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Molecular Breeding of Transgenic Tomato Plants Expressing the δ-Endotoxin Gene of *Bacillus thuringiensis* subsp. *tenebrionis*

Seong-Lyul Rhim

Department of Genetic Engineering, Hallym University, Chuncheon, 200-702, Korea

Abstract : The transgenic tomato plants showing the insecticidal activity against the coleopteran insect larvae have been bred to the 4th generation (R_4). The *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*)-toxin gene and the expression were detected in the R_4 transgenic plants. The expression of the toxin gene conferred a coleopteran insect larvae tolerance to the transgenic tomato plants. The ploidy levels of the R_4 transgenic plants were diploid. The results indicated that the toxin gene was inherrited to the next generation and expressed. Such a molecular breeding can provide a method for a permanent control of insects of agronomic relevance.(Received March 4, 1998; accepted April 20, 1998)

Introduction

The *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) was first described by Krieg *et al.* (1983) and shown to produce insecticidal proteins directed against coleoptera larvae. The insecticidal crystal of *B.t.t.* differs from those of other *B.t.*-strains in shape and in the size of the protein subunits. *B.t.t.* produces very characteristic flat plate-like crystals being quadrangular to rhomboidal in outline (Krieg *et al.*, 1987). The *B.t.t.*-toxin gene was cloned and mutagenized at the 5'-end region (Rhim *et al.*, 1990) for the construction of a chimeric gene with the *B.t.t.*-toxin gene. The chimeric *B.t.t.*-toxin gene was introduced to tomato plant cells. Tomato is also a host plant for the colorado potato beetle larvae (Coleoptera) and can be readily transformed and regenerated in a leaf disk system. The transgenic tomato plants expressed an insecticidal protein of 74 kDa against the coleopteran insect larvae and were determined as diploid (Rhim *et al.*, 1995). The expression caused a significant insecticidal activity of the transgenic tomato plants against colorado potato beetle larvae. In this report, the inheritance of the toxin gene and the toxin activity was tested in the R_4 generation of the transgenic plants. From this result, it was observed that the toxin gene could be inherited and the insecticidal activity was maintained in the R_4 transgenic tomato plants.

Materials and Methods

Reagents and enzymes

Restriction endonucleases, DNA polymerase, DIG (Dioxigenin) for DNA labelling and detection-kits were supplied by Boehringer Mannheim. Nitrocellulose paper membranes from Schleicher & Schuell. The other biochemicals were purchased from Sigma Chemical Co.

Breeding of transgenic plants

The transgenic plants were transplanted from the MS-salt (Murashige and Skoog, 1962) agar culture media to soil pots via the water culture for seven days long at room temperature. The plants were grown in the greenhouse and self-pollinated. The seeds were collected and asceptically germinated on the MS-salt agar medium containing kanamycin (100 μg/ml). This procedure was repeated for three times, obtaining R_4 plants. The R_4 plants were used to assay for the determination of the inheritance and expression of the toxin gene.

Determination of ploidy level

The ploidy level of the transgenic plants was determined by counting the number of chloroplasts per guard cell pair in leaf epidermal tissue (Koorneef *et al.*, 1989). The epidermal tissue was peeled from the lower surface of the leaves, mounted in 30 mM KCl, 10 mM K^+-Mes (pH 6.0) solution on a slide glass, and observed. The number of chloroplasts was counted under a microscope (Zeiss Jena, Germany) with 100 x objective lens immersed in oil.

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 used for

Key words : Molecular breeding, R_4 transgenic tomato line, Gene inheritance, *Bacillus thuringiensis* subsp. *tenebrionis*-toxin gene.

the PCR analysis. The two primers complement with *B.t.t.*-toxin gene in both termini were 20mer oligonucleotides (btt1, GCA-TAGAATTCAATTTCACA; btt2, TAATGAATTCTAGCTCG-ATA). PCR amplification was performed in 100 μl of total volume. Each reaction mixture contained to 1 μg of genomic DNA template, 100 μM each dNTP, 1 pmole of each two primers and 1.5 unit DNA polymerase. The mixture was subjected for optimal results at 25 cycles, 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 seperated 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 EcoRI in the *B.t.t.*-toxin gene) labeled with dioxigenin(DIG) at 68oC for 16h, which was washed stringently at 68oC. After the washing, the fragment of DNA was colorized by using the DIG-detection kit.

Immuno-blot analysis of transgenic plant protein

For extraction of total protein from the tomato plants with the *B.t.t.*-toxin gene, leaf tissue was frozen in liquid N2, powdered and sonicated in 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). The total protein extract was used for electrophoresis on a 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).

Insecticidal activity tests

Tomato plants were tested for lethality to colorado potato beetle larvae (*Leptinotarsa decemlineata*) belong to Coleoptera with a leaf feeding assay. After the feeding, the mortality of the larvae was scored. The insect bioassays were performed in duplicate or triplicate.

Results and discussion

Selection and breeding of transgenic tomato plants with the *B.t.t.*-toxin gene

Tomato plants were transformed with the plasmid vector pBinAR-Btt, consisting of the 35S promoter of the cauliflower mosaic virus (CaMV), a modified *B.t.t.* gene and the 3'-end of the octopine synthase gene from the Ti-plasmid of *Agrobacterium*, and three transgenic plant lines were obtained as previously described (Rhim *et al.*, 1990, 1995). All the three transgenic plants (R_0) showed no difference in the morphology between these transgenic plants and the nontransgenic parent plants. The test of ploidy level indicated that the transgenic plants were diploid. The R_0 transgenic plants were grown in the green house and self-pollinated. The seeds from one of the three transgenic plants were collected and asceptically germinated on the MS-agar medium containing kanamycin. The resistance to kanamycin indicated that the progeny plants contained the transgene including the neomycin phosphotransferase II gene. The progeny plants were grown in the greenhouse and self-pollinated. Such a procedure was repeated for three times to get the R_4 progeny plants.

Detection of *B.t.t.*-toxin gene in the R_4 transgenic tomato plants

The *B.t.t.*-toxin gene was detected by using the PCR followed by Southern- blot hybridization with the DIG-labeled *Eco*RI-fragment (676 bp) of pBinAR-Btt. For three random selected R_4 progny lines, total DNA was isolated, and the region of the *Eco*RI-fragment was amplified by 25 cycles using the PCR method. The products were separated on the agarose gel and blotted on the nylon filter. The hybridization was performed with the DIG-labeled *Eco*RI-fragment (Fig. 1). The *B.t.t.*-toxin gene was detected in the R_4 transgenic tomato plants (Fig. 1, lanes 2, 3 and 4), but not from DNA of

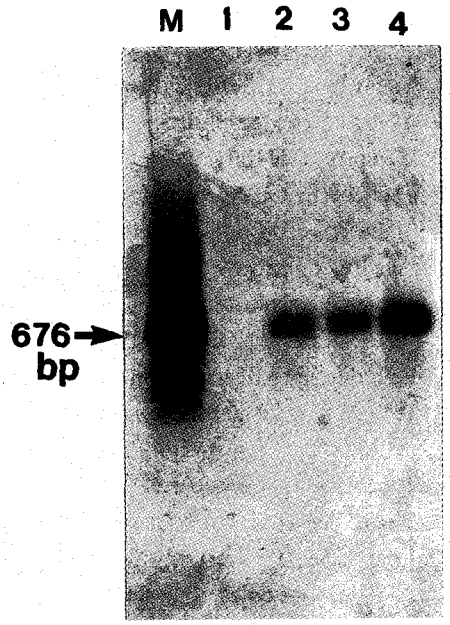

Fig. 1. Detection of *B.t.t.*-toxin gene in the R_4 transgenic tomato plants. Total DNA was isolated from the three random selected R_4 progeny plants. The *B.t.t.*-toxin gene was detected by using PCR amplification followed by Southern hybridization with the DIG-labeled EcoRI-fragment (676 bp) of pBinAR-Btt (Rhim *et al.*, 1995). Lane M: PCR amplification of 676 bp of the EcoRI-fragment. Lane 1: PCR amplification of normal tomato plant (wild type, control).Lanes 2-4: PCR amplification of the R_4 transgenic tomato plants.

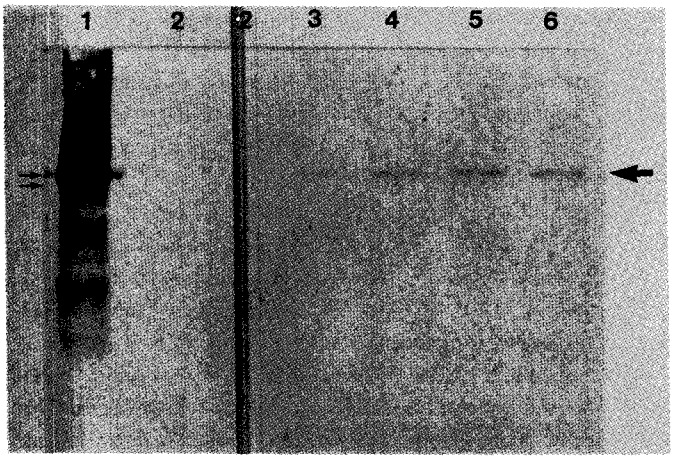

Fig. 2. Immuno-blot analysis of the R_4 transgenic tomato plants. Total protein was extracted from the leaf of the transgenic plants. The extract was separated on a 7% SDS-polyacrylamide gel and blotted on nitrocellulose paper, and detected with antiserum against the *B.t.t.*-toxin. The small arrows indicate the 68 and 74 kD toxin in E. coli transformed with *B.t.t.*-toxin gene. The large arrow indicates the 74 kD toxin expressed in R_4 progeny tomato plants. Lane 1: *B.t.t.*-toxin (10 μg). Lane 2: wild type of tomato plants (100 μg). Lanes 3-6: R_4 progeny tomato plants (100 μg).

a wild type plant (Fig. 1, lane 1). This result indicated that the toxin gene was inherited to the R_4 plant lines.

Immuno-blot analysis of the R_4-transgenic tomato

To analyze the expression of *B.t.t.*-toxin gene, the total proteins were extracted from the fresh leaf tissues and separated on a SDS-polyacrylamide gel. After transfer on nitrocellulose paper, immunohybridization was performed with *B.t.t.*-toxin antibody. Only one protein band of 74 kDa of the expected size of the toxin was observed in the transgenic plants (Fig. 2). Based on staining intensities and comparison with *B.t.t.*-produced toxin, we estimated that approximately 0.5 μg toxin protein was present in 100 μg of total protein. In other words, the toxin produced in the transgenic tomato plant was about 0.5% of the total soluble protein or 0.0005% of the fresh leaf tissues. The same expression patterns had been observed in the R0 transgenic plants (Rhim *et al.*, 1995). In contrast, no toxin was detected in wild type plants.

Determination of ploidy level

The ploidy level of the R_4-transgenic plants was determined by counting the number of chloroplasts per guard cell pair in leaf epidermal tissue (Koorneef *et al.*, 1989). The number of the chloroplasts ranged between seven and nine per guard cell pair in the R_4 progeny transgenic tomato plants, indicating that the transgenic plants were diploid (Fig. 3).

Insecticidal activity tests

The R_4 whole plants were tested for toxicity by using a feeding assays with 10 neonate larvae of *L. decemlineata* for three days, which showed that there was little feeding damage to the plants (Fig. 4). No mortality of larvae was observed in a control experiment. The result of the insecticidal activity tests reported here can provide a method for a permanent control of insects of agronomic relevance in contrast to the sporadic pest controls. Genetically engineered insect tolerant crop plants could thus provide a valuable addition to current strategies which mainly rely on whole bacteria of *B.t.* strains.

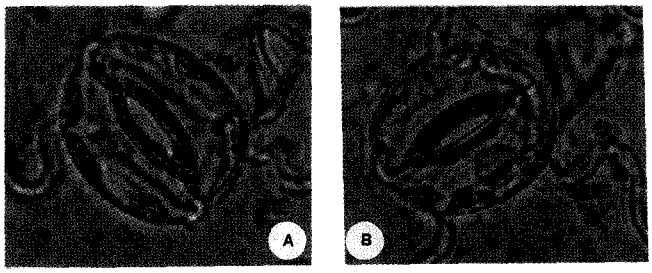

Fig. 3. Chloroplasts in guard cell pair observed by light microscopy for the determination of the ploidy level in one of the R_4 progeny tomato plants(x1,000). A: R0 transgenic plant B: R_4 progeny plant.

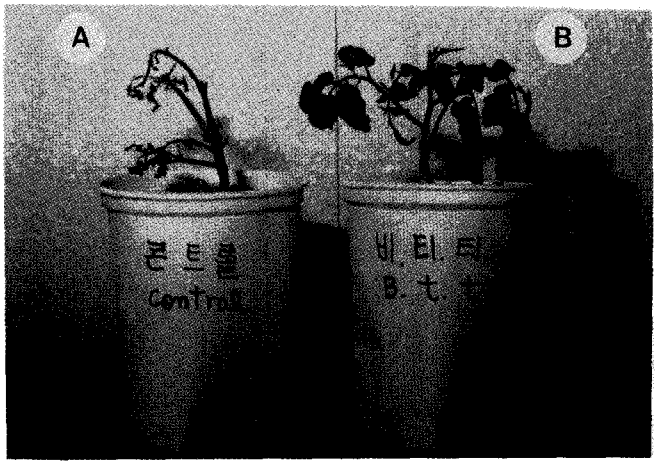

Fig. 4. Insecticidal activity tests to colorado potato beetle. The R_4 progeny whole tomato plants were tested for the insecticidal activity by using a feeding assays with 10 neonate colorado potato beetle larvae for three days. The picture shows one of the results of the feeding bioassays with the whole R_4 transgenic tomato plants. A: nontransgenic tomato plant B: The R_4 progeny tomato plant.

Acknowledgement

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살충성 형질 전환 토마토 식물체의 분자 육종

임성렬(한림대학교 유전공학과)

초 록 : 딱정 벌레목 유충에 살충성을 나타내는 형질 전환 토마토 식물체를 4세대까지 분자 육종을 하였다. 분자 육종된 4세대 식물체에서 살충성 유전자인 *B.t.t.* 독소 유전자와 유전자가 발현되는 것을 확인하였다. 이 4세대 형질 전환 식물체는 독소 유전자 발현에 의해 딱정 벌레 유충에 살충성을 나타내고 있음이 확인 되었고, 4세대 형질 전환 식물체는 염색체는 2 배수체로 확인되어 정상적인 토마토 식물체임이 증명 되었다. 이 결과들은 형질 전환에 사용된 독소 유전자가 다음 세대로 안정되게 유전되고 있음을 나타내고 있다는 증거이다. 이러한 형질 전환된 식물체의 분자 육종은 농업에 있어서 현재의 일시적인 해충 방제에 비해 장기적인 해충 구제에 대한 새로운 방법의 가능성을 제시하고 있다고 하겠다.

찾는말 : Molecular breeding, R_4 transgenic tomato line, Gene inheritance, *Bacillus thuringiensis* subsp. *tenebrionis*-toxin gene