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The Occurrence of Endopolyploid Cells During Seedling Development of *Allium fistulosum* L.

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Abstract

The endopolyploidy during seedling development of Welsh onion (*Allium fistulosum* L.) is described. Flow cytometric measurements of relative nuclear DNA contents showed that certain cells became polyploid. Endopolyploidy was not present in the nuclei of embryos during imbibition. Endopolyploidization had taken place in all organs tested. Leaf segments of *in vivo* grown 3 month-old plants had elevated ploidy levels and contained nuclei of 2C, 4C, 8C, and 16C, where C is the haploid nuclear genome complement. The endopolyploid nuclei fall into clear ploidy series (2C, 4C, 8C, and 16C). Therefore, the process of endopolyploidy appears to correspond to endoreduplication.

Key words: *Allium fistulosum* L., DAPI, endopolyploidy, endoreduplication, flow cytometry, Welsh onion.

Abbreviations

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

Endopolyploidy (or somatic polyploidy) has been described in many eukaryotes (Nagl, 1976; Brodsky and Uryvaeva, 1977; Barlow, 1978). The occurence of endopolyploidy is very frequent in insects. A variety of cell types in *Drosophila* larval tissues undergo endopolyploidization (Sauer *et al.*, 1995). In animals, endopolyploidy is observed in specific cell types such as some molluscan neurons, mammalian megakaryocytes and plancental trophoblast cells (Chase and Tolloczko, 1987; Zybina and Zybina, 1996). The occurrence of endopolyploidy is common in plants (Barlow, 1978). However, endopolyploidization events in plants are poorly understood (Trass *et al.*, 1998). Endopolyploidy is tightly coupled to cell differentiation (Nagl, 1976). Furthermore, clear relationship was observed between endopolyploidy and cell size (Melaragno *et al.*, 1993; Kudo and Kimura, 2002b).

Allium is a cosmopolitan genus with more than 600 species distributed in the northern hemisphere (Novák *et al.*, 1986). They comprise major vegetable crops and many wild relatives. *A. fistulosum* L. (Welsh onion or Japanese bunching onion) is one of the most important horticultural crops in the genus *Allium*. *A. fistulosum* is widely cultivated from Siveria to Tropical Asia and shows morphological variability in Japan (Haishima *et al.*, 1993). *A. fistulosum* is a diploid (x = 8) biennial herbaceous plant, flowering in the second season of growth (Inden and Asahira, 1990). *A. fistulosum* has no well-developed bulb and shortened stem-plate from which roots and shoots arise.

The development of endopolyploidy has recently been described in economically important crops, such as *Cucumis sativus* (Gilissen *et al.*, 1993), *Lycopersicon esculentum* (Smulders *et al.*, 1994), several *Brassica* crops (Kudo and Kimura, 2001a, b), *Raphanus sativus* (Kudo and Kimura, 2002a) and *Portulaca grandiflora* (Mishiba and Mii, 2000). So far, very little information is available on the development of endopolyploidy in *A. fistulosum*. The objective of this study is to determine whether endopolyploidization occurs in seedling development of *A. fistulosum*.

In vitro plants of *A. fistulosum* cv. Nebuka were grown from seeds and used for the present study. Seeds were surface-sterilized for 20 min with 2% 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, soli-

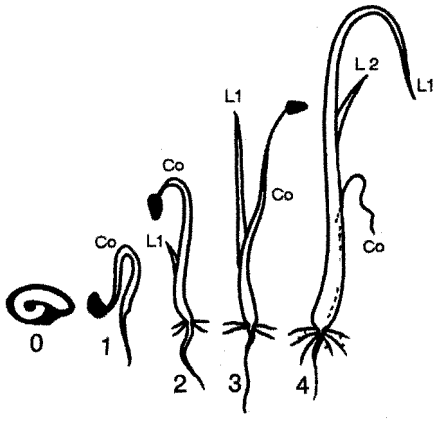

Fig. 1 Five developmental stages from seed imbibition to seedling establishment of *Allium fistulosum* cv. Nebuka under *in vitro* conditions. *Stage 0*: embryo 4 h after imbibition with no sign for germination; *Stage 1*: 1 day-old seedling, outgrowth of the radicle and upward elongation of the cotyledon; *Stage 2*: 10 day-old seedling, appearance of the first leaf; *Stage 3*: 20 day-old seedling, development of the first leaf; *Stage 4*: 30 day-old plant, development of the second leaf, expansion of the first leaf, and wilting of the cotyledon.
Co = cotyledon, L1 = the first leaf, and L2 = the second leaf.

dified 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* plants were cultivated in a greenhouse. Leaf segments were harvested after three months of culture.

DNA content of nuclei from embryos and plants at different developmental stages was determined by flow cytometry (FCM). The developmental stages of *in vitro* plants are classified into five categories according to their morphology distinguished from embryos to 30 day-old plants (**Fig. 1**). Nuclei were extracted and stained with 4', 6-diamidino-2-phenylindole (DAPI) using a high resolution kit (PARTEC High Resolution Kit type P, Partec GmbH, Münster, Germany).

For the FCM analysis on *in vitro* plants, tissue samples were harvested from four embryos which were carefully excised from seeds after 4 h of culture (stage 0), and from seedlings at different developmental stages (stage 1-stage 4) by dissecting into several parts, i. e., leaves, cotyledon, and roots. The leaves were numbered from bottom to top, with the oldest leaf as number one.

For *in vivo* plants, the segments (1.0 cm^2) of central portion of the outer leaf were harvested and used for the analysis.

The samples were chopped with a razor blade in a few drops of nuclei extraction buffer (solution A of the kit). A 1-2 ml of staining solution containing DAPI (solution B of the kit) was added and the sample was passed through a 100-μm Cell Trics filter (PARTEC). Fluorescent microscopic observations confirmed that isolated nuclei were not clumping. The analyses were performed with a PAS flow cytometer (PARTEC) equipped with an HBO 100 mercury arc lamp. The signals of nuclei within each peak were determined by FlowMax software (PARTEC). Mean and coefficient of variation of fluorescence peaks were estimated with WinMDI software (version 2.8, copyright © 93-99 Joseph Trotter). In analysis of each sample, a minimum of 3,000 nuclei were totally counted at a rate of 10-20 nuclei s^{-1}. Measurements of nuclear DNA content were carried out with at least 6 replications. To determine the position of 2C peak, nuclei of *in vitro* young leaves were analyzed twice on each measurement. The data were plotted on a semi-logarithmic scale, so that the histograms from 2C to 16C were evenly distributed along the abscissa. The data were presented as percentage of the total amount of nuclei in all peaks of the histogram.

In vivo plants were harvested after three months of culture and the abaxial epidermis of the most outer leaves was used for the microscopic obeservation of nuclei. The peeled epidermis was placed on a glass slide and immersed in a drop of DAPI at a concentration of 5 μg ml^{-1} for 5 min. The epidermis was then washed in distilled water and observed under epifluorescence microscopy (model BHS-RFC, Olympus) using the x20 objectives of a microscope.

In most cases, FCM histograms were sharp, and coefficients of variation (CV%) lay between 1.3 and 4.9. Because *A. fistulosum* is a diploid species, 2C DNA level corresponds to the diploid state of the genome found in the G1 phase, while 4C DNA level coincides with G2 phase of dipolid which results from the S phase duplication of chromatids, and thus, an indicator of the capacity of cells to enter mitosis. However, the 4C value may not be the G2 state of diploid, but may represent an endopolyploid form of the genome. In this case, the presence of 8C nuclei is an indicator of the capacity of cells to enter endopolyploidization events.

FCM histograms from embryonic nuclei at stage 0 showed a large 2C peak (85% of total nuclei counted) and a second smaller peak (15%) with twice the amount of fluorescence, corresponding to nuclei with replicated 4C DNA content (**Fig. 2A**).

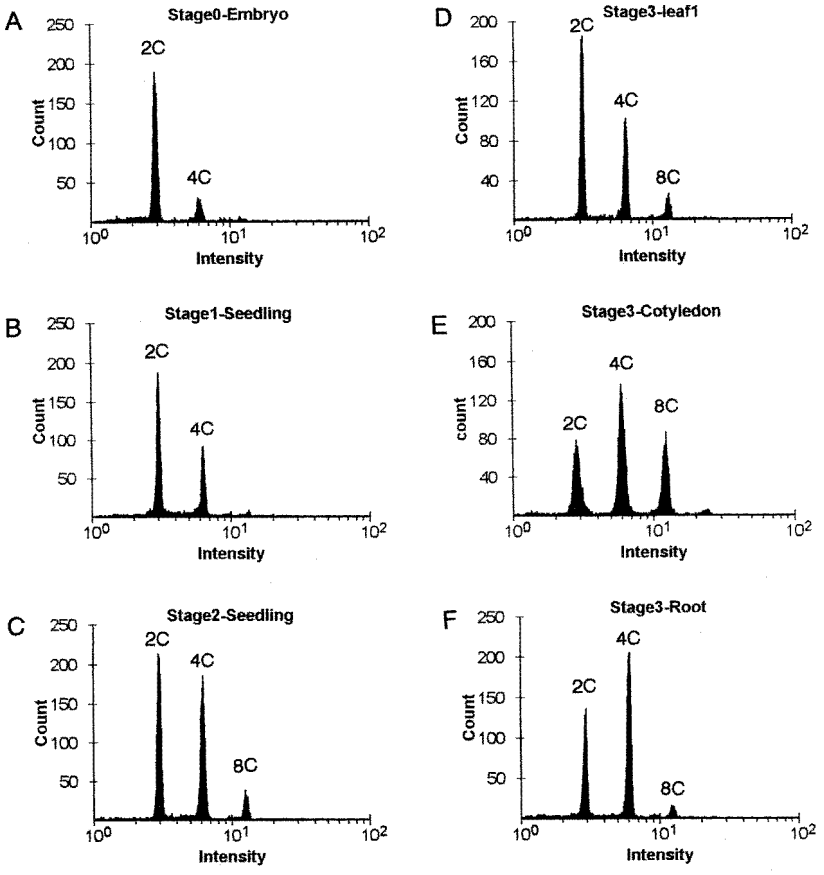

Fig. 2 Characteristic histograms of nuclei distribution from Stage 0 to Stage 3. of *Allium fistulosum* cv. Nebuka. (A) Embryo at Stage 0, (B) Whole seedling at Stage 1, (C) Whole seedling at Stage 2, (D) Leaf1 at Stage 3, (E) Cotyledon at Stage 3, (F) Root at Stage3.

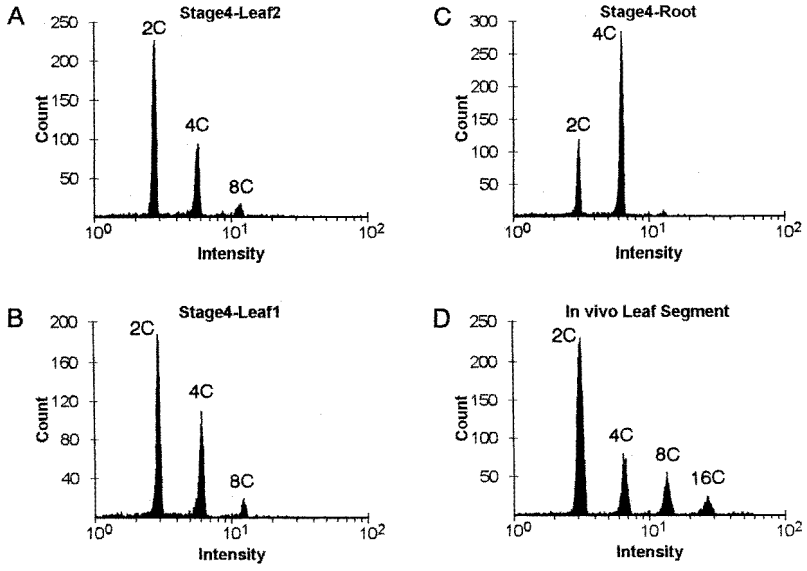

Fig. 3 Characteristic histograms of nuclei distribution from organs of in vitro plants at Stage 4 and leaf segments of *in vivo* plants of *Allium fistulosum* cv. Nebuka. (A) Leaf 2 at Stage 4, (B) Leaf 1 at Stage 4, (C) Root at Stage 4, (D) Leaf segment of *in vivo* plants.

During imbibition, the elevated water content of the seeds may trigger the initiation of cell cycle events. Germinating seedlings at stage1 had increased the frequency of 4C nuclei (**Fig. 2B**). 25% of somatic cells in the seedlings had already 4C nuclei. The first cycle of endopolyploidization of nuclear DNA had taken place at stage 2 as indicated by the appearance of a small population of 8C nuclei (**Fig. 2C**). The whole seedlings at this stage contained about 10% of 8C cells. At stage3, endopolyploidy was observed in the somatic cells of all organs tested (**Fig. 2D-F**). The percentage of nuclei with

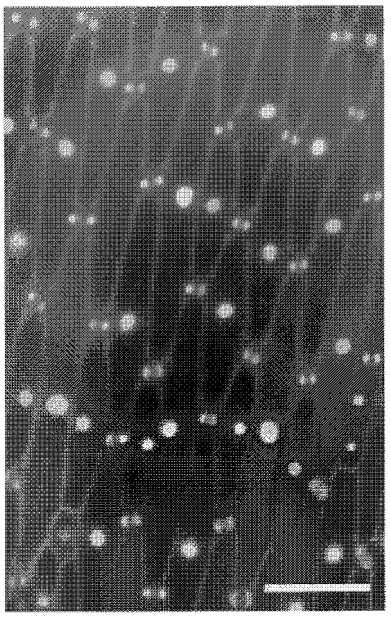

Fig. 4 Fluorescence of DAPI-stained nuclei in the abaxial epidermis of the outer leaf of *in vivo* plants in *Allium fistulosum* cv. Nebuka. Bar = 100 μm.

8C level varied from 4% to 25% of the total nuclei counted among the organs. Cotyledon had 25% of 8C nuclei (**Fig. 2E**) and leaf 1 had 8% (**Fig. 2D**) while root had only 4% 8C (**Fig. 2F**). At stage 4, multiploidy was detected in leaf 1 and leaf 2 (**Fig. 3A, B**). Leaf 1 and leaf 2 contained 10% and 7% of endopolyploid nuclei (8C), respecitvely. In leaf 1, the frequency of 8C cells slightly increased. In contrast to the endopolyploid organs, root tissues reduced their ploidy level and had virtually only diploid (2C-4C) nuclei (**Fig. 3C**).

Endopolyploidy was also detected in leaf seg ments of *in vivo* plants. The third round of endopolyploidization (16C) was observed (**Fig. 3D**).

The observation of DAPI-stained epidermal tissues revealed apparent differences in nucleus size in relation to cell size (**Fig. 4**). Guard cells had small nuclei whereas large elongated epidermal cells had large nuclei.

In the present study, *A. fistulosum* is revealed to be a species with endopolyploidy up to 16C. The endopolyploid nuclei fall into clear ploidy series (2C, 4C, 8C, and 16C). Therefore, the process of endopolyploidy appears to correspond to endoreduplication. Since the cells with 8C content are not found in the germinationg embryo but appear with the initiation of first leaf, it might be possible that endopolyploidization begins with some cells of cotyledon and leaves. A similar change in the pattern of endopolyploidy has been observed previously in other plant species such as *Cucumis sativus* (Gilissen *et al.*, 1993), and *Brassica oleracea* (Kudo and Kimura, 2001b). Therefore, the development of endopolyploidy during the early stage of germination might be a common feature in a wide range of plant species. Because a strong correlation was found between cell size and nuclear size in leaf epidermal cells of *A. fistulosum* (**Fig. 4**), endopolyploidy of this species might be associated with cell differentiation as described previously (Kondorosi *et al.*, 2000; Kudo and Kimura, 2002b). Although the physiological role of endopolypolidy in *A. fistulosum* is still unclear, it is possible that gene expression might be increased in these endopolyploid cells with increase in the available DNA templates (Nagl, 1976; Baluška and Kubica, 1992; Larkins *et al.*, 2001). Further studies are required to clarify the physiological role of the endopolyploidization in this important crop.

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