

Effect of Acetosyringone on *Agrobacterium*-mediated Transformation of *Eustoma grandiflorum* Leaf Disks

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Abstract

Eustoma grandiflorum is a popular ornamental flower worldwide. The production of transgenic *Eustoma* is expected to help in the study and modification of its traits, such as growth, flowering time, flower color, and flower shape. *Agrobacterium*-mediated transformation of leaf disks is a frequently used method of plant transformation. To improve the transformation efficiency of *Eustoma*, I focused on two steps: the inoculation of *Agrobacterium* into the leaf disks, and subsequent co-cultivation. The effect of detergent in the inoculation buffer was tested. Given its marginal effect on the viability of the disks, Tween 20 was determined to be a better candidate for use in the buffer. Acetosyringone is known to improve transformation efficiency by up-regulating the virulence of *Agrobacterium*. In previous studies using *Eustoma*, the effect of this compound has only been tested during inoculation. However, I studied the effect of acetosyringone by adding it to the inoculation as well as the co-cultivation medium. When added to the co-cultivation medium, acetosyringone significantly increased the number of transformed calli harboring a marker transgene. More than 0.7 callus per leaf disk was obtained by adding acetosyringone, whereas less than 0.03 callus per disks were obtained in its absence. Thus, the mediated incorporation of a transgene into the *Eustoma* genome can be intensified by the addition of acetosyringone to the co-cultivation medium.

Discipline: Horticulture

Additional key words: *Agrobacterium tumefaciens*

Introduction

Eustoma grandiflorum, a member of the family Gentianaceae, has become a popular ornamental flower worldwide. However, there are some problems, such as heat-induced rosetting (Ohkawa et al. 1991), reduced rate of flowering under low temperature (Nakano et al. 2011), and flower bud necrosis under low light intensity (Ushio and Fukuta 2010), which reduce the productivity of *Eustoma*. To study the growth and flowering habits of *Eustoma* by introducing a gene of interest, it is important to develop an efficient transformation method. Transgenic breeding is employed in ornamental flower plants for altering the flower shape, color, and other properties. In a study aimed at improving the disease resistance in *Eustoma* by the stable integration of a candidate gene, Isuzugawa et al. (2002) evaluated the factors affecting the transformation of *Eustoma*. Thiruvengadam and Yang (2009) also reported a transformation method for altering the flowering time and flower shape in *Eustoma*.

It is important to establish an efficient transformation technique that would aid further research and breeding studies on *Eustoma*.

Agrobacterium-mediated transformation of plant leaf disks is widely used to study the biological function of genes and produce crops with novel traits. This transformation involves infecting leaf disks with *Agrobacterium*, the cells of which subsequently die along with the transformed calli being selected and regenerated into plants. For *Eustoma*, the growth environment and medium composition required for callus formation and plant regeneration have already been well standardized (Isuzugawa et al. 2002). Although transformation efficiency was not calculated in all previous studies, generally 2-3 transformants are obtained per 100 explants (Semeria et al. 1996; Ledger et al. 1997; Isuzugawa et al. 2002). In this study, we focused on the infection step, which entails the inoculation of *Agrobacterium* into the leaf disks and co-cultivation for enhanced infection efficiency.

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Materials and methods

1. Growth conditions of plants and bacteria

Aseptically cultured *Eustoma* plants were used as the experimental material. Seeds of *E. grandiflorum* ‘Tenryu White’ (Miyoshi, Tokyo, Japan) and ‘Shihai’ (Fukkaen, Nagoya, Japan) were surface-sterilized by washing with 70% ethanol for 1 min and 5% sodium hypochlorite for 10 min. The seeds were subsequently washed three times with distilled water and inoculated on Murashige-Skoog (MS) medium containing 8 g L⁻¹ of agar and 5 g L⁻¹ of sucrose. The plants were grown in a clear box (W × D × H = 6 × 6 × 9 cm) at 24°C for about 12 weeks, with transplantation to fresh medium every 3 weeks. The plants were provided continuous light (80 μmol m⁻² s⁻¹) using fluorescent tubes. Disks (5 mm in diameter) were punched out from the leaves by using a sterilized cork borer. *Agrobacterium tumefaciens* strain LBA4404 harboring the vector pBI121 (Clontech, California, USA) was cultured overnight at 28°C in LB medium supplemented with 50 mg L⁻¹ of kanamycin and 50 mg L⁻¹ of streptomycin. The bacterial cells were collected by centrifugation at 5,000 × g for 5 min at 20°C, and then resuspended in inoculation medium as described below.

2. Effects of detergents on leaf disk viability

Leaf disks were soaked in MS liquid medium supplemented with Triton X-100, Tween 20, or Silwet L-77, at concentrations of 0.01 or 0.1% for 15 min. The disks were blotted on filter paper and then grown on callus induction MS (CIM) medium consisting of 1× MS salts, 8 g L⁻¹ of agar, 20 g L⁻¹ of sucrose, 1.0 mg L⁻¹ of 6-benzylaminopurine, and 1.0 mg L⁻¹ of 1-naphthaleneacetic acid for 7 days. The disks whose cut surface stayed green were counted as viable. A total of 20 leaf disks was used for each treatment.

3. Effect of acetosyringone on transformation

The *A. tumefaciens* pellet was resuspended in MS medium containing 0, 0.1, or 1.0 mM of acetosyringone (AS) to an optical density of 0.5 at 600 nm. The leaf disks were soaked in the bacterial suspension for 30 min and then blotted on filter paper for about 5 min. The disks were then placed on the co-cultivation medium (CIM medium supplemented with 0, 0.01, or 0.1 mM of AS). After co-cultivation at 25°C for 2 days in the dark, the disks were placed on CIM medium supplemented with 500 mg L⁻¹ of carbenicillin for 2 days under continuous lighting to kill the *Agrobacterium* cells (Isuzugawa et al. 2002). To select the transformed cells using kanamycin resistance, the disks were transferred onto CIM medium

supplemented with 100 mg L⁻¹ of carbenicillin and kanamycin each, and then incubated for 4 weeks.

4. Histochemical β-glucuronidase staining

β-Glucuronidase (GUS) activity was tested according to Jefferson et al. (1987). The leaf disks were incubated in 50 mM sodium phosphate buffer, pH 7.0, containing 10% methanol, 10 mM of EDTA, 1 mM of potassium ferricyanide, 1 mM of potassium ferrocyanide, 0.2 mg ml⁻¹ of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt (X-Gluc), and 0.1% Triton X-100 at 30°C. Termination of staining and bleaching were performed by soaking the disks in 80% ethanol.

5. Genomic PCR

Genomic PCR was performed by using KOD FX (TOYOBO, Osaka, Japan). Genomic DNA was extracted from the leaves (5 mm × 5 mm) of wild-type and putatively transgenic plants by boiling, as per the manufacturer's instructions. A 903 bp fragment of the *GUS* gene (AAC53706) was amplified with a primer set (5'-CGATGCGGTCCTCATTACG-3' and 5'-CTGTAAGTGCGCTTGCTGAG-3'). *ACTIN* gene was amplified as described previously (Nakano et al. 2011), as a positive control of DNA extraction.

Results and discussion

1. Effects of detergents in inoculation buffer on leaf disk viability

Given the water-repelling surface of the *Eustoma* leaf, the leaf disks stay afloat on the buffer during inoculation. The addition of a detergent to the inoculation buffer was considered to possibly improve infection efficiency, as reported in *Arabidopsis thaliana* (Kim et al. 2009). However, as some detergents are toxic to plant cells (Buchanan 1965), their effect on leaf disk viability was studied. Tween 20 and Triton X-100 are widely used detergents in transformation experiments (e.g. Kim et al. 2009). Silwet L-77 is used in *Agrobacterium*-mediated transformation of *Arabidopsis* using the floral dip method (Clough and Bent 1998). Almost all the disks soaked in Tween 20 were viable after 7 days (Table 1). Conversely, not all disks were viable after soaking in Triton X-100 or Silwet L-77 at 0.01%. Nearly half of the disks turned brown after being dipped in a medium supplemented with 0.1% Triton X-100. Thus, Tween 20 could be used for inoculation without any negative effect.

2. Effect of acetosyringone on generation of transformed calli

Next, I studied the effect of acetosyringone (AS) on infection efficiency. AS induces the *vir* gene in the Ti plasmid of *Agrobacterium* and enhances infection (Baker et al. 2005). AS is used for the transformation of monocots, such as *Oryza sativa*, which do not produce

AS-like phenolic compounds (Hiei et al. 1994). In *Eustoma* transformation, AS is only used during the inoculation step (Isuzugawa et al. 2002; Thiruvengadam and Yang 2009). Here, I studied the effect of AS during inoculation and co-cultivation. After co-cultivation, transformed cells were selected on the medium supplemented with kanamycin. GUS activity derived from the vector pBI121 was detected by histochemical staining of the calli. No callus formation was observed on the cut surface of the leaf disks from non-transformed wild type plants (data not shown). Adding AS to the inoculation buffer did not affect the formation of kanamycin-resistant or GUS-stained calli (Table 2), as reported by Isuzugawa et al. (2002). In contrast, adding AS to the co-cultivation medium increased the number of kanamycin-resistant and X-Gluc-stained calli (Fig. 1 A and B; Table 2). In wheat, 0.1 mM of AS in the co-cultivation medium resulted in a sevenfold increase of transformed calli (McCormac et al. 1998). The addition of AS into the co-cultivation medium seems effective in *Agrobacterium*-mediated transformation of *Eustoma*, as well as monocots. Some calli were kanamycin-resistant, but were not stained by X-Gluc (Fig. 1A and B; Table 2). This was probably because the expression of *GUS* was insufficient for detection in these calli or AS induced the formation of non-transformed calli.

Table 1. Effect of detergents added to the inoculation buffer on leaf disk viability in *Eustoma* ‘Tenryu White’

Detergent	Concentration in inoculation buffer (%)	Viability* (%)
No detergent	-	100a
Tween 20	0.01	100a
	0.1	98 ± 2ab
Triton X-100	0.01	80 ± 9bc
	0.1	43 ± 8d
Silwet L-77	0.01	92 ± 8ab
	0.1	78 ± 6bc

* A total of 20 leaf disks was used for each treatment.

Values are means ± SE (n = 3).

Different lowercase letters indicate significant differences among the treatments (Tukey’s test, $P < 0.05$).

Table 2. Effect of acetosyringone on *Agrobacterium*-mediated transformation of *Eustoma*

Cultivar	AS concentration in inoculation buffer (mM)	AS concentration in co-cultivation medium (mM)	No. of kanamycin-resistant calli/ No. of leaf disks*			No. of X-Gluc stained calli/ No. of leaf disks*			No. of X-Gluc stained calli/leaf disk**
			Replicate			Replicate			
			1	2	3	1	2	3	
Tenryu White	0	0	0/44	0/45	1/47	0/44	0/45	1/47	< 0.01d
	0.1	0	0/45	1/48	1/47	0/45	1/48	1/47	< 0.01d
	1	0	3/43	0/45	1/48	3/43	0/45	1/48	0.03 ± 0.02c
	0.1	0.01	36/31	68/34	36/31	32/31	60/34	26/31	1.21 ± 0.28b
	0.1	0.1	98/46	128/30	62/32	92/46	82/30	58/32	2.18 ± 0.28a
Shihai	0	0	0/42	0/45	0/42	0/42	0/45	0/42	0c
	0.1	0	0/45	0/48	0/47	0/45	0/48	0/47	0c
	1	0	0/35	1/47	1/44	0/35	1/47	1/44	< 0.01b
	0.1	0.01	38/38	36/33	24/31	34/38	26/33	16/31	0.73 ± 0.11a
	0.1	0.1	36/36	58/34	38/35	24/36	50/34	36/35	1.06 ± 0.23a

*Leaf disks contaminated with molds or bacteria were omitted.

**Values are means ± SE (n = 3). Different lowercase letters indicate significant differences among the treatments (Tukey’s test, $P < 0.05$).

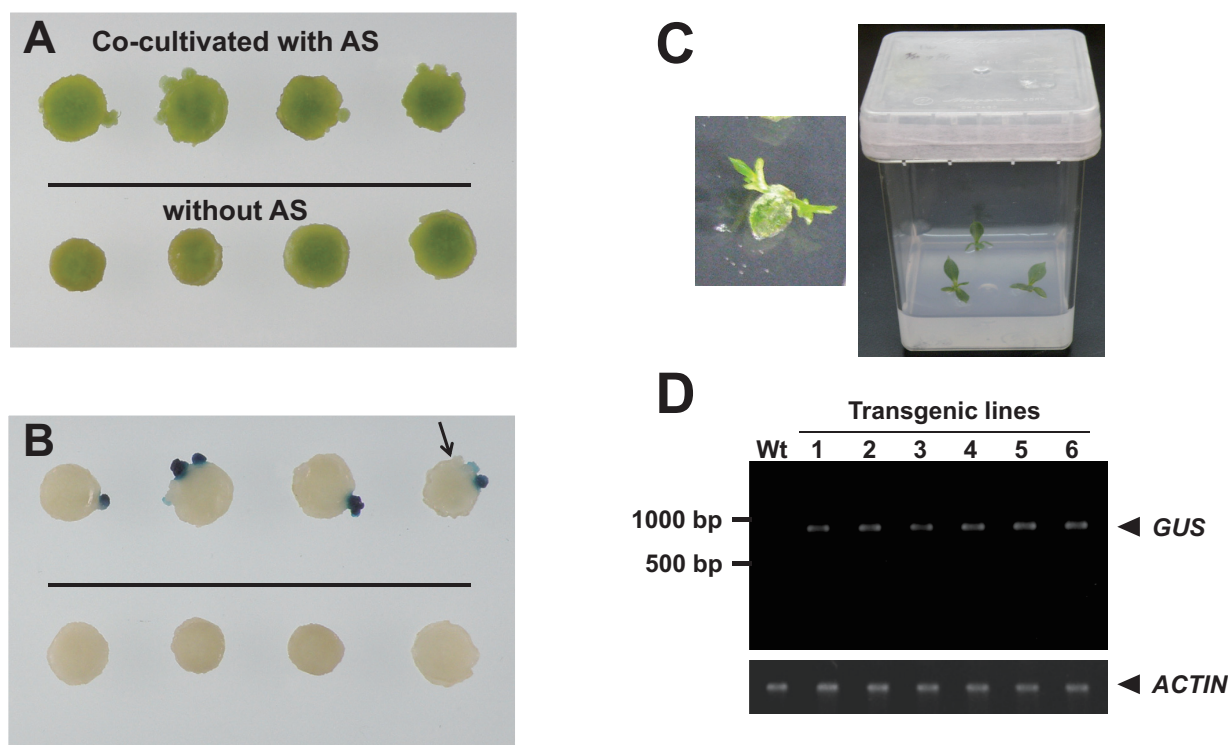


Fig. 1. Effect of acetosyringone (AS) on *Agrobacterium*-mediated transformation of *Eustoma*.

A: Leaf disks were co-cultivated with *Agrobacterium* harboring the vector pBI121 on a medium supplemented with AS (upper row) or without it (lower row). After co-cultivation and eradication, the transformed cells were selected on CIM containing kanamycin for 28 days. B: GUS staining of the disks shown in A. The arrow indicates a kanamycin-resistant callus lacking GUS activity. C: Shoots were successfully re-differentiated from the transformed calli by using the shoot induction medium described by Isuzugawa et al. (2002). D: Genomic PCR of *GUS* gene. Wild-type (Wt) and six randomly selected putative transgenic plants were analyzed. The *ACTIN* gene was used as a positive control of DNA extraction.

Table 3. Effects of detergents and acetosyringone on *Agrobacterium*-mediated transformation of *Eustoma* cv. Tenryu White

Tween 20 concentration in inoculation buffer (%)	AS concentration in co-cultivation medium (mM)	No. of X-Gluc stained calli/leaf disk*	No. of kanamycin-resistant shoots/leaf disk**
0	0	0c	0c
0.01	0	0c	0c
0.10	0	0c	0c
0	0.01	0.54 ± 0.05b	0.37 ± 0.03b
0.01	0.01	1.00 ± 0.27a	0.58 ± 0.04a
0.10	0.01	0.87 ± 0.32ab	0.62 ± 0.07a

AS concentration in the inoculation buffer was 0.1 mM.

*Twenty leaf disks were grown on CIM containing kanamycin for 4 weeks. Values are means ± SE (n = 3).

**Twenty leaf disks were grown on CIM for 1 week and on the shoot induction medium for 6 weeks in the presence of kanamycin. Values are means ± SE (n = 3).

Different lowercase letters indicate significant differences among the treatments (Tukey's test, $P < 0.05$).

3. Efficient transformation of *E. grandiflorum*

Finally, the combined effect of Tween 20 and AS was tested. Different combinations of the inoculation buffer containing 0, 0.01, or 0.1% Tween 20 and co-cultivation medium containing 0 or 0.01 mM of AS were used. A total of 20 leaf disks was used for each treatment. The presence of AS in the co-cultivation medium strongly enhanced infection efficiency (Table 3). The addition of detergent in the inoculation buffer slightly improved the transformation efficiency in *Arabidopsis* (Kim et al. 2009). Tween 20 alone did not affect the transformation efficiency in *Eustoma* (Table 3). Tween 20 present in the inoculation buffer slightly increased the number of transformed calli only in combination with AS (Table 3). When the leaf disks were selected on the shoot regeneration medium described previously (Isuzugawa et al. 2002), transformed shoots could be successfully obtained (Fig. 1C; Table 3). Genomic PCR of six randomly selected shoots confirmed that *GUS* gene was successfully integrated into their genome (Fig. 1D). In conclusion, the incorporation of a gene of interest into the *Eustoma* genome by *Agrobacterium*-mediated transformation can be intensified by adding AS during co-cultivation.

References

- Baker, C.J. et al. (2005) Involvement of acetosyringone in plant-pathogen recognition. *Biochem. Biophys. Res. Commun.*, **328**, 130-136.
- Buchanan, G.A. (1965) Patterns of surfactant toxicity to plant tissues. In *Retrospective theses and dissertations*, Paper 3905, Iowa state University, Iowa, USA, 7-14.
- Clough, S.J. & Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*, **16**, 735-743.
- Hiei, Y. et al. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.*, **6**, 271-282.
- Isuzugawa, K. et al. (2002) *Agrobacterium*-mediated transformation of Lisianthus (*Eustoma grandiflorum* Griseb.). *Bull. Yamagata Hortic. Exp. Stn.*, **14**, 1-11 [In Japanese].
- Jefferson, R.A. et al. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.*, **6**, 3901-3907.
- Kim, M.J. et al. (2009) Optimization of conditions for transient *Agrobacterium*-mediated gene expression assays in *Arabidopsis*. *Plant Cell Rep.*, **28**, 1159-1167.
- Ledger, S.E. et al. (1997) Transformation of Lisianthus (*Eustoma grandiflorum*). *Plant Cell Rep.*, **16**, 853-858.
- McCormac, A.C. et al. (1998) The use of visual marker genes as cell-specific reporters of *Agrobacterium*-mediated T-DNA delivery to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). *Euphytica*, **99**, 17-25.
- Nakano, Y. et al. (2011) Characterization of *FLC*, *SOC1* and *FT* homologs in *Eustoma grandiflorum*: effects of vernalization and post-vernalization conditions on flowering and gene expression. *Physiol. Plant.*, **141**, 383-393.
- Ohkawa, K. et al. (1991) Effects of air temperature and time on rosette formation in seedlings of *Eustoma grandiflorum* (Raf.) Shinn. *Sci. Hortic.*, **48**, 171-176.
- Semeria, B. et al. (1996) Genetic transformation of *Eustoma grandiflorum* by *Agrobacterium tumefaciens*. *Plant Cell Tissue Organ Cult.*, **47**, 67-72.
- Thiruvengadam, M. & Yang, C.H. (2009) Ectopic expression of two MADS box genes from orchid (*Oncidium Gower Ramsey*) and lily (*Lilium longiflorum*) alters flower transition and formation in *Eustoma grandiflorum*. *Plant Cell Rep.*, **28**, 1463-1473.
- Ushio, A. & Fukuta, N. (2010) Effects of nitrogen fertilization levels in nutrient solution applied before/after flower budding on blasting in winter-flowering of *Eustoma grandiflorum* (Raf.) Shinn. *Hortic. Res.*, **9**, 191-196 [In Japanese].