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Interconversion between Protocorm–like–bodies (PLBs) and Rhizomes in *Cymbidium*

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Rhizome explants of three temperate terrestrial *Cymbidium* species, *C. sinense*, *C. ensifolium* and *C. kanran*, produced protocorm–like–bodies (PLBs) by the addition of 10 mgl^{-1} 6–benzylaminopurine (BA). PLB explants of *Cymbidium* Melody Fair 'Marilyn Monroe', a hybrid of tropical species, induced rhizomes under shaded and continuous dark conditions, and the conversion was promoted by the supplement of 0.1 and 1 mgl^{-1} a–naphthaleneacetic acid (NAA). The structure of the induced PLBs and rhizomes was very similar to that of the true PLBs and rhizomes, respectively, except for negative geotropism and less starch accumulation in the induced rhizomes. These results indicate that the interconversion between rhizome and PLB is possible in *Cymbidium* species and this organogenesis may be regulated by auxins and cytokinins.

INTRODUCTION

Cymbidium is one of the most commercially important orchids and used for cut flowers and potted plants. They are traditionally classified into tropical and temperate oriental groups in horticultural uses. Tropical *Cymbidium* species have large flowers with diverse color patterns, and generally include epiphyte species originated in southern and northeastern India, Sri Lanka, Thailand, Malaysia and Philippines. Several thousand hybrids of the tropical group are popular in worldwide markets. Temperate oriental *Cymbidium* species, which are found in China, Taiwan, Korea and Japan (Du Puy and Cribb, 1988), are mostly terrestrials with small flowers. They have a long history of cultivation in eastern Asia because of their wide variation in leaf shapes and flower colors and forms.

Since *in vitro* micropropagation system is a widely used method for clonal multiprication of *Cymbidium*, *in vitro* organogenesis has been studied in both tropical (Wimber, 1963; Morel, 1985) and temperate (Sawa and Torikata, 1968; Paek and Murthy, 2002) species. It has been revealed that the process of shoot formation in tropical and temperate species is different. Tropical *Cymbidium* species and their hybrids form protocorms after seed germination or produce protocorm–like–bodies (PLBs) from the apical meristem in tissue culture (Knudson, 1922; Morel, 1960). The protocorms or PLBs readily produce shoots and roots within short–term culture (Kohl, 1962; Morel, 1985). Temperate terrestrial species develop rhizomes following protocorm formation after seed germination or directly from apical meristem in *in vitro* culture. Rhizomes constantly grow with branching and produce shoots (Sawa and Torikata, 1968; Ueda and Torikata, 1968).

Although the two developmental types have been shown by many works (Segawa, 1929; Ueda and Torhikata, 1969; Shimasaki and Uemoto, 1987), several exceptions were also reported. Wang (1990) reported that the rhizomes of *Cymbidium ensifolium*, a temperate species, produced PLBs. Paek and Murthy (2002) reviewed that the development of PLBs in *Cymbidium kanran*, another temperate species, was shown by Paek *et al.* (1989). This exceptional organogenesis might have the potential of establishing new methods for mass production. *In vitro* propagation of temperate species is extremely limited due to low frequency of shoot regeneration and slow growth of rhizomes, whereas tropical *Cymbidium* species were easily multiplied via PLB culture. Because Wang (1990) successfully multiplied the PLBs of *C. ensifolium* in liquid rotating culture, it is expected that *in vitro* mass propagation via PLBs would be applied to temperate *Cymbidium* species. However, the regulatory factors that trigger PLB development in temperate *Cymbidium* species are still unclear.

Rhizome formation in tropical *Cymbidium* species was also found by Hasegawa (1987). He reported that the shoot tips of *C. insigne*, a tropical species, developed rhizomes under dark condition. Rhizome development was also observed in the tropical species of *Cymbidium aloifolium* (Nayak *et al.*, 1998) and *Cymbidium dayanum* (Chang *et al.*, 2005). Such interconversion between rhizomes and PLBs is particularly interesting when considering the evolutional events of tropical and temperate *Cymbidium* species. Molecular phylogenic analysis of *Cymbidium* species showed that the ancestral life form of *Cymbidium* was an epiphyte and all terrestrial species were evolved from epiphytic plants (Yukawa *et al.*, 2002). Thus, rhizomes of terrestrial species have evolved from protocorm of

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tropical epiphytic plants. To identify the regulatory factors for the organogenesis is necessary for understanding how rhizome evolved from protocorm, thus, the evolution of terrestrial species from epiphyte species.

The balance of auxins and cytokinins has been shown to have crucial roles in plant development (Skoog and Miller, 1957; Heide, 1965). The interconversin between PLBs and rhizomes in *Cymbidium* species seems to be regulated by auxin and cytokinins, because PLB development from rhizomes was achieved with cytokinins and auxins by Wang (1990) and Paek *et al.* (1989). It has been recognized that auxins promote the development and growth of rhizomes in temperate *Cymbidium* species (Shimasaki and Uemoto, 1990; Paek and Yeung, 1991).

We examined the effects of auxin, cytokinin and light conditions on PLB and rhizome development to clarify the regulatory factors of interconversion between PLBs and rhizomes. Histological observation of the converted PLBs and rhizomes was also performed in comparison to the original organs.

MATERIALS AND METHODS

Plant materials

Rhizomes of *C. sinense*, *C. ensifolium* and *C. kanran* and PLBs of *C.* Melody Fair 'Marilyn Monroe', were used. The rhizomes of *C. sinense* and *C. kanran* were derived from *in vitro* germination in 1997 and 1978, respectively. The rhizomes of *C. ensifolium* and the PLBs were kindly supplied by Dr. K. Shimasaki, Kochi University, and Dr. M. Tanaka of Kagawa University, respectively.

Preparation of explants

Aseptic rhizomes and PLBs were pre–cultured before supplying as the source of explants. To obtain the rhizomes of a uniform age, 5 to 10 mm long apical segments of the rhizomes were excised and cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 30 gl^{-1} sucrose and 8 gl^{-1} agar for three to four months. The PLBs were sectioned in pieces of 3 mm diameter and cultured in liquid MS medium with 20 gl^{-1} sucrose by rotating (2 r.p.m.) for two to four weeks. The apical rhizomes from 5–10 mm long or newly formed PLBs of 3 mm diameter were excised and used as explants.

Culture conditions

An MS medium supplemented with 30 gl^{-1} sucrose and 8 gl^{-1} agar was used as a basal medium. The pH was adjusted to 5.6 before adding agar, and the medium was autoclaved for 15 minutes at 121 °C. Thirty ml of the medium was put into 100 ml Erlenmeyer flasks. Cultures were maintained at 25±2 °C. The PLB explants were cultured in continuous light (39.1 μmol m^{-2} s^{-1}), shaded (1% of the light) and constant dark conditions to examine the effects of light condition. To examine the effects of auxin, the PLB explants were cultured on MS medium supplemented with 0, 0.1, 1 or 10 mgl^{-1} of α–naphthaleneacetic acid (NAA) in dark condition for three to four months. Rhizome explants of the temperate species were cultured on MS medium supplemented with 0, 0.1, 1 or 10 mgl^{-1} of 6–benzylaminopurine (BA) in continuous light condition. Four PLB explants and five rhizome explants were cultured in each flask. To observe the development of converted PLBs after excision, the PLBs induced from the rhizomes of *C. sinense* were excised and cultured in liquid MS medium with 20 gl^{-1} sucrose and 0 or 1 mgl^{-1} BA by rotating.

Histological analysis

Rhizomes of *C. sinense* and *C. ensifolium* were cultured on MS medium in dark condition for the comparison purpose. The rhizomes induced from the PLB explants of *C.* Melody Fair 'Marilyn Monroe' in the dark, the dark–grown rhizomes of *C. sinense* and *C. ensifolium*, and the PLBs induced from the rhizome explants of *C. sinense* with 10 mgl^{-1} BA were fixed in FAA (30% ethanol: formaldehyde: acetic acid=90:5:5, v/v/v), dehydrated in a butyl alcohol series and embedded in paraffin wax. Sections were cut at 10 μm thickness and stained with hematoxylin or iodine solution. The samples were observed under a light microscope.

RESULTS

Conversion from rhizome to PLB

The rhizomes of all three temperate species produced PLBs by the addition of 1 and 10 mgl^{-1} BA (Table 1). The supplement of 10 mgl^{-1} BA strongly enhanced PLB formation in all species, while that of 1 mgl^{-1} BA mainly increased shoot formation in *C. sinense* and *C. kanran*. The converted PLBs were globular shape and had a leaf primordium in their top as true PLBs of tropi-

Table 1. Effects of BA on development of the rhizome explants in *C. sinense*, *C. ensifolium* and *C. kanran*

	BA (mgl^{-1})	Number of explants cultured	PLB formation (%) [z]	Shoot formation (%) [y]
C. sinense	0	42	0	2
	0.1	42	0	0
	1.0	42	17	81
	10.0	42	91	43
C. ensifolium	0	60	0	0
	0.1	55	0	0
	1.0	55	20	2
	10.0	60	83	5
C. kanran	0	50	0	0
	0.1	55	0	0
	1.0	55	9	29
	10.0	45	96	18

[z] (number of explants forming PLBs / number of explants survived)×100.
[y] (number of explants forming shoots / number of explants survived)×100.
Data were recorded after 2 months of culture. Survival rates of the explants were 98–100%.

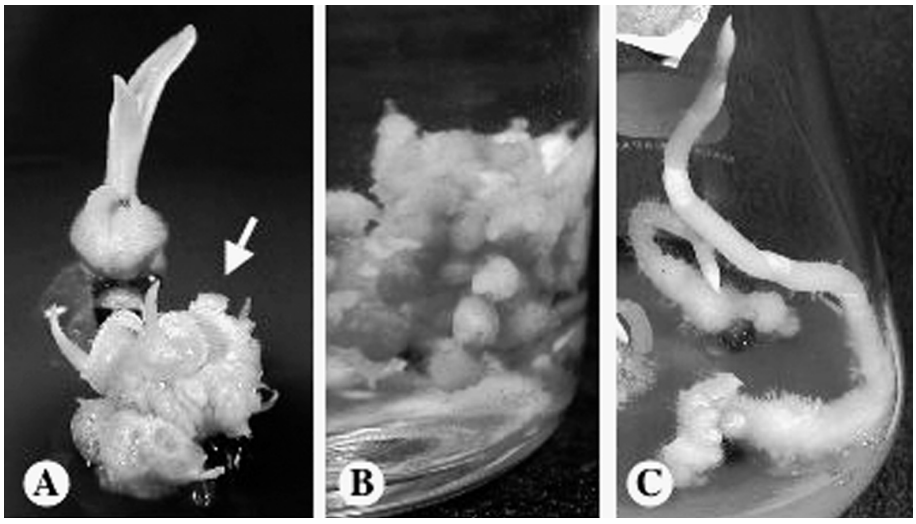

Fig. 1. A, PLB (*arrow*) formation from the rhizome explants of *C. sinense* on MS medium with 10 mgl^{-1} BA. B, PLB proliferation in *C. sinense* cultured in liquid MS medium with 1 mgl^{-1} BA. C, Rhizome formation from the PLB explants of *C*. Melody Fair 'Marilyn Monroe' in dark condition.

cal hybrids (Fig. 1A). The induced PLBs of *C. sinense* were successfully multiplied by liquid shaking culture with or without BA (Fig. 1B).

Conversion from PLB to rhizome

Both shaded and dark conditions induced rhizomes from the PLB explants of *C*. Melody Fair 'Marilyn Monroe' (Table 2), whereas constant lighting increased only shoot formation rate. The highest value (83%) was obtained in the shaded condition. Rhizome formation from the PLB explants was promoted with 0.1 and 1 mgl^{-1} NAA in dark condition (Table 3) with the highest vales of 86% by the addition of 1 mgl^{-1} NAA. The converted rhizomes grew upwards and did not show gravitropism (Fig. 1C), whereas the rhizomes of terrestrial species generally grew downwards into the medium. The converted rhizomes had no branch but scale leaf in each node and trichomes on internodes (Fig. 1C).

Table 2. Effects of light condition on rhizome and shoot formation from the PLB explants of *C*. Melody Fair 'Marilyn Monroe'

Light condition	Number of explants cultured	Rhizome formation (%) [z]	Shoot formation (%) [y]
Light	48	0	74
Shaded (1% of light)	48	83	0
Dark	48	60	0

[z] (number of explants forming rhizomes / number of explants survived) × 100.
[y] (number of explants forming shoots / number of explants survived) × 100.
Data were recorded after 3 months of culture. Survival rates of the explants were 96–100%.

Table 3. Effects of NAA on rhizome formation from the PLB explants of *C*. Melody Fair 'Marilyn Monroe' in dark condition

NAA (mgl^{-1})	Number of explants cultured	Rhizome formation (%) [z]
0	64	58
0.1	20	75
1.0	20	86
10.0	20	55

[z] (number of explants forming rhizomes / number of explants survived) × 100.
Data were recorded after 4 months of culture.

Histology of transformed organs

One large vascular bundle showing cylinder–like structure was observed in the central portion of the tissue both in the rhizome of *C. sinense* (Fig. 2A) and in the transformed rhizome of *C*. Melody Fair 'Marilyn Monroe' (Fig. 2B). Four to five phloem clusters irregularly surrounded the central xylem were also observed in both vascular bundles (Figs. 2C, D). Starch grains were observed not only in the cells of cortex but also in those close to the apical meristem in *C. sinense* (Fig. 2E), whereas they were only in the cells around the vascular bundle in *C*. Melody Fair 'Marilyn Monroe' (Fig. 2D). The starch grains in the cells around apical meristem were biased towards gravity in *C. sinense* (Fig. 2E).

The converted PLBs from the rhizomes of *C. sinense* are shown in Fig. 2F. Several vascular bundles developed from the base to the apical meristem and leaf primordia developed well. The structure of the PLBs of *C*. Melody Fair 'Marilyn Monroe' was also observed (data not shown), showing their structure was quite similar to that of the transformed PLBs.

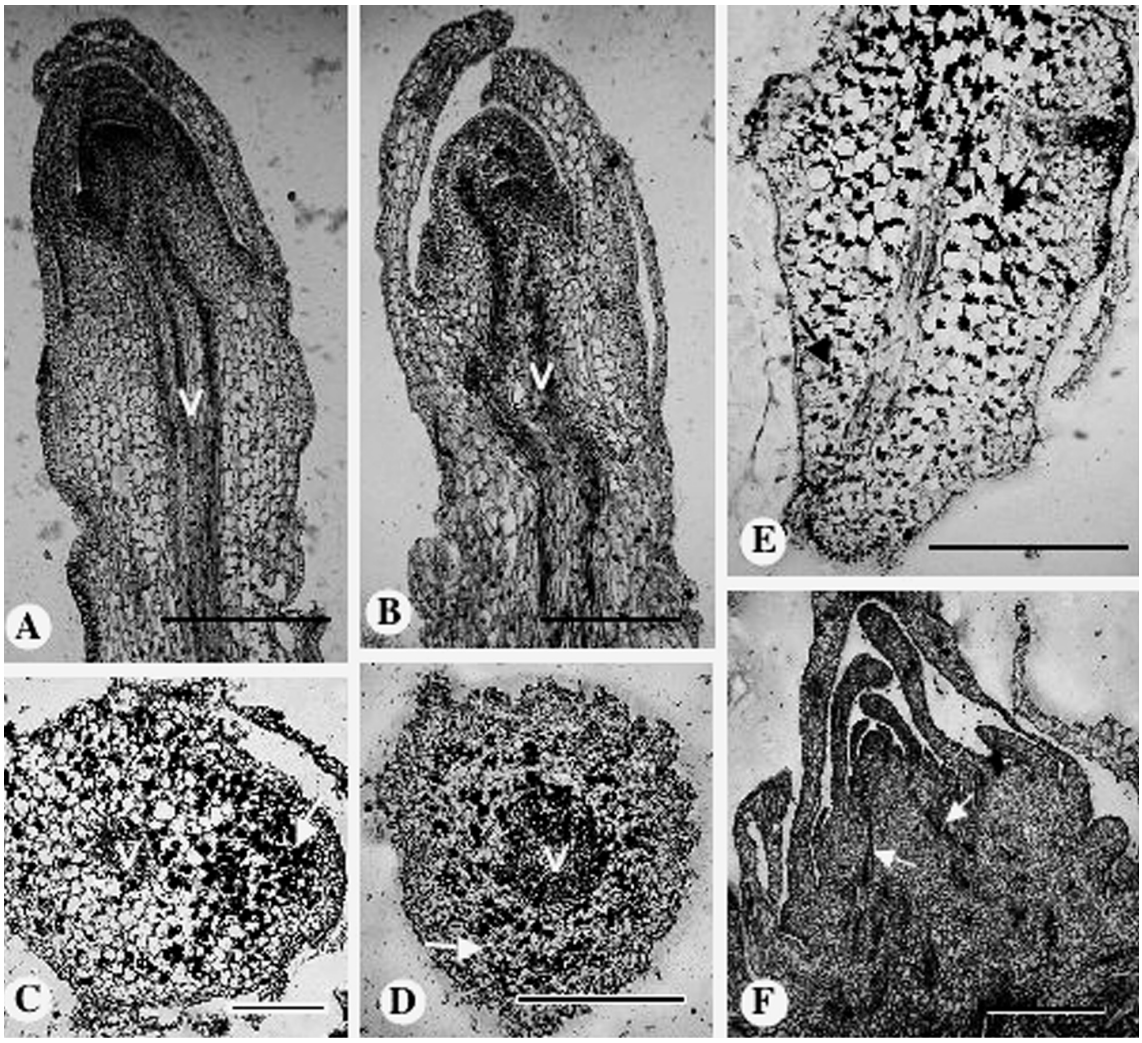

Fig. 2. A, B; Longitudinal section of dark-grown rhizome of *C. ensifolium* (A) and rhizome induced from the PLB explant of *C.* Melody Fair 'Marilyn Monroe' (B) in dark condition. A cylinder of vascular bundle (V). C, D; Cross sections of rhizomes of *C. sinense* (C) and *C.* Melody Fair 'Marilyn Monroe' (D). Accumulation of starch grains (*arrows*). E; Sedimentation of starch grains (*arrows*) in rhizome of *C. sinense*. F; Longitudinal section of PLB induced from the rhizome explant of *C. sinense* on MS medium with 10 mgl^{-1} BA (*arrows* show vascular bundles). The sections were stained with hematoxylin (A, B, F) and iodine solution (C, D, E). Bars = 0.5 mm.

DISCUSSION

The transformation from rhizome to PLB was regulated by the concentration of cytokinin in the temperate species examined (Table 1). Wang (1990) induced the PLBs from shoot meristem and axillary buds of *C. ensifolium* with 10% coconut milk and 5 mgl^{-1} NAA in 1/2 MS medium. Paek *et al*. (1989) cultured the PLBs of *C. kanran* on MS medium supplemented with 5 mgl^{-1} BA and 0.3 mgl^{-1} NAA (Paek and Murthy, 2002). The addition of 10 mgl^{-1} BA induced PLBs and 1 mgl^{-1} BA did shoots in this study. High concentration of cytokinin would probably stimulate PLB transformation and mild concentration may induce shoot formation. The PLB transformation was achieved in all the three temperate species, showing that PLB formation would be universal organogenesis in temperate *Cymbidium* species. The converted PLBs of *C. sinense* were rapidly propagated in liquid MS medium after derivation from the rhizomes (Fig. 1B) as Wang (1990) reported in *C. ensifolium*. Considering these results together, the multiplication via PLBs might be available in other temperate *Cymbidium* species.

The transformation from PLB to rhizome was regulated by light condition (Table 2) and promoted by auxin (Table 3). The growth and proliferation of the rhizomes was stimulated by addition of NAA, indole–3–acetic acid and indole–3–butyric acid in *C. aloifolium* (Nayak *et al*., 1998). The addition of auxin has been shown to promote the development and growth of rhizomes in temperate *Cymbidium* species; the rhizome development from pseudobulbs was enhanced by 1 to 100 mgl^{-1} NAA, IBA or 2,4–D in *C. goeringii* and *C. kanran* (Shimasaki and Uemoto, 1987) and the flower bud of *C. goeringii* formed rhizomes by the treatment with 1 or 10 mgl^{-1} NAA (Shimasaki and Uemoto, 1991). Auxin would universally promote rhizome development in both temperate and tropical *Cymbidium* species. Cytokinin seems to increase PLB development in both tropical and tem-

perate species, because the addition of BA inhibited the conversion from PLB to rhizome and increased PLB proliferation in the dark and shaded conditions (data not shown).

The histological observation revealed that the structure of vascular bundle of the converted rhizome was quite similar to that of temperate species (Figs. 2A, B, C, D). Hasegawa (1987) compared the structure of the rhizomes of *C. insigne* and *C. goeringii* and reported the structural similarity in vascular bundles. The converted rhizome, however, was completely different in the less accumulation of starch grains and geotropism. Amiloplasts sedimentation triggers gravitropic sensing in roots of higher plants (Caspar and Pickard, 1989; Sack, 1991). The rhizome of *C. sinense* contained starch grains that sediment in the cells around apical meristem (Fig. 2E), while the converted rhizome of *C.* Melody Fair 'Marilyn Monroe' did not. The large accumulation of starch grains may contribute to gravitropism in the rhizomes of temperate species. The structure and morphological feature of converted PLBs were quite similar to the PLBs of tropical hybrids (Fig. 2F). These structures are also shown in the PLBs of tropical *Cymbidium* hybrid, *C.* Mini Dream 'Golden Color' (Kanase *et al.*, 1993).

Taking the evolutionary process into consideration, the conversion from rhizome to PLB is the reversion back to the morphology of protocorm. This may explain why the converted PLBs from rhizome had the high homology with the PLBs of tropical species in their structures. The converted rhizomes from the PLBs, on the other hand, were different from the rhizome of temperate species in the accumulation of starch grains and gravitropism. These results suggest that such functions may have changed during the evolution from protocorm to rhizome. Ueda and Torikata (1970) showed that the cytokinin activities of the extracts from *C. goeringii* were considerably lower than those from *C. insigne* and *C. pumilum* in the cultures from shoot meristems. Our results suggest that cytokinin affects PLB formation and auxin is effective in rhizome development. Considering these results, lowering cytokinin and rising auxin in concentration or activities would have contributed to the evolution from protocorm to rhizome in *Cymbidium* species.

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