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ORIGINAL PAPER

Efficient somatic embryogenesis and *Agrobacterium*-mediated transformation of pothos (*Epipremnum aureum*) 'Jade'

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Abstract Leaf and petiole explants of monocotyledonous pothos (*Epipremnum aureum*) 'Jade' were cultured on Murashige and Skoog basal medium supplemented with *N*-(2-chloro-4-pyridl)-*N*′-phenylurea (CPPU) or *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea (TDZ) with α-naphthalene acetic acid (NAA). Somatic embryos appeared directly from explants after 4–8 weeks of culture; 9.1 μM TDZ with 1.1 μM NAA induced 61.1 % leaf discs and 94.4 % of petiole segments to produce plantlets through embryo conversion. Using this established regeneration method and an enhanced green fluorescent protein (GFP) gene (*egfp*) as a reporter marker, an *Agrobacterium*-mediated transformation procedure was developed. Leaf discs and petiole segments were inoculated with *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pLC902 that contains novel bi-directional duplex promoters driving the *egfp* gene and hygromycin phosphotransferase gene (*hpt*), respectively. The explants were co-cultivated with strain EHA105 for 3, 5, and 7 days, respectively prior to selective culture with 25 mg l^{-1} hygromycin. A 5-day co-cultivation led to 100 % of leaf discs to show transient GFP expression and 23.8 % of the discs to produce stable GFP-expressing somatic embryos. A 7-day co-cultivation of petiole explants resulted in the corresponding responses at 100 and 14.3 %, respectively. A total of 237 transgenic plants were obtained, and GFP fluorescence was observed in all plant organs. Regular PCR and quantitative real-time PCR analyses confirmed the presence of 1 or 2 copies of the *egfp* gene in analyzed plants. The highly efficient regeneration and transformation systems established in this study may enable genetic improvement of this vegetatively propagated species through biotechnological means.

Keywords *Agrobacterium tumefaciens* · GFP · *Epipremnum aureum* · Somatic embryogenesis · Transgenic pothos

Abbreviations

AS	Acetosyringone
CaMV 35S	Cauliflower mosaic virus 35S promoter
CPPU	*N*-(2-chloro-4-pyridl)-*N*′-phenylurea
GFP	Green fluorescent protein
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
QRT-PCR	Quantitative real-time PCR
TDZ	*N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea

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Introduction

Epipremnum aureum (Linden and Andre) Bunt., commonly known as pothos, is an important species in the monocotyledonous family Araceae or aroids. It is an herbaceous evergreen native to Southeast Asia and the Solomon Islands (Huxley 1992). Because of its showy foliage and ability to grow under interior conditions with low levels of light and humidity, this species has been grown as hanging

baskets or potted plants used widely for interior decoration (Chen et al. 2005) and also for reducing indoor volatile organic compounds through phytoremediation (Oyabu et al. 2003). Pothos was first commercially produced in the United States in the 1920s (Smith and Scarborough 1981) and ranked second in wholesale dollar value in the foliage plant industry from the 1950s to 1960s (McConnell et al. 1989) but its value dropped to twelfth in 2007 (USDA 2009). The decreased industry value of pothos is primarily attributed to the limited number of cultivars in commercial production (Henny et al. 2009a, b).

Pothos does not flower under greenhouse or interior environments and seldom flowers even in its native habitat (Henny and Chen 2003). Due to the difficulty in flower induction for breeding and development purposes, there have been only four cultivars available on the market: Golden Pothos, Marble Queen, Jade, and Neon in 90 years of pothos production (Bown 2000; Chen et al. 2002). As a result, induced mutation methods have been used for new cultivar development. Two recent pothos cultivars Pearls and Jade (Henny et al. 2009a) and Green Genie (Henny et al. 2009b) were released from selections of irradiated 'Marble Queen' plants. Induced mutation, however, is a random event and occurs at low frequency; its use in breeding does not meet the pace of market demand for new pothos cultivars (Zhao et al. 2012b).

Genetic engineering offers an alternative approach for developing new cultivars via transferring single or multiple genes of interest into target plants. Transgenic aroid plants have been reported in *Anthurium* (Chen and Kuehnle 1996; Chen et al. 1997; Kuehnle et al. 2004; Fitch et al. 2011), *Caladium bicolor* (Li et al. 2005), *E. aureum* 'Golden Pothos' (Kotsuka and Tada 2008), and *Zantedeschia elliottiana* (Yip et al. 2007). Kotsuka and Tada (2008) were the first to report the transformation of 'Golden Pothos' with a β-glucuronidase reporter gene (*gus*) using *Agrobacterium tumefaciens* strain EHA105. The report, however, did not explicitly define the regeneration method, transformation efficiencies, and the number of transgenic plants recovered. As a result, the challenge for developing efficient and reliable regeneration and transformation systems critical for pothos improvement through biotechnology still remains.

A key limitation in *Agrobacterium*-mediated transformation of monocotyledonous species is the reliability of plant regeneration system (Frame et al. 2002). Micropropagation of pothos was initially documented in the 1970s when Miller and Murashige (1976) propagated pothos lateral buds using node sections. Qu et al. (2002) established a regeneration method for *E. aureum* 'Jade' through indirect shoot organogenesis. However; organogenesis through a callus phase often results in somaclonal variation in aroids (Chen et al. 2003). Somatic embryogenesis, particularly direct somatic embryogenesis, has advantages over both shoot culture and organogenesis because a large number of plantlets can be produced and this method can potentially scale-up propagation with bioreactors and produce synthetic seeds (Rani and Raina 2000). Somatic embryos provide ideal materials for genetic transformation (Parimalan et al. 2011; Sivanesan et al. 2011). Recently, efficient regeneration methods via direct somatic embryogenesis were established in *E. aureum* 'Golden Pothos' (Zhang et al. 2005) and 'Marble Queen' (Zhao et al. 2012b). However, regeneration of *E. aureum* 'Jade' through somatic embryogenesis has not been reported.

Reporter genes play an important role in selection of transformed cells and recovery of transgenic plants. The *gus* gene is the most widely used reporter gene in various plant species. However, the histochemical GUS assay is destructive and therefore not suitable for assaying primary transformants. Green fluorescent protein gene (*gfp*) from the Pacific Northwest jellyfish (*Aequorea victoria*) is a non-destructive reporter gene and has been successfully used to facilitate visual selection of transformed plants (Haseloff and Siemering 2006). However, there have been no reports on successful utilization of the *gfp* gene in pothos transformation studies.

In the present study, we evaluated critical culture conditions and established an efficient method for regenerating *E. aureum* 'Jade' through direct somatic embryogenesis. Based on the established regeneration method, we developed an efficient transformation procedure for this cultivar using a binary vector pLC902 containing novel bi-directional duplex promoters respectively driving an enhanced green fluorescent protein reporter gene (*egfp*) as a reporter marker and a hygromycin phosphotransferase gene (*hpt*) as a selective marker.

Materials and methods

Plant materials and culture media

Shoot tips (about 6 cm long) with young leaves were excised from greenhouse-grown *E. aureum* 'Jade' plants. After washing the tips thoroughly in tap water, leaves and petioles were isolated and surface-sterilized using 70 % (v/v) ethanol for 1 min followed by immersing them in 1.5 % (w/v) sodium hypochlorite solution for 20 min with agitation. After rinsing four times with sterilized distilled water, leaves were cut into 4 cm^2 pieces (2 × 2 cm) and petioles cut into 1 cm long segments as explants.

A basal medium consisting of MS mineral salts and vitamins (Murashige and Skoog 1962) with 3.0 % (w/v) sucrose and 0.7 % (w/v) agar (Phytotechnology Laboratories, Shavnee Mission, KS) was used in this study. The pH

of the medium was adjusted to 5.8 with 1 M KOH solution prior to autoclaving at 121 °C for 25 min. Plant growth regulator stock solutions were filter-sterilized and added to autoclaved basal medium when temperature dropped to 50 °C. Growth regulators *N*-(2-chloro-4-pyridl)-*N*′-phenylurea (CPPU) at 4.0, 8.1, and 12.1 μM or *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea (TDZ) at 4.5, 9.1, and 13.6 μM respectively with 1.1 μM α-naphthalene acetic acid (NAA) were tested.

Somatic embryogenesis and plant regeneration

All explants were cultured in 100 × 15 mm Petri dishes (Fisher Scientific Inc., Pittsburgh, PA) containing 20 ml of medium. Leaf discs were placed with their adaxial surface up and petiole segments placed horizontally on the medium surface. Each Petri dish contained 6 explants, and there were 6 replicates per treatment. Cultures were initially maintained in darkness at 26 °C for 8 weeks. Explants with somatic embryos were recorded, and the induction frequency as the percentage of explants that produced embryos was calculated. Explants yielding somatic embryos were sub-cultured onto fresh medium containing the same growth regulators as the initial culture and placed under light with a 16-h photoperiod and a photosynthetic photon flux density of 80 μmol m^{-2} s^{-1} provided by cool-white fluorescent light bulbs. After 4 weeks of culture, explants with somatic embryos that converted into plantlets were recorded, and embryo conversion frequency was calculated as the percentage of explants with converted embryos over the total number of cultured explants per dish. Plantlets derived from embryo conversion were transferred to baby-food jars containing the same medium without growth regulators for continuous plant growth. Four weeks later, individual plantlets were separated and transferred to basal medium for use as explant sources for transformation or were transplanted into a soilless substrate (40 % sphagnum peat, 25 % pine bark, 25 % coconut coir, and 10 % Styrofoam by volume) for plant establishment in a shaded greenhouse.

Agrobacterium-mediated transformation

Agrobacterium tumefaciens strain EHA105 with a binary vector pLC902 (Fig. 1) containing novel bi-directional duplex promoters for *hpt* gene and *egfp* gene was used in this study. The *egfp* gene was controlled by a double-enhanced cauliflower mosaic virus (CaMV) 35S promoter (Li et al. 2001; Li et al. 2004). *Agrobacterium* culture was initiated in 25 ml of MG/L medium (Garfinkle and Nester 1980) containing 100 mg l^{-1} kanamycin and 20 mg l^{-1} rifampicin. After culturing over night at 28 °C on a rotary shake at 200 rpm, the bacterial suspension was centrifuged at 5,000 rpm for 5 min. The resultant pellet was re-suspended in liquid somatic embryo induction medium containing 100 μM acetosyringone (AS) and cultured under the same conditions until an OD_{600} of 0.8–1 was reached. Leaf discs and petiole segments taken from the plantlets regenerated through the aforementioned somatic embryogenesis method were inoculated with the bacterial culture for 10 min at room temperature. Inoculated explants were blotted dry on sterile filter paper to remove excess bacterial solution and placed on the upper surface of double-layered filter paper moistened with liquid somatic embryo induction medium containing 100 μM AS in Petri dish. Cultures were maintained in the dark at 26 °C for 3, 5, and 7 days, respectively for determining the duration of cultivation on the transformation efficiency. There were 7 leaf discs or petiole segments per Petri dish and 3 dishes per treatment. At the end of each culture period, the explants were washed with liquid somatic embryo induction medium and blotted dry on sterile filter paper.

Regeneration of transgenic plants and GFP expression

The co-cultivated explants were transferred onto solid somatic embryo induction medium with 9.1 μM TDZ and 1.1 μM NAA supplemented with hygromycin at 25 mg l^{-1} and carbenicillin and cefotacime respectively at 200 mg l^{-1}. The explants were maintained in the dark at 26 °C and sub-cultured 4 weeks later on fresh medium with the same levels of antibiotics. After 8 weeks of culture, hygromycin-resistant explants with somatic embryos were transferred onto the same fresh medium containing 9.1 μM TDZ and 1.1 μM NAA as well as hygromycin at 25 mg l^{-1} and cefotacime and carbenicillin respectively at 200 mg l^{-1} for embryo conversion. Cultures were kept under light (80 μmol m^{-2} s^{-1}) with a 16-h photoperiod for 4 weeks. Plantlets regenerated from embryo conversion were transferred into baby-food jars containing MS basal medium supplemented with hygromycin at 25 mg l^{-1} and cefotacime and carbenicillin respectively at 200 mg l^{-1} for continuous growth. Plantlets with well-developed shoots and roots were transplanted to the soilless substrate and grown in a shaded greenhouse.

The transient GFP expression in leaf and petiole explants was recorded 10 days after inoculation using a Leica MZFLIII stereomicroscope equipped with epi-fluorescence illumination and detection systems (Leica Microscopy System Ltd., Heerbrugg, Switzerland). Percent transient GFP expression was calculated as the number of leaf discs or petiole segments exhibiting GFP fluorescence divided by the total number of explants co-cultivated with *Agrobacterium*. Stable transformation frequency was calculated as the percentage of leaf or petiole explants producing a GFP positive SE over the total number of

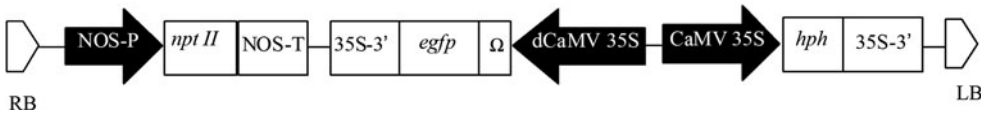

Fig. 1 Schematic diagram of T-DNA region of the binary vector pLC902 containing bi-directional duplex promoters driving *egfp* reporter gene and *hph* gene conferring hygromycin resistance, respectively. *RB* right border, *LB* left border, *dCaMV 35S* double enhanced (2x-419 to −90) CaMV 35S promoter with the Ω leader sequence of tobacco mosaic virus. CaMV 35S, promoter sequence of CaMV 35S transcript; 35S-3′, the termination site and polyadenination signal of the CaMV 35S transcript

explants. The number of transgenic plantlets was recorded after 4 weeks of culture in baby-food jars.

Transgene detection via PCR analysis

Total genomic DNA was extracted from young leaves of transgenic and non-transformed plants using the DNeasy® plant mini kit (Qiagen sciences, Valencia, CA, USA). A 717 bp coding region of the *egfp* gene was PCR-amplified using a forward primer EG-51 (5′ATGGTGAGCAAGGGC GAGGAGCTGT3′) and a reverse primer EG-32 (5′CTTGT ACAGCTCGTCCATGCCGAGA3′). The amplification reaction was performed using a DNA Engine (Bio-Rad Laboratories, Hercules, CA) under the following conditions: 1 cycle at 95 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final cycle at 72 °C for 8 min. Plasmid DNA used in transformation served as a positive control while DNA from non-transformed plants was used as a negative control. The amplified products were separated by electrophoresis on a 1 % of agarose gel with 0.5 mg l^{-1} ethidium bromide.

The 1C genome size of *E. aureum* 'Jade' was calculated using the PARTEC PA cytometer and the protocol reported by Zhao et al. (2012a). Transgene copy number was determined according to the absolute quantification method described by Bubner and Baldwin (2004) using QRT-PCR analysis in a LightCycler 480® instrument equipped with a 96-well plate Therma-base and software release v1.5 (Roche Molecular Biochemicals, Indianapolis, IN, USA). Oligonucleotide primers for amplification of a 340-bp fragment from the *egfp* gene included a forward primer ERT-51: 5′CCAT CCTGGTCGAGCTGGAC3′ and a reverse primer ERT-32: 5′TTCAGCTCGATGCGGTTCAC3′. All reactions were carried out in sample wells containing 2 µl sample DNA (6 ng total DNA), 10 µl 2 × SYBR Green I Master PCR mix, 2 µl each of primer (0.5 µM) and 4 µl sterile water. A standard curve was obtained by mixing the *Eco*RI linearized *egfp* gene-containing plasmid pU203 with 6 ng genomic DNA from a non-transgenic plant. The amount of plasmid DNA needed to be mixed with the genomic DNA was calculated based on the ratio of the transgene copy number over the *E. aureum* 'Jade' genome size. A standard set of mixtures representing 1, 2, 3, 4, and 5 copies of transgene equivalence was used to establish the standard curve. QRT-PCR conditions were as follows: 95 °C for 10 min to activate the DNA polymerase, then 45 cycles of 95 °C for 5 s, 56 °C for 10 s, and 72 °C for 40 s. The melting curves of the PCR products were acquired by incubating at 95 °C for 5 s, lowering the temperature to 50 °C and maintaining for 1 min, and then heating to 95 °C. Samples were replicated three times, and experiments were repeated twice.

Fluorescence signals were analyzed by the "absolute quantification analysis using the fit points method" (Roche Molecular Biochemicals, Indianapolis, IN, USA). Transgene copy number (*egfp* gene) in transgenic plants was determined based on the comparison of crossing point (Cp) of extrapolated concentrations with standard curves.

Statistical analysis

All experiments for plant regeneration were arranged in completely randomized design. Each Petri dish was considered an experimental unit. The frequency of somatic embryo occurrence and embryo conversion were analyzed using one way analysis of variance (ANOVA). When significance occurred, mean differences were separated using Fisher's Protected Least Significant Differences (LSD) at $P \leq 0.05$ level.

Results

Direct somatic embryogenesis

Somatic embryos appeared directly at the cut edges of both leaf and petiole explants after 4-8 weeks of culture in the dark. Embryos were white and globular, appearing individually or in clusters. Somatic embryogenesis was asynchronous and slower in leaf explants. After cultures were placed under light, embryo conversion occurred producing a bipolar structure, i.e. root and shoot on opposite ends. Explant types, growth regulator combinations and concentrations significantly affected somatic embryogenesis. Petiole explants had much higher frequencies in somatic embryo induction and embryo conversion than leaf explants regardless of CPPU or TDZ concentrations. For example, CPPU at 8.1 µM with NAA at 1.1 µM led to

Table 1 Frequencies of somatic embryo occurrence and embryo conversion from leaf and petiole explants of *E. aureum* 'Jade' cultured on Murashige and Skoog basal medium supplemented with *N*-(2-chloro-4-pyridl)-*N*'-phenylurea (CPPU) or *N*-phenyl-*N*′-1, 2, 3-thiadiazol-5-ylurea (TDZ) with μM α-naphthalene acetic acid (NAA)

Growth regulator concentration (μM)			Leaf explant		Petiole explant	
CPPU	TDZ	NAA	Frequency of explants with embryos (%)	Frequency of explants with embryo conversion (%)	Frequency of explants with embryos (%)	Frequency of explants with embryo conversion (%)
4.0		1.1	27.8 ± 8.6 d[z]	13.9 ± 6.8 e	55.6 ± 8.6 c	47.2 ± 6.8 c
8.1		1.1	38.9 ± 8.6 bc	30.5 ± 6.8 c	94.4 ± 8.6 a	88.9 ± 8.6 a
12.1		1.1	22.2 ± 8.6 d	19.5 ± 6.8 de	69.5 ± 6.8 b	55.6 ± 8.6 bc
	4.5	1.1	30.5 ± 6.8 cd	25.0 ± 9.1 cd	58.4 ± 9.1 c	52.8 ± 6.8 bc
	9.1	1.1	72.2 ± 8.6 a	61.1 ± 8.6 a	97.2 ± 6.8 a	94.4 ± 8.6 a
	13.6	1.1	44.4 ± 8.6 b	41.7 ± 9.1 b	75.0 ± 9.1 b	61.1 ± 8.6 b

[z] Means followed by different letters within a column represent significant difference at $P \leq 0.05$ level analyzed by Fisher's Protected Least Significant Difference test

94.4 % of petiole explants producing somatic embryos and 88.9 % of explants showing embryo conversion (Table 1); whereas, the corresponding frequencies for leaf explants were only 38.9 % and 30.5 %, respectively. In contrast to CPPU, TDZ with NAA induced more leaf explants to produce somatic embryos and more explants with converted embryos. Among the growth regulators tested, MS medium containing 9.1 μM TDZ and 1.1 μM NAA induced 72.2 % of leaf explants and 97.2 % of petiole explants to produce somatic embryos and 61.1 % of leaf explants and 94.4 % petiole explants with embryo conversion. All plantlets regenerated from somatic embryos produced well-developed roots and grew vigorously in baby-food jars or in the soilless substrate in a shaded greenhouse. This established regeneration medium was then used for developing *Agrobacterium*-mediated transformation system.

Effects of co-cultivation time and explant types on transient GFP expression and stable transformation

The frequency of transient GFP expression varied greatly with explant types and the duration of co-cultivation. In a 3-day co-cultivation, 71.4 % of leaf discs and 52.4 % petiole explants exhibited transient GFP expression (Fig. 2). A co-cultivation of 5 days led to 100 % of leaf explants and 90.5 % of petiole explants to show transient GFP expression. Transient GFP expression was observed in all explants after 7 days co-cultivation.

Hygromycin-resistant embryos and stable transformation frequencies also differed with explant types and the co-cultivation period (Fig. 2). A 5-day co-cultivation appeared to be the optimal duration for leaf explant transformation since 23.8 % of cultured leaf explants were able to produce hygromycin-resistant and GFP positive somatic embryos, whereas 3- and 7-day co-cultivation led to only 9.5 and 4.8 % of explants producing GFP positive somatic embryos (Fig. 2a). On the other hand, 7-day co-cultivation of petiole explants resulted in a stable transformation frequency of 14.3 % which was better than 4.8 % obtained by co-cultivation of 3 and 5 days (Fig. 2b).

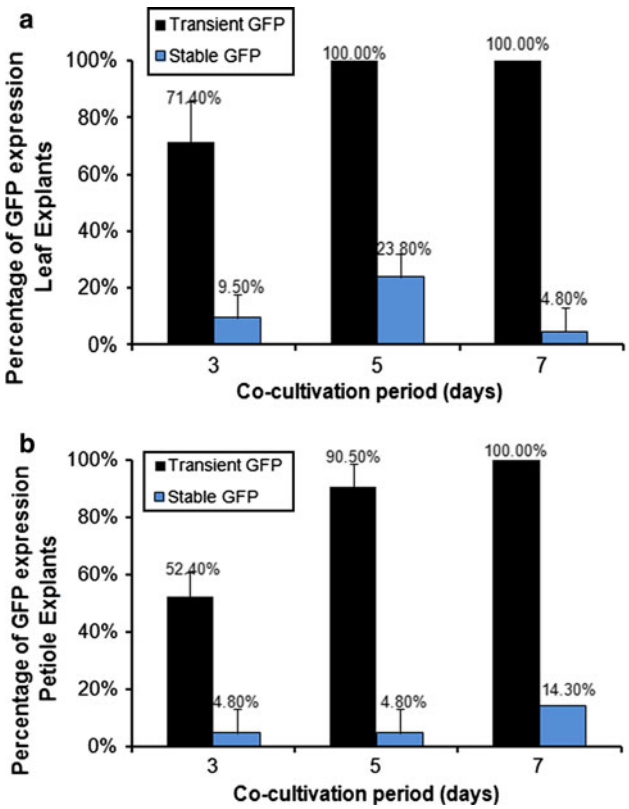

Fig. 2 Effects of co-cultivation days on the frequencies of transient GFP expression and stable transformation in innoculated leaf and petiole explants of *E. aureum* 'Jade'. *Data* represent the mean percentage of three replicate samples, each replicate consisting of 7 leaf or petiole explants. *Error bars* represent standard errors

Regeneration of transgenic plants

The *egfp* reporter gene allowed real-time monitoring of transgene expression after transformation. GFP expression was initially weak and only observed in a few cells on the edge of leaf discs and petiole segments 3 days after *Agrobacterium* inoculation. After 7–10 days of inoculation, stronger transient GFP expression was observed at the edges of leaf discs and segments (Fig. 3 a, b). Hygromycin-resistant and GFP-expressing somatic embryos appeared individually or in clusters (Fig. 3 c, f). Embryos proliferated into larger embryo masses by producing secondary embryos which were also GFP positive (Fig. 3 d, g). All the hygromycin-resistant embryos expressing GFP were able to convert and produce GFP-positive bipolar structure GFP (Fig. 3 e, h). GFP expression was observed in entire plants including leaves and petioles (Fig. 3i, k) as well as roots (Fig. 3 j, l). A total of 237 transgenic plantlets were regenerated, of which 168 were regenerated from leaf explants and 69 from petiole explants. An average of 2.7 transgenic plants was produced from leaf discs compared with a mean of 1.1 from petiole segments. All plantlets regardless of the explant sources, grew vigorously after transplanting to the soilless substrate in the shaded greenhouse (Fig. 3m), and no somaclonal variants were observed from transgenic plants over 1.5 years of growth.

Molecular analysis of transformants

Genomic DNA extracted from 13 randomly selected transgenic and a non-transformed control plants were used for amplification of the *egfp* gene. Regular PCR analysis showed that an amplicon at about 717 bp corresponding to the *egfp* gene was obtained from all the transgenic plants (Fig. 4, lanes 3–15) but not from the non-transformed plant (Fig. 4, lane 2) or the water control (Fig. 4, lane 1). Lane P was the positive control of the plasmid.

The mean nuclear DNA content for *E. aureum* 'Jade' analyzed by the PARTEC PA cytometer was 8.586 pg $2C^{-1}$. The estimated genome size was 4.198×10^9 bp $1C^{-1}$. The amount of *Eco*RI linearized plasmid pU203 corresponding to 1 copy of the *egfp* gene was calculated as 6 ng (genomic DNA amount) $\times$ 5.352×10^3 bp (size of pU203)/4.198×10^9 bp (1C genome size of *E. aureum* 'Jade') = 0.0076 pg. A set of reaction mixtures representing 1–5 copies of the *egfp* gene was used to establish a standard curve for evaluation of transgene copy number (Table 2, plasmid:1–5 copy). Eight plants selected randomly from the regular PCR-analyzed 13 transgenic plants were analyzed by QRT-PCR. Extrapolated Cp values revealed that there was 1 copy of the *egfp* gene in 5 plants, but 2 copies occurred in 3 plants (Table 2). No targeted gene amplification was detected from the non-transformed plant. Melting curve analysis of QRT-PCR products showed that all the plasmid copy controls and transgenic plants produced a single-peak profile, indicating that the fluorescence signals were derived from target-specific amplification.

Discussion

Somatic embryogenesis

This is the first report of regeneration of *E. aureum* 'Jade' through direct somatic embryogenesis. Leaf or petiole explants were cultured on MS basal medium supplemented with 9.1 μM TDZ and 1.1 μM NAA in darkness for 8 weeks for embryo induction. Explants with somatic embryos were sub-cultured on the same medium under light conditions for embryo conversion. Four weeks after sub-culture, plantlets derived from the embryo conversion were transferred to MS medium devoid of growth regulators for continuous growth. After another 4 weeks, plantlets were either be transplanted to a soilless substrate for plant establishment in a shaded greenhouse or transferred to MS medium devoid of growth regulators in baby-food jars for plant line maintenance. This established method is simple and convenient because leaves and petioles are abundant sources for producing explants, and the same culture medium is used for both embryo induction and conversion. The time length required from culture initiation to embryo conversion is only 12 weeks. This protocol is highly efficient with up to 61.1 and 94.4 % of cultured leaf and petiole explants showing embryo conversion (Table 1). No somaclonal variants were observed from regenerated plants grown in the shaded greenhouse. This is in agreement with previous findings that direct somatic embryogenesis has low probability of somaclonal variation (Ahloowalia 1991; Merkle 1997; Chen and Henny 2006).

Leaf explants of *E. aureum* 'Jade' were less effective in somatic embryogenesis compared to petiole explants (Table 1). CPPU or TDZ with NAA induced 55.6–97.2 % of petiole explants to produce somatic embryos and 47.2–94.4 % of explants showed embryo conversion; whereas, the corresponding frequencies for leaf explants were 22.2–72.2 and 13.9–61.1 %, respectively (Table 1). Similar results were also observed in *E. aureum* 'Marble Queen' (Zhao et al. 2012b). TDZ appeared to be more effective than CPPU in inducing somatic embryogenesis, which is consistent with the results of *E. aureum* 'Marble Queen' (Zhao et al. 2012b), but different from *E. aureum* 'Golden Pothos' (Zhang et al. 2005). The varied frequencies in somatic embryo induction and conversion reflect cultivar differences in response to TDZ and CPPU induction. This might also be related to different levels of

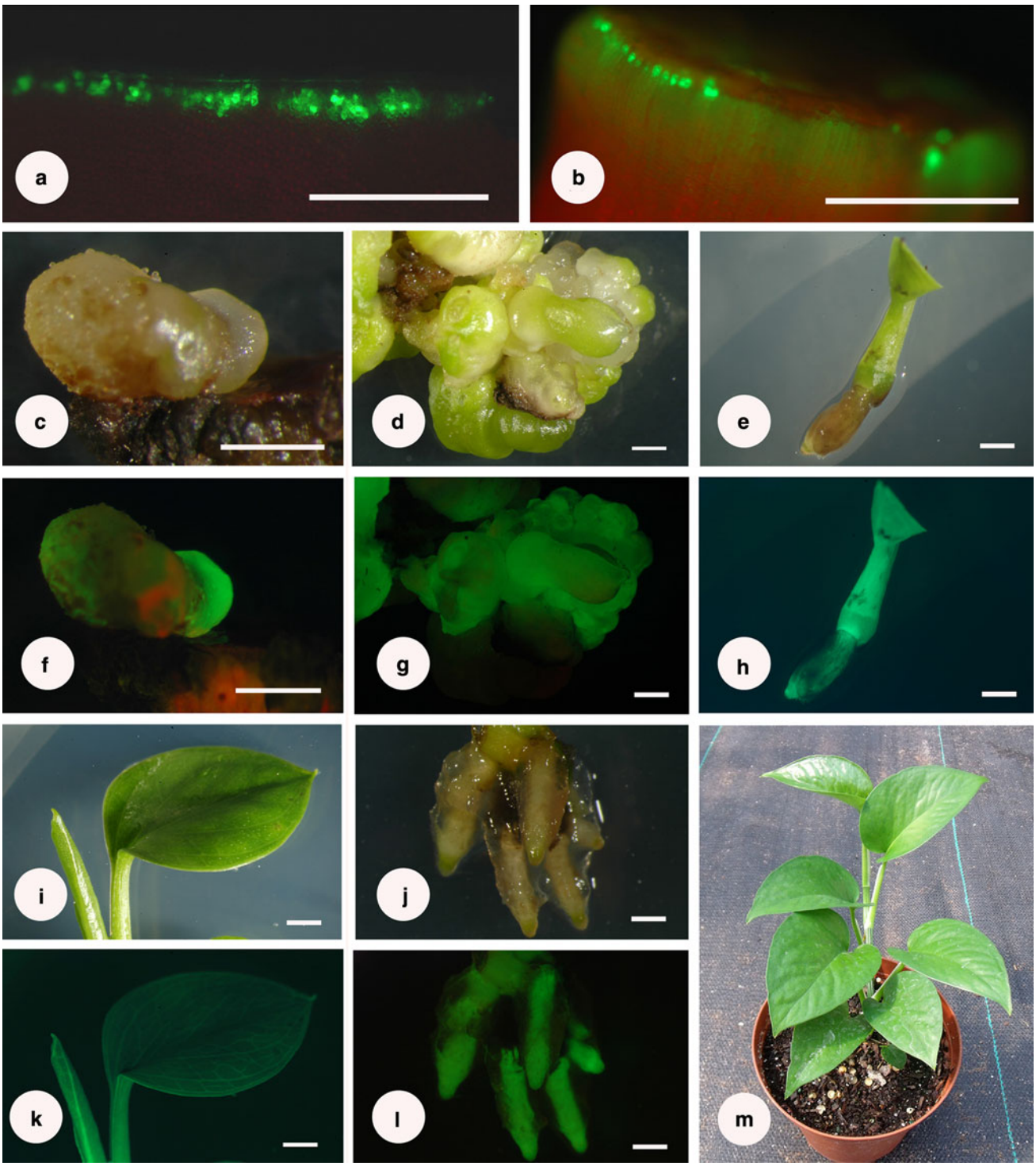

Fig. 3 Regeneration of transgenic plants of *E. aureum* 'Jade' illustrated by transient GFP expression in leaf discs (**a**) and petiole segments (**b**) 10 days after co-cultivation with *Agrobacterium*. Transient GFP expression occurred in somatic embryos at globular (**c**, **f**) and scutellum (**d**, **g**) stages and converted embryos with bipolar structure (**e**, **h**). Regenerated plantlets expressed GFP in leaves (**i**, **k**) and roots (**j**, **l**), and a greenhouse-grown potted plant (**m**). *All bars* 1 mm

endogenous hormones in leaves or petioles and different modes of action between CPPU and TDZ or interactions of both factors. In general, TDZ has higher biological activities than CPPU even though both are urea-type cytokinins (Ricci and Bertoletti 2009). TDZ is highly stable and resistant to degradation by cytokinin oxidase

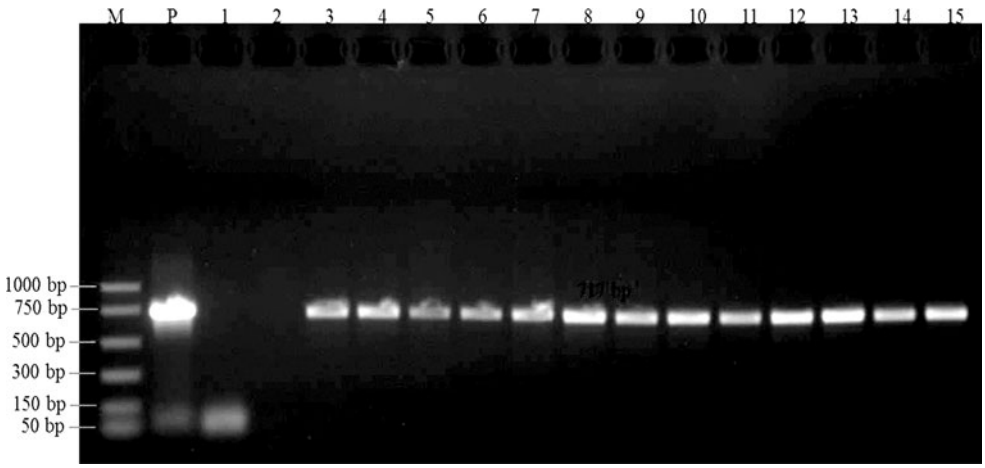

Fig. 4 PCR analysis of transgenic *E. aureum* 'Jade' plants using *egfp* gene. *Lane M* as Marker; *lane P* is plasmid control; *lane 1* is water control; *lane 2* is untransformed plant; *lanes 3–15* are randomly selected transgenic plants

Table 2 The *egfp* gene copy number in transgenic plants of *E. aureum* 'Jade' determined using quantitative real-time PCR

Sample name	Mean Cp[a]	STD Cp[b]	Mean conc[c]	STD conc	Copy number
Plasmid: 1 copy	21.587	0.125	0.971	0.082	1
Plasmid: 2 copy	20.591	0.213	1.984	0.277	2
Plasmid: 3 copy	20.182	0.054	3.183	0.091	3
Plasmid: 4 copy	19.699	0.055	4.001	0.137	4
Plasmid: 5 copy	19.256	0.132	5.380	0.453	5
GP-1	21.468	0.057	1.052	0.042	1
GP-2	21.473	0.205	1.055	0.151	1
GP-3	20.559	0.090	1.976	0.121	2
GP-4	21.616	0.170	0.954	0.114	1
GP-5	20.453	0.099	2.129	0.146	2
GP-6	21.693	0.067	0.900	0.043	1
GP-7	21.185	0.143	1.295	0.112	1
GP-8	20.814	0.138	1.658	0.159	2

[a] Average values of crossing pints (Cp) from three sample replicates

[b] Standard deviation values for Cp

[c] Average values of extrapolated relative to a single transgene copy

(Mok et al. 1987) and is capable of inducing both auxin and cytokinin responses (Gill and Saxena 1993).

Genetic transformation

Based on the established somatic embryogenesis and plant regeneration method, an efficient protocol for producing a larger number of transgenic plants was developed using *Agrobacterium*-mediated transformation. The average transformation efficiencies for petiole and leaf explants were 14.3 and 23.8 %, respectively (Fig. 2), which were much higher than many reported *Agrobacterium*-mediated transformation of other monocotyledonous species. For example, the highest transformation efficiency in sorghum (*Sorghum bicolor* Moench) was reported to be 8.3 % (Gurel et al. 2009). A transformation efficiency of 10 % was recently documented in rice (*Oryza sativa* L.) (Wakasa et al. 2012). The reported percentage in transformation of *E. aureum* 'Golden Pothos' varied from 5 to 30 %; based on their data, a calculated mean was 17.25 % (Kotsuka and Tada 2008). In addition to the efficient regeneration method, the higher transformation efficiency achieved in the present study may be attributed to the novel bi-directional duplex promoters that drive the *egfp* and *hpt* genes, respectively. Comparative analysis of bi-directional promoters in grape and tobacco showed higher reporter gene expression than a unidirectional expression system incorporating similar enhancer and core-promoter complexes (Li et al. 2004). This may suggest that the manipulation of transcription machinery is more efficient in a bi-directional mode than in an unidirectional mode, thus, transformation efficiency was enhanced in this studied monocotyledonous species.

Hygromycin resistance gene is an effective selectable marker for genetic transformation of *E. aureum* 'Jade'.

Thus far, no non-transformant escaped from hygromycin selection at 25 mg l^{-1}. Kotsuka and Tada (2008) tested the effects of hygromycin, kanamycin, and geneticin on the regeneration of stem explants of *E. aureum* 'Golden Pothos' and reported that hygromycin was the most suitable selection antibiotic. For transformation of monocotyledons, kanamycin is less effective as a selective agent especially in *Gramineae* (Hauptmann et al. 1988). Hygromycin is one of the most commonly used selection agents for transformation in monocotyledons, including maize (Walters et al. 1992), rice (Zheng et al. 1991), wheat (Ortiz et al. 1996), and switchgrass (Mann et al. 2012).

Factors including co-cultivation time, explant type, and culture medium affect T-DNA delivery and its integration into the plant genome (Cheng et al. 2004; Sood et al. 2011; Dutt and Grosser 2009). According to Sood et al. (2011), a co-cultivation of 2–3 days is generally considered to be suitable for *Agrobacterium*-mediated transformation in monocotyledonous plant species. Kotsuka and Tada (2008) reported that a 1 day pre-culture followed by 3 days co-cultivation was the best for transformation of stem explants of *E. aureum* 'Golden Pothos'. In the present study, no pre-culture was needed, and a transformation efficiency of 23.8 % was achieved for leaf explants after they were co-cultivated for 5 days. Petiole transformation efficiency was comparably lower; a 7-day co-cultivation resulted in a transformation efficiency of 14.3 % (Fig. 2). Anuradha et al. (2006) reported that a prolonged co-cultivation resulted in necrosis of explants of peanut (*Arachis hypogaea* L.). No necrosis was observed in any explants of this study. Petiole explants as documented in Table 1 had higher frequencies in somatic embryogenesis than leaf explants, but a lower efficiency in transformation was evident (Fig. 2). Similar results were reported in alfalfa (*Medicago sativa* L.) (Chabaud et al. 1988) and strawberry (*Fragaria vesca* L.) (Alsheikh et al. 2002). The higher transformation efficiency in leaf explants could be attributed to the probability that leaf discs may have more wounded sites than petiole explants for *Agrobacterium* infection.

The *egfp* reporter gene provides a useful visual marker for monitoring genetic transformation in plants. It permits direct observation of transgene expression in living cells, without treatment with any exogenous chemicals, which simplifies and improves the evaluation of transformation events in real time. Therefore, it has been used as a GUS gene replacement for genetic transformation (Stewart 2001). In the present study, the high level of GFP expression was observed in different stages of embryogenesis and also all plant organs (Fig. 3). This is the first report of genetic transformation of a monocotyledonous *E. aureum* with both *egfp* and *hpt* marker genes that were previously designed for transformation in grape (Li et al. 2001) and citrus (Dutt and Grosser 2009).

Determining transgene copy number is an important step in characterization of transgenic plants. Southern blot analysis has been the most commonly used method for copy number assessment. However, southern blot analysis is time-consuming, laborious, and requires large amounts of genomic DNA. QRT-PCR analysis has been adapted as an alternative method for copy number determination (Song et al. 2002; Dutt et al. 2008). Our studies indicate that QRT-PCR was able to determine *egfp* gene copy numbers in transgenic *E. aureum* 'Jade' plants. The tested transgenic *E. aureum* 'Jade' plants contained either 1 or 2 copies of *egfp* gene, which indicated that *Agrobaterium*-mediated transformation had a lower copy number of transgenes (Smith and Hood 1995).

In summary, this study established a protocol for efficient regeneration of *E. aureum* 'Jade' via direct somatic embryogenesis and developed a reliable procedure for *Agrobacterium*-mediated transformation of the *egfp*. The achieved highly efficient regeneration and transformation systems offer a new avenue for improving this monocotyledonous species. In addition to enhancing its aesthetic value as an important ornamental crop, engineering its capacity to reduce indoor volatile organic compounds could greatly expand its commercial production and utilization as an interior plant. Because pothos does not flower in nature, genetic engineering will not cause genetic contamination resulting from gene flow. Furthermore, the stable GFP expression demonstrated in this study may provide a valuable tool for investigating developmental aspects of somatic embryogenesis for this species.

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Conflict of interest The authors declare that they have no conflict of interest.

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