Polysomaty in *Cymbidium*

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Abstract. Nuclear DNA content in various parts of *Cymbidium* plants was measured by flow cytometry. Two types of *Cymbidium*, protocorm-like body (PLB)-propagated epiphytic hybrids and rhizome-propagated terrestrial *C. kanran* Makino demonstrated polysomaty. Small shoots on PLBs of *Cymbidium* hybrids showed two peaks (2C and 4C) while PLBs showed four peaks, estimated to be 2C, 4C, 8C, and 16C. Roots and floral organs excluding ovaries of hybrids were highly polysomatic as were the rhizomes and roots of *C. kanran*. The patterns of polysomaty development were organ and developmental stage specific. Young leaves taken from *in vitro* plants are suitable material for determining the ploidy levels of *Cymbidium* plants by flow cytometry.

Cymbidium is among the most successful orchids as pot plants and cut flowers. Large numbers of new genotypes are released each year to meet consumer's demands. Recent genotypes of epiphytic *Cymbidium* hybrids are mostly triploid or tetraploid. The basic chromosome number of *Cymbidium* plants is $2n = 40$ with some exceptions (Aoyama, 1989). Speedy and reliable methods for ploidy determination are required for an effective breeding program. Precise information on ploidy levels is also beneficial to propagators and growers. Clonal multiplication of *Cymbidium* *in vitro* is achieved through protocorm-like body (PLB) proliferation in epiphytic *Cymbidium* and through rhizome proliferation in terrestrial *Cymbidium* (Arditti, 1977; Hasegawa, 1987). Propagators must pay attention to the genetic instability of propagules including altered ploidy levels. Growers will benefit from information on ploidy levels of new genotypes, which affect growth characteristics.

Flow cytometry is a powerful tool to determine the ploidy level of large numbers of samples within a short time (Dolezel, 1997). Recently, polysomaty has been reported in the orchids *Phalaenopsis* and *Dendrobium* by using flow cytometry (Jones and Kuehnle, 1998; Mii et al., 1997). Nagl (1972) reported DNA amplification in PLB parenchyma cells in *Cymbidium*. To determine the ploidy level of a plant by flow cytometry, the understanding of polysomaty development in each organ is required. The aim of this work is to characterize polysomaty in various organs of *Cymbidium* and assess which are most suitable for ploidy determination by flow cytometry.

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Materials and Methods

Two *Cymbidium* hybrids [Flower Dance 'Christmas Kiss' (4x) and Twilight Moon 'Day Light' (2x)], were obtained from a commercial source (Boi-U Co., Kagawa, Japan) as both flowering plants and *in vitro* cultures. PLBs were cultured on medium containing half strength MS salts, 100 g·L⁻¹ green banana fruit and 20 g·L⁻¹ sucrose. PLBs with small shoots, leaves, pseudo bulbs and roots of *in vitro* young plants and leaves, flower stalks, pedicels, ovaries (premature, green), pollinia and petals of flowering plants grown in a greenhouse were collected from both genotypes. Rhizomes, roots and plantlets of *C. kanran* Makino were collected from rhizome cultures maintained on medium containing 3 g·L⁻¹ Hyponex (complete soluble fertilizer, 6.5 N–6 P–19 K, Hyponex Co., Tokyo), 3 g·L⁻¹ Bacto peptone, 1 mg·L⁻¹ 1-naphthaleneacetic acid (NAA), 30 g·L⁻¹ sucrose and 2 g·L⁻¹ gellan gum (Hasegawa, 1987) at Kagawa Univ. Farm, Japan.

To isolate nuclei, 25 mm² of leaf and petal tissues, cross sections (1–2 mm thickness) of flower stalk, pedicel and green ovary, 1 mm root tips and 5 mm root tissues were prepared. Samples were collected from at least three independent plants and PLBs. Each sample tissue was chopped with a razor blade in a plastic dish with 0.5 mL of ice-cool buffer (solution A of high resolution DNA kit, Partech, Münster, Germany) and incubated on ice for 4 min. After filtration through a 20 µm nylon mesh, 2.5 mL of the staining solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM MgCl₂, 1% (w/v) PVP K-30 (Wako Chemicals, Tokyo), 0.1% (v/v) Triton X-100 and 2 mg·L⁻¹ 4',6-diamidino-2-phenylindole (DAPI), pH 7.5 (Mishiba et al., 2000) was added to the nuclei sample. After 1 min of incubation on ice, the fluorescence of the nuclei was measured using Ploidy Analyzer PA (Partech, Münster, Germany) equipped with a mercury lamp and filter combinations of KG1, BG38, UG1, TK420, and GG435.

At least 8000 nuclei of three different samples were measured for each position of the plants. Data were represented as histo-

grams showing relative nuclear DNA content of samples. The area of histogram peaks represented the number of nuclei for each ploidy level. Proportions of nuclei with different C-values were calculated and subjected to analysis of variance. The C-value of *Cymbidium* was determined by the measurement of the relative nuclear DNA content of pollen. *Hordeum vulgare* 'Bonus' (2C=9.41pg, Kankanpa et al, 1996) or *Triticum aestivum* 'Chikugo-izumi' leaf tissue were used as an internal standard to obtain reproducible results.

Results

Flow cytometric analysis of pollinia resulted in two peaks (Fig. 1). One was a vegetative nucleus and another was a generative nucleus in which DNA had replicated and arrested in the G2 stage, typical of bicellulate pollen (Bino et al. 1990), these two peaks representing 1C and 2C values of the *Cymbidium* 'Day Light' genome.

Small shoots (5 mm in height) produced on PLBs of 'Day Light' (diploid) showed two peaks, one large and one small, representing G0/G1 (2C) and G2 (4C) stage cells, respectively. On the other hand, PLBs showed four peaks estimated to be 2C, 4C, 8C and 16C from the relative nuclear DNA contents (Fig. 1). The proportion of 2C cells was only 20% while 4C and 8C cells were 40% and 32%, respectively. No difference was observed in the ploidy distribution when PLBs were divided into upper and lower parts. Samples of PLB epidermis that were excised carefully under a binocular microscope displayed only two peaks (2C and 4C), while the inner tissue showed four peaks (2C–16C) indicating high levels of polysomaty (data not shown).

Proportions of cells with each ploidy in different organs are summarized in Fig. 2. Leaves of *in vitro* plantlets (≈5 cm in height) and small shoots on PLBs had ≈90% and 80% of 2C cells, respectively, while the rest were 4C. The basal part of the plantlet, which develops into a pseudobulb, had 2C to 8C cells. Root tips (1 mm in length) of *in vitro* plantlets had 2C to 16C cells. Roots taken from 1 to 3 cm above the root tip had no 2C cells. Proportions of higher ploidy cells (8C and 16C) were significantly increased ($P = 0.01$) as the distance from the root tip increased.

'Christmas Kiss' (4x) showed the same developmental pattern of polysomaty as 'Day Light' if 4C–32C in the tetraploid is considered to correspond to 2C–16C in the diploid. 'Christmas Kiss' had a higher percentage of cells with lower ploidy levels than 'Day Light' (Fig. 2).

Flower stalks, pedicels and petals of 'Day Light' showed polysomaty having 2C to 16C cells (Fig. 3). On the other hand, ovaries had a significantly larger ($P = 0.01$) proportion of 2C cells. 'Christmas Kiss' showed a similar pattern of polysomaty development.

Rhizomes of *C. kanran* also showed polysomaty while shoots regenerated from rhizomes had no polysomaty (Fig. 4). Older parts of the rhizome consisted of cells with

significantly higher ($P=0.01$) ploidy levels in comparison with younger parts. Roots of *C. kanran* had polysomaty as observed in other *Cymbidium* hybrids (data not shown).

Discussion

Endoreduplication is cyclic DNA synthesis without cell division, leading to the existence of cells with different ploidy levels in a tissue or organ, which is termed polysomaty. Despite the appearance of polysomaty in a wide range of plant species (Cavallini et al., 1988; De Rocher et al., 1990; Galbraith et al., 1991; Gilissen et al., 1993; Mishiba and Mii 2000; Pijnacker et al., 1989; Smulders et al.,

1995), the function of polysomaty is not well understood. It has been claimed that polysomaty is related to nuclear genome size (De Rocher et al., 1990), interaction between nuclear and organelle genomes (Galbraith et al., 1991), cellular dimension (Melaragno et al., 1993), and auxin-induced cell elongation (Gilissen et al., 1993).

In this study both diploid and tetraploid genotypes showed a similar developmental pattern of polysomaty. The results showed that the patterns of polysomaty development were organ and developmental stage specific, agreeing with previous reports in various species (Galbraith et al., 1991; Gilissen et al., 1993; Smulders et al., 1994). The proportion

of lower ploidy cells in the tetraploid genotype 'Christmas Kiss' was much higher than that in the diploid genotype 'Day Light' as also reported in tomato (Smulders et al., 1994) and *Portulaca* (Mishiba and Mii, 2000).

Genome size of diploid *Cymbidium* 'Day Light' estimated from the genome size of *H. vulgare* 'Bonus' ($2C=9.41\text{pg}$, Kankapää et al., 1996) was $16.5\text{pg}/2C$. The estimated DNA value from DAPI-stained samples depends on the base composition of the plant because DAPI binds preferentially to AT-rich regions of DNA (Dolezel et al. 1998). The limitation of DAPI-stained samples in this experiment must be taken into account to compare the value of genome size estimated by PI stain.

Fig. 1. Histograms of fluorescence intensity of nuclei. **A)** pollinia, T; wheat leaf as an internal standard, **B)** shoots on PLBs, H; barley as an internal standard, and **C)** PLBs lacking shoots.

Fig. 2. Frequency of nuclei at different C-values measured in various parts of *Cymbidium* 'Day Light' (upper) and 'Christmas Kiss' (lower). Leaf (F-plant); mature leaf from flowering plants, root 3 cm, root 1 cm; root tissue 1 or 3 cm apart from root tip, root tip; about 1 mm root growing tip, pseudo bulb; base of in vitro plant, leaf (in vitro); leaf from in vitro plant, PLB; PLB excluding shoots, shoots on PLB; 5 mm shoots on PLB. 4 to 32C in the tetraploid 'Christmas Kiss' is considered to correspond to 2 to 16C in the diploid 'Day Light'.

But the obtained 2C value of 'Day Light' still does not support the idea that the occurrence of polysomaty relates to small genome size (De Rocher et al., 1990).

Shoots regenerated from PLBs were 2C/4C as expected whereas PLBs showed more frequent polysomaty. This means that a meristematic center of PLBs remains euploid. In the micropropagation of epiphytic *Cymbidium* hybrids, PLBs are cut into small pieces and each piece produces some new protocorms on an appropriate medium. PLBs start to produce shoots if the cutting is postponed. New PLBs regenerate from epidermal cells, not from parenchyma cells inside of PLBs (Kim and Kako, 1984). In our study, epidermal tissue of PLBs showed only 2C and 4C peaks. Fujii et al. (1999) also reported that most cells in the outer tissue of *Cymbidium* PLBs had a DNA content of 2C while Nagl et al. (1972) observed that DNA amplification was restricted to the green assimilating parenchyma cells near the epidermis. Maintenance of euploidy in epidermal cells may be a reason why change of ploidy levels did not occur at a high frequency in micropropagated *Cymbidium* through PLB division. Begum et al. (1994) reported callus induction and PLB regeneration from the inner tissue of *Cymbidium* PLBs without de-

scription of characteristics of the regenerants. Change of ploidy levels may occur at a higher frequency in this case. Increasing ploidy level in suspension culture of *Doritaenopsis* (Orchidaceae) was observed when 2,4-D or picloram was added to the culture medium (Mishiba et al., 2001). The strong selection of euploidy in regenerated plants has been reported in some callus and cell cultures having polyploidy and aneuploidy (Hahne and Hoffmann, 1986; Swedlund and Vasil, 1985), while considerable changes of chromosome numbers in regenerants was also observed in potato callus cultures (Ramulu and Dijkhuis, 1986).

Roots of *Cymbidium* were highly polysomatic as reported in *Phalaenopsis* (Mii et al., 1997) and *Dendrobium* (Jones and Kuehnle, 1998). No polysomaty was reported in *Hosta* roots (Zonneveld and Van Iran, 2000), but low and high levels of polysomaty were reported for potato (Ramulu and Dijkhuis, 1986) and tomato (Smulders et al., 1995) roots, respectively. Leaves taken from flowering *Cymbidium* plants were slightly polysomatic. This is considerably different from that in *Phalaenopsis* and *Dendrobium* which showed a high level of polysomaty in mature leaves. Floral organs of *Cymbidium* consisted of higher

ploidy cells except for the ovary. Development of polysomaty in floral organs, especially the perianth, has been reported in *Portulaca* (Mishiba and Mii, 2000).

The present results suggest that shoot regeneration arises from euploid cells and that endoreduplication occurs in cells without a potential for shoot regeneration.

In practice flow cytometry is a very useful tool to determine the ploidy level of plant materials. The present study showed that leaves from *in vitro* plants are suitable materials for flow cytometric analysis in *Cymbidium* as described in *Dendrobium* (Jones and Kuehnle, 1998).

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Fig. 3. Frequency of nuclei at different C-values measured in various floral parts of *Cymbidium* 'Day Light'.

Fig. 4. Frequency of nuclei at different C-values measured in various parts of *C. kanran* Makino. Leaf: leaf from *in vitro* plants, rhizome tip; ≈ 0.5 mm growing tip, rhizome 1-2 cm; rhizome tissue with 1-2 cm tip removed.

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