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Systemic Endopolyploidy in Development of Spinach (*Spinacia oleracea* L.)

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Abstract

Occurrence of endopolyploid cells in somatic tissues of spinach (*Spinacea oleracea* L.) was investigated by flow cytometry. Endopolyploidy was not present in embryos during imbibition of seeds. Rapid endopolyploidization occurred in seedlings during germination. Spinach appears to become endopolyploid by repeated rounds of replication of its entire genome. Spinach contained cells with six ploidy levels that correspond to 2C, 4C, 8C, 16C, 32C and 64C, where C is the haploid nuclear genome complement. The endopolyploid nuclei fall into clear ploidy series (2C, 4C, 8C, 16C...). Therefore, the process of endopolyploidy corresponds to endoreduplication.The patterns of endopolyploidy was characteristic of tissue type and developmental stage. However, endopolyploidy was not observed in apical meristematic tissues. Endopolyploidization may give rise to genetic plasticity in spinach.

Key words: Endopolyploidy, Endoreduplication, Flow cytometry, Nuclear DNA content, *Spinacea oleracea* L.

Abbreviations

DAPI: 4', 6-diamidino-2-phenylindole · *FCM*: Flow cytometry.

Endopolyploidy occurs in a wide variety of multicellular organisms including plants (Brodsky and Uryvaeva, 1977; Barlow, 1978; Traas *et al.*, 1998), Although the biological significance of endopolyploidy is not yet clear in plants. Recent flow cytometric data revealed that endopolyploidy is commonly found in economically important plant speices belonging to different genera, such as *Brassica oleracea* (Kudo and Kimura, 2001a, b), *B. rapa* (Kudo and Kimura, 2001a), *Raphanus sativus* (Kudo and Kimura, 2002a), *Lycopersicon esculentum* (Smulders *et al.*, 1994), *Cucumis sativus* (Gilissen *et al.*, 1993), and *Allium fistulosum* (Kudo *et al.*, 2003).

Spinach (*Spinacia oleracea* L.) is an important vegetabe crop belonging to the family Chenopodiaceae. So far, none of previous investigations quantified nuclear DNA content in relation to development of spinach plants. Therefore, ploidy profiles was determined by flow cytometry during the different stages of development.

In vitro plants of *Spinacia oleracea* cv. A-Pa-Re were grown from seeds and used for the present study. Seeds were surface-sterilized for 20 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 gl^{-1} sucrose, solidified with 2.5 gl^{-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-hour day light/8-hour dark photoperiod.

DNA content of nuclei from plants was determined by flow cytometry (FCM). For the FCM analysis, tissue samples were harvested from the plants at the different developmental stages (Stage 0 -Stage 3) as shown in Fig. 1, ranging from an embryo excised from a seed after 16 hours of imbibition (Stage 0), to the seedlings at each developmental stages (Stage 1-Satge 3). Each seedling was dissected into several parts, i. e. leaves, cotyledon, hypocotyl, and roots. Leaves were numbered from old to young, with the oldest outer leaf as

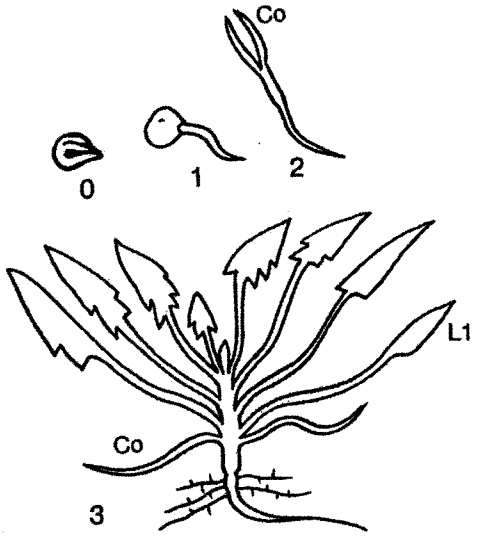

Fig. 1 Four developmental stages from seed imbibition to seedling establishment of *Spinacia oleracea* under *in vitro* conditions. *Stage 0*: embryo 16 h after imbibition with no sign for germination; Stage 1: germinated seedling, outgrowth of the radicle; *Stage 2*: 2 d-old seedling, upward elongation of cotyledon; *Stage 3*: 40 d-old plant, development of leaf 5 to leaf 8, expansion of leaf 1 to leaf 4; Co = cotyledon, L1 = leaf 1.

number one.

Nuclei were extracted and stained using a high resolution kit (PARTEC CyStain UV Plant DNA Analysis Kit Precise P, Partec GmbH, Münster, Germany). The samples were chopped with a razor blade in a 0.4-ml of nuclei extraction buffer (nuclei extraction solution of the kit). A 1-2 ml of staining solution containing the dye 4′, 6-diamidino-2-phenylindole (DAPI, stain solution of the kit) was added and the sample was passed through a 100-μm Cell Trics filter (PARTEC). 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). Measurements of nuclear DNA content were carried out with at least 4 replications. At a single analysis, a minimum of 3,000 nuclei were counted for each sample. To determine the standard peak position of 2C nuclei, the 2C peak from nuclei of *in vitro* young leaves was analyzed 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 were presented as percentage of the totoal amount of nuclei in all peaks of the histogram.

Because spinach is a diploid species, the 2C DNA level corresponds to cells in G1. The 4C population encompasses normal cells in G2 and cells that have gone through a single round of endopolyploidization. Therefore, the presence of 8C population is an indicator of cells that must have all undergone endopolyploidization events.

FCM histograms from nuclei at stage 0 showed a large 2C peak (63% of total nuclei counted) and a second smaller peak (34%) with twice the amount of fluorescence, corresponding to nuclei with replicated 4C DNA content (**Fig. 2A**). Imbibition of the seeds may trigger the progression of cell cycle events, as shown in our previous study, in which cell cycle activity arrested at G1 in most embryonic cells of matured dry cabbage seeds initiated to shift to G2 (4C) upon imbibition (Kudo and Kimura, 2001b).

Rapid endopolyploidization occurred in germinating seedlings at stage 1 (**Fig. 2B**). The cells of the germinating seedlings considerably increased their C-values to four ploidy levels (2C -16C). Del Nero-Buffalino and Witkus (1984) also observed endopolyploid 8C nuclei in root cells in germinated seeds of *Spinacia oleracea* using Feulgen staining. At stage 2, extensive endopolyploidization was found in cells of all organs tested (**Fig. 2C-E**). Cotyledon (**Fig. 2C**) and hypocotyl (**Fig. 2D**) contained cells with four ploidy levels (2C-16C). Cells of radicle tissues went through up to four rounds of endopolyploidization and reached a maximum of 32C level (**Fig. 2E**).

Fig. 3 shows the patterns of endopolypoloidy in plants of stage 3. The ploidy patterns of the stage 3 progressively increased in cotyledon (**Fig. 3E**), and hypocotyl (**Fig. 3F**) compared with satge 2. The signals of the 32C were detected in these organs. Main root (**Fig. 3G**) contained cells of five ploidy levels. Lateral root exhibited the four multiple peaks (**Fig. 3H**). In fully expanded leaf 1, the distributions of endopolyploid nuclei depended on tissue type (**Fig. 3B-D**). Nuclei from midrib (**Fig. 3C**) and petiole (**Fig. 3D**) showed much higher levels of endopolyploidy than those from leaf blade (**Fig. 3B**). The fifth round of DNA replication (64C) was specific to the petiole tissues (**Fig. 3D**). Stability of the diploid level was observed in the apical meristematic tissues (**Fig. 3A**). Values for DNA contents higher than 64C were never observed in any tissues at this stage.

The results of flow cytometric analysis of the ploidy level in *Spinacia oleracea* showed that this species is polysomatic type of plants. Most somatic cells of *S. oleracea* undergo several rounds of endopolyploidization, resulting in the cells with multiple polyploidy levels. Spinach plants contained a mixture of cells with clear six ploidy series from 2C to 64C, which corresponds to nuclear DNA

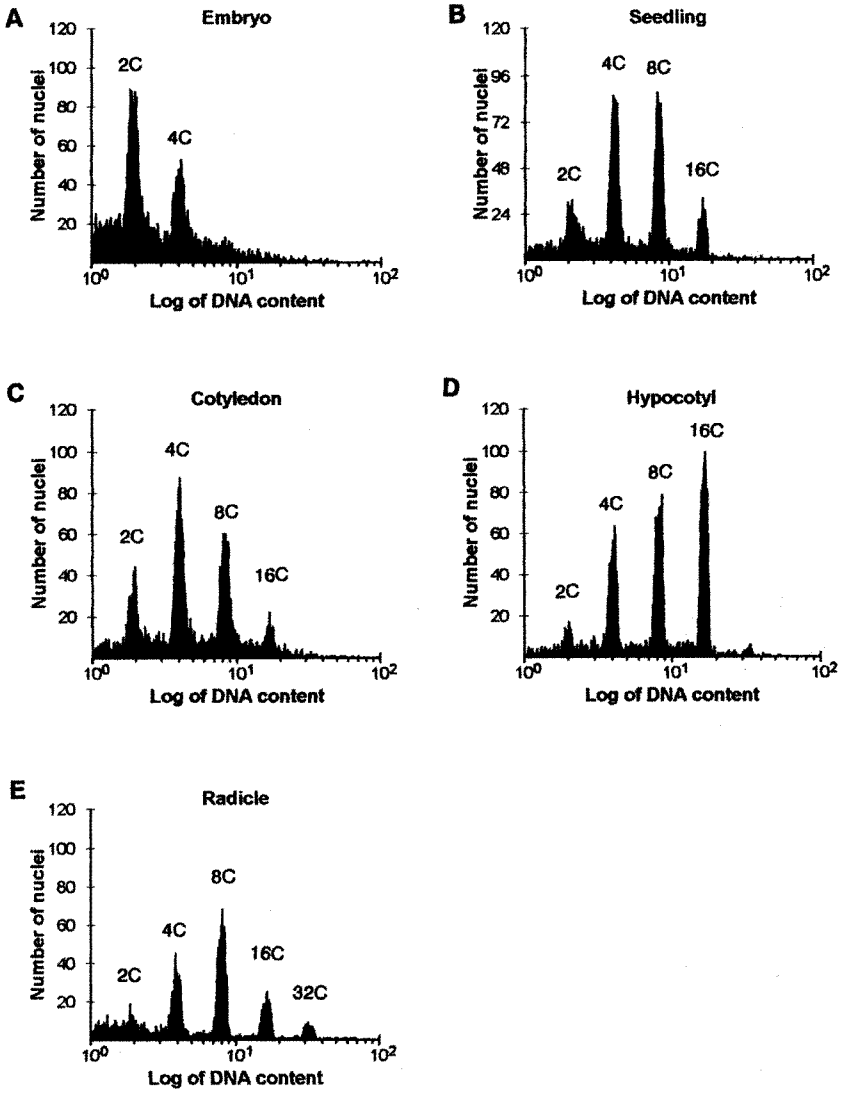

Fig. 2 Characteristic histograms of nuclei distribution from Stage 0 to Stage 2. (A) Embryo at Stage 0, (B) Whole seedling at Stage 1, (C) Cotyledon at Stage 2, (D) Hypocotyl at Stage 2, (E) Radicle at Stage 2.

endoreduplication.

The results indicated that distribution of endopolyploid cells in spinach plants is tissue-specific. The degree and extent of endopolyploidy tend to increase with increasing age of plants. Generally, older tissues exhibited higher levels of endopolyploidy than younger tissues. However, during the development of the plant, apical meristematic tissues were maintained at normal diploid (2C) level. In cabbage plants, Kudo and Kimura (2001b) also observed that apical meristematic tissues were maintained at the diploid level. Endopolyploidization of the cells may be repressed to ensure the stability of the genetic line, because meristem cells are functionally analogous to animal stem cells (Fletcher, 2002). Our previous studies showed that somatic cells in *Brassica oleracea* (Kudo and Kimura, 2001a), *B. rapa* (Kudo and Kimura, 2001b), *Raphanus sativus* (Kudo and Kimura, 2002a) and *Allium fistulosum* (Kudo *et al.*, 2003) undergo several rounds of endoreduplication. Systemic control of endopolyploidy has also been described in several plant species such as *Arabidopsis* thaliana (Galbraith *et al.*, 1991), *Cucumis sativus* (Gilissen *et al.*, 1993), and *Lycopersicon esculentum* (Smulders *et al.*, 1994). Endopolyploidy may be a common feature in many economically important crops. These findings, together with our data in spinach, indicate that nuclear DNA content in plant cells is not static; rather than a great amount of variation in the course of thier differentiation.

The growth of plant cells is linked to the increase of their DNA content (Meralagno *et al.*, 1993; Kudo and Kimura, 2002b). Plant cell size is correlated with nuclear size (Kondorosi *et al.*, 2000). Endoreduplication can augment further the growth capacity and the extent of cell enlargement. The regulation of endoreduplication may allow cells to reach extraordinary sizes (Cebolla *et al.*, 1999). This process probably provide a means to manipulate cell size or

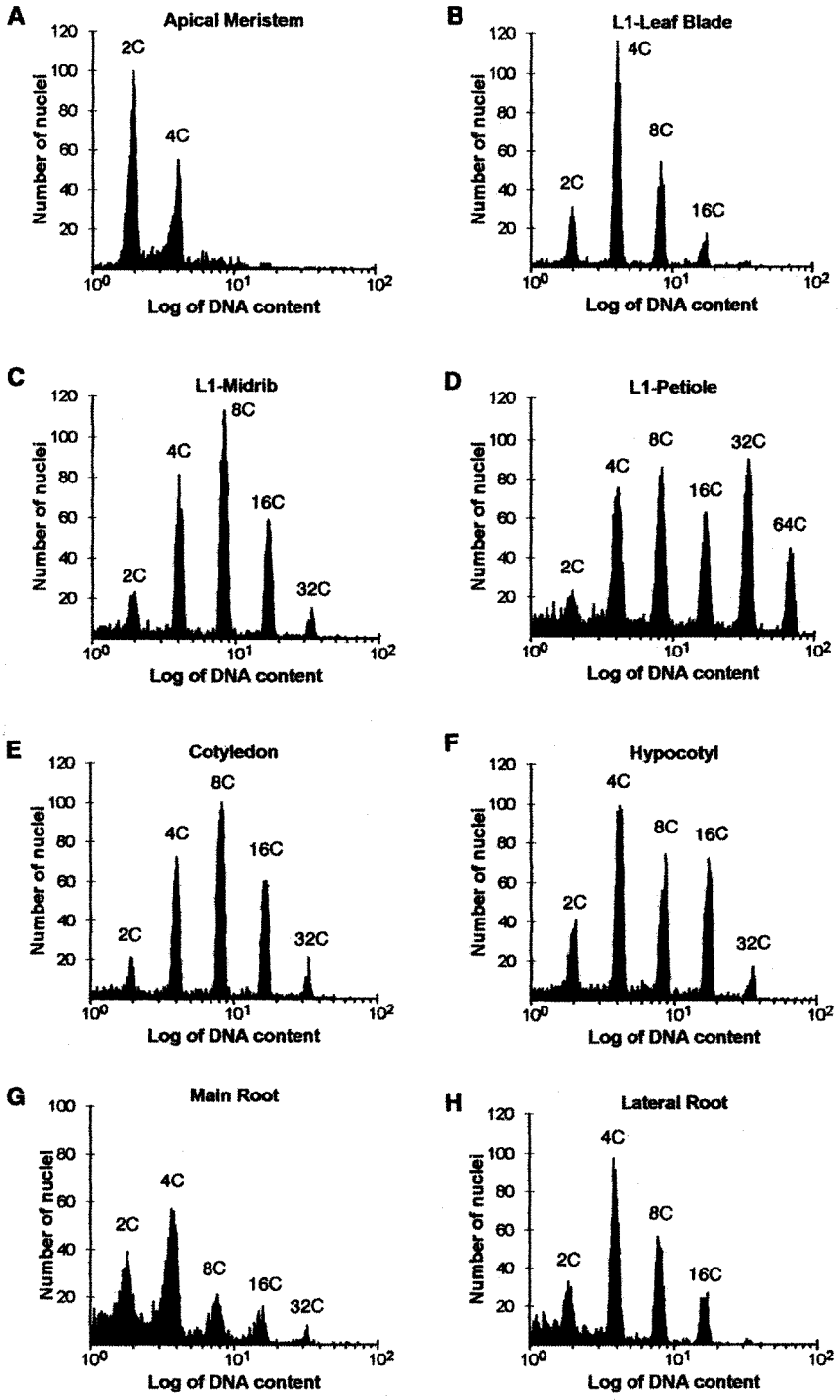

Fig. 3 Characteristic histograms of nuclei distribution from tissues at Stage 3.
(A) Apical meristem, (B) Leaf blade of leaf 1, (C) Midrib of leaf 1, (D) Petiole of leaf 1, (E) Cotyledon, (F) Hypocotyl, (G) Main root, (H) Lateral root.

organ size in many agronomically important crops.

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