**Agrobacterium-mediated Production of Transgenic Plants in Tricyrtis hirta** (Liliaceae)

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**Abstract**

A system for producing transgenic plants has been developed for the Liliaceous geophyte *Tricyrtis hirta* via *Agrobacterium*-mediated transformation. Tepal-derived embryogenic calluses were co-cultivated with *A. tumefaciens* EHA101/pIG121Hm, which harbored the binary vector carrying the neomycin phosphotransferase II (NPTII), hygromycin phosphotransferase (HPT) and intron-containing β-glucuronidase (GUS-intron) genes in the T-DNA region. The duration of co-cultivation and acetosyringone (AS) treatment during co-cultivation affected the frequency of transient expression of the GUS gene: the best result was obtained when embryogenic calluses were co-cultivated for 7 days in the presence of 50 mg l\(^{-1}\) AS. Following co-cultivation, the calluses were transferred to a medium lacking plant growth regulators (PGRs) but containing 40 mg l\(^{-1}\) hygromycin and 300 mg l\(^{-1}\) cefotaxime, on which several hygromycin-resistant (Hyg\(^{r}\)) somatic embryos were produced 8 weeks after transfer. These embryos were transferred to the same medium but containing lower concentrations of antibiotics (20 mg l\(^{-1}\) hygromycin and 100 mg l\(^{-1}\) cefotaxime) to promote germination. Finally, Hyg\(^{r}\) embryo-derived plantlets were established on a medium without both PGRs and antibiotics. Most of them were verified to be stable transformants by GUS histochemical assay and PCR analysis.

**INTRODUCTION**

*Tricyrtis hirta*, a Liliaceous geophyte native to Japan, has recently become popular as an ornamental plant for pot and garden uses. Differently from most Liliaceous species, *T. hirta* requires only one year from in vitro regeneration to flowering. Therefore, this species seems to be suitable as a model for molecular biological studies in Liliaceae.

Recently we established highly regenerable callus cultures of *T. hirta* (Nakano et al., 2004). In several Liliaceous species including *Muscari armeniacum* (Suzuki and Nakano, 2002), *Agapanthus praecox* (Suzuki et al., 2001) and *Lilium* cv. Acapulco (Hoshi et al., 2004), regenerable callus cultures have been used as a target material for *Agrobacterium*-mediated transformation. Thus, in the present study, we examined the development of an efficient system for *Agrobacterium*-mediated transformation in *T. hirta* by utilizing the established regenerable callus cultures.

**MATERIALS AND METHODS**

**Plant Material and Agrobacterium Strain**

Tepal-derived embryogenic calluses of *T. hirta* (Nakano et al., 2004) were monthly subcultured at 25°C in the dark onto half-strength MS medium (Murashige and Skoog, 1962) containing 1 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum. They developed numerous somatic embryos following transfer to half-strength MS medium lacking plant growth regulators (PGRs) but containing 30 mg l\(^{-1}\) sucrose and 2 g l\(^{-1}\) gellan gum. Four- to six-month-old callus cultures were used for transformation.

*A. tumefaciens* strain EHA101/pIG121Hm was used in the present study. The T-DNA region of the binary vector pIG121Hm contains the neomycin phosphotransferase
II (NPTII) gene under the control of the nopaline synthase (NOS) promoter, the hygromycin phosphotransferase (HPT) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and the β-glucuronidase gene with an intron (GUS-intron) fused to the CaMV35S promoter (Ohta et al., 1990) This bacterial strain was inoculated into liquid YEP medium (An et al., 1988) containing 50 mg l⁻¹ kanamycin (kanamycin monosulfate) and 50 mg l⁻¹ hygromycin (hygromycin B), and incubated for more than 24 h at 28°C with reciprocal shaking (150 rpm).

**Inoculation, Co-cultivation and Selection of Putative Transformants**

Cultured Agrobacterium cells were collected by centrifugation (2,000 g, 10 min) and suspended to an OD₆₀₀ of 0.2 in liquid inoculation media which consisted of half-strength MS medium, 1 mg l⁻¹ 2,4-D and 30 g l⁻¹ sucrose with or without 50 mg l⁻¹ acetosyringone (AS). After blotting the embryogenic calluses on sterile filter papers to remove excess culture medium, they were immersed into the bacterial suspension for 3 min. Calluses were then transferred to the same medium as for inoculation but solidified with 2 g l⁻¹ gellan gum. They were incubated at 25°C in the dark for 3, 5, 7 or 14 days.

Co-cultivated calluses were transferred to half-strength MS medium lacking PGRs but containing 40 mg l⁻¹ hygromycin, 300 mg l⁻¹ cefotaxime (Cliform; Hoechst), 30 mg l⁻¹ sucrose and 2 g l⁻¹ gellan gum for selecting transgenic cells and tissues. They were subcultured every 2 weeks onto fresh medium of the same composition at 25°C under continuous illumination. After 8 weeks, to promote germination, developed somatic embryos were transferred to half-strength MS medium lacking PGRs but containing 20 mg l⁻¹ hygromycin, 100 mg l⁻¹ cefotaxime, 30 mg l⁻¹ sucrose and 2 g l⁻¹ gellan gum, and cultured under the same conditions. Four to five weeks later, regenerated plantlets were transferred to half-strength MS medium lacking both PGRs and antibiotics but containing 30 mg l⁻¹ sucrose and 2 g l⁻¹ gellan gum, and cultured under the same conditions.

**GUS Histochemical Assay and PCR Analysis**

Histochemical localization of GUS gene expression in co-cultivated calluses and transgenic plantlets was detected according to Suzuki et al. (2001). PCR analysis using total genomic DNAs isolated from leaf tissues as a temperate and the GUS gene primer set, which yields a 0.45 kbp fragment inside of the GUS gene (Anzai et al. 1996), was performed according to Suzuki et al. (2001).

**RESULTS AND DISCUSSION**

**Transient Expression of the GUS Gene**

Effects of the co-cultivation period and the addition of AS to both inoculation and co-cultivation media on the number of blue spots, which may result from transient expression of the GUS gene, per 0.5 g fresh weight (FW) of embryogenic calluses were initially examined (Table 1). Our preliminary experiments indicated that no endogenous GUS activity was observed in the control, non-co-cultivated calluses, and leaf and root tissues of the control plantlets. In the absence of AS, no blue spots could be detected in the calluses irrespective of the co-cultivation period. In the presence of AS, blue spots started to be detected 5 days after co-cultivation, and the number increased thereafter. The highest number, over 350 spots per 0.5 g FW of calluses, was obtained 7 days after co-cultivation (Fig. 1A). Positive effects of AS treatment on the efficiency of transient expression of a reporter gene and of stable transformation have already been reported for Agrobacterium-mediated transformation of several Liliaceous plants such as Agapanthus praecox ssp. orientalis (Suzuki et al., 2001) and Muscari armeniacum (Suzuki and Nakano, 2002). In the present study, AS treatment during co-cultivation was essential for transient expression of the GUS gene. Based on these results, embryogenic calluses of T. hirta were co-cultivated for 7 days in the presence of AS in subsequent experiments. Seven-day co-cultivation was also performed in the other Liliaceous plants, A. praecox ssp. orientalis (Suzuki et al., 2001) and Lilium sp. (Hoshi et al., 2004).
Production of Transgenic Plants

Embryogenic calluses co-cultivated for 7 days were successively subcultured onto PGR-free medium containing 40 mg l⁻¹ hygromycin and 300 mg l⁻¹ cefotaxime. Our preliminary experiment indicated that this level of hygromycin was sufficient to inhibit the formation of somatic embryos from the control, non-co-cultivated embryogenic calluses of T. hirta. Co-cultivated embryogenic calluses turned brown within 4 weeks on a medium containing 40 mg l⁻¹ hygromycin, but hygromycin-resistant (Hyg⁺) somatic embryos, white to light yellow in color, started to develop thereafter (Fig. 1B). These Hyg⁺ somatic embryos were selected and transferred to the same medium but containing lower concentrations of antibiotics (20 mg l⁻¹ hygromycin and 100 mg l⁻¹ cefotaxime), on which over 90% of them germinated after 4-5 weeks (Fig. 1C). Small plantlets thus obtained were further transferred to a medium without both PGRs and antibiotics, on which Agrobacterium-free plantlets with a well-developed root system were established within 4 weeks (Fig. 1D).

Hyg⁺ somatic embryo-derived plantlets were subjected to PCR analysis for confirmation of their transgenic nature (Fig. 2). PCR using the GUS gene primer set showed the expected band of 0.45 kbp in 64 out of 93 plantlets analyzed. No amplified fragments were detected in the control, non-transgenic plantlets. These results indicated that the GUS gene was present in about 70% of the plantlets regenerated from Hyg⁺ somatic embryos. On average, about 10 independent transgenic plantlets could be obtained per 1 g FW of co-cultivated embryogenic calluses. Leaf and root segments of 10 independent transgenic plantlets were subjected to GUS histochemical assay: 8 plantlets showed GUS gene expression in both leaves and roots (Fig. 1E, F), whereas no GUS gene expression could be detected in the remaining 2 plantlets. The lack of GUS gene expression in these plantlets might be a result of genomic position effects, deletion of the promoter or GUS coding region, or gene silencing due to DNA methylation as suggested by Suzuki and Nakano (2002) for Agrobacterium-mediated transformation of M. armeniacum.

In the present study, we have succeeded in the development of an efficient system for producing transgenic plants of T. hirta via Agrobacterium-mediated transformation. Over 30 independent transgenic plants have so far been transplanted to the greenhouse (Fig. 1G). All of them exhibited normal phenotype at least at early stage of growth. Production of transgenic T. hirta plants with genes involved in the flavonoid biosynthesis pathway and with MADS-box genes are now in progress by using the transformation system developed here.

ACKNOWLEDGEMENT
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Literature Cited
Nakano, M., Mizunashi, K., Tanaka, S., Godo, T., Nakata, M. and Saito, H. 2004. Somatic embryogenesis and plant regeneration from callus cultures of several species in the


**Tables**

Table 1. Effects of the co-cultivation period and AS treatment on the number of blue spots resulting from transient expression of the GUS gene per 0.5 g FW embryogenic calluses of *Tricyrtis hirta*.

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<tr>
<th>AS</th>
<th>Co-cultivation period (days)</th>
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<td>3</td>
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<td>-</td>
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1 Values represent the mean ± standard error of at least 3 independent co-cultivation experiments.

2 + and - represent the presence and absence of 50 mg l⁻¹ AS, respectively, in both inoculation and co-cultivation media.
Fig. 1. Production of transgenic plants of *Tricyrtis hirta* via *Agrobacterium*-mediated transformation of embryogenic calluses. (A) Embryogenic calluses showing transient expression of the GUS gene after 7 days of co-cultivation in the presence of AS. Bar = 2 mm. (B) *Hyg*<sup>+</sup> somatic embryos (arrowheads) developed on a medium containing 40 mg l<sup>-1</sup> hygromycin. Bar = 2 mm. (C) Germination of *Hyg*<sup>+</sup> somatic embryos on a medium containing 20 mg l<sup>-1</sup> hygromycin. Bar = 1 cm. (D) A plantlet regenerated from a *Hyg*<sup>+</sup> somatic embryo. Bar = 1 cm. (E, F) GUS histochemical assay of leaf (E) and root (F) segments of transgenic (lower) and the control, non-transgenic (upper) plantlets. Bars = 5 mm. (G) A transgenic plantlet established in the greenhouse. Bar = 1 cm.

Fig. 2. PCR analysis for detecting the GUS gene in transgenic plantlets of *Tricyrtis hirta*. Lane M, 100-bp DNA ladder as molecular markers; lane 1, binary plasmid pIG121Hm as a positive control; lane 2, non-transgenic plantlet as a negative control; lanes 3-6, independent transgenic plantlets. Numerals indicate kbp.