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Original Paper

Agrobacterium-mediated transformation of the wild orchid species *Phalaenopsis amabilis*

Endang Semiarti[1,2], Ari Indrianto[1], Azis Purwantoro[3], Sulastri Isminingsih[2], Nilo Suseno[1], Takaaki Ishikawa[4,5], Yasushi Yoshioka[4], Yasunori Machida[4], Chiyoko Machida[5,6,*]

[1] Faculty of Biology, Gadjah Mada University, Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia; [2] Center Study of Biotechnology, Gadjah Mada University, Barek, Yogyakarta 55281, Indonesia; [3] Faculty of Agriculture, Gadjah Mada University, Sekip Selatan, Yogyakarta 55281, Indonesia; [4] Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan; [5] Plant Biology Research Center, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan; [6] College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan
*E-mail: cmachida@isc.chubu.ac.jp Tel&Fax: +81-568-51-6276

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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.

Key words: *Agrobacterium*, *KNOX*, multiple shoots, *Phalaenopsis amabilis*, transgenic orchid.

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. 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. 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.

Recently, successful genetic transformation of *Phalaenopis* orchids has been reported, as well as that of

Abbreviations: kb, kilo base-pairs; NAA, naphthalene acetic acid; NP medium, new Phalaenopsis medium; PLBs, protocorm-like bodies; SAM, shoot apical meristem; 35S, 35S RNA from the cauliflower mosaic virus; 2-IP, N^6- (Δ^2-isopentenyl) adenine.
This artic can be found at http://www.jspcmb.jp/

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; Chai 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. We first examined shoot formation conditions on shoot induction medium containing N^6-(Δ^2-isopentenyl) adenine (2-IP) and naphthalene acetic acid (NAA) for *P. amabilis* protocorms. The transformed protocorms regenerated plants under the conditions that showed the highest frequency of shooting. 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.

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 (Islam 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 (Asano et al. 2004). 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 LBA4404 (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′-ATGAAGATTAAGGTCGT-GGCA-3′; ACTR1, 5′-TCCGAGTTTGAAGAGGCTAC-3′.

Results

Shoot formation in Phalaenopsis amabilis

The life cycle of *P. amabilis* is about 2.5 years. *Phalaenopsis* seeds mature at 16 weeks after pollination. Transverse sections of *Phalaenopsis* seeds show that they consist of an embryo surrounded by a transparent layer of dead cells, called a testa (TE) (Figure 1A). The seed contains no endosperm. For germination in a laboratory environment, seeds need to be planted on a sterile artificial medium (see Materials and methods). Figures 1A and 1B show the series of developmental changes from seed to mature plant. At 2 weeks after seed planting (WASP), a group of small cells that stain deeply with toluidine blue and contain large nuclei is seen at one pole of the embryo. At 3 WASP, the formation of the shoot apical meristem (SAM) is initiated and absorbing hairs (AH) form at the basal part of the developing embryo. At 3 WASP, a single leaf primordium emerges apically from the embryo. The developing embryo at this stage is called a protocorm. From 4 to 6 WASP, leaves successively emerge from the stem, and the normal monopodial shoot system is formed. At 36 WASP the plant can be transferred from medium into a community pot in the glasshouse. When a plant has produced 4 to 6 leaves, it becomes capable of producing an inflorescence. In *Phalaenopsis*, a flowering plant still continues to grow, so flowering is not a separate stage. At 53 WASP, the adult orchid plant starts producing an inflorescence, which carries 10 to 20 flower buds. At this time, leaves reach their maximum length and have the shape of a simple elongated ellipse. The stem is entirely covered by overlapping leaf sheaths. Adult plants usually have a short stem, about 5 to 6 cm in length. The elongated, branched inflorescence emerges from a leaf axil, puncturing the leaf-sheath, and remains in bloom for about 3 months.

Conditions for shoot formation

First we analyzed the conditions for shoot formation of *P. amabilis* from protocorms. Addition of cytokinin and auxin to shoot-induction medium increased the frequency of shooting from protocorms. Table 1 shows that the highest frequency of shooting (98%) from protocorms was observed with 5 μM 2-IP and 0.15 μM NAA treatment. This condition was used for regenerating transgenic orchids.

Efficiency of transformation

We used a medium containing 200 mg l^{-1} kanamycin and

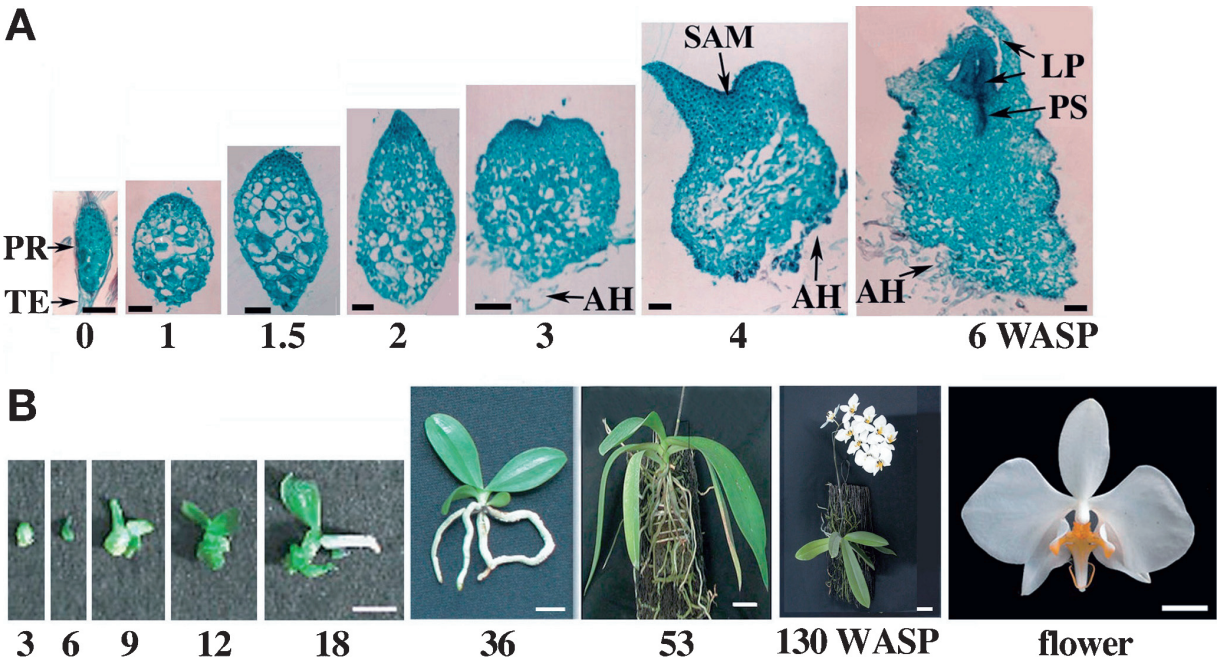

Figure 1. Shoot development of *P. amabilis*. (A) Development of orchid embryos *in vitro* after planting of seeds on modified NP medium. Longitudinal sections of embryos at 0–6 weeks after seed planting (WASP). PR, placental ridge; TE, testa; SAM, shoot apical meristem; AH, absorbing hair; LP, leaf primordium; PS, procambial strand. (B) *In vitro* cultured orchid protocorms and seedlings (3–36 WASP), adult plants (53–130 WASP) and a flower. Scale bars: 20 μm for 0 WASP, 40 μm for 1–3 WASP, 50 μm for 4–6 WASP in (A). 5 mm for 3–18 WASP, 1 cm for 36 WASP, 5 cm for 53 and 130 WASP and 2 cm for flower in (B).

Table 1. Optimal conditions for shooting from protocorms

Concentration of NAA (μM)	Number of protocorms examined	Number of protocorms producing shoots (frequency of shoot formation)						
		Concentration of 2-IP (μM)				Concentration of kinetin (μM)		
		0	1	3	5	1	3	5
0	60	12 (20%)	22 (37%)	27 (45%)	35 (58%)	15 (25%)	20 (33%)	33 (55%)
0.05	60	17 (28%)	25 (42%)	26 (43%)	32 (53%)	22 (37%)	30 (50%)	19 (32%)
0.10	60	6 (10%)	25 (42%)	32 (53%)	41 (68%)	30 (50%)	33 (55%)	27 (45%)
0.15	60	4 (7%)	35 (58%)	46 (77%)	**59 (98%)***	24 (40%)	43 (72%)	36 (60%)
0.20	60	4 (7%)	30 (50%)	43 (72%)	39 (65%)	15 (25%)	25 (42%)	20 (33%)

* Highest frequency of shoot formation.

Table 2. 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	0
	2	1557	0	0.0	0
pG35S	1 (3 days)**	350	0	0.0	0
	2 (7 days)	500	0	0.0	0
	3 (4 days)	1150	20	1.7	20
	4 (4 days)	1000	15	1.5	15
pG35SKNAT1	1 (4 days)	1850	2	0.1	57
	2 (4 days)	3425	10	0.3	82

* Frequency of transformation was measured from the number of protocorms producing shoots per total protocorms examined.
** Number in parenthesis indicates the period for *Agrobacterium* cocultivation with orchid protocorms.

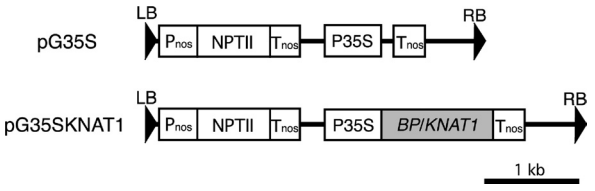

Figure 2. Structure of the T-DNA of plasmids pG35S and pG35SKNAT1. A binary plasmid pG35S was used as vector. The binary plasmid pG35SKNAT1 harbours the coding region of the 1.2 kb *Arabidopsis BP*/*KNAT1* gene under the control of the 35S promoter of cauliflower mosaic virus (CaMV). Right border, RB; left border, LB; promoter of the nopalin synthase gene, Pnos; polyadenylation site of the nopaline synthase gene, Tnos; neomycin phosphotransferase gene, NPTII; 35S promoter of CaMV, P35S.

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 (Figure 2). The experiments were performed 4 times. We obtained no transformants in the first two experiments, when protocorms were cocultivated with *Agrobacterium* harbouring pG35S for 3 and 7 days (Table 2, lines 3, 4). Since overgrowth of *Agrobacterium* was seen on the medium after cocultivation for 7 days, we shortened the time for cocultivation to 4 days to prevent overgrowth of *Agrobacterium.* In the third and fourth experiments, the protocorms that had been cocultivated for 4 days with *Agrobacterium* harbouring pG35S produced shoots at frequencies of 1.7 and 1.5% (Table 2, lines 5, 6) 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 3D, 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 2, lines 7, 8) on medium containing kanamycin and carbenicillin. When the protocorms were transformed with pG35SKNAT1, however, multiple kanamycin-resistant shoots formed simultaneously from each protocorm (Figures 3G, H). Careful observation of the surface of the protocorms showed that developing protocorms had many protrusions (Figures 3I, J, K). All of the shoots developed into intact plantlets. The 12 starting protocorm lines have been regenerated into 139 plantlets (Table 2, Table 3). These plants were confirmed to be transgenic by cultivation on kanamycin selection medium.

Molecular analyses of transformants

We first examined the presence of the kanamycin-resistance gene in the genome of all plantlets that were regenerated on plates containing kanamycin using PCR. The fragments were amplified in all plantlets we examined (data not shown). Then the presence of the

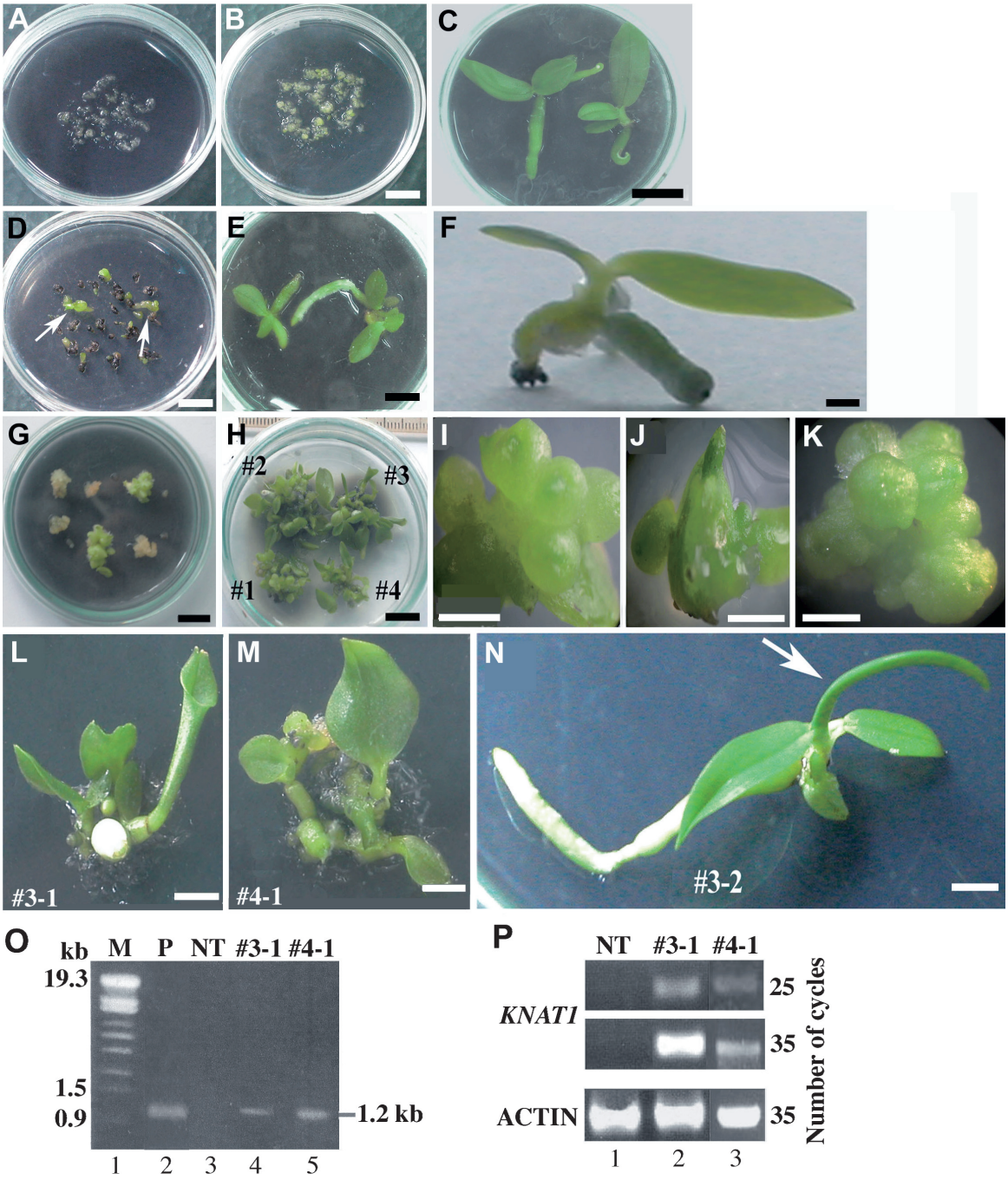

Figure 3. Phenotype of transgenic *P. amabilis* plants harboring 35S::*BP*/*KNAT1*. (A) Unregenerated protocorms on medium containing 200 mg l^{-1} kanamycin. (B) Developing protocorms on antibiotic-free medium. (C) Normal seedling regenerated from a protocorm. (D) Regenerated protocorms (white arrows), 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 and 300 mg l^{-1} carbenicillin 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–N) 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).

Table 3. Phenotype of 35S::*BP/KNAT1* transformants of *Phalaenopsis amabilis*

Protocorms producing Shoot	Number of regenerated plants	Shape of leaves *
35S #1	1	Normal
35S #2	1	Normal
35S #3	1	Normal
35S::*BP*/*KNAT1* #1	28	Altered (T, R)
35S::*BP*/*KNAT1* #2	29	Altered (T, R, M)
35S::*BP*/*KNAT1* #3	11	Altered (T, R, M)
35S::*BP*/*KNAT1* #4	6	Altered (T, R)
35S::*BP*/*KNAT1* #5	9	Altered (T, R, M)
35S::*BP*/*KNAT1* #6	4	Altered (R)
35S::*BP*/*KNAT1* #7	22	Altered (T, R, M)
35S::*BP*/*KNAT1* #8	4	Altered (R, M)
35S::*BP*/*KNAT1* #9	8	Altered (T, M)
35S::*BP*/*KNAT1* #10	5	Altered (T, R, M)
35S::*BP*/*KNAT1* #11	8	Altered (T, M)
35S::*BP*/*KNAT1* #12	5	Altered (R, M)

* T=Trumpet-like; R=Rectangular, M=Mediolaterally unopened leaf blade. Leaf shape of transgenic plants was observed 12 months after transformation.

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 3O, only 1.2 kb DNA fragments were amplified in #3-1 and #4-1 plantlets. When we used primers specific for 0.7 kb fragments outside of the T-DNA border of the Ti plasmid vector, no fragments were amplified (data not shown). These data suggest that the *BP/KNAT1* gene was inserted into the genome of these 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 3P). 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 Table 3 and Figures 3L–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 3C) and plantlets transformed with pG35S were of normal phenotype (Figures 3D–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) The same medium used for optimal induction of shoots from protocorms (a medium containing 5 μM 2-IP and 0.15 μM NAA), was used for regeneration of transformed protocorms, so medium changes are minimized. (4) A kanamycin resistance gene can be used as a selective marker. (5) The 35S::*BP*/*KNAT1* construct is useful as a visible marker for transformation because it alters leaf shape. 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. Some of transformants that had severe defects of leaves might not have grown. 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 known that the overexpression of class 1 *KNOX* genes results in abnormal morphology in both dicot and monocot plants (Lincoln et al. 1994; Williams-Carrier et al. 1997; Tamaoki et al. 1997; Nishimura et al. 2000; Sentoku et al. 2000). Since overexpression of these genes results in the formation of ectopic shoots or organs with ectopic shoot apices (Lincoln et al. 1994; Nishimura et al. 2000; Sentoku et al. 2000), these genes are thought to have an ability to control the indeterminate state of leaf cells in both plant species. Since effects of overexpression of class 1 *KNOX* genes on the phenotypes in monocotyledonous plants are, however, slightly different from those of overexpression of these genes in dicotyledonous plants, it has been proposed that there might be a fundamental difference in the developmental plasticity of the leaf cells of both plant species (Williams-Carrier et al. 1997). Transgenic rice plants transformed with *OSH1*, a *KNOX* gene of rice, form green organs with shoot apices on the leaves, bladeless leaves, and normal leaf sheaths and blades without ligules and show diffusion of the blade–sheath boundary in the leaves, which is explained by the concept that ectopic expression of the *KNOX* genes induces more proximal sheath-like characteristics (the more indeterminate state) in the distal blade region (Sentoku et al. 2000). In the present study, transgenic *Phalaenopsis* protocorms that were transformed with *BP*/*KNAT1* produced multiple shoots with mediolaterally unopened blade of leaves (Figure 3N), which are similar to the bladeless leaves of the rice transgenic plants (Sentoku et al. 2000). The phenotype of the mediolaterally unopened blade can be similarly explained by a meristematic function of *BP*/*KNAT1*. The transgenic protocorms also produced multiple shoots with trumpet-like and rectangular-shaped leaf blades, which were newly observed in the present study (Figure 3L and M). These phenotypes might have been generated by over- and ectopic-expression of the *KNOX* gene from the heterologous system in *Phalaenopsis* plants. The trumpet-like leaf blade is observed when cell fate determination along the adaxial-abaxial polarity of leaves is disrupted (Xu et al. 2003). It has yet to be known that a *KNOX* gene is involved in the establishment of adaxial-abaxial polarity. It is interesting to investigate whether endogenous *KNOX* genes in this plant species might have additional functions.

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