Full Length Research Paper

Enhancement of α-tocopherol content in transgenic *Perilla frutescens* containing the γ-TMT gene

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We report an efficient procedure for transformation of young leaf disc explants of *Perilla frutescens* using *Agrobacterium tumefaciens* strain 4404 harboring the binary vector pYB1130, which contains T-DNA incorporating the γ-TMT gene driven by the CaMV 35S promoter and the neomycin-phosphotransferase (*npt* II) gene for kanamycin selection. Explants were cultured on modified MS medium supplemented with 0.1 mg/l TDZ, 30 g/l sucrose, 100 mg/l kanamycin and 250 mg/l cefatoxime. Transformed shoots emerged from the basal part of the leaf after 4 to 5 weeks and had normal phenotypes when grown in the field. To improve transformation efficiency, the following factors were examined; plant growth regulators, kanamycin sensitivity, inoculation time and co-cultivation period. The highest number of transformants was obtained when leaf explants were co-cultivated for 3 days with *Agrobacterium* in the dark at 24°C. The optimum time of inoculation was 7 min, resulting in 17 transgenic plants. Stable integration of T-DNA into the genome of the putative transgenic plants was confirmed by polymerase chain reaction amplification (PCR) and Northern blot analysis. This method can be efficiently used for stable transformation of *P. frutescens* in which the α-tocopherol content was enhanced by the overexpression of this gene.

Key words: Co-cultivation, overexpression, *Perilla frutescens,* γ-TMT. α-tocopherol.

INTRODUCTION

Perilla frutescens Britt, commonly known as the Beefsteak mint plant, is a self-compatible annual herb in the Labiatae. It is widely cultivated in Asia (Korea, Japan, China, northeast India and the Himalayan hills) for its essential oil and anthocyanin content and is used as a spice, colorant and in Chinese medicine (Honda et al., 1990; Lee et al., 2001; Longvah et al., 1991). The seeds and leaves are used as flavoring and as a nutritional source in combination with vegetables in various Korean foods (Shin and Kim, 1994). *Perilla* oil is used as a salad oil or in cooking (Shin and Kim, 1994). *Perilla* oil is rich in polyunsaturated fatty acids, including linolenic (56.8%) and linoleic (17.6%) acids (Longvah et al., 1991). However, the high linolenic content in *Perilla* oil leads to rapid oxidation during processing and storage, resulting in a relatively short shelf life. The plant is used widely in prevention or treatment of depression (Takeda et al., 2002), vascular diseases (Makino et al., 2002) and in-

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Abbreviations: CTAB, Cetyltrimethylammonium bromide; **HPLC,** high performance liquid chromatography; **MS medium,** Murashige-Skoog medium; **NPT II,** neomycin phosphotransferase gene; **TDZ,** thidiazuron; **γ-TMT,** γ-tocopherol methyltransferase.

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digestion (Koezuka et al., 1985). Recently, many other important pharmaceutical properties of *Perilla* have been reported, including antioxidant activity (Kim et al., 2007), cytotoxicity (Toshihiro et al., 2006), anti-inflammatory and anti-allergic activity (Ueda et al., 2002). The leaf and/or stem of this herb have detoxicant, antitussive, antibiotic and antipyretic properties, and are used in a folk medicine for treating intestinal disorders (Nakazawa and Ohsawa, 2000).

Tocopherols (vitamin E) have a number of functions in plants, including preventing oxidative stress, protecting fatty acids in membranes from oxidation (Mckersie et al., 1990) and scavenging reactive oxygen species derived from photosynthesis (Fryer, 1992). The major tocopherol in *Perilla* is γ-tocopherol (92%), whereas α-tocopherol constitutes only a minor fraction of the total tocopherol pool (Shin and Kim, 1994). In the tocopherol biosynthesis pathways, γ-tocopherol methyltransferase (γ-TMT) is the final enzyme that catalyzes the conversion of γ-tocopherol into α-tocopherol, the most bioactive species of tocopherol (Shintani and DellaPenna, 1998; D'Harlingue and Camara, 1985; Koch et al., 2003). Hence, there is huge scope for converting γ-tocopherol to α-tocopherol by overexpression of the γ-TMT gene in *P. frutescens* to improve the nutritional and medicinal value. Some atempts have been made to alter the tocopherol composition of plants by manipulating tocopherol biosynthesis. For example, overexpression of γ-TMT in *Arabidopsis thaliana* was reported to increase vitamin E activity nine-fold over the wild-type control (Shintani and DellaPenna, 1998). Similar results were reported in other plants such as *Brassica* (Yusuf and Sarin, 2007), lettuce (Cho et al., 2005) and soybean (Tavva et al., 2007). Genetic transformation of *P. frutescens* has also previously been reported (Lee et al., 2005; Kim et al., 2004).

The objective of the present study was to determine an efficient transformation system for *P.frutescens* by optimizing key parameters that enhance genetic transformation and to increase the α-tocopherol content through overexpression of the γ-TMT gene from *A. thaliana*.

MATERIALS AND METHODS

Plant material

Seeds of *P. frutescens* Britt, genotype Kocf 47, were used in this experiment. Seeds were obtained from the Bioherb Research Institute, Kangwon National University, Korea and rinsed in running tap water for 5 h. Seeds were sterilized in 70% (v/v) ethyl alcohol for 1 min, followed by rinsing twice with sterile deionized water and subsequently soaked in (5% v/v) sodium hypochlorite (NaOCl) solution supplemented with two drops of Tween 20 and agitated on a mechanical shaker for 15 min. After thorough rinsing in sterilized water, the surface-sterilized seeds were planted in MS medium supplemented with 30 g/l sucrose and 0.8% agarose (w/v), pH 5.8 and cultures were kept under a 16 h photoperiod at 25°C under standard cool white fluorescent tubes at a photon flux density of 45 μmol m^{-2} s^{-1}. Germination started within 3 to 4 days of sowing. Lami-

nas were excised from 3 to 4 week-old *in vitro* grown seedlings and aseptically cut into pieces of approximately 0.5 cm in diameter for use in optimizing shoot regeneration and genetic transformation.

Kanamycin resistance test of normal leaves

For the efficient selection of putative transgenic plants during the transformation process, a kanamycin resistance test was performed. Leaves excised from normal plants were cultured on MS salt containing 0.1 mg/l TDZ, 3% sucrose and 0.8% agar, pH 5.8, with 0, 25, 50, 75, 100 or 150 mg/l kanamycin. The percentages of surviving leaf explants were determined after 4 weeks.

Vector construction and *Agrobacterium*-mediated plant transformation

Agrobacterium tumefaciens strain LBA 4404 harboring the binary vector pYB1130 was used. This vector contains the neomycin phosphotransferase gene (*npt* II) directed by the nos promoter as a selectable marker (Figure 1a). A single colony of *A. tumefaciens* strain LBA 4404 was grown for 24 h at 28 ± 1°C with shaking (150 rpm) in 20 ml of liquid Luria-Bertani (LB) medium (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7.0) containing 100 mg/l kanamycin. The *A. tumefaciens* cells were centrifuged for 10 min at 7000 x g at 4°C and resuspended in liquid inoculation medium (MS salt containing 20 g/l of sucrose) to obtain a final O.D $_{600}$ of 1.0 for plant infection.

To investigate the influence of inoculation time, the leaf explants were then soaked in the bacterial culture of different growth phases (OD$_{600}$ 0.5, 0.6, 0.8, 1.0 and 1.2) for 3, 5, 7, 10 and 15 min. The infected explants were dry-blotted using sterile filter paper and transferred to co-cultivation medium containing 0.1 mg/l TDZ, 3% sucrose and 0.8% agar, pH 5.8. To investigate the influence of co-cultivation period, leaf explants were then transferred to co-cultivation medium for 1, 2, 3, 4 or 5 days in the dark at 25°C.

After co-cultivation, the explants were washed in liquid MS medium supplemented with filter-sterilized 250 mg/l cefatoxime, blotted dry with sterile filter paper and transferred to selection medium, consisting of MS salt containing 0.1 mg/l TDZ, 3% sucrose and 0.8% agar, pH 5.8, supplemented with 100 mg/l kanamycin for selection and 250 mg/l cefotaxime to eliminate bacterial growth. The cultures were kept under a 16 h photoperiod at 45 μmol m^{-2} s^{-1} and 25 ± 2°C and then transferred into fresh selection medium and allowed to regenerate for 30 days with sub-culturing every 2 weeks. The primary regenerated shoots from the leaf explants were then transferred in MS supplemented with 50 mg/l kanamycin for root induction. Healthy rooted plantlets were separated, washed to remove agar and transferred to 100 x 85 cm diameter plastic pots containing sterilized soil mixture (vermiculite and perlite 1:1). Each pot was covered with a polythene bag to maintain high humidity and was placed in a growth chamber for 1 week. The polythene bags were gradually opened and after 6 weeks, the plantlets were moved to a greenhouse.

DNA isolation and polymerase chain reaction amplification (PCR) analysis

To verify the presence of the *npt* II and γ-TMT genes in regenerated plants, total DNA was extracted from young leaves excised from kanamycin-resistant shoots as well as from control plants by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The primers for a 1070 bp fragment of the γ-TMT were; TMT-1 (5-GAA-TTC-ATG-AAA-GCA-ACT-CTA-GC-3) as a sense primer and TMT-2 (5-TAA-TCG-ATT-AGA-CTT-AGA-GTG-GCT-TC-3) as an antisense primer. Amplification conditions for the
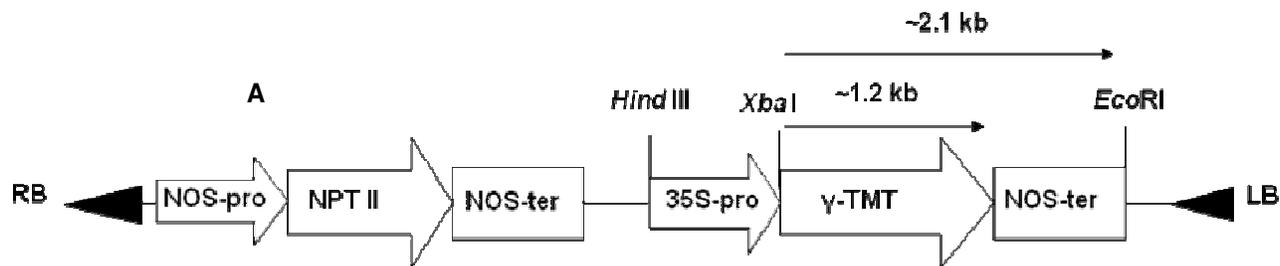
Figure 1a. Vector construction and regeneration of putative transgenic plants of *P. frustescens* via *Agrobacterium* mediated transformation of leaf explants. LB, left border; RB, right border; p35S, CaMV 35S promoter; Tnos, transcription terminator of nopaline synthase gene; Pnos, promoter of nopoaline synthase gene; *npt* II, coding region of neomycin phosphotransferase II gene; γ-TMT, γ-tocopherol methyltransferase gene.
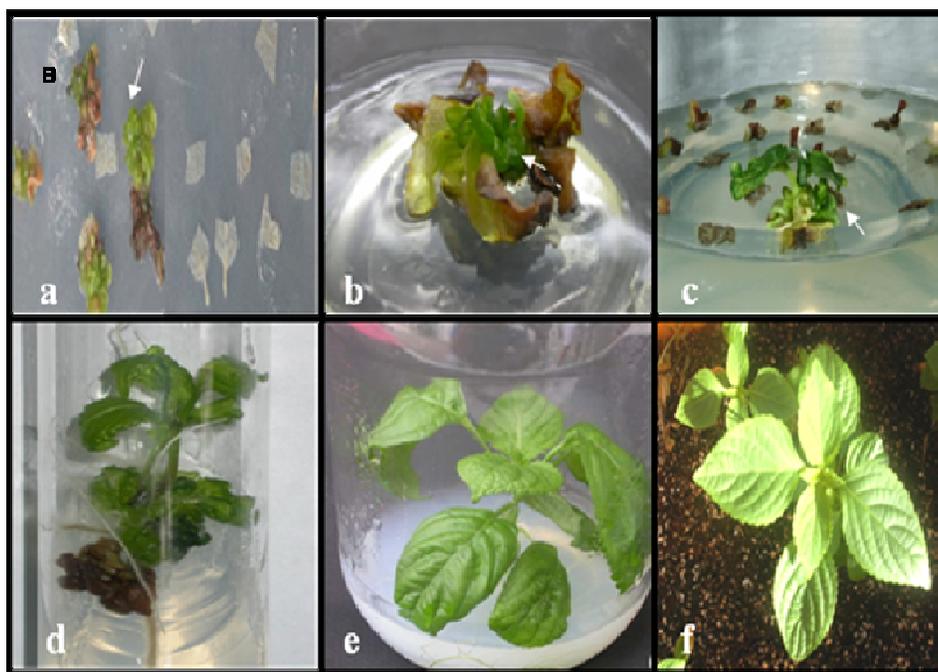

Figure 1b. (a) Embryogenic callus developed from the leaf explants on kanamycin containing medium 4 weeks after inoculation with *A. tumefaciens*; (b) regeneration of putative transgenic shoots from the embryogenic callus of leaf explants; (c) clusters of putative transgenic shoots on selection medium; (d) elongation of regenerated shoot; (e) rooting of elongated putative transgenic plantlets on a selection medium; (f) transgenic plants growing in the greenhouse.

γ-TMT gene fragment were 35 cycles, each consisting of 50 s at 94°C, 50 s at 57°C, 1 min at 72°C, post elongation at 72°C for 3 min and a final extension at 72°C for 10 min. Amplification products were analyzed on 1% (w/v) TAE agarose gels, stained with ethidium bromide and visualized with UV light.

Northern blot analysis

Total RNA was extracted from the leaves of putative transgenic *P.frutescens* lines grown in a greenhouse, using the procedure of Yi et al. (1999). Of the total RNA, 20 µg were separated on form-aldehyde-containing agarose gels and blotted onto nylon membranes, following the standard procedures of Sambrook et al. (1989) and using the hybridization buffer of Church and Gilbert (1983). Equal loading of RNA was verified by visualizing ribosomal RNA stained with ethidium bromide and the prepared nylon mem-brane was hybridized with full-length γ-TMT cDNA labeled with (a-32P).

HPLC analysis

The young leaves collected from control and T_0 plants of transgenic *P. frutescens* were extracted and analyzed for tocopherol content using high performance liquid chromatography (HPLC) with an ultraviolet absorbance detector, following the method of Carpenter (1979). Approximately 1 g of freeze-dried tissue was ground, and the resultant powder was mixed vigorously with 10 ml 80% MeOH and stored at room temperature for 1 day. The extracts were filtered
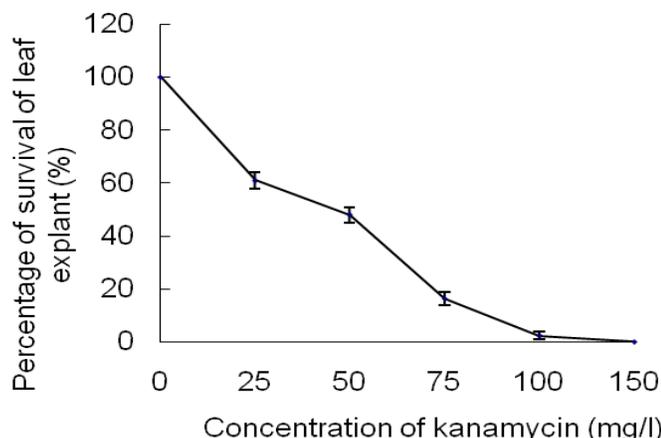
Figure 2. Effect of various concentration of kanamycin on the regeneration ability of leaf explants of *P. frustescens*. Each value represents the mean of three independent experiments with 30 explants per treatment.

through filter paper (Whatman No.42) to remove debris. The extracts were evaporated at 40°C and resuspended in 10 ml n-hexane and iso-propyl alcohol (98.5:1.5) and the supernatant was used for HPLC (Shimadzu 20 AT, Japan). Tocopherols were separated on a micro-Bondpack C18 column (3.9 x 300 mm) with a water symmetry C18 (3.9 x 10 mm) guard column. The mobile phase was an initial linear gradient of acetonitrile in water (from 95 to 100% for 10 min) followed by 100% acetonitrile for 10 min; the flow rate was 1 ml/min. The column temperature was 40°C and was scanned at 295 nm. Samples were kept in an autosampler at room temperature and the injection volume was 20 µl. Identification and quantification of tocopherols was done by comparing retention time and peak area to tocopherol standards. Standards for α- and γ-tocopherol were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Statistical analysis

All data reported are mean ± S.E. and each experiment was replicated three times with 30 explants in each treatment. The data were analyzed using analysis of variance (ANOVA) and significant differences were determined at a probability level of 0.05.

RESULTS AND DISCUSSION

Production of transgenic plants

The establishment of an integrated transformation method involves many factors, such as choice of regenerable explants, culture condition and transformation technique (Cui et al., 2004). In the present study, different factors responsible for T-DNA transfer into the plant were evaluated by studying expression of the γ-TMT gene after co-cultivation of leaf explants with *Agrobacterium* strain LBA 4404.

We have standardized plant regeneration system in *P. frutescens* earlier by using leaf explants (Lee et al., 2004). Regenerated shoots were obtained from leaf explants cultures on solid MS medium containing different concentrations of cytokinins and auxin. After 45 days of culture, green multiple shoot buds formed along the cut surface of the leaves on the MS medium containing 0.1 mg/l TDZ supplemented with 30 g/l sucrose and 0.8% agarose (w/v), pH 5.8. This combination was routinely used for the transformation experiments.

Kanamycin resistance test on *P. frutescens* transformation

Leaf discs from non-transformed plants were placed on regeneration medium supplemented with kanamycin at various concentrations (0, 25, 50, 75, 100 or 150 mg/l) to investigate the resistance of leaf explants to the antibiotic. On media containing 50 and 75 mg/l kanamycin, the shoot regeneration percentages were 48 and 16.33%, respectively. In contrast, 2.33% shoot regeneration occurred on medium containing 100 mg/l kanamycin. Therefore, 100 mg/l kanamycin was used as the minimal concentration required for selection of transgenic *P. frutescens* (Figure 2).

Kanamycin-resistant explants cultured on shoot-induction medium were transferred into fresh medium every 7 days. Adventitious shoots were regenerated 3 weeks after *Agrobacterium* infection on regeneration medium containing 0.1 mg/l TDZ, 100 mg/l kanamycin and 250 mg/l cefatoxime. Numerous knob-like adventitious shoots of putative transformants were observed on the cut surfaces of explants and these later developed into new plants (Figure 1a, b). Among the regenerated plants, many shoots gradually turned white and died 3 to 4 weeks after infection, whereas putative transformed shoots survived and grew normally (Figure 1B,b). Healthy regenerated shoots were excised from explant cultures and transferred to elongation medium supplemented with kanamycin and cefatoxime (Figure 1B,d). Shoots reached a length of 4 to 5 cm within 2 weeks. Elongated shoots were transferred into rooting medium supplemented with kanamycin and cefatoxime (Figure 1B,e). Root initiation occurred within 8 days and a good root system developed after 4 weeks of culture. The rooted plantlets were transferred to a glass greenhouse for acclimatization (Figure 1B,f).

Effect of inoculation time

Inoculation of explants into an *Agrobacterium* suspension enhances the attachment of *Agrobacterium* to the explant (Yong et al., 2006). Inoculation times of 0, 3, 5, 7, 10 or 15 min were tested to improve the transformation efficiency of *P. frutescens*. Higher transformation efficiencies were obtained when leaf discs were inoculated with bacterial solution for 7 min, following co-cultivation for 3 days. However, an increase in inoculation time of more than 7 min resulted in overgrowth by *Agrobacterium* and a reduction in transformation efficiency (Figure 3). Similar
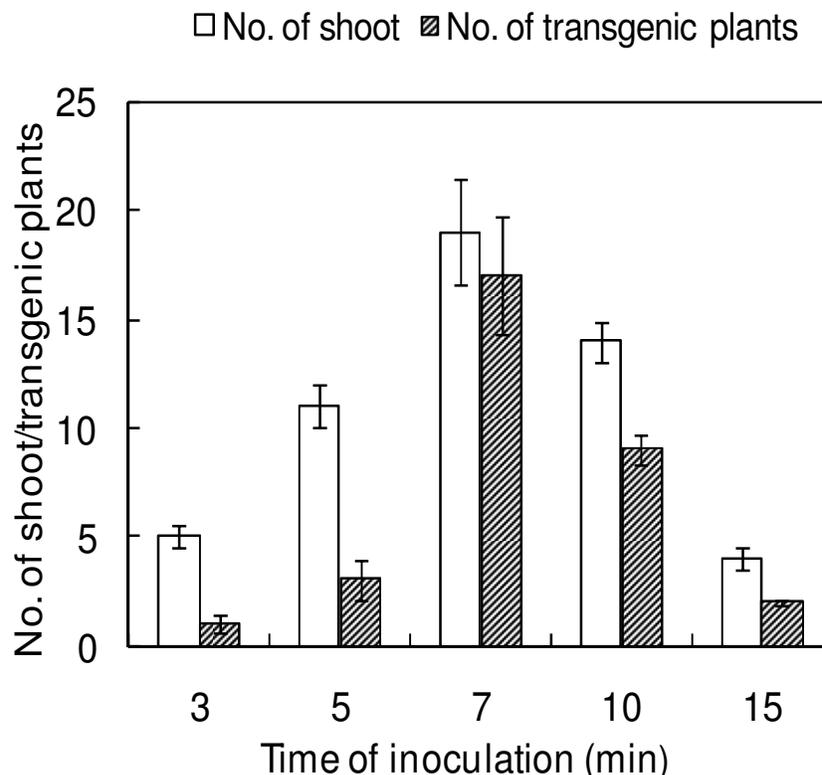
☐ No. of shoot ▨ No. of transgenic plants

Figure 3. Effect of inoculation time on plant regeneration and transformation efficiencies using leaf explants of *P.frutescens*. Each value represents the mean of three independent experiments with 30 explants per treatment.

results were also reported for *Cyclamen persicum* (Aida et al., 1999), while prolonged inoculation (15 min) was successful for efficient transformation of trifoliate orange (Kaneyoshi et al., 1994; Drake et al., 1997).

Effect of co-culture period

The effect of co-culture was investigated by co-cultivating the explants in the dark from 0 to 5 days at 24 to 26°C. The highest transformation efficiency of explants was obtained after co-cultivation for 3 days in the dark at 24 to 26°C. No γ-TMT gene expression was detected in explants immediately after inoculation. The transformation efficiency was very low after co-cultivation for 1 day but was similar after co-cultivation was extended for more than 3 days due to overgrowth of bacteria in the broth (Figure 4). Explant co-cultivation with *Agrobacterium* for 2 to 4 days is commonly used in genetic transformation of plants (Shrawat et al., 2007; Park et al., 2002; Lievre et al., 2005); however, co-cultivation for more than 3 days in the dark resulted in overgrowth of *Agrobacterium* around the explants and decreased the transformation efficiency. There was no reduction in the transformation rate after 3 days of co-cultivation due to a change in virulence of the *A. tumefaciens* strain, but to overgrowth of bacteria,

which killed the leaf explants. However, prolonged co-cultivation periods of more than 3 days have been successfully used in *Pyrus communis* and *Kalanchoe laciniata* (Mourgues et al., 1996; Jia et al., 1989).

PCR and northern blot analysis

DNA isolated from kanamycin-resistant plants, non-transformed plants and plasmid pYB1130 was used as a template for PCR amplification of the γ-TMT gene. The presence of a band of 1070 bp in samples from transformed shoots (Figure 5a, Lanes 1 to 9) confirmed the integration of the γ-TMT gene. Amplification of the γ-TMT gene was not observed in control plants (Lane c). Amplified *npt* II fragments of the expected size of 700 bp were obtained from transformed plant, indicating the integration of the *npt* II gene (Figure 5b, Lanes 1 to 5). No amplification of the *npt* II fragment from non-transformed plants samples was observed (Figure 5b, Lane c). To examine tissue expression of the gene in transgenic *P. frutescens* plants, total RNA was isolated from different PCR-positive transformants and probed with γ-TMT. The amount of γ-TMT transcript expression varied significantly among the different transgenic lines (Figure 5c). The results indicated that, the transgene was successfully
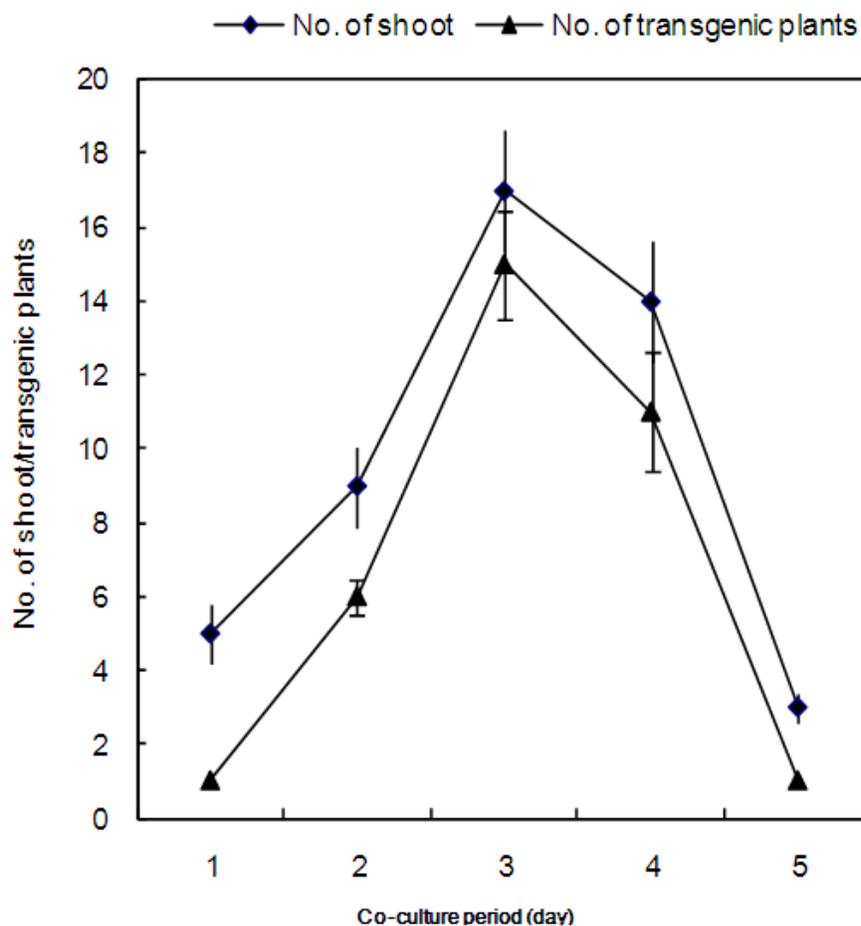
Figure 4. Effect of co-cultivation period on plant regeneration and transformation efficiencies of leaf explants of *P. frustescens*. Leaf explants were inoculated with *Agrobacterium* (OD_{600} of 1.0) for 7 min prior to co-cultivation in the dark. Each value represents the mean of three independent experiments with 30 explants per treatment.

expressed in transgenic *P.frutescens* plants.

Agronomic characteristics of transgenic plant

The change in tocopherol composition did not have any significant variation on agronomic characters of transgenic lines in the field, when compared with control plants (Table 1). The height of transgenic lines was ranging from 102.20 ± 3.86 to 152. 20 ± 2.44 cm, while plant height of control line was 117.10 ± 3.71 cm. The branch number of transgenic plants was similar to the corresponding non-transgenic (control) plant, except the T-6 transgeic lines, which showed increased branched number (10.60 ± 1.30). Among the transgenic lines, T-1 showed smaller leaf size and leaf stalk length (33.17 ± 2.75 cm^2 and 4.22 ± 0.90 cm, respectively) when compared with control plant. Czubacka and Doroszewska (2010) and Huo et al. (2010) noticed some changes in plant morphology of transgenic plants, however, Du et al., (2008) and Guan et al., (2000) reported no significant

agronomic changes between transgenic rapeseed and control plants.

HPLC analysis

HPLC profiles from extracts of transgenic plants revealed an increase in the α-tocopherol peak and a decrease in the γ-tocopherol peak (Figure 6). The tocopherol content and composition of transgenic lines and control plants were investigated using HPLC (Figure 5). The content of α-tocopherol and the α/γ-tocopherol of control plants was 48.60 and 8.24 µg/g tissue, respectively and in transgenic leaves the α-tocopherol content increased about 1.81 fold to 87.8 µg/g tissue (Table 2) and the α/γ-tocopherol ratio increased 86.61 fold in transgenic leaves. This suggests that, γ-tocopherol had been converted into α-tocopherol by overexpression of the γ-TMT gene. Similar results were reported for *A. thaliana*, where overexpression of γ-TMT caused an 80 fold increase in seed α-tocopherol levels (Shintani and Della, 1998). In other studies,
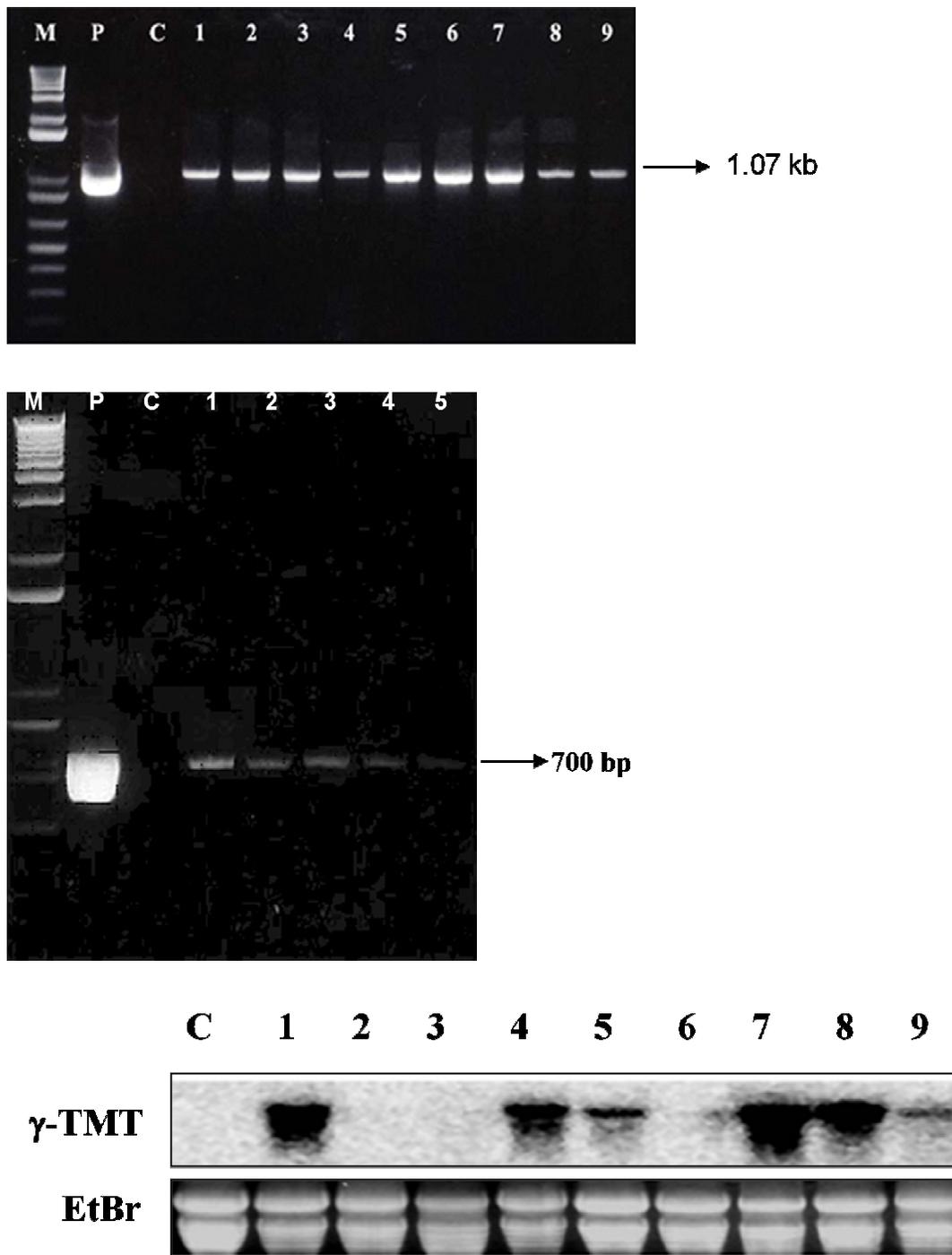
Figure 5. PCR and northern blot analyses of transgenic plants for the detection of mRNA transcripts of γ-TMT. (a) PCR analysis from leaves of transgenic and control plants in *P. frutescens* using specific primers of amplification of the 1070 bp fragment of γ-TMT gene. M, Molecular marker; P, the plasmid pYB1130 as a positive control; C, the genomic DNA from untransformed plants (Negative control). Lane 1 to 9, the genomic DNA of T_0 transgenic plants transformed with γ-TMT. (b) PCR analysis from leaves of transgenic *P. frutescens* using specific primers of amplification of 700 bp *npt II* gene in agarose gel. M, Molecular marker; P, the plasmid pYBI130 as a positive control; C, the genomic DNA from untransformed plants (Negative control); lane 1 to 5, the genomic DNA of T_0 transformant. (c) Northern blot analyses of primary transformants for the detection of mRNA transcripts of γ-TMT. Lane 1, untransformed plants (Negative control); lane 2 to 11, the transgenic plants transformed with γ-TMT. The picture of the ethidium bromide-stained gel shown below the blot demonstrates equal loading of RNA.
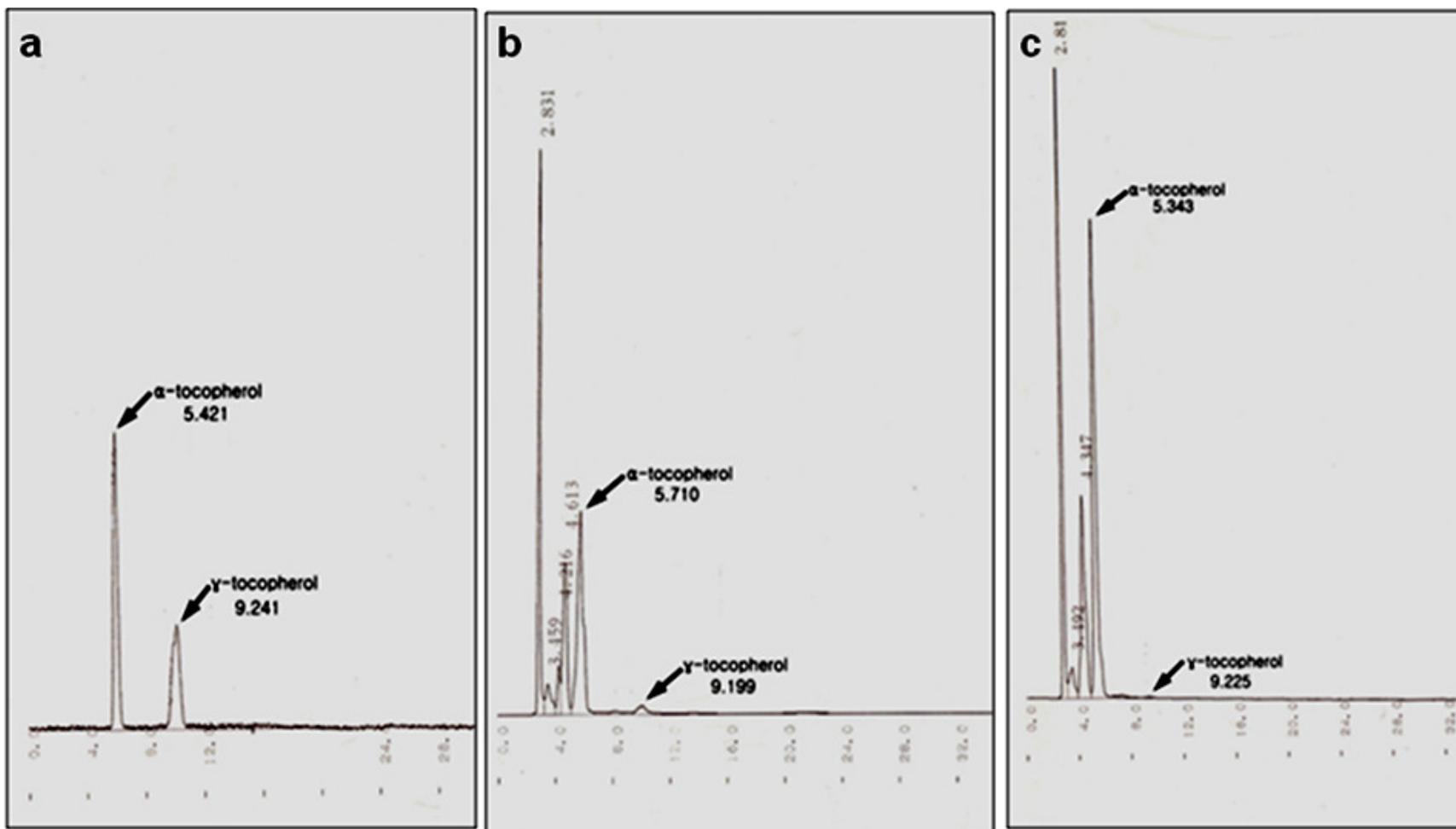
Figure 6. HPLC profiles of tocopherol contents in the leaves collected from control and transgenic *P.frutescens*; a, Standard; b, control plant, c, transgenic plant. Retentions time for α-tocopherol = 3.4; Retention time for γ-tocopherol = 3.1.

over-expression of γ-TMT caused six fold increases in α-tocopherol levels in *Brassica juncea* (Yusuf and Sarin, 2007), a two-fold increase in lettuce (Cho et al., 2005) and a 10.4 fold increase in soybean (Tavva et al., 2007).

Conclusion

In this study, we developed a simple and efficient system for the production of transgenic *P. frutescens* using *Agrobacterium*-mediated trans-

formation to increase the α-tocopherol content. Co-cultivation period and infection time were found to be important factors for increased transformation efficiency. Using this transformation protocol, morphologically normal plants were

Table 1. Agronomic characteristics of transgenic *P. frutescens*.

Plant line	Plant length (cm)	Branch number	Leaf size (cm^2)	Leaf stalk length (cm)
[a]C	117.10 ± 5.71	8.60 ± 3.40	56.50 ± 4.39	7.52 ± 1.33
[b]T-1	120.50 ± 4.63	8.00 ± 1.82	33.17 ± 2.75	4.22 ± 0.90
[b]T-2	121.00 ± 3.84	8.25 ± 2.89	51.48 ± 1.83	6.01 ± 1.06
[b]T-3	102.20 ± 3.86	6.50 ± 1.95	42.30 ± 4.56	5.94 ± 1.14
[b]T-4	118.00 ± 2.39	6.60 ± 1.54	49.40 ± 1.43	6.30 ± 1.26
[b]T-5	152.20 ± 2.44	7.00 ± 2.10	71.17 ± 4.12	7.64 ± 0.84
[b]T-6	151.30 ± 5.99	10.60 ± 1.30	66.75 ± 4.97	7.24 ± 0.83

[a]C, Control plant; [b]T-1, [b]T-2, [b]T-3, [b]T-4, [b]T-5, [b]T-6, transgenic plant. Each value is the mean of at least three independent experiments ± Standard deviations (S.D).

Table 2. Tocopherol content estimated by HPLC analysis from leaves collected from control and transgenic *P. frutescens*.

Plant line	Contents (µg)/ex. hexane-DMSO (100 µg)			
	α-Tocopherol (µg/ml)	γ-Tocopherol (µg /ml)	Total (µg/ml)	Ratio of α/ γ
[a]C	48.60 ± 1.25	5.90 ± 1.11	54.50 ± 1.36	8.24
[b]T	87.80 ± 2.31	0.12 ± 1.43	87.90 ± 2.43	731.67

[a]C, Control; [b]T, transgenic plant. Values are the means of eight replicates ± standard deviation for individual plants.

regenerated that over-expressed the γ-TMT transgene. Moreover, overexpression of the γ-TMT gene significantly increased the α-tocopherol content and α/γ-tocopherol ratio. Thus, the protocol presented here may provide guidelines for further stable transformation of *P. frutescens* with agronomically useful traits.

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