Note

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

Satoshi KUSHIKAWA[1]*, Kazumitsu MIYOSHI[2] and Masahiro MII[3]

[1]*Section of Plant Biotechnology, Gunma Horticultural Experiment Station, 493 Nishiobokata, Sawa-azuma, Gunma 379-2224, Japan*

[2]*Crop Science Lab., Faculty of Bioresource Sciences, Akita Prefectural University, Akita City, Akita 010-0195, Japan*

[3]*Faculty of Horticulture, Chiba University, 648 Matsudo, Matsudo, Chiba 271-8510, Japan*

**Corresponding author* E-mail address: kushi@aic.pref.gunma.jp

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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 hardiness, 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 ℃ 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 acetosyringone, the inoculation period was changed from 5 to 20 min, and the bacterial suspension was diluted by a factor of ten (= based upon the OD_{600} 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 acetosyringone (0-500 μM), and maintained for 48 h in the dark at 20 ℃. 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-methyl-umbelliferyl 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 OLYMPUS). Results are shown in **Fig. 1** and **2**.

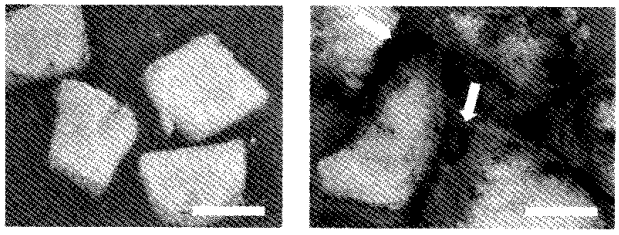

Fig. 1 Typical pictures of the foci from leaf cells of *S. ionantha* Wendl. 'Mitchell' expressing GUS gene. Leaf segments were cultured for 0 (A) to 3 (B) weeks prior to inoculation for 20 min with *A. tumefaciens* EHA105 (pIG121Hm) after which they were co-cultivated for 48 h in the presence of 100 μM acetosyringone and then histochemically assayed for GUS expression. Arrows indicate shoot primordia. Bar = 5 mm.

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 transfomation (**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 acetosyringone 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

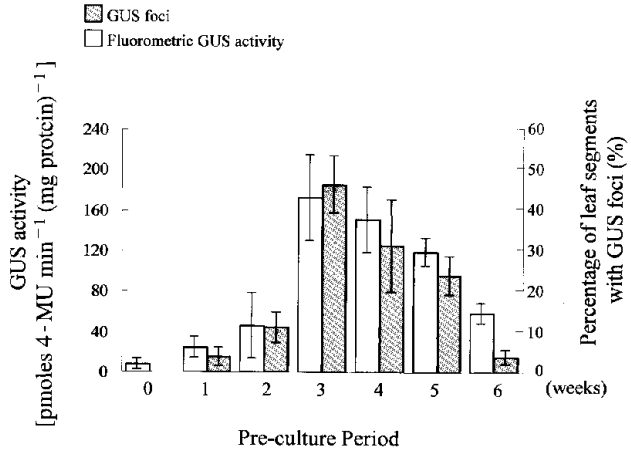

Fig. 2 Effect of pre-culture on transient GUS expression in leaf segments of *S. ionantha* Wendl. 'Mitchell' after *Agrobacterium* infection. Leaf segments were cultured for 0 to 6 weeks prior to inoculation for 20 min with *A. tumefaciens* EHA105 (pIG121Hm), after which they were co-cultivated for 48 h in the presence of 100 μM acetosyringone and then assayed for GUS expression. Vertical bars represent standard deviations (n=3).

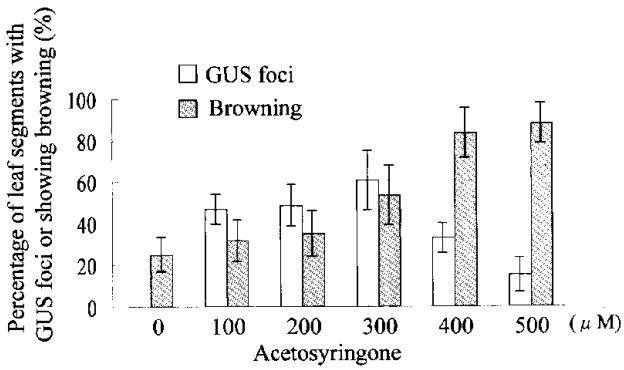

Fig. 3 Effects of various concentration of acetosyringone added in the co-cultivation medium upon the transient GUS expression and browning of leaf segments of *S. ionantha* Wendl. 'Mitchell'. Segments were pre-cultured for 3 weeks, inoculated for 20 min with *A. tumefaciens* strain EHA105 (pIG121Hm), 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).

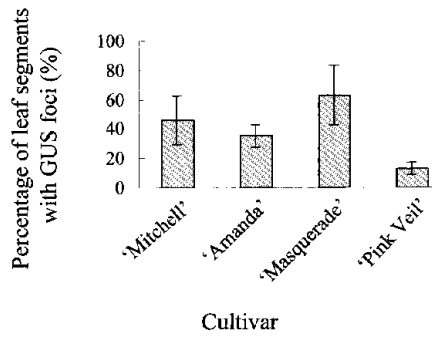

Fig. 4 GUS expression in leaf segments of four cultivars of *Saintpaulia*. Leaf segments of each cultivar were pre-cultured for 3 weeks, inoculated for 20 min with *A. tumefaciens* strain EHA105 (pIG121Hm), co-cultivated for 48 h in the presence of 100 μM acetosyringone, and then subjected to a histochemical GUS assay. No GUS expression was observed in the segments that had not been cultured prior to *Agrobacterium* inoculation. 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 sonication 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

Clough, J. S., Bent, A. F., 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J., **16**: 735-743.

Ducrocq, C., Sangwan, R. S., Sangwan-Norreel, B. S., 1994. Production of *Agrobacterium*-mediated transgenic fertile plants by direct somatic embryogenesis from immature zygotic embryos of *Datura innoxia*. Plant Mol. Biol., **25**: 995-1009.

Geier, T., 1983. Induction and selection of mutants in tissue cultures of Gesneriaceae. Acta Hort., **131**: 329-337.

Grout, B. M. W., 1990. African violet. In: Amirato P. V., Evance D. A., Sharp W. R., Bajaji Y. P. S. (Eds.): Handbook of Plant Cell Culture, Vol.15, pp.181-205. Mac-Graw Hill Publishing Company.

Hood, E. E., Gelvin, S. B., Melchers, L. S., Hoekema, A.,

1993. New *Agrobacterium* helper strains for gene transfer to plants. Transgenic Res., **2**: 208-218.

Hoshino, Y., Nakano, M., Mii, M., 1995. Plant regeneration from cell suspension-derived protoplasts of *Saintpaulia ionantha* Wendl. Plant Cell Rep., **14**: 341-344.

Janssen, B. J., Gardner, R. C., 1989. Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Mol. Biol., **14**: 61-72

Jefferson, R. A., Kavanah, T. A., Arai. Y., 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., **6**: 3901-3907.

Kushikawa, S., Hoshino, Y., Mii, M., 2001. *Agrobacterium*-mediated transformation of *Saintpaulia ionantha* Wendl. Plant Sci., **161**: 953-960.

Mercuri, A., Benedetti, L. D., Burchi, G., Schiva, T., 2000. *Agrobacterium*-mediated transformation of African violet. Plant Cell, Tissue Organ Cult., **60**: 39-46.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. **15**:473-497.

Mϕlgaard, J. P., Roulund, N., Deichmann, V., Irgens-Mϕller, L., Andersen, S. B., Farestveit, B., 1991. In vitro multiplication of Saintpaulia ionantha Wendl. by homogenization of tissue cultures. Sci. Hort., **48**: 285-292.

Ohta, S., Mita, S., Hattori, T., Nakamura, K., 1990. Construction and expression in tabacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol., **31**: 805-813.

Sangwan, R. S., Bourgeois, Y., Sangwan-Norreel, B. S., 1991. Genetic transformation of *Arabidopsis thaliana* zygotic embryos and identification of critical parameters influencing transformation efficiency. Mol. Gen. Genet., **230**: 475-485.

Santarem, E. R., Trick, H. N., Essig, J. S., Finer, J. J., 1998. Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. Plant Cell Rep., **17**: 752-759.