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Agrobacterium-mediated transformation of Perilla (*Perilla frutescens*)

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Abstract

Agrobacterium tumefaciens-mediated transformation system for perilla (*Perilla frutescens* Britt) was developed. *Agrobacterium* strain EHA105 harboring binary vector pBK I containing *bar* and γ-*tmt* cassettes or pIG121Hm containing *npt*II, *hpt*, and *gus*A cassettes were used for transformation. Three different types of explant, hypocotyl, cotyledon and leaf, were evaluated for transformation and hypocotyl explants resulted in the highest transformation efficiency with an average of 3.1 and 2.2%, with pBK I and pIG121Hm, respectively. The *Perilla* spp. displayed genotype-response for transformation. The effective concentrations of selective agents were 2 mg l^{-1} phosphinothricin (PPT) and 150 mg l^{-1} kanamycin, respectively, for shoot induction and 1 mg l^{-1} PPT and 125 mg l^{-1} kanamycin, respectively, for shoot elongation. The transformation events were confirmed by herbicide Basta spray or histochemical GUS staining of T_0 and T_1 plants. The T-DNA integration and transgene inheritance were confirmed by PCR and Southern blot analysis of random samples of T_0 and T_1 transgenic plants.

Abbreviation: γ-*tmt* – γ-*tocopherol methyltransferase gene; bar* – bialaphos resistance gene; *gusA* – β-*glucuronidase gene; hpt* – hygromycin phosphotransferase gene; *hpt II* – neomycin phosphotransferase gene; PPT – phosphinothricin

Introduction

Perilla (*Perilla frutescens* Britt) is an annual self-fertilizing plant and extensively cultivated in some Asian countries including Korea, Japan, Northeast China and Nepal (Honda et al., 1990; Kim et al., 2002a, b). As one of the most important oil crops in Korea, its seeds and seed oil are used for condiments and leaves are used as a fresh vegetable (Lee et al., 2003). Perilla seeds are also used for tea and soup (Honda et al., 1990). Perilla contains about 45% oil that is composed of over 90% of unsaturated fatty acids such as oleic, linoleic and linolenic acid (Park et al., 2000). High linolenic acid content in perilla oil (over 60%) causes a relatively short shelf-life. Although many attempts have been made through conventional breeding to improve shelf-life of perilla oil, no success has been achieved to date. Therefore, genetic engineering may provide a breakthrough in developing new perilla varieties with a long shelf-life of oil as well as other improved agronomically important traits.

We have previously reported an efficient regeneration protocol for perilla (Lee et al., 2003). This regeneration process is based on indirect organogenesis with a very short intermediate callus stage. Several genotypes and different types of explants

were explored for efficient regeneration with the leaf explants of several genotypes displaying the highest regeneration responses. It was also found that the MS basal medium (Murashige and Skoog, 1962) supplemented with 1 mg l^{-1} 6-benzylaminopurine (BA), 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA) and 3% (w/v) sucrose was most effective in promoting normal shoot induction.

We report here the successful transformation of perilla using *Agrobacterium*-mediated transformation and we demonstrate that transgenes have been integrated into the perilla genome and stably inherited.

Materials and methods

Plant materials

The Korean perilla genotypes Yeupsil (Lee et al., 1989) and Daesil (Park et al., 2002) were used. Perilla seeds were surface-sterilized with 70% ethanol for 1 min, washed three times with sterile distilled water, and then sterilized with 1% sodium hypochlorite solution for 10 min. After washing three times with sterile distilled water the seeds were germinated on agar (0.55%) solidified MS basal medium in the Vitro Vent containers (Duchefa, The Netherlands) and placed at 25 °C under a 16-h photoperiod (13.40 ± 1.5 W m^{-2}). Hypocotyl (5–8 mm in length) and cotyledon explants (5–8 mm^2 in size) were excised from 6 to 8 day-old seedlings, and leaf explants (5–8 mm^2 in size) were excised from 12 to 14 day-old seedlings for *Agrobacterium* inoculation.

Bacterial strain and plasmids

The *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) carrying either binary vector pBK I or pIG121Hm. pBK I contains the herbicide resistance *bar* gene under the control of cauliflower mosaic virus (CaMV) 35S promoter and the γ-tocopherol methyltransferase (γ-TMT) gene (Kim et al., 2002a, b) driven by the seed specific vicillin promoter (Higgins et al. 1988). pIG121Hm (Ohta et al., 1990) contains the neomycin phosphotransferase gene (*npt* II) under the control of the nopaline synthase (NOS) promoter, the hygromycin phosphotransferase gene (*hpt*) and intron inserted β-glucuronidase gene (*gus*A) (Vancanneyt et al., 1990), each driven by CaMV 35S promoter (Figure 1).

Inoculation and co-cultivation

Agrobacterium strain EHA 105 harboring either pBK I or pIG121Hm was inoculated into 200 ml of YEP broth (5 g l^{-1} Yeast Extract, 10 g l^{-1} peptone, and 5 g l^{-1} NaCl, pH 7.0) medium supplemented with 50 mg l^{-1} kanamycin and grown at 28 °C on a rotary shaker (250 rpm) until the optical density (OD_{600}) reached 0.6 to 1. Bacterial cells were harvested by centrifugation at 3000 rpm for 5 min in a 50 ml sterile centrifuge tube (Corning, USA) and then resuspended in 30 ml of liquid inoculation medium (MS medium supplemented with 3% sucrose and 100 mM of acetosyringone) in the 50 ml tube.

The explant tissues were inoculated with the *Agrobacterium* suspension in the 50 ml tube for 30 min at 25 °C in darkness with gentle shaking.

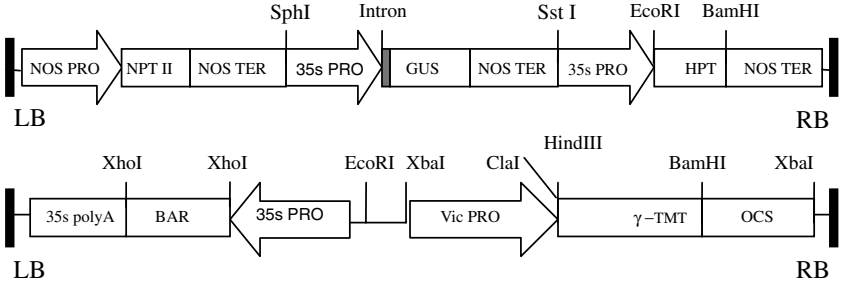

Figure 1. Schematic representation of T-DNA region of binary vectors pIG121Hm (top) and pBK I (bottom). LB, left border; RB, right border; NOS PRO, nopaline synthase promoter; NPT II, neomycin phosphotransferase II; NOS TER, nopaline synthase gene terminator; 35S PRO, cauliflower mosaic virus 35S promoter; GUS, β-glucuronidase gene; HPT, hygromycin phosphotransferase; 35S poly A, 35S terminator; BAR, gene encoding phosphinothricin acetyl transferase; Vic PRO, vicillin promoter; OCS, octopine synthase terminator.

The explants were then blotted dried on a sterile filter paper for 5 min to remove excess solution before transferred to solidified co-cultivation medium (MS medium with 3% sucrose and 0.55% agar) and incubated for 3 days at 25 °C in darkness.

Selection and regeneration of transgenic perilla

Preliminary experiments were performed to determine the optimum concentrations of the PPT and kanamycin for selecting transgenic plants. Different amounts of PPT (0, 0.5, 1 and 2 mg l^{-1}) and kanamycin (0, 100, 125 and 150 mg l^{-1}) were added to the regeneration medium (MS basal medium supplemented with 1 mg l^{-1} BA, 0.1 mg l^{-1} NAA and 3% sucrose). Explants were cultured on each selection medium for 3 weeks at 25 °C under 16-h photoperiod. This experiment was performed with three replications. After 3 weeks, the number of explants producing callus was calculated.

Based on the preliminary experiments, the minimal concentrations of PPT and kanamycin for selection were found to be 1 and 125 mg l^{-1}, respectively. For more stringent selection 2 mg l^{-1} PPT and 150 mg l^{-1} kanamycin were used. The shoots were excised after 9–11 weeks when they started to emerge and became 2–3 cm long, transferred to MS basal medium supplemented with either 1 mg l^{-1} PPT or 125 mg l^{-1} kanamycin as well as 500 mg l^{-1} carbenicillin and grown for 3 weeks for shoot elongation and further selection. The PPT or kanamycin resistant shoots were subsequently transferred to rooting medium containing MS basal medium without selective agents to allow development of roots for 4 weeks. Rooted plantlets were transferred to Vitro Vent container filled with vermiculite. These plantlets were acclimatized at 25 °C under 16-h photoperiod for 2 weeks in the culture room and transferred to pots (17×20 cm in size) filled with a vermiculite and sand at 1:1 ratio and grown under greenhouse conditions.

Confirmation of transformation

Herbicide application and histochemical GUS staining

To confirm the expression of *bar* in transgenic T_0 and T_1 perilla plants, 0.3% Basta was sprayed twice weekly onto the mature stage or seeding stage, respectively. GUS expression was confirmed by GUS staining of the first leaf tissues of the putative transgenic T_0 plants and of a whole T_1 seedling plant. The tissues and the plants were stained overnight with X-Gluc staining solution (Jefferson, 1987).

Polymerase chain reaction (PCR) and Southern blot analysis

Total genomic DNA was isolated from leaf tissues of young transgenic T_0 and T_1 perilla plants using a modified CTAB procedure (Murray and Thompson, 1980). In order to verify the presence of the *bar* gene of pBK I in regenerated perilla plants, a PCR primer set (5′-TCTGCACCATCGTCAACCACTACAT and 5′-CTGAAGTCCAGCTGCCAGAAACCCA-3′) was designed to amplify a 0.5 kb PCR fragment from the *bar* ORF within the pBK I. In order to detect GUS gene from pIG121Hm, a primer set (5′-AATTGATCAGCGTTGGTGG-3′ and 5′-GGTGTAGAGCATTACGCTGC-3′) was designed to amplify a 0.5-kb fragment from the *gus*A ORF within the pIG121Hm. Each PCR reaction mixture contained 100 ng of template DNA, 0.5 M of each primer, 0.1 mM dNTP mixture, 5 µl Taq DNA polymerase reaction buffer and 1 unit Taq DNA polymerase (Bioneer, Korea) in a total reaction volume of 50 µl. The PCR reaction was performed as follows: 96 °C for 30 s, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s of annealing, and 72 °C for 30 s of extension, and a final extension at 72 °C for 7 min. Amplified products were electrophoresed on 0.9% agarose–EtBr gels and visualized under UV light.

For Southern blot (Southern, 1975), 10 µg of genomic DNA from each transgenic plant was digested with *Eco*RI restriction endonuclease (Bioneer, Korea), subjected to electrophoresis on 0.8% agarose gel and transferred to positively charged nylon membrane (Roche, Germany). The *Cla*, I–*Bam*, HI fragment of pBK I including vicilin promoter or the 0.5 kb fragment of PCR-amplified *gus*A ORF was used as probe for detecting the presence of a part of pBK I or *gus*A gene, respectively, in transgenic perilla. Probe labeling, pre-hybridization, hybridization

and subsequent luminescent detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) according to the manufacturer's instruction.

Results

Optimum concentrations of PPT and kanamycin for perilla transformation

Leaf, hypocotyl and cotyledon explants were cultured on shoot induction medium supplemented with different concentrations of PPT and kanamycin. The regeneration response was evaluated using percent callus induction, i.e. the number of explants showing callus induction over the total number of explants to start with under the selection conditions (Table 1). At a concentration of 0.5 mg l^{-1} PPT about 13.3% of hypocotyl explants produced calluses and no callus was induced from leaf and cotyledon explants. No callus was initiated from any explants cultured at concentrations higher than 1 mg l^{-1} PPT. The 100 mg l^{-1} kanamycin resulted in no callus initiation from leaf and cotyledon explants but 12.5% of hypocotyl explants formed callus. At a concentration of 125 mg l^{-1} no callus initiation was observed from any explants examined. Therefore, 1 mg l^{-1} PPT and 125 mg l^{-1} kanamycin were minimal concentrations for selection. To make the selection stringent, 2 mg l^{-1} PPT or 150 mg l^{-1} kanamycin was used for selecting transgenic perilla in all subsequent recovery experiments.

Regeneration of transgenic perilla

After 3 days of co-cultivation, the explants were transferred onto the shoot induction and selection medium composed of MS basal medium containing 1 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 500 mg l^{-1} carbenicillin, and 2 mg l^{-1} PPT or 150 mg l^{-1} kanamycin. Perilla regeneration involved indirect organogenesis with short intermediate callus stage. About 20 days after plating, shoots began to emerge. A total of 9–11 weeks were needed from callus initiation to excisable shoots (2–3 mm), which were then transferred to shoot elongation medium (MS basal medium containing 3% sucrose, 500 mg l^{-1} carbenicillin, and 1 mg l^{-1} PPT or 125 mg l^{-1} kanamycin) and cultured for three additional weeks. Most shoots that survived this selection stage were transgenic and were transferred to the rooting medium (MS basal medium without hormones and selection agents) and cultured for 4 weeks. The rooted plantlets were transferred to pots, acclimated for 2 weeks in the culture room, and moved to the greenhouse (Figure 2).

Confirmation of the transgenic plants

Herbicide application

The putative transgenic T_0 plants with pBK I were sprayed 0.3% Basta twice a week at the mature stage. Transgenic T_0 plants displayed tolerance to

Table 1. Callus induction rate from perilla explants on media supplemented with different amount of PPT and kanamycin

Selection marker	Concentration (mg l^{-1})	Callus formation (%)[a]		
		Cotyledon	Hypocotyl	Leaf
PPT	0	100	100	100
	0.5	–	13.3 ± 1.8	–
	1	–	–	–
	2	–	–	–
Kanamycin	0	100	100	100
	100	–	12.6 ± 3.6	–
	125	–	–	–
	150	–	–	–

Data were from three independent experiments and in each experiment 28–30 explants were cultured on regeneration medium with each concentration of selective agent. Callus formation was investigated three weeks after plating explants from perilla cultivar Yeupsil.

[a]The callus formation was the number of explants developing callus over the total number of explants to start with (100%).

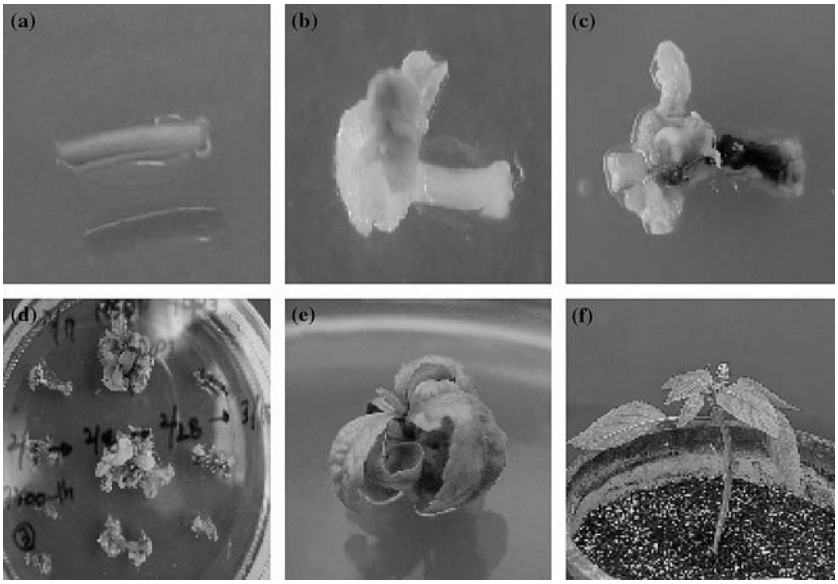

Figure 2. Plant regeneration from perilla hypocotyls transformed with pBK I on MS medium supplemented with 1 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 2 mg l^{-1} PPT and 500 mg l^{-1} carbenicillin. (*a*): 1 day, (*b*): 4 weeks, (*c*): 6 weeks, (*d*): 9 weeks, (*e*): 10 weeks, and (*f*): 20 weeks after co-cultivation.

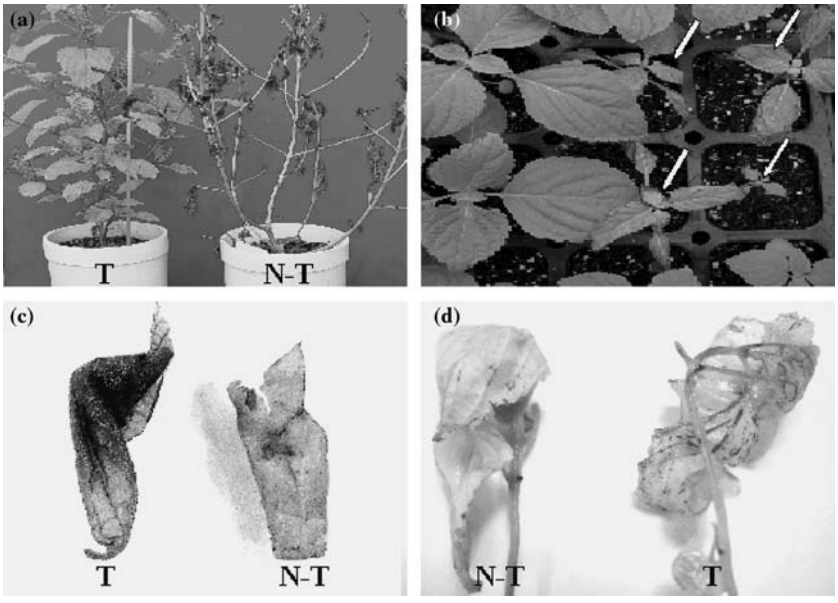

Figure 3. Confirmation of transformation by 0.3% Basta treatment (a and b) and GUS staining (C and D). (a) Comparison of transgenic T_0 perilla plant (T) showing Basta resistance with untransformed counterpart (N-T), (b) Transgenic T_1 plants showing segregation of Basta resistance (arrow indicates the progenies that do not carry *bar* gene and were susceptible to Basta), (C) Comparison of *gus*A gene expression in leaves of transgenic T_0 perilla plant (T) with that of untransformed counterpart (N-T), (D) Comparison of *gus*A gene expression in transgenic T_1 perilla seedling (T) with that of untransformed counterpart (N-T).

Basta; whereas the untransformed control plants were severally damaged and died following Basta treatment. The transgenic T_1 plants derived from a selfied T_0 plant were also sprayed with 0.3% Basta at the seedling stage (1 month after germination) to confirm the inheritance of the herbicide resistance

gene. Of 48 T_1 progeny plants, 39 showed tolerance to Basta spraying (Figure 3) suggesting that the transgene was stably inherited to the progeny and segregated among T_1 plants (Figure 3).

GUS assay

The GUS expression of the transgenic perilla transformed with pIG121Hm was confirmed by GUS staining of leaf tissue of T_0 plants and whole seedlings of T_1 progeny. As shown in Figure 3, transgenic tissues and plants exhibit GUS expression clearly distinguishable from those of the control (Figure 3).

PCR and Southern blot analysis

Transformation was also confirmed by PCR analysis for T_0 plants and Southern blot anlaysis of T_1 plants. As shown in Figure 4, the expected amplified bands for *bar* (Figure 4a) and *gus*A gene (Figure 4b) were present for transgenic T_0 plants whereas no band was observed in control plants.

The inheritance of transgenes was confirmed by Southern blot analysis of some T_1 progenies. The *Cla* I–*Bam* HI fragment of pBK I including vicillin promoter region was used as a probe for detecting the transformed gene present in transgenic perilla. The 0.5 kb fragment of PCR-amplified *gus*A gene was labeled and used as the probe for *gus*A gene transformants. As shown in Figure 5, the DNA fragment either from vicilin promoter region (Figure 5a) or from *gus*A ORF (Figure 5b) was present in T_1 plants, suggesting that the transformed gene was stably inherited to T_1 progenies (Figure 5). As for the transformants with pBK I, two inheritance patterns of transgene were shown; four copies of transgene were stably inherited in the progenies (Lane B4–6) or three copies of the transgene seemed to be segregated out in the T_1 progenies (Lane B2–3).

Transformation efficiency

The transformation efficiency in this study ranged from 0 to 3.1% depending on genotypes, types of explant, and plasmid vectors used (Table 2). It was not possible to obtain transformants from the genotype Daesil. Among the types of explant used, hypocotyl was the best for perilla transformation with an average transformation efficiency of 3.1 and 2.1% for vectors pBK I and pIG121Hm,

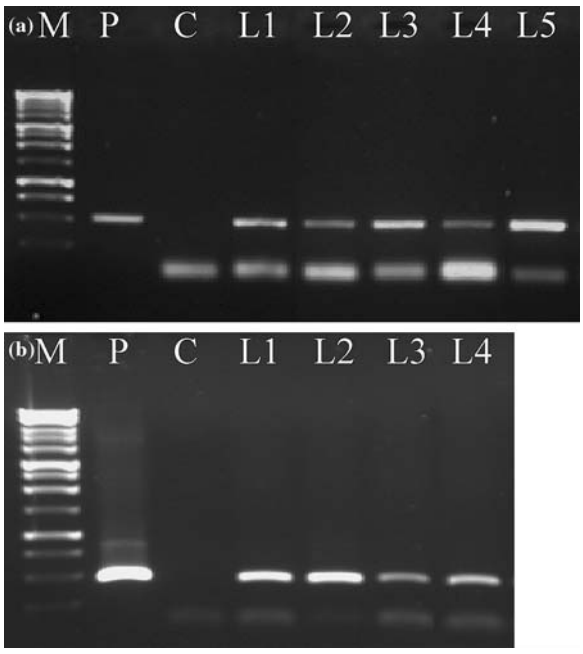

Figure 4. Confirmation of transgenic T_0 plants by PCR. (*a*) PCR amplified bar gene in transgenic perilla plants (L1–5 in upper lanes) transformed with pBK I (M, molecular marker; P, positive control pBK I; C, negative control, untransformed counterpart), (*b*) PCR amplified GUS gene in transgenic perilla plants (L1–4 in upper lanes) transformed with pIG121Hm (M, molecular marker; P, positive control pIG121Hm; C, negative control untransformed counterpart).

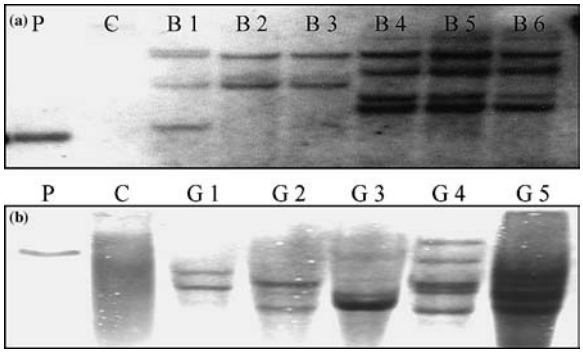

Figure 5. Southern blot analysis of genomic DNA isolated from transgenic T_1 plants transformed with pBK I (*a*) and pIG121Hm (*b*). The *Cla*I-*Bam*HI fragment of pBK I including vicillin promoter or PCR-amplified *gus*A gene was used as probe, respectively. (*a*) P, positive control (20 pg of *Xba*I restricting fragment of pBK I); C, untransformed Control; B1–6, transgenic T1 plants (B1–3 derived from one T_0 plant and B4–6 derived from another T_0 plant), (*b*) P, positive control (20 pg of *Eco*RI digested fragment of pIG121Hm); C, untransformed control; G1–5: transgenic T_1 plants derived from different T_0 plants.

respectively. When leaf explant was used, the transformation efficiency was low (0.3%) with pBK I vector or no transformant was obtained with pIG121Hm. It was not possible to obtain transgenic plants when cotyledon explants were used for transformation regardless of the plasmids used.

Discussion

Perilla (*Perilla frutescens*) was successfully transformed by *Agrobacterium*-mediated transformation and the inheritance of transgenes was confirmed by Southern blot analysis (Figure 5). Of the two genotypes, Yeupsil and Daesil, only Yeupsil was transformed. Among the types of explant examined, hypocotyl and leaf explants could be successfully used for perilla transformation though hypocotyls explant gave better transformation efficiency. Genotypic variation has been recognized in soybean (Somers et al., 2003) and wheat (Wu et al., 2003). Since there was no significant difference in shoot regeneration ability between perilla genotypes Yeupsil and Daesil (Lee et al., 2003), genotypic variation in transformation seems to be existed in perilla.

The concentrations of selecting agent PPT used (2 and 1 mg l^{-1}) for transgenic perilla recovery in this study were lower than those used in other crops such as potato at 10 mg l^{-1} (Barrell et al., 2002), soybean at 3.3 mg l^{-1} (Zhang et al., 1999) and rice at 4 mg l^{-1} (Enriquez-Obergon et al., 1999). Higher concentrations of kanamycin were necessary in this study than those used in cotton at 50 mg l^{-1} (Sunilkumar and Rathore, 2001) and potato and canola as well as tomato at 100 mg l^{-1} (Barrell et al., 2003; Khan et al., 2003; Cortina and Culianez-Macia, 2004). The transformation efficiency of perilla ranged 0–3.1% depending on the genotypes, no transgenic plants were obtained from Daesil cultivar and among explants used, highest average transformation efficiency was obtained with hypocotyl explants. Only two transformants were obtained using leaf explants.

Table 2. Transformation of perilla Yeupsil

Vector	Type of explant	Number of explants	Number of transformants	Transformation efficiency (%)
pBKI	Hypocotyl	217.7 ± 2.5	6.7 ± 3.8	3.1 ± 1.8
	Leaf	219.3 ± 1.2	0.7 ± 1.2	0.3 ± 0.5
	Cotyledon	218.3 ± 1.5	0	0
pIG121Hm	Hypocotyl	217.0 ± 5.2	4.7 ± 2.1	2.1 ± 0.9
	Leaf	215.7 ± 4.0	0	0
	Cotyledon	217.3 ± 3.0	0	0

Data were from three independent experiments.

Although the leaf explants were generally better for obtaining plantlets by *in vitro* culture (Lee et al., 2003) hypocotyl explants turned out to be better material for perilla transformation. The perilla transformation system developed here can be used routinely for perilla transformation.

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