Construction of Citrus Transgenic Plant with Fatty Acid Desaturase Gene

Seong-Beom Jin, Kyung-Hwan Boo, Do-Seung Lee, Hyun-Byung Chae, Sung-Jun Song[1] and Key-Zung Riu*

Department of Plant Environment & Biotechnology, [1]Applied Radioisotope Research Institute, Cheju National University, Cheju 690-756, Korea

Abstract

The transgenic plant of *Citrus* species (*Citrus aurantium* L.) was constructed with a fatty acid desaturase gene using microprojectile bombardment transformation system. The DNA of a fatty acid desaturase gene, *fad7*, constructed in pBI121 was coated onto tungsten particles (1.1 μm) and introduced into callus cells by bombarding with 1100 psi of helium pressure, 1/4 in of gap distance, 7.0 cm of target distance and 27 in Hg of chamber vacuum. The bombarded cells were selected on the medium containing kanamycin. The selected cells were successfully regenerated into plantlets via somatic embryogenesis on the media containing plant growth regulators. The results of polymerase chain reaction analysis of genomic DNAs from the putative transformants showed that the introduced DNAs of *fad7* were present in both the selected callus cells and the regenerated plantlets.

Key words : Citrus, orange, transformation, microprojectile bombardment, transgenic plant, fatty acid desaturase, *fad7*

Abbreviations : BA, benzyladenine; CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; NAA, naphthalene acetic acid; NOS, nopaline synthase; NPTII, neomycin phosphotransferase II; PCR, polymerase chain reaction

* Corresponding author
Phone : 82-64-754-3344
Fax : 82-64-756-3351
Email : kzriu@cheju.cheju.ac.kr

Introduction

The fatty acid composition of plant cell membrane is characterized by a high level of trienoic fatty acids such as hexadecatrienoic acid (16 : 3) and α-linolenic acid (18 : 3).[1] The trienoic fatty acids appear to have at least two important physiological roles in plants. First, trienoic fatty acids are proposed to be important for low temperature fitness in higher plants.[2,3] Second, linoleic acid (18 : 2) and linolenic acid (18 : 3) serve as precursors for several fatty acid-derived signalling molecules such as traumatic acid and jasmonic acid.[4] These imply that some regulations of the level of trienoic fatty acids are involved in the defensive response of higher plants to environmental stresses including low temperature, wounding, and pathogen invasion.[2-4]

The syntheses of trienoic fatty acids are catalyzed by fatty acid desaturases. Now seven genetic loci, *fad2* through *fad8*, have been identified in higher plants that control fatty acid desaturation.[5,6] Of these loci, *fad2* and *fad3* are involved in desaturation by endoplasmic reticulum desaturases, whereas the others by chloroplast membrane bound desaturases.[5] The composition of unsaturated fatty acids in membrane lipids can be altered by modification of these genes.[7]

Many reports revealed that modifications of the genes encoding fatty acid desaturases altered not only the composition of polyunsaturated fatty acids in membranes but also physiological characteristics of plants. According to Tasaka *et al.*[7] and Ishizaki-Nishizawa *et al.*[8], the transformation of plants with the fatty acid desaturase genes increased trienoic fatty acids in membrane and promoted growth and photosynthesis, especially at

low temperatures. Recently, Martin *et al.* showed the reduction in trienoic acids contents lowered the expression of wound-inducible proteinase inhibitor II in the leaves and tubers of transgenic potato.[9] Therefore, transformation with fatty acid desaturase genes seems to be useful for breeding plants resistant to stress, especially to cold.

Cold tolerance has been a prime objectives in breeding of citrus species in many countries including United States of America, Japan and China.[10] The conventional crossing has been exploited as a basic method for breeding of cold tolerant citrus species.[10] However, recent advances in biotechnology, especially in transgenic plant technique, offer new possibilities for genetic improvement in citrus. In this study we attempted to construct orange transgenic plants with a fatty acid desaturase gene for breeding citrus which is suitable for growing at lower temperature.

Materials and Methods

Plant materials and tissue culture. A callus cell line of orange (*Citrus aurantium* L.) established in the Citrus Research & Education Center, University of Florida was used. The callus cells were cultured basically on a medium containing minerals/vitamins of Murashige-Tucker (MT) medium[11] and 50 g/l of sucrose. The medium was solidified with 8.0 g/l of agar and the pH was adjusted to 5.8 prior to autoclaving at 120°C for 15 min. For the preparation of selective medium, kanamycin solution was filtered through 0.2 μ membrane and added to the autoclaved medium just before solidification. All cultures were subcultured every 3-4 weeks and maintained under 5000 lux of white fluorescence light at 25±2°C with a 16 h photoperiods.

Genes and vectors. The *fad7* gene isolated from *Arabidopsis thaliana* kindly provided by Dr. K. Iba[5] at the Kyushu University, Japan was used as a fatty acid desaturase gene.[5] This gene was reconstructed into *SmaI* site of pBI121 (Clontech Lab, San Francisco, USA) harboring CaMV 35S promoter, NOS terminator, and NPTII gene. The *gus* constructed in pBI121 was used as a reporter gene for optimizing the condition of DNA introduction into citrus cells with microprojectile bombardment system. The plasmids were amplified in *E. coli* JM109. Mega-prep kit (Promega Co., USA) was used for isolation and purification of plasmid DNA.

DNA introduction into cells. Tungsten particles were used as a DNA microcarrier. The microcarrier particles were prepared essentially as described previously.[12] The prepared microcarriers were stored at -20°C and used within two weeks after preparation. DNAs were coated onto the microcarriers according to the procedure described in the previous paper.[12] The DNA-coated microcarriers were transferred onto the macrocarrier disk and bombarded to cells with PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA). The bombardment conditions were as follows : gap distance, 1/4 in; target distance, 7.0 cm; chamber vacuum, 27 in Hg; helium pressure, 1100 psi. The efficiency of DNA introduction into cells was evaluated by GUS assay. GUS assay was performed as described previously.[13]

Genomic DNA analysis. Polymerase chain reaction (PCR) was used for identification of the introduced gene in plant cells. The genomic DNAs of callus and plant were extracted and purified as described.[14] The PCR primers specific for *nptII*[15] and *fad7*[5] were designed based on the DNA sequence of each gene. The sequences of primer pair for *nptII* were 5'-ATG ATT GAA CAA GAT GGA TT-3' and 5'-TCA GAA GAA CTC GTC AAG AA-3', and the expected size of DNA fragment to be amplified was 795 bp. The

sequences of primer pair for *fad7* were 5'-ATG GCG AAC TTG GTC TTA TCA-3' and 5'-TAC CAA GGA AGC TTA TCT TCA-3', and the expected size of DNA fragment to be amplified was 1.0 kb. The PCR kit including DNA polymerase was purchased from Takara (Shiga, Japan). The PCR was begun with denaturation of template DNA's at 96°C for 5 min, and thirty cycles of amplification were performed with denaturation at 96°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. The extension time at final cycle was 10 min. The PCR products were analyzed on a 1.0% agarose gel electrophoresis.

Results and Discussion

Gene introduction into callus cells. Transformation of biological material using the microprojectile bombardment process has been known to be a valuable technique for delivering DNA into the cells of plant,[16] animal,[17] and microbial species,[18] as well as into subcellular organelles.[19] However, achieving high rates of transformation requires optimizing some parameters involved in this process. In order to establish the condition for citrus transformation using the microprojectile bombardment process, we optimized two important parameters, the size of DNA-carrier particle and the helium pressure for accelerating particles.

Table 1 shows the effects of microcarrier size and helium pressure on the efficiency, expressed as GUS score, of DNA introduction into callus cells. The GUS scores of the cells bombarded with M17 particles (1.1 μm) were relatively high compared to other type of particles. However, even with the microcarrier of same size, the cells gave different GUS scores when bombarded under different helium pressure. The highest GUS score was obtained where the M17 particles were used with 1100 psi of helium pressure. Therefore, we used the tungsten particles M17 as a DNA microcarrier and bombarded the cells under 1100 psi of helium pressure to introduce DNA into citrus cells, with 1/4 in of gap distance, 7.0 cm of target distance, and 27 in Hg of chamber vacuum as described in *Materials and Methods*.

Table 1. Effects of DNA-carrier particle size and helium pressure on the efficiency of DNA introduction into citrus callus cells.*

Particle Type (diameter)	Helium Pressure (psi)	GUS Score**
M5 (0.4 μm)	900	+
	1100	+
	1300	+++
M10 (0.7 μm)	900	+++
	1100	++
	1300	+
M17 (1.1 μm)	900	+++
	1100	++++
	1300	++
M20 (1.3 μm)	900	+
	1100	+
	1300	+
M25 (1.7 μm)	900	-
	1100	-
	1300	+++

*The cells were bombarded with tungsten particles coated with the DNA of pBI121 harboring GUS gene. The gap distance, target distance, and chamber vacuum were 1/4 in, 7.0 cm and 27 in Hg, respectively.

**The GUS score is a semi-quantitative estimation of the level of GUS expression in callus cells in a bombarded plate. Each score is the average of the data obtained from at least three plates. Each plate was given a low (-, +), intermediate (++, +++), or high (++++) value based on the number and density of the blue spots which were visible on the surface of the cells.

For bombardment, new cultures of callus cells cultured for 3 weeks under light condition were used. The cells were transferred to dark and kept for 24 h prior to bombardment. The cells were then bombarded with DNA as described above. Figure 1 shows the GUS expression in the citrus callus cells transformed with pBI121 harboring *gus* gene using this procedure.

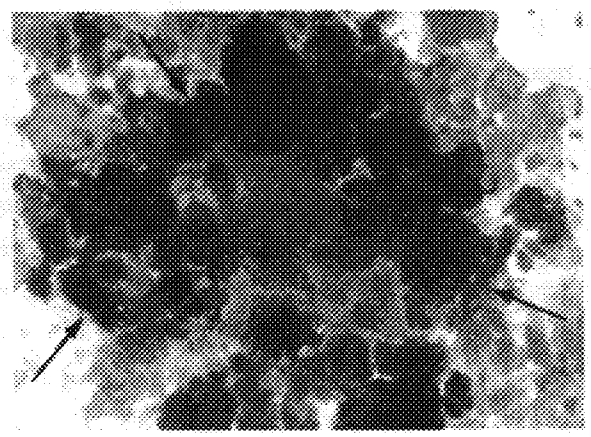

Fig. 1. Transient expressions of GUS gene in the citrus callus cells bombarded with the DNA of pBI121 harboring *gus*. Some of the cells expressing GUS gene are indicated by arrows.

Transformant screening. The callus cells of citrus bombarded with the DNA of pBI121 harboring *nptII* and *fad7* genes were kept in dark for 2 days to stabilize. After stabilization the cells were transferred onto a screening medium. We began screening of transformants with low concentration of kanamycin in medium and then increased the concentration of kanamycin. At first, the bombarded and stabilized cells were cultured on a medium containing 50 mg/l of kanamycin for 2 weeks. On this medium, some of the callus cells showed continuous growth (Fig. 2).

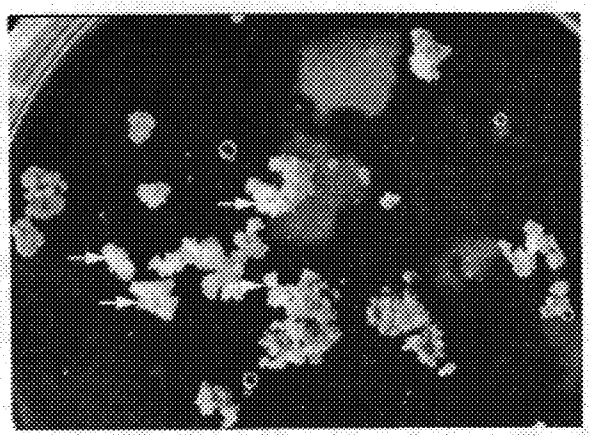

Fig. 2. Selective growth of citrus callus cells bombarded with pBI121 harboring *nptII* and *fad7* genes on a medium containing 50 mg/l of kanamycin. Some of the bombarded cells show continuous growth on a selective medium as indicated by arrows, while others do not.

The clones selected on the first screening medium were transferred onto the second medium containing 100 mg/l of kanamycin and cultured for 3 weeks. The cells showing continuous growth were subcultured on a new second medium for another 3 weeks. After screening twice on the second medium, the cells were transferred onto the third medium containing 200 mg/l of kanamycin and cultured for 4 weeks. The cells selected by the serial screening showed vigorous growth on the medium containing 300 mg/l of kanamycin (Fig. 3).

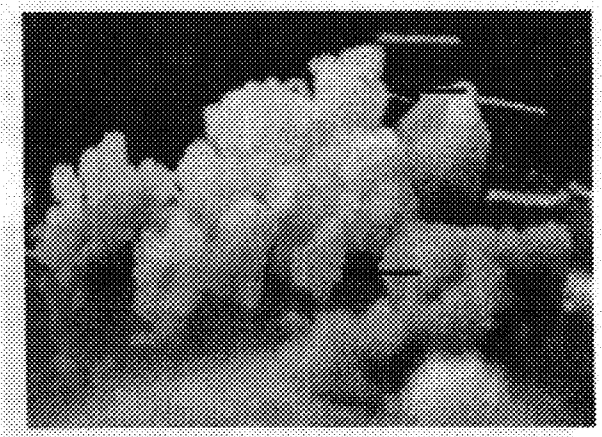

Fig. 3. Vigorous growth of the selected cells on a medium containing 200 mg/l of kanamycin after serial screening. Arrows indicate the selected callus cells growing vigorously on a medium of high concentration of kanamycin.

Plant regeneration. The callus cells screened on the kanamycin medium were transferred to a kanamycin-free medium for inducing embryogenesis. The embryogenesis induction medium contained 6% of sucrose and 1.6% of agar instead of 5% and 0.8% of each in the culture medium for callus cells as described in *Materials and Methods*. Somatic embryos were formed from the callus cells about two weeks after culturing on the embryogenesis induction medium. Figure 4 shows the initial stage of somatic embryogenesis. When the cells were cultured on the medium for about 4 weeks, many cells were developed into green shiny embryos (Fig. 5)

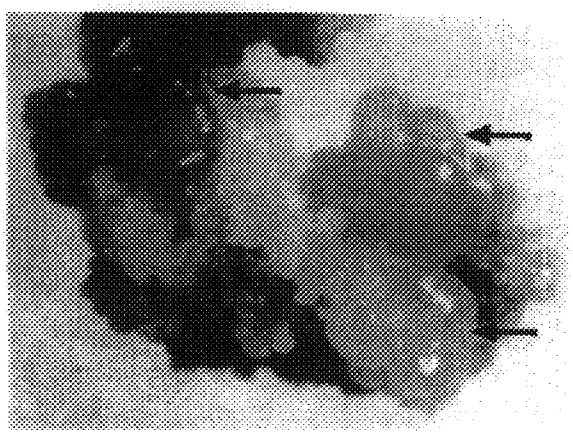

Fig. 4. Initial stage of somatic embryogenesis in the transformed cells of citrus. Some of the transformed callus cells developed into yellow-green embryos (arrows) on a embryogenesis induction medium containing 6% of sucrose and 1.6% of agar.

Fig. 5. Further growth of the transformed cells of citrus after embryogenesis. Arrows indicate active growths of somatic embryos cultured on a embryogenesis induction medium for 4 weeks.

In order to establish the condition for plant regeneration from the somatic embryos, we examined the inductions of shoot and root from the embryos when cultured on the embryogenesis medium containing different levels of auxin and cytokinin (Table 2). At 5.0 mg/l or higher concentration of NAA, the embryos degenerated to callus cells and formed neither shoot nor root. Between 0.1 and 2.0 mg/l of NAA, some embryos formed shoots and others formed roots. However, many embryos degenerated to callus at this concentration of NAA. Unlike NAA, BA induced only shoots without root or callus. The highest shoot induction was obtained at 1.0 mg/l of BA. Therefore, the shoots were induced on the embryogenesis medium supplemented with 1.0 mg/l of BA.

Root induction from the regenerated shoots is not indispensable for molecular breeding in citrus because citrus species can be propagated by grafting. However, the plants with roots grow faster and are the better for propagation. For root induction, the regenerated shoots were transferred onto a cytokinin-free medium containing auxin. The effect of auxin on the root induction in the regenerated shoots of citrus is shown in Table 3. The root induction was highest at 0.1 mg/l of NAA. The plantlets with roots in Fig. 6 were obtained from the shoot cultures on a medium with 0.1 mg/l of NAA and without cytokinin.

Table 2. Effect of growth regulators on the induction* of callus, shoot and root from the somatic embryos of citrus.

Growth regulators	NAA							BA						
Concentration (mg/L)	0	0.1	0.5	1.0	2.0	5.0	10	0	0.1	0.5	1.0	2.0	5.0	10
Callus	-	+	++	++	++	++++	+++	-	-	-	-	-	-	-
Shoot	-	-	+++	+++	+++	-	-	-	+++	+++	++++	++	+	-
Root	-	+	++++	+++	+++	-	-	-	-	-	-	-	-	-

*Degree of induction : -, none; +, very poor; ++, poor; +++, good; ++++, very good

Table 3. Effect of auxin level of culture medium on root formation in the shoots regenerated from the transformed embryos of citrus.

NAA Concentration (mg/l)	Rooting*
0	-
0.1	++++
0.5	+++
1.0	++
2.0	+
5.0	-

*Degree of rooting : -, none; +, very poor; ++, poor; +++, good; ++++, very good

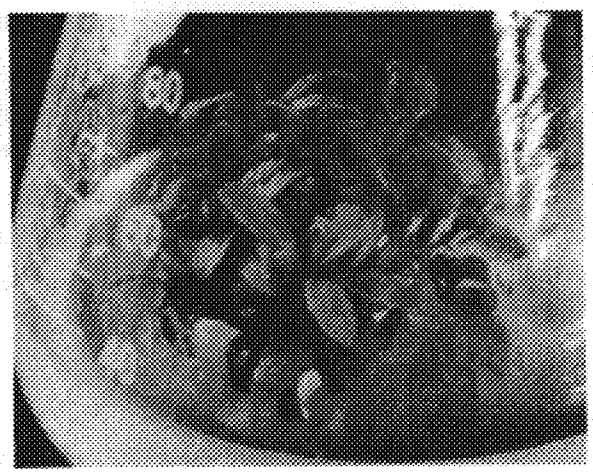

Fig. 6. The Plantlets regenerated from the citrus callus cells transformed with *fad7* gene.

Identification of the introduced gene. The genomic DNAs extracted from the selected clones on the kanamycin medium were analyzed by PCR with the primer pair specific for each gene of *nptII* and *fad7*. The results in Figure 7 showed two among five tested clones had both genes of *nptII* and *fad7*, while the other three had none of these genes.

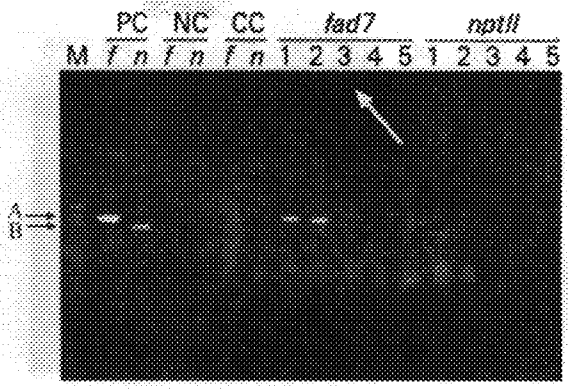

Fig. 7. PCR-amplified DNA fragments of *nptII* and *fad7* genes from the genomic DNA of the citrus callus clones bombarded with pBI121 harboring both of *nptII* and *fad7*. PCR was performed with template DNA of each *fad7* and *nptII* gene for positive control (PC), and without template DNA for negative control (NC) to check contamination in PCR. The genomic DNAs of five clones of bombarded callus (1 - 5) were analyzed with each primer pair specific for *fad7* and *nptII*. One clone of non-bombarded callus (CC) was used as an another negative control. A, 1.0 kb DNA amplified from *fad7*; B, 795 bp DNA amplified from *nptII*; M, size marker DNA; *f*, PCR with fad7-specific primers; *n*, PCR with *nptII*-specific primers.

The genomic DNAs extracted from the regenerated plantlets were also analyzed by PCR with the primer pair specific for each gene of *nptII* and *fad7*. The results in Figure 8 showed one among five tested clones had both genes of *nptII* and *fad7*, while the other four had none of these genes. Although many plants regenerated from the selected callus clones did not have the target gene, we could obtain several transgenic plants carrying *fad7* gene. This is the first report on the construction of transgenic plant of citrus species transformed with fatty acid desaturase gene. Analyses on the expression of *fad7* gene and the physiological characteristics of the transgenic plants are pending further experiments.

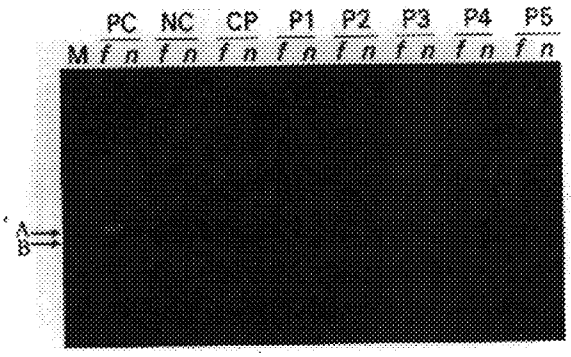

Fig. 8. PCR-amplified DNA fragments of *nptII* and *fad7* genes from the genomic DNA of the transgenic plants regenerated from the cells bombarded with pBI121 harboring both of *nptII* and *fad7*. In order to check contamination, PCRs were performed for positive (PC) and negative control (NC) as described in the legend of Figure 7. The genomic DNAs of five clones of putative transgenic plant (P1 – P5) were analyzed with each primer pair specific for *fad7* and *nptII*. One clone of non-transformed plant (CP) was used as a negative control. A, 1.0 kb DNA amplified from *fad7*; B, 795 bp DNA amplified from *nptII*; M, size marker DNA; *f*, PCR with fad7-specific primers; *n*, PCR with *nptII*-specific primers.

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