DEVELOPING AN *AGROBACTERIUM*-MEDIATED TRANSFORMATION SYSTEM FOR *LILIUM* X *FORMOLONGO* USING THIN CELL LAYER OF BULB SCALES

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ABSTRACT

An Agrobacterium-mediated transformation system for *Lilium x formolongo*, a new lily variety with white flowers and high commercial value, has been developed. In vitro bulb scales were sliced into thin layers of 2 mm thick and were cultured on MS medium + 1mg/l IBA + 6% sucrose for about 6 weeks in the dark at 25^{0}C to induce callus or bulblets. These explants were then inoculated and co-cultivated for 7 days with a cell suspension of *A. tumefaciens* strain EHA105 harboring the plasmid pIITB02 containing the nptII gene for kanamycin resistance driven by a nos promoter and *GUS* gene coding for ß-glucuronidase and the bar gene, coding for phosphinothricin acetyltransferaza. Cefotaxime at 500 mg/l and PPT at 1.5 mg/l showed to be the most optimal selection concentrations. Putative transformants which regenerated on selection media were subject to a GUS assay. GUS assay analysis showed 4% of the explants expressing GUS. The transformation system discussed in the present study allows the introduction of useful genes in the *Lilium x formolongo* genome.

Key words: *Agrobacterium*, *Lilium x formolongo*, GUS assay, transformation, bulb scales, thin cell layers

1. INTRODUCTION

The lily is one of the most important crops in the world flower bulb industry. In Viet nam, lilies are the most important ornamental crop. The return from lily cut flowers are 10 to 15 times higher than from chrysanthemum, rose, carnation, cala lily (just after orchids). However, lily production and commerce in Vietnam has not been developed despite its great potential. There is almost no research programe on lily breeding that have been conducted in the country. In the world, a wide range of researche has been succsessfully conducted on lily breeding, using both traditional and modern techniques. Crosses between species of different sections of *Lilium* are often hampered by crossing barriers including pollen tube inhibition in the style; or if embryos have been formed, the production of hybrids is often hindered by embryo abortion. Various methods to overcome gametophytic self incompatibility and embryo abortion using pre-fertilization (cut style, grafted style) have been done by Asano *et al.* (1977, 1980), Stewart (1981), Kanoh *et al.* (1988), Chi (2002), Van Tuyl *et al*. (1990). Besides, researche on gene transformation have been carried by Van Creij *et al.* (1999). Watad *et al.* (1998); Suzuki and Nakano (2002); Mercuri (2003); Ahn (2004); Hoshi *et al*. (2004); In - Har Park and Hee-Sung Park (2002). While most of the reported studies are on gene transformation using callus as explants for transformation

experiments, we investigated the potential of using thin cell layers of bulb scales for production of trangenic bulblets in *Lilium* x *formolongo*, a new lily variety with a white flower and high commercial value (Nguyen Quang Thach *et al*., 2005), via *Agrobacterium*. The established system will be used for further research on gene transformation in lily for desired traits.

2. MATERIALS AND METHODS

Plant materials and Agrobacterium Strain

In vitro bulb scales were sliced into thin layers of 2 mm thick and were cultured on the medium MS+ 1ppm IBA+ 6% sucrose for about 6 weeks in the dark at 25 ^{0}C to induce callus or protocorm like bodies.

A. tumefaciens strain EHA105 harboring the plasmid plITB2c, kindly provided by Prof. Dr. Nguyen Van Uyen from the Institute of Tropical Biology, Vietnam was used in the present study. The plasmid plITB2c contained the neomycin phosphotransferase II (NPTII), coding for kanamycin resistance, a NOS promoter, a GUS gene coding for ß-glucuronidase and a bar gene, coding for *phosphinothricin acetyltransferaza* (Figure 1). This *Agrobacterium* strain was inoculated into liquid YEB medium containing 20 mg/l acetosyringone, 50 mg l^{-1} Kanamycin at 28^{0}C for 48 hours with reciprocal shaking at 200 rpm.

Agrobacterium cultures were pelleted at 5,000 rpm for 10 min, and resuspended in liquid co-cultivation medium which consisted of MS medium plus with 1mg/l IBA + 20mg/l acetosyringone, 6% sucrose, pH 5.8 to OD600 of 0.3 for use in the inoculations. Thin layers of bulb scale showing embryogenic calluses or bulblets were immersed into the bacterial suspension for 30 min. They were then co-cultivated with *Agrobacterium* at 28 ^{0}C for 7 days in the dark on the co-cultivation medium described above. Then explants were washed twice with MS hormone-free liquid medium containing 500 mg/l cefotaxime, and plated on a filter paper to dry up and then put on the solidified MS selection medium containing 1mg/l IBA, 500mg/ l cefotaxime, 6% sucrose. Explants were subcultured weekly onto MS selection medium in the dark at 25±1◦C until bulblets or shoots were formed.

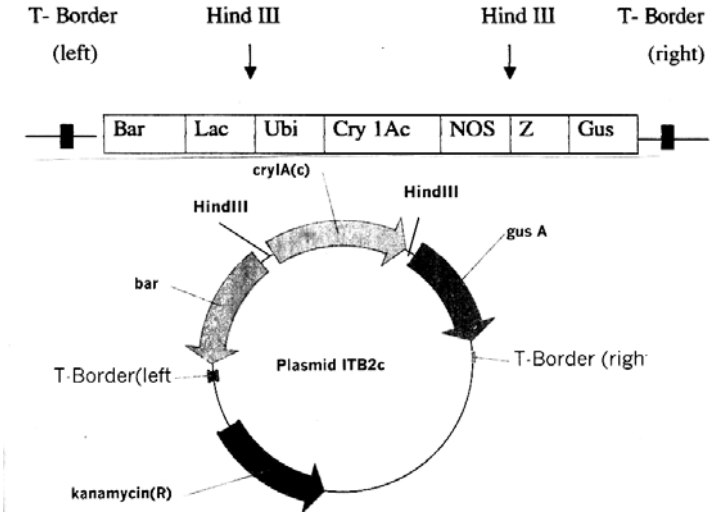

Fig. 1. The contruct of Plasmid plIBTB2c

GUS histochemical assay

Histochemical localization of *GUS* gene expression was investigated in co-cultivated calluses of the control and putatively transformed materials. The calluses developed on selection media were incubated overnight at 37^0C in X-gluc solution made up of 10 mM EDTA, 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% (w/v) X-gluc. Chlorophyll was removed by treating the tissue in ethanol 70 %. A blue colour was observed in transformed calluses in comparison to a white colour in untransformed control material.

3. RESULTS AND DISCUSSION

Production of optimal plant material for transformation

An efficient regeneration system is a prerequisite for successful application of the gene transformation techniques for any plant including lily. We examined the regeneration capacity of different explant sources (receptacle, bulb scale and *in vitro* leaf) on MS medium supplemented with 1ppm IBA and 6% sucrose (Table 1). Bulb scales appeared to be the most optimal material for callus and bulblet formation. The percentage of callus and bulblet induction from bulb scale explants was 41.7% and 38.2%, respectively.

Table 1. Effect of explant sources on their morphogenesis (after 6 weeks of culture).

Explant sources	% forming Callus	% forming bulblets
Bulb scale	41,7	35,2
Receptacle	20,0	13,3
in vitro leaf	27,8	14,8

Effect of the cefotaxime concentration on the growth of thin layers of bulb scale

Bulb scales were sliced into 2 mm thick layers and cultured on MS basic medium + 1ppm IBA + 6% sucrose (pH 5.7) and supplemented with either 0; 100; 500; 700 or 900 mg/l cefotaxime to examine for the effective concentration for eliminating the bacterium after inoculation of the explants.

Table 2. Effect of the cefotaxime concentration on the growth of thin layers of bulb scale.

Cefotaxime (ppm)	% brown	% callus induction	% bulblet formation
0	37,0	63,0	81,5
100	63,0	51,9	81,5
500	63,0	48,1	63,0
700	100	44,4	63,0
900	100	40,7	59,3
LSD 5%	1,7		
CV%	1,2		

Cefotaxime caused a negative effect on the growth and morphogenesis capacity of the explants. After 4 weeks of culture on the medium supplemented with cefotaxime, the explant became brown and then died. The increase of cefotaxime concentration from 100 to 900 mg/l resulted in the decrease of survival rate from 63 % to 0 % and the percentage of explant inducing callus and bulblets decreased from 63,0% to 40,7%, and from 81,5 to 59,3%, respectively. At cefotaxime concentration of 700 mg/l and above, all explants died. Thus, a concentration of 500 mg/l cefotaxime later was used to eliminate the bacterial cells.

Effect of the Phosphinothricin (PPT) concentration on the growth of thin layers of bulb scale

To identify the concentration of PPT used to select the putative transformants, PPT was added into basal medium at concentrations of 0; 0.5; 1.0; 1.5 or 2.0 mg/l. As shown in Table 3, 0.5 mg/l or more PPT was sufficient to inhibit the growth of non-co-cultivated thin bulb scale layers. The PPT concentration that completely inhibited the growth of thin bulb scale layers was 1.5 mg/l or more and PPT concentration of 1.5 mg/l was used for selecting putative transformants of *Lilium* x *formolongo* in the subsequent transformation experiments.

Table 3. Effect of the PPT concentration on the growth of thin layers of bulb scale.

PPT (mg/l)	Percentage of dead explants (%)			Percentage of regeneration (%)		
	After 2 weeks	After 4 weeks	After 6 weeks	After 2 weeks	After 4 weeks	After 6 weeks
0	0	7.9	11.1	5.8	19.1	22.2
0.5	11.1	30.2	34.9	0	17.5	11.1
1.0	12.7	30.2	55.5	0	11.1	9.5
1.5	22.2	35.0	100	0	6.3	0
2.0	25.4	35.0	100	0	6.3	0
LSD 5%			2.7			
CV%			2.5			

Selection and regeneration of transformed plants of *Lilium* x *formolongo*

Following co-cultivation on the selective medium supplemented with 1.5 mg/l PPT, we obtained 48 out of 400 explants. Among these 48, only 38 explants showed bulblet and shoot regeneration. These might be the PPT resistant materials after transformation. Twenty seven samples from 38 regenerated explants were subjected to GUS histochemical assay. No endogenous the *GUS* activity was detected in the control. Only one sample (4 %) showed *GUS* gene expression, implying a successful transformation of *Lilium* x *formolongo* by *Agrobacterium* using thin cell layers of bulb scales.

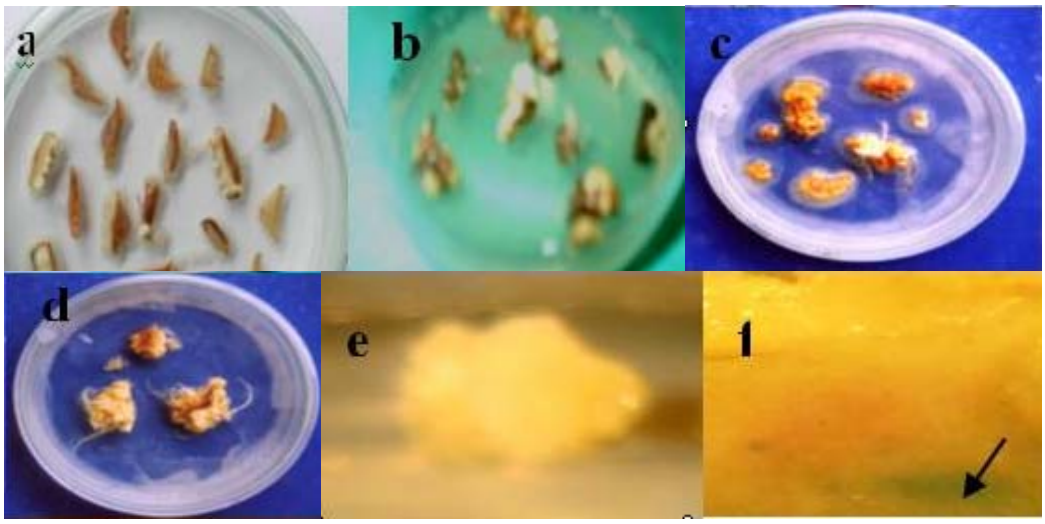

Fig. 2. Results of transformation via *Agrobacterium* using thin cell layers of bulb scales of *Lilium* x *formolongo*. a, b- thin cell layers of bulb scales cultured on MS + 1 mg/l IBA + 6% sucrose after 6 weeks. c- callus co-cultivated with *Agrobacterium* after 7 days; d- surviving calluses on selection medium supplemented with 1.5 mg/l PPT; e,f- GUS histochemical assay of transgenic calluses (arrowhead) (f) and the control (e).

It is obvious that transformation for lilies is still difficult with very low transformation efficiency. Efficient production of transgenic plants via *Agrobacterium*-mediated transformation in the Liliaceous ornamentals was affected by several factors such as, target material for *Agrobacterium* inoculation, kind of *Agrobacterium* strain, duration of the co-cultivation period, and surfactant (Tween 20) and acetosyringone treatment during co-cultivation (Suzuki and Nakano, 2002). Our results confirmed that the PPT concentration of 1.5 mg/l and cefotaxime of 500 mg/l were effective to select the putative transformants. In the report of Ahn *et al.* (2004), PPT at a concentration of 2 mg/l successfully selected the transgenic plants in *Lilium longiflorum.* Suzuki and Nakano (2002) also used a medium containing 500 mg/l cefotaxime for selecting transformed tissues in *Lilium formosanum*.

Since several callus lines, which were resistant to cefotaxime and PPT but did not express the *gus* gene, were obtained, further experiments should be carried out to clarify the correlation between the transgene copy number and the activity of transgene expression in the transformed materials. In the future, the methodology for lily transformation from other cultivars and different *Agrobacterium tumefaciens* strains should be examined. Hereafter, several valuable genes for herbicide tolerance, resistance to diseases, insects or viruses, or alteration of flower color or plant form should be introduced by using the established systems into *Lilium* x *formolongo* for its genetic improvement.

4. CONCLUSIONS

Thin cell layers of bulb scales 2 mm thick of *Lilium* x *formolongo* showed to be the optimal materials for transformation with *Agrobacterium tumefaciens*.

PPT at 1.5 mg/l and cefotaxime at 500 mg/l were found to be an optimal selection concentration.

Lilium x *formolongo* can be transformed by *Agrobacterium for* desired traits.

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