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INDUCTION OF EMBRYOGENIC CALLUS AND CELL SUSPENSION CULTURE FROM SHOOT TIPS EXCISED FROM FLOWER STALK BUDS OF *PHALAENOPSIS* (ORCHIDACEAE)

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Summary

Embryogenic calluses were induced from 73% of *Phalaenopsis* shoot-tip explants excised from flower stalk buds by culturing for 7 mo. on New Dogashima Medium (NDM) containing 0.5 μ*M* α-naphthaleneacetic acid (NAA), 4.4 μ*M* 6-benzylaminopurine and 29.2 m*M* sucrose. The sucrose concentration was increased to 58.4 m*M* 4 mo. after initiation of the callus culture. These calluses were successfully subcultured as cell suspension cultures in liquid NDM supplemented with 5.4 μ*M* NAA and 58.4 m*M* sucrose. By simply reducing the sucrose concentration to 29.2 m*M*, the cells grew into plantlets through a developmental process similar to that of *Phalaenopsis* seedlings. The occurrence of somaclonal variants was less than 10% in six out of eight genotypes examined. These results suggest that the embryogenic callus and cell suspension culture could be utilized as the materials for micropropagation and breeding of *Phalaenopsis* orchids.

Key words: embryogenic callus induction; sucrose concentration; suspension cell culture; shoot-tip culture; flower stalk buds; *Phalaenopsis*.

Introduction

Since Morel (1960) reported shoot-tip cultures of *Cymbidium*, efficient micropropagation systems using tissue culture techniques have been reported in many genera of the Orchidaceae (Arditti and Ernst, 1993). Most of the orchids of commercial importance have been propagated using tissue culture through the formation of protocorm-like body (PLB) except for some recalcitrant species such as *Paphiopedilum*.

In many plant species, regeneration of plants from callus tissue, especially highly totipotent embryogenic callus, has been recognized as one of the essential techniques for micropropagation and biotechnological applications. In orchids, successful reports on plant regeneration from embryogenic callus have been limited. Sajise and Sagawa (1991) first reported embryogenic callus formation in *Phalaenopsis* without a detailed description of the method for callus induction. However, there have been no reports on the induction of embryogenic callus in other orchid species. In *Phalaenopsis*, callus induction was achieved from PLB which was induced by culturing shoot tips of lateral buds on flower stalks (Ichihashi, 1992) and leaf segments (Ishii et al., 1998). However, there have been no reports on the direct induction of embryogenic callus from explants obtained from plants grown in the greenhouse. In the present study, we show the successful results on the direct induction of embryogenic callus from shoot-tip explants of flower stalk buds, the establishment of a fine cell suspension, and plant regeneration through somatic embryogenesis in *Phalaenopsis*.

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Materials and Methods

Callus induction. Greenhouse-grown plants of nine *Phalaenopsis* genotypes were used as plant material (Tables 1 and 2). Shoot-tip explants were obtained from the plants by the method previously reported (Tokuhara and Mii, 1993) and placed on New Dogashima Medium (NDM) supplemented with 0.5–10.7 μ*M* α-naphthaleneacetic acid (NAA), 4.4 μ*M* 6-benzylaminopurine (BA), 29.2 or 58.4 m*M* sucrose and solidified with 2 g l^{-1} gellan gum. The pH of the media was adjusted to 5.4 prior to autoclaving for 15 min at 120°C. Each explant was inoculated into 15 ml medium in a 24 mm × 100 mm test tube capped with aluminum foil. The cultures were incubated at 23 ± 1°C under 14-h photoperiods provided by fluorescent lamps at 33 μmol m^{-2} s^{-1}. These cultures were transferred to the same fresh medium at monthly intervals. After 4 mo. of culture, half of the explants cultured on medium with 0.5 μ*M* NAA, 4.4 μ*M* BA and 29.2 m*M* sucrose were transferred to the same fresh medium with the sucrose concentration increased to 58.4 m*M* sucrose. Nine to 11 explants were cultured for each treatment and the experiment was repeated three times. After 7 mo. of culture, the rates of callus and PLB formation of explants were recorded.

Establishment of cell suspension culture. The calluses were induced from shoot-tips of vegetative buds on the flower stalk of *Phalaenopsis* [(Baby Hat × Ann Jesica) × *equestris*] (PM292) on NDM containing 0.5 μ*M* NAA, 4.4 μ*M* BA, 58.4 m*M* sucrose and 2 g l^{-1} gellan gum. About 200 mg calluses were transferred into a 100-ml Erlenmeyer flask containing 40 ml liquid medium and incubated on a reciprocal shaker at 80 rpm, 23 ± 1°C under a 14-h light photoperiod provided by fluorescent lamps at 33 μmol m^{-2} s^{-1}. After several subcultures at 2-wk intervals, the cell suspension cultures were used for testing the optimum concentration of plant growth regulators (PGR). For this study, the cells were collected by centrifugation at 200 × ***g*** for 5 min and 1 ml packed cell volume (PCV) (*ca.*

TABLE 1

EFFECTS OF NAA AND SUCROSE CONCENTRATION ON CALLUS INDUCTION AND PLB FORMATION FROM SHOOT TIPS EXCISED FROM FLOWER STALK BUDS OF *P.*[(BABY HAT×ANN JESSICA)×*EQUESTRIS*] AFTER 7 MO. OF CULTURE

NAA (μ*M*)	Sucrose (m*M*)	No. of explants	Survival (%)	PLB formation (%)	Callus formation (%)	Fresh weight of callus (mg)
0.5	29.2	27	76.7 ± 3.2[b]	44.4 ± 10.4	24.4 ± 9.6	73.6 ± 30.1
0.5	29.2/58.4[a]	27	78.9 ± 0.8	5.0 ± 3.5	73.4 ± 4.7	200.0 ± 68.1
0.5	58.4	28	3.7 ± 3.0	0	0	0
5.4	29.2	32	40.9 ± 5.7	6.1 ± 5.0	9.4 ± 4.3	10.7 ± 4.7
10.7	29.2	32	31.2 ± 2.2	3.3 ± 2.7	16.1 ± 7.1	14.1 ± 7.2
Significance			*	*	*	*

BA at 4.4 μ*M* was added to all culture media.
[a] Sucrose concentration was changed from 29.2 to 58.4 m*M* after 4 mo. of culture.
[b] Means of three replications±standard error.
* Significance at $P < 0.05$ by ANOVA.

500 mg FW) were transferred into a 100-ml Erlenmeyer flask containing 40 ml of liquid NDM supplemented with NAA (0, 0.5, and 5.4 μ*M*), BA (0, 0.4, and 4.4 μ*M*) and 58.4 m*M* sucrose. After 4 wk of culture, the cells (1 ml PCV) in each medium (40 ml) were subcultured in the same fresh medium and the PCV of these cells was recorded after 4 wk of culture. Five flasks were made for each treatment.

Plant regeneration and somaclonal variation. After proliferation in liquid medium, embryogenic cells of eight genotypes (one genotype of *P.* Hanaboushi, one genotype of *P.* Snow Parade, one genotype of *P.* Little Steve, three genotypes of *P.* Wedding Promenade, one genotype of *P.* Wedding March and one genotype of *P.* Reichentea) induced from shoot-tip culture of vegetative buds on flower stalks, on medium containing 0.5 μ*M* NAA, 4.4 μ*M* BA, 58.4 m*M* sucrose and 2 g l^{-1} gellan gum, were subcultured twice at 1-mo. intervals. For each genotype, 50–100 mg of the calluses were transferred onto a 9 cm Petri dish containing 25 ml NDM supplemented with 29.2 m*M* sucrose and 2 g l^{-1} gellan gum, and cultured for 5 mo. for PLB formation under the same environmental conditions used for callus proliferation. Then the PLBs induced were cultured for 10 mo. on PGR-free medium containing 10 g l^{-1} Potato Granules (Basic American Foods, Walnut Creek, CA, USA), 10 g l^{-1} apple juice (Kyoei Co. Ltd. Kuroishi, Aomori, Japan), 58.4 m*M* sucrose and 2 g l^{-1} gellan gum until plantlets with four or five leaves and three or four roots were obtained. Finally, 14–119 plantlets were established in pots for each genotype for further growth in the greenhouse until flowering. Abnormalities in flower and leaf characteristics were investigated during the full bloom period in the first year for each genotype.

Results and Discussion

Callus induction. In the presence of 4.4 μ*M* BA, both the survival of the explants and callus formation after 4 mo. of culture were affected by the concentrations of both NAA and sucrose. On NDM media containing 29.2 m*M* sucrose, excised shoot-tips turned pale yellow or necrosed when 5.4 or 10.7 μ*M* NAA was added, whereas the shoot-tips remained green and induced pale yellow granular calluses at the base of the explants on a lower concentration, 0.5 μ*M*, of NAA. PLBs were also induced in several explants on this medium. However, the use of a higher concentration, 58.4 m*M*, of sucrose resulted in complete necrosis of most explants even at the low NAA concentration after 7 mo. of culture. Viable explants on this medium induced neither PLB nor callus. In contrast, the survival rate of the explants on NDM medium containing 0.5 μ*M* NAA and 4.4 μ*M* BA with 29.2 m*M* sucrose after 7 mo. of culture was 77% (Table 1). The change of sucrose concentration from 29.2 to 58.4 m*M*, 4 mo. after culture initiation, had no effect on survival rate. Most of the explants cultured on 29.2 m*M* sucrose throughout the culture period became green and 44% of the explants induced PLB and 24% formed callus. On the other hand, explants which were transferred onto medium with

TABLE 2

SOMACLONAL VARIATIONS IN FLOWER, LEAF AND INFLORESCENCE CHARACTERS OBSERVED IN THE PLANTS DERIVED FROM EMBRYOGENIC CALLUSES USING SHOOT-TIP CULTURE OF VEGETATIVE BUDS ON FLOWER STALK IN *PHALAENOPSIS*

Genotypes	*P.* Hanaboushi PP625	*P.* Snow Parade PP1068	*P.* Little Steve PP1674	*P.* Wedding Promenade PP1954	*P.* Wedding Promenade PP1985	*P.* Wedding Promenade PP2352	*P.* Wedding March PP2182	*P.* Reichentea PP2439
No. of regenerants	14	21	41	33	30	36	40	119
No. of variants	2 (14.2)[a]	0 (0)	0 (0)	2 (6.1)	3 (10)	2 (5.6)	3 (7.5)	57 (47.9)
Creased leaf	2							1
Narrow leaf				2	2			2
Thick leaf and flower							2	5
Labelum-like lateral sepal						1		
Dwarfed flower					1			2
Shortened inflorescence axis[b]						1		1
Shortened internode of inflorescence[c]							1	
Thickened top part of inflorescence axis								46

[a] Numbers in parentheses show the percentage for surviving plants.
[b] Shortening of the inflorescence axis from base to the node with the first flower.
[c] Shorter internodes between each flower.
P. Hanaboushi = *P.* Carol Ai Killips × *P.* Otohime; *P.* Snow Parade = *P.* Crescent × *P.* Musashino; *P.* Little Steve = *P.* Steven Ai × *P. equestris*; *P.* Wedding Promenade = *P.* Cosmetic Art × *P. equestris*; *P.* Wedding March = *P.* Hanaboushi × *P. equestris*; *P.* Reichentea = *P. fasciata* × *P. gigantea.*

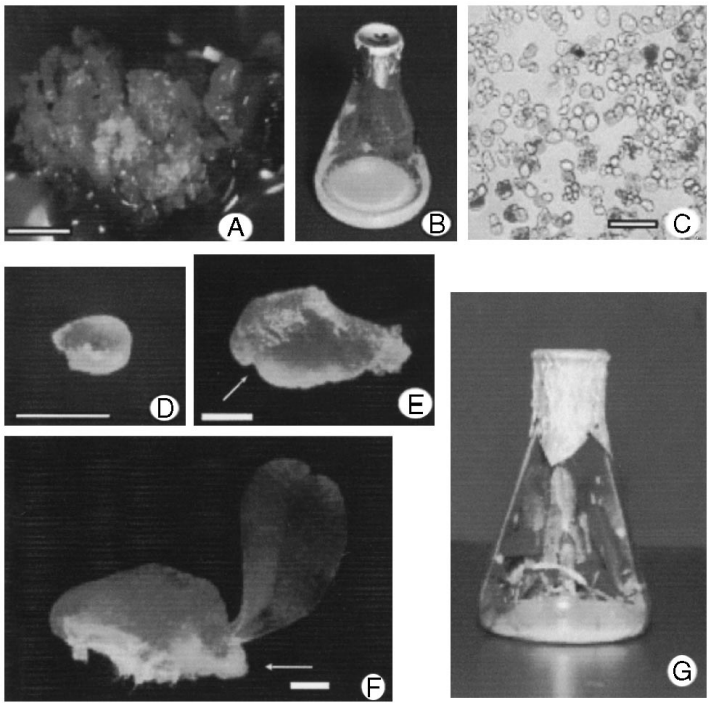

FIG. 1. Regeneration of plantlets from embryogenic callus-derived PLB. *A*, Embryogenic callus derived from the shoot tip of a flower stalk bud after 7 mo. of culture. *B*, Cell suspension culture. *C*, Suspension cells derived from embryogenic callus. *D*, PLB derived from suspension cells. *E*, Elongated PLB with a meristem which is shown as the depression (*arrow*). *F*, Root development from PLB at the base of elongating leaf (*arrow*). *G*, Plantlets derived from suspension cells 10 mo. after formation of PLB. *Bar* = 1 mm; except for *C*, where *bar* = 0.1 mm.

58.4 m*M* sucrose from that with 29.2 m*M* sucrose after 4 mo. of culture became pale yellow and induced callus at 73% instead of forming PLBs. The calluses induced by this treatment showed the highest fresh weight (200 mg per explant) after 7 mo. of culture and were friable (Fig. 1A). In contrast, survival rates of the explants on media containing high concentrations of NAA at 5.4 and 10.7 μ*M* were 41 and 31%, respectively. However, most of the surviving explants only showed slight swelling with browning and the frequencies of PLB and callus formation were low in these two media.

There have been several reports on callus formation from the tissue culture of *Phalaenopsis*. Sajise and Sagawa (1991) first reported embryogenic callus formation in *Phalaenopsis*, but they did not describe a detailed method for callus induction. Furthermore, Kobayashi et al. (1993) utilized callus as the source for protoplasts and first succeeded in plant regeneration from protoplasts in *Phalaenopsis*, however, details for callus formation were not described. Ichihashi (1992) reported that callus-like masses were induced by culturing shoot tips of young flower stalk buds, in which excised explants were initially converted into a callus-like mass followed by development into PLBs. Recently, calluses of *Phalaenopsis* were successfully induced on medium supplemented with 116.9 m*M* sucrose and 20% coconut water by culturing PLB segments derived from leaf segments from *in vitro*-cultured plants (Ishii et al., 1998). In most of these reports, explants were cultured without changing the sucrose concentration throughout the whole culture period. In the present study, the frequency of callus induction was increased threefold by changing the sucrose concentration from 29.2 to 58.4 m*M* 4 mo. after initiation of culture. In our previous study, white and yellowish PLBs were induced on media containing 58.4 m*M* sucrose, and most of the PLBs died following repeated subculture without inducing callus on the same medium (Tokuhara and Mii, 1993). A similar phenomenon has also commonly been observed by commercial growers in seed germination of *Phalaenopsis*, in which most of the green protocorms turned yellow and then died on media containing 58.4–87.6 m*M* sucrose. The browning of protocorms could be reduced by decreasing the sucrose concentration and most of the protocorms remained green and grew into plantlets on media containing 29.2 m*M* sucrose (data not shown). Possibly shoot-tip explants were similar to protocorms and a high concentration of sucrose was detrimental to the explants. Once the explants survived and stabilized their physiological condition at the low concentration, 29.2 m*M*, of sucrose during the initial 4 mo. of culture, they could react to a high sucrose concentration (58.4 m*M*) which might be favorable for callus formation. A high concentration of sucrose might act as an osmotic stress (George, 1993) or to inhibit chlorophyll formation to induce embryogenic callus formation.

Establishment of cell suspension culture. The calluses induced from shoot-tip explants were successfully converted into cell suspension cultures by transferring into liquid medium on a reciprocal shaker at 80 rpm (Fig. 1B, C). Proliferation of the cells was affected by PGR concentrations, although the color of callus was pale yellow without necrosis in all the culture media tested. The highest proliferation rate (4.6 times increase in PCV) was obtained

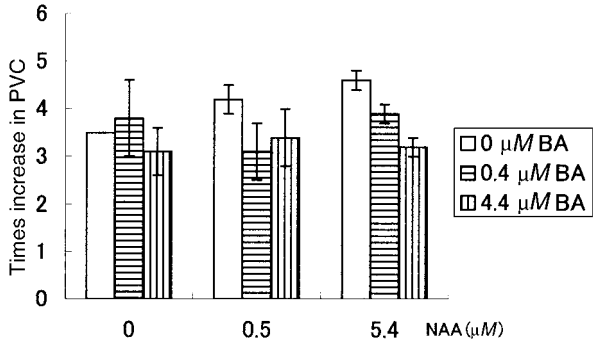

FIG. 2. Effects of NAA and BA on proliferation rate of cell suspension culture of *P.* [(Baby Hat×Ann Jessica)×*equestris*]. PCV increase of suspension cells after 4 wk. PCV was measured by centrifugation at 200× *g* for 5 min.

in medium containing 5.4 μ*M* NAA alone for 4 wk, although it is possible that the optimum concentration may be higher (Fig. 2). The proliferation rate decreased with increasing BA concentration and was the lowest (3.1 times increase in PCV) in medium containing 4.4 μ*M* BA alone (Fig. 2).

Plant regeneration and somaclonal variation. Embryogenic cells of all eight *Phalaenopsis* genotypes examined could readily develop into PLBs 5 mo. after transfer onto medium containing a reduced sucrose concentration, 29.2 m*M* (Fig. 1D). They grew into plantlets through a similar developmental process to *Phalaenopsis* seedlings when maintained on NDM containing 10 g l^{-1} Potato Granule, 10 g l^{-1} apple juice, 58.4 m*M* sucrose and 2 g l^{-1} gellan gum 10 mo. after PLB formation (Fig. 1E–G). Fourteen to 119 regenerated plants were successfully transferred into pots with sphagnum moss for each cultivar or genotype. Somaclonal variations observed in leaf, flower and inflorescence characteristics of some cultivars were classified into eight categories (Table 2). There were variants in leaf characteristics such as creased leaf (Fig. 3A), narrow leaf (Fig. 3B) and thick leaf (Table 3). The variant with thick leaves and thick flowers was confirmed to be octaploid by flow cytometric analysis. Two types of flower characteristics were observed: labellum-like lateral sepals and dwarfed flowers, both of which had been observed in our previous study (Tokuhara and Mii, 1998). The remaining three types of variants were those with an abnormal inflorescence axis. One of the inflorescence variants had a shorter internode between each flower without any change in inflorescence axis length from the base to the node with the first flower. The second type of variant was characterized by the shortening of the inflorescence axis from the base to the node with first flower. These two types of variants were also found in our previous study (Tokuhara and Mii, 1998). The other type of variant, which was found in almost half of the regenerants in PP2439, had a thicker inflorescence axis from the first flower node to the top than normal plants (Fig. 3C). The frequency of somaclonal variants except for this one was 0–14.2% (Table 2), which was similar to the values observed in the plants derived from the shoot-tip culture of vegetative buds on flower stalks through the micropropagation of PLBs without callus formation (Tokuhara and Mii, 1998).

In the present study, we established a highly efficient system of embryogenic callus formation from flower stalk buds through shoot-

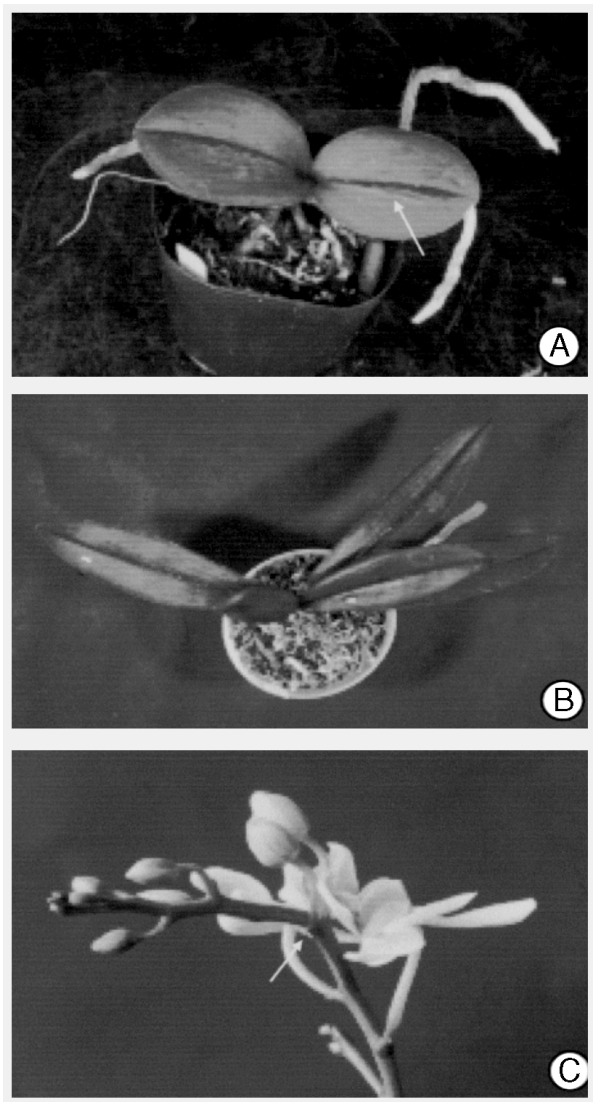

FIG. 3. Somaclonal variants derived from embryogenic callus of *Phalaenopsis*. *A*, Variant with creased leaves (*arrow*). *B*, Variant with narrow leaves. *C*, Variant with thickened inflorescence axis at the top part (*arrow*).

TABLE 3

COMPARISON OF SEVERAL CHARACTERISTICS BETWEEN A NORMAL PLANT AND TWO LEAF VARIANTS, ONE WITH NARROW LEAVES AND THE OTHER WITH THICK LEAVES REGENERATED FROM CALLUS OF *P.* REICHENTEA (PP2439)

		Variant	
Characteristic	Normal	Narrow leaf	Thick leaf
Length (mm)	92.5 ± 1.5	65.9 ± 0.7	78.2 ± 5.3
Width (mm)	44.8 ± 0.7	24.5 ± 3.6	40.6 ± 2.6
Length/width	2.08 ± 0.03	2.81 ± 0.38	1.9 ± 0.07
Thickness (mm)	1.8 ± 0.0	1.9 ± 0.1	2.6 ± 0.1

On the length of inflorescence axis from base to node with first flower, normal plants showed 11.2 ± 0.4 cm but variant showed 8.2 cm.

tip culture. The method is expected to be utilized as an efficient method for micropropagation of *Phalaenopsis* orchids because of the high proliferation rate of both the callus and cell suspension culture without loss of embryogenic potential for at least 5 yr. The embryogenic callus or cell suspension culture will also be efficiently utilized as the target material for biotechnological studies such as somatic hybridizaion and genetic transformation, as shown in our recent study (Belarmino and Mii, 2000).

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