Nuclear DNA endoreduplication during petal development in cabbage: relationship between ploidy levels and cell size

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Abstract
The development of cabbage petals comprises two distinct phases: a cell division phase and a consecutive phase of cell expansion until the onset of opening. In this study, cytological changes characterizing the two phases of petal development were analysed. First, the mitotic activity and the surface area of epidermal cells during petal development were investigated. The DNA content of isolated nuclei from the different stages of petal tissues was determined by flow cytometric analysis. The results show that cell differentiation, leading to expanded cells, is characterized by endoreduplication. In the proximal part of the petal, after cell division arrest, differentiation frequently involves endoreduplication and cell enlargement. By contrast, normal diploid nuclei remained in the distal part of the lamina in the mature petal. It is suggested that the developmental programmes of the cabbage petal may be a trigger for the initiation of endoreduplication. Correlation between ploidy levels and cell size is also discussed.

Key words: Brassica oleracea L., endopolyploidy, endoreduplication, flow cytometry, petal development.

Introduction
Endoreduplication is an alternative form of cell cycle that occurs in a wide variety of organisms (Nagl, 1976; Barlow, 1978). During endoreduplication, nuclear DNA is replicated without mitosis or cytokinesis, resulting in cells with DNA content much greater than 2C (C is the haploid DNA content). The occurrence of endoreduplication is very frequent in arthropods. Endoreduplicated chromosomes (polytene chromosomes) is a nuclear differentiation mechanism in larval and adult Diptera (Pearson, 1974). A variety of cell types in Drosophila larval tissues undergo endoreduplication events (Sauer et al., 1995). In animals, endoreduplication is only observed in specific cell types such as some molluscan neurons, mammalian megakaryocytes and placental trophoblast cells (Chase and Tolley, 1987; Zybina and Zybina, 1996). The function of endoreduplication is unknown, although some of the proposed roles include gene amplification, radiation resistance and cell differentiation (Nagl, 1976; Barlow, 1978; Galbraith et al., 1991).

The occurrence of endoreduplication is common in plants (Barlow, 1978). Ploidy level of plant cells has been determined by using conventional cytophotometric methods since the early 1950s (D’Amato, 1984). Many informative investigations have been made in specific cell types that are highly specialized, such as suspensor cells in Phaseolus (Nagl, 1978), basal cells of the hairs on Bryonia anthers (Barlow, 1975) and storage parenchyma cells in cotyledons of several legume species (Smith, 1971; Millerd and Whitfield, 1973; Davies, 1976). The former two types of cells represent examples of polytene chromosomes. However, the cytophotometric analyses have been limited to a relatively small number of nuclei and simply showed the presence or absence of one or two genomic copies of DNA (DeRocher et al., 1990). Flow cytometry has opened the way for rapid and accurate determination of C levels from a large number of nuclei (Galbraith, 1983). Recently, flow cytometric data for the systemic control of endoreduplication have been accumulated in economically important crops, such as cucumber (Gilissen et al., 1993), tomato (Smulders et al., 1994) and Brassica crops (Kudo and Kimura, 2001a). A more recent investigation reported detailed patterns of endopolyploidy in various vegetative tissues in cabbage plants (Kudo and Kimura, 2001b). In this case, the number of endoreduplication

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cycles (endocycles) is tissue-specific and is characteristic of the stage of development.

Numerous observations have been made of endoreduplication, but the function of this process has not been thoroughly investigated. Recently, there were studies directed at the molecular mechanisms responsible for the endoreduplication cycle in maize endosperm. Grafi and Larkins have shown that endoreduplication of maize endosperm involves the inhibition of the M-phase-promoting factor and the induction of S-phase-related protein kinase. This implies that in plants as well as animals, cell proliferation and also endoreduplication (Grafi et al., 1995).

In this study, the aim was to document endoreduplication events in the course of petal development of cabbage. The temporal and spatial regulation of the mitotic activity of epidermal tissues was investigated and nuclear DNA synthesis was measured as determined by flow cytometry during the different stages of petal development. It was shown that differentiating cells in the proximal part of the petal become more polyploid as the mitotic activity of the petal epidermis decreases, unlike in the distal part of the petal. The differentiated epidermal cells toward the base of the petal are large and elongated, having extremely large nuclei. A relationship was proposed between ploidy levels and cell size during petal development.

Materials and methods

Plant materials

Cabbage (Brassica oleracea L. var. capitata cv. Ri-Nen) was used. Plants were grown to maturity in a greenhouse under natural conditions. When the plants flowered, buds of different developmental stages were removed from the main inflorescence and the petals were dissected from the flower bud under a binocular microscope.

Cell size measurement

Petals were fixed in a solution of 3 parts 95% ethanol to 1 part acetic acid for 4 h at room temperature and stored in 70% ethanol at 4 °C. Fixed tissues were soaked in water and then incubated overnight in chloral hydrate solution (25 g in 25 ml). Adaxial epidermal cells were examined using a confocal laser-scanning microscope (model Fluoview-BX50, Olympus). Cell surface areas were determined using a Fluoview image analysis system (Olympus).

DNA staining and mitotic index determination

Mitotic indexes were determined on the adaxial epidermis of petals harvested at different stages of development. The epidermis was placed on a glass slide and immersed in a drop of 4’,6-diamidino-2-phenylindole (DAPI) at a concentration of 1 μg ml⁻¹ for 20 min. The epidermis was then washed in distilled water. Stained DNA was then visualized by epifluorescence microscopy (model BHS-RFC, Olympus) using the ×20 and ×40 objectives of a microscope. A total of 1000 nuclei were counted for each developmental stage.

Nuclei isolation and flow cytometric analysis

Nuclei were prepared from petals harvested at different developmental stages with a high resolution kit (Partec GmbH, Münster, Germany) according to the manufacturer’s instructions. Whole petals or dissected tissues were 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 (Partec), 0.5–2.0 ml of staining solution containing the dye DAPI (solution B of the kit) was added. The analyses were performed with a PAS flow cytometer (Partec) equipped with an HBO lamp for UV excitation. Fluorescence peaks were estimated with the WinMDI software (version 2.8, copyright © 93–99 Joseph Trotter). Cytometric analysis was performed on a minimum of 10⁴ nuclei (total count). Measurements of nuclear DNA content were carried out with at least six replications originating from different buds and mature flowers. To determine the standard peak position of 2C nuclei, the 2C peak from nuclei of in vitro young leaves was analysed at least twice on each measurement. The data were plotted on a semi-logarithmic scale, so that the histogram peaks from 2C to 32C were evenly distributed along the abscissa. The data were presented as a percentage of the total amount of nuclei in all peaks of the histogram.

Results

Development of cabbage petals

The developmental stages of cabbage petals are shown in Fig. 1. Five stages were morphologically distinguished from young petals (stage 1) to mature petals (stage 5). At stage 1, petals have formed small, flat laminae, which reached to about two-thirds of stamen height. At stage 5,
the structure of the fully expanded petals was relatively simple and a vascular array was apparent in the petals (Fig. 1). Analysis of epidermal cell area in relation to position in the mature petal (Fig. 2) showed that in the flattened distal part of the petal, the epidermal cells were small and homogeneous. By contrast, the epidermal cells toward the base of the petal were large and extremely elongated. These two types of epidermal cells each occupied about 50% of the petal length and the point of transition of epidermal cell shape is quite marked (Fig. 2). The elongate morphology of the proximal epidermal cells appear to relate to their rapid expansion which pushes the petal out of the bud as it opens. The distal epidermal cells did not expand significantly during the latter stages of petal development (stage 3 and stage 5), whereas the proximal epidermal cells increased, on average, approximately 6-fold in cell surface area (Fig. 2). The mitotic index in the petal epidermis sharply decreased during petal development (Fig. 3). The mitotic index in the distal part of the petal remained at stage 3. From these observations of mitotic figures between stage 3 and full expansion (stage 5), there was no evidence for cell division in the proximal part of the petal (data not shown), and the increase in petal size during this period appears to be mainly as a result of cell expansion in the proximal part of the petal.

Fig. 2. The relationship between the surface area of epidermal cells and their distance from the petal base at different developmental stages. (▲), stage 3 and (○), stage 5.

Fig. 3. The mitotic index in epidermal cells in cabbage petal development. (A) Whole petals between stage 1 and stage 2, and (B) the distal part of the petal between stage 3 and stage 5.

Fig. 4. Fluorescence of DAPI-stained nuclei in epidermal cells of mature cabbage petals. (A) Nuclei from the distal part of the petal. (B) Nuclei from the proximal part of the petal. Bars = 20 μm.
The observation of DAPI-stained petal tissues revealed apparent differences in nucleus size in relation to position in the mature petal (Fig. 4). Epidermal cells in the distal part of the petal contained small nuclei (Fig. 4A), whereas elongated epidermal cells in the proximal part of the petal had large nuclei (Fig. 4B).

Increase of ploidy level during petal development

Figure 5 shows the evolution of the ploidy level during petal development. For earlier stages (stage 1 and stage 2), whole petals were used for nuclear preparations, as the petals were too small to separate the different parts. From stage 3 to stage 5, the petals were dissected into two parts, the proximal part and the distal part. In very young developing stages (from stage 1 to stage 2), petals exclusively contained nuclei with the 2C and 4C DNA levels, accounting for 46% (2C) and 54% (4C) of total nuclei at stage 1, and 37% (2C) and 63% (4C) of total nuclei at stage 2. Because cabbage is a diploid species (2n = 18), the 2C DNA level corresponds to the diploid state of the genome found in the G1 phase, whereas the 4C DNA level results from the S-phase doubling of chromatids found in the G2 phase, and thus is an indicator of the capacity of cells to enter mitotic cycles. Therefore, the major 2C and 4C cells suggest that the tissue is in a dividing state. Later in development (from stage 3 to stage 5), cells in the proximal parts of the petal significantly increased their ploidy level, but cells in the distal part of the petal maintained diploid level. The first cycle of endoreduplication of nuclear DNA had taken place at stage 3 as indicated by a small proportion of polyploid 8C nuclei. Further endoreduplication had occurred at stage 4. At this stage, the proximal part of the petal tissue contained cells with 16C levels, but nuclei at the 2C DNA level were still detectable. An additional round of endoreduplication was detected at stage 5. About 5% of cells of the basal part of the petal had undergone four rounds of endoreduplication cycles, reaching the 32C ploidy level. The flow cytometric profiles displayed a small but reproducible 32C peak from nuclei in the proximal part of the petal at stage 5 (Fig. 6B).

Fig. 5. The evolution of the ploidy level during cabbage petal development. (A) Early stages (stage 1 and stage 2) of petal development, (B) late stages (stage 3, stage 4, and stage 5) of petal development, the distal part of petal, and (C) late stages of petal development, the proximal part of petal.
At this stage, in contrast, the number of nuclei at the 2C DNA level decreased dramatically and became undetectable (Fig. 6B). However, nuclei from the distal part of the petal gave the 2C and 4C peaks from stage 3 to stage 5 (Figs 5, 6A).

Discussion

The study of cabbage petals may help in providing information about the relationship between endoreplication and cell growth during plant organ development. In the present study, one of the most striking features of petal cells is the uneven increase of their DNA content during development. This cytometric data showed that, in the proximal part of the petal, cells become more endopolyploid with up to a 32C ploidy level, whereas cells in the distal part of the petal maintained the diploid level throughout petal differentiation. At stage 5, flow cytometric profiles for the distal part of the petal is characterized by a high proportion of nuclei at the 2C and 4C levels, indicating that the cells never endoreduplicate and their mitotic cell cycle is arrested either in the G1 phase or in the G2 phase of the diploid cell cycle, even at the mature stage of the flower. The results suggest that the occurrence of endoreduplication may be not only temporally, but also spatially regulated in a whole petal. At the mature stage, epidermal cells in the proximal part of the petal are large and extremely elongated, but epidermal cells in the distal part of the petal are small and highly homogeneous. These findings, together with microscopic observations of nuclei, indicate that the formation of large differentiated cells is accompanied by an increased ploidy level. The correlation between the cell size and the degree of endopolyploidy supports the idea that the nuclear DNA content might play a key role in controlling cell size. Endoreduplication in cabbage petals might be a major driving force for cell differentiation. By measuring the mitotic index of epidermal cells of petals, it was shown that the distribution of mitotic activity is determined not only temporally, but also spatially within a whole petal and the results suggested that DNA synthesis continues in the basal petal cells after cell division has ceased. The correlation between DNA contents and cell size is not known: however, on the basis of these results, a possible scenario explaining the correlation between ploidy levels and cell size during petal development is presented in Fig. 7. Petal tissues can be considered as being composed of cells that simultaneously react upon developmental cues. During petal development, at least two consecutive developmental signals provoke two simultaneous responses of the cells; the first one triggering the arrest of the cell cycle, and the second one triggering the onset of endoreduplication. These signals may be related to endogenous positional cues causing the distinct zonation. In the distal part of the petal, cells never endoreduplicate. Upon receiving the arrest signal of the cell cycle, the cells will increase in size until cell expansion levels.

Fig. 6. Characteristic histograms of nuclei distribution at stage 5. (A) Nuclei from the distal part of the petal, (B) nuclei from the proximal part of the petal.

Fig. 7. Scenario explaining the correlation between ploidy levels and cell size during development of cabbage petal. (A) In the distal part of the petal, cells never endoreduplicate, and (B) in the proximal part of the petal, cells can switch to endoreduplication.
is arrested simultaneously. This results in a homogeneous final cell size distribution, as observed in the distal part of the petal (Fig. 7A). In the proximal part of the petal, cells can switch to endoreduplication. The switch to endoreduplication cycles is not necessarily synchronous for neighbouring cells. As a result, size differences will have built up by the time the cells receive the arrest signal of the cell cycle. These differences will be amplified during the petal expansion phase, and as a result, the final cell size will be correlated with ploidy levels (Fig. 7B).

Extensive endoreduplication has recently been detected in cabbage flowers (Kudo and Kimura, 2001c). The investigators showed the presence of endopolyploidy in different organs of cabbage flowers. For instance, filament tissues contain cells with six ploidy levels (2C–64C) and carpel tissues have cells with three ploidy levels (2C–8C). The 2C–32C range of nuclear ploidy in cells of cabbage petal has been also described. These findings, together with the data presented in this study, indicate that endoreduplication is a common feature in the development of cabbage flowers. However, endoreduplication is not obvious in Arabidopsis thaliana, a related crucifer to Brassica species. A. thaliana lacks somatic polyploidy in floral organs in which all cells are at the 2C level (Galbraith et al., 1991). A coupling between cell size and nuclear DNA content has been documented in many eukaryotic organisms. Cells that have undergone endoreduplication are larger than comparable cells that have not (Larkins et al., 2001). In budding yeast, Saccharomyces cerevisiae, diploid cells are larger than haploid cells (Sherman, 1991). In fission yeast, Schizosaccharomyces pombe, mutant cells that have undergone successive endoreduplication rounds become gigantic (Moreno and Nurse, 1994). In plants, there is a body of evidence that the size of cells is linked to their DNA content (Kondorosi et al., 2000). This is illustrated by the correlation between cell surface and nuclear DNA levels in neighbouring leaf epidermal cells of Arabidopsis (Melaragno et al., 1993) and maize (Cavallini et al., 1997). Arabidopsis mutants that increase or reduce the ploidy levels in trichomes invariably show an increase or a reduction of the final cell size (Folkers et al., 1997). These results also support the idea that endoreduplication is coupled with cell size. Endoreduplication levels could determine cell size in that endoreduplication occurs before the onset of the massive expansion of petals in flower opening. However, endoreduplication is not always coupled with an increase in cell size and can be, at least partially, uncoupled from cell elongation: when Arabidopsis seedlings are allowed to germinate, endoreduplication takes place before the elongation of hypocotyl cells (Gendreau et al., 1997).

The correlation between ploidy levels and cell size indicates that endopolyploid nuclei might be required for the formation of large cells. The physiological role of developmentally programmed polyploidy is, however, elusive. Multiplication of the genome has been proposed to increase metabolic activity, rRNA synthesis and transcriptional activity (Baluska and Kubica, 1992). Endopolyploid nuclei can be advantageous for specialized functions. During maize endosperm development, cells shift from mitotic cycles to endoreduplication cycles, driving the massive synthesis of storage proteins (zein) and starch (Lur and Setter, 1993). The extent of endopolyploidy correlates with the yield of maize grain (Kowles et al., 1992; Cavallini et al., 1995). Endoreduplication in the pea cotyledons is related to the accumulation of storage products (Liu et al., 1996).

Endopolyploidy may be important for understanding the regulation of gene expression in differentiated tissues. Galitski et al. described the impact for polyploidy on gene expression in isogenic budding yeast S. cerevisiae strains that varied in ploidy from haploid to tetraploid (Galitski et al., 1999). The authors showed that a subset of genes was induced or repressed in a ploidy-dependent manner. Recent work on Medicago root nodules provided molecular evidence for the regulation of cell size by endopolyploidy (Cebolla et al., 1999). The work demonstrated a gene ccs52, a plant homologue of the anaphase-promoting complex activators that are involved in mitotic-cyclin degradation. Overexpression of this gene in fission yeast resulted in the arrest of mitosis, and induced endoreduplication and cell enlargement.

The developmental signals that control the exit from the cell cycle or transform the mitotic cycle to endocycle during cell differentiation have not yet been identified (Kondorosi et al., 2000). However, plant cells can elect different options for cell growth during differentiation (Melaragno et al., 1993; Valente et al., 1998). Upon differentiation signals, plant cells can exit from the mitotic cycle both in the G1 and G2 phases with 2C and 4C DNA content, respectively. One option is to differentiate with original 2C and 4C ploidy level (G1 and G2 differentiation). The growth potential and metabolic activity of 4C cells during G2 differentiation is greater than those of 2C cells during G1 differentiation. The other option is to enter the endocycle, resulting in the same nucleus with increased ploidy levels. Duplication of the genome can augment further the growth capacity and the extent of cell enlargement. The control of endoreduplication may allow cells to reach extraordinary sizes (Cebolla et al., 1999). This process probably provides a means to manipulate the cell size in agronomically important crops.

References


