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Optimization in *Agrobacterium*-mediated transformation of *Anthurium andraeanum* using GFP as a reporter

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Abstract Although *Agrobacterium*-mediated transformation protocols for many economically important plant species have been well established, protocol for a number of flowering plants including *Anthurium andraeanum* remains challenging. In this study, we report success in generating transgenic *Anthurium andraeanum* cv Arizona using *Agrobacterium* GV3101 strain harboring a binary vector carrying *gfp* as a reporter gene. The possibility of facilitating the screening process for transgenic plants expressing functional proteins using *gfp* marker was explored. In order to realize high transformation efficiency, different explant sources including undifferentiated callus pieces and petioles were compared for their regeneration efficiency and susceptibility to *Agrobacterium*-mediated transformation. We also optimized the concentration of AS added to co-cultivation media. Genomic PCR revealed that 11 of the 22 resistant plantlets regenerated on selective medium were successfully transformed. Green fluorescence was observed using a fluorescence microscope in 7 of the 11 PCR-positive plants, indicating GFP was expressed stably in the transformed *Anthurium andraeanum*. The highest transformation efficiency obtained in this study was 1.71% (percentage of explants with transgenic shoots in total explants) when callus explants were used as starting material and 125 µmol l^{-1} AS was added during the co-cultivation process.

Keywords: cocultivation, gene transfer, green fluorescent protein, regeneration

INTRODUCTION

Anthurium, a member of the family *Araceae* (monocotyledon), is found in tropical America and is highly prized as an ornamental plant with its beautiful flowers and exotic foliage (Dufour and Guerin, 2003). It is economically desirable to breed new cultivars of *Anthurium andraeanum* and the most common breeding method is sexual hybridization. Genetic engineering can be used to introduce heterogenous genes that are not present in the gene pool of host plant, thus allowing novel phenotypes to be

generated. Among the numerous methods for plant transformation, the *Agrobacterium*-mediated method is preferable because it generates a high proportion of independent transformants with single or low transgene copy numbers, which is usually favourable for expression of foreign genes (Dai et al. 2001; Shou et al. 2004).

In the past decades, tissue and organ culture systems for *Anthurium andraeanum* have been successfully established and plantlets were regenerated from both calli and somatic embryos. These methods facilitated the establishment of genetic transformation system of *Anthurium andraeanum* (Kuehnle et al. 1992; Vargas et al. 2004; Beyramizade et al. 2008; Yu et al. 2009). Success in *Anthurium* transformation was firstly reported in 1996 (Chen and Kuehnle, 1996), in which study the etiolated internode segments of *Anthurium* were co-cultured with *Agrobacterium tumefaciens* LBA4404 carrying chimeric genes. Root explants from several *Anthurium* cultivars were used for transformation research and their transformation efficiency was evaluated (Chen et al. 1997). Since then, *Agrobacterium*-mediated genetic transformation of *Anthurium* has been reported by several groups (Fitch et al. 2005; Fitch et al. 2006; Khaithong et al. 2006; Khaithong et al. 2007). However, literatures on *Anthurium* transformation are limited, and the transformation efficiency was fairly low. Therefore, it is desirable to optimize a transformation protocol with higher efficiency and easier selection method for *Anthurium* based on previously published methods.

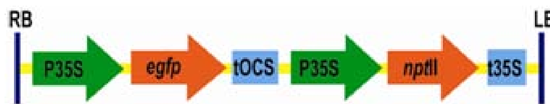

Fig. 1 Schematic diagram of a part of the T-DNA region of pJCGLOX,0. RB, right border; P35S, 35S promoter of cauliflower mosaic virus; e*gfp*, enhanced green fluorescent protein reporter gene; tOCS, octopine synthase terminator; *npt*II, gene for neomycin phosphotransferase; t35S, terminator of cauliflower mosaic virus; LB, left border.

Green fluorescent protein (*gfp*) gene from the jellyfish (*Aequiorea victoria*), as a reporter gene, has been successfully applied to visual selection of transformed plants (Stewart, 2005). Transformation efficiency of a specific plant can be evaluated conveniently with the *gfp* gene as a result of a simple assay using a fluorescence microscope. We therefore utilized a binary vector pJCGLOX,0 (Joubès, 2004) that contains a modified *gfp* gene (e*gfp*) as a reporter and neomycin phosphotransferase II (*npt*II) as a selectable marker to develop a transformation protocol for *Anthurium andraeanum*. The effects of explant sources and AS concentrations on transgenic *Anthurium andraeanum* yield are also discussed.

MATERIALS AND METHODS

Plant materials and explants

The explant materials for axenic tissue culture were surface-sterilized petioles previously described (Yao et al. 2006). Callus clumps with adventitious shoots were maintained in 1/2 MS media containing 1.0 mg l^{-1} 6-BA and 0.1 mg l^{-1} 2,4-D under

greenhouse conditions (natural photoperiod extended to 16 hrs as required by giving a photon flux density of 5000 lux, temperature of 25ºC). Minced calli and petioles cut from these shoots were used for the following transformation procedure.

Optimization of regeneration media and kanamycin sensitivity of explants

To establish an efficient regeneration system, 1/2 MS media with different 6-BA concentrations (0.3, 0.5, 0.7, 1.0, 1.3, 1.5, 2.0 mg l^{-1}) were used to test its efficacy in inducing shoots while the concentration of 2,4-D was kept constant at 0.1 mg l^{-1}. The callus was cut into pieces (0.5 cm^3) with all the adventitious shoots on the surface removed. About 35-40 minced callus explants were included in each treatment which was triplicated. Two months later, the morphogenetic characteristics of these explants were recorded and the numbers of adventitious shoots were counted.

To test the effects of kanamycin on *Anthurium andraeanum*, minced calli and petioles (32-65) were cultured in callus inducing medium (CIM) (Table 1) supplemented with kanamycin at different concentrations (0, 50, 75,100, 125, 150 mg l^{-1}). Each treatment was repeated 3 times. After 4 weeks, the explants were transferred onto the same medium. After another four weeks, new calli and adventitious shoots were counted and recovery rate (proportion of explants callusing) was determined.

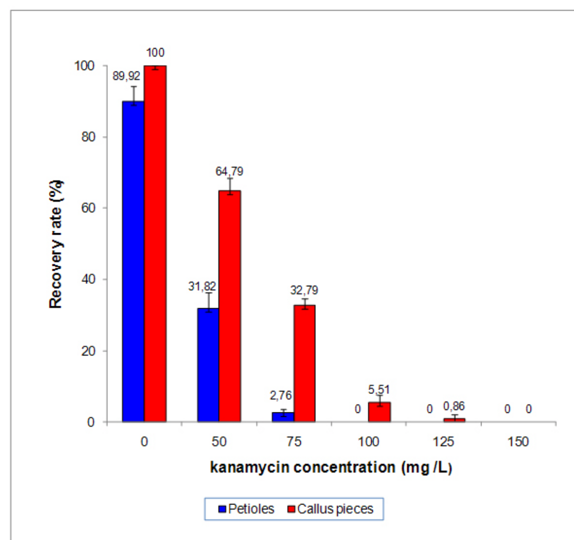

Fig. 2 Callus induction rate decrease with increasing concentrations of kanamycin. Error bars represent one standard error (n = 3).

***Agrobacterium* strains and plasmid**

Agrobacterium tumefaciens strain GV3101 (pMP90RK) was used as the T-DNA donor (Koncz and Schell, 1986). Both *gfp* and *npt*II genes were driven by CaMV 35S promoters in plasmids pJCGLOX,0 (Figure 1) (Joubès, 2004).

Transformation and regeneration

The *Agrobacterium* cells carrying the binary vector pJCGLOX,0 were grown in YEP liquid medium supplemented with 25 mg ml^{-1} chloromycin and 50 mg ml^{-1} gentamicin in a rotating (200 rpm) incubator at 28ºC for about 24 hrs. When the OD_{600} value reached 0.6-0.8, the cultures were centrifuged for 10 min at 4000 g and the bacterial pellet was re-suspended in liquid CCM (Table 1). Explants from callus pieces (40-63) and petioles (52-101) were then submerged in the inoculum for 30 min, then transferred to solid CCM containing acetosyringone at different concentrations (0, 50, 75, 100, 125 μmol l^{-1}). Each treatment was repeated 3 times. After incubation for 3 days in the dark, the explants were washed twice in sterile ddH_2O with 500 mg ml^{-1} cefotaxime and placed on RM (Table 1) for 5 days in light. The explants were transferred to CIM-CK (Table 1) containing 400 mg l^{-1} and 125 or 100 mg l^{-1} kanamycin for calli and petioles, respectively. The explants were transferred to fresh CIM-CK every 3 weeks.

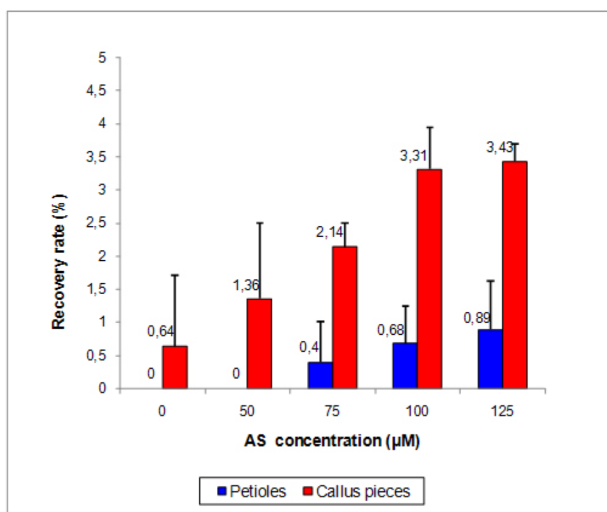

Fig. 3 Recovery rate of both explant sources rise with increasing AS concentration. Error bars represent standard error (n = 3).

Six months later, some rounded, green-yellow and newly formed calli appeared from the wounded callus pieces and petioles while other explants became yellow, dark and gradually died. The number of putative transformants was recorded for each treatment according to their explant source and AS concentration applied. Recovery rate (proportion of explants callusing on selective medium) was calculated accordingly. Resistant calli were transferred to shoot inducing medium (SIM) for shoot induction and regenerated shoots were rooted in root inducing medium (RIM) (Table 1).

Data regarding differentiation, recovery rate and transformation efficiency were analyzed with ANOVA; mean comparisons were made using Duncan's test ($P < 0.05$).

DNA isolation and genomic PCR analysis

Genomic DNA was isolated from putative transformants and wild type plants using the CTAB method (Chaudhry et al. 1999). The DNA was tested by *gfp* primer pairs: 5'-ACGTAAACGGCCACAAGTTC-3' and 5'-TAGCTCAGGTAGTGGTTGTCG-3'. PCR products were electrophoresed in agarose gel, stained with ethidium bromide and visualized under ultraviolet light (Sambrook and Russell, 2001).

Visualization of GFP expression

The expression of GFP was observed in callus tissue and regenerated plants under a fluorescent microscope (Olympus BX51, Japan) equipped with a fluorescence module consisting of a 100-W mercury lamp and GFP Plus excitation and emission filters. This system (excitation filter 480/40 nm; dichroic mirror 505 nm LP; barrier filter 510 nm LP) permits visualization of GFP following excitation by blue light.

RESULTS AND DISCUSSION

Media for callus induction and shoot regeneration

That callus or **s**omatic embryo can be induced *in vitro* on explants by incorporation of plant hormones into a growth medium has been well established (Kuehnle et al. 1992; Vargas et al. 2004). For many plants, regeneration of transgenic shoots from callus is the bottleneck for genetic engineering. This is especially true when the callus originated from a few transformed cells with different regeneration requirements on selective media (Miguel and Oliveira, 1999). Cytokinin plays an important role in this response, therefore it was necessary to test the function of 6-BA in *Anthurium* regeneration.

Table 1. Media composition.

Medium	Composition
CIM (callus inducing medium)	1/2MS + 6-BA 0.5mg l^{-1} + 2,4-D 0.1 mg l^{-1} + sucrose 3% + agar 0.7%
CCM (co-cultivation medium)	1/2MS + 6-BA 0.5 mg l^{-1} + 2,4-D 0.1 mg l^{-1} + sucrose 3% + agar 0.7% + AS (0-125) μmol l^{-1}
RM (recovery medium)	1/2MS + 6-BA 0.5 mg l^{-1} + 2,4-D 0.1 mg l^{-1} + sucrose 3% + agar 0.7% + Cef 400 mg l^{-1}
CIM-CK	1/2MS + 6-BA 0.5 mg l^{-1} + 2,4-D 0.1 mg l^{-1} + sucrose 3% + agar 0.7% + Cef 400 mg l^{-1} + kan (100/125) mg l^{-1}
SIM (shoot inducing medium)	1/2MS + 6-BA 1.0 mg l^{-1} + 2,4-D 0.1 mg l^{-1}+ sucrose 3% + agar 0.7% + Cef 400 mg l^{-1} + kan (100/125) mg l^{-1}
RIM (root inducing medium)	1/2MS + 2,4-D 0.5 mg l^{-1} + sucrose 3% + agar 0.7% + Cef 400 mg l^{-1}

Based on our previous study, we maintained 2,4-D at 0.1 mg l^{-1} and changed the ratio of cytokinin to auxin by adjusting the concentration of 6-BA. Calli can be observed about 4 weeks after initiation of the cultures, however there were great differences in the morphogenetic response among the treatments (Table 2). Soft, quickly growing, pale or green yellow calli appeared in media with a lower 6-BA concentration (0.3 and 0.5 mg l^{-1}). By the end of 8 weeks, these calli were larger than others and this could have result from quickly dividing cells. However, no shoots were generated. With increasing concentrations of 6-BA, shoots were induced on these explants while the growth rate of calli decreased gradually. When the 6-BA concentration was 2.0 mg l^{-1}, callus formation was totally inhibited and no shoots appeared by the end of 8 weeks.

This study indicated that a lower ratio of cytokinin to auxin led to rapid division of the cells on the explant wounds, but these calli were not always regenerable. Although a higher 6-BA concentration resulted in slower cell proliferation, it resulted in shoot regeneration. Shoots were induced on the calli in media when the ratio of cytokinin to auxin was around 10. However, an even higher 6-BA was not beneficial for regeneration, instead it appeared to suppress cell division and inhibit shoot formation.

Based on above experiment, we chose 0.5 mg l^{-1} 6-BA with 0.1 mg l^{-1} 2,4-D for the callus inducing medium and 1.0 mg l^{-1} 6-BA with 0.1 mg l^{-1} 2,4-D for the shoot inducing medium for transformation.

Kanamycin sensitivity

Elucidating the effects of kanamycin at various concentrations on the growth of *Anthurium andraeanum* is a preliminary task for transformation research. When nontransformed explants from petioles and calli were maintained on CIM medium without kanamycin, 100% of the calli pieces and 89.92% of petioles produced new calli on the wounds, respectively. With increasing concentrations of kanamycin, both percentages decreased dramatically (Figure 2). No callus growth or shoot regeneration occurred in the medium containing 125 mg l^{-1} and 100 mg l^{-1}kanamycin or higher for explants from callus clumps and petioles, respectively. Therefore, selection by kanamycin at 125 and 100 mg l^{-1} was used for selecting transformants from callus and petiole tissues, respectively.

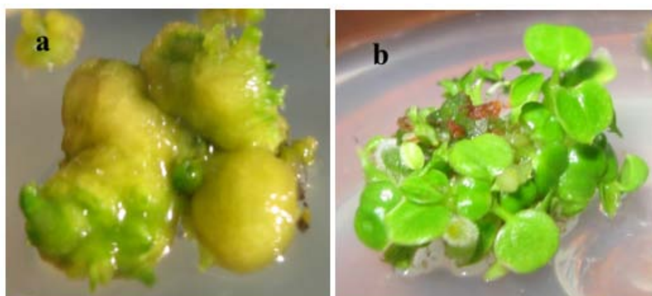

Fig. 4 Calli formation and shoots regeneration during selection step. (a) Transformed callus selected 6 months after co-cultivation of primary explants. (b) Shoots were regenerated on the transformed callus.

Effect of AS and explant sources on transformation

The transfer of T-DNA is mediated by the virulence genes in *Agrobacterium*. Transcription of these genes is induced by various phenolic compounds released by wounded plant cells. Induction of *vir* genes can also be achieved *in vitro* by co-cultivation of *Agrobacterium* with wounded plant cells or tissues in media containing signal molecules such as AS (Stachel et al. 1986). AS has been commonly utilized in most transformation research for both dicotyledon and monocotyledon plants to enhance transformation efficiency (Frame et al. 2002; Olhoft et al. 2003; Kant et al. 2007). However, the effects of AS on *Agrobacterium*-mediated transformation is known to vary according to the plant species. There is even evidence to suggest that AS may suppress virulence in some strain/plant species interactions (Godwin et al. 1991). It was unclear whether or not AS enhanced transformation of *Anthurium andraeanum*. Elucidating the effects of AS on *Anthurium* transformation was an indispensable step to obtaining an optimal protocol.

Table 2. Morphogenic response of explants.

Hormone composition		Morphogenic response	
6-BA	2,4-D	Callus description	Average #Shoots/Callus
0.3 mg/L	0.1 mg/L	Pale yellow, soft, quickly growing	0d*
0.5 mg/L		Yellow green, soft, quickly growing	0d
0.7 mg/L		Yellow green, quickly growing	2.00ab
1.0 mg/L		Yellow green, moderately growing	2.08a
1.3 mg/L		Green, hard, slowly growing	1.78bc
1.5 mg/L		Dark green, slowly growing	1.75c
2.0 mg/L		Difficult to form callus on the wound	0d

*Different letters in the same column indicate significant differences, 5% level, Duncan's multiple range test.

For petiole explants, no resistant callus was generated with AS lower than 75 µmol l^{-1} in the co-cultivation media (Figure 3). The recovery rates increased with higher AS concentrations which reached 0.89% when the AS concentration was 125 µmol l^{-1}. On the other hand, callus-derived explants tended to produce a higher proportion of kanamycin resistant calli than those from petioles at the same AS concentration. The recovery rate was 0.64% even on AS-free co-cultivation media and increased to 3.43% in co-cultivation media with 125 µmol l^{-1} AS. The data demonstrated that AS enhanced the interaction between GV3101 and *Anthurium* cells, with the optimal AS dose used in this study was 125 µmol l^{-1}. Meanwhile, callus explants appear to be more suitable than petiole explants for transformation. This could be the result of differences in the morphogenetic competence and differential sensitivity of cells in different tissues (Yildiz et al. 2002; Jabeen et al. 2005; Nuñez et al. 2007).

Selection strategy and transformant regeneration

T-DNA transfer between *Agrobacterium* and the host plant is achieved during the co-cultivation period and the plant cells become transformed. The length of the co-

cultivation period depends on the plant species and explants involved. In this research, three days was chosen for the co-cultivation period, because this length produced the most kanamycin-resistant calli in our previous study. After co-cultivation, the explants were transferred to media without selective stress to allow for recovery from the agro-inoculation. This step is indispensable for *Anthurium andraeanum* transformation. No resistant callus was obtained without this step. The recovery step allows some time for the selective gene to express and accumulate to sufficient levels for transformants to grow in the selective media (Xu et al. 2009).

About two months later, some small, green yellow spots appeared on the wounded callus pieces and bases of petioles, while other explants became chlorotic and blackened. The new calli were cut from the original explants and inoculated to new CIM. Six months after co-cultivation, five putative transformants from petiole explants and seventeen from calli were obtained. When the resistant calli reached a suitable size (about 2 cm^3) in CIM, they were transferred to SIM to induce shoot formation for a period of 6 to 8 months (Figure 4). Regenerated shoots were rooted in RIM.

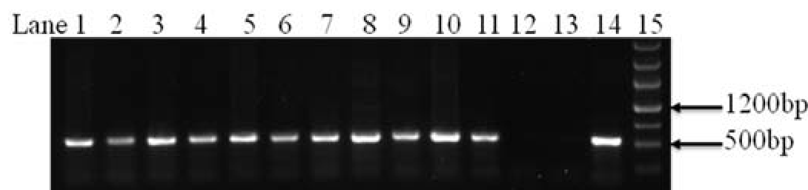

Fig. 5 Electrophoresis of PCR products. Lane 1-11, transformed plants, PCR products of expected size (547 bp) corresponding to e*gfp* were amplified; Lane 12, wild type plant; Lane 13, plant transformed with an empty vector (pJCGLOX,0 without *gfp*); Lane 14, vector pJCGLOX,0 as a positive control, corresponding product was amplified. Lane 15, Marker.

PCR analysis and GFP detection

After selection for 6 months with kanamycin, the presence or absence of *gfp* in all putative transformants was determined by genomic PCR analysis. PCR products of the expected size (547 bp) corresponding to e*gfp* were amplified from 11 of the 22 kanamycin- resistant plants, whereas DNA from wild type plants and plants transformed with an empty vector (a same vector without *gfp*) did not yield such a product (Figure 5). All 5 plants from petiole explants were PCR positive, while only 6 of the 17 plants from callus were PCR positive. Statistical analysis reveals that both explant source and AS concentration affect transformation efficiency.

Transient expression was detected in explants about 2 days after co-cultivation, but the fluorescence disappeared in most materials in the following course. Stable expression of GFP in regenerated plants was observed with a fluorescence microscope. Despite the presence of the e*gfp* gene in 11 transgenic lines, only 7 of the lines expressed GFP in young calli and stems (Figure 6). No GFP activity was detected in tissues from wild type plants and plants transformed with an empty vector, indicating that the green fluorescent areas were specific to GFP. The lack of protein expression is probably the result of gene inactivation. DNA methylation of the promoter region or some kind of gene silencing as a consequence of multiple insertions could be involved (Paszkowski and Whitham, 2001). Out of a total of 22

regenerated plants, 11 (50%) plants were PCR-positive and GFP was expressed in 7 (63.64%) of these transformants. It is noteworthy that about 40% of plants shown to possess transgenes by PCR, did not express functional proteins. Therefore, GFP can be valuable to facilitate the screening process for transgenic plants that express functional proteins, if they are fused to the target gene. This may be important especially when kanamycin (Km) is used for selection since it was recently shown that the proportion of “escapes” may be very high under Km-selection (Kuvshinov et al. 1999).

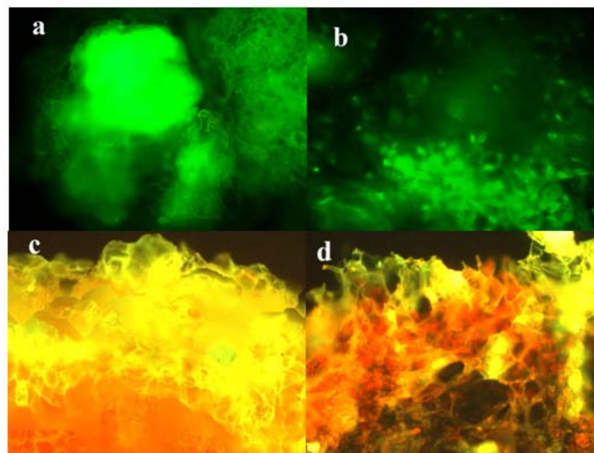

Fig. 6 Stable expression of eGFP in transformed callus (a) and stem cells (b), no green fluorescence was observed in wild type callus (c) and (d) callus transformed with empty vector (pJCGLOX,0 without gfp).

The *gus*A gene is another widely used reporter to detect transformed plants and cells. However, the assay for GUS activity requires histochemical staining with exogenous substrate and is destructive to plant tissues. The GFP gene has substantial advantages over other reporter and selectable genes for it is suitable for monitoring the time course of gene expression in living cells or for rapid screening of primary transformants (El-Shemy et al. 2004). Successful expression of GFP in *Anthurium* proved the feasibility of utilizing this novel gene in its transformation research.

As a monocot, *Anthurium* is not very susceptible to *Agrobacterium*-mediated transformation. Transformation efficiency is fairly low and cultivar-dependent, methods should be contrived to circumvent the problem. Chen and his colleagues got transformation efficiency of 1.3% (Chen et al. 1997). Since then, little work was reported about improvement of the data. The optimal transformation efficiency in this study was 1.71% using callus explants as starting material and 125 µmol l^{-1} AS during the co-cultivation process, which is significantly different from other treatments.

The production of transgenic plants is fundamental to investigations of gene function as well as to the improvement of horticultural traits in economically important flowers. *Agrobacterium tumefaciens*-mediated plant transformation has been used to generate transformants for both purposes. In the present study, we report an efficient protocol

for shoot regeneration and *Agrobacterium tumefaciens*-mediated transformation from callus and petiole explants of *Anthurium andraeanum*. *Agrobacterium*-mediated transformation of *Anthurium andraeanum* was confirmed by PCR analysis and fluorescence microscopy. To date, we have not observed any phenotypic alterations in the transformed plants as a result of transformation. The development of transformation protocols will make it possible to engineer new traits into this promising ornamental species.

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