Plant Pathol. J. 19(3) : 162-165 (2003)

Transformation of Fuji Apple Plant Harboring the Coat Protein Gene of *Cucumber mosaic virus*

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(Received on April 30, 2003; Accepted on June 16, 2003)

Transformation of Fuji apple (*Malus domestica* 'Fuji') was performed using *Agrobacterium tumefaciens* harboring a coat protein (CP) gene of *Cucumber mosaic virus* (CMV). A plasmid DNA containing the virus CP and NPT II genes was introduced into the leaves of apple by the *Agrobacterium*-mediated transformation procedure. Regenerated transformants of the apple were obtained by kanamycin resistance conferred by the introduced NPT II gene. PCR analysis showed that 3 out of 20 putatively selected R0 plant lines contain the CMV-CP gene. Nine putative transgenic lines out of 20 lines were investigated with the PCR analysis; 5 regenerants produced a 450 bp DNA band and 3 regenerants showed a 671 bp DNA band for the NPT II and CMV-CP genes, respectively. Southern hybridization results demonstrate the successful integration of the CMV-CP gene into the genome of the apple. This is the first report on the generation of useful virus resistance source of transgenic apple for molecular breeding program.

Keywords **:** Apple, Fuji, coat protein, CMV, NPT II gene, transformation, virus resistance

Vegetatively propagated clones of horticultural plants have contributed much to production reduction. Horticulturists and orchardists of the 1600s and 1700s believed that fruit cultivars tended to "run out" due to aging and repeated propagation, hence, many cultivars have been replaced periodically with new seedling-produced scions. Some problems in the decline of production were attributed to virus diseases. However, scientists have developed molecular breeding strategies to overcome these circumstances since the discovery of transgenic plant resistant to *Tobacco mosaic virus* in 1986. Molecular breeding is a promising alternative approach in introducing specific characteristics to certain plant species and varieties.

Malus transformation has been achieved in some laboratories by various methods, including the direct uptake of naked DNA by protoplasts (Lee et al., 1995), and the *Agrobacterium*-mediated transformation of leaf segments (De Bondt et al., 1996; James et al., 1989; Maheswaran et al., 1992; Norelli et al., 1994; Martin et al., 1990 and Song et al., 2000). Although these transgenic apples confer reporter and antibiotic resistance genes, the introduction of virus gene(s) useful for pathogen resistance has not been reported yet.

Cucumber mosaic virus (CMV), the type species of the genus *Cucumovirus* (family: *Bromoviridae*), has a particularly wide host range, infecting more than 800 species in over 70 families of mono- and dicotyledonous plants, and it is transmitted efficiently by more than 60 aphid species. Worldwide, CMV causes important diseases in vegetables, ornamentals, legumes, and other important crops. Many strains of CMV have been described and classified into Sub-groups I and II according to the sequence similarity of the genomic RNAs (Palukaitis et al., 1992).

In this study, CMV gene was introduced to apple and the integration of the transgene in transgenic 'Fuji' apple (*Malus domestica*) was confirmed by the *Agrobacterium*-mediated transformation procedure.

Materials and Methods

Plant material. Leaf material used in the experiment was taken from 21-day-old *in vitro*-cultured axillary shoots of 'Fuji' apple. The three or four youngest fully expanded leaves were excised and stored in petri-dishes on sterile filter paper moistened with deionized distilled water. One-third of basal part from each leaf was dissected and placed abaxial side up directly on apple regeneration medium or on N6 medium after *Agrobacterium* co-cultivation.

Explants were incubated in the dark for 21 days at 26-28C and then transferred to 16-hour daily photoperiod condition provided by white fluorescent tubes at 40 $\mu ol \cdot m^{-2} \cdot s^{-1}$.

Agrobacterium strains and co-cultivation. *Agrobacterium tumefaciens* strain LBA4404 harboring a plasmid (pCMASCP 121-123) (Fig. 1) that contain a CMV-CP gene was used as a plant transformation vector in this experiment. The binary vector

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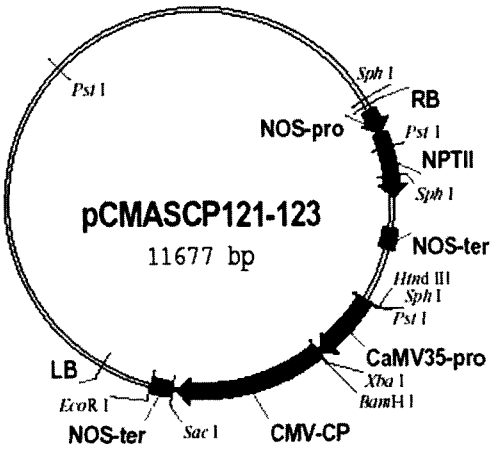

Fig. 1. Schematic diagram of plant transformation vector (pCMASCP121-123) used for apple transformation in this study. RB: right border of T-DNA; LB: left border of T-DNA; NOS-P: nopaline synthase promoter; NOS-T: nopaline synthase terminator; NPTII: Neomycin phosphotransferase II; CaMV35S: cauliflower mosaic virus 35S promoter; CMV-CP: CMV-as coat protein gene.

pCMASCP121-123 consisted of a kanamycin-selectable modification of pBI121, where the GUS gene was replaced with the CMV-CP gene (Ryu and Park, 1995). Leaf explants were dipped in *A. tumefaciens* suspension, blotted on sterile filter paper saturated with modified N6 liquid medium containing 20 μM acetosyringone. After a 24- to 72-hour co-cultivation, leaf explants were washed in N6 liquid medium, blotted, and transferred to media with or without antibiotics.

Selection of putative transformants. After explants were co-cultivated with *Agrobacterium* for 1-3 days, they were transferred to selection medium (regeneration medium supplemented with 200 μg/ml cefotaxime to inhibit further growth of bacteria and 25 μg/ml kanamycin for selection).

Polymerase chain reaction. Subsequently, PCR was used to detect specific NPT II gene sequences from small amount of putatively transformed plant tissue (Moore et al., 1992). Two CMV-specific oligonucleotides derived from the CMV-CP gene were also used as primers for PCR analysis (Ryu and Park, 1995). Genomic DNA was prepared by the procedure of Kim et al. (1997). The PCR was carried out in 20 μl containing 2.5 mM $MgCl_2$, 50 mM KCl, 1.0 mM each of dNTPs, 50 pmol primers, and 1 unit of Taq DNA polymerase (Takara CO.). The PCR reaction was performed in a thermal cycler (MJ Research) with an initiation denaturation step of 5 minutes at 95°C, and subsequently 30 cycles of 30 seconds at 94°C, 1 minute at 55°C, and 2 minutes at 72°C, followed by a final step of 10 minutes at 72°C.

Southern hybridization. Ten μg of DNA from each line was digested with *Hind* III restriction enzyme, fractionated on a 0.9% agarose gel, and transferred onto Hybond-N (Amersham) membrane by the alkaline blotting method (Sambrook et al., 1989). A 25-ng portion of CMV-CP probe was digoxigenen (DIG) labeled using the DIG DNA labeling and detection starter kit II (Boehringer Mannheim). Southern hybridization was made according to the standard protocols given in the kit.

Results and Discussion

Plasmid DNA containing the cucumber mosaic virus coat protein (CMV-CP) and NPTII genes was introduced into the leaves of 'Fuji' apple by the *Agrobacterium*-mediated transformation procedure. In earlier experiments, many of the shoots that regenerated on selective medium were "escape shoots". Hence, a major objective of the present experiments was to minimize the number of escape shoots produced.

PCR was performed on all CMV-CP+ shoots regenerated to confirm the presence of either the NPTII and/or CMV-CP gene in the genomes of putatively transformed apples. This was done while the shoots were still small (<50 mg fresh weight) leaf pieces from regenerated shoots and were subjected to PCR analysis. Thus, this analysis gave a further indication of transformation in harvested shoot. Nine putative transgenic lines out of 20 lines were investigated with the PCR analysis; 5 regenerants produced a 450 bp DNA band (with NPTII primer set) (Fig. 2) and 3 regenerants showed a 671 bp DNA band (with CMV-CP primer set) (Fig. 3). Recent reports with *Agrobacterium*-mediated transformation of the commercial cultivars 'Delicious' (Sriskandarjah et al., 1994) and 'Royal Gala' (Yao et al., 1995), indicated that the regeneration of GUS- and NPT II- positive plants per total number of explants

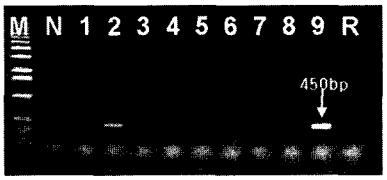

Fig. 2. Analysis of PCR products of NPT II gene in putatively transformed shoots of 'Fuji' apple. Lane M: 1 kb ladder (Gibco BRL Co.); Lane N, R: non-transformed shoots; Lane 1-9: putatively transformed shoots.

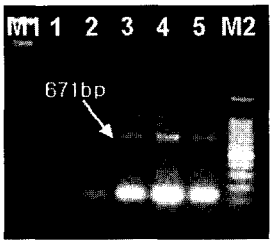

Fig. 3. Agarose gel electrophoresis of PCR products of CP gene of CMV in putatively transformed shoots of 'Fuji' apple. Lane M1: 1 kb ladder (Gibco BRL Co.); Lane M2: 100 bp ladder (Promega Co.); Lane 1: negative control; Lane 2: control; Lane 3-5: putatively transformed shoots.

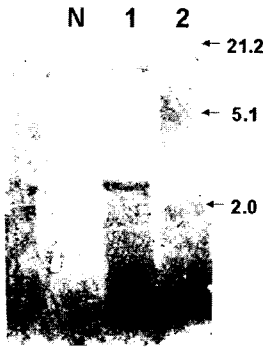

Fig. 4. Southern hybridization analysis of transgenic 'Fuji' apple. Genomic DNA of putative transgenic plants were digested with *Hin*d III and electrophoresed on agarose gel. Lane N: Non-transformed shoots; Lanes 1, 2: transgenic shoots.

varied from 1.5 to 8.7%.

To confirm the integration of T-DNA in transgenic apple plants, Southern blot analysis of two independent transgenic plants and one untransformed plant was performed. Genomic DNA was isolated from untransformed 'Fuji' *in-vitro* propagated plants (Fig. 4, lane N) and putative transformed 'Fuji' clones (Fig. 4, lanes 1, 2). DNA was digested with *Hin*d III and hybridized sequentially with DIG-labeled CMV-CP probe. As expected, the CMV-CP probe hybridized to fragments longer than 2.0 kb in the T-DNA region and the nearest *Hin*d III site in the genomic DNA flanking the insertion sites. The different fragment sizes for each line indicate that each plant arose from separate transformation events (Maximova et al., 1998). The results successfully demonstrated the introduction of the T-DNA insert containing the NPTII gene and CMV-CP gene into the genomes of R0 transgenic plants.

Although some initially regenerated shoots and plants derived from the transgenic plants are chimeric in nature, plants that appear solidly transformed have maintained gene expression for up to 5 years and protein expression for up to 4 years. In conclusion, this study demonstrates that the *Agrobacterium*-mediated transformation of apple tree is established. Actually, CMV is not infected in cultivated apple trees (Lee et al., 2002), and apple-infecting viruses such as *Apple mosaic virus* (ApMV) and *Apple stem grooving virus* (ASGV) are the major pathogens for the plants in Korea. Recently, Lee et al. (2002) reported the sequence information of CP genes of the Korean isolates of ApMV. Transformation of the plant with the newly constructed plasmid vector containing ApMV CP gene is now in progress to make the virus-resistant transgenic apple tree (Lee et al., 2002). This is the first report on the generation of useful virus resistance source of transgenic apple for molecular breeding program in Korea.

Acknowledgment

This study was supported in part by the Genetic Engineering Research Project (Grant #1998-019-G00026) from the Korea Research Foundation in Korea.

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