In Vitro Cell.Dev.Biol.—Plant (2011) 47:702–709
DOI 10.1007/s11627-011-9370-7

MICROPROPAGATION

In vitro shoot induction and plant regeneration from flower buds in *Paphiopedilum* orchids

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Received: 4 October 2010 / Accepted: 29 April 2011 / Published online: 3 June 2011 / Editor: D. Tomes

Abstract *Paphiopedilum* species are recalcitrant in tissue culture, and no explant from mature plants has been successfully mass propagated *in vitro*. This study was aimed at inducing shoots and regenerating plants from the flowering plants of a sequentially flowering *Paphiopedilum* Deperle and a single floral *Paphiopedilum* Armeni White. By using cross-sectioned flower buds (FBs), we found that in both species, only sections that contained the base tissue of FBs were able to produce shoots and plants. We have also found that sections of FBs between 1.5 and 3.0 cm from *Paphiopedilum* Deperle were able to produce shoots, but only sections of FBs >2.5 cm from *Paphiopedilum* Armeni White were regenerable. Our microscopic observations revealed that the small bract at the FB base harbored a new miniature FB, which further harbored a primitive FB with dome-shaped meristem-like tissues that presumably led to the plant induction. The reiteration of this pattern resulted in a scorpioid cyme inflorescence architecture in the multifloral *Paphiopedilum* species, and its failure to reiterate resulted in a single flower. The induction rates were 57–75%, and all plants survived in a greenhouse. This method is potentially applicable for the micropropagation and conservation of slipper orchids.

Keywords *Paphiopedilum* orchids · *In vitro* shoot induction · Flower bud base · Inflorescence architecture · Micropropagation

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Introduction

Species of *Paphiopedilum*, commonly known as slipper orchids, are terrestrial orchids belonging to Cypripedioideae. Their flowers are extremely diverse in color, shape, and size, attracting orchid lovers worldwide. Because of overexploitation and habitat destruction, *Paphiopedilum* has become an endangered species, and all of its genera are prohibited from international trades among the fauna and flora formulated by the Committee for International Trade in Endangered Species (CITES 2008). Nearly all slipper orchids are traded as potted plants through *in vitro*-germinated seedlings from seed capsules of hybrids having their parental origins from South and Southeast Asia (Teob 1989). Although the vegetative propagation through lateral bud division is possible, it is extremely unproductive and unreliable for commercialization or conservation purposes. Micropropagation has the ability to massively produce plants at low costs and has been successfully employed for the commercialization of *Phalaenopsis* at an industrial level that dominated the potted orchids market of approximately US $140 million wholesale values in 2008 in the USA (USDA 2009). Because *in vitro*-propagated materials are exempt from the trade prohibition by CITES, micropropagation through tissue culture is an absolute requirement for the commercialization of slipper orchids beyond the level of small-scale private trades. The ability to

preserve the germplasm by tissue culture will also help the conservation of many wild slipper orchids that are close to extinction in their natural habitats. Unfortunately, there is a complete lack of *in vitro* propagation techniques suitable for the commercialization or conservation of slipper orchids because of the inability to regenerate mature plants.

The following two major problems contribute to the unsuccessful micropropagation of slipper orchids: (1) explants from mature plants of Cypripedioideae species are recalcitrant to shoot induction and plant regeneration (Arditti and Ernst 1993); (2) it is difficult to obtain aseptic explants from mature plants through normal surface sterilization steps for *in vitro* culture (Chugh *et al*. 2009) because of the long maturation time and high humidity growth conditions that are conducive to microbial contamination. To circumvent these obstacles, immature seeds have been used for shoot induction (Tomita and Tomita 1997). Unfortunately, these seeds easily lose their germinability within a short time once capsules are harvested (De Pauw and Remphrey 1993). Mature seeds can maintain their germinability for a long time (Lauzer *et al*. 1994; Whigham *et al*. 2006), and the resultant germinated seedlings can be kept sterile in culture. For these reasons, seeds and seedlings have been the most favorable explant sources for experimentation in tissue culture regeneration. Various tissues from *in vitro*-germinated seedlings have been successfully induced to produce callus, shoots, and plants (Huang 1988; Lin *et al*. 2000; Huang *et al*. 2001; Chen *et al*. 2004; Ng and Saleh 2010; Ng *et al*. 2010). Recently, callus and protocorm-like bodies capable of regenerating into plants were also induced directly from seeds of several *Paphiopedilum* species (Hong *et al*. 2008; Long *et al*. 2010) and a threatened Mexican orchid (Santos Díaz and Álvare 2009). However, tissue culture clones derived from seeds or seedling tissues often suffer a serious drawback because their genotypes are highly variable and produce phenotypes that are unpredictable and lack uniformity. Furthermore, it commonly takes 5 yr of greenhouse care to produce mature plants before their flower phenotypes are revealed. Even then, the few selected mature plants that are deemed valuable cannot be micropropagated.

The induction of callus and a few plants from the shoot apex of mature *Paphiopedilum* plants has been reported, but tissues did not survive upon subculture (Stewart and Button 1975). In any case, shoot tip culture requires sacrifice of the lateral bud, and it could stunt the growth of mother plants (Philip and Nainar 1986). Orchid plants produced from long *in vitro* culture periods through calluses are also highly vulnerable to somaclonal variation and prone to contamination as with *Phalaenopsis* (Chen *et al*. 1998).

The goal of this investigation was to explore the possibility of using dispensable flower buds from mature flowering *Paphiopedilum* plants as the explant source for tissue culture regeneration. We report here high-frequency direct induction of shoots and regeneration of plants from sectioned young flower buds (FBs) in two different *Paphiopedilum* hybrids. We have also analyzed the gross structure of the FB base and provide hypotheses of where the shoots or plants originate and how the inflorescence architecture of the *Paphiopedilum* species forms.

Materials and Methods

Plant materials. Potted plants of *Paphiopedilum* Armeni White (=*Paphiopedilum delenatii* × *Paphiopedilum armeniacum*) and *Paphiopedilum* Deperle (=*Paphiopedilum primulinum* × *P. delenatii*) were grown from *in vitro*-germinated seedlings for approximately 4–6 yr to the flowering stage in greenhouses. *Paphiopedilum* Armeni White plants produced single flowers about 7.6 cm in size that often last for 3 mo. Flowers appear as butter cream frosting white during the initial opening stage and become pure porcelain white with yellow marks on the staminode at full bloom. *Paphiopedilum* Deperle plants have mottled foliage and generally produce about three to five flowers also about 7.6 cm in size on an inflorescence that blooms sequentially for up to 3 mo.

Young FBs at various developmental stages before bud opening were severed approximately 5 mm below the base of FBs. FB lengths were measured from the bud tips to the cut ends and sorted into small (1.5–2 cm), medium (2.0–2.5 cm), and large (2.5–3 cm) size groups. Because of the scarcity of the flowering plants and the unavailability of a technique to synchronize the flowering time in slipper orchids, all available flowering plants were screened to select FBs that fit within the above size ranges.

Surface sterilization, sectioning, and initial culture of explants. The FBs were washed for 3 min in tap water, immersed in 1% sodium hypochlorite solution containing 0.1% Tween-20 in a glass beaker, and immediately sonicated at full power for 15 min in an ultrasonic cleaner (40 KHz Bransonic 8210R-DTH, Danbury, CT). This sonication step was critical in producing sterile explants for tissue culture. It was reported that low-frequency ultrasonication can greatly enhance the biocidal efficiency of sodium hypochlorite solution (Duckhouse *et al*. 2004), and our sonication condition was chosen after a variety of parameters were tested (unpublished data). The FBs were then rinsed three times with autoclaved distilled water, transferred to a sterile Petri dish (15 × 150 mm), and dried briefly in a laminar flow hood for several minutes. Beginning at the tip, each bud was cut perpendicularly to its axis into 0.5-cm cross-sections with a sharp scalpel. Sections were divided into the following

three tissue groups by visual examination: (1) bud—sections that contained only the FB without the bud base where the FB and the stalk joins; (2) base—sections that contained the FB base and that included at least one third of the bottom portion of the small bract; (3) stalk—sections that contained only the new flower stalk under the bud base without any bud base tissue.

The cross-sections were placed into Petri dishes containing a MS-¼N medium, which is a modified Murashige and Skoog (1962) culture medium by reducing the NH_4NO_3 and KNO_3 salts to 412.5 and 475 mg/l, respectively, and sucrose to 2%. The pH was adjusted to 5.5±0.1 and the medium solidified with 0.8% (*w/v*) agar (bacteriological grade; Sigma-Aldrich, St. Louis, MO). The medium was supplemented with 4.43 μM benzyl adenine (Sigma) and 4.52 μM 2,4-dichlorophenoxyacetic acid (Sigma) for the culture of *Paphiopedilum* Armeni White explants and with 44.39 μM benzyl adenine and 26.85 μM α-naphthaleneacetic acid (Sigma) for the culture of *Paphiopedilum* Deperle explants. The culture media, including all supplements, were autoclaved for 15 min under 1.05 kg/cm^2 at 121°C. All explant cultures were incubated at 25±1°C with 55–60% relative humidity in the dark for 6 mo.

Subculture of shoots and plants induced from initial flower bud explants. After the initial dark culture period, shoots or plants were transferred to 50-ml glass tubes (Kimble, Vineland, NJ; 22×150 mm, borosilicate glass) containing 10 ml of slanted MS-¼N medium with 1 gl^{-1} activated charcoal (Taipei Chemical Industry Co., Hsinchu, Taiwan) without any plant growth regulators, and the tubes were covered with two layers of aluminum foil at the top before autoclaving. The shoots or plants were placed vertically into the culture medium after removing the brown part of original explant tissues. The cultures were maintained at 25±1°C, 55–60% relative humidity, and 16-h photoperiod at 30 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Sylvania, Danvers, MA). After 12 wk without subculturing, the plants were transferred to 3-in. plastic pots with loosely packed New Zealand sphagnum and maintained in a greenhouse at 27±2°C with 70±10% relative humidity.

Anatomical study of flower buds in Paphiopedilum species. Young FBs were removed from the inflorescence and dissected for observation under a stereomicroscope (model SCW, Carton Optical Industries, Tokyo, Japan). Three different *Paphiopedilum* species were used: (1) *Paphiopedilum* Deperle; (2) *Paphiopedilum* Delrosi, which is a multiple floral-type primary hybrid from a cross between a multiple floral *Paphiopedilum rothschildianum* and a single floral *P. delenatii*; and (3) *Paphiopedilum* In-Charm Flame × *P. urbarnianum*, a single floral Maudiae-type hybrid.

Results

Shoot induction and plant regeneration from Paphiopedilum Armeni White and Paphiopedilum Deperle. The 15 young FBs from inflorescences of *Paphiopedilum* Armeni White collected at different flowering stages were relatively uniform in appearance within the three size groups (Fig. 1*a*). Each bud has three distinguishable tissue parts: bud, base with a large (removed before sectioning) and a small bract, and stalk. Under dark, explants from the base tissue of the large FB size group developed long etiolated shoots (Fig. 1*b*, bold arrow) or plants that consisted of both shoots and rudimentary roots (thin arrow). No callus formation was observed. The original explants turned dark brown, and brown exudates were observed in the medium surrounding the explant. The brown tissues were removed and the whitish shoots or plants transferred to MS-¼N medium without plant growth regulators. After incubation for 8 wk under light, plants with two green leaves and well-developed roots were clearly visible (Fig. 1*c*). Some explants produced two or three etiolated shoots (Fig. 1*d*, arrows), and they all became green plants upon transferring to the new medium and exposure to light (Fig. 1*e*). These plants developed normal roots with white root hairs (Fig. 1*f*, thin arrow) when subcultured in fresh medium and showed the characteristic pigmentation pattern of young leaves (bold arrow). When the cultures were exposed to light for 12 wk and then transferred to pots, the plants developed into normal plants with fully expanded leaves (Fig. 1*g*), with a survival rate of 100% as potted plants.

The young FBs of *Paphiopedilum* Deperle also had three distinct parts (Fig. 2*a*): bud (arrowhead), base with a large and a small bract (thin arrow), and stalk (bold arrow). Explants from the base tissue group produced etiolated plants (Fig. 2*b*, bold arrow) with rudimentary roots (thin arrow) after the initial dark incubation period; the original tissues turned dark brown. As observed in *Paphiopedilum* Armeni White, no callus was produced. After transferring to fresh medium and incubating under light for 8 wk, plants with expanded green leaves and enlarged roots with white root hairs were formed (Fig. 2*c*). Some explants produced etiolated plants with multiple leaves (Fig. 2*d*, bold arrows) and roots (thin arrow), and they looked phenotypically normal upon transfer to fresh medium and incubation under light (Fig. 2*e*).

Effects of tissue type and flower bud size on plant induction. In *Paphiopedilum* Armeni White, only explants from the base tissue group of the large FBs (2.5–3 cm) produced shoots or plants, and the induction rate was 60% (Table 1). In *Paphiopedilum* Deperle, induction also occurred only from the bud base tissue group; however, all three FB sizes were able to produce shoots or plants.

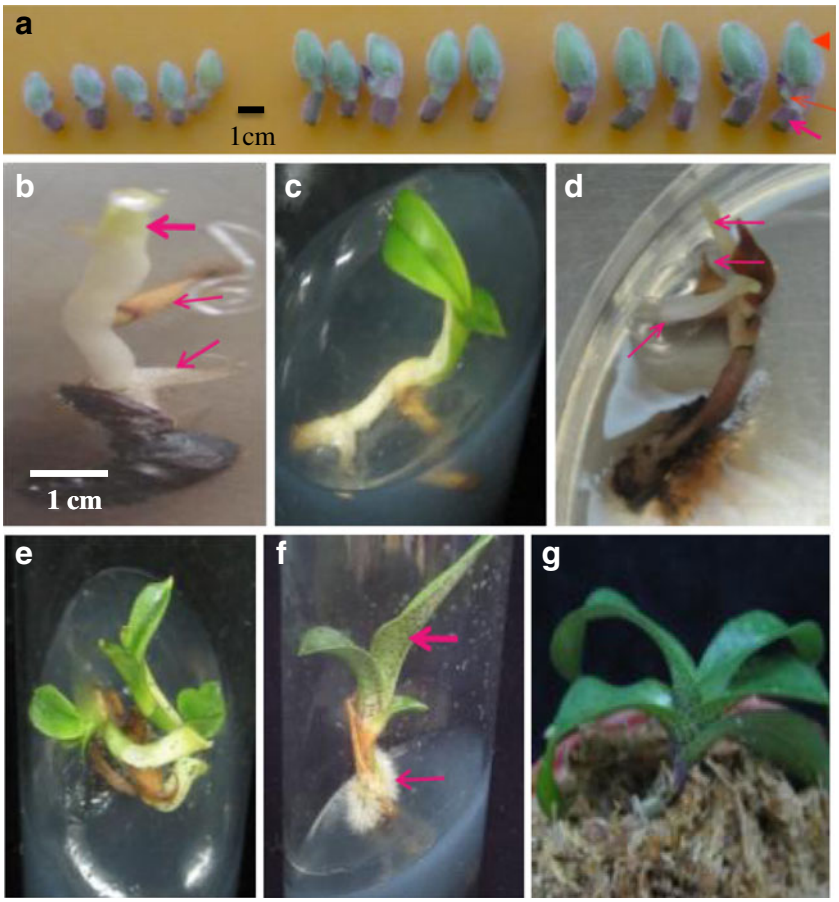

Figure 1. Induction of shoots and plants from *Paphiopedilum* Armeni White. (*a*) *Left*, *middle*, and *right panels* represent five buds each of small-, medium-, and large-sized groups, respectively; the positions of the three tissue types of bud, base, and stalk are indicated by *arrowhead*, *thin arrow*, and *bold arrow*, respectively. (*b*) Etiolated shoot (*bold arrow*) with roots (*thin arrows*) induced during the dark incubation period. (*c*) Green plant after incubation of an etiolated plant under light for 8 wk. (*d*) Multiple shoots (*arrows*) that were developed. (*e*) Green plants after exposing the etiolated shoots to light. (*f*) Well-developed plant with white airy root hairs (*thin arrow*) and pigmentation patterns on leaves (*bold arrow*). (*g*) Plant after transferring to a pot and grown in the greenhouse for 2 mo.

Large FBs had a higher induction rate (75%) than medium (57%) and small (60%) ones. In both hybrids, no shoot or plant was induced from either the bud or the stalk tissue group that did not contain bud base tissue where the small bract was located.

Gross anatomy of flower bud base. Since only sections that contained the FB base were able to produce shoots or plants, the FBs were dissected to analyze the gross structure of the bud base. In *Paphiopedilum* Deperle, the young FB (Fig. 3*a*, arrowhead) is located lateral to the base of the long ovary of an older FB that was about to open, and it has two unequal-sized bracts (Fig. 3*b*) with the base of the small bract sitting inside the large bract. The large bract is a simple leaf-like organ that later becomes the permanent bract for the FB when it blooms. The small bract had a new miniature FB sitting at its base (Fig. 3*c*), and the small bract later became the large bract (Fig. 3*c*, bold arrow) of the new miniature FB (arrowhead), which had its own miniature small bract (thin arrow). The color of this miniature FB ranged from whitish to light green, with the small ones lighter in color. Removing the new miniature small bract exposed a smaller and younger FB (Fig. 3*d*, empty arrowhead) at its base. Again, the new miniature small bract itself became the large bract (Fig. 3*d*, bold arrow) of this youngest FB due to the presence of an even more miniature-sized small bract (thin arrow), which appeared to emerge out of the base. The top of the youngest miniature FB appeared as a dome-shaped meristematic tissue (empty arrowhead).

The gross anatomical views of the multiple-flower-bearing-type *Paphiopedilum* Delrosi were similar to those of the sequentially flowering *Paphiopedilum* Deperle. There were also two unequally sized bracts at the node around the pedicel of a blooming flower (Fig. 3*e*). The large one (bold arrow) was a terminally differentiated tissue. The small one (thin arrow) contained a new miniature FB with a meristem-like, dome-shaped bud top (Fig. 3*f*, empty arrowhead) and a miniature small bract (thin arrow). Again, the small bract of the older FB became the large bract for the newer FB. This flower development pattern repeated until the number of flowers reached its limit of approximately five. Thus, the main stalk terminated in the first flower and the lateral meristem at the base of the small bract reiterated this pattern, forming a scorpioid cyme inflorescence architecture (Fig. 3*g*, the numbers 1, 2, 3 indicate the first, second, and third terminal flowers, respectively). Each repeat produced a noticeably thinner

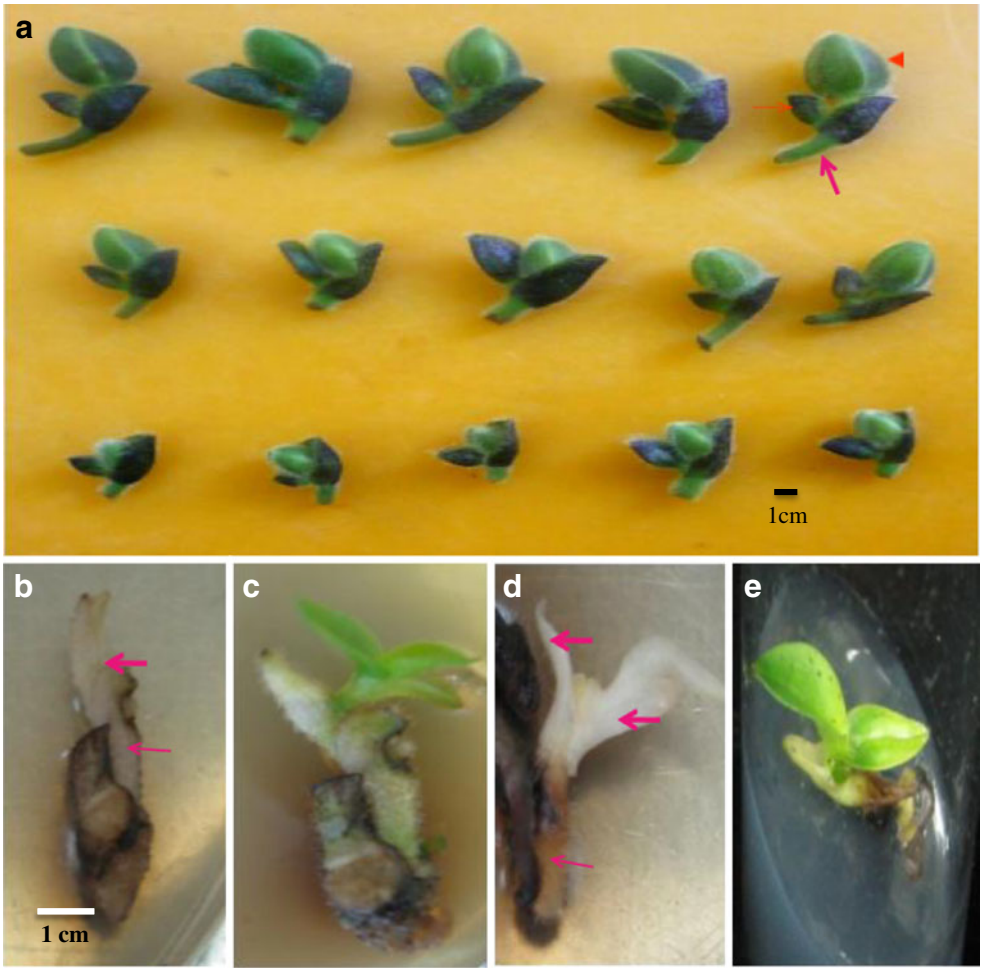

Figure 2. Induction of shoots and plants from *Paphiopedilum* Deperle. (*a*) *Upper*, *middle*, and *bottom panels* represent five buds each of the small-, medium-, and large-sized groups, respectively; the positions of the three tissue types of bud, base, and stalk are indicated by *arrowhead*, *thin arrow* and *bold arrow*, respectively. (*b*) Etiolated shoot (*bold arrow*) with rudimentary roots (*thin arrow*) that was induced during the dark incubation period. (*c*) Green plant with roots after incubating an etiolated plant under light for 8 wk. (*d*) Multiple etiolated leaves (*bold arrows*) that were developed from an explant. (*e*) Green plant after exposing an etiolated shoot to light.

stalk that terminated in the next flower. By the time the third FB bloomed, its stalk diameter was reduced to approximately one half that of the main stalk.

In the single floral species of Maudiae-type slipper orchid, a large bract wrapped around the pedicel at the flower node (Fig. 3*h*, bold arrow) in which a small bract (thin arrow) existed. Partially peeling off the large bract revealed the small bract (Fig. 3*i*, thin arrow), which harbored at its base a new miniature FB similar to those observed in the two other species described above. However, the flower parts of this miniature FB were less distinguishable and appeared to remain undeveloped or degenerated when the first FB bloomed. Two meristem-like, dome-shaped bud tops (Fig. 3*j*, empty arrowheads)

Table 1. Effect of tissue types and bud sizes on shoot induction and plant regeneration in two *Paphiopedilum* hybrids

	Tissue type	Bud size								
		2.5–3 cm			2–2.5 cm			1.5–2 cm		
		Total buds	No. with shoot/plantlet	Shoot Induction rate (%)	Total buds	No. with shoot/plantlet	Induction rate (%)	Total buds	No. with shoot/plantlet	Induction rate (%)
Paphiopedilum Armeni White	Bud	5	0	0	5	0	0	5	0	0
	Base	5	3	60	5	0	0	5	0	0
	Stalk	5	0	0	5	0	0	5	0	0
Paphiopedilum Deperle	Bud	8	0	0	7	0	0	5	0	0
	Base	8	6	75	7	4	57	5	3	60
	Stalk	8	0	0	7	0	0	5	0	0

Data were recorded after 6 mo of incubating the sectioned explants in MS-¼N medium

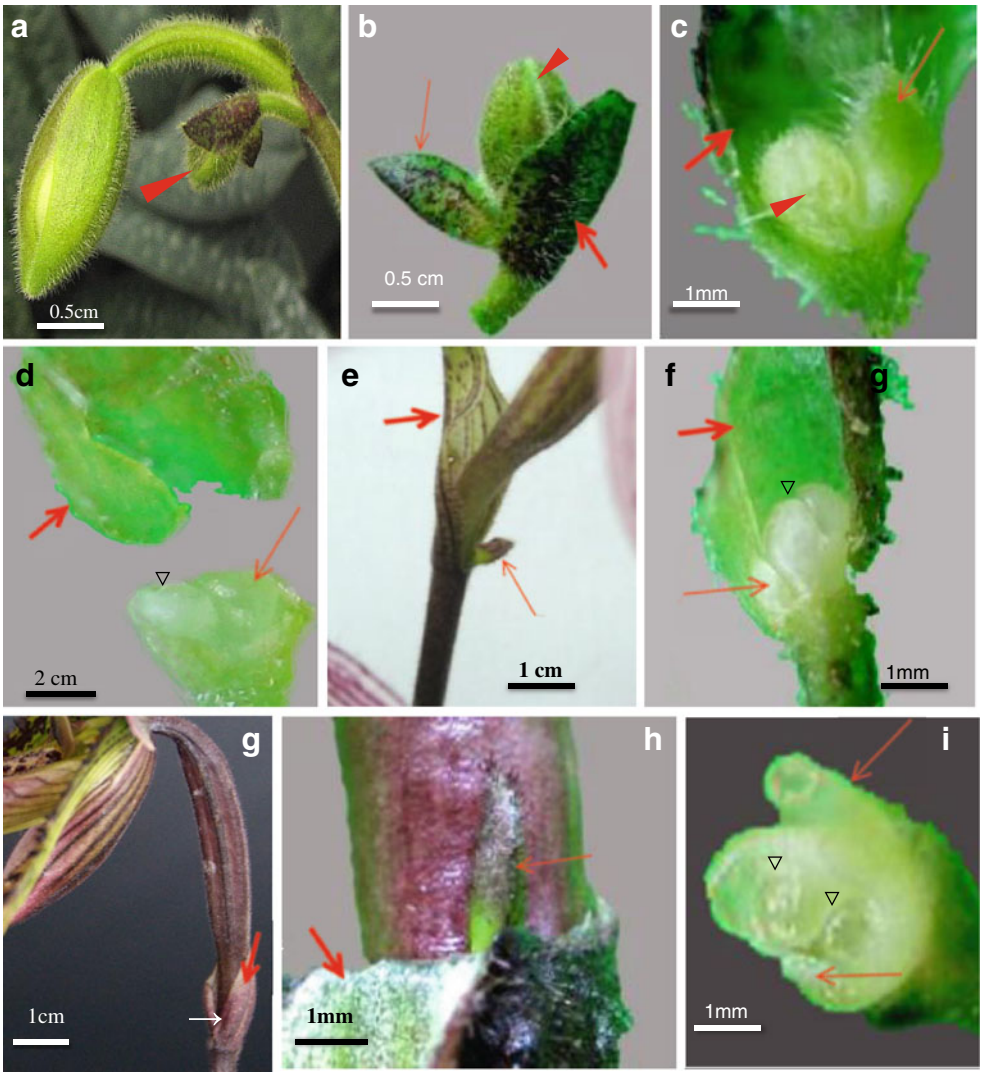

Figure 3. Gross anatomical views of three *Paphiopedilum* species. (*a–d*) *Paphiopedilum* Deperle. (*e*, *f*) *Paphiopedilum* Delrosi. (*g*) Scorpioid cyme inflorescence architecture of *Paphiopedilum* Deperle; *the numbers on the flowers* indicate the sequence of the terminal flowers that appeared. (*h–j*) *Paphiopedilum* In-Charm Flame × *P. urbarnianum*. *Solid arrowhead*, *thin arrow*, *bold arrow*, and *empty arrowhead* indicate FB, small bract, large bract, and dome-shaped meristem-like tissues, respectively.

with one larger than the other were observed. Each bud top had a primitive small bract (thin arrow) connected to it at the base as if there were two underdeveloped miniature FBs.

Discussion

Orchid tissue culture has been a leader in *in vitro* plant propagation when it began more than a century ago with the germination of *Phalaenopsis* seeds *in vitro* (Yam and Arditti 2009). However, the micropropagation of *Paphiopedilum* species from mature plants has not been successful, perhaps because of the scarcity of the materials available for experimentation, the long maturation time, the difficulty to decontaminate the explants, and the difficulty in regeneration of the plants. We believe this is the first report for a highly efficient and rapid method to directly induce shoots and plants from explant sections containing the FB base of fully matured, flowering *Paphiopedilum* plants. Because the plants were produced directly from the meristems without going through the callus stage, problems caused by somaclonal variation frequently found in callus-derived shoots can be avoided, and they are expected to have the same genetic traits as their mother plants. The phenotypes of the regenerated plants are therefore predictable and consistent, which is particularly important for commercial production and conservation. The induced plants are an ideal explant source for micropropagation because their aseptic nature will ensure sterility and their meristem-derived juvenility status renders them responsive to tissue culture regeneration.

Slipper orchids available on the market come almost exclusively from *in vitro*-germinated seeds of hybrid species; therefore, their traits are highly variable and unpredictable. For example, about half of the plants from a hybrid can be albino (Koopowitz 2008). The phenotype of plants must be predictable and uniform to be commercially viable on a large scale, but the seedling-derived

micropropagated clones from hybrid crosses have neither of these two basic requirements. In addition, the germination rates of *Paphiopedilum* seeds are generally low in comparison to other commercialized orchids, and the seedlings need a long time to reach the maturation stage before flowering (Arditti *et al*. 1982). In fact, some orchids require 10 or even 16 yr for the first bloom (Koopowitz 2008). Since the orchid plants are selected and traded based on their flower appearances, the uncertainty about traits from seedling-derived plants severely hinders their marketability. The highly selected few elite slipper orchids with prize-winning flowers that command extremely high prices cannot be mass propagated and are generally used as parental lines in crosses to produce new hybrid seeds. Our plants directly induced from the FB base take about 6 mo from a flowering orchid and can be immediately used for clonal micropropagation to produce mature plants that should be predicable, stable, and uniform in their genetic traits. In addition, unlike seedling, the original plants or main meristem need not be sacrificed.

Among the bud, base, and stalk tissue groups divided from cross-sections of young FBs, only the base tissue group produced shoots and plants. There were two unequal-sized floral bracts at the node of FB in all the hybrids we examined. The small bract of the first FB was a lateral bract that harbored at its base a new miniature-sized FB with its own miniature-sized small bract, which further harbored a primitive FB. This pattern reiterated, resulting in multiple flowers. We believe that the plants were derived from these new dome-shaped, meristem-like FBs. This would explain why only the base tissue group was capable of producing the plants because it was the only group that contained such structures.

The initiation of a floral meristem and its subsequent formation of multimeric complexes that interact at transcriptional levels lead to the development of plant sexual organs (Airoldi 2010). A transient mechanism allows the transition of a meristem from newly initiated to established status that reiterates to form the inflorescence architectures of plants (Prusinkiewicz *et al*. 2007). While meristem types vary among different species, the lateral meristem is the primary indeterminate meristem *versus* the determinate apical floral meristem that later develops into all the floral parts (McSteen and Leyser 2005). We observed that while the first FB itself later became a terminal flower, the small bract contained both apical and lateral meristems at its base. The apical meristem would later develop into another terminal FB, whereas the lateral meristem would form a new apical and a new lateral meristem. This pattern reiterated to form the scorpioid cyme inflorescence architecture in multiflora *Paphiopedilum* species. When the flower numbers reach the limit for a specific genotype, which are generally five in *Paphiopedium*, the lateral meristem would cease its ability to produce new meristems. Depending on the lag time between each reiteration, the flowers opened successively as in *Paphiopedilum* Deperle, or near simultaneously as in *Paphiopedilum* Delrosi. Even in the single floral type of *Paphiopedilum* In-Charm Flame × *P. urbanianum*, similar meristem-like tissues existed but apparently failed to develop further, resulting in the production of a single flower. Thus, in all three *Paphiopedilum* hybrids, multi-flower type is more primitive than single-flower type. The continued presence of these meristem-like tissues at the FB base may be the reason that plants were induced from all sizes of the FBs in *Paphiopedilum* Deperle, whereas the lack of such continuation in *Paphiopedilum* Armeni White resulted in plant induction only from the large FB that contained a sufficient meristem tissue mass necessary for regeneration. Our ability to induce plants from all these genotypes suggests the existence of indeterminate, totipotent meristem tissues at the FB base that are competent for plant induction *in vitro* when placed in a suitable culture medium and environmental cues (*e.g.*, in darkness). We have tested a number of culture conditions by changing the medium and plant growth regulator constituents (unpublished data) before adapting the current protocol, but further improvement of the culture conditions for direct micropropation from FBs is likely.

In conclusion, considering the extremely recalcitrant nature of mature plants, our 57–75% successful rate of *in vitro* plant induction offers an efficient and rapid method to provide an aseptic explant source for both the micropropagation and preservation of slipper orchids that are precious, rare, threatened, or already extinct in their natural habitats. We have also provided insight into the origin of the tissue which gives rise to the shoots and plants. We further revealed a special floral architecture in *Paphiopedilum* in that the small bract located at the FB base of slipper orchids harbored new miniature FBs with meristem-like tissues in which the shoots and plants presumably originated.

Acknowledgment We thank Dr. Michael Fine of Virginia Commonwealth University for reviewing the manuscript and for advice on data analysis.

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