Agrobacterium-Mediated Transformation of Easter Lily (*Lilium longiflorum* 'Nellie White')

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Abstract

Conditions were optimized for transient transformation of *Lilium longiflorum* 'Nellie White' using *Agrobacterium tumefaciens*. Bulb scale and basal meristem explants were inoculated with *A. tumefaciens* strain AGL1 containing the binary vector pCAMBIA 2301 which has the *uidA* gene that codes for *β*-glucuronidase, GUS expression. Transformed bulb scales showed transient GUS expression when they had been precultured 11 days on Murashige and Skoog's (MS) medium supplemented with 2 mg/L dicamba. The outer, larger-sized bulb scales were not infected nearly as well as the inner, smaller bulb scales. Maximum GUS expression occurred when bulb scales had been obtained from plants that had been grown in the dark for at least 2 months rather than in the light indicating the importance of growing plants in the dark for *Agrobacterium*-mediated transient transformation of bulb scales. Basal meristems taken from plants grown 4 months in the dark showed 3 times as much GUS expression as basal meristems from plants grown in a 12 h light photoperiod. The frequency of transient GUS expression achieved in this study indicated that it should be possible to achieve stable transformation of 'Nellie White' which is the cultivar that dominates the US market of Easter lilies. Experiments for stable transformation are in progress.

INTRODUCTION

In the US, Easter lilies are an important holiday pot plant with a wholesale value of $ 27 million (National Agriculture Statistics Service-Floriculture Crop, 2011). *Agrobacterium tumefaciens* has been used by others to transform Oriental lilies (Hoshi et al., 2004; Ogaki et al., 2008; Krens et al., 2009; Núñez de Cáceres et al., 2011), *Lilium longiflorum* × *L. formosanum* (Li et al., 2008), and *Lilium* ×*formolongi* (Ogaki et al., 2008), but only two cultivars of *Lilium longiflorum*, 'Snow Queen' and 'Tiepao', have been transformed using *Agrobacterium* (Mercuri et al., 2003; Liu et al., 2011). Transformation is genotype-dependent, and this study was done to determine the optimum conditions for transient transformation of *L. longiflorum* 'Nellie White' using the *uidA* gene that codes for GUS expression. This report shows the importance of growing 'Nellie White' plants in the dark rather than light for transient transformation of bulb scales using *Agrobacterium*. Verification of transient transformation is important before spending the many months needed to achieve stable transformation.

MATERIALS AND METHODS

Plant Materials

Lilium longiflorum 'Nellie White' plants grown in vitro on Murashige and Skoog's medium (MS-Murashige and Skoog, 1962) served as the source of bulb scale and basal meristem explants. Plants were grown under a 12 h light photoperiod at 25°C. Explants were precultured on MS medium containing 2 mg/L dicamba for 11 days in the dark at 25°C.

Transformation

A. tumefaciens strain AGL1 containing pCAMBIA2301 was cultured for 16 h at 25°C in liquid LB medium supplemented with 50 mg/L kanamycin. After the bacteria were centrifuged at 8,000 rpm at 4°C for 5 min, their OD_{600} was adjusted to 0.6 using inoculation medium (MS medium containing 30 g/L sucrose, 2 mg/L dicamba, and 100 µM acetosyringone, pH 5.8). Bulb scales that had been precultured for 11 days on MS medium containing 2 mg/L dicamba were cut in half immediately prior to inoculation with *A. tumefaciens* for 20-25 min. Basal meristems were not cut in half. Inoculated explants were then blotted on filter paper to remove excess *Agrobacterium* and cultured on co-cultivation medium (MS medium with 1.9 g/L 2-(N-morpholino)ethanesulfonic acid buffer, 3% sucrose, 2 mg/L dicamba, and 100 µM acetosyringone) for 2-3 d in the dark at 25°C. Explants were then rinsed 3X in sterile water, blotted on filter paper, and then cultured on co-cultivation medium containing 500 mg/L cefotaxime. After one day on co-cultivation medium in the dark, explants were weighed. GUS expression was then determined by incubating the explants for 16 h at 37°C in staining solution with substrate before counting the number of blue spots that indicate transient GUS expression (Jefferson et al., 1987).

Treatments

The length of time in darkness needed for lily plants that served as a source of bulb scales was determined. Lily plants were cultured in the dark at 25°C on MS medium for 15-110 days, and bulb scales from these plants were used for *Agrobacterium* infection.

A comparison was made between bulb scales precultured on MS medium with either 2 mg/L dicamba or 1 mg/L picloram. Bulb scales were precultured for either 16 h, 9, 15, or 30 days in the dark before being used for *Agrobacterium* infection.

Bulb scales were precultured in the dark or light on MS medium with 2 mg/L dicamba for either 16 h, 9, 11, or 15 days before they were inoculated with *A. tumefaciens*.

GUS expression was compared for the larger bulb scales (1.2-1.7 mm) or smaller bulb scales (0.7-1.1 mm) taken from either the inner part of the bulb or from relatively smaller bulbs. Bulb scales were precultured on MS medium with 2 mg/L dicamba prior to inoculation with *A. tumefaciens*. Plants that were used as the source of bulb scales were grown on MS medium in the dark for 4 months or under a 12 h light photoperiod.

Basal meristems (approximately 2 mm in height) were taken from lily plants that had been grown on MS medium for 4 months in the dark or under a 12 h light photoperiod. The meristems were precultured 11 days in the dark on MS medium with 2 mg/L dicamba before Agro infection.

Statistics

All experiments were replicated twice using three plates of bulb scales or basal meristems for each replication. Ten bulb scales or meristems were cultured on each petri plate. The number of GUS positive blue spots were counted for each plate, and the bulb scales or bulb meristems were weighed to determine the number of blue spots/g fresh weight (FW). An analysis of variance ($P \leq 0.001$) followed by Dunn's multiple comparison with a 95% confidence interval ($P \leq 0.05$) was carried out using SIGMASTAT (SPSS, Chicago, IL, US) for comparing the means of the treatments.

RESULTS AND DISCUSSION

'Nellie White' plants required a long period of dark incubation (2 months) before there was significant GUS expression from their bulb scales (Fig. 1). In comparison, Li et al. (2008) achieved stable transformation of *L. longiflorum* × *L. formosanum* using bulb scale explants taken from plants that had been grown in a 16 h light photoperiod.

Bulb scale explants showed twice as many GUS positive blue spots when the bulb scales had been precultured in the dark on MS medium with 2 mg/L dicamba rather than 1 mg/L picloram (Fig. 2). Bulb scales precultured on MS medium with 2 mg/L dicamba

for 9-15 days in the dark showed GUS expression, but a 16 h preculture period did not result in any blue spots. It was also important that the 11 day preculture be done in the dark rather than under a 12 h light photoperiod (data not shown).

Smaller bulb scales from the inner region of the bulb or from a small bulb showed significantly more blue spots than the larger bulb scales (Fig. 3). The larger bulb scales had to be sliced lengthwise into 2-3 pieces for GUS positive blue spots to occur indicating that wounding was necessary for Agro infection. The smaller bulb scales were also sliced into 2 pieces if the size was feasible. Smaller bulb scales are also thinner than the larger bulb scales, and this appeared to help infection by *A. tumefaciens*. Smaller bulb scales showed 6X as many blue spots when the bulb scales had been taken from plants grown 4 months in the dark as compared to plants grown under a 12 h light photoperiod (Fig. 3).

GUS expression occurred following Agro infection of 'Nellie White' basal meristems, and there were large regions of GUS expression following histochemical staining (Fig. 4). There were 5X as many blue spots when the basal meristems had been taken from plants cultured 4 months in the dark as compared to plants grown under a 12 h light photoperiod. Two groups have used basal meristems of *L. longiforum* 'Tiepao' and the Oriental hybrid 'Stargazer' for stable transformation (Liu et al., 2011; Núñez de Cáceres et al., 2011). 'Tiepao' plants were grown under a 16 h light photoperiod, and the basal stems from 'Stargazer' were taken from adventitious bulblets cultured in the dark 30-40 days on MS medium with 2 mg/L picloram and 1 mg/L BAP to induce shoot formation, and basal meristems were collected from the shoots. This 30-40 days of darkness is similar to that required by 'Nellie White' although there was no GUS expression when 'Nellie White' basal meristems were cultured in the dark on picloram with either kinetin or BAP. It appears that cytokinins inhibit GUS expression in 'Nellie White' basal meristems (Table 1).

CONCLUSIONS

The important factors that affected transient GUS expression were: 1) preculture of the lily plants in the dark when using bulb scales and basal stem explants, 2) preculture of both bulb scales and basal meristem explants in the dark on MS medium with 2 mg/L dicamba , and 3) using the inner, smaller bulb scales rather than the outer, larger ones.

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Literature Cited

Hoshi, Y., Kondo, M., Mori, S., Adachi, Y., Nakano, M. and Kobayashi, H. 2004. Production of transgenic lily plants by *Agrobacterium*-mediated transformation. Plant Cell Rep. 22:359-364.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS-fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Krens, F.A., Menzel, T.R., Liu, C., Dees, D.C.T. and van Kronenburg, B.C.E. 2009. Oriental lily hybrids engineered to resist aphid attack. Acta Hort. 836:253-257.

Li, Q.-H., Hong, B., Tong, Z., Ma, C., Guan, A.-N., G., Yu, J.-J. and Gao, J.-P. 2008. Establishment of regeneration system and transformation of *Zm401* gene in *Lilium longiflorum* × *L. formosanum*. China Agricult. Univ. 5:113-119.

Liu, J., Zhang, J., Xu, B., Jia, C., Zhang, J., Tan, G. and Jin, Z. 2011. Regeneration and production of transgenic *Lilium longiflorum* via *Agrobacterium tumefaciens*. In Vitro Cell. Dev. Biol.-Plant 47:348-356.

Mercuri, A., Benedetti, L.D., Bruna, S., Bregliano, R., Bianchini, C., Foglia, G. and Schiva, T. 2003. *Agrobacterium*-mediated transformation with *rol* genes of *Lilium longiflorum* thunb. Acta Hort. 612:129-136.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid assays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
National Agriculture Statistics Service-Floriculture Crop. 2011. http://usda.mannlib.cornell.edu/MannUsd.
Núñez de Cáceres, F.F., Davey, M.R. and Wilson, Z.A. 2011. A rapid and efficient *Agrobacterium*-mediated transformation protocol for *Lilium*. Acta Hort. 900:161-117.
Ogaki, M., Furuichi, Y., Kuroda, K., Chin, D.P., Ogawa, Y. and Mii, M. 2008. Importance of co-cultivation medium pH for successful *Agrobacterium*-mediated transformation of *Lilium* × *formolongi*. Plant Cell Rep. 27:699-705.

Tables

Table 1. Transient GUS expression when basal meristem explants were precultured 11 days on MS medium in the dark that contained picloram and either BAP or kinetin.

Hormone (mg/L)	No blue spots/g tissue ±SE
1 Picloram	14±4.3 a
1 Picloram/1 BAP	0.2±0.2 b
1 Picloram/1 kinetin	0±0 b
2 Picloram/2 BAP	0±0 b
2 Picloram/1 kinetin	0±0 b

Means with different letters are significantly different at $P \leq 0.05$ according to Dunn's multiple comparison.

Figures

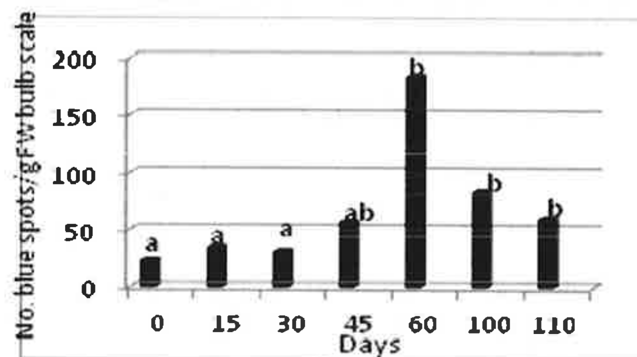

Fig. 1. Transient GUS expression (no. blue spots/g FW bulb scale explant) when lily plants were precultured on MS medium for the indicated number of days in the dark. Bars with different letters are significantly different at $P \leq 0.05$ according to Dunn's mutiple comparison.

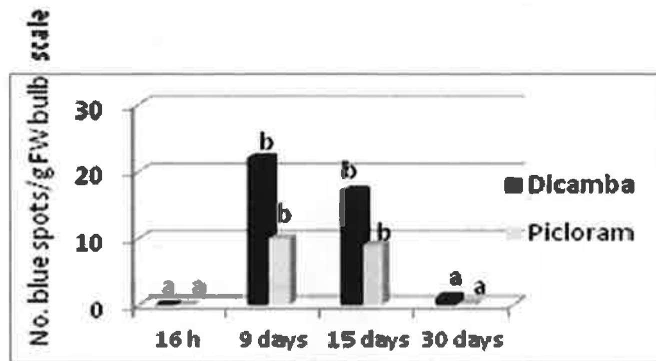

Fig. 2. Transient GUS expression (no. of blue spots/g FW) for bulb scales precultured on MS medium with either 2 mg/L dicamba or 1 mg/L picloram for various lengths of time. Bars with different letters are significantly different at $P \leq 0.05$ according to Dunn's multiple comparison.

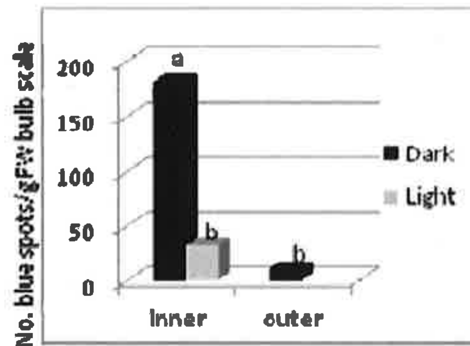

Fig. 3. Transient transformation (no. GUS positive blue spots/g FW) for larger, outer and smaller, inner bulb scales precultured 11 days on MS medium with 2 mg/L dicamba. Bars with different letters are significantly different at $P \leq 0.05$ according to Dunn's multiple comparison.

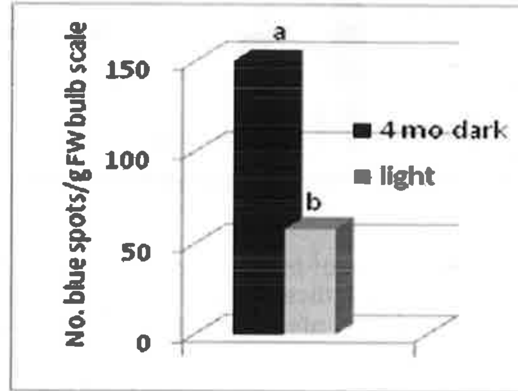

Fig. 4. Transient GUS expression for basal meristems collected from lily plants grozn on MS medium for 4 months in the dark under a 12 h photoperiod. Basal meristems were then precultured 11 days in the dark on MS medium with 2 mg/L dicamba prior to infection with *A. tumefaciens*. Bars with different letters are significantly different at P≤0.05 according to Dunn's multiple comparison.