Pre-Culture Treatment Enhances Transient GUS Gene Expression in Leaf Segment of *Saintpaulia ionantha* Wendl. after Inoculation with *Agrobacterium tumefaciens*

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Abstract

We studied the effects of various treatments given before or at inoculation with *Agrobacterium tumefaciens* strain EHA105 (pIG121Hm) on the transient GUS gene expression in leaf segments of *Saintpaulia ionantha* Wendl. Sonication and vacuum infiltration treatments in the presence of *Agrobacterium* had no positive effect on GUS expression. In contrast, the explants cultured for 3 to 5 weeks on shoot induction medium containing 0.5 mg l⁻¹ NAA and 0.5 mg l⁻¹ BA, prior to *Agrobacterium* inoculation markedly increased transient GUS expression. The addition of acetosyringone to the co-cultivation medium enhanced the GUS expression. A similar enhancing effect was observed in all of the 4 cultivars examined, suggesting the wide applicability of the pre-culture treatment.

Keywords: *Agrobacterium tumefaciens*, GUS expression, pre-culture treatment

*Saintpaulia* is an important ornamental plant and a popular indoor plant. Many varieties have been developed by conventional breeding methods. However, the scope and flexibility of these techniques are limited. As a result, no cultivars with commercially important traits, such as yellow or true red colored flowers, gray mold disease resistance, cold hardness, have yet been produced. Biotechnological methods such as genetic transformation are now expected to provide an alternative route to obtaining varieties incorporating such traits.

Recently, we have succeeded in producing transgenic plants using *Agrobacterium*-mediated transformation of *Saintpaulia ionantha* Wendl. This method succeeded, by using suspension cells that have high regenerative ability (Kushikawa et al., 2001). However, this method is not applicable to other cultivars because the induction of cell suspension culture is difficult with them. Mercuri et al. (2000) have succeeded in producing transgenic plants of a cultivar 'Rapsody' by inoculating the petiole segments with oncogenic type of *A. tumefaciens* strain A281 containing pKIWI105 (Janssen et al., 1989). However, they were unable to succeed in the transformation when they used leaf segments. The reason for this is still unclear (Mercuri et al., 2000). We have encountered the same difficulty with leaf segments in our previous transformation trials using other cultivars of *Saintpaulia* (Kushikawa et al., unpublished results).

Since *Saintpaulia* has a high potential to regenerate multiple shoots from leaf cuttings (Geier, 1983; Grout, 1990; Mølgaard et al., 1991), we thought that the leaf cutting system must be a useful approach for the genetic transformation of this plant. In the present study, therefore, we studied the effects of sonication (Santarem et al., 1998), vacuum infiltration (Clough et al., 1998), and pre-culture treatment (Sangwan et al., 1991) of leaf segments of *S. ionantha* on the transient expression of β-glucuronidase (GUS) induced by inoculation with *A. tumefaciens*. We used a disarmed strain of *A. tumefaciens* EHA105 (pIG121Hm) (Hood et al., 1993). The vector plasmid used (pIG121Hm) possessed an intron-GUS construct as a reporter gene under the control of a 35S cauliflower mosaic virus.
promoter which is not expressed in *Agrobacterium* but in plant cells (Ohta et al., 1990).

Four cultivars of *Saintpaulia*, 'Mitchell', 'Amanda', 'Masquerade' and 'Pink Veil' were propagated *in vitro* by culturing shoot sections at 20°C under continuous illumination (35 μmol m⁻² s⁻¹) as described in Hoshino et al. (1995). From 2-month-old plantlets of these cultivars, the forth to tenth leaves were harvested, cut into 5 mm x 5 mm segments, and used for study.

Prior to the inoculation with *Agrobacterium*, leaf segments were cultured on shoot induction medium which consisted of Murashige and Skoog (MS) (1962) basal medium supplemented with 0.5 mg l⁻¹ α-naphthaleneacetic acid (NAA), 0.5 mg l⁻¹ benzyladenine (BA), 20 g l⁻¹ sucrose and 2 g l⁻¹ Gellan Gum (Wako Pure Chemical Industries, Ltd.) for up to 6 weeks.

The segments, once the culture was established, were then inoculated with *Agrobacterium* as described previously (Kushikawa et al., 2001) except that the bacterial inoculation medium was replaced with liquid MS medium containing no acetylsyringeone, the inoculation period was changed from 5 to 20 min, and the bacterial suspension was diluted by a factor of ten (= based upon the OD₅₀₀ of the suspension). After the bacterial inoculation, leaf segments were transferred onto the co-cultivation medium which was MS basal medium supplemented with 20 g l⁻¹ sucrose, 2 g l⁻¹ Gellan Gum and varied concentrations of acetylsyringeone (0–500 μM), and maintained for 48 h in the dark at 20°C. The leaf segments were then subjected to a GUS assay according to Jefferson et al. (1987). Determination of the fluorometric GUS enzyme activity was made using 4-methylumbelliferyl β-D-glucuronide (4-MUG) as the substrate. Using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), the number of leaf segments with GUS foci was counted under a binocular microscope (SZH10 OLUMPU). Results are shown in Fig. 1 and 2.

By contrast to the treatments with sonication and infiltration, the pre-culture treatment markedly improved transient GUS gene expression of *Saintpaulia* cells by *Agrobacterium*-mediated transformation (Fig. 1 and 2). The optimum period of the pre-culture was found to be 3 to 5 weeks, at which numerous shoot primordia were observed on the leaf segments (data not shown). Without pre-culture treatment, no GUS foci were observed on the segments (see Fig. 1 and 2). We also found that the addition of acetylsyringeone to the co-cultivation medium was essential to obtain transient GUS expression as shown in Fig. 3. The concentrations of 100–300 μM showed the best results, where 50–60% of leaf segments displayed GUS foci. Beyond these optimal concentrations, about 80% of the segments showed evidence of necrosis (leaf browning) (see Fig. 3).

In addition, freshly prepared leaf segments were transferred into an *Agrobacterium* suspension, to which either sonication or vacuum infiltration treatment was performed. Sonication was done at 40 kHz with a sonicator (Elma transonic DIGITALS) for 0, 1, 2, 3, 5, 10, 20 and 30 s. Vacuum infiltration was performed using an aspirator (ASPIRATOR A-3S Tokyo RIKAKIKAI Co., Ltd.) for 0, 10, 30, 60, 180 and 300 s. Vacuum infiltration for up to 300 s

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**Fig. 1** Typical pictures of the foci from leaf cells of *S. ionantha* Wendl. 'Mitchell' expressing GUS gene. Leaf segments were cultured for 0 (A) to 3 (B) weeks prior to inoculation for 20 min with *A. tumefaciens* EHA105 (pG121Hm) after which they were co-cultivated for 48 h in the presence of 100 μM acetylsyringeone and then histochemically assayed for GUS expression. Arrows indicate shoot primordia. Bar = 5 mm.

**Fig. 2** Effect of pre-culture on transient GUS expression in leaf segments of *S. ionantha* Wendl. 'Mitchell' after *Agrobacterium* infection. Leaf segments were cultured for 0 to 6 weeks prior to inoculation for 20 min with *A. tumefaciens* EHA105 (pG121Hm), after which they were co-cultivated for 48 h in the presence of 100 μM acetylsyringeone and then assayed for GUS expression. Vertical bars represent standard deviations (n=3).
Fig. 3 Effects of various concentration of acetosyringone added in the co-cultivation medium upon the transient GUS expression and browning of leaf segments of S. ionantha Wendel. 'Mitchell'. Segments were pre-cultured for 3 weeks, inoculated for 20 min with A. tumefaciens strain EHA105 (pPG121Hm), co-cultivated for 48 h in the presence of 0–500 μM acetosyringone, and then subjected to a histochemical GUS assay. Vertical bars represent standard deviations (n=3).

had almost no effect upon the transient GUS expression of the leaf segments regardless of the acetosyringone concentration in the co-cultivation medium. Vacuum infiltration longer than 30 s was observed to have a detrimental effect on explants, resulting in death of tissues within two weeks. Although sonic treatment for more than 3 s was effective for obtaining GUS expression in limited regions, the explants were seriously damaged, thus they became necrotic and eventually died even on a non-selective medium. Consequently, pre-culture treatment was the only effective method to obtain leaf segments competent to A. tumefaciens transformation.

Two weeks after inoculation with A. tumefaciens, the leaf segments that were infected just after cutting did not show any bacterial growth, whereas those infected after pre-culture for 3 weeks showed signs of bacterial growth within 3 days of co-cultivation. Therefore, the recalcitrance of Saintpaulia leaf segments with no treatment might originate from some inhibitory factors in the leaf cells or the absence of competent cells to A. tumefaciens infection. Transient GUS expression observed as foci on leaf segments pre-cultured for 3 weeks seemed to be limited to the shoot primordia produced de novo (Fig. 2), which would contain actively dividing cells. The correlation between cell division activity and Agrobacterium-mediated transformation was also described by Ducrocq et al. (1994). The high competence of the actively dividing cells, such as suspension cultured cells, for the Agrobacterium infection observed in our previous study (Kushikawa et al., 2001) may agree with our present observation. For obtaining transgenic plants, it is essential to induce secondary adventitious shoots from the transgenic cells scattered on the surface of the shoot primordia under the selection conditions.

In the present study, we confirmed that a relatively high transient GUS expression can be obtained by pre-culturing the leaf segments for 3–5 weeks on MS medium supplemented with 0.5 mg l⁻¹ NAA and 0.5 mg l⁻¹ BA prior to Agrobacterium infection. Since this treatment was proved to be effective for all of the cultivars examined with little variation (Fig. 4), it will be applicable to a wide range of Saintpaulia cultivars. Further experiments are now in progress to produce stable transgenic plants using the methodologies described in this report.

References


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