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SHORT COMMUNICATION

Multiple Regeneration Pathways via Thin Cell Layers in Hybrid *Cymbidium* (Orchidaceae)

Jaime A. Teixeira da Silva* and Michio Tanaka

Department of Horticultural Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761–0795, Japan

Abstract

Protocorm-like body (PLB) and subsequent shoot development in hybrid *Cymbidium* Twilight Moon 'Day Light' can be established *in vitro* via 3 pathways: PLBs, PLB thin cell layers (TCLs), or embryogenic callus (EC). Traditionally *Cymbidium* hybrids are mass-produced commercially through the *neo*-formation of secondary PLBs (2° PLB) from initial or primary PLBs (1° PLB) or PLB segments, or from PLB TCLs, resulting in a moderate number of 2° PLBs (average 4.46 2° PLBs/1° bisected PLB, or 1.12 2° PLBs/ PLB TCL). This study shows that EC can be induced from 1° PLBs or PLB TCLs. Thereafter, resulting 2° PLBs (average 22.1 2° PLBs/EC cluster derived from 1° PLB) form directly from the EC on the same medium or following the transfer of EC onto PGR-free medium. By flow cytometry and PCR-RAPD analysis, the cytogenetic stability of 1° PLBs, of resulting 2° PLBs and EC, and plants derived therefrom was demonstrated.

Key words: Callogenesis; Caulogenesis; Flow cytometry; RAPD; Vitron®

Introduction

The effective tissue culture of somaclonally invariant *Cymbidium* is considered to be a priority to preserve this ornamental commodity's germplasm. The tissue culture of *Cymbidium* shoot tips incidentally also marked the birth of tissue culture per se in 1960 (Morel 1960). Since then, protocols for the tissue culture of *Cymbidium* from flower stalks, pseudobulbs, flower buds, shoot tips, or protocorm-like bodies (PLB) have been described. The term PLB, first coined by Morel (1960), describes structures that resemble protocorms but that are formed by tissue explants and/or callus *in vitro*. Studies on callus induction are scarcer, likely because of their slow growth and necrotic tendency (Begum and others 1994a). Callus has also been shown to be inducible from pseudobulb sections, rhizomes, and roots of *C. ensifolium* seedlings, a terrestrial orchid species (Chang and Chang 1998). In general, though, callus induction is slow, difficult, time-dependent, and inconsistent.

This study was designed to investigate the capacity to induce secondary PLBs (2° PLBs) primarily via

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*Corresponding author; e-mail: jaimetex@yahoo.com

three developmental pathways: primary PLBs (1° PLB), 1° PLB-derived embryogenic callus (EC), or 1° PLB-derived thin cell layers (TCLs), all of which lead to the same cytogenetically stable 2° PLBs and shoots. Moreover resulting 2° PLBs, plantlets, and EC were investigated for cytological and genetic variability or stability, with the effect of variation on subsequent *in vitro* plantlet growth and acclimatization.

This is the first report ever on the regeneration of *Cymbidium* via TCLs or via TCL-derived EC, resulting in up to as many as five times the number of PLBs as conventional methods reported in previously published (conventional) protocols. We further demonstrate that EC is both re- and sub-culturable, and can be induced to form PLBs by the simple removal of sucrose from the medium, making this protocol extremely practical, and valuable for commercial growers and breeders. Moreover, we were able to show, through comparative ploidy analyses, that somatic embryos and PLBs, until now loosely defined as being separate morphological entities, are in fact one and the same organ.

Materials and Methods

Plant Material, Culture Conditions, (*In Vitro*) Acclimatization

Primary PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan) originated from shoot-tip culture were subcultured every 2 months on modified Vacin and Went medium (Vacin and Went 1949) supplemented with 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin, 2 g l^{-1} tryptone, and 20 g l^{-1} sucrose ($VW_{PLB.TCL}$), and solidified with 8 g l^{-1} Bacto agar (Difco Labs., USA). Embryogenic callus induction and proliferation medium (VW_{EC}) was similar to $VW_{PLB/TCL}$, except that thidiazuron (TDZ) was used instead of kinetin. All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Shoots (n = 25; 4–5 cm in length) derived from 2° PLBs from either the PLB, EC, or TCL pathways were transferred to an OTP® film culture vessel, the Vitron®, embedded in a 25-hole rockwool Multiblock (Grodania®, Denmark), prior to which 200 ml agar-less $VW_{PLB/TCL}$ medium was evenly distributed. In addition, and as a control, twenty 1° PLB-derived shoots originating from either the PLB, EC, or TCL pathways were placed on Hyponex® (6.5:6:19; 3 gl^{-1}, water-soluble fertilizer, Hyponex, USA), and 3% sucrose medium (100 ml) in a glass bottle (n = 5; 75 × 130 mm). Stock cultures were kept on 40 ml medium in 100 ml Erlenmeyer flasks, double-capped with aluminium foil, at 25 ± 1°C, under a 16 h photoperiod with a light intensity of 45 μmol m^{-2} s^{-1} provided by plant growth fluorescent lamps (Homo Lux, Japan), conditions under which all culture vessels were placed.

Rooted shoots (on rockwool in the Vitron®; Figure 1H) can be individually separated and used directly in the greenhouse, without the need for special acclimatization procedures.

PLB versus EC versus TCL Pathways

Longitudinally bisected hybrid *Cymbidium* Twilight Moon 'Day Light' 1° PLB (3–4 mm in diameter) segments (Figure 1A) were used as explants for 2° PLB and EC induction. In the PLB pathway (Figure 1), 1° PLB segments were made to induce 2° PLBs (Figure 1B) on $VW_{PLB/TCL}$. Secondary PLB segments derived from 1° PLBs in the PLB pathway could be used once again as 1° PLBs (Figure 1C and 1D). In the EC pathway, EC was induced (Figure 1E) on VW_{EC}. Embryogenic callus (40 mg, 2 months old) was removed from 1° PLB segments in the EC pathway, and subsequently transferred to PGR-free medium (faster method; Figure 1G), or maintained on the same medium (slower, unconventional method) for the formation of 2° PLBs (Figure 1G). In the TCL pathway, 2° PLBs derived from 1° PLBs in the PLB pathway can be prepared either as transverse TCLs (tTCLs; 200–500 μm thick, 2–4 mm in diameter; Figure 1I) or longitudinal TCLs (lTCLs; 200–500 μm thick, 2 mm^2; Figure 1K), resulting in the formation of 2° PLBs (Figure 1J and 1L, respectively). Longitudinal TCLs are primarily epidermal with few subepidermal layers; that is, they are of a single cell type, whereas tTCLs have multiple cell types. In PLB, EC, and TCL pathways, 2° PLBs can give rise to shoots and roots (Figure 1H) when left indefinitely on the same medium. Two minor pathways, created using root tips or leaf blades (Figure 1M, 1N, and 1O, respectively), result in little or no 2° PLB formation, respectively. All cultures were kept under flask, temperature and light conditions as described above.

Morphogenic, Photosynthetic, Flow Cytometric and Genetic Analyses

Plantlet growth was quantified by the number of new leaves and roots, plant height, and the fresh and dry weight of shoots and roots. Chlorophyll content in the third leaf (counting downward from the top) of the plantlets was measured as the SPAD value (measure of photon capture and chlorphyll content) by a chlorophyll meter (SPAD-502, Minolta, Japan).

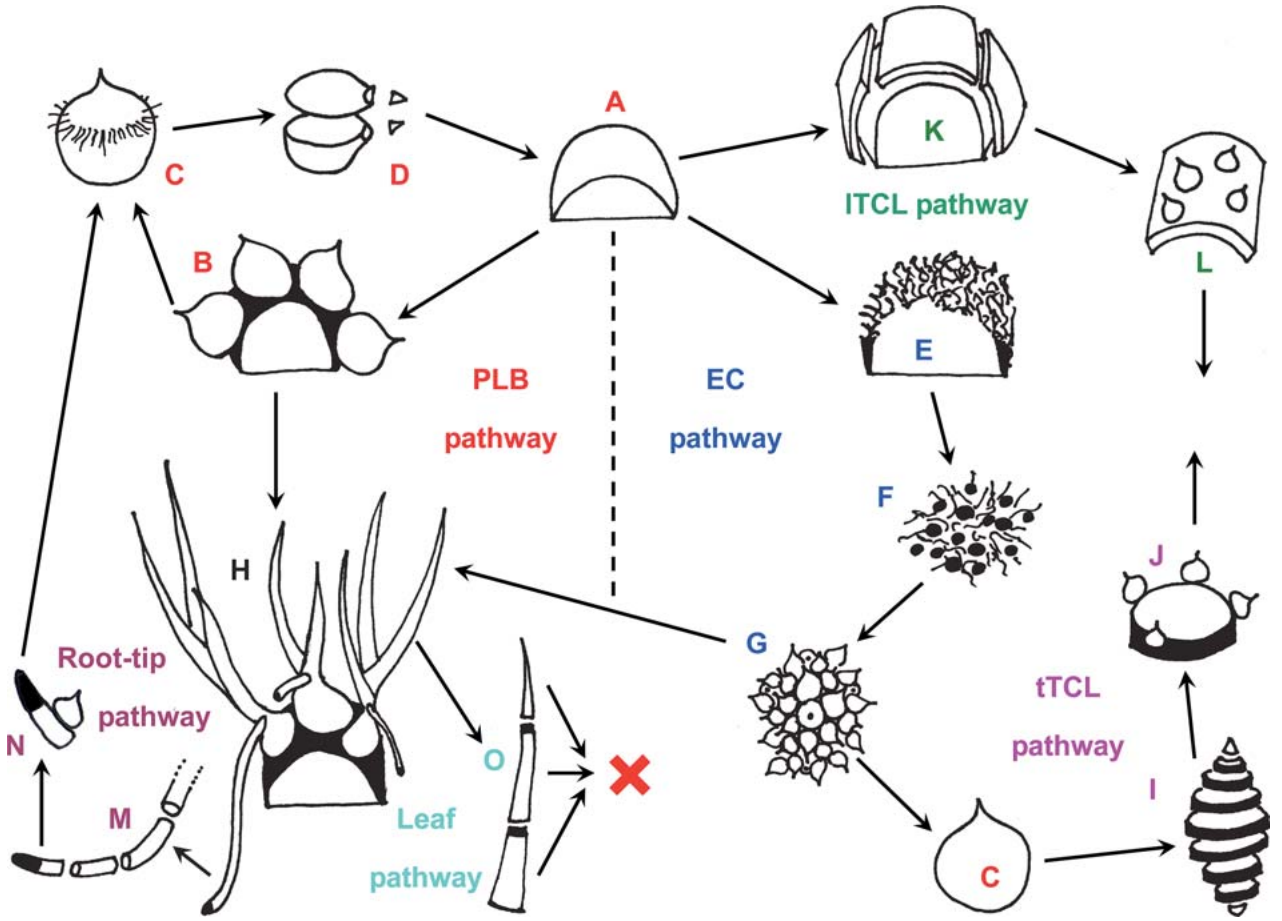


Figure 1. The induction of embryogenic callus (EC) and protocorm-like bodies (PLBs), and subsequent development of shoots by different induction pathways. Longitudinally bisected hybrid *Cymbidium* Twilight Moon 'Day Light' 1° PLB (3–4 mm in diameter) segments (A) are used as explants for 2° PLB and EC induction. In the PLB pathway, 1° PLBs are made to induce 2° PLBs (B) on $VW_{PLB/TCL}$. Secondary PLBs derived from 1° PLBs in the PLB pathway can be used again as 1° PLBs (C, D). In the EC pathway, EC is induced (E) on VW_{EC}. Embryogenic callus is removed from 1° PLBs in the EC pathway and subsequently transferred to PGR-free medium or maintained on the same medium for the formation of 2° PLBs (G). In the TCL pathway, 2° PLBs derived from 1° PLBs in the PLB pathway can be prepared either as transverse TCLs (I) or longitudinal TCLs (K), resulting in the formation of 2° PLBs (J and L, respectively). In PLB, EC, and TCL pathways 2° PLBs can form shoots and roots (H) when left indefinitely on the same medium. Two minor pathways, using root tips or leaf blades (M, N, and O, respectively), result in little or no 2° PLB formation, respectively.

Nuclei were isolated from 0.5 cm^2 of material (all steps in Figure 1) by chopping in a few drops of nucleic acid extraction buffer (Partec Cystain UV Precise P, Germany) followed by digestion on ice for 5 min. The nuclear suspension was filtered through 30-µm mesh size nylon filter (CellTrics®) and five times the volume of Partec Buffer A (2 µg ml^{-1} 4,6-diamidino-2-phenylindole (DAPI), 2 mM $MgCl_2$, 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, 0.1% Triton-X, pH 7.5) and left at room temperature for 5 min. Nuclear fluorescence ($\leq$ 5,000 nuclei) was measured in three samples with a Partec® Ploidy Analyser, and relative fluorescence intensity of the nuclei was analyzed when the coefficient of variation was less than 3%.

DNA was isolated from *in vitro* PLBs or callus of *Cymbidium* Twilight Moon 'Day Light' using Qiagen DNeasy® (U.S.A.) according to the manufacturer's instructions and following recommendations for DNA extraction and polymerase chain reaction (PCR) analysis of orchids (Teixeira da Silva and Tanaka 2005). DNA was quantified using a Shimadzu UV-1200 spectrophotometer, and purity was established from 260:280 nm ratios.

Three PCR/mtDNA primer sets were used: 5SP3/5SP4, which amplifies a portion of the 5S-rRNA gene clusters; LFY3 and trnh, which encode for a portion of the homeotic gene, *LEAFY* intron; and *trnD-trnT* locus of cpDNA, respectively. Polymerase chain reaction was performed as

Table 1. Effect of Choice of Regeneration Pathway on PLB and Callus Formation, and Ploidy in *Cymbidium* Twilight Moon 'Day Light'

Regeneration pathway	Explant	% PLBs*[5]	No PLBs*[6]/ explant	% Callus*[7]	FC*[8]	FC*[9] PLB	FC*[10] Callus
EC	Callus*[1]	100[a]	22.1 ± 1.16[a]	31[a]	66:32:t:0	66:33:t:0	60:36:4:0
PLB	Whole*[2]	96.4[a]	8.41 ± 0.72[b]	2.3[c]	72:28:0:0	68:30:2:0 (*pooled*)	56:38:3:3 (*pooled*)
	Bisected	89.2[ab]	4.46 ± 0.23[c]	6.1[b]	64:31:3:0		
	tTCL	81.1[b]	1.12 ± 0.21[d]	0[d]	66:30:4:0		
	lTCL	23.7[c]	0.26 ± 0.08[e]	0[d]	61:39:0:0		
Leaf	Tip	0[d]	0[e]	0[d]	91:9:0:0	66:33:1:0 (*pooled*)	61:37:2:0 (*pooled*)
	Sheath	0[d]	0[e]	0[d]	73:25:t:0		
Root	Proximal tip*[3]	0.4[d]	0.12 ± 0.01[e]	0[d]	75:17:6:t	70:29:1:0 (*pooled*)	62:33:3:t (*pooled*)
	Distal end*[4]	0[d]	0[e]	0[d]	96:4:0:0		

*FC: flow cytometry; TCL: thin cell layer. *[1]Reversion to PLBs can occur easily on the same medium or on PGR-free medium; *[2]with shoot tip removed; *[3]non-sectioned explant that includes meristem and root cap initials in which cell division and expansion occurs, that is, terminal 2 mm; *[4]c/s taken from at least 1 cm behind the proximal tip; *[4]sterile distilled water in which PLBs were dissected: 10 ml per 100 PLBs; *[5]% of explants forming PLBs on PLB induction medium; *[6]numbers of PLBs formed on PLB explants when on PLB induction medium; values presented as mean ± SE; different letters within a column indicate significant differences at $p < 0.05$ according to Duncan's New Multiple Range test; *[7]% of explants forming yellow or white, friable, or hard (embryogenic) callus. *[8,9,10] Relative amounts (2C:4C:8C:16C); a minimum of three samples per explant source (t: trace, ≤ 2%); *[8]ploidy level for specific explant source; *[9,10]ploidy level for resulting PLB or callus, respectively.*

previously described (Teixeira da Silva and Tanaka 2005).

For scanning electron microscopy, samples were fixed in 30% FAA for 2 days, 50% FAA for 2 days, and 70% FAA for 2 days. Fixed specimens were dehydrated through an ethanol/acetone series, critical point dried, coated with Platinum (Pt), and examined in a scanning electron microscope (Hitachi S-2150, Japan).

Statistical Analyses

Growth, photosynthetic, and ploidy experiments were organized according to a randomized complete block design (RCBD) with three blocks of 20 replicates per treatment. Data were subjected to analysis of variance (ANOVA) with mean separation ($p \leq 0.05$) by Duncan's New Multiple Range test (DMRT) with SAS® version 6.12 (SAS Institute, Cary, NC, USA).

Results and Discussion

In Vitro Organogenesis and Plantlet Formation

Plant regeneration from orchid callus is usually achieved through PLB formation (Begum and others 1994b; Huan and Tanaka 2004). Callus induction has been achieved from both the inner (Begum and others 1994a) and outer tissues (Huan and Tanaka 2004) of PLBs, or from pseudobulbs, rhizomes, and root explants of terrestrial orchid, *Cymbidium ensifolium* (Chang and Chang 1998). In the latter induction studies, callus production was slow (12–18 months) and it required high levels of PGRs. Huan and Tanaka (2004) reported that the induction of callus from PLB half-segments occurred within a month, and on single PGR (NAA or 2,4-D)-supplemented medium, or in combination with TDZ.

Primary PLBs can form 2° PLBs in the PLB pathway (Figure 1B). The number of 2° PLBs formed is as follows: 8.41, 4.46, 1.12, and 0.26 per whole (apical meristem excised), bisected, tTCL or lTCL from 1° PLBs, respectively (Table 1). These 2° PLBs can in turn be used as 1° PLBs (explant material; Figure 1A) or to form shoots and roots (Figure 1H) if left on the same medium indefinitely, proving that PLBs are in fact somatic embryos in *Cymbidium*. In the EC pathway, EC can be induced from 1° PLBs (Figure 1E) at 22.1 PLBs per EC cluster (40 mg, 2 months old, following the subculture of initial EC on VW_{EC}; Table 1). Transfer of this EC to PGR-free medium results in the formation of large numbers of 2° PLBs (Figure 1F and 1G), which can also form shoots and roots (Figure 1H) if left on the same medium indefinitely.

Using the tTCL or the lTCL pathways (Figure 1I and 1J; 1K and 1L, respectively), 2° PLBs are formed on the surface, that is, PLB epidermal tissue. Secondary PLBs can also be induced from root tips (Figure 1M and 1N) but cannot be formed from any part of the leaves (Figure 1O). Embryogenic callus can be maintained indefinitely on the

same medium, and can be continuously proliferated for more than 1 year without cytogenetic variation.

The use of thin cross sections or TCLs of plant parts, designed to control morphogenesis more strictly than regular multi-tissue/organ explants (Teixeira da Silva 2003), especially from 6–7 week-old PLBs whose cells at this stage are highly meristematic, can be used to hasten and improve plant regeneration in *Cymbidium*. This is so because of the availability of nutrients and growth-promoting substances at the site of regeneration (Teixeira da Silva and others 2005b) and because of the elimination of correlative control imposed by other tissues and organs (Teixeira da Silva and others 2005a). Studies on the clonal propagation of *Cymbidium* from leaf primordia concluded that the optimal size of explants is about 0.5 mm including 3–4 leaf primordia, and that PLBs arise at the base of leaf primordia (Lavrentyeva 1986).

The production of 2° PLBs can be hastened by the use of tTCLs or lTCLs as compared to whole or bisected PLBs. In both these TCL types, the origin of 2° PLBs is from the epidermal tissue of 1° PLBs, easily confirmed by the use of lTCLs, which are covered by 2° PLBs, or by the use of tTCLs, in which 2° PLBs form only on the edge of the explant (Figure 2B), that is, epidermal tissue. The increased production of 2° PLBs can be achieved by inducing EC first (Table 1). Secondary PLBs may form directly from EC maintained on the same medium, as a result of the total consumption of PGRs by the EC, whereas when EC is transferred to PGR-free medium, the same developmental pathway results. The addition of sucrose to PGR-free medium results in EC, but the removal of sucrose results in PLB formation (data not shown). This, together with the fact that *neo* PLB formation—that is, 2° PLBs can only form from the peripheral tissue of tTCLs, or from the entire surface of lTCLs—demonstrates that, until now, EC and immature PLBs were considered to be separate entities. We propose that somatic embryogenesis and PLB formation are essentially identical processes—at least at the histological and cytogenetic levels, as shown by Teixeira da Silva and others (2005a). We will conduct gene expression studies on embryogenesis-associated genes to confirm our claims.

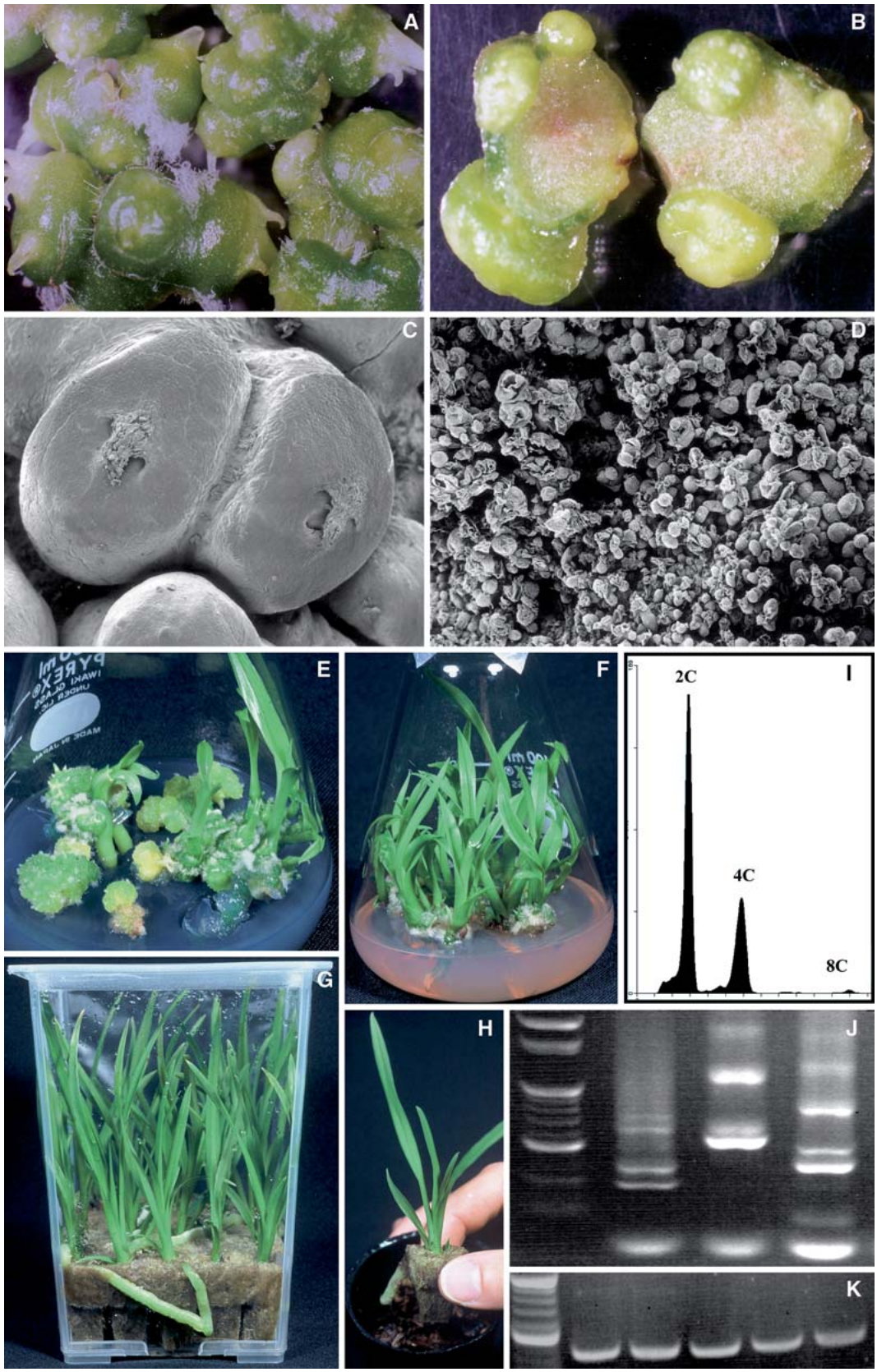
Cytogenic Variation Analysis

In both the PLB and EC pathways, little cell division was observed in the mid-section and base of adventitious shoots and roots. Contrastingly, active cell division (4C) was observed in 2° PLBs, EC and in EC-derived 2° PLBs (Table 1). High levels of endoreduplication (8–16C) were observed in root tips of both methods, in induced (Figure 1E) and proliferated (Figure 1F) EC and in the early stages of EC-derived 2° PLB formation (Figure 3).

Results of this study indicate that *Cymbidium* plantlets can be induced from either 1° PLBs via 2° PLB formation (PLB pathway), or via 1° PLB-induced-EC-derived 2° PLBs (EC pathway). Despite differences in ploidy levels between different steps (Figure 3), the resultant ploidy of plantlets (Figure 1H) is both similar and stable, with no endoreduplication.

Whereas Fukai and others (2002) showed that PLB epidermal tissue had only 2C and 4C peaks, Fujii and others (1999) claimed that most cells in the outer tissue of *Cymbidium* PLBs had a 2C DNA content, but following an NAA treatment, nuclear DNA contents increased up to 16C (that is, the cells showed higher nuclear polyploidy), correspondent to increased cell size. Protocorm-like body–propagated epiphytic *Cymbidium* hybrids and rhizome-propagated terrestrial *C. kanran* Makino demonstrated polysomaty from 2C to 16C; roots and floral organs, excluding ovaries of hybrids, were highly polysomatic, as were the rhizomes and roots of *C. kanran* (Fukai and others 2002). In *Cymbidium*, ploidy ebbs and flows between pathways (Figure 1), and despite radical differences in endopolyploidy between developmental stages, the resulting plantlets are cytogenetically stable (Figures 1 and 3; Table 1). The incidence of endoreduplication and

Figure 2. *In vitro* growth and acclimatization, and histological and cytogenetic analyses of hybrid *Cymbidium* Twilight Moon 'Day Light'. (A) 2° PLBs derived from 1° PLBs in the PLB pathway; (B) 2° PLBs forming on the epidermal edge of tTCLs; (C) SEM of 'twin' PLBs formed on $VW_{PLB/TCL}$; (D) SEM of EC formed on VW_{EC}; (E) mixed organogenesis (PLBs, EC, shoots); (F) shoots rooting after 65 days on Hyponex®; (G) well-rooted and developed plants after 90 days on rockwool in the Vitron®, which can be easily transferred into the greenhouse as individual units (H) without the need for acclimatization; (I) histogram of typical PLB tissue, exhibiting low levels (8C) of endopolyploidy: x-axis (staining intensity), y-axis (nuclear count); (J) RAPD-PCR using 5S rDNA primers: lane 1 (100 bp molecular marker), lane 2 (PLB from PLB pathway), lane 3 (EC from EC pathway), lane 4 (root+PLB in root-tip pathway); (K) RAPD-PCR using *LFY3* primers: lane 1 (100 bp molecular marker), lane 2 (PLB from PLB pathway), lane 3 (EC from EC pathway), lane 4 (root+PLB in root-tip pathway), lane 5 (leaf from PLB-derived shoot), lane 6 (lTCLs).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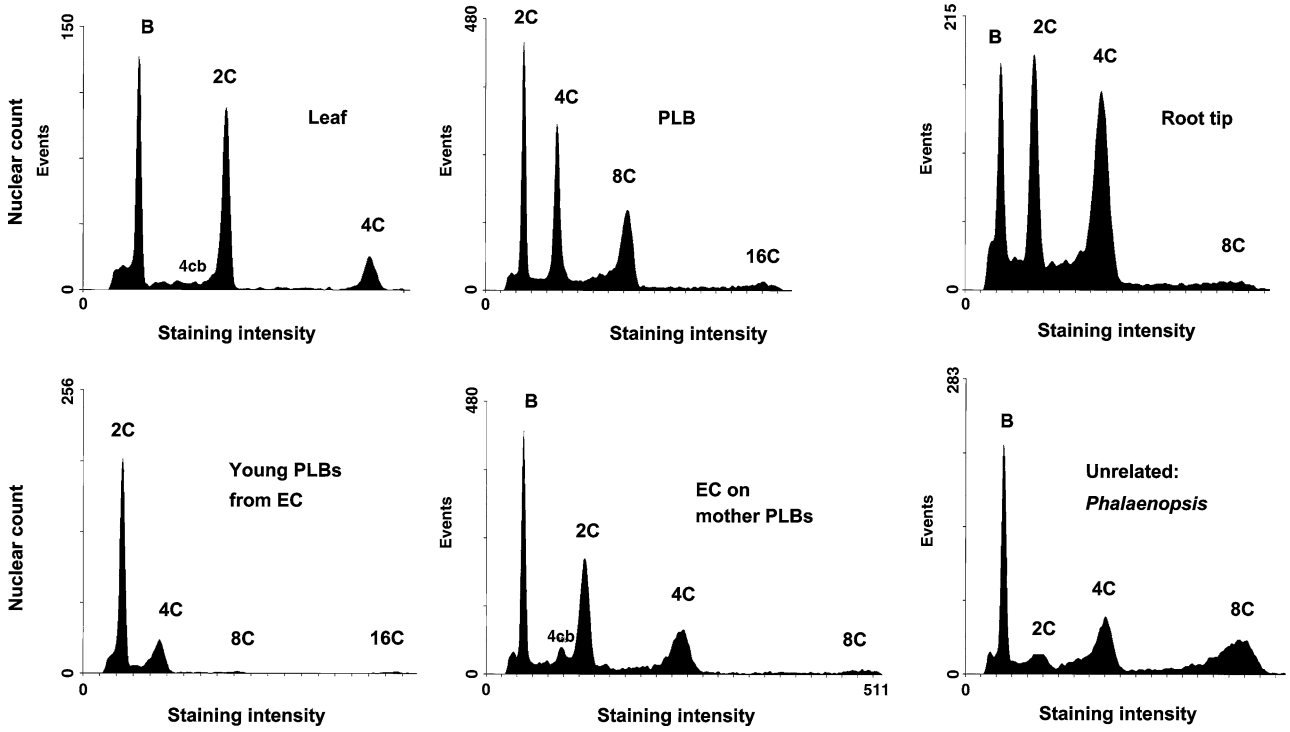

Figure 3. The location, lack, or incidence of endoreduplication in different parts of the plant and stages of development. B: diploid barley.

the link to regeneration capacity may be related to the cell cycle phase, the ploidy level, the position of the cell, and cellular development.

RAPDs were previously used to distinguish 36 *Cymbidium* cultivars (Obara-Okeyo and Kako 1998), whereas in our study, because of the choice of explant source and regeneration pathway, use of chloroplast-based and mitochondrion-based RAPD-PCR allowed for the detection of changes in the photosynthetic/mitochondrial machinery and genomes (Figure 2J and 2K).

Provisional experiments done on eight other *Cymbidium* hybrids (Aroma Candle 'Hot Heart', Pretty Poetry 'Malachite', Alice Beauty '№ 1', Spring Night '№ 12', Dream City '№ 1', Call Me Love 'Snow Princess', Energy Star '№ 4', Sweet Moon '№ 2') indicate that the PLB or callus formation follow the same developmental patterns in response to the same factors, although in a quantitatively different way.

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