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Flow Cytometric Analysis for Systemic Endopolyploidy in Development of Radish (*Raphanus sativus* L.)

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Abstract

Systemic endopolyploidy during development of radish (*Raphanus sativus* L.) is described. Flow cytometric measurements of relative nuclear DNA contents revealed that a majority of somatic cells in radish plants undergoes several rounds of endoreduplication, resulting in tissues that contain cells with multiple ploidy levels (also called 'endopolyploidy' or 'polysomaty'). The distribution of endopolyploid cells is organ-specific. Endopolyploidy was not present in the embryos in dry seeds. Rapid and extensive endoreduplication occurred in the radicle and the hypocotyl of the embryos during germination of seeds. Endoreduplication events were detected in all tissues except shoot tip tissues. *In vitro* grown plants contained cells with five ploidy levels that correspond to 2C, 4C, 8C, 16C and 32C. Tuberous root tissues of *in vivo* grown plants also showed highly polysomatic nature. It is probably that growth of radish plants does not depend on cell proliferation alone. Under normal developmental programs, most somatic cells undergo a transition from proliferating state to endoreduplicating state. The biological significance of endoreduplication in radish plants is discussed.

Keywords: Endopolyploidy, endoreduplication, flow cytometry, nuclear DNA content, *Raphanus sativus* L.

Abbreviations

DAPI, 4'.6-diamidino-2-phenylindole; FCM, Flow cytometry.

Introduction

The variation of cellular ploidy levels designated 'endopolyploidy' or 'somatic polyploidy' is a common feature that has been described in many eukaryotes (Brodsky and Uryvaeva, 1977; Barlow, 1978). Endopolyploidy is estimated to occur in over 90% of angiosperms and endoreduplication is the most common mode of endopolyploidization (D'Amato, 1989; Joubès and Chevalier, 2000). Endoreduplicating nuclei retain the capability of DNA replication without passing through mitosis (Grafi, 1998). During endoreduplication cycles, chromosomes with 2^n chromatids are produced without changing in the chromosome number (Levan and Hauschka, 1953; Joubès and Chevalier, 2000). In plants, endoreduplication has been intensively studied in maize endosperm (Kowles and Phillips, 1985; Kowles *et al.*, 1990). Recently, it has been shown that endoreduplication of maize endosperm involves inhibition of M-phase-promoting factor and induction of S-phase-related protein kinase (Grafi and Larkins, 1995).

Flow cytomerty (FCM) has recently opened the way for accurate determination of C levels (C is the haploid DNA content per nucleus) of large number of nuclei in plants (Galbraith *et al.*, 1983; Michaelson *et al.*, 1991). FCM has progressively replaced Feulgen microspectrophotometry, which was previously widely used (Bennett *et al.*, 1982). FCM has successfully been applied to analyses for endopolyploidization events (DeRocher *et al.*, 1990; Galbraith *et al.*, 1991; Mishiba *et al.*, 2001). Flow cytometric data for systemic control of endoreduplication have been described in plants, such as succulent *Mesembryanthemum crystallinum* (DeRocher *et al.*, 1990), *Arabidopsis thaliana* (Galbraith *et al.*, 1991), cucumber (Gilissen *et al.*, 1993), tomato (Smulders *et al.*, 1994) and *Brassia* crops (Kudo and Kimura, 2001a, b).

Radish is a major root crop in the world wide, in particular in East Asia. The genus *Raphanus* is considered to belong to a cytogenetic category,

Brassica coeonospecies on the basis of morphological and cytogenetical similarities (Gómez-Campo, 1999). *Raphanus* probably shares a common ancestry with *B. rapa* and *B. oleracea* as indicated by the close chromosome homology between *B. oleracea* and *Raphanus* genomes - up to 7 bivalents in *R. sativus* × *B. oleracea* and up to 7 bivalents in *R. sativus* × *B. rapa* (Gómez-Campo and Prakash, 1999). On the basis of chloroplast DNA restriction site variations, *Raphanus* is closely related to the *B. rapa* and *B. oleracea* genomes (Warwick and Black, 1991; Pradhan *et al.*, 1992).

Because nucleus is the center of the developmental activity in plant cells, one questions the nature of the nuclei themselves. Do they, during all the developmental activity, maintain a constant amount of DNA? The question was motivated by the fact that plant cells are known to increase nuclear DNA content during differentiation (Barlow, 1978; D'Amato, 1989). To our knowledge, none of previous investigations quantified DNA levels in relation to development of radish plants. Therefore, DNA synthesis was determined by flow cytometry during the different phases of radish development. Our results showed that most somatic cells of radish go through several rounds of endoreduplication, suggesting that the endopolyploidization events are developmentally regulated.

Materials and Methods

Plant material

Plant material included four radish cultivars: 'Ten-Sui', 'Natsu-Nobu', 'Toki-Nasi', 'Yu-Tou-Sei'. 'Ten-Sui' was most intensively studied.

In vitro plants were grown from seeds. Seeds were surface-sterilized for 15 min in 1% sodium hypochlorite solution and washed three times with sterile distilled water. Two seeds were plated on half-strength MS medium (Murashige and Skoog, 1962) containing 20 g l^{-1} sucrose, solidified with 2.5 g l^{-1} gelrite in a 500-ml glass jar; pH of the medium was adjusted to 5.8 before autoclaving at 121 ℃ for 20 min. Plants were grown at 25 ℃ under cool white fluorescent lights (50 μmol m^{-2} s^{-1}) with a 16-h day light/8-h dark photoperiod. Seeds were also sown in soil and *in vivo* radish plants were cultivated in a greenhouse. Tuberous roots (including hypocotyls) were harvested after three months of culture.

Flow cytometry

DNA content of nuclei from embryos and plants were determined by flow cytometry. Nuclei were extracted and stained using a high resolution kit (PARTEC High Resolution Kit type P, Partec GmbH, Münster, Germany) according to the manufacturer's instructions. Embryos were carefully excised from dry seeds with a fine dissecting needle. An individual seedling at each developmental stage was dissected into several parts; taproot (the former radicle), hypocotyl, cotyledons, and leaves. The leaves were numbered from bottom to top, with the oldest leaf as number one. Tuberous roots were harvested at the size of about 8 cm in diameter of *in vivo* culture. Hand-cut transverse sections (0.5 cm^3) of 0.5 mm thickness were made from the central region and the periphery region of the tuberous root tissues. The samples were individually chopped with a sharp razor blade in 0.5 ml of nuclei extraction buffer (solution A of the kit). After filtration through a 30-μm Cell Trics filter, 2.0 ml of staining solution containing the dye 4'.6-diamidino-2-phenylindole (DAPI, solution B of the kit) was added. The measurements were performed with a PAS flow cytometer (Partec) equipped with an HBO lamp for UV excitation. The signal intensity of each peak was determined by FlowMax software (Partec). Fluorescence peaks were estimated with the WinMDI software (version 2.8, copyright (c) 93-99 Joseph Trotter). Within one sample, a minimum of 3000 particles (total count) was analyzed. Measurements of nuclear DNA content were carried out of at least 6 replications originating from different embryos or seedlings. To determine the standard peak position of 2C nuclei, the 2C peak from nuclei of *in vitro* young leaves was analyzed at least twice on each measurement. The data were plotted on a semi-logarithmic scale, so that the histograms from 2C to 64C were evenly distributed along the abscissa. The data was presented as percentage of the total amount of nuclei in all peaks of the histogram.

Results

Flow cytometry

FCM is a rapid and accurate way to determinate the C levels (C is the haploid DNA content per nucleus) of large number of nuclei: six replications are enough for reproducible measurement. The peak positions showed some variation between the samples from different parts of individual seedling. However, this variation did not interfere with the assignment of peaks. Another problem was the presence of the background signals. This noise was predominantly located in the lower channel numbers. For instance, high background signals were found in the samples from cotyledon (**Fig. 2B**) and radicle (root) tissues (**Fig. 2D** and **Fig. 4E**). The noise partly hampered the flow cytometric analysis

of these samples (**Fig. 2D**).

Developmental stages in radish seedling grown in vitro

The description of successive developmental stages of seedlings of the radish cultivar cv. Ten-Sui is given in **Fig. 1**. Four developmental stages were morphologically distinguished from the dry seed (Stage 0) to the seedling (Stage 3). Germination of seeds started within 1 day. After imbibition of the seed, the outgrowth of the radicle and hypocotyl elongation (Stage 1) was observed. The majority of the seedlings reached Stage 2 after 7 days. The seedlings developed at Stage 3 within 25-30 days.

Endopolyploidization in in vitro grown seedlings

Because radish is a diploid species, 2C DNA level corresponds to the diploid state of the genome found in the G1 phase, while 4C DNA level results from the S phase duplication of chromatids found in the G2 phase, and thus, an indicator of the capacity of cells to enter mitosis. Therefore, the major 2C and 4C peaks indicate that the tissues are in a dividing state. However, the 4C value may not be the G2 intermediate state of ploidy, but may represent a multiploid form of the genome. The presence of 8C nuclei is an indicator of the capacity of cells to enter endoreduplicatoin cycles.

Nuclei from embryos at stage 0 produced a large 2C peak (**Fig. 2A**). This suggested that cells of quiescent embryos are arrested in the presynthetic

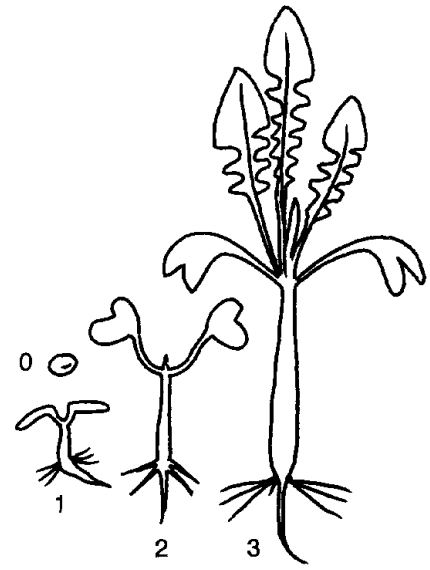

Fig. 1 Stages of seed germination and plant development of *Raphanus sativus* cv. Ten-Sui. *Stage 0*: dry seed; *Stage 1*: upward elongation of the hypocotyl and appearance of cotyledons, but the cotyledons not fully expanded; *Stage 2*: opening of the cotyledons and development of leaf 1; *Stage 3*: development of leaf 3 to leaf 5, expansion of leaf 1 and leaf 2, and slightly wilting of cotyledons.

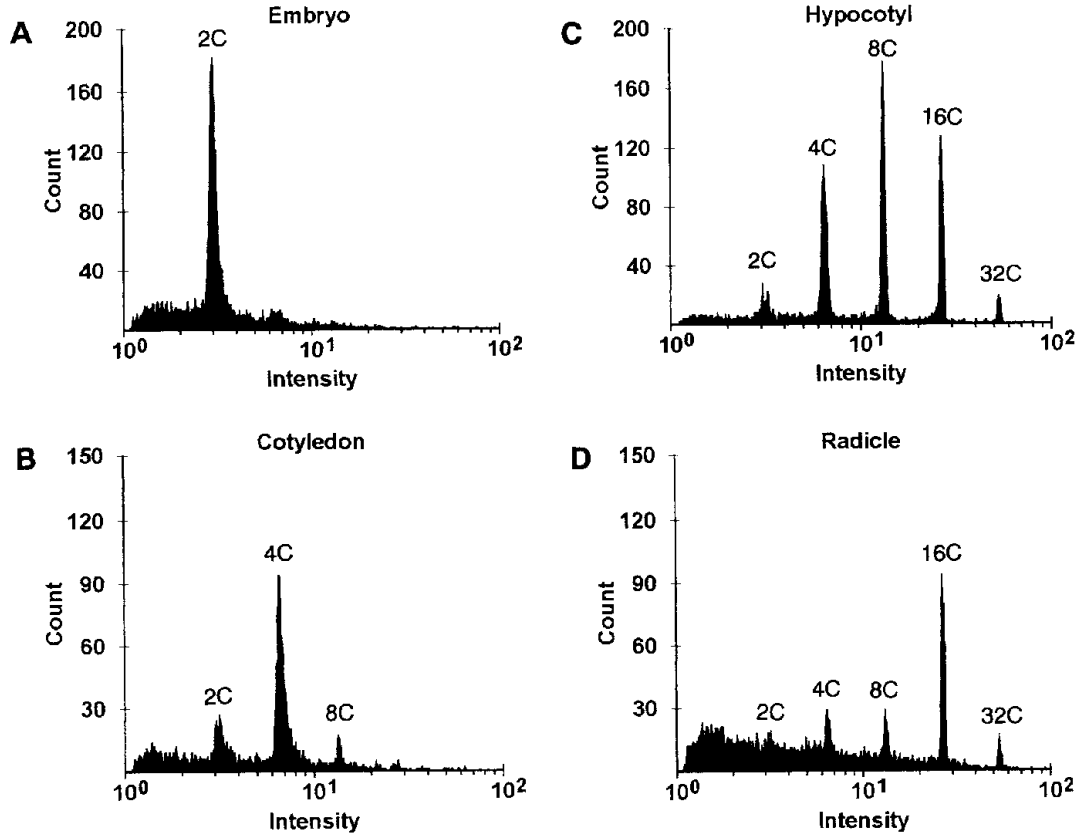

Fig. 2 Characteristic histograms of nuclei distribution at Stage 0 (A) and Stage 1 (B-D) in *Raphanus sativus* cv. Ten-Sui. (A) embryo, (B) cotyledon, (C) hypocotyl, (D) radicle.

G1 phase of nuclear division. At stage 1, endoreduplication occurred in all tissues tested. Cotyledon cells gave a large amount of 4C signals and a small amount of 2C and 8C signals (**Fig. 2B**), indicating that certain cells in the cotyledons undergo one cycle of endoreduplication. In contrast, cells of hypocotyls and radicles considerably increased C-levels (**Fig. 2C, D**). Extensive endoreduplication of

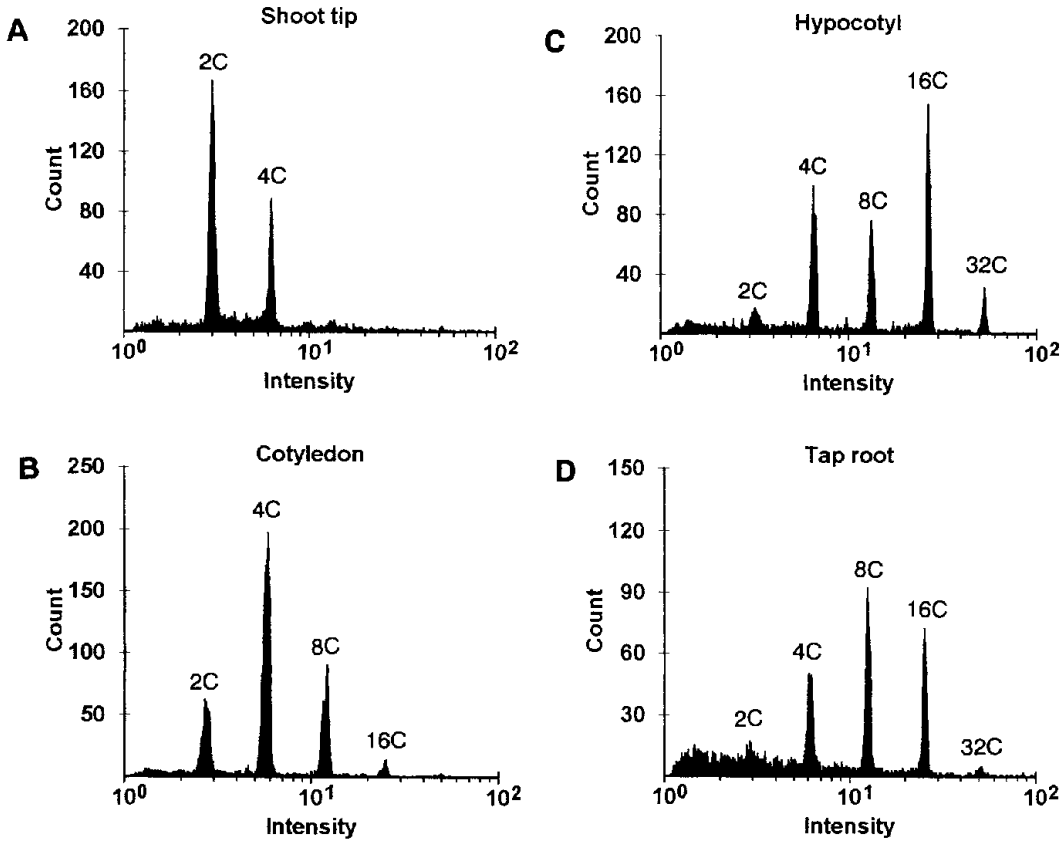

Fig. 3 Characteristic histograms of nuclei distribution at Stage 2 in *Raphanus sativus* cv. Ten-Sui. (A) shoot tip, (B) cotyledon, (C) hypocotyl, (D) taproot (former radicle).

Table 1 Ploidy patterns in different *Raphanus sativus* cultivars at stage 3

Cultivar	Tissue type	Ploidy patterns (% of nuclei populations*)				
		2C	4C	8C	16C	32C
Ten-Sui	Shoot tip	82.2 ± 2.2	17.8 ± 2.2			
	Leaf 1	62.1 ± 1.3	33.4 ± 1.5	4.5 ± 2.6		
	Cotyledon	18.5 ± 1.6	52.1 ± 2.2	27.2 ± 1.8	2.2 ± 1.7	
	Hypocotyl	12.9 ± 1.8	23.4 ± 3.1	23.3 ± 2.4	32.3 ± 1.0	8.1 ± 1.5
Natsu-Nobu	Shoot tip	79.1 ± 2.3	20.9 ± 2.3			
	Leaf 1	58.5 ± 1.3	35.9 ± 3.3	5.6 ± 2.5		
	Cotyledon	23.4 ± 1.9	49.3 ± 2.2	25.5 ± 3.0	1.8 ± 1.3	
	Hypocotyl	15.0 ± 2.2	25.2 ± 3.8	24.3 ± 1.3	28.5 ± 1.6	7.0 ± 2.0
Toki-Nasi	Shoot tip	84.4 ± 3.3	15.6 ± 3.3			
	Leaf 1	66.2 ± 2.5	29.6 ± 2.4	4.2 ± 1.7		
	Cotyledon	19.8 ± 0.8	50.6 ± 2.9	27.6 ± 3.5	2.0 ± 1.8	
	Hypocotyl	10.3 ± 2.2	28.0 ± 3.8	23.3 ± 2.5	29.4 ± 2.4	9.0 ± 1.0
Yu-Tou-Sei	Shoot tip	76.7 ± 2.2	23.3 ± 2.2			
	Leaf 1	60.2 ± 1.5	35.5 ± 1.8	4.3 ± 1.0		
	Cotyledon	20.0 ± 2.8	50.3 ± 3.0	27.2 ± 1.3	2.5 ± 1.3	
	Hypocotyl	13.5 ± 5.3	26.4 ± 1.5	22.2 ± 2.1	30.5 ± 0.5	7.4 ± 1.8

*For each value:means ± standard error

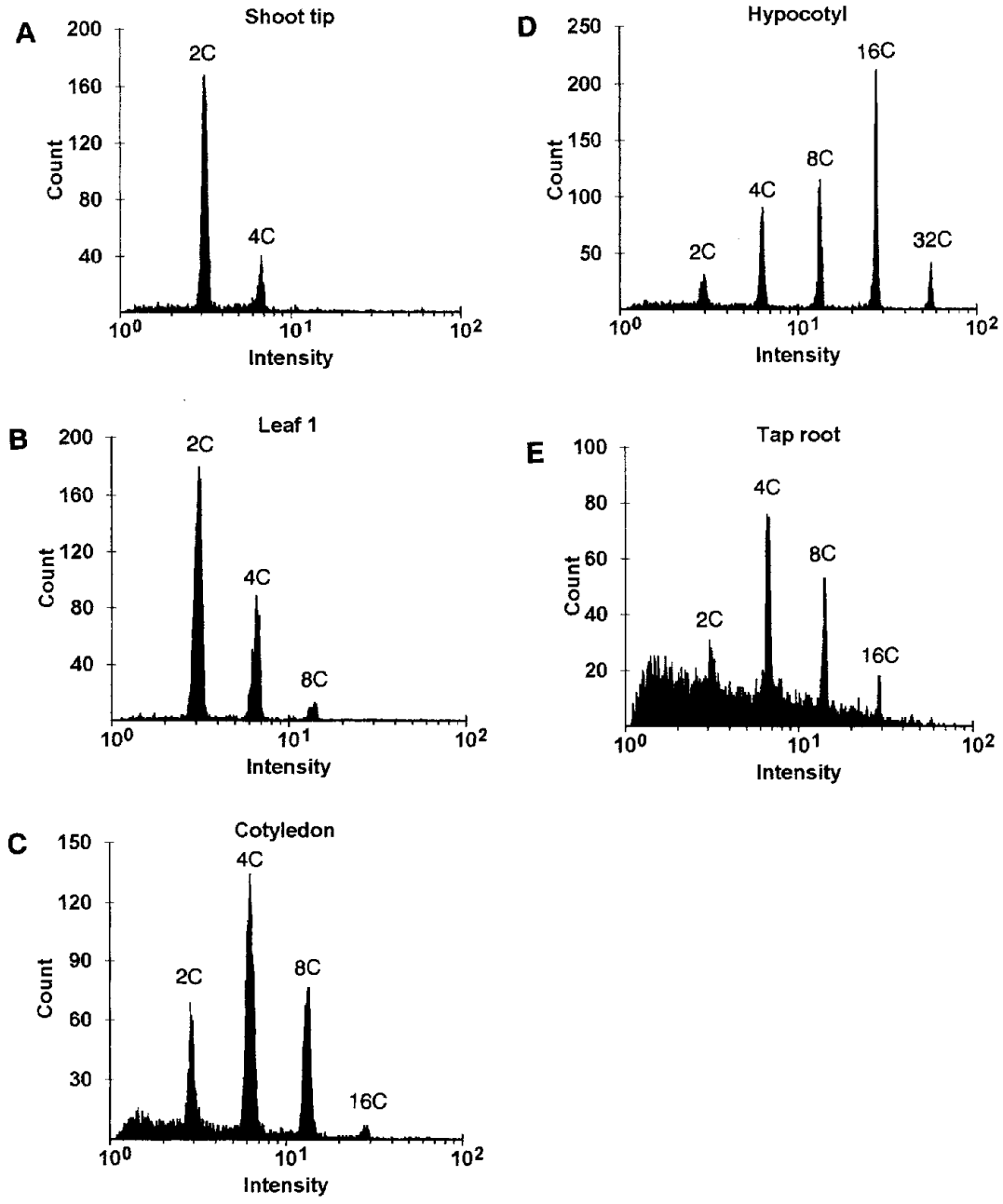

Fig. 4 Characteristic histograms of nuclei distribution at Stage 3 in *Raphanus sativus* cv. Ten-Sui. (A) shoot tip. (B) leaf 1, (C) cotyledon, (D) hypocotyl, (E) taproot.

the nuclear DNA had taken place in these tissues. In hypocotyl tissues contained cells with five ploidy levels, 2C, 4C, 8C, 16C and 32C (**Fig. 2C**). At this time point, no exponential growth of hypocotyl had occurred. Therefore, endoreduplication had preceded hypocotyl elongation. Cells of radicle tissues went through up to four rounds of endoreduplication and reached a maximum of 32C level (**Fig. 2D**).

At stage2, the ploidy patterns of the stage 1 had progressively changed in cotyledons (**Fig. 3B**) . Signals of the 16C nuclei occurred in the cotyledons. In hypocotyl and taproot, the degree of endopolyploidy was maintained, where the 8C, 16C and 32C peaks were detected (**Fig. 3C, D**). Shoot meristematic cells had 2-4C DNA contents, indicating that they are diploid, as would be expected for G1, S, and G2 contents of a diploid cell line (**Fig. 3A**).

Endoreduplication in cotyledons occurred gradually and their ploidy patterns are progressive at stage 3 (**Fig. 4C**). The cotyledons displayed a mixture of multiple polyploid cells with four ploidy levels (2C -16C), where a small but reproducible 16C peak was present. Stability of the diploid level was observed in shoot tips (**Fig. 4A**). Fully expanded leaf 1 was found to have 8C nuclei in addition to 2C and 4C nuclei (**Fig. 4B**). Hypocotyl cells exhibited the five multiple peaks (**Fig. 4D**). Taproot decreased the level of ploidy (**Fig. 4E**). Values for DNA contents higher than 32C were never observed in any tissues at this stage.

All radish cultivars tested showed similar ploidy

patterns (**Table 1**). These plants contained cells with five ploidy levels that correspond to 2C, 4C, 8C 16C and 32C.

Endopolyploidization in tuberous roots of in vivo plants

Extensive endoreduplication had also taken place in tuberous root tissues. Nuclei from central region (**Fig. 5B**) showed higher levels of polyploidy than nuclei from peripheral region (**Fig. 5A**) within the same tuberous root. The fifth round of DNA replication (64C) was specific in the central reigion. In addition, nuclei at the 2C DNA level were undetectable (**Fig. 5B**). Values for DNA contents higher than 64C were never detected in tuberous roots.

Besides Ten-Sui, multiple polyploidy was also detected in other radish cultivars (**Table 2**). However, values of maximum DNA content per nucleus (32C-64C) depended on the cultivars.

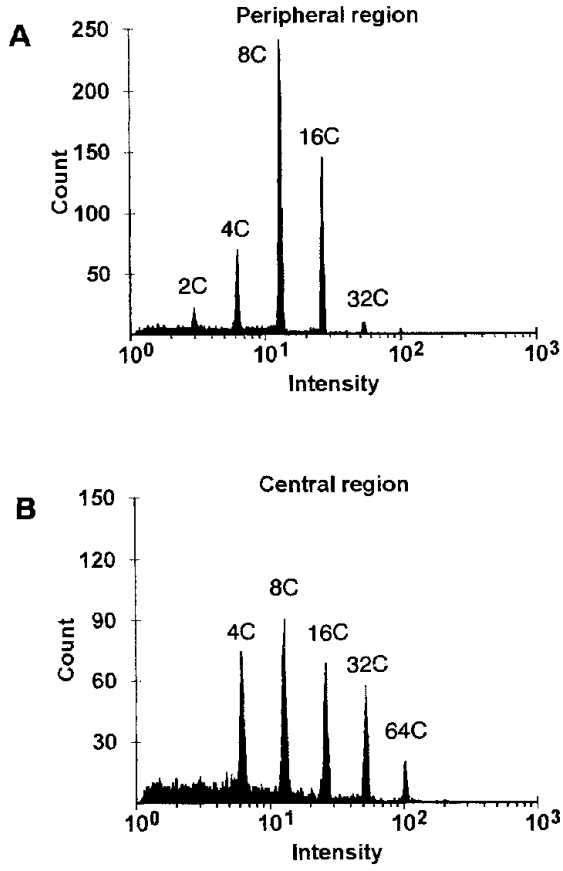

Fig. 5 Characteristic histograms of nuclei distribution in teberous root samples in *Raphanus sativus* cv. Ten-Sui. (A) periphery region, (B) central region.

Discussion

By flow cytometry, the ploidy profiles during development of radish plants were characterized and our results demonstrated that most somatic cells undergo several rounds of endopolyploidization, resulting in the cells with multiple ploidy levels. A striking feature of radish cells is their uneven ploidy levels during differentiation. Tissues in radish plants are composed of ordered collections of various cell types in their DNA content. Developmental programs may control the switch from cell proliferation to endopolyploidization. Indeed, certain cells are able to achieve DNA contents of up to 64C, where C is the haploid DNA content per nucleus. The endopolyploid nuclei fall into clear ploidy series (2C, 4C, 8C, 16C...), corresponding to nuclear DNA endoreduplication. In general, older tissues exhibited higher levels of endopolyploidy than younger tissues within the same plants. The distribution of endopolyploid cells is organ-specific. Our previous studies showed that most of somatic cells in several *Brassica* crops undergo several rounds of endoreduplication and that this process is developmentally regulated (Kudo and Kimura, 2001a, b). In both *B. oleracea* and *B. rapa*, the seedlings become endo-

Table 2 Ploidy patterns for tuberous roots in different *Raphanus sativus* cultivars

Cultivar	Position in tuberous roots	Ploidy patterns (% of nuclei populations*)					
		2C	4C	8C	16C	32C	64C
Ten-Sui	Peripheral	11.8 ± 2.4	30.7 ± 2.8	50.7 ± 1.2	6.2 ± 0.9	0.6 ± 1.5	
	Central		26.6 ± 1.7	31.7 ± 2.2	22.2 ± 1.5	14.9 ± 1.6	4.6 ± 1.4
Natsu-Nobu	Peripheral	14.1 ± 3.6	24.3 ± 2.2	59.3 ± 1.8	2.3 ± 1.1		
	Central		23.0 ± 3.5	40.6 ± 1.9	28.2 ± 0.6	8.2 ± 0.8	
Toki-Nasi	Peripheral	12.2 ± 3.3	32.5 ± 1.9	48.6 ± 1.9	5.8 ± 1.5	0.9 ± 1.2	
	Central		18.1 ± 3.0	37.7 ± 2.4	30.0 ± 1.8	13.5 ± 2.5	0.7 ± 0.7
Yu-Tou-Sei	Peripheral	14.4 ± 2.5	34.5 ± 3.3	42.3 ± 3.0	7.8 ± 1.9	1.0 ± 0.6	
	Central		16.4 ± 4.6	38.4 ± 2.7	33.0 ± 2.5	10.7 ± 0.5	1.5 ± 1.5

*For each value:means ± standard error

polyploid during repeated rounds of endoreduplication. At the present study we extended the earlier observations. It is probable that endopolyploidy is very common feature *in Brassica* coenospecies.

Endopolyploidy was not present in the embryos from dry seeds. However, the embryos apparently contain certain cells that are programmed to enter endoreduplication cycles but remain in a quiescent state until they receive a developmental signal during germination. Rapid and extensive endoreduplication can occur in radicle and hypocotyl cells at the early germination stage. Endoreduplication in these cells takes place before exponential growth, and appears to provide the growth potential for the subsequent cell elongation. Further endoreduplication cycles were detected in all tissues except those of the shoot tip regions.

Plants can sense and control the DNA content of a cell and regulate its ploidy level during development (Traas *et al.*, 1998). Nuclear DNA content may be controlled by a hierarchy of regulatory systems, which operate at cellular, tissue, organ and organism levels. In reality, the growth of a given cell is influenced by interactions with neighboring cells and is under the control of higher regulatory systems. Co-ordination of these networks leads to the formation of endoploiploid cells in which a single cell co-exists with a multitude of identical and different cell types. Systemic control for endoreduplication has been recently described in several plant species such as *Arabidopsis thaliana* (Galbraith *et al.*, 1991). Nuclear DNA content is not static; rather than a great amount of variation in the course of development.

Endoreduplication is often observed in association with cell growth, and in plants a strong correlation between cell sizes (Kondorosi *et al.*, 2000). The differentiation of plant cells is linked to the increase of thier DNA content (Meralagno *et al.*, 1993) .The physiological role of endoreduplication is, however, elusive as it might contribute to or be a consequence of cell differentiation programs. Multiplication of the genome has been proposed to increase metabolic activity, ribosome RNA synthesis and transcriptional activity (Nagl, 1976; Baluska and Kubica, 1996). Moreover, by causing a short-circuit in the cell cycle endoreduplication may provide a faster way than cell proliferation to increase the tissue or organ size in the developmental programs (Maralagno *et al.*, 1993).

Endoreduplication has been described in storage organs such as endosperm of maize kernels (Kowles *et al.*, 1990) and tomato fruits (Bergervoet *et al.*, 1996; Joubès *et al.*, 1999). In these cells, endoreduplication can be advantageous for specialized functions. During development of maize endosperm, cells shift from mitotic divisions to endoreduplication events, driving the massive synthesis of storage proteins and starch (Lur and Setter, 1993). Tuberous roots and endosperm have in common that both tissues function as a sink of metabolites during development. The sink function of the radish tuberous roots is possibly facilitated by endoreduplication of nuclear DNA.

Understanding the mechanisms for regulation of endoreduplication can provide new insights into the evolution of polyploid genomes in plants. Unlike in animals, where polyploidy is rare, it has been estimated that up to 80% of angiosperms have polyploids in their lineages (Leitch and Bennett, 1997). Many economically useful species are polyploid (e.g. wheat, maize, sugarcane, potato and cotton). These species have undergone polyploidization events during thier evolution (Ayala *et al.*, 2000; Gaut *et al.*, 2000). In particular, a distinctive feature of the evolution and speciation of flowering plants is allopolyploidy- the combination of genetically different diploid nuclear genomes from two or more different ancestral species (Leitch and Bennett, 1997). For instance, *Brassica napus* has been considered to be allotetraploid that has originated in nature from spontaneous duplication of entire genome following hybridization between *B. oleracea* and *B. rapa* (Gómez-Campo, 1999). Duplication of entire genome is common in plant genome evolution. It may be that the evolution of plant genome was the prerequisites for expansion of genomes by endoreduplication at all scales.

In conclusion, with this study we provide a detailed view of DNA synthesis in the course of radish development. Changes in DNA content appear to be fundamental events in normal development programs of radish plants. Growth of radish plants probably does not depend on cell proliferation alone. Appropriate molecular tools for the control of endoreduplication are necessary for improvement of economically important crops. Control of endoreduplication may provide a means to manipulate the growth of organs of agronomic interest.

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