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Ectopic Expression of *Arabidopsis Rotundifolia* 3 Gene in Perilla (*Perilla frutescens* L.)

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ABSTRACT

The *Rotundifolia* 3 (*Rot* 3) gene regulates narrow leaf shape in *Arabidopsis thaliana*. We introduced *Rot* 3 into perilla (*Perilla frutescens* L.) by *Agrobacterium*-mediated transformation, and analyzed the leaf shape of transgenic plants. Cv. 'Manbaek' showed the highest performance in shoot induction (27.3%) compared with that of six tested cultivars. Thirteen transgenic plants were obtained from cotyledon tissues of 'Manbaek' with a 9.2% of transformation efficiency. Southern blot analysis revealed that the average copy of the integrated *Rot* 3 was 1 or 2 in T_1 transgenic plants. The high level of *Rot* 3 mRNA resulted in elongated leaves. On the other hand, *Rot* 3 transcript levels were lower in the lines of round leaves than in those from the elongated leaves. These results suggest that leaf shape can be artificially modified and that the heterologous expression of the leaf shape-regulating gene can be used as a new breeding strategy for improving leaf design in perilla.

Keywords: *Agrobacterium*-mediated transformation, leaf shape, *Rot* 3
Abbreviations: BA, 6-benzyladenine; **NAA**, α-naphthalene acetic acid; **PCR**, polymerase chain reaction; ***Rot* 3**, *Rotundifolia* 3; **RT-PCR**, reverse transcriptase-PCR

INTRODUCTION

The leaves of Perilla (*Perilla frutescens* L.) contain abundant antioxidant chemicals such as anthocyanins and polyunsaturated fatty acids (Shin and Kim 1994). In recent years, for this reason, the consumption of perilla leaves has increased dramatically and has been regarded as health food in Korea. Perilla leaves are not only served with "Sashimi", grilled meat, and salad, but are also used for decoration in traditional foods in Korea. In terms of leaf shape, most perilla leaves are heart-shaped with dented margins with narrow genetic diversities (http://www.ars-grin.gov).

Leaf shape is determined by asymmetrical cell growth of the proximo-distal axis (longitudinal), left-right axis (transverse), and adaxial–abaxial axis (thickness) (Bowman *et al*. 2002). Leaf length and width are mainly controlled by two different genes *ANGUSTIFOLIA* and *ROTUNDIFOLIA* 3 (Tsuge *et al*. 1996). The *ANGUSTIFOLIA* (AN) gene controls leaf shape through polarity-specific elongation of cells in leaf width. In the mutant, the failure of cell elongation in the leaf-width direction reduces the formation of intracellular spaces (Tsuge *et al*. 1996). The *ROTUNDIFOLIA* 3 (*Rot* 3) gene controls polar-cell elongation of the leaf longitudinal (leaf-length) direction in *Arabidopsis thaliana* (Tsuge *et al*. 1996; Kim *et al*. 2002). Phytohormons such as gibberellic acid (Zeevaart *et al*. 1993) and brassinolides (Takahashi *et al*. 1995) have been shown to be involved in proximo-distal elongation of cells. The *Rot* 3 gene encodes cytochrome P-450, which is homologous to putative steroid hydroxylases and biosynthesis of brassinosteroid (BR) (Tsukaya 2002). Change of leaf shape will be in demand to satisfy the ornamental values of people's preferences and to improve harvest index.

Here we report the high efficiency of *Agrobacterium*-mediated transformation using 'Manbaek' out of six other cultivars. Moreover, ectopic expression of *Rot* 3 resulted in a narrow leaf shape in transgenic perilla, indicating that biodesign of perilla can be used to meet customers' preference.

MATERIALS AND METHODS

Plant materials

Perilla seeds (*Perilla frutescens* L., cv. 'Manbaek') were surface-sterilized for 20 min in 20% (v/v) commercial bleach (5.25% sodium hypochlorite). After three additional 5-min washes in sterile water, the seeds were germinated on MS medium (Murashige and Skoog 1962) containing 30 g l^{-1} sucrose, 5 g l^{-1} gelrite. Surface-sterilized seeds were incubated for 1 week at 26°C under a 16/8-h (day/night) photoperiod with light condition (20 mE s^{-1} m^{-2}).

Agrobacterium strain and infection

Agrobacterium strain LBA 4404 harboring the *Rot* 3 gene was provided by Dr. GT Kim (accession no. U57411, Department of Molecular Genetics, Dong-A University, Busan, Korea). *Agrobacteria* were cultured for 60 h in LB medium supplemented with 50 mg l^{-1} kanamycin (Sigma, USA) and 250 mg l^{-1} cefotaxime (Sigma, USA) in a rotary shaker (200 rpm) at 30°C. The well-grown cells were harvested by centrifugation at 2,500 rpm and resuspended in a one-tenth-strength MS medium containing 0.1 mg l^{-1} α-naphthalene acetic acid (NAA), 2.0 mg l^{-1} 6-benzyladenine (BA), 50 mg l^{-1} kanamycin, 250 mg l^{-1} cefotaxime, and 30 g l^{-1} sucrose, pH 5.8. Cotyledons were dissected from the pre-cultured plantlets and infected with the *Agrobacterium* ($O.D._{600}$ = 0.3) by immersion for 30 min.

Selection and plant regeneration

Explants infected with *Agrobacterium* were subsequently washed with sterile water and transferred into a MS medium containing 30 g l^{-1} sucrose, 0.1 mg l^{-1} NAA, 2.0 mg l^{-1} BA, 5 g l^{-1} Gelrite and 50 mg l^{-1} kanamycin (Sigma, St. Louis, Mo., http://www.sigmaaldrich.com) for 1 week. Shoots were transferred to MS medium supplemented with 30 g l^{-1} sucrose, 0.1 mg l^{-1} NAA, 2.0 mg l^{-1} BA, 50 mg l^{-1} kanamycin, 250 mg l^{-1} cefotaxime, and 5 g l^{-1} Gelrite and cultured for 3 weeks in at 26°C under a 16/8-h (day/night) photo-

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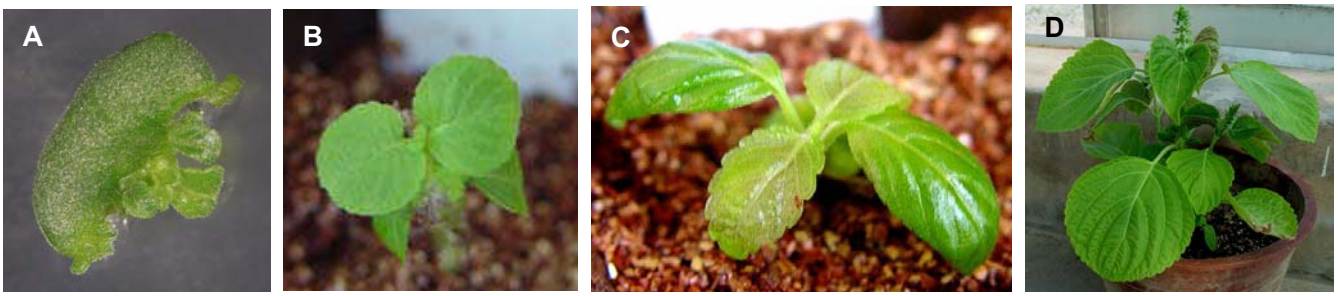

Fig. 1 Regeneration of *Agrobacterium*-mediated transgenic perilla. (**A**) Induction of cotyledon-derived shoot on modified MS medium. (**B**) Round shape on co-cultivation of perilla tissue indicating the *Agrobacterium* vector (*Rot* 3). (**C**) Long-round shape on co-cultivation of perilla tissue indicating the *Agrobacterium* vector (*Rot* 3). (**D**) Transgenic plants transplanted in a pot and ripening stage of the transgenic plants.

period with light intensity of 20 mE s^{-1} m^{-2}. The regenerated shoots were transferred to a bottle (6 × 16 cm) containing fresh medium and when the shoots reached the top of the box, the plantlets were transferred to soil for generation advance.

Molecular analysis of putative transgenic plants

DNA was extracted from putative transgenic plants for PCR DNA gel blot analysis by the CTAB method (Maniatis *et al*. 1982). PCR assay was carried out with a *Rot* 3-specific primer set (forward; 5′-TGAGGCCTCGGTTGTTCTCA-3′, reverse; 5′-GTCTTCCTCAG CCGTCCA-3′). PCR conditions were 5 min at 94°C for pre-denaturation, followed by 30 cycles of polymerization reaction each, consisting of denaturation for 30 s at 94°C, annealing for 30 s at 5°C, and extension for 30 s at 72°C. A final extension step was run for 7 min at 72°C. The expected fragment size was approximately 1.0 kb. DNA gel blot analysis, 10 μg of genomic DNA was digested with *Kpn*I and then electrophoresed on a 0.8% agarose gel. The DNA fragments were subsequently transferred into a nylon membrane and hybridized with *Rot* 3-specific [^{32}P] labeled probe. The hybridization pattern was identified on X-ray film. The *Rot* 3 gene expression patterns of putative transgenics as determined by DNA assay were analyzed by Northern analysis. Total RNAs were isolated from putative perilla plants (3rd leaf) using guanidine thiocyanate as described previously (Kawai *et al*. 1992). Hybridization was performed the same as described in DNA gel blot analysis. The membrane was exposed overnight and analyzed using an Imaging Plate Scanner BAS 1500 (Fujifilm, Japan, http://www.fugifilm.co.jp).

Analysis of leaf shape

The leaf shapes of transgenic plants were measured from higher than the 5th emerging leaves of each T_0 and T_1 plants in a greenhouse. The leaf length and width of 10 leaves was measured for each plant with 3 replications using a digimatic caliper (Mituto, Japan). The leaf index (LI) was calculated from the formula:

LI = leaf width (cm) / leaf length (cm) × 100

Data showed tendency that leaf shape is round if is near to absolute value 1.

RESULTS

Establishment of *in vitro* culturing system, *Agrobacterium*-mediated transformation, and the selection of transgenic perilla lines

To establish an efficient *in vitro* system for Perilla, a modified MS medium (MS basal medium containing 0.1 mg l^{-1} NAA, 2.0 mg l^{-1} BA, 30 g l^{-1} sucrose and 5 g l^{-1} Gelrite) was evaluated for initiating shoot regeneration (**Fig. 1A**). Modified MS medium showed reproducible results of shoot regeneration from cotyledons (**Fig. 1A**). Non-transgenic tissues gradually turned to brown or whitish brown during antibiotic selection, and kanamycin-resistant tissue could develop the third or fourth leaf successfully during the selection period (**Fig. 1B, 1C**). Putatively transgenic tissue was maintained and proliferated on selection medium until the fourth round of subculture. Sixteen kanamycin-resistance plants were obtained from 130 cotyledons and the ratio of young rooted plants on kanamycin medium was 12.3%. Young plantlets were transferred into soil and grown in a greenhouse (**Fig. 1D**). Finally we collected 473 T_1 seeds from 12 (75.0%) events (**Fig. 1D**).

Polymerase chain reaction and DNA gel blot analysis of transgenic plants

Sixteen putative transgenic plants (T_0) were analyzed by PCR and DNA gel blot assay (**Fig. 2A-C**). Thirteen of the 16 kanamycin-resistance lines amplified the expected band (1 kb) successfully by *Rot* 3-specific primers in the PCR

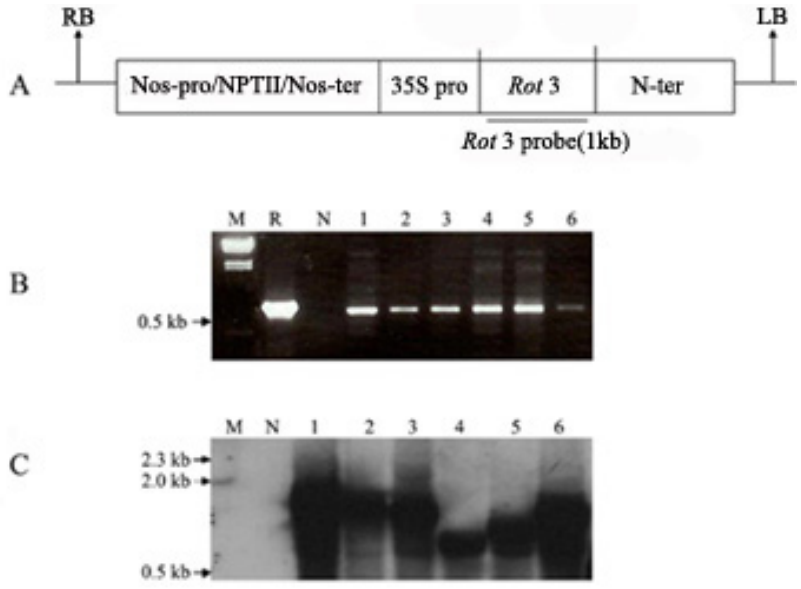

Fig. 2 DNA gel-blot analysis. (**A**) Schematic diagram of part of the T-DNA region of vector. Nos-pro, promoter of nopaline synthase gene; NPT II, neomycin phosphotransferase II gene; Nos-ter, terminator of nopaline synthase gene; 35S pro, CaMV 35S promotor. (**B**) PCR analysis of genomic DNA from non-transgenic and transgenic plants (T_0). Lanes: M, λ DNA/*Hind*III-digested marker; R, *Rot* 3 vector; N, non-transgenic; 1-6, transgenic plants. (**C**) Perilla genomic DNAs (10 μg) digested with *Kpn*I fractionated on 0.8% agarose gels, and transferred to nylon membranes. Lanes M, λ DNA/*Hind*III-digested marker; N, non-transgenic plant; 1~6, T_0 transgenics.

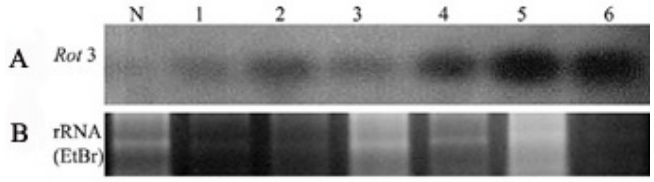

Fig. 3 Northern blot analysis of RNA (A) and total RNA (B) extracted from T_0 transgenic plants. Lanes: N, non-transgenic; 1~6, transgenic plants. Total RNA was extracted from 4-week-old transgenic plants. Lanes: N, non-transgenic; 1~6, transgenic plants.

assay (**Fig. 2A**). To determine the copy number of the introduced gene, we performed DNA gel blot analysis with a *Rot* 3-specific probe. The hybridization pattern showed that the *Rot3* gene was introduced to one to two copies in the perilla genome (**Fig. 3**). The transformation efficiency, calculated from cotyledon-derived tissue positive for both PCR and Southern analysis, was about 9.2%.

Transcription of the *Rot* 3 gene in transgenic plants

To determine transcription of the *Rot* 3 gene in the T_1 generation, we extracted total RNA from the leaves of the *Rot* 3 transgenic plants. RNA was analyzed by semi-quantitative RT-PCR and Northern analysis. The *Rot* 3 gene was successfully transmitted to the T_1 generation and the expression patterns were different from individuals to individuals. High transcription levels of *Rot* 3 gene were detected in the transgenic, which had long leaves (lanes 4, 5, 6, **Fig. 3A**) and relatively low transcription appeared in the round type leaf (lanes 1, 2, 3, **Fig. 3A**). Two types of leaf shape alternations were observed in progenies of *Rot* 3 transgenic plants (**Fig. 3**). The physiological role of the *Rot* 3 gene is the polar elongation of cells causing a decreased or an increase in the dimensions of cells (Tsuge *et al*. 1996). It is hypothesized that dividing cells in leaves synthesize some factors by the *Rot* 3 protein to communicate with elongating cells for regulating the direction of cell elongation (Tsukaya 1994). Despite the increased amounts of *Rot* 3 transcript in these transgenic lines, no significant change in plant growth or development was detected (**Fig. 3**). In *A. thaliana* the *Rot* 3 gene seems to be necessary at various stages during the development of leaves but not during the development of roots and stems (Kim *et al*. 1998). In the future, is guessed that other expression by leaf shape must try study more concretely.

Analysis of leaf shape and genetic behavior of T_1 progenies

The leaf shape of T_1 progenies (60 plants) of *Rot 3* transgenic plants showed that ~75% of plants developed round to oblong leaves compared to wt perilla and the segregation ratio fitted a Mendelian inheritance ratio (**Table 1**). The modification of leaf shape was also confirmed by the leaf area index (LAI). The average LI of 6 non-transgenic plants was 1.3 ± 0.21 and LI of transgenic varied from 1.01 to 1.66 (**Table 2**).

DISCUSSION

Although the successful *Agrobacterium*-mediated transformation of neomycin phosphotransferase II gene into perilla (*Perilla frutescens*) has been reported, the transformation efficiency has remained under the plant tissue's difference and transformation method (Kim *et al*. 2004; Lee *et al*. 2005). In the present study, we did not screen for direct factors affecting transformation efficiency such as pre-culture of explants, duration of co-cultivation, and kinds of *Agrobacterium* strain. The transformation efficiency using cv. 'Manbaek' cotyledons was 9.2% higher than our previous report of 1.4% (Kim *et al*. 2004). A pre-culturing step prior to *Agrobacterium* inoculation often has positive or negative effects on the transformation efficiency according to the plant species (Lee *et al*. 2000; Costa *et al*. 2002; Kim *et al*. 2004). The optimal duration of co-cultivation with *Agrobacterium* has been reported to be no longer than 4 days in many crops (Zhang *et al*. 1999; Khan *et al*. 2003; Cortina and Culianez-Macia 2004). Virulence of *Agrobacterium* strains also is one of the major factors for transformation. Higher virulence tends to lead to an increased level of expression of the *vir*G (Jin *et al*. 1987). Kim *et al*. (2002) reported that the *Agrobacterium* strain EHA105 showed high transformation performance than LBA 4404. Significantly, in the present study, 10% of the transformation ratio was evaluated using LBA4404 as the *Agrobacterium* host strain (**Figs. 1, 2**). PCR products for the *Rot* 3-specific region were detected in 12 out of the 16 kanamycin-resistant lines (**Fig. 3**). The results show that an antibiotics selection marker is quite efficient for preliminary selection of putative perilla transgenics.

The segregation ratio of leaf shape in the T_1 generation was 3:1, the same as Mendelian segregation ratio (**Table 1**). A high level of *Rot* 3 transcript was detected in transgenic lines showing longer leaves while transgenic lines with a relatively lower level of *Rot* 3 mRNA appeared to have rounder type leaves similar to those of non-transgenic plants (**Fig. 3**). Different levels of transgene expression have been explained by epigenic aspects of "positional effect" in chromosome, copy number of introduced gene, and repeated sequence (Matzke and Matzke 1998; Kooter *et al*. 1999; Selker 1999).

In summary, a reproducible protocol for *A. tumefaciens*-based perilla transformation using super-virulent strain LBA4404 was established. This reliable genetic transformation system facilitates the application of molecular techniques and new breeding strategy for perilla species.

Table 1 Segregation ratio of leaf shapes in T_1 perilla plants transformed with *Rot* 3.

T_1	Leaf shape		Ratio	X^2 value (P value)
	№ of varied type	№ of donor type		
60	44	16	3 : 1	0.114 (0.5<P<0.95)

Table 2 Leaf shape of transgenic plants with *Rot* 3 in perilla.

Plants	Leaf length (mm, A)	Leaf width (mm, B)	Leaf index[x] (A/B)
NT[z]	94.04 ± 1.03[y]	71.83 ± 3.04	1.30 ± 0.21 c
T_1-1	91.74 ± 4.20	90.16 ± 5.42	1.01 ± 0.04 d
T_1-2	92.29 ± 4.92	88.73 ± 3.92	1.04 ± 0.13 d
T_1-3	89.92 ± 2.71	87.65 ± 1.07	1.02 ± 0.33 d
T_1-4	97.58 ± 3.31	87.02 ± 3.81	1.12 ± 0.27 d
T_1-5	90.53 ± 4.02	85.87 ± 1.52	1.05 ± 0.40 d
T_1-6	89.73 ± 5.73	86.51 ± 5.17	1.03 ± 0.07 d
T_1-7	91.74 ± 4.31	55.16 ± 4.61	1.66 ± 0.37 a
T_1-8	92.29 ± 6.04	58.73 ± 2.62	1.57 ± 0.16 ab
T_1-9	89.92 ± 3.73	60.65 ± 1.23	1.48 ± 0.41 b
T_1-10	97.58 ± 6.75	67.02 ± 1.68	1.45 ± 0.72 b
T_1-11	90.53 ± 3.32	55.87 ± 3.08	1.62 ± 0.16 a
T_1-12	93.73 ± 2.80	56.51 ± 5.43	1.65 ± 0.09 a

[z]NT: non-transgenic plant, T_1-1~T_1-12: transgenic plant. [y]mean±SD. 6 plants used in statistics of each line. [x]In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

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