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Transformation of *Perilla frutescens* var. *crispa* Using an *Agrobacterium*-Ri Binary Vector System

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Abstract

Transgenic hairy roots of *Perilla frutescens* var. *crispa* were obtained using an *Agrobacterium*-Ri binary vector system. The chimeric *uidA* gene encoding β-glucuronidase (GUS) flanked by the TR2′ promoter for mannopine synthase was introduced in the plant genome by *Agrobacterium*-mediated transformation by means of scratching stems of young plants. The adventitious roots which arose on the infected sites were excised, and the several clones of root tissue could be maintained on hormone-free agar medium. The transgenic states of these clones were confirmed by Southern blot hybridization. Expression of the *uidA* gene in the transgenic tissues was indicated by *in vitro* enzyme assay and histochemical staining of GUS activity. More than half of hairy roots which emerged were confirmed to be doubly transformed with T-DNAs from Ri and binary Ti plasmids.

1. Introduction

Perilla frutescens var. *crispa*, a perennial herb widely cultivated in Japan, has been used in traditional Chinese medicine, and also for food and food coloring [1]. In this species, there are many cultivars as to difference in anthocyanin content, such as cv. Akajiso (red form) and cv. Aojiso (green form), and in pattern of major essential oils. For further elucidation on a molecular basis for characterizing the difference in secondary metabolism in these forms, it is necessary to establish a method for the genetic transformation of this plant. So far, no investigation has been reported for genetic transformation of *P. frutescens* var. *crispa*.

The binary vector system based on an *Agrobacterium*-Ri plasmid has been used to produce transgenic hairy roots containing the T-DNAs of helper Ri plasmids and second binary vectors [2]. This technique depends on the fact that the T-DNA derived from a Ti plasmid can be mobilized in *trans* by *vir* gene products of the Ri plasmid. We have been exploring the Ri binary vector system for the genetic transformation of pharmaceutically important plants [3-9]. In most cases, doubly-transformed tissues integrated with both T-DNAs derived from an Ri plasmid and a binary vector were obtained in a high frequency without any selection of transformed cells by the traits conferred by binary vectors. In the present study, we have investigated stable transformation of *P. frutescens* var. *crispa* using Ri binary vector system.

2. Materials and Methods

2. 1 *Plasmids, bacteria and plants*

In a binary vector, pGSGluc1 (from Plant Genetic Systems, Gent, Belgium), the *uidA* gene encoding GUS was placed under the transcriptional control of TR2′ promoter for mannopine synthase and flanked by the 3′ region of gene 7 from a Ti plasmid (indicated in **Fig. 4**). The pGSGluc1 was introduced into *Agrobacterium rhizogenes* harboring pRi15834, a wild agropine-type Ri plasmid, as described previously [9]. The seeds of *Perilla frutescens* var. *crispa* cv. Akajiso (red form) and cv. Aojiso (green form) purchased from Sakata Co., (Yokohama, Japan) were sterilized with 2.5 % sodium hyperchrolite solution and germinated on A1 medium (half-strength Murashige and Skoog salts [10], 1% sucrose and 0.8% agar, pH 5.7). Sterile plants were maintained on same medium at 25°C under illumination (16 hr / day, 2,000 lux).

2. 2 *Plant transformation and tissue culture*

Agrobacterium rhizogenes harboring both pRi15834 and pGSGluc1 was cultured in YEB medium (5 g/*l* beef extract, 1 g/*l* yeast extract, 5 g/*l* peptone, 5 g/*l* sucrose, 2 mM $MgSO_4$) supplemented with 50 mg/*l* rifampicin, 300 mg/*l* streptomycin and 100 mg/*l* spectinomycin for 2 days at 25°C. Precultured *A. rhizogenes* was inoculated onto the stems of one-month-old sterile plants by scratching with a needle. The adventitious roots which emerged were excised

from the infected sites and transferred to B5 agar medium [11] containing 200 mg/*l* Claforan® (Hoechst) to remove *A. rhizogenes* for several weeks. The root tissues were cultured at 25°C in the light (16 hr / day, 2000 lux) and transferred onto fresh B5 medium every 2 weeks.

2. 3 Opine assay

The extracts of tissues were obtained by homogenizing fresh root tissues in micro tubes. Mannopine and agropine in the extracts were detected by high-voltage paper electrophoresis as described previously [12].

2. 4 Genomic DNA isolation, polymerase chain reaction (PCR) and Southern hybridization

Plant total DNA was extracted by modified CTAB method [13]. For PCR-Southern blot hybridization, ten ng of DNA was amplified by PCR with the primer set, the forward primer (5′-CCTGTAGAAACCCCAACCCG-3′) and the reverse primer (5′-TGTTTGCCTCCCTGCTGCGG-3′), which was expected to yield the 1. 8 kb *uidA* fragment. The amplification was performed in 50 μl of reaction mixture with *Taq* DNA polymerase (Toyobo, Japan) using Zymoreactor II (Atto Tokyo, Japan). Each PCR cycle consisted of denaturation at 94°C for 1 min., annealing at 36°C for 2 min., and extension at 72°C for 3 min.; and for the final cycle only, the duration of the extension was 10 min. The amplification was performed for 40 cycles. The PCR products were electrophoresed on 1. 0 % agarose gel and transblotted onto a nylon filter (Hybond N^+, Amersham, Bucks, UK) and then hybridized with random labeled ^{32}P-probes (Takara, Japan) by the protocols recommended by the suppliers. The 4. 2 kb *Bam*HI-*Hin*dIII fragment of pGSGluc1 was used as the probe for *uidA* gene.

For Southern blot analysis, two μg of genomic DNA was digested with *Bam*HI and *Hin*dIII or only with *Bam*HI. The resulting DNA fragments were subjected to electrophoresis in 0. 8 % agarose gel and transferred onto Hybond N^+ filter and hybridized with radio-labeled probes of *uidA* gene and the TL-DNA region obtained by *Bam*HI digestion of pLJ1.

2. 5 Histochemical staining and in vitro *assay of GUS activity*

Histochemical analysis and *in vitro* assay of GUS activity were carried out as described by Jefferson *et al.* [14] with some modifications. The root tissues of *ca.* 15 mm length were incubated in 100 mM sodium-phosphate buffer (pH 7. 3) containing 0. 5 mg/m*l* 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc; Sigma), at 37°C for 2 hours in the dark. After incubation, tissues were rinsed in 100 mM sodium-phosphate buffer (pH 7. 3) and transferred to a microscope slide in a drop of clearing reagent, a 2: 1: 2 (v/v) mixture of chloral hydrate, lactic acid and phenol. After 24 hr of clearing, tissues were tested under a microscope (Olympus BX50). The GUS activity *in vitro* was determined with 4-methylumbelliferyl-β-glucuronide as the substrate as described previously [14].

2. 6 Extraction and determination of anthocyanin

Plant tissues (10-30 mg) were immersed in methanol containing 1% hydrochloride for 24 hours at 4°C. The absorbance at 517 nm of percolated solution was determined. The concentration of anthocyanin was estimated as malonylshisonin [15] using the following equation: C mg/g = (843. 7 g/mol) / (27, 700 mol^{-1}) · A_{517} · D · (W g)$^{-1}$ (C, concentration of anthocyanin; A_{517}, absorbance at 517 nm; D, dilution ratio; W, fresh weight of plant tissue; 843. 7, molecular weight of malonylshisonin; 27,700, molar extinction coefficient for malonylshisonin).

3. Results and Discussion

3. 1 Transformation and screening of hairy roots

Upon infection of *Agrobacterium rhizogenes* on young seedlings of *Perilla frutescens* var. *crispa*, adventitious roots appeared on stems at the scratched sites after 5 days of inoculation. The root tips of *ca.* 5 mm length were excised and transferred onto B5 agar medium containing Claforan® to remove *A. rhizogenes*. We isolated a number of independent adventitious root clones, 11 clones from the green form and 3 clones from the red form (**Fig. 1**). In 13 clones out of these 14 clones, mannopine accumulated (**Table 1**). Additionally, agropine accumulated in only one of these 13 clones. The production of opines such as mannopine and agropine in transgenic plant tissue indicates the expression of mannopine synthase gene (*mas1′* and *mas2′*) and agropine synthase gene (*ags0′*), which were located in TR-DNA of pRi15834 [12]. Agropine synthesis from mannopine is catalized with agropine synthase. Some deletion and/or rearrangement of *ags0′* might occur in hairy root clones which produced only mannopine, as reported previously [16].

Histochemical analysis of GUS activity in putative transgenic hairy roots indicated the integration and expression of *uidA* gene in the 9 out of 13 opine producing clones (69. 2 %) as summarized in **Table 1.** Generally, doubly transformed hairy roots that integrated both T-DNAs from an Ri plasmid and a binary plasmid have been obtained in a high frequency without any selection of transformed cells using the Ri binary vector system [3, 4, 8, 9]. The GUS activity was observed in root tips and the tissues surrounding root meristem (**Fig. 2**). This expression pattern

Table 1.
Opine production and GUS activity in putative transformed hairy roots.

Mother plant	Number of hairy roots obtained	Number of clones Opine (+)/Total	GUS (−)/Opine (+)
Aojiso (Green form)	11	10/11	6/10
Akajiso (Red form)	3	3/3	3/3
Total	14	13/14	9/13

Opine production was determined by paper electrophoresis, and GUS activity was assayed by histochemical staining as described in Materials and Methods.

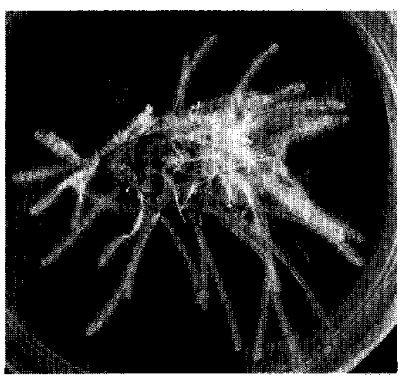

Fig. 1 Hairy roots of *Perilla frutescens* var. *crispa* (clone G544) on B5 agar medium.

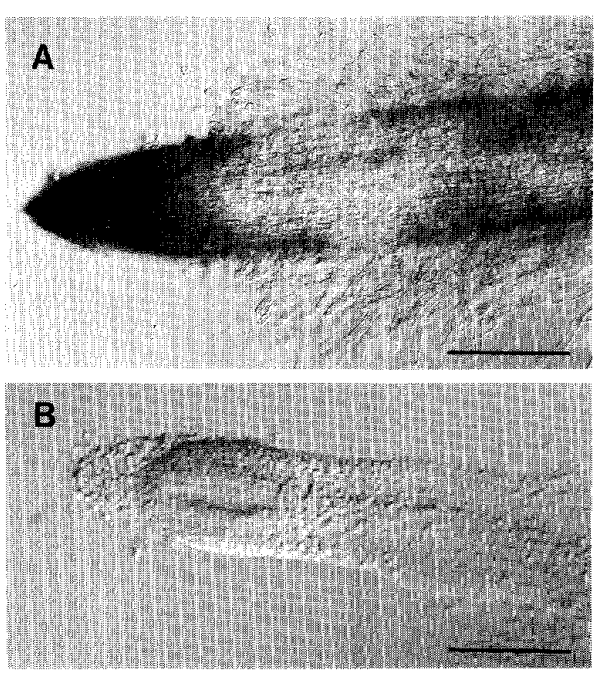

Fig. 2 Histochemical staining of GUS activity.
The GUS activity was histochemically assayed using X-gluc as a substrate as described in Materials and Methods. The dark parts are blue in color. A, positive clone G151; B, negative clone G541. The bars are 300 μm.

driven by TR2′ promoter in hairy roots of *P. frutescens* var. *crispa* is essentially the same as observed for other plant species [5]. Finally, 8 clones of the green

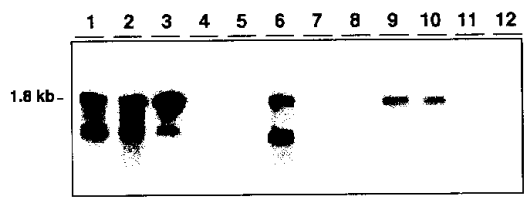

Fig. 3 PCR-Southern hybridization of hairy roots.
Extraction of DNA, amplification by PCR and DNA blot analysis were carried out using *uidA* gene as a probe as described in Materials and Methods. Lanes 1-10, hairy root clones G151, G311, G32, G341, G421, G422, G541, G544, R712, R01 respectively; lane 11, nontransformed plant 'Aojiso'; lane 12, nontransformed plant 'Akajiso'

form (G151, G311, G32, G351, G421, G422, G541 and G544) and 2 clones of the red form (R712 and R01) were established on B5 agar medium. In this study, it was shown that the Ri binary vector system could be applied to genetic engineering of *P. frutescens* var. *crispa*.

3. 2 Molecular analysis of hairy roots

In ten clones of hairy root, the presence of integrated T-DNAs of pGSGluc1 in genome DNA was analyzed by PCR-Southern hybridization (**Fig. 3**). The 1.8 kb fragment of *uidA* was amplified by PCR and then blotted onto a filter. The filter was hybridized with ^{32}P-labeled *Bam*HI-*Hin*dIII fragment (4.2 kb) of pGSGluc1 covering the *uidA* gene as a probe. An amplified 1.8 kb fragment could be detected in the DNA of six clones, G151, G311, G32, G422, R712 and R01, which showed GUS activity in the histochemical assay. Additionally, a shorter fragment was detected in these positive clones. This fragment is thought to be partially amplified *uidA* gene. However, the 1.8 kb fragment was not amplified in clone G544 which showed a weak signal in histochemical GUS staining.

Further analysis of integrated genes was performed for representative 4 clones, G151, G422, G544 and R712 by Southern blotting of the total DNA of hairy roots (**Fig. 4**). The results of analysis for these four clones are summarized in **Table 2.** The total DNA double-

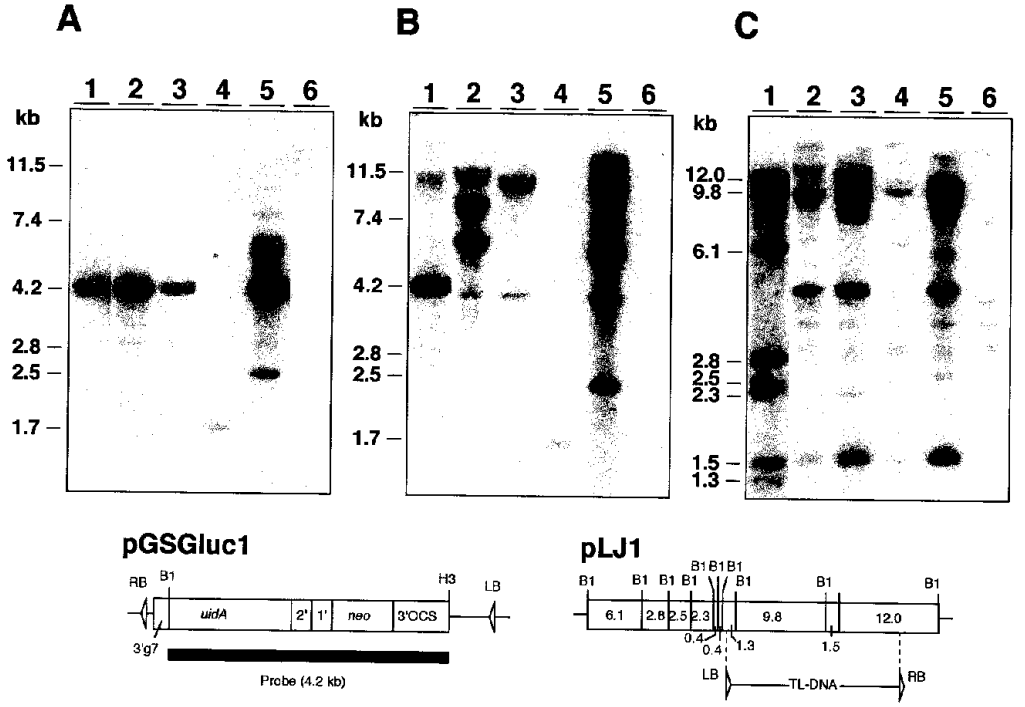

Fig. 4 Southern blot analysis of *uidA* gene in hairy roots.
Genomic DNAs isolated from hairy roots and nontransformed control were doubly digested with *Bam*HI and *Hind* III (A) or only with *Bam*HI (B and C). Two μg of genomic DNA was loaded on the gel, and the gel blot was hybridized with ^{32}P-labeled probe of 4.2 kb fragment of pGSGluc1 (A and B) and *Bam*HI digested fragments of pLJ1 (C). Lane 1, positive controls, 10 pg of *Bam*HI-*Hind*III fragment of pGSGluc1 for A and B, and 500 pg of pLJ1 digested with *Bam*HI for C; lanes 2, 3, 4, 5, hairy root clones G151, G422, G544, R712, respectively; lane 6, nontransformed control plant. The T-DNA regions of pGSGluc1 and pLJ1 were schematized at the bottom. 1′ and 2′, TR1′ and 2′ promoters; 3′g7, 3′ region of gene 7 from Ti plasmid; *neo*, neomycin phosphotransferase II gene ; 3′ OCS, 3′ region of octopine synthase gene; B1, *Bam*HI site; H3, *Hind*III site; LB and RB, left and right border repeat of T-DNAs; The size of *Bam*HI-fragments in pLJ1 was indicated in kb.

digested with *Bam*HI and *Hind*III was hybridized with ^{32}P-labeled *uidA* probe. The intact *uidA* fragment (4.2 kb) was detected in clones, G151, G422 and R712, which were positive both in PCR analysis and GUS activity. However, the *uidA* fragment in clone G544, which was negative in PCR analysis and gave a weak signal in GUS staining, was detected as a truncated fragment of 1.7 kb. From the analysis of single cut DNA with *Bam*HI, it was shown that one to several copies of *uidA* gene were integrated in the genome DNA of hairy roots.

Detection of TL-DNA of pRi15834 was performed by Southern blot analysis. Three clones, G151, G422 and R712, contained the whole region of TL-DNA; however, clone 544 possessed a partial fragment of TL-DNA. It is noted that the genomic DNA of non-transformed *P. frutescens* var. *crispa* contained DNA sequences exhibiting weak homology with the pLJ1 DNA, being judged from the hybridization signals of lane 6 in **Fig. 4-C**.

3.3 Anthocyanin content in hairy roots

The anthocyanin content in transformed hairy roots was determined as summarized in **Table 2**. These amounts were less than 20 μg/g fresh weight (malonylshisonin equivalent) and were at almost the same level as that in normal roots and less than 0.2 % of that (11.8±1.7 mg/g fresh weight) in red leaf of *P. frutescens* var. *crispa*. No significant difference in anthocyanin contents was observed between hairy roots derived from the red and green forms.

4. Conclusions

In the present study, we have established a protocol for transfer of a foreign gene into the genome of *P. frutescens* var. *crispa* using an *Agrobacterium*-Ri binary vector. Although all trials have been unsuccessful for the regeneration of plantlets from hairy roots of this plant so far, the present method can be applied to incite transgenic root tissues for the study of biotechnological applications in *P. frutescens* var. *crispa*. Anthocyanin accumulation was at almost the same low level as that in normal roots. It was suggested that the hairy roots of *P. frutescens* var. *crispa* could be used for expression study of engineered genes involved in anthocyanin biosynthesis [17] with low back ground.

Table 2.
Summary of analyses of transformed hairy roots.

Clones	Opine*1	GUS staining	GUS activity*2 (nmol/mg/min.)	Copy number*3 of inserted *uidA* gene	Anthocyanin content*4 (μg/fresh weight)
G 151	m	++	175±38	3	19±8
G 422	m	+	57±9	1	12±3
G 544	m	±	not detected	1 (truncated)	17±10
R 712	m, a	+	68±9	>4	5±5

*1 Detected opines (m, mannopine; a, agropine) were indicated.
*2 GUS activity was analyzed using 4-methylumbelliferyl-β-glucuronide as a substrate. Data are mean ± SD from three determinations.
*3 Copy number of inserted *uidA* gene was estimated from the hybridization of *Bam*HI-digested DNA with radiolabeled *uidA* probe.
*4 Anthocyanin content was estimated as malonylshisonin. Data are mean ± SD from three determinations.

Acknowledgments

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