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ORIGINAL ARTICLE

Variations in endopolyploidy level during the short period of the early growing stage in the roots and leaves of maize (*Zea mays*) seedlings

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Abstract We used a flow cytometer to investigate the variations in endopolyploidy (the frequencies of nuclei with DNA contents equivalent to 4C through 16C) during the short period of the early growing stage in vigorously growing young tissues of maize seedlings. We examined different portions of the root and leaves that had been growing for 7 (day 7) and 13 (day 13) days after germination. Endoreplication showed two opposing phenomena without aging. In one case, the endopolyploidy of the first leaf was higher on day 13 than on day 7. In the latter case, endopolyploidy decreased, as clearly revealed by a comparison of the endopolyploidy of the second leaves and the 160–170 mm portion of the seminal root on days 7 and 13. Endopolyploidy was also lower in the top of the leaf. In roots, endopolyploidy was increased by the exogenous application of abscisic acid for only 1 day. The levels of endopolyploidy increased without an increase in cell size in the roots. These results showed that endoreplication occurs in actively growing and young tissue and that the variation can be induced in the short period examined.

Keywords Cell size · Endopolyploidy · Flow cytometry · Root · Leaf · *Zea mays* L.

Introduction

Endopolyploidy is a phenomenon whereby chromosomal DNA is replicated without the formation of a somatic spindle, and repeated replication of DNA results in multiples of the normal DNA content of the nucleus of affected cells (Wilhelm et al. 1995; Grafi 1998; Kudo and Kimura 2001; Bertin et al. 2007). Nuclear endopolyploidy is a common feature of eukaryotes (Nagl 1978). In particular, in seed plants, endopolyploidy has been frequently reported in annual herbaceous species but rarely in woody species (Barrow and Meister 2003).

Flow cytometry has been used extensively for measuring the DNA content of various tissues of many plant species and has revealed systematic endopolyploidy in plants at different developmental stages and in plants grown under different environmental conditions (DeRocher et al. 1990). Variations in the extent of endopolyploidy during the growth of shoots have been reported in *Raphanus sativus* (Kudo and Kimura 2002), *Allium fistulosum* (Kudo et al. 2003), *Brassica oleracea* (Kudo and Kimura 2001), several species of legume (Lagunes-Espinoza et al. 2000), *Lycopersicon esculentum* (Smulders et al. 1994), *Arabidopsis thaliana* (Galbraith et al. 1991), and *Cucumis sativus* (Gilissen et al. 1993). In *L. esculentum*, continuous flow cytometric analysis during seed germination and seedling development demonstrated endopolyploidy with a nuclear DNA content of 128C in aged leaves and petioles (Smulders et al. 1994). In *B. oleracea*, at five developmental stages during seed germination and the establishment of seedlings, rapid endopolyploidization was observed in the radicle and hypocotyl of embryos, and a consistent nuclear DNA level of 2C and 4C was preserved only in shoot tips (Kudo and Kimura 2001). However, detailed information on endopolyploidy, for example, the frequency and distribution of cells with various ploidies in roots, is not yet available, though endopolyploidy in some portions of roots has been observed in a few studies (Lim and Loh 2003; Yang and Loh 2004).

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Endoreplicated cells are formed when the somatic spindle is disabled during the cell cycle, as occurs with aging (Wilhelm et al. 1995; Grafi 1998; Kudo and Kimura 2001; Bertin et al. 2007). In *Mesembryanthemum crystallinum* (DeRocher et al. 1990), the nuclear DNA content (endopolyploidy) of young leaves was equivalent to 2C and 4C, while that of mature leaves ranged from 32C to 64C. Similarly, considerable endopolyploidy was reported in various tissues of *Zea mays* (Rayburn et al. 1989; Kowles et al. 1990, 1997; Wilhelm et al. 1995), *Vicia faba* (Borisjuk et al. 1995), common beans (Bino et al. 1993), *A. thaliana* (Galbraith et al. 1991; Melaragno et al. 1993), *C. sativus* (Gilissen et al. 1993), *L. esculentum* (Smulders et al. 1994), and *B. oleracea* (Kudo and Kimura 2001). Cell size was increased by endoreplication and aging (Bertin 2005 and references therein). In contrast, endopolyploidy occurred in the hypocotyl, cotyledon, and radicle immediately after germination in *R. sativus* (Kudo and Kimura 2002). Similarly, in *C. sativus*, endopolyploidy was observed in the peg portion, radicle, and proximal portion of the cotyledon (Gilissen et al. 1993). We could postulate from these early investigations that endoreplication occurred in vigorously growing young tissues irrespective of aging. However, information on the variations of endopolyploidy during the short period of the early growing stage is fragmented and obscure.

In our preliminary experiment, endopolyploidy was confirmed in six F_1 hybrid cultivars of *Z. mays*. In the present study, we used one cultivar, 'White Pop', as a model plant (Ogawa et al. 2005, 2009; Ogawa and Yamauchi 2006). The purpose of the present study was to clarify the variations in endopolyploidy during the short period at the early growing stage in the root and leaves of maize seedlings. Furthermore, we investigated the effect of abscisic acid (ABA), a plant hormone that regulates plant growth (reviewed by Milborrow 1974), on the variations of endopolyploidy during a short period of 24 h. The mechanisms responsible for variations in endopolyploidy of maize plants are discussed on the basis of our observations.

Materials and methods

Plant materials

Three days after the germination of seeds of *Z. mays* L. 'White Pop' (Sakata Seed Corporation, Yokohama, Japan) in darkness at 30°C in Petri dishes, seedlings with a seminal root and approximately 8 mm in length were transplanted onto plastic nets (3.2 × 3.2 mm mesh), which were floated on a nutrient solution. The solution contained 1.5×10^{-3} M KNO_3, 1.0×10^{-3} M $Ca(NO_3)_2{\cdot}4H_2O$, 2.5×10^{-4} M $NH_4H_2PO_4$, 5.0×10^{-4} M $MgSO_4{\cdot}7H_2O$, 1.3×10^{-5} M Fe-EDTA, 2.3×10^{-6} M $MnCl_2{\cdot}4H_2O$, 1.2×10^{-5} M H_3BO_3, 1.9×10^{-7} M $ZnSO_4{\cdot}7H_2O$, 7.9×10^{-8} M $CuSO_4{\cdot}5H_2O$, and 7.5×10^{-9} M $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. Eight germinated seeds were placed on each net and cultivated in a beaker that contained 1,000 ml of this solution. The solution was aerated by continuous bubbling with air (1,000 ml air/min) provided by an aerator (HPα10000; Nisso, Japan). The bubbles did not travel as far as the root axis, and root growth was not affected by this procedure (Ogawa et al. 2005, 2009; Ogawa and Yamauchi 2006). The nets and walls of beakers were covered with aluminum foil to exclude light and to stimulate root growth. The plants were illuminated with a 12-h photoperiod of white light and maintained at 28 ± 0.2°C with a relative humidity of approximately 70% in a growth chamber (MLR-350H; Sanyo, Japan). The photon flux density of photosynthetically active radiation (PAR; 400–700 nm) at the top of each plant was 320 μmol m^{-2} s^{-1}.

Sampling for measurements of DNA content

Four and 10 days after transplanting (7 and 13 days after germination; hereafter referred to as day 7 and day 13, respectively), seedlings were subjected to flow cytometric analysis. Sections of approximately 1 cm^2 of leaf tops of the first, second, and third leaves, which were numbered from the bottom to the top, were consecutively sampled for DNA content by flow cytometry. In addition, in the case of the third leaf on day 13, sections of approximately 1 cm^2 (in which cell division was occurring) were taken from the middle (120–130 mm from the top) and from the base (240–250 mm from the top) of the leaf. Seminal and lateral roots were also sampled. Three sections from different portions of the seminal roots were collected, namely, two sections without lateral roots (0–10 and 80–90 mm from the root apex) and one section with lateral roots (160–170 mm from the root apex). Lateral roots of 1 cm in length, which emerged from the seminal root between 160 and 170 mm from the root apex, were also excised for flow cytometry.

Seedlings that had been grown for 3 days in the nutrient solution and then for 1 day in the same solution supplemented with 1 ppm ABA (Sigma, Japan) were sampled to investigate the effects of ABA on endopolyploidy in five replicates. The concentration of ABA in the medium was determined according to the method described in a previous study of *Triticum aestivum* by Jones et al. (1987).

Measurement of DNA content by flow cytometry

The DNA content of nuclei from each sample was determined by flow cytometry (Ploidy Analyzer; Partec, Germany) equipped with an HBO lamp for UV excitation.

The samples were individually chopped with a sharp razor blade in approximately 0.5 ml of extraction buffer (CyStain® UV precise P Nuclei Extraction Buffer; Partec). The extraction solution containing nuclei was filtered through a nylon sieve (30-μm pores). After the addition of 2 ml of staining solution, which contained 4′, 6-diamidino-2-phenylindole-2-HCl (DAPI; CyStain® UV precise P staining Buffer; Partec), the residue was discarded. For each sample, at least 3,000 nuclei (total count) were analyzed (Kudo and Kimura 2001). Data were plotted on a linear scale from channels 20 to 512. The nuclei detected from channels 48 to 52 were considered to have a DNA content of 2C, from 95 to 104 of 4C, from 192 to 211 of 8C, and from 381 to 420 of 16C. The number of nuclei in each peak was counted, and the percentage of nuclei in each peak relative to the total number of nuclei in all peaks was calculated.

Measurement of cell size

We compared the size of cells in seminal root sections (80–90 mm from the apex) on days 7 and 13 (Ogawa and Yamauchi 2006). Sections were fixed in FAA solution (formalin:acetic acid:50% ethanol = 1:1:18 v/v), dehydrated in a water–ethanol–butanol series, and embedded in paraffin (melting point 58–60°C). Both transversal and longitudinal sections of dehydrated samples were cut at 10-μm thickness with a rotary microtome (HM 360; Microm International, Germany) and stained with hematoxylin and safranin. Profiles of the sections were recorded with a light microscope (BX 51; Olympus, Japan) equipped with a digital camera (Camedia C5050 Zoom; Olympus). Cell areas in transverse sections and cell lengths in longitudinal sections of roots were calculated from the images with imaging software (Lumina Vision; Mitani., Japan) and a computer (Mebius PCXJ820R; Sharp, Japan). Five sections, which had been appropriately cut to reveal cell layers, were examined to determine the cell size of each sample. Mean cell size was obtained from measurements of 213–297 cells that were in the center of each section.

Statistical analysis

The significance of the differences in endopolyploidy and in cell size was determined by either Student's *t* tests or Duncan's tests.

Results

The frequencies of cells with a nuclear DNA content equivalent to 2C, 4C, 8C, and 16C (2C, 4C, 8C, and 16C cells) in the roots and leaves were determined, and endopolyploidy was confirmed in both roots and leaves of maize seedlings (Fig. 1). Cell endopolyploidy in the roots ranged from 2C to 16C, while that in leaves ranged from 2C to 8C. In the first and second leaves, the frequencies of 4C showed the highest values (65%), and the frequency of 2C showed the highest in the third leaf (65%). Various endopolyploidy patterns were detected in different portions of the root. On the seminal root axis, higher frequencies of 4C or higher cells were found in the basal portion (160–170 mm portion from the root apex). The frequencies of 4C showed the highest, namely 68%, in the apex (0–10 mm) and 55% in the 80–90 and 160–170 mm portions from the root apex. In lateral roots, the pattern of endopolyploidy was similar to that in the apex of the seminal root. Higher frequencies of 4C cells were detected in the oldest leaf (the first leaf).

Table 1 shows the variations in patterns of endopolyploidy in the roots and leaves of seedlings at different developmental stages, namely, on day 7 and on day 13. The frequency of 2C cells in the first leaf at day 13 (24%) was significantly lower than that on day 7 (31%), and the extent of endopolyploidy increased from day 7 to day 13. In contrast, the extent of endopolyploidy in the second leaf on day 13 was lower than on day 7, with the frequency of 2C cells increasing and that of 4C cells decreasing significantly. The same tendency in terms of variations in the extent of endopolyploidy was observed in the third leaf. Overall, the endopolyploidy (the frequencies of 4C cells through 16C cells) of older portions of the seminal root axis, such as the 0–90 mm portion, was greater on day 13 than on day 7. The frequencies of 4C or higher cells in the 0–10 mm portion and those of 8C or higher cells in the 80–90 mm portion increased. In the 160–170 mm portion, the frequencies of 8C and 16C cells decreased significantly, while the frequency of 4C cells increased.

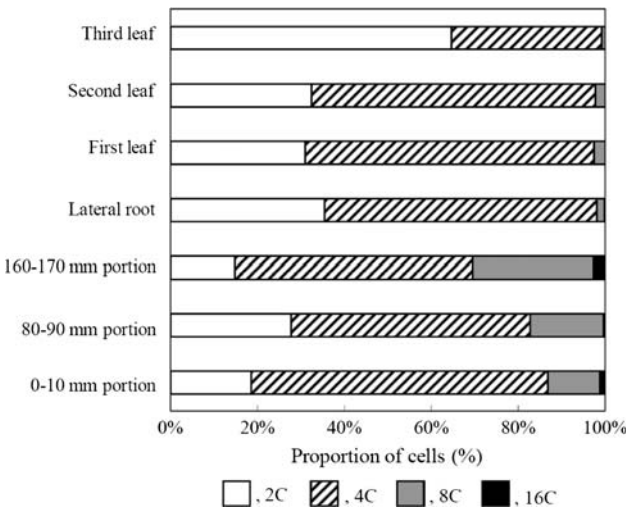

Fig. 1 Frequencies of 2C, 4C, 8C, and 16C cells in the roots and leaves *Z. mays* L. 'White Pop' as determined 7 days after germination. Each value is the mean of results from three replication

Table 1 Endopolyploidy in the leaves and seminal roots of *Z. mays* L. 'White Pop' on day 7 and day 13

Portion	Endopolyploidy (% of nuclei)			
	2C	4C	8C	16C
Leaves				
First leaf (day 7)	30.9 ± 2.1*	66.5 ± 2.2	2.6 ± 0.3	0.0 ± 0.0
First leaf (day 13)	24.1 ± 1.5	71.8 ± 1.3	4.0 ± 0.7	0.1 ± 0.0
Second leaf (day 7)	32.4 ± 1.7***	65.3 ± 1.6***	2.2 ± 0.2	0.1 ± 0.0
Second leaf (day 13)	43.7 ± 1.2	54.5 ± 1.3	1.7 ± 0.3	0.0 ± 0.0
Third leaf (day 7)	64.3 ± 3.7	35.2 ± 3.8	0.5 ± 0.1	0.0 ± 0.0
Third leaf (day 13)	72.9 ± 1.9	26.8 ± 1.9	0.3 ± 0.1	0.0 ± 0.0
Seminal root				
0–10 mm portion (day 7)	18.5 ± 1.5*	68.3 ± 1.4	12.0 ± 0.8	1.2 ± 0.1
0–10 mm portion (day 13)	12.5 ± 1.8	72.0 ± 1.2	14.4 ± 2.8	1.1 ± 0.1
80–90 mm portion (day 7)	27.7 ± 1.2***	55.0 ± 3.9	16.9 ± 3.5	0.4 ± 0.0
80–90 mm portion (day 13)	19.8 ± 0.9	54.8 ± 4.0	24.9 ± 4.5	0.5 ± 0.1
160–170 mm portion (day 7)	14.7 ± 1.1	54.8 ± 1.3	27.8 ± 1.6*	2.7 ± 0.9*
160–170 mm portion (day 13)	17.3 ± 1.3	62.9 ± 2.4	19.4 ± 3.1	0.4 ± 0.1

Each value is the mean of results from five replicates ± standard error

*, *** Significant difference between day 7 and day 13 at $P < 0.05$ and $P < 0.001$, respectively, as determined by Student's *t* test

Table 2 shows the variations in endopolyploidy in different portions of the third leaf on day 13. The frequencies of 2C–8C cells differed significantly among three samples collected from different positions in the leaf. Endopolyploidy was lower at the top of the leaf, which was the oldest portion of the leaf. The frequencies of 2C cells decreased toward the base of the leaf, being 73% at the top, 53% at the middle, and 45% at the basal portions of the leaf. In contrast, the frequencies of 4C and 8C cells increased. The frequencies of 4C cells were 27% (top), 45% (middle), and 51% (base), and those of 8C cells were <1% (0.3%; top), 2% (middle), and 4% (base).

Table 3 shows the variations in endopolyploidy in the roots and leaves of 3-day-old seedlings with and without ABA treatment for 1 day. Endopolyploidy in leaves and roots was influenced differently by ABA. In all portions of the root, endopolyploidy in all ABA-treated samples was significantly higher than that in controls. The variations in endopolyploidy after ABA treatment were most significant in the lateral root, and the frequency of 2C cells decreased from 35 to 19%, while the frequencies of 4C and 8C cells increased from 63 to 74% and from 2 to 7%, respectively. In contrast, there was no significant variation in endopolyploidy after ABA treatment in leaves, with the exception of the frequency of 16C cells in the first leaf, which was 3% in the control and 4% in ABA-treated groups.

We found no significant differences between seedlings on day 7 and day 13, in terms of respective cell areas in transverse sections and respective cell length in longitudinal sections, when we compared portions of root located at a distance of 80–90 mm from the root apex (Table 4).

Table 2 Endopolyploidy in the top, middle, and basal portions of the third leaf of 'White Pop' on day 13

Portion	Endopolyploidy (% of nuclei)			
	2C	4C	8C	16C
Top	72.9 ± 1.9 a	26.8 ± 1.9 a	0.3 ± 0.1 a	0.0 ± 0.0 a
Middle	52.7 ± 1.3 b	45.2 ± 1.3 b	2.1 ± 0.2 b	0.1 ± 0.0 a
Basal	44.9 ± 1.7 c	50.8 ± 1.5 c	4.2 ± 0.7 c	0.1 ± 0.1 a

Each value is the mean of results from five replicates ± standard error

Different letters in the columns indicate a significant difference as determined by Duncan's test ($P < 0.05$)

Discussion

We attempted to clarify the variations in endopolyploidy during the short period of the early growing stage of maize seedlings. Our results suggest that endoreplication may increase or decrease during the short period in the vigorously growing young tissues without the effects of aging. In 6 days, between day 7 and day 13, we confirmed that endopolyploidy increases in the first leaf and in the seminal root in the 0–10 mm and 80–90 mm portions (Table 1). However, in the 160–170 mm portion of the seminal root, we observed a decrease in endopolyploidy during the same period. Additionally, cell division in root occurred at the root apex, and extensive division and elongation of cells in leaves of *Z. mays* occur in the portions 5 cm above the ligules (Barlow 1986). Comparing identical tissues, the endopolyploidy level increased in the seminal root (Fig. 1) and decreased in the third leaf (Table 2). The seminal root elongated during the 7 days after germination, and the third leaf elongated for a few days after initiation. These results also demonstrated that a variation in endopolyploidy could

Table 3 Endopolyploidy in leaves and root with and without ABA treatment sampled on day 7

Portion	Endopolyploidy (% of nuclei)			
	2C	4C	8C	16C
Leaves				
First leaf (control)	30.9 ± 2.1	66.5 ± 2.2	2.6 ± 0.3*	0.0 ± 0.0
First leaf (ABA)	24.5 ± 1.9	71.8 ± 1.3	3.6 ± 0.7	0.1 ± 0.0
Second leaf (control)	32.4 ± 1.7	65.3 ± 1.6	2.2 ± 0.2	0.1 ± 0.0
Second leaf (ABA)	39.1 ± 2.8	58.7 ± 2.6	2.2 ± 0.3	0.1 ± 0.0
Third leaf (control)	64.3 ± 3.7	35.2 ± 3.8	0.5 ± 0.1	0.0 ± 0.0
Third leaf (ABA)	71.1 ± 1.3	28.4 ± 1.3	0.5 ± 0.1	0.0 ± 0.0
Seminal root				
0–10 mm portion (control)	18.5 ± 1.5**	68.3 ± 1.4*	12.0 ± 0.8**	1.2 ± 0.1
0–10 mm portion (ABA)	10.4 ± 1.6	73.1 ± 1.5	14.9 ± 0.3	1.6 ± 0.2
80–90 mm portion (control)	27.7 ± 1.2***	55.0 ± 3.9	16.9 ± 3.5	0.4 ± 0.0
80–90 mm portion (ABA)	15.3 ± 1.0	57.8 ± 1.7	26.1 ± 2.1	0.7 ± 0.2
160–170 mm portion (control)	14.7 ± 1.1**	54.8 ± 1.3	27.8 ± 1.6*	2.7 ± 0.9
160–170 mm portion (ABA)	7.6 ± 1.7	52.1 ± 1.7	38.2 ± 2.9	2.1 ± 0.4
Lateral root (control)	35.4 ± 1.8***	62.7 ± 1.7**	1.8 ± 0.2***	0.2 ± 0.0
Lateral root (ABA)	19.1 ± 1.1	73.8 ± 1.4	6.9 ± 0.6	0.2 ± 0.1

Each value is the mean of results from five replicates ± standard error

*, **, *** Significant difference between controls and ABA-treated samples at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, as determined by Student's t test

Table 4 Cell sizes on day 7 and day 13 in the portion 80–90 mm from the root apex

	Day 7	Day 13	
Cell area in cross section (μm^2)	263.9 ± 17.5 (n = 222)	267.0 ± 17.7 (n = 213)	NS
Cell length in longitudinal section (μm)	146.1 ± 3.0 (n = 297)	148.1 ± 3.9 (n = 261)	NS

Values are shown as means ± standard errors

NS No significant difference between day 7 and day 13 as determined by Student's t test

occur during the short period in the early growing stage without aging.

During flow cytometric analysis, two 2C nuclei can physically adhere to each other and be counted as a 4C nucleus. In a preliminary experiment, we confirmed that the physical adhesion of two 2C nuclei hardly ever occurred in our system (i.e., Partec; data not shown). Nuclei with a DNA content of 4C were derived from endoreduplicated cells and from cells at specific stages (i.e., the S phase to the anaphase of the M phase) of the cell cycle. Endoreplicated cells are formed when the somatic spindle is disabled during the cell cycle, as occurs with aging (Wilhelm et al. 1995; Grafi 1998; Kudo and Kimura 2001; Bertin et al. 2007). Repeated DNA replication without cell division increases endopolyploidy in cells. In the present study, we showed that the endopolyploidy level increased during the short period in the early growing stage. This was caused by an active cell cycle with a disabled somatic spindle in the short period examined.

We observed a decrease in the endopolyploidy level (Tables 1, 2). The operation of the cell cycle was inhibited by aging (Gahan and Hurst 1976; Fountain et al. 2003). It is possible that cell division eventually occurs in parts of roots and leaves resulting in a relative increase in 2C cells and a decrease in endoreduplicated cells.

The extent of endopolyploidy was markedly increased in the roots when seedlings were treated with ABA for a single day (Table 3). In previous studies, investigations of the relationship between phytohormones and endopolyploidy were restricted to specific types of cells, such as endosperm cells and cells cultured in vitro. Mambelli and Seter (1998) showed that the extent of endopolyploidy was reduced in the cells of the apical pericarp surface of intact kernels of *Z. mays* by ABA at 1,000 μM. Lur and Setter (1993) showed that an increase in the level of endogenous indole acetic acid in the endosperm of *Z. mays* coincided with an increase in DNA content. Mishiba et al. (2001) showed that treatment of cultured cells derived from a tetraploid cultivar of *Doritaenopsis* with 2,4-dichlorophenoxyacetic acid (2,4-D) increased the relative proportion of 8C cells. In the endosperm of *Z. mays*, the exogenous application of 2,4-D increased the nuclear DNA content (Lur and Setter 1993). Lim and Loh (2003) showed that the frequency of 4C and 8C nuclei in embryos of the hybrid orchid *Vanda* Miss Joaquim that had been cultured with 10^{-5} M Gibberellin for 4 weeks was significantly higher

than that in controls. In the present study, exogenous ABA increased endopolyploidy in roots, irrespective of the position and type of root (Table 3). ABA is considered a major promoter of plant growth (Milborrow 1974). Therefore, the application of exogenous ABA for a short period might have affected the growth of seedling tissues, which eventually led to an increase in the extent of endopolyploidy in the root.

We looked for a correlation between cell size and endopolyploidy in the root. There have been reports of such a correlation in various plant organs and tissues (Bertin 2005 and references therein). Bertin et al. (2003) reported that there was no relationship between DNA endoreduplication and cell size in *L. esculentum* during fruit development. In the present study, even though the extent of endopolyploidy was greater on day 13 than on day 7 in the root (Table 1), cell sizes showed no significant variations during the 6 days (Table 4). From these results, we could postulate that the endopolyploidy level increased without variations in cell size in the early growing stages of plants over a relatively short period, other than the increase due to plant aging.

The physiological significance and the control of endopolyploidy are poorly understood (Larkins et al. 2001). Further studies are required to clarify the significance and mechanism of endopolyploidy, which occurs over a relatively short period and is associated with plant aging in different species and cultivars under various culture conditions.

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