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Agroinfiltration for transient gene expression in floral tissues of *Dendrobium* Sonia'Earsakul'

Pinthong, R., Sujipuli, K. and Ratanasut, K.*

Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand

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Agroinfiltration is an *Agrobacterium*-mediated transformation method used for transient gene expression in specific tissues or organs. It has been developed and applied to several plant species. Here we report the development of the agroinfiltration method for transient gene expression in the sepals and petals of *Dendrobium* Sonia 'Earsakul', which is an important economic cut-flower orchid in Thailand. Two *A. tumefaciens* strains EHA105 and AGL1 harboring pCAMBIA1304 containing the *gusA* gene were evaluated for their ability to infect the floral tissues by the GUS assay. The result showed that almost no significant differences in GUS activity could be observed between the *A. tumefaciens* strains EHA105 and AGL1. This indicates that both EHA105 and AGL1 can infect the floral tissues of *D.* Sonia 'Earsakul'. Determination of two other factors, bacterial density and co-cultivation period, that could affect transient GUS expression, revealed that the bacterial densities between OD_{600} 0.5-2.0 and the co-cultivation time for 3 d are suitable for bacterial infection and GUS expression in the sepals and petals of *D.* Sonia 'Earsakul'. Our results demonstrate that transient gene expression in floral tissues of *D.* Sonia 'Earsakul' via agroinfiltration allows the rapid characterization of both flower-specific genes and constitutively expressed genes of interest, for example, anthocyanin biosynthetic genes.

Keywords: *Agrobacterium tumefaciens*, orchid, GUS, transient transformation

Introduction

Dendrobium hybrids are important economic cut-flower orchids in Thailand. Thailand exports cut-flower orchids worldwide valued at approximately 2,500 million Baht each year (data from Centre for Agricultural Information Office of Agricultural Economic, Ministry of Agriculture and Co-operatives, Thailand). *Dendrobium* hybrids account for about 80% of total orchid exports from Thailand (data from Department of Agriculture, Thailand). To increase the export value in the future, new varieties, especially new colors and

* Corresponding author: Ratanasut, K.; e-mail: kumropr@nu.ac.th

forms addressing customer demands, must be added to world markets. Breeding of *Dendrobium* hybrids by cross-pollination has limitations in production of new colors in the same flower form.

Molecular plant breeding is an advanced technique used to develop traits of interest by producing transgenic plants via genetic engineering. Biotechnology has potential for breeding new flower colors via metabolic engineering of the anthocyanin biosynthetic pathway. In stable plant transformation, either *Agrobacterium*-mediated transformation or by biolistics (particle bombardment) is combined with tissue culture to regenerate transgenic plants.

Orchid transformation has been extensively studied since 1990s (Teixeira da Silva *et al*., 2011). Regeneration of the transgenic orchid from the protocorm generally requires tissue culture and is a lengthy process. Color modification of transgenic orchids takes at least 3-4 years from the transformed protocorms to the flowering plants. If transgenic plants do not produce a desired trait as expected, for example, flower color, both time and money are lost. Transient transformation is an alternative to the generation of stably transformed plants and provides rapid and facile functional analysis of transgenes.

Agroinfiltration is a transient transformation method for transferring T-DNA into plant cells by *Agrobacterium tumefaciens.* Transient expression of a transgene can be analyzed directly in infiltrated tissues within a few days. The reporter gene *uidA* (*GUS*) driven by the CaMV35S promoter has been used extensively for indicating the success of plant transformation including orchids (Teixeire da Silva *et al*., 2011).

In this study, we report the achievement of a transient gene expression system via agroinfiltration in floral tissues of *Dendrobium* Sonia 'Earsakul'. The *CaMV35S::gusA* construct was used for transformation and analysis of GUS expression was performed by the GUS assay.

Materilas and methods

Plant material

Fully-opened flowers of *D.* Sonia 'Earsakul' were used in this study. Flowers were freshly collected from plants for agroinfiltration. The *D.* Sonia 'Earsakul' plants were obtained from commercial orchid farms in Nakornpathom province, Thailand.

Agrobacterium tumefaciens strains

A. tumefaciens strains EHA105 and AGL1, and the *Agrobacterium* EHA105 and AGL1 strains harboring the binary vector pCAMBIA1304, named

as EHA105-1304 and AGL1-1304, respectively, were used in this study. The pCAMBIA1304 binary vector carries the T-DNA region containing the *hygromycin phosphotransferase II* (*hptII*) gene and the reporter genes, *gusA* and *gfp* driven by the CaMV35S promoter (CSIRO, Australia) (Figure1).

Agrobacterium was grown in Luria broth (LB) liquid and on agar media supplemented with rifampicin (20 µg ml^{-1}). In addition, kanamycin (50 µg ml^{-1}) was added to EHA105 and AGL1 cultures for the selection of pCAMBIA1304.

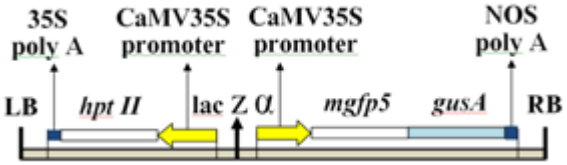

Fig. 1. Schematic representation of the pCAMBIA1304 T-DNA construct used for agroinfiltration of *D.* Sonia 'Earsakul'. The *hpt II* and reporter genes, *gusA* and *gfp*, are under the control of CaMV35S promoter

Agroinfiltration

A single colony of *Agrobacterium* strainsEHA105 or AGL1 was grown in 5 ml of LB broth medium supplemented with 20 µg ml^{-1} rifampicin, whereas a single colony of EHA105-1304 or AGL1-1304 was grown in 5 ml of LB broth medium supplemented with 20 µg ml^{-1} rifampicin and 50 µg ml^{-1} kanamycin. The *Agrobacterium* cells were cultured at 28°C and 250 rpm for 2 days. Then 1 ml of the *Agrobacterium* cultures was refreshed in 5 ml of new LB broth medium supplemented with the appropriate antibiotics as indicated above and incubated at 28°Covernight. The *Agrobacterium* cells were harvested by centrifugation at 5600 rpm for 3 min and resuspended in deionized water with 100 µM acetosyringone (3,5-Dimethoxy-4-hydroxyaceto-phenone) to a final OD_{600} of 0.5 according to the methods of Yasmin and Debener (2010).

The fully-opened flowers of *D.* Sonia 'Earsakul' were infected by pricking the center of the sepals and petals using a sterile syringe-needle, and infiltrated with 0.5 ml of *Agrobacterium* suspension using a 1-ml needleless syringe. The infiltrated flowers were incubated at 25°C in the dark in microtubes containing water and enclosed in a transparent, rectangular covered box. The samples were then assayed for GUS expression. Each experiment was repeated three times.

Histochemical GUS assay

The histochemical GUS assay was performed according to the procedures of Jefferson *et al*. (1987). The infiltrated petals and sepals were immersed in 10

ml of GUS staining solution containing 0.5 mg ml^{-1} X-gluc, 10 mM ml^{-1} EDTA, 100 mM ml^{-1} NA_2PO_4 (pH 7.0), 0.5 mg ml^{-1} $K_4(F_4(CN)_6)$, 0.5 mg ml^{-1} $K_3(Fe(CH)_6)$, 0.5% triton X-100, and 20% Methanol. The GUS staining samples were placed in a vacuum chamber at 37°Covernight. The staining solution was removed on the next day, and plant tissues were decolorized using 70% ethanol.

Results and discussions

We used an agroinfiltration method (modified from Schöb et al. 1997) in sepals and petals to establish a transient gene expression system in floral tissues of *D*. Sonia 'Earsakul'. Two preliminary experiments were conducted to clarify the effects of three factors: *Agrobacterium* strain, bacterial concentration, and co-cultivation time, on transient *GUS* expression in infiltrated sepals and petals.

Influence of the Agrobacterium strain

Two *Agrobacterium* strains EHA105 and AGL1 harboring the vector pCAMBIA1304 were evaluated for their ability to infect the sepals and petals of *D*. Sonia 'Earsakul'. No difference in GUS expression could be observed due to the use of either EHA105 or AGL1 in assays conducted under the same conditions (Figure 2C and 2D). On the other hand, Men *et al*. (2003) reported slight differences in GUS expression in protocorm-like bodies of *D. nobile* infected with *Agrobacterium* strains EHA105 and AGL1 carrying pCAMBIA1301. Our results indicated that both *Agrobacterium* strains were effective bacteria that could be used to infect the floral tissues of *D*. Sonia 'Earsakul' via agroinfiltration. *Agrobacterium* strain EHA105 was also effective in infecting the protocorm-like bodies of *Oncidium* orchid 'Sherry Baby cultivar OM8' (Liau *et al*., 2003). No GUS activity was detected in the sepals and petals infiltrated with the *Agrobacterium* EHA105 which did not carry pCAMBIA1304 (Figure 2B). This indicated that there was no endogenous GUS activity in the sepals and petals of *D*. Sonia 'Earsakul'. *Agrobacterium* strain EHA105-1304 was selected for further studies.

Effect of bacterial density

To determine the optimal concentration of bacteria for GUS expression, bacterial suspensions were adjusted to OD_{600} 0.2, 0.5, 1.0 and 2.0. GUS expression was observed at all tested bacterial concentrations. The GUS activity of bacterial density OD_{600} 0.2 was lower than other concentrations, and detected only at the injected area (Figure 3B). For bacterial densities between

OD_{600} 0.5-2.0, no significant differences of GUS activity could be detected (Figure 3C and 3E). Yasmin and Debener (2010) also reported no significant effect of the density of *Agrobacterium* suspensions over a broad range of OD_{600} 0.5-4.0 on GUS activity in the rose petals infiltrated with the *Agrobacterium* GV3101 harboring pBINPLUS::GUS-Intron, but with densities less than 0.5 GUS expression could not be detected. However, *Agrobacterium* suspensions OD_{600} 0.6-1.0 have been commonly used in *Agrobacterium*-mediated transformation of orchids, for example, *D. nobile* (Men *et al*., 2003), *D. phalaenopsis* (Cao et al. 2006), *Oncidium* (Liau et al. 2003) and *Phalaenopsis* (Mishiba *et al*., 2005).

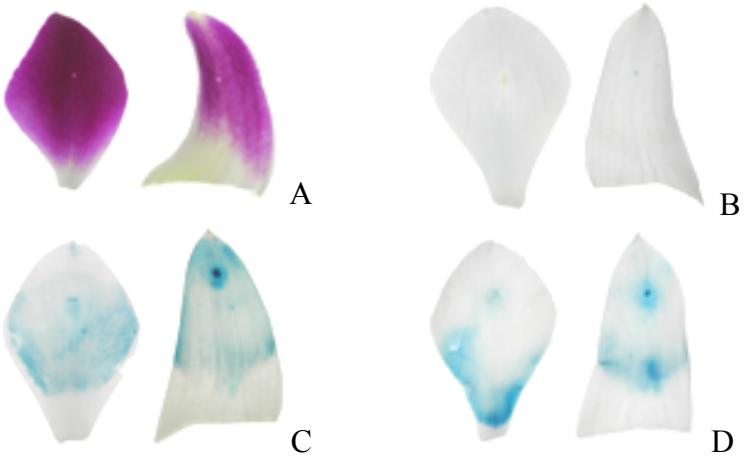

Fig. 2. Effect of *Agrobacterium* strain on the expression of GUS in the sepals and petals of *D*. Sonia 'Earsakul': the sepals (right) and petals (left) infiltrated with the *Agrobacterium* EHA105 before (A) and after (B) GUS assays, the sepals (right) and petals (left) infiltrated with *Agrobacterium*s trains EHA105-1304 (C) and AGL1-1304 (D).

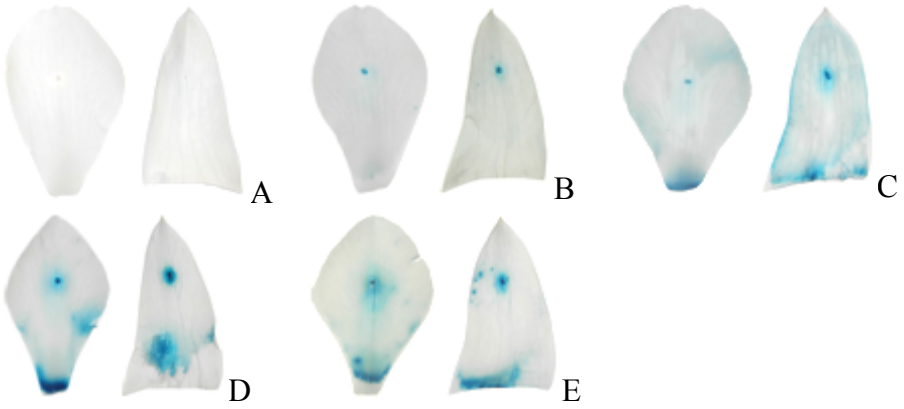

Fig. 3. Effect of bacterial density on the expression of GUS in the sepals and petals of *D*. Sonia 'Earsakul': the sepals (right) and petals (left) infiltrated with the *Agrobacterium* EHA105 with OD_{600} of 0.5(A), the sepals (right) and petals (left) infiltrated with the *Agrobacterium* EHA105-1304 suspensions OD_{600} of 0.2(B), 0.5(C), 1.0(D) and 2.0(E).

Effect of co-cultivation time

To determine the optimal co-cultivation period, the EHA105-1304-infiltrated flowers were co-cultivated at 25°C for 2, 3, 4 and 5 d. GUS expression was detectable with all tested conditions. The highest GUS activity was detected at day three, after which the activity decreased (Figure4). Our results agree with the co-cultivation time for *Agrobacterium*-mediated transformation that has commonly been used,3 d in orchids, for example, *D. phalaenopsis* (Cao et al. 2006), *Oncidium* (Liau et al. 2003) and *Phalaenopsis* (Mishiba et al. 2005). Previously, Belarmino and Mii (2000) reported that extending the co-cultivation period up to 5-7 d for *Phalaenopsis* transformation did not increase GUS transient activity but caused necrosis and death of cells.

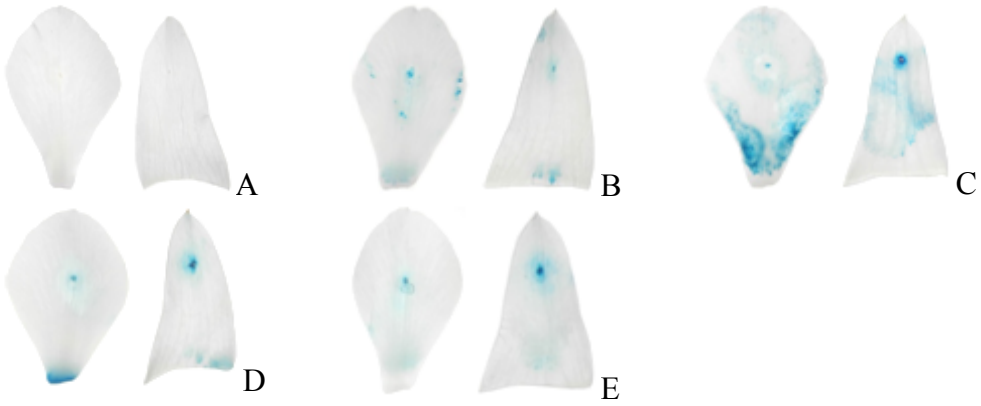

Fig. 4. Effect of co-cultivation time on the expression of GUS in the sepals and petals of *D.* Sonia 'Earsakul': the sepals (right) and petals (left) infiltrated with the *Agrobacterium* EHA105 and co-cultivated for 3 d(A). The sepals (right) and petals (left) infiltrated with the *Agrobacterium* EHA105-1304 and co-cultivated for 2(B), 3(C), 4(D) and 5(E) d.

Conclusion

We report here the development of agroinfiltration for transient gene expression in the sepals and petals of *D.* Sonia 'Earsakul' using *Agrobacterium* strains EHA105 and AGL1 harboring pCAMBIA1304. Our results indicate that both *Agrobacterium* EHA105 and AGL1 are effective in infecting the floral tissues of *D.* Sonia 'Earsakul' via agroinfiltration. Testing two other factors affecting transient GUS expression, bacterial concentration, and co-cultivation time, indicated that the bacterial densities between OD_{600} 0.5-2.0 and the co-cultivation time for 3 d are optimal for GUS expression. Transient gene expression in floral tissues of *D.* Sonia 'Earsakul' rapidly allows the

characterization of both flower-specific genes and constitutively expressed genes of interest, particularly genes involved in the anthocyanin production.

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