Development of a Highly Efficient and Simple Micropropagation System for *Phalaenopsis* Using Elongated Protocorm-like Bodies Induced by Skotomorphogenesis under Dark Conditions

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A highly efficient and simple micropropagation system for *Phalaenopsis* was developed using elongated protocorm-like bodies (ePLBs) obtained by skotomorphogenesis. When normal protocorm-like bodies (nPLBs) without growing point excision were cultured under different light conditions (dark: $0 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, low light: $2 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, high light: $80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, of photosynthetic photon flux density respectively), PLB proliferation efficiency was higher under dark than high light and low light conditions. In addition, shoot formation percentage was lower under dark conditions (8.0%) than under low light (66.0%) and high light conditions (68.2%) and few PLBs developed shoots during culture under dark conditions. The Secondary ePLBs obtained after culturing under dark conditions were approximately twice as long as nPLBs. After acclimation under low light conditions for 2 weeks, the ePLBs were transferred to high light conditions after making a partial incision in their apical parts. Under high light conditions, a large number of secondary PLBs were obtained from ePLBs, 6 times as many as from nPLBs treated with the same partial incisions. The findings of this study showed that culturing PLBs in dark conditions suppresses shoot formation that might interfere with PLB proliferation, and that a large number of secondary PLBs could be obtained from these ePLBs compared with nPLBs after exposure to high light intensity.

Key Words: elongated PLB (ePLB), micropropagation, *Phalaenopsis*, protocorm-like-bodies (PLBs), skotomorphogenesis.

Introduction

Phalaenopsis is an orchid genus containing 62 species (Christenson, 2001). *Phalaenopsis* orchids are distributed throughout tropical and subtropical regions of Southeast Asia, from the Himalayan Mountains, southern China, and Taiwan to northern Australia (Chiba, 2002). Their beautiful, colorful, and long-lasting flowers, combined with the extensive development of numerous artificial hybrids, have contributed to their considerable popularity in the orchid trade.

In vitro culture techniques, such as aseptic germination on artificial media, have been used extensively in the cultivation of *Phalaenopsis* (Knudson, 1922), as germination of *Phalaenopsis* seeds under natural conditions is difficult due to their small size and absence of an endosperm. In other orchid genera, mericlinal propagation

has been extensively employed in the commercial production of superior clones using protocorm-like bodies (PLBs) induced from shoot apices. However, because *Phalaenopsis* species have a stunted monopodial shoot, excision of the shoot apex may result in the death of the plant. Consequently, many clonal propagation methods using PLBs induced from flower stem buds (Ichihashi, 1992; Tokuhara and Mii, 1993), leaves (Tanaka and Sakanishi, 1977), and roots (Park et al., 2003) have been investigated.

PLBs can be rapidly proliferated by separating secondary PLBs from parental PLBs. Each of the resulting PLBs can then easily be differentiated into a plantlet. The proliferation efficiency at this stage can be increased considerably by dividing PLB clusters into pieces (Amaki and Higuchi, 1989) and injuring PLB meristems (Nii et al., 2004).

However, these proliferation techniques are highly time and labor consuming because small PLBs need to be excised and separated by hand. In addition, PLBs bearing shoots that have developed from their meristems are not

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likely to form secondary PLBs, making it necessary to perform complicated operations, such as removal of the growing point of the PLB and/or inhibition of shoot formation. Compared to other orchid groups, *Phalaenopsis* PLBs have a tendency to turn into shoots relatively easily, which means that in the absence of these operations (i.e. excision and separation), the proliferation efficiency in the micropropagation stage is markedly decreased.

We therefore considered that operations such as separation of PLBs in the micropropagation stage could be simplified by using elongated PLBs (ePLBs) that can be obtained by culturing under dark conditions due to skotomorphogenesis. We also considered that using ePLBs would increase the efficiency of micropropagation since the number of secondary PLBs on one parental PLB may increase because ePLBs are longer and larger than normal PLBs (nPLBs). This study was therefore conducted to establish a simple and efficient micropropagation method utilizing ePLBs with their detailed characterization.

Materials and Methods

Plant materials

Phal. Double Delight 'Hawaiian Spots' that had been purchased in a flower market and cultivated in a greenhouse was used in this study.

Induction of plantlets and PLBs

Induction of plantlets and PLBs by flower stalk culture and leaf segment culture was carried out according to the method of Tanaka (1992). We used PSR medium (see *Culture media* section below) to induce plantlets by flower stalk culture, and the plantlets were then used for PLB induction using leaf segment culture medium for *Phalaenopsis* (Tanaka, 1992). PLBs thus obtained were subcultured on PSR medium and used for the experiments.

nPLBs and ePLBs

We used both normal PLBs (nPLBs) and elongated PLBs (ePLBs) for the experiments. nPLBs measuring 4–5 mm in length were produced and maintained under high light conditions, whereas ePLBs measuring 1 cm in length were produced by growing under low light and dark conditions.

Culture media

PSR medium developed by the authors was used for shoot cultures of *Phalaenopsis* in the present study (Table 1). PSR medium was prepared as follows: 200 g potato was cut into lengths measuring 5 mm × 5 mm × 50 mm, and placed in a beaker containing 1 L distilled water. After gently boiling for 30 min, the potato segments were removed and filtered through cotton gauze. All the constituents listed in Table 1 except for gellan gum were added to the broth and its volume was adjusted to 1 L with distilled water. This basic broth

Table 1. Components of PSR medium.

Medium components (mg·L ⁻¹)	
Macro elements	
NH ₄ NO ₃	825
KNO ₃	950
CaCl ₂ ·2H ₂ O	220
MgSO ₄ ·7H ₂ O	185
KH ₂ PO ₄	85
Na ₂ -EDTA	18.65
FeSO ₄ ·7H ₂ O	13.9
Micro elements	
H ₃ BO ₃	3.1
MnSO ₄ ·7H ₂ O	11.15
ZnSO ₄ ·7H ₂ O	4.3
KI	0.415
Na ₂ MoO ₄ ·2H ₂ O	0.125
CoCl ₂ ·6H ₂ O	0.0125
CuSO ₄ ·5H ₂ O	0.0125
Vitamins and amino acids	
myo-Inositol	100
Glycine	2
Pyridoxine hydrochloride	0.5
Nicotine acid	0.5
Thiamine hydrochloride	0.1
Other	
NAA	1
BAP	1
Adenine	10
Potato	200 g
Coconut water	200 mL
Sucrose	20 g
Gellan gum	2.5 g

could be frozen and stored. Prepared PSR medium was used as a semi-solid medium. PSR medium was autoclaved (121°C, 20 min) and 20 mL was poured into a Petri dish (diam.: 90 mm). Leaf segment culture medium was prepared according to Tanaka (1992).

Culture conditions

PLB cultures were kept at 25°C under the following 3 light intensities with white fluorescent lamps as the light source in this study; dark condition: 0 μmol·m⁻²·s⁻¹, low light condition: 2 μmol·m⁻²·s⁻¹, high light condition: 80 μmol·m⁻²·s⁻¹, of photosynthetic photon flux density respectively. Except for the dark condition, the cultures were kept under a photoperiod regime of 14L:10D.

Effect of lighting conditions on proliferation and length of PLBs (Experiment 1)

PLBs obtained from leaf segment cultures were cultured for 6 months under 6 different conditions; 3 lighting conditions (dark, low light, and light conditions) in combination with or without growing point excision. Growing point excision treatment was conducted by

excising off the top one-third of the PLBs every month. These PLBs were subcultured every 2 weeks to produce secondary PLBs. Secondary PLBs measuring more than 4 mm were separated from parental PLBs and plated as independent PLBs as with subcultured parental PLBs. At the beginning of this experiment, 5 PLBs were plated onto PSR medium in a Petri dish and cultured under each of the 6 experimental conditions. These PLBs were subcultured on PSR medium, with 16 PLBs per Petri dish for each condition. After 6 months of culture, the number of total individuals (Nt), number of PLBs (Np), and shoot formation percentage $[(Nt - Np)/Nt \times 100]$ were observed under each condition. The value for Nt included both primary and secondary PLBs and PLBs with developed shoots. Np refers to the number of PLBs without shoots among Nt. Then, for each experimental light condition, 200 individual PLBs for each condition were randomly selected from all of the PLBs without shoots (50 samples only in low light with no treatment), including secondarily proliferated PLBs, during subculture. Overall lengths of the 200 PLBs were measured to 0.1 mm with a ruler and the average length of the obtained PLBs was determined for each light condition.

Effect of light acclimation on ePLB proliferation (Experiment 2)

ePLBs measuring approximately 1 cm in length that were produced by culturing under dark conditions were transferred to either low light or dark conditions for an additional 2 weeks. Then, the PLBs from both conditions were subjected to partial incision treatment as described by Nii et al. (2004) before a further 2 weeks of culture under high light conditions. After 2 weeks, percentage survival [(number of surviving samples/total number of samples) $\times 100$], percentage secondary PLB formation [(number of samples produced secondary PLBs/the number of samples) $\times 100$] and average number of secondary PLBs [total number of secondary PLBs subjected to each condition/number of samples] were calculated for both treatments. Twenty ePLBs were used for each

treatment, each of which was performed in triplicate.

Comparison of nPLB and ePLB proliferation with partial incision treatment (Experiment 3)

To investigate the effect of partial incision on nPLB and ePLB propagation, an experiment consisting of 4 treatment conditions was performed, i.e. nPLB and ePLB with or without partial incision were used in this experiment. Partial incision was only conducted at the beginning of the experiment for each sample. For ePLBs with partial incision treatment, those kept under dark conditions were transferred to low light conditions for 2 weeks as acclimation treatment before transferring to high light conditions to use for the experiment. For ePLB samples, moreover, partial incision treatment was applied to the top one-third of the enlarged part of the ePLB. nPLBs and ePLBs were then cultured under high light conditions for 2 months with subculturing every 2 weeks. Then, the percentage survival [(number of surviving samples/number of samples) $\times 100$], shoot formation percentage [(number of samples that developed shoots/number of samples) $\times 100$], percentage secondary PLB formation [(number of samples that formed secondary PLBs/number of samples) $\times 100$], and the average number of secondary PLBs [the total number of secondary PLBs/the number of samples] in each condition were calculated. Twenty nPLBs or ePLBs were used for each treatment, each of which was performed 5 times.

Results

Effect of lighting conditions on the proliferation and length of PLBs (Experiment 1)

Table 2 shows the effect of lighting conditions on shoot formation, proliferation, and length of PLBs with or without growing point excision treatment. Under conditions without growing point excision, Nt decreased in the following order: light, dark, and low light conditions, and Np: dark, light, and low light conditions. Shoot formation frequency under both high light and low light conditions was approximately 70%, and plant-

Table 2. Effect of light condition and growing point excision treatment on shoot formation, PLB proliferation, and PLB length.

Light condition	Light intensity ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	No treatment				Growing point excision treatment			
		Nt	Np	Shoot formation ^z percentage (%)	PLB length ^y (mm)	Nt	Np	Shoot formation ^z percentage (%)	PLB length ^y (mm)
Light	80	840	267	68.2	4.5 $\pm$ 0.1	2178	1252	42.5	4.5 $\pm$ 0.1
Low light	2	156	53	66.0	6.2 $\pm$ 0.1	934	481	48.5	6.4 $\pm$ 0.2
Dark	0	791	728	8.0	9.6 $\pm$ 2.0	283	280	1.1	8.8 $\pm$ 0.3

^z Percentage of shoot formation = $[(Nt - Np)/Nt \times 100]$. Nt: number of total individuals. Np: number of PLBs.

^y Mean $\pm$ SE (n = 200 or 50).

Five PLBs were used for each light condition at the beginning of the experiment. Growing point excision treatment was conducted by excising off the top one-third of the PLBs every month. The PLBs were subcultured every two weeks and secondary PLBs of more than 4 mm were separated from parental PLBs at subculture, with inoculation of 16 PLBs per Petri dish for each condition. Excision treatment of these secondary PLBs was conducted only at their first excision time. After 6 months, the total number of individuals (Nt), which included PLBs both with and without shoot formation, and number of PLBs without shoot formation (Np), was counted, and the rate of shoot formation was calculated for each condition. The average length of PLBs was determined by measuring lengths of 200 PLBs (50 PLBs for low light and no treatment culture), which were randomly selected from all of the PLBs without shoots and with or without growing point excision treatment for each light condition.

lets developed on more than half of all individual samples. Conversely, shoot formation frequency in the dark was only 8%. Figure 1A–C shows PLBs cultured under each of the 3 light conditions without excision treatment. Under high light conditions, many PLBs formed shoots and roots and developed into plantlets. Under low light conditions, some PLBs formed leaves. Under dark conditions, many long and narrow PLBs developed from the basal part of the parental PLBs (Fig. 1E). The shoots that developed from PLBs under dark conditions were pale yellowish-white and thin, and did not expand their leaves.

In the PLBs subjected to growing point excision, Nt and Np were higher under high light conditions followed by low light and dark conditions (Table 2). Under high and low light conditions, shoots developed from approximately 40% of all individuals after 6 months of culture when growing point excision treatment was performed every month, whereas the frequency of shoot

formation in the dark was about 1%. PLB length was 4.5, 6.2, and 9.6 mm under high light, low light, and dark conditions without excision treatment and 4.5, 6.4, and 8.8 mm under high light, low light, and dark conditions with excision treatment, respectively (Table 2). Thus, as shown in Figure 1D, PLBs became elongated in both low light and dark conditions. In addition, the color of the PLBs obtained under high light conditions was green, while those obtained under low light and dark conditions were yellow-green and pale yellowish-white, respectively.

Effect of light acclimation on ePLB propagation (Experiment 2)

The surface color of ePLBs kept under dark conditions was pale yellowish-white, while that of ePLBs after light acclimation treatment was yellow-green (Fig. 2). Although non-treated ePLBs were elongated and rod-shaped, ePLBs subjected to acclimation showed the same structures except for their tops, which were enlarged and spherical.

Thus, in order to investigate the effect of light acclimation on ePLB proliferation, ePLBs with and without acclimation pre-treatment were transferred to high light conditions for 2 weeks (Table 3). Percentage survival did not differ between the two conditions, but both the rate of ePLBs with secondary PLB formation and the number of secondary PLBs per initially cultured PLB were significantly higher than those of non-treated ePLBs ($P < 0.01$, and $P < 0.05$, respectively).

Comparison of nPLB and ePLB proliferation with partial incision treatment (Experiment 3)

Partial incision treatment damaged nPLBs and decreased the survival rate (57%), but no apparent reduction in percentage survival was observed in nPLBs without partial incision and ePLBs with or without partial incision treatment ($P > 0.05$) (Table 4). Partial inci-

Fig. 1. Difference in growth of PLBs after culture under different lighting conditions with or without growing point excision treatment. A–C: PLB cultures without growing point excision under (A) high light, (B) low light, and (C) dark conditions. Under high light conditions, formation of leaves and roots from many PLBs was observed. Under low light, some PLBs formed leaves, but most parental PLBs developed secondary PLBs arising from their basal parts. Under dark conditions, many long and narrow PLBs developed from the basal part of the parental PLBs. These PLBs cultured under dark conditions were pale yellowish-white and thin, and did not expand their leaves, which are features of skotomorphogenesis. D: Difference in lengths of secondary PLBs produced from PLBs with growing point excision treatment after culture under high light (nPLB) (left), low light (center), and dark (ePLB) (right) conditions. PLB length increased as light intensity decreased. E: Some examples of ePLB proliferation after culture without growing point excision under dark conditions. Arrowheads show parental ePLBs and arrows show secondary ePLBs. Long and narrow ePLBs developed from the basal part of the parental ePLBs. Scale bars, 1 cm for A to E. Observations were made after 6-month culture.

Fig. 2. Morphological changes in ePLB in response to light acclimation. A: With acclimation. B: Without acclimation. Bar = 1 cm. During the acclimation period of 2 weeks under low light conditions, the tops of the ePLBs became enlarged and turned yellow-green from pale yellowish-white (A) compared with ePLBs maintained under dark conditions (B).

Table 3. Effect of light acclimation treatment of ePLBs on secondary PLB propagation.

Acclimation treatment	Percentage survival (%)	Secondary PLB formation (%)	No. of secondary PLB
–	100	55.0 ± 4.7 ^z	1.3 ± 0.2
+	100	88.3 ± 2.4	2.8 ± 0.4
Significance ^y	NS	**	*

^z Mean ± SE (n = 3).

^y NS, * and ** indicate a non-significant and a significant difference with $P < 0.05$ and a significant difference with $P < 0.01$ by the t-test, respectively. ePLBs produced by dark condition culture were transferred to either low light or dark conditions for an additional 2 weeks. Culture under low light conditions was performed as light acclimation treatment. After 2 weeks of culture with or without light acclimation, the PLBs were subjected to partial incision treatment and transferred to high light conditions. After 2 weeks of culture to high light conditions, percentage survival, percentage secondary PLB formation, and average number of secondary PLBs per initially placed PLB were calculated. Twenty ePLBs were used for each treatment, each of which was performed in triplicate.

Table 4. Effect of cutting treatment of PLBs on PLB cultures.

Sample type	Cut treatment	Percentage survival (%)	Shoot formation percentage (%)	Secondary PLB formation (%)	No. Secondary PLB
nPLB	–	95.0 a ^z	88.0 a	40.0 b	4.0 a
	+	57.0 b	54.0 ab	42.0 b	5.6 a
ePLB	–	100.0 a	96.0 a	86.0 a	9.8 a
	+	89.0 a	21.0 b	89.0 a	31.9 b

^z Different letters indicate significant differences at $P < 0.05$ according to Tukey's test (n = 5).

Twenty nPLBs or ePLBs were used for each treatment. ePLBs were acclimated for 2 weeks under low light conditions before cutting treatment. Each treatment was performed 5 times. After 2 months of culture with subculturing every 2 weeks, observation was made.

sion generally suppressed shoot formation. For example, the percentage shoot formation of ePLBs with partial incision was significantly lower than the shoot formation percentage of both nPLBs and ePLBs without partial incision ($P < 0.05$). Percentage secondary PLB formation was observed to vary between the types of PLB; for example, the percentage in ePLBs was significantly higher than that in nPLBs ($P < 0.05$). Partial incision had no significant effect on percentage secondary PLB formation ($P > 0.05$). Of the 4 conditions, the number of secondary PLBs obtained from ePLBs with partial incisions was significantly higher than that obtained from the other 3 conditions.

Figure 3 shows the results of secondary PLB formation for each of the experimental conditions in Experiment 3. In nPLB without partial incision, shoots, roots, and plantlets developed from nPLBs and secondary PLB formation was hardly observed (Fig. 3A). In nPLB with partial incision, although some development of plantlets was observed from parental nPLBs, a large number of secondary PLBs were formed at the base of the nPLBs (Fig. 3B). In ePLB without partial incision (Fig. 3C), development of plantlets from the top of each ePLB was observed, with abundant secondary PLBs (9.8 per one ePLB) forming from the base of ePLBs. In ePLB with partial incision (Fig. 3D), a few plantlets were observed at the top of ePLBs, with numerous secondary PLBs (31.9 per one ePLB) arising from the entire ePLB surface.

Discussion

Tanaka (1987) suggested that PLBs of *Phalaenopsis* form shoots considerably easier than those of other

Fig. 3. Effect of partial incision treatment on secondary PLB formation from the surface of PLBs. A: nPLB (without partial incision). B: nPLB (with partial incision). C: ePLB (without partial incision). D: ePLB (with partial incision). Bar = 1 cm. PLBs were cultured as described in Experiment 3 of Materials and Methods. In comparison with nPLBs (A, B), many secondary PLBs were formed from the surface of ePLBs (C, D). Shoot formation from the top of ePLBs with partial incision (D) was hardly observed and formation of numerous secondary PLBs was confirmed on most of the surface of ePLBs.

orchid species, such as members of *Cymbidium*. This is important because the capacity of PLBs to proliferate decreases after shoot formation. Consequently, numerous methods for promoting PLB proliferation have been examined to date, including growing point excision (Amaki and Higuchi, 1989), inhibition of shoot formation (Tanaka, 1997), and cutting PLB into pieces before shoot development (Murdad et al., 2006; Tomuro et al., 1994).

In the present study, we examined the effect of growing point excision treatment on secondary PLB proliferation from PLBs grown under 3 different light conditions (Exp. 1). Without excision treatment, PLBs grown under dark conditions did not form shoots and had much higher PLB proliferation (Np) than those grown under high light and low light conditions. However, growing point excision treatment greatly reduced PLB proliferation in the dark culture, whereas the treatment greatly enhanced both the suppression of shoot formation and PLB proliferation in the culture under light conditions, especially under high light conditions (Table 2).

We then considered that a large number of secondary PLBs could be also obtained from dark-grown ePLBs if they were subjected to growing point excision treatment after turning green. Consequently, the effect of greening treatment of ePLBs on secondary PLB formation was examined (Exp. 2). Partial incision treatment was applied instead of complete excision treatment of growing points before transfer to high light conditions because the former treatment was reported to cause less damage to PLBs (Nii et al., 2004).

During the acclimation period, PLBs enlarged at their top and turned yellow-green from pale yellowish-white (Fig. 2). As a result, acclimated ePLBs with low light intensity gave a much higher percentage of secondary PLB formation and a higher number of secondary PLBs than ePLBs directly transferred to high light conditions after culture under dark conditions without reducing the survival rate (Table 3). These results suggest that injury of the shoot apex by partial incision treatment is also effective for enhancing secondary PLB formation from ePLBs after they have turned green by exposing to light conditions.

The final comparison of secondary PLB formation between nPLB and ePLB with or without partial incision treatment revealed that ePLB with partial incision treatment gave the highest efficiency of secondary PLB production, i.e., 32 PLBs were formed per inoculated PLB after 2 months of culture (Table 4). The reasons for the effectiveness of partial incision of ePLBs on secondary PLB formation were considered to be as follows.

Firstly, the percentage survival of nPLBs with partial incision was significantly lower than the percentage survival of nPLBs without partial incision and ePLBs with or without partial incision (Table 4). The partial incisions made a cutting injury at approximately one-third the length of nPLBs. Small nPLBs could not survive the

wound response to the partial incision since the wound response of *Phalaenopsis* is severe compared to other orchids and sometimes causes necrosis. Partial incisions in the ePLBs were made to the top one-third of the enlarged region that developed after light acclimation (Fig. 2). This injury was less influential since ePLBs are larger than nPLBs, and there is enough tissue in an ePLB that is not affected by the wound. It is considered that these differences between nPLBs and ePLBs in the damage caused by partial incision affected the observed difference in the percentage survival of the two types of PLB.

Secondly, a significantly low shoot formation percentage (21%) was only observed in ePLBs with partial incisions, which was markedly lower than the shoot formation percentage of 70.0–96.7% reported for nPLB by Nii et al. (2004). Although a decrease of shoot formation percentage was also observed in nPLBs with partial incision, the decrease was not significantly different from those in nPLBs without partial incision and ePLBs without partial incision.

Finally, the percentage of secondary PLB formation of ePLBs was higher than that of nPLBs, but partial incision treatment did not affect the percentage of secondary PLB formation. It has been reported that the percentage of secondary PLB formation is higher in the basal parts than in the top parts of nPLBs (Amaki and Higuchi, 1989; Tanaka, 1987). In particular, when partially incised ePLBs had been subjected to light acclimation before culturing under high light conditions, a large number of secondary PLBs were formed from the basal half of the enlarged spheroidal part produced during the light acclimation period as well as the entire elongated rod-shaped part produced during dark culture (Fig. 3C, D). Therefore, it is suggested that the parts corresponding to the basal parts of nPLBs, which are highly productive with respect to secondary PLBs, are enlarged in ePLBs, which contributed to the increase in the percentage of secondary PLB formation and the number of secondary PLBs. It is therefore suggested that ePLBs with partial incision yield more secondary PLBs than nPLBs because of the relatively small decrease in the percentage survival, increase of proliferation ability through a decrease in the shoot formation percentage, and an increase in the PLB productive surface.

In summary, the present study revealed that the proliferation of PLBs was facilitated by suppressing shoot formation by culturing under dark conditions (Exp. 1), which did not require complicated conventional operations, such as dividing PLBs and/or growing point excision. Since the PLBs obtained from dark condition cultures were elongated by skotomorphogenesis, application of the partial incision method reported by Nii et al. (2004) for ePLBs produced 6 times as many secondary PLBs as nPLBs with partial incision and 8 times as many as nPLBs without partial incision (Exp. 3).

From the results of this study, we propose a highly

Fig. 4. Schematic diagram of a simple and efficient PLB proliferation system. Open and shaded squares indicate high light and dark conditions, respectively. In this system, by subculturing under dark conditions in process I, ePLBs can be produced and maintained with suppressed shoot formation. Subsequently, after light acclimation, numerous secondary PLBs (nPLBs) can be obtained by culturing the ePLBs with partial incision under light conditions in process II.

efficient and simple micropropagation system using ePLBs, as shown in Figure 4. In this system, by subculturing under dark conditions, ePLBs can be produced and maintained while suppressing shoot formation. In addition, the method does not require a labor-intensive process, such as cutting. Subsequently, after light acclimation, numerous secondary PLBs can be obtained by culturing ePLBs with partial incision under light conditions. Further, induction of ePLBs from these secondary PLBs is also possible by returning them to dark conditions for culturing. Using this system, 728 ePLBs can be obtained from 5 PLBs under dark culture conditions for six months using process I (Table 2). Then, by subjecting the obtained ePLBs to process II, about 32 secondary PLBs can be formed on an ePLB in two and a half months (Table 4). By a simple calculation, it is thus possible to produce more than 20 thousand plantlets from 5 PLBs within a year. This system is well suited to commercial nursery clonal plant production of *Phalaenopsis*.

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