Effects of Benzyladenine and α - Naphthaleneacetic Acid on Cell Division and Nuclear DNA Contents in Outer Tissue of *Cymbidium* Explants Cultured *In Vitro*

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Summary

Differentiation of a protocorm-like body (PLB) *in vitro* from the outer tissue (OT) of *Cymbidium* PLBs was studied. Cell division frequency was high in the first to third layers of OT explants. The frequency of anticlinal cell division was dominant in outer cell layers, whereas both periclinal and anticlinal cell divisions occurred in the inner cell layers. Cell division frequency in a group of dividing cells was activated in the center of a protuberance (PLB initials). These results trace the process of globular PLB formation.

There were differences between NAA and BA effects on the cells of OT cultured tissue. Cell division activity was high in the BA treatment whereas dividing cells were not observed in most cultures of NAA treatment. The cell size was larger in NAA-treated explants; it was smaller in BA-treated ones. These results show that BA stimulated cells division whereas NAA affected cell maturation. The nuclear DNA contents were stable and constant on both hormone-free and BA supplemented media. In NAA-treated explants the DNA contents changed gradually with time toward the levels of 8c and 16c.

NAA did not inhibit DNA synthesis but inhibit mitosis. NAA may affect the cell cycle between the S and M phases. The data on the cell division activity and the cell size indicate that plant growth regulators also may affect the length of the G1 phase and induce somaclonal variations during plant culture distorting the cell cycle.

Key Words: *Cymbidium*, DNA contents, morphogenesis, protocorm-like body (PLB).

Introduction

In cultures of outer tissue (OT) segments from protocorm-like body (PLB) of *Cymbidium*, we found that BA promoted PLB formation from OT explants, but NAA inhibited it (Fujii et al., in press).

Plant growth regulators affect morphogenesis of cultured tissue by controlling cell division frequency and cell the cycle (Kim and Kako, 1984; Paek and Yeung, 1991; Begum et al., 1994). Changes of morphogenesis and cell cycle often parallel the pattern of DNA synthesis and plant growth (Alvarez, 1968; Nagl et al., 1972; Altamura et al., 1987; Fosket and Short, 1973; Galbraith et al., 1991). Nagl and Rücker (1974) observed that shoot growth from the cultured protocorm tissue of *Cymbidium* was inhibited by kinetin and that DNA replication had shifted from diploid in mitotically cycling cells to polyploid in endomitotically cycling cells. In other plant, endoreduplication prior to mitosis was induced by a synthetic nutrient medium supplemented with cytokinin and auxin (Libbenga and Torrey, 1973); mature endomitotic plant cells were induced to a mitotic cell cycle by cytokinin (Torrey, 1961). Thus, plant growth regulators seem to vary cell cycle, depending on

hormone type, plant species, and cultivars.

In this trial, we elucidated the effects of plant growth regulators on cell division activities and nuclear DNA contents of the outer tissue (OT) of *Cymbidium* PLB.

Materials and Methods

1. Plant materials and culture medium

PLB (3–4 mm in diam.) from *Cymbidium* Lucky Rainbow ‘Lapine Dancer’ and *Cym.* Thanksgiving ‘Nativity’ was aseptically dissected under a zoom stereomicroscope and the outer tissue (OT) excised. Buds, any outgrowth, and vascular system of PLB were removed, leaving 0.5–1 mm thick OT explants composed of epidermal and cortical cells.

Explants culture were on MS solid medium containing 3% sucrose and 0.8% agar supplemented with 5.0 μ M NAA or 5.0 μ M BA; a hormone-free MS medium was used as control. The pH of the medium was adjusted to 5.7–5.8 before autoclaving at 121 °C for 15 minutes. Explants in culture flasks were incubated at 25 $\pm$ 1 °C under a light intensity of a 16 μ mol.s⁻¹.m⁻² provided by white fluorescent lamps (FL 20 S. W. Toshiba, Tokyo) and a 16-hr photoperiod. The OT explants were cultured for 4 weeks to observe histological differences of the growth process in the three treatments.

2. Histological observations

To observe formation and development of PLB from OT explants, and to measure nuclear DNA contents in the developmental stage of PLB, explants were cultured for 0, 7, 14, 21, and 28 days. They were fixed in FAA, dehydrated in n-butyl alcohol, embedded in paraffin, and cut longitudinally into 10 μm sections. The sections were hydrolyzed by 1N HCl for 8 minutes, stained with Schiff's reagent, and observed under a light microscope.

To follow the formation of globular shapes of PLB from OT, OT explants were cultured for 14 days, then the cell division activity (metaphase to anaphase) and the direction of anticlinal or periclinal cell division were recorded.

To compare the effects of hormones, the explants were cultured in each medium for 7, 14, 21 and 28 days. Sections were prepared as above and cell division frequency, direction of anticlinal and periclinal cell division, cell size, and nuclear DNA contents were examined.

Cell division frequency (%) on each cell layer was

calculated according to the formula of Begum et al.(1994); $\text{frequency} = \text{DC}/\text{T} \times 100$, DC=number of dividing cells, and T=total number of cell in a layer. The cell sizes (cell length and cell width) of meristematic cells, 1 to 3 cell layers below the epidermal cell, were measured under a light microscope. The same cells were used to measure nuclear DNA contents under a microspectrophotometer.

Nuclei DNA contents of randomly selected dividing cells were measured weekly. The relative nucleus DNA content was based on the cell nuclei of explants at 0 days as 2c.

Results and Discussion

Effects of NAA and BA on cell division activity, and direction of cell division

The frequencies of cell division measured weekly for 28 days of culture (Figs.1 and 2) revealed that in both *Cym. Thanksgiving 'Nativity'* and *Cym. Lucky Rainbow 'Lapine Dancer'*, cell division occurred from outer cell layers inward and that the frequency of cell division

Fig. 1. Cell division activity of outer tissue (OT) of *Cymbidium Thanksgiving 'Nativity'* explants with culture time on hormone-free or 5.0 μM BA MS medium. Cell division frequency represents both anticlinal and periclinal division.

Fig. 2. Cell division activity of outer tissue (OT) segments of *Cymbidium* Lucky Rainbow 'Rapine Dancer' with culture time on hormone-free or 5.0 μ M BA supplemented MS medium. Cell division frequency represents both anticlinal and periclinal division.

gradually increased with culture time in BA and hormone-free media. The frequency of anticlinal cell division was dominant in the outer three cell layers, whereas periclinal cell division was higher in the inner cell layers.

After 7 days of culture, the maximum cell division frequency in 'Nativity' was 1.8% in the first (outer most) cell layer on a hormone-free medium, and 2.3% in the third cell layer in the BA treatment (Fig.1). In 'Lapine Dancer', the maximum frequency was 1.8% in the first and third cell layers in a hormone-free medium, and 2.7% in the first and second cell layers in the BA medium (Fig.2).

After 28 days of culture, the highest cell division frequency in 'Nativity' was 4.1% in the third cell layer on a hormone-free medium and 5.3% in the first cell layer on a BA medium (Fig.1). In 'Lapine Dancer', the highest frequency was 2.4% at the second cell layer, and 5.8% at the first cell layer for the hormone-free and BA media (Fig.2).

Kim and Kako (1984) reported that PLB formation in the cultured shoot apex of *Cym.* Sazamani 'Haru-no-

umi' and cell division activity were highest in the epidermal cells. Epidermal cells divided anticlinally whereas other cells divided both anticlinally and periclinally. We found the same results in this study.

In a NAA supplemented medium, no cell division occurred in most explants during the 28 days of culture, resulting in low PLB formation (Fig. 3). On the contrary, BA promoted cell division, resulting in high PLB formation. Cell division activities are proportional to the PLB formation.

The cell division pattern of PLB formation and the frequency of cell division in the dividing cell areas for the hormone-free and BA media after 7 days of culture are presented in a three dimensional graph (Fig. 4, 5). The protuberance (PLB initials) was divided into three sections, left (side L), center, and right (side R). The apical regions of each section were further divided into three sub-sections, R, C, and L (Fig. 6). Cell division activity is represented graphically for each sub-section within a given cell layer. In both hormone-free and BA media, the highest cell division occurred in the center sections for both cultivars. BA treatment promoted cell

Fig. 3. Longitudinal sections of OT cultured for 28 days on hormone-free or hormone supplemented medium in *Cymbidium* Lucky Rainbow 'Lapine Dancer'. A: hormone-free MS medium; B: 5.0 μ M BA; C: 5.0 μ M NAA. AM=apical meristem, LP=leaf primordia. Scale bar: A, B, C = 100 μ m.

Fig. 4. Three-way plot of cell division activity of outer tissue (OT) segments of *Cymbidium* Thanksgiving 'Nativity' after 7 days of culture in hormone-free or 5.0 μ M BA supplemented MS medium. Protuberance was divided three parts (see text and Fig. 6).

Fig. 5. Three-way plot of cell division activity of outer tissue (OT) segments of *Cymbidium* Lucky Rainbow 'Lapine Dancer' after 7 days of culture in hormone-free or 5.0 μ M BA MS medium. Protuberance was divided three parts (see text and Fig. 6).

division over the hormone-free treatment in most cell layers (Fig. 4, 5). That more anticlinal cell division occurred in the outer epidermal cells, whereas more periclinal cell division took place in the inner cell layers, indicates that the surface of the globular bodies stretched

while the inner tissue expanded in volume in forming the PLB.

Effects of NAA and BA on cell size

Cell length and width measurements of the first to

Table 1. Effects of plant growth regulators on cell size^z with culture time in *Cymbidium*.

Treatment	Cell size in μm^y							
	7 days		14 days		21 days		28 days	
	Length	Width	Length	Width	Length	Width	Length	Width
'Nativity'								
Hormone-free MS	37.5b	24.0b	28.0a	18.5a	25.3a	15.8a	26.5b	15.8a
+NAA (5.0 μM)	44.0c	26.5b	64.0b	38.0b	66.8b	38.3b	75.0c	38.3b
+BA (5.0 μM)	26.0a	21.5a	22.3a	14.0a	22.5a	14.0a	20.1a	14.0a
'Lapine Dancer'								
Hormone-free MS	26.3b	18.3b	25.9b	18.6a	19.5a	14.8b	21.0b	13.0a
+NAA (5.0 μM)	57.9c	34.1c	74.8c	41.8c	87.0b	56.5c	87.8c	55.5b
+BA (5.0 μM)	19.3a	14.0a	15.5a	11.1a	14.2a	9.8a	14.6a	9.6a

^z At zero day, the initial cell sizes, length and width, were 50 and 30 μm in Cym. Thanksgiving 'Nativity', and 60 and 32.5 μm in Cym. Lucky Rainbow 'Lapine Dancer', respectively.

^y Values with different letters in the same column are significantly different at 5% level of probability according to Duncan's multiple range test.

third cell layers in all three treatments during 28 days of culture reveal that they were 50 μm and 30 μm , respectively at 0 days in 'Nativity', whereas they were 60 μm and 32.5 μm , respectively in 'Lapine Dancer'. The smallest cell was observed in the BA treatment where cell division was most active. The largest cell was in the NAA treatment which evoked few cell divisions. Cell size also gradually decreased with time in both hormone-free and BA treatments, but increased in the NAA treatment (Table 1). After 28 days culture, the mean cell sizes for 'Nativity' was 20.1 x 14.0 μm on BA treatment, and 75.0 x 38.3 μm in the NAA treatment; those for 'Lapine Dancer' were 14.6 x 9.6 μm in the BA treatment, and 87.8 x 55.5 μm in the NAA treatment

(Table 1).

Hence, cell division in the hormone-free and BA media resulted in small young cells, whereas cells matured in NAA medium without cell division. In the BA treatment, the small cell size is attribute to a shortened G1 phase of the cell cycle which inhibited cell elongation and promoted formation of small, immature cells. On the other hand, in the NAA treatment, the G1 phase of the cell cycle may have been longer.

Fig. 6. Three-way plot of PLB protuberance developed from outer tissue segment.
Center, Side L, Side R, C, L and R : indicate the position of cell division frequency was checked.

Fig. 7. Nuclear DNA contents of *Cymbidium* Lucky Rainbow 'Lapine Dancer' after 28 days of culture in hormone-free or 5.0 μM BA in MS medium.

Change in nuclear DNA contents

The nuclear DNA contents of OT explants on both hormone-free and BA media did not vary much during the 28 days of culture. Most cells that were observed had DNA content of 2c. But a few cells had 4c. However, there were a moderate number of cells with DNA content levels between 2c and 4c. The nuclear DNA

content pattern was stable and constant over the 28 days culture period (Fig. 7).

In the NAA treatment, after 7 days of culture the peak of 2c was highest nuclear DNA contents; no 4c peak was present. The number of 4c nuclei was the largest among three treatments. In addition, some nuclei with over 4c DNA content were observed (Fig. 8, 9). The peak of the nuclear DNA contents shifted gradually with

Fig. 8. Weekly changes in nuclear DNA contents of *Cymbidium* Thanksgiving 'Nativity' during 28 days of culture in MS medium with 5.0 μ M NAA.

Fig. 9. Weekly changes in nuclear DNA contents of *Cymbidium* Lucky Rainbow 'Rapine Dancer' during 28 days of culture in MS medium with 5.0 μ M NAA.

time (Fig. 8, 9). The frequency of 2c nuclei decreased, whereas those of 4c and 8c nuclei increased. Nuclei of up to 8c DNA content were observed in 'Nativity' after 21 days (Fig. 8). In 'Lapine Dancer', nuclear DNA contents increased up to 16c level after 14 days (Fig. 9).

Nagl (1972) and Melaragno et al. (1993) suggested that nuclear polyploidy was proportional to cell size. In our study, the cell size decreased with time in both the hormone-free and BA media, but increased in the NAA medium. Our results may suggest that the possibility that cell size increase in the NAA medium is due to a duplication of nuclear DNA contents.

Increased kinetin concentration inhibited the development of protocorms to plantlets in *Cym.*, which Nagl and Rücker (1974) attributed to a decrease in the diploid cells group of protocorm and an increase in cells exhibiting an endomitotic cell cycle. We observed that auxin increased nuclear DNA contents. Libbenga and Torrey (1973) reported that endoreduplication varied with species or variety, and that hormone sensitivity among cultivars varied. They induced endoreduplication in *Pisum sativum* with combination of auxin and kinetin. We found that NAA inhibited PLB formation but promoted DNA content in the nuclei. Nagle and Rücker (1974) showed that endoreduplication was accompanied by inhibition of plantlet growth. This response indicates that when plant growth regulators inhibit growth, endoreduplication of nuclear DNA is promoted.

We hypothesize that a) auxin does not inhibit DNA synthesis but inhibits mitosis in the culture of outer tissue from PLB explants, and b) plant growth regulators may affect morphological changes not only through cell division activity but also the cell cycle. A somaclonal variation during *in vitro* culture may be caused by a distortion of the cell cycle from the effect of plant growth regulators. Specifically, plant growth regulators may affect on cells between the S and M phases, which may also affect the length of the G1 phase. Plant growth regulators may exert their effects morphologically by affecting DNA, RNA, protein synthesis, and metabolism.

Literature Cited

- Altamura, M. M., P. Bassi, A. Cavallini, G. Cionini, R. Cremonini, B. Monacelli, G. Pasqua, O. Sassoli, K. Tran Thanh van. and P. G. Cionini. 1987. Nuclear DNA changes during plant development and the morphogenetic response *in vitro* of *Nicotiana tabacum* tissue. *Plant Science* 53 : 73–79.
- Alvarez, M. R. 1968. Quantitative changes in nuclear DNA accompanying postgermination embryonic development in *Vanda* (Orchidaceae). *Amer. J. Bot.* 55 : 1036–1041.
- Begum, A. A., M. Tamaki and S. Kako. 1994. Formation of protocorm-like bodies (PLB) and shoot development through *in vitro* culture of outer tissue of *Cymbidium* PLB. *J. Japan. Soc. Hort. Sci.* 63 : 663–673.
- Fosket, D. E. and K. C. Short. 1973. The role of cytokinin in the regulation of growth, DNA synthesis and cell proliferation in cultured soybean tissues (*Glycine max* var. Biloxi). *Physiol. Plant.* 28 : 14–23.
- Galbraith, D. W., K. R. Harkins and S. Knapp. 1991. Systemic Endopolyploidy in *Arabidopsis thaliana*. *Plant Physiol.* 96 : 985–989.
- Kim, K. W. and S. Kako. 1984. Morphological and histochemical studies on protocorm-like bodies formation and explant development in the *Cymbidium* shoot apex culture *in vitro*. *J. Kor. Soc. Hort. Sci.* 25 (2) : 156–163.
- Libbenga, K. R. and J. G. Torrey. 1973. Hormone induced endoreduplication prior to mitosis in cultured pea root cortex cells. *Amer. J. Bot.* 60 : 293–299.
- Melaragno, J. E., B. Mehrotra and A. W. Coleman. 1993. Relationship between endopolyploidy and cell size in epidermal tissue of *Arabidopsis*. *The Plant Cell* 5 : 1661–1668.
- Nagl, W. 1972. Evidence of DNA amplification in the orchid *Cymbidium in vitro*. *Cytobios* 5 : 145–154.
- Nagl, W., J. Hendon and W. Rücker. 1972. DNA amplification in *Cymbidium* protocorms *in vitro*, as it relates to cytodifferentiation and hormone treatment. *Cell Diff.* 1 : 229–237.
- Nagl, W. and W. Rücker. 1974. Shift of DNA replication from diploid cells in cytokinin-controlled differentiation. *Cytobios* 10 : 137–144.
- Paek, K. Y. and E. C. Yeung. 1991. The effects of 1-naphthaleneacetic acid and N⁶-benzyladenine on the growth of *Cymbidium forrestii* rhizomes *in vitro*. *Plant Cell, Tissue and Organ Culture* 24 : 65–71.
- Torrey, J. G. 1961. Kinetin as a trigger for mitosis in mature endopolyploid plant cells. *Expl. Cell Res.* 23 : 281–299.

シンビジウムの PLB 外部表皮系組織片の培養における NAA と BA の細胞分裂と核 DNA 量への影響

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摘 要

PLB 形成に関与する表皮系組織切片 (OT) の細胞分裂は, OT の外側の細胞層 (表皮側) から始まった. 細胞分裂頻度は外側の細胞層 (第 1 層から第 3 層) が盛んで, 分裂方向は外側の細胞層が主に垂層分裂, 内側の細胞層が垂層分裂と並層分裂を行っていた. また分裂細胞群の分裂頻度は中央部で活性化し, これらのことから OT 外植体からの球状体の PLB 形成の様相が推察された.

OT 外植体の分裂性細胞に対する植物ホルモンの影響は, NAA と BA との間で大きく異なった. 細胞分裂活性は BA を添加した処理区で培養期間を通して高くなったが, NAA を添加した処理区ではほとんどの培養体において分裂活性が確認できなかった. 細胞の大きさは, BA 区で最小化し, NAA 区で最大化した. このことから NAA は細胞を成熟させ, BA は若い分裂細胞へと変化させるも

のと考えられた. また核 DNA 含量を調査した結果, BA 区や対照区では培養期間を通して 2c 核と 4c 核とで安定していたが, NAA 区では培養期間が進むにつれて核 DNA 量が 8c 核や 16c 核まで増加した. 以上のことから植物ホルモンは細胞周期に大きく影響を及ぼしているものと考えられた. 即ち NAA は DNA 生成を抑制はしないものの有糸分裂を抑制していると考えられ, S 期から M 期かけて大きな影響を及ぼすと推察された. また細胞分裂活性と細胞の大きさの結果から植物ホルモンは G1 期の長短にも影響を与えるようである. これらのことから, 植物ホルモンによって引き起こされる培養中の変異は, その原因の一つとして細胞周期の乱れから生じるように考察された.