Establishment of High-frequency Genetic Transformation Method of Indonesian Orchid Species Mediated by *Agrobacterium tumefaciens*

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ABSTRACT

The development of an efficient methodology for the genetic transformation of orchids is needed in order to support the genetic engineering of orchids. It is therefore important to identify those factors affecting the transformation process. Previously, we reported a convenient method for the transformation of *Phalaenopsis amabilis* using *Agrobacterium tumefaciens*, in which intact protocorms were used.We also found that embryos cultured on a medium containing tomato extract grew more rapidly than those cultured on a medium with coconut water.When we used protocorms grown on a medium containing tomato extract, we obtained regenerated shoots that had been transformed with a kanamycin resistance gene at relatively high frequencies (7 – 17%).These results suggest that the rate of growth of pre-cultured protocorms may be important for the successful regeneration of transformed shoots. We also obtained regenerated shoots that had been transformed with the green fluorescent protein (GFP) gene at a high frequency (10 – 14%). Both the presence and expression of these transgenes were confirmed in transformed plants by molecular analyses and by the detection of green fluorescence following excitation with blue light. We further applied this method to Indonesian orchid species.

INTRODUCTION

It is important and of practical value for the orchid industry to generate novel traits such as improved floral characters to satisfy consumer demand and appreciation for aesthetics and novelty. A common trend in current orchid biotechnology is the application of molecular techniques for orchid improvement, which allows the introduction of desirable traits by introducing specific genes. The core component of the molecular breeding of orchids is the need to create efficient and reproducible gene transformation systems. A reproducible methodology for the genetic transformation of orchids, and better recognition of the factors affecting the transformation process, are needed in order to support this objective. Previous studies have reported orchid transformation either directly through the delivery of marker genes such as those encoding *Escherichia coli* β -glucuronidase (*GUS*) and *Aequorea victoria* green fluorescent protein (*GFP*) into plant cells by particle bombardment (Anzai *et al*. 1996), or indirectly through the use of *Agrobacterium tumefaciens* (Belarmino and Mii, 2000; Chia *et al*., 1994; Mishiba *et al*., 2005; Chan *et al*., 2005; Sjahril *et al*., 2006; Sjahril and Mii, 2006). Recently, we have developed a convenient method for genetic modification of *Phalaenopsis amabilis* orchid using *A. tumefaciens* (Semiarti *et al*., 2007) in which intact protocorms (young orchid seedlings) were used for transformation.This method is also simple, reproducible, and applicable in other species. However, the transformation efficiency was ≤2%, and further studies were needed to improve this.

For *in vitro* germination of orchid seed, organic substances such as coconut water and tomato extract are commonly used as media supplements. The presence of anti-oxidants such as

vitamin C, sugars, and other compounds in tomato extracts could promote the germination and growth of protocorms (Arditti and Ernst, 1993). Perl *et al*. (1996) determined that a combination of polyvinylpolypyrrolidone (PVPP) and dithiothreitol (DTT) as anti-oxidants improved plant viability. Tissue necrosis in *Agrobacterium*-treated embryogenic calli of grapevine plants was inhibited completely by using these anti-oxidants, while the virulence of *Agrobacterium* remained unaffected. These treatments enabled the recovery of stable transgenic grapevine plants resistant to hygromycin.

In the present study, in order to improve the frequency of *Agrobacterium*-mediated transformation of *P. amabilis*, we pre-cultured the protocorms on medium containing an extract from fully-ripe tomato fruit and investigated the effect of this pre-culturing treatment on improving the efficiency of regeneration of transformed shoots.

MATERIALS AND METHODS

Plant material, growth conditions, and culture medium

Adult plants of *Phalaenopsis amabilis* (L.) Blume from Java were obtained from Royal Orchids (Prigen, East Java, Indonesia). Seeds were derived from crosspollinated plants that had been sown on a modified, new *Phalaenopsis* (NP) medium (Islam *et al*., 1998) and maintained under continuous white light. Adult plants were maintained in a glasshouse at room temperature. Seeds were sown on modified NP medium with various concentrations of coconut water (50 – 150 ml l–1) and/or tomato extract (50 – 200 mg l–1) and grown for 3 weeks to produce protocorms, which were used for transformation. Coconut (*Cocos nucifera* from Java) and the tomato (*Lycopersicon esculentum*) cultivar 'Arthaloka' from West Java were obtained from local markets. Tomato fruit extract was prepared by cutting tomatoes into 1 cm3 cubes, homogenising them, and filtering the homogenate through a steel mesh with a 150-µm pore size. The nutrient compositions of the coconut water sample and the tomato extract were analysed by high performance liquid chromatography at the Food and Product Technology Laboratory of the Faculty of Agricultural Technology of Gadjah Mada University.

Developmental stages of P. amabilis

To determine the growth rates of orchid embryos and protocorms, the sizes, colours, and shapes of the embryos or protocorms were evaluated as described by Dressler (1981). At Stage 0, each intact seed (270 – 400 µm-long) with its embryo (100 – 200 µm-long) is coated by a layer of net-like cells, the testa. At Stage 1, the testa spreads apart and the embryo swells into an ovoid-shaped mass of cells. At Stage 2, the seed coat cracks and the mass of cells grows outside the coat (0.5 – 1.0 mm-long).At Stage 3, the mass of cells elongates gradually into a coneshaped body (1.0 – 1.4 mm-long). At Stage 4 (the protocorm), root hairs emerge from the basal portion of the cone-shaped body, which turns green. At Stage 5, the photosynthetic protocorm forms a leafy shoot at its apex and forms new root hairs. After Stage 5, seed germination is complete, two leaves gradually emerge and roots form.

Plasmid vector and bacterial strain

Using the binary plasmid vector pBI121 (Clontech Laboratories Inc., Otsu, Japan), containing a kanamycin resistance gene and the 35S CaMV promoter with the 3' *nos* terminator, a PCR-amplified fragment containing the entire coding region of the *GFP* gene was used to generate a plasmid that we designated pBI121-p35S::GFP. This construct was introduced into the disarmed, octopine-type *A. tumefaciens* strain LBA4404 (Hoekema *et al*., 1983).

Nucleic acid isolation and purification

Nucleic acids (genomic DNA, total RNA,mRNA, and cDNA) were prepared according to Semiarti *et al*. (2007).

Transformation and transformant regeneration

Transformants were obtained and regenerated using the methods described by Semiarti *et al*. (2007). Genomic DNA from putative 35S::GFP transformants was analysed by PCR using the following selective forward (F) and reverse (R) primers to detect both the kanamycin resistance gene (neomycin phosphotransferase II; *NPTII*) and the *GFP* gene: NPTIIF1 (5'-CCTGCCCATTCGACCACCAA-3') and NPTIIR1 (5'- AGCCCCTGATGCTCTTCGTC-3') for the *NPTII* gene; and GFPF1 (5'-ATGGTGAGCAAGGGCGAGGA-3') and GFPR1 (5'-GTCCATGCCGTGAGTGATCC-3') for the *GFP* gene. PCR was performed with 30 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 90 s. As an internal control, genomic DNA was amplified using primers for the *ACTIN* gene, as described by Semiarti *et al*. (2007). To detect *GFP* gene expression in the transformants, seedlings or

plant tissues were excited with blue light (495 nm) using a Nikon Diaphot 300 microscope (Nikon Corp., Tokyo, Japan) equipped with a B2 filter, which distinguished the red autofluorescence of chlorophyll from the fluorescence of GFP. The images were captured using a Nikon Cool Pix 5000 digital camera system, with adaptor for microscopy (Nikon Corp.).

DNA analysis by Southern hybridisation

Genomic DNA from 9-month-old leaves of five independent transgenic lines of *P. amabilis* that expressed GFP fluorescence was digested using the restriction enzymes *Eco* RI and *Hin*d III. These plants also yielded the predicted 360-bp PCR product using a primer pair designed for the *GFP* coding region. The digested genomic DNA fragments were transferred to a nylon membrane (Amersham Hybond-N+; GE Healthcare, Cambridge, UK) and hybridised with a digoxigeninlabelled probe for the *GFP* gene derived from the plasmid pBI121-GFP (12.6 kbp) using the DIG DNA Labeling Kit (Roche Diagnostics, Tokyo, Japan). The hybridised DNA fragments were visualised using the DIG Luminescent Detection Kit (Roche Diagnostics) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

***Effect of tomato extract on the formation of shoots from protocorms of* P. amabilis**

We tested coconut water and tomato extract as potential supplements to accelerate the growth of *Phalaenopsis* embryos, especially at the early developmental Stages, using embryos grown on NP medium with or without either supplement. Based on the growth Stage classification described above, we determined the optimal concentration of tomato extract based on the number of growing embryos and protocorms found at each Stage (Table I). The number of seeds developing to Stage 4 was increased at higher concentrations of tomato extract in the NP medium, achieving an optimal number at 100 – 150 mg l–1 tomato extract. Therefore, 100 mg l–1 tomato extract was used in the following experiments.

We also analysed growth rates on NP medium with or without coconut water and tomato extract (Figure 1A). The fastest rate of embryo development was observed on NP medium supplemented with both coconut water and tomato extract. Protocorms cultured on NP medium containing tomato extract alone appeared to change from yellow to green more rapidly than those cultured on NP medium containing coconut water alone. Tomato extract thus appeared to affect the growth rate at all Stages of embryo development, including the formation of the shoot apical meristem prior to the emergence of the leaf primordia. The tomato extract contained carotene, vitamin C, and other anti-oxidants which were not detected in coconut water (Table II). These components could affect growth of the embryo.

Oladiran and Iwu (1992) showed that fully-ripe tomato fruit contained basic nutrients and essential vitamins, as well as trace elements. Among these, carotenoids with cyclic end-groups were essential components of all photosynthetic membranes and played several roles, including protection against photo-oxidation (Cunningham *et al*., 1996). These are potential candidates for the growth-promoting compounds in the tomato extract, as it was rich in carotenoids. We therefore tested a single carotenoid, lycopene, for its possible effects on growth promotion, but found no significant effect at concentrations typically found in tomato extracts [≤ 0.1% (w/w)], while high concentrations of lycopene inhibited seed growth (data not shown). Further studies on other components found in tomato extract are needed to determine whether any single compound has an effect, or several compounds have a synergistic effect, on the growth and development of *P. amabilis* seed.

***Effect of pre-culture of protocorms on NP medium containing tomato extract on the transformation frequency of* P. amabilis**

Protocorms were pre-cultured on NP medium supplemented with coconut water and/or tomato extract, prior to transformation, to determine the effects of pre-culture supplementation on the frequency of transformation (Table III). The transformation efficiency was determined based on the percentage of protocorms that produced shoots on the selective medium out of the total number of protocorms examined.The transformation frequency of regenerated shoots was increased from 1.2% on NP medium with coconut water alone to 13.2% on NP medium containing 100 mg l–1 tomato extract alone, and to between 6.8 – 16.6% on NP medium containing both coconut water and tomato extract (Table III; Figure 1B, Panel D).These results were higher than the frequency of transformed regenerated shoots on medium containing coconut water alone (1.2%; Table III), confirming the observations made by Semiarti *et al*. (2007).

Table I. Growth stages of P. amabilis cultured on NP medium supplemented with different concentrations of tomato extract for 21 days after sowing

Concentration of tomato extract (mg l^{-1})	Total no. embryos examined	Experiment No.	Embryo Stage 0 (%)	Embryo Stage 1 (%)	Embryo Stage 2 (%)	Swollen embryo (protocorm) Stage 3 (%)	Green protocorm Stage 4 (%)	Protocorm with shoot apical meristem Stage 5 (%)
0		1	23.9	12.2	3.6	20.8	39.1	0.5
	806	2	18.7	5.4	7.4	30.5	36.9	1.0
		3	24.4	2.2	4.9	40.1	28.3	0.0
50		1	22.2	3.6	4.3	40.2	29.3	0.4
	1148	2	27.5	6.0	8.8	28.4	28.4	0.9
		3	28.0	6.9	4.0	28.7	31.5	0.9
100		1	24.9	4.7	5.5	13.0	51.1	0.8
	1577	2	31.3	1.9	6.1	9.0	51.2	0.5
		3	36.0	4.1	2.0	15.8	42.1	0.0
150		1	46.0	4.8	3.7	9.5	36.1	0.0
	1417	2	26.2	1.8	5.6	14.6	51.8	0.0
		3	35.5	2.9	4.4	15.0	42.3	0.0
200		1	49.6	3.1	2.8	9.7	34.7	0.0
	1583	2	62.6	7.8	3.6	6.0	20.1	0.0
		3	52.5	5.7	1.1	17.0	23.7	0.0

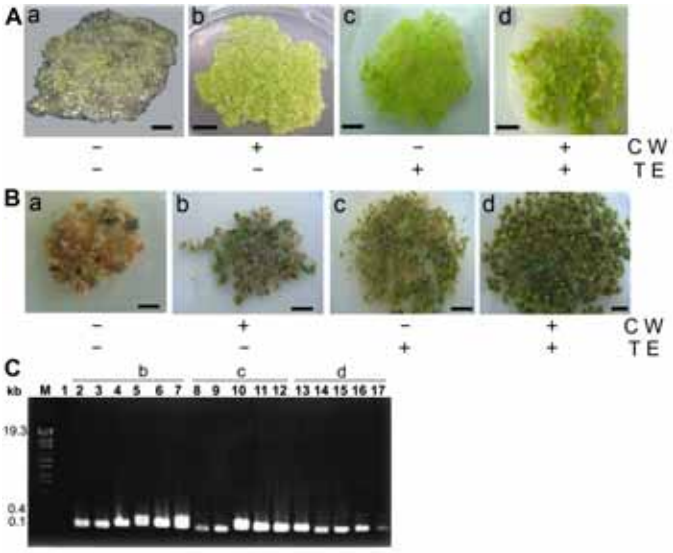

Fig. 1 Main Panel A, growth of protocorms of *P. amabilis* on various culture media 3 weeks after sowing. Sub-Panel a, NP medium; sub-Panel b, NP medium supplemented with 150 ml l–1 coconut water (CW); sub-Panel c, NP medium supplemented with 100 mg l–1 tomato extract (TE); and sub-Panel d, NP medium supplemented with 150 ml l–1 CW and 100 mg l–1 TE. Main Panel B, development of shoots from protocorms of *P. amabilis* that had been cultured on NP medium supplemented with coconut water (CW) and/or tomato extract (TE) for 3 weeks. Protocorms were selected from NP medium containing 200 mg l–1 kanamycin after *Agrobacterium*-mediated transformation with pBI121 after 5 weeks. Sub-Panel a, unregenerated protocorms on medium containing 200 mg l–1 kanamycin (Km); sub-Panels b–d, kanamycin-resistant seedlings produced from protocorms that had been transformed with pBI121 containing the kanamycin resistance gene (*NPTII*). Main Panel C, PCR detection of the kanamycin resistance gene (*NPTII*) in putative transgenic orchid plants harbouring pBI121. Fragments from a *Sty* I digest of phage DNA were used as size markers (M). No amplified DNA fragments from the kanamycin resistance gene were seen in DNA from untransformed *P. amabilis* orchid plants (lane 1). The specific 105-bp PCR fragment of the *NPTII* gene was amplified from DNA of putative transgenic orchid seedlings from sub-Panels b (lanes 2 –7), c (lanes 8 – 12), and d (lanes 13 – 17) in Main Panel A. Bars = 5mm.

Table II. Components in coconut water and tomato extract used in this study

Component	Coconut water	Tomato extract
Ash	0.55%	0.31%
Lipid	0.05%	0.47%
Total protein	0.19%	1.78%
(soluble protein)	(0.17%)	(1.46%)
Total sugars (reducing sugars)	3.22% (3.02%)	3.70% (3.39%)
Total carotene	Nd	1.84%
Antioxidants (DPPH)*	Nd	0.024%
Vitamin C	Nd	0.042%
Crude fibre	Nd	1.05%
Phosphate (P_2O_5)	0.013%	0.13%
Inorganic ions		
Mg^{2+}	0.0058%	0.0081%
Mn^{2+}	0.00021%	0.000029%
Na^{+}	0.046%	0.0090%
K^{+}	0.23%	0.16%
pH	5.16	4.34

*An ingredient that prevents oxidation; DPPH, 1,1-diphenyl-2-picrylhydrazyl. The samples used were coconut water from *Cocos nucifer*a from Java and the tomato cultivar Arthaloka (*Lycopersicon esculentum* from West Java). All values are in percentage (w/w). All samples contained 91 - 95% (w/w) H_2O. Values are based on the average of duplicate samples. Nd, not detectable.

In the case of transformation with pBI121-p35S::GFP, transformed regenerated shoots were produced at frequencies of 9.8 – 13.5% following pre-culture on NP medium supplemented with both coconut water and tomato extract (Table III). Overall, the transformation frequencies of protocorms pre-cultured on NP medium supplemented with tomato extract alone, or with both coconut water and tomato extract were higher than that of protocorms pre-cultured on NP medium supplemented with coconut water alone, suggesting that the growth rate of protocorms was related to the pre-culture conditions which are therefore important for the regeneration of transformed shoots.

Several studies have examined the use of rich sources of nutrients, vitamins, and phytohormones, including coconut water, carrot, maize, or potato extracts, as possible supplements for stimulating the germination of various orchid species (Arditti and Ernst, 1993; Raghavan, 1997; Islam *et al*., 2003; Mishiba *et al*., 2005; Chansean and Ichihashi, 2007). More studies on other sources of nutrients may be required to establish an optimum method for transformation.

Effect of pre-culture of protocorms on NP medium containing tomato extract on the transformation frequency of Vanda tricolor var suavis

We used this transformation method to Pandanus orchid (*Vanda tricolor var Suavis* Form Merapi). Protocorms were pre-cultured on NP medium supplemented with coconut water and tomato extract, prior to transformation. The transformation frequency of regenerated shoots was 20.3%. This result indicates that the transformation method may be useful for many other orchid species.

Molecular analysis of putative transformants

First, we examined the genomic DNA from *P. amabilis* plantlets regenerated on agar plates containing 200 mg l–1 kanamycin for the presence and expression of the kanamycin resistance gene (*NPTII*) using PCR. The predicted 105-bp fragment was amplified from all putative transformants in each treatment (Figure 1C).

The plantlets that regenerated after transformation with the plasmid pBI121-p35S::GFP were examined for the presence of the *GFP* gene by PCR amplification of the 360-bp fragment from the GFP coding region (Figure 2B). Of the 210 plantlets examined, 191 were positive for the *GFP* gene fragment.

To confirm the presence of the *GFP* gene, and to assess the gene copy number in plants that also showed kanamycin resistance, we performed Southern hybridisations. Hybridisation using an anti-sense probe for the 3' end of the *GFP* gene (Figure 2A) showed twoto- four copies of the *GFP* gene in each transgenic line (Figure 2C). Since the genomic DNA of each putative transgenic plant showed uniquely-sized bands hybridising to the *GFP* anti-sense fragment, this T-DNA fragment was confirmed to be inserted into the genome at different independent sites, and in multiple copies in each putative transgenic plant line.

For further analysis, we purified total poly(A)+ RNA from individual leaves of an untransformed wild-type plant, a plantlet transformed with pBI121, and three lines transformed with pBI121-p35S::GFP. We quantified the relative levels of *GFP* gene transcripts (mRNA) using RTPCR with the primers specific for *GFP*. PCR products were

detected in all three lines of plantlets transformed with pBI121-p35S::GFP, but not in the untransformed plantlet or the plantlet transformed with pBI121 alone (Figure 2D). Thus, transcripts of the *GFP* gene had accumulated in the leaves of the transformants, confirming expression of the *GFP* transgene in these plants. Plantlets transformed with pBI121-p35S::GFP showed green fluorescence after excitation with blue light (Figure 2E), whereas untransformed plantlets did not (Figure 2F).

Taken together, the molecular analyses of the transformants strongly suggests that supplementation using tomato extract during pre-culture in NP medium improved the transformation efficiency of *P. amabilis* several-fold.

Table III. *Transformation frequency of* P. amabilis *protocorms following 3 weeks of pre-culture on NP medium supplemented with tomato extract and coconut water*

Plasmid	Coconut water	Tomato extract	Total no. protocorms examined	No. protocorms producing shoots (% of total)
None	+	+	1557	0 (0%)
pBI121 (vector)	+	-	1200	14 (1.2%)
pBI121 (vector)	-	+	1200	159 (13.2%)
pBI121 (vector)	+	+	1557†	260 (16.6%)
			1500†	102 (6.8%)
pBI121-p35S::GFP	+	+	1557‡	210 (13.5%)
			1500‡	147 (9.8%)

†, ‡Data are from two independent transformation experiments in each case.

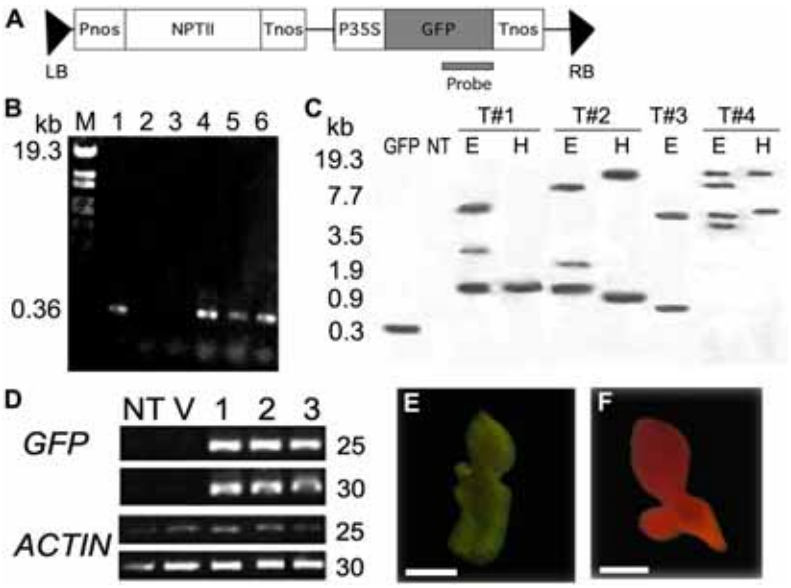

Fig. 2. Analysis of putative *GFP* transformants of *P. amabilis*. A: Schematic representation of the T-DNA region of the binary plasmid pBI121-p35S::GFP. The binary plasmid pBI121-p35S::GFP contained the 720-bp *GFP* gene, which encodes the jellyfish green fluorescent protein under control of the 35S promoter of cauliflower mosaic virus (CaMV). RB, right border; LB, left border; Pnos, promoter of the nopaline synthase gene; Tnos, polyadenylation site of the nopaline synthase gene; NPTII, neomycin phosphotransferase gene; P35S, 35S promoter of CaMV. A 360-bp fragment from the 3' end of the *GFP* gene was used as a probe during Southern hybridisation. B: PCR analysis of the *GFP* transgene in putative transgenic orchids. Fragments from a *Sty*I digest of λ phage DNA were used as size markers (M), the specific 360-bp DNA fragment was amplified from the plasmid pBI121-p35S::GFP (lane 1), and fragments were amplified from DNA of an untransformed *P. amabilis*

plant (lane 2), a plant transformed with the empty vector pBI121 (lane 3), and three plants independently transformed with pBI121-p35S::GFP (lanes 4 - 6). No fragment was amplified from DNA from untransformed plants (lane 3) or empty vector-transformed plants (lane 4). C: Southern blot analysis. Lane 1: Fragments from a *Sty*I digest of λ phage DNA as the size marker. GFP: GFP probe; NT: genomic DNA from transformant plant was digested with *Eco*RI; T#1, T#2, T#3 and T#4: genomic DNA (5 μg) from transformed plants was digested with *Eco*RI (E) or *Hind*III (H). Fragments were fractionated in a 1% agarose gel, then blotted and hybridised with a digoxigenin (DIG)-labelled *GFP* gene probe. D: Expression of the *GFP* gene in putative transgenic *P. amabilis* plants. RT-PCR analysis of transcripts of the *GFP* gene in a wild-type plant (NT), in a plant transformed with the empty vector pBI121 (V), and in pBI121-p35S::GFP-transformed plantlets (1 - 3). The number of cycles is indicated at the right of each panel. Amplified DNA fragments were separated by electrophoresis in an agarose gel and visualised with ethidium bromide. As a control, the same samples were amplified with primers specific for the *ACTIN* gene transcript. See the Materials and Methods for details about RT-PCR. E and F: Detection of GFP expression in putative 35S::GFP transformant plantlet (E) and untransformed plantlet (F) under blue light. Bars, 1 cm.

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アグロバクテリウム法によるインドネシアのラン科植物の高頻度遺伝子導入法の確立

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摘要

ラン科植物は一般に花が咲くまでの期間が長いため、高頻度の形質転換系を確立することは、応用研究においても、また基礎研究の上でもきわめて重要である。また、インドネシアでは、過剰採取と生息地の破壊によって絶滅の危機に瀕している種も多く、種の保全においても、種々のラン科植物に応用可能な形質転換系の確立が望まれている。我々は、既にアグロバクテリウム法を用いて *Phalaenopsis amabilis* の播種後３週目の若い実生であるプロトコームを用いた平易な形質転換体作製法を開発し報告した。今回、さらに、培地にトマト抽出液を加えることにより、生育速度を高く保った若い実生を形質転換に用いたところ、約 10%の高い効率で形質転換体が得られることがわかった。これらの形質転換体について、確かに遺伝子が導入されていることを PCR 法とサザンブロット法で確認した。また RT-PCR 法と蛍光タンパク質を指標として発現を確認した。さらに、*Vanda tricolor* においても高頻度で形質転換体が得られることがわかった。この方法は、今後、多くのラン科植物に応用できると期待される。