This procedure enabled removal of bacteria and growth and embryogenesis of nucelli. Complete bacterial elimination and sufficient growth of nucelli required about 3 months of alternating between the two media.

PCR (polymerase chain reaction)-mediated Southern blot analysis

Isolation of total DNA from plants was performed by phenol extraction (Rogers and Bendich 1988). The DNA was analysed by PCR using two primers complementary with *B.t.t.*-toxin gene in both termini were 20mer oligonucleotides (btt1, GCATAGAATTCAATTTCACA; btt2, TAATGAATTCTAGCTCGATA). PCR amplification was performed in 100 μL of total volume. Each reaction mixture was contained 1 μg of genomic DNA template, 100 μM each dNTP, 1 pmole each of the two primers and 1.5 unit DNA polymerase. The mixture was subjected for optimal results at 25 cycles in each consisting of 94℃ for 1 min, 54℃ for 2 min. 72℃ for 3 min. The samples following the PCR-analysis were stored at 4℃ for further experiments. The PCR products were separated on 0.8% agarose gel and blotted onto nylon membrane (Sambrook et al. 1989). The membrane was hybridized with DNA probe (676 bp fragment of *Eco*RI in the *B.t.t.*-toxin gene) labeled with DIG at 68℃ for 16 h, which was washed stringently at 68℃. After the washing, the fragment of DNA was stained by using the DIG-detection kit.

Immunohybridization of transgenic plant protein

For extraction of total protein from the citrus plants with the *B.t.t.*-toxin gene, 5 grams of leaf tissue was frozen in liquid N_2, powdered and sonicated in 2 mL of the extraction buffer (50 mM·Tris-HCl, 100 mM NaCl, 0.5% NP40, 1% 2-mercaptoethanol, 4% SDS, 2.5 mM phenylmethylsulfonyl fluoride, pH 12.5) as described by Barton et al. (1987). 100 μL of total protein extract was used for electrophoresis on 7% SDS-polyacrylamide gel and for immunoblot hybridization. The immunohybridization was performed with a monospecific *B.t.t.*-toxin antibody purified on a substrate column using conjugated alkaline phosphatase as previously described (Rhim et al. 1990).

Results and Discussion

Transformation and regeneration of citrus plants

The cultures, when freed from bacteria, were allowed to undergo embryogenesis by further culturing in antibiotic-free medium for four months, with the medium being refreshed every month. Ultimately, the nucelli proliferated into clusters composed of embryos. Ten of the original nucelli had developed clusters that were at least 2 cm in diameter. The ten clusters were divided into 5 mm portions and equal amounts of each cluster were subcultured for another three months on media with or without 20 mg/L kanamycin sulfate. The tissues were weighed and the media were refreshed at monthly intervals. Putative transformants were selected at the end by comparing the fresh weights of embryo clusters from kanamycin vs. kanamycin-less media. Clusters that showed equal and better growth on kanamycin medium were tentatively concluded as having been transformed. However, only one cluster that gave better growth in kanamycin-containing than in kanamycin-less medium, and originating in one nucellus was designated line 8. The cluster line was retained for further investigation. The embryogenic tissues from line 8 were maintained in fresh media until shoots

emerged. The shoots were excised when about 1 cm tall and grafted onto Troyer citrange (*Poncirus trifoliata* x *Citrus sinensis*) seedlings *in vitro* to obtain transplantable plants.

Shoots were not rooted directly because the process occurred too slowly. Only three of twelve plants survived when potted in soil and were available for verification of transformation.

Detection of *B.t.t.*-toxin gene

Figure 1. depicts the *B.t.t.*-toxin gene isolated from transgenic citrus plants (lanes 2, 3, 4). The gene was not detected in the nontransformed citrus plant (lane 1). The results showed that the *B.t.t.*-toxin gene was incorporated into the three transformed citrus plants.

Expression of *B.t.t.*-toxin gene in citrus plants

Expression of *B.t.t.*-toxin gene was detected in the transgenic citrus plants. The expected 74 kDa toxin protein band was observed in the three transformed plants (Figure 2, lanes 2, 3, 4). Based on staining intensities and comparison with *B.t.t.*-produced toxin (Rhim et al. 1990, 1995), we estimate that approximately 0.13 μg toxin protein was present in 100 μL of the extract of the plant, i.e., the toxin yield of the transgenic plant was about 0.00013% per gram fresh leaf tissue. No toxin was detected in the untransformed control plants (Figure 2, lane 1). The result indicated that the chimeric construct of the *B.t.t.*-toxin gene was successfully expressed in citrus plants.

We transformed citrus plants, which are very important as income source of many farmers in Asia. The longicorn beetle, *Anaplophora maculata* Thomson, has been a devastating pest of citrus trees. The adult beetle feeds on the leaves, whereas the larvae burrow and tunnel through the stems. It might be possible to protect the trees if transgenic plants with resistance against the insect can be achieved. The *B.t.t.*-toxin gene was chosen for that purpose. We first established a transformation system based on nucellus culture. The binary vector pBinAR-Btt containing the chimeric toxin gene was then transferred to the nucelli by infection with *Agrobacterium*. Three transgenic plants that regenerated from thirty nucelli were selected on basis of a kanamycin resistance. The transformation efficiency was about 10%. The *B.t.t.*-toxin gene was detected in all three transgenic plants by PCR mediated Southern blot analysis and its expression was determined by toxin protein assay. Based on the findings it is proposed that nucellar culture and the vector pBinAR-Btt may be useful for transformation of a variety of citrus and possibly other woody fruit trees. The PCR mediated Southern blot analysis, newly adopted in these experiments, maybe sensitive enough to detect the toxin gene in the cell. However, it is still not known that the expression of the toxin gene in the citrus plants is enough to protect the citrus trees against the longicorn beetle. The bioassay to correlate *B.t.t.*-toxin expression with insect mortality is in progress. The toxin produced by the transgenic citrus plant was about 0.00013% per gram fresh leaf tissue. The expression level was lower than in tomato plants. The amount of recombinant *B.t.t.*-toxin in the transgenic tomato plants was about 0.001% of the fresh

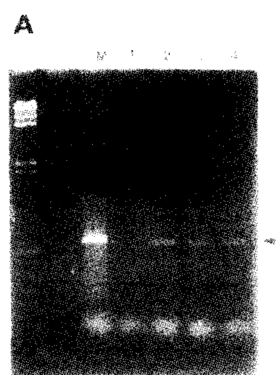

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Figure 1. Detection of *B.t.t.*-toxin gene in transgenic citrus plants. A: The *B.t.t.*-toxin gene was detected by using PCR amplification from total DNA of the transgenic citrus plants. B: The amplified DNA was hybridized with with the DIG-labeled *Eco*RI-fragment (676 bp) of pBinAR-Btt. Lane M: PCR amplification of 676 bp of the *Eco*RI-fragment (marker), Lane 1: normal citrus plant (wild type, control); Lanes 2, 3, 4: transgenic citrus plants. Arrows indicate the 676 bp of the *Eco*RI-fragment.

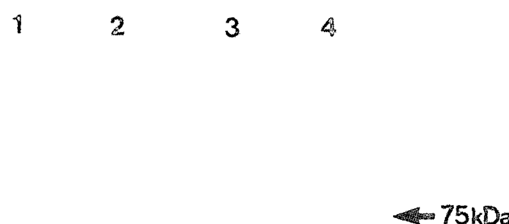

Figure 2. Immunohybridization analysis of transgenic citrus plants. Total protein was extracted from the leaf of the wild type or transgenic citrus plants. The extract (100 μL) was separated on a 7% SDS-polyacrylamide gel and blotted onto nitrocellulose paper for the hybridization with antiserum against the *B.t.t.*-toxin. Lane 1: normal citrus plant; Lanes 2, 3, 4: transgenic citrus plants. Arrows indicate the *B.t.t.*-toxin of 75 kDa.

weight of tissue (Rhim et al. 1995). Assuming 10 g protein per gram leaf tissue, the expression of *B.t.t.*-toxin was high compared to the *B.t.k.*-toxin, which was found at 0.001% of the total callus protein (Barton et al. 1993) or about 50 ng per gram of fresh leaf tissue (Fischhoff et al. 1987). During the screening for putatively transgenic plants, it was observed that nucelli infected with *Agrobacterium* containing the plasmid pBinAR-Btt were very sensitive to kanamycin. Kanamycin may have a significant inhibitory effect on differentiation of citrus nucellus. It will be interesting to analyze the roles of kanamycin during the embryogenesis and differentiation of the citrus nucelli.

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