アグロバクテリウムを用いた *Phalaenopsis amabilis* の高頻度形質転換法の開発と応用

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Development of methods for *Agrobacterium*-mediated transformation of the wild orchid species *Phalaenopsis amabilis* and its application

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Abstract

Phalaenopsis hybrids constitute a major ornamental crop. An important parent species for many of these hybrids is *Phalaenopsis amabilis*. We developed a convenient method for the genetic modification of *P. amabilis* using *Agrobacterium tumefaciens*. The transformed intact protocorms, which are young orchid seedlings of *P. amabilis,* regenerated plants under the same conditions that showed the highest frequency of shooting. A kanamycin resistance gene under the control of the 35S promoter can be used as a selective marker. In addition, T-DNA vectors containing the *Arabidopsis* class 1 *KNOX* gene, *BP*/*KNAT1,* were successfully introduced into protocorms. Shoots were generated with an abnormal leaf shape that was easily distinguished from that of normal shoots, indicating that *BP*/*KNAT1* can be used as a visible marker gene. Furthermore, the protocorms transformed with *BP/KNAT1* produced multiple shoots. Both the presence and expression of the transgene in transformed plants were confirmed by molecular analysis. We further apply this method for generation of useful *Phalaenopsis* orchid plamts.

Introduction

Orchidaceae, popularly known as orchids, are the largest family of flowering plants. The numerous hybrids of mainly tropical origin are of great horticultural significance. The wild ancestor species of many of these hybrids are now endangered because of habitat destruction, especially through the loss of tropical lowland and montane primary forest (Dressler 1981). With respect to vegetative architecture, two main types of shoot organisation are often distinguished in orchids (even though these are not always clear-cut): sympodial and monopodial. Somewhat simplified, it can be stated that sympodial orchids consist of an indeterminate sequence of modules, each of which is of determinate growth, whereas monopodial orchids consist of a single module of indeterminate growth (Dressler 1981).

The monopodial orchid genus *Phalaenopsis* is a case in point. Hybrids of this genus are of great economic value as house and garden plants as well as cut flowers. At the same time, many wild species of *Phalaenopsis* are extremely rare in nature because of habitat loss as well as overcollection. The well-known *P. amabilis*, with its large white flowers, is one of the most important ancestor species of *Phalaenopsis* hybrids（Figure 1）. These hybrids are usually clonally propagated. A problem in this respect is the circumstance that seedlings initially form only a single vegetative shoot (Dressler 1981). However, additional shoots induced from cut protocorm-like bodies (PLBs) can be efficiently obtained using new *Phalaenopsis* medium, which contains a high concentration of nitrogen (Islam et al. 1998).

Development of a method for improving *Phalaenopis* orchids through genetic modification could be extremely valuable for horticulture and, indirectly, also for conservation. Establishment of transformation methods for *P. amabilis* is important to understand functions of genes and to manipulate them in *Phalaenopis* orchids.

Successful genetic transformation of *Phalaenopis* orchids has been reported, as well as that of other orchids (Belarmino and Mii 2000;

Knapp et al. 2000; Yu et al. 2001; Chai et al. 2002; Liau et al. 2003; Men et al. 2003; Mishiba et al. 2005; Chan et al. 2005). Although Agrobacterium-mediated transformation of *Phalaenopsis* hybrids has been established using protocorms (Chai et al. 2002; Mishiba et al. 2005; Chan et al. 2005), transformation of *P. amabilis,* which is the parent species for many *Phalaenopis* hybrids, has not been described.

Furthermore, only the hygromycin-resistance and β-glucuronidase genes were applicable as markers in *Phalaenopsis* hybrids (Belarmino and Mii 2000; Cai et al. 2002; Mishiba et al. 2005; Chan et al. 2005). It is also useful to develop selective markers containing a visible marker, which can be used to easily distinguish transformants from escaped normal shoots. The *BP*/*KNAT1* gene is primarily expressed in the region around the shoot apical meristem (SAM) and regulates SAM development in *Arabidopsis thaliana* (Kerstetter and Poethig 1998, Byrne et al. 2002). Since *Arabidopsis* plants transformed with the *BP*/*KNAT1* gene driven by the 35S promoter produce highly abnormal leaf morphology, including severely lobed leaves (Lincoln et al. 1994, Chuck et al. 1996), *BP*/*KNAT1* can be used as a visible marker for transformation experiments.

In this study we report a method for genetic transformation mediated by *Agrobacterium tumefaciens* using *P. amabilis* protocorms as the source of plant material. The kanamycin resistance gene and the *Arabidopsis BP*/*KNAT1* gene driven by the 35S promoter were successfully used as marker genes. In addition, protocorms transformed with the *BP/KNAT1* gene formed multiple shoots from a single embryo. We discuss the development of transformation methods based on our results and its application.

Materials and methods

Plant materials and growth conditions

Phalaenopsis amabilis (L.) Blume (Java form) was used in this study. The plant material was obtained from Royal Orchids (Prigen, East Java, Indonesia). For phenotypic analyses, seeds were sown on modified new Phalaenopsis (NP) medium (Ishii et al. 1998). The cultures were maintained under continuous white light at 25°C. Adult plants were maintained in a glasshouse.

Plasmid vector and bacterial strain

To generate pG35S, two DNA fragments, one containing the promoter for 35S RNA from the cauliflower mosaic virus (P35S) and the other the terminator of the nopaline synthase gene, were amplified from pTH-2 (Chiu et al. 1996) by PCR. These two amplified fragments were introduced into the multiple cloning sites of the binary vector pGreen-BAR. The PCR-amplified fragment containing the entire coding region of *BP/KNAT1* cDNA was cloned in a pG35S binary vector to generate pG35SKNAT1. This construct was introduced into disarmed octopine type *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema et al. 1983).

Nucleic acid isolation and purification

Nucleic acids were isolated from the protocorms and leaves of wild-type or putative transgenic plants by the method of Semiarti et al. (2001) using the QIAGEN DNA purification kit for isolation of genomic DNA and QIAGEN RNeasy mini kit (QIAGEN GmbH, Germany) for isolation of total RNA. mRNA was isolated from total RNA using Dynabeads Oligo $(dT)_{25}$ (DYNAL, Norway) and cDNA was synthesized from the mRNA using the TimeSaver cDNA synthesis kit (Amersham Biosciences, USA). The cDNA was used as template for RT-PCR analysis.

Transformation and regeneration of transformants

Overnight cultures of *Agrobacterium* were diluted 1:4 (v/v) using NP liquid medium supplemented with 15% (v/v) coconut water, 2% (w/v) sucrose, 5 µM benzyladenine and 0.01% silvet L-77 (Nippon Unicar Co. Ltd., Tokyo). Prior to modification, orchid protocorms were cultured for 4 days in modified NP solid medium containing 1 mg l^{-1} 2,4-D, then immersed in the diluted culture of *Agrobacterium* for 30 min. These protocorms were transferred onto sterile filter paper to dry and then onto modified NP medium containing 1 mg l^{-1} 2,4-D without antibiotics. After 4 days, the explants were transferred onto solid NP medium containing 1 mg l^{-1} 2,4-D and 300 mg l^{-1} carbenicillin, which inhibits the growth of *Agrobacterium*, and cultured for 4 weeks. Protocorms that grew were rinsed thoroughly with liquid NP medium containing 200 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin, then transferred onto medium containing 5 µM 2-IP, 0.15 µM NAA, 200 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin. The developing protocorms were transferred onto new medium every 3 weeks for further selection of transformants. Developing shoots were screened by PCR using a *BP*/*KNAT1*-specific primer to confirm that they were transformants. When the shoot and roots had grown sufficiently, the plantlets were transferred onto NP medium supplemented with 100 mg l^{-1} kanamycin and 50 mg l^{-1} carbenicillin.

Polymerase chain reaction analysis of transformants

Genomic DNA from the putative 35S::*BP*/*KNAT1* transformants was analyzed by PCR using primers KNAT1F1 and KNAT1R1, which are specific for the *BP*/*KNAT1* gene: KNAT1F1, 5'-CTTCCTAAAGAAGCACGGCAG-3';

KNAT1R1, 5'-CCAGTGACGCTTTCTTTGGTT-3'. PCR was performed with 30 cycles of 94˚C for 1 min, 60˚C for 30 s, and 72˚C for 1 min 30 s. PCR products were separated in a 0.7% agarose gel, stained with ethidium bromide, and visualized under UV-transillumination. To detect expression of *BP*/*KNAT1* transcripts in putative 35S::*BP*/*KNAT1* transgenic plants, RT-PCR was performed using the primers specific for the *BP*/*KNAT1* gene. As an internal control for RT-PCR, the cDNA was amplified using primers for the *ACTIN* gene (accession number AY134752): ACTF1, 5'-ATGAAGATTAAGGTCGTGGCA-3'; ACTR1, 5'-TCCGAGTTTGAAGAGGCTAC-3'.

Results

Efficiency of transformation

We used a medium containing 200 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin, which appeared to be optimal (data not shown) for the selection of transformants. The first transformation used the construct pG35S, which contains the kanamycin resistance gene. The experiments were performed 2 times. The protocorms that had been cocultivated for 4 days with *Agrobacterium* harbouring pG35S produced shoots at frequencies of 1.7 and 1.5% (Table 1, lines 3, 4) on medium containing kanamycin. We obtained 35 shoots out of 2,150 protocorms on medium containing kanamycin and carbenicillin after cocultivation with *Agrobacterium* harbouring pG35S in these experiments. The thirty-five shoots were independent, since each protocorm produced only one shoot (Figures 2D, E).

Next, we constructed the plasmid pG35SKNAT1 containing the *BP/KNAT1* gene of *Arabidopsis*, which is a member of the class 1 *KNOX* homeobox gene family. When protocorms were cocultivated with *Agrobacterium* harboring pG35SKNAT1 for 4 days, shoots were produced at frequencies of 0.1 and 0.3% (Table 1, lines 5, 6) on medium containing kanamycin and carbenicillin. When the protocorms were transformed with pG35SKNAT1, however, multiple kanamycin-resistant shoots formed simultaneously from each protocorm (Figures 2G, H). Careful observation of the surface of the protocorms showed that developing protocorms had many protrusions (Figures 2I, J, K). All of the shoots developed into intact plantlets. The 12 starting protocorm lines have been regenerated into 139 plantlets (Table 1). These plants were confirmed to be transgenic by cultivation on kanamycin selection medium.

Molecular analyses of transformants

The presence of the *BP/KNAT1* gene was examined by PCR amplification of a 1.2 kb fragment in the coding region of the *BP/KNAT1* gene. As shown in Figure 2O, only 1.2 kb DNA fragments were amplified in #3-1 and #4-1 plantlets. For further analysis, we purified poly $(A)^+$ RNA in individual leaves of wild type, #3-1 and #4-1 plantlets, and attempted to quantify relative levels of transcripts for *BP/KNAT1* using RT-PCR with primers specific for the *BP/KNAT1* cDNA sequence. PCR products were detected from #3-1 and #4-1 plantlets but not the wild type (Figure 2P). Thus, transcripts of the *BP/KNAT1* gene had accumulated in leaves of the #3-1 and #4-1. These results suggest that the introduced *BP/KNAT1* gene was expressed in these putative transgenic plants.

Phenotypes in transgenic orchid plants that express the BP gene

All 35S::*BP*/*KNAT1* transgenic plants showed altered leaf shape. As shown in Figures 2L-N, we observed three types of altered leaf shape, namely trumpet-like (T), rectangular (R) and mediolaterally unopened (M) leaves. In contrast, untransformed *Phalaenopsis* plantlets (wild type) (Figure 2C) and plantlets transformed with pG35S were of normal phenotype (Figures 2D-F). The transformed multiple shoots continued to grow, maintaining their abnormal shoot organization by producing a set of leaves and adventitious roots (data not shown). These results suggest that abnormal leaf organization is generated by the expression of *BP*/*KNAT1* in *Phalaenopsis* leaves.

Discussion

Phalaenopsis hybrids have recently become a valuable ornamental crop, and to increase their potential, orchid breeding programs that can depend on the development of a method for genetic modification show great promise**.** Genetic transformation of plants by *Agrobacterium* has been successfully applied to various plants belonging to widely separated clades. Nevertheless, it is still difficult to apply this method to certain horticultural plants that cannot easily be clonally propagated. We have reported here a method of *Agrobacterium*-mediated transformation using protocorms of *P. amabilis*, the parent species of *Phalaenopsis* hybrids. The protocol described in this article is simple and reproducible. The improvements over previously published methods can be summarized as follows. (1) Intact protocorms were used for transformation. This is a simpler approach than methods using chopped and subcultured protocorms or PLBs as described elsewhere (Liau et al. 2003; Men et al. 2003; Mishiba et al. 2005). (2) A kanamycin resistance gene can be used as a selective marker. (3) The 35S::*BP*/*KNAT1* construct is useful as a visible marker for transformation because it alters leaf

shape. Some of *35S::BP/KNAT1* transgenic plants, which formed relatively normal leaves, were transferred from medium into a community pot in the glasshouse, although they do not produced an inflorescence yet. Phenotype of flowers and seed formation of these plants should be investigated in future.

Unexpectedly, multiple shoots were generated with this gene. However, in any event this phenotype is useful for producing clonal transgenic orchids at the T1 generation, since many clonal shoots may be regenerated from one protocorm. We, however, have to confirm all the plantlets are clonal or not. If 35S::*BP*/*KNAT1* could be removed by a site-specific recombination system, such as R-*RS* (Onouchi et al. 1995; Toriyama et al. 2003) and cre-*loxP* (Albert et al. 1995; Vergunst et al. 2000; Zuo et al. 2001; Srivastava et al. 2004; Sreekala et al. 2005), the plant presumably would be able to develop leaves of normal shape.

Antisense *DOH1* expression also causes abnormal multiple shoot development in *Dendrobium* orchids, indicating a role for *DOH1*, another member(s) of the class 1 *KNOX* family, in their basic plant architecture (Yu et al. 2001). In addition, both *DOH1* sense and antisense transformants exhibit defects in leaf development (Yu et al. 2000; 2001). Since the transformation frequency using pG35SKNAT1 was one-seventh than that using pG35S, the *BP/KNAT1* gene might somehow affect efficiency of transformation. It may be possible that some of transformants that had defects of leaves severely did not grow. Although the function of members of the class 1 *KNOX* family is not known in *P. amabilis* plants, further studies using such transformed *P. amabilis* plants are also expected to lead to a better understanding of the function of genes that are involved in developmental processes, including shoot and leaf development.

It is well known that the overexpression of class 1 *KNOX* genes often results in abnormal plant morphologies in both dicot and monocot plants (Sinha et al. 1993; Lincoln et al. 1994; Tamaoki et al. 1997; Williams-Carrier et al. 1997; Sentoku et al. 2000). The transgenic Arabidopsis plants with *35S::BP/KNAT1* produced highly lobed leaves and leaves containing ectopic shoots (Lincoln et al. 1994; our unpublished results). While rice plants that were transformed with class 1 *KNOX* genes formed green organs with many shoot apices on their adaxial and bladeless leaves with normally developed leaf sheaths (Sentoku et al. 2000; Nagasaki et al. 2001). Difference between dicotylenonous plants and monocotyledous plants may reflect a fundamental difference in the plasticity of leaf cells (Williams-Carrier et al. 1997). In this experiment we showed that *Phalaenopsis* protocorms transformed with the *KNOX* gene of *Arabidopsis* produced multiple shoots with trumpet-like, rectangular, and mediolaterally unopened blade of leaves that were not observed in rice. Some of these phenotypes might be due to overexpression and ectopic expression of the *KNOX* gene. Alternatively these phenotypes might be produced by suppression of the function of endogenous genes by ectopic expression of the *Arabidopsis KNOX* gene. These possibility should be investigated in future by functional analysis of the class 1 *KNOX* genes of *P. amabilis*.

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Figure 1. *Phalaenopsis Amabilis.*
A 2.5-year-old adult plant. Bar, 5 cm.

Table 1. Frequency of transformation of *Phalaenopsis amabilis*

	Experiment	Number of protocorms examined	Number of protocorms producing shoots	Frequency of transformation (%)*	Number of regenerated plants
Non-transformant	1	100	0	0	0
	2	1557	0	0	0
pG35S	1	1150	20	1.7	20
	2	1000	15	1.5	15
pG35SKNAT1	1	1850	2	0.1	57
	2	3425	10	0.3	82

* Frequency of transformation was measured from the number of protocorms producing shoots per total protocorms examined

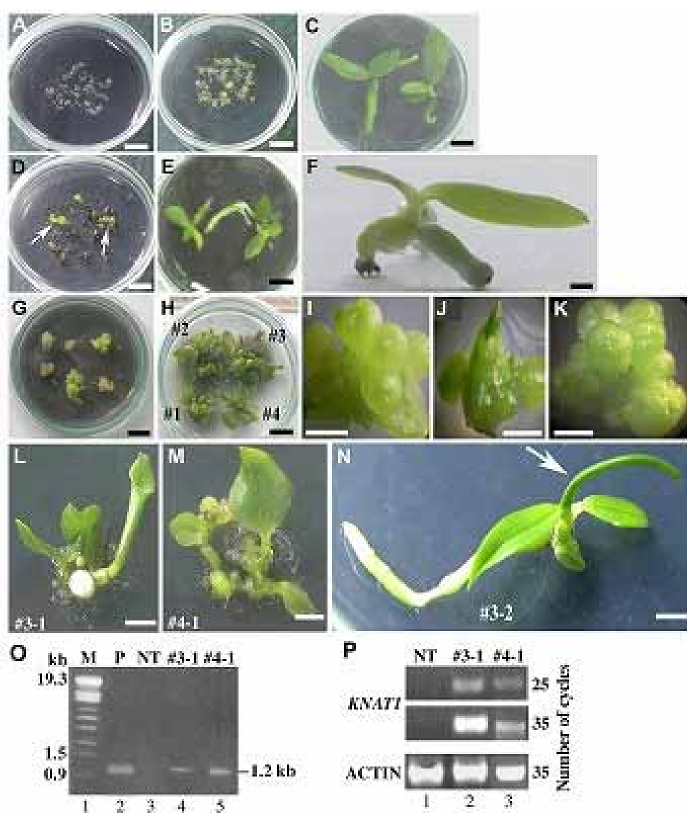

Figure 2. Phenotype of transgenic *P. amabilis* plants harboring 35S::*BP/KNAT1*.

(A) Unregenerated protocorms on medium containing 200 mg l^{-1} kanamycin. (B) Developing protocorms on antibiotic-free medium. (C) Normal seedling regenerated from a protocorm. (D) Regenerated protocorms (white arrows), 2 months after infection by *Agrobacterium* containing pG35S, growing on medium containing 200 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin. (E) Kanamycin-resistant seedlings produced from protocorms that were transformed with the pG35S vector, 6 months after planting. (F) Side view of a kanamycin-resistant seedling produced from protocorms that were transformed with the pG35S vector, 6 months after planting. (G) Shoot development from kanamycin-resistant PLB on medium containing 200 mg l^{-1} kanamycin after *Agrobacterium*-mediated infection with pG35SKNAT1, 2 months after transformation. (H) 4 lines that produced numerous shoots, 6 months after transformation. (I-K) Higher magnified views of H. (L-M) 35S::*BP/KNAT1*

putative transgenic *P. amabilis* lines #3-1, #4-1 and #3-2, which exhibit a trumpet-like leaf (L), a rectangular leaf (M) and a mediolaterally unopened leaf blade (white arrow) (N), respectively. Photographs were taken 6 months after transformation. (O) PCR analysis of the *BP/KNAT1* transgene in putative transgenic orchid. Fragment from a *Sty* I digest of λ phage DNA as size maker (lane 1), the specific 1.2-kb DNA fragment amplified from the plasmid pG35SKNAT1 (lane 2), #3-1 and #4-1 *P. amabilis* plant DNA (lanes 4 and 5, respectively). No fragment was amplified from untransformed plant DNA (lane 3). (P) Expression of the *BP/KNAT1* gene in putative transgenic *P. amabilis* plants. Reverse transcription-PCR analysis of transcripts of the *BP/KNAT1* gene in a wild-type *Phalaenopsis* plant (NT, lane 1), and in *Phalaenopsis* #3-1 and #4-1plantlets (lanes 2 and 3, respectively). The number of cycles is indicated at the right of each panel. Amplified DNA fragments were separated by electrophoresis in an agarose gel and visualized with ethidium bromide. Products of a control PCR were amplified with primers specific for the actin gene transcript. See Materials and Methods for details of the RT-PCR. Scale bars: 1 cm in (A-E, G, H), 2.5 mm in (F), 3 mm in (I-K), 5 mm in (L-N).

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Phalaenopsis amabilis は多くのコチョウラン栽培種の親株として使われている。我々は、*Agrobacterium tumefaciens* による *P. amabilis* 形質転換体の作製法を確立した。遺伝子導入には若い実生であるプロトコームを用いた。35S プロモーター制御下のカナマイシン耐性遺伝子が選択マーカーとして使えることを示した。さらに、シロイヌナズナの茎頂メリステムの維持に関わる class 1 *KNOX* 遺伝子群の一つである *BP/KNAT1* 遺伝子を導入したところ、野生型とは異なる異常な形態の葉をもつシュートが一つの実生から多数形成された。これらの形質転換体について、確かに導入遺伝子が発現していることを確認した。今後、有用な *Phalaenopsis amabilis* の作製を試みる。