Role of DNA endoreduplication, lipotubuloids, and gibberellic acid in epidermal cell growth during fruit development of *Ornithogalum umbellatum*

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Abstract

Cytophotometry of individual nuclei was used to examine the level of endoreduplication in epidermal cells from the upper and lower parts of the ovary during *Ornithogalum umbellatum* flower and fruit development. An increase in DNA content from 2–4C to 2–8C in both parts of the ovary was observed, while the epidermal cell surface area grew about 6-fold and 15-fold in the lower and upper parts of the ovary, respectively. However, the correlation between mean epidermal cell size and ploidy was distinct during epidermis growth. Lipotubuloids became bigger in the upper than in the lower part during ovary and fruit development. In addition, more dynamic growth of the epidermal cells of the upper than of the lower part of the ovary was connected to the higher content of gibberellic acid.

A hypothesis has been put forward that the role of DNA endoreduplication in epidermal cell growth was modulated by the function of lipotubuloids and the gradient of gibberelin.

Key words: Endoreduplication, gradient of gibberellic acid, lipid bodies, lipotubuloids, ovary, regulation of cell growth.

Introduction

Endoreduplication, a modified cell cycle in which chromosomal DNA is successively duplicated in the absence of mitosis, is widespread among Eukaryota, but prevails most in plants (Nagl, 1976; Joube`s and Chevalier, 2000; Larkins et al., 2001; Sugimoto-Shirasu and Roberts, 2003).

Endoreduplication often occurs during the differentiation of cells that are highly specialized in their morphology or metabolism. *Arabidopsis thaliana* trichomes, large branched cells, generally have DNA contents of 32C (Hülskamp, 2004). *Zea mays* endosperm cells, which accumulate starch and storage proteins, usually undergo multiple successive endocycles during seed development, with some attaining DNA contents as high as 96C and 192C (Leiva-Neto et al., 2004; Bauer and Birchler, 2006). *Arabidopsis* cotyledons and leaf epidermis pavement cells have ploidy levels from 2C to 32C and from 2C to 16C, respectively (Galbraith et al., 1991; Melaragno et al., 1993). Moreover, the ploidy levels of hypocotyls vary depending on growth conditions, with levels of 2–8C under normal light conditions and 2–16C in darkness (Gendreau et al., 1998). In nearly 100 mono- and dicotyledonous plant species, differentiation of root parenchyma cells is preceded by DNA endoreplication. However, there are exceptions concerning species with high contents of 2C DNA (Olszewska and Osiecka, 1982, 1983, 1984).

Genetic studies suggest that the succession of endocycles is genetically regulated either positively or negatively. Trichomes of one class of *Arabidopsis* mutants, including *triptychon* and *kaktus*, have DNA contents lower and higher than the wild type, respectively (Esch et al., 2003; Hülskamp, 2004).

Recently the role of various proteins in regulation of endoreduplication has been investigated. Weinl et al. (2005) suggest that plant cyclin-dependent kinase inhibitors (ICK1/KRP1) can block entry into mitosis but allow S-phase progression, which makes endoreduplication of *Arabidopsis* trichomes concentration dependent. On the other hand, during *Arabidopsis* leaf development, the cyclin-dependent kinase inhibitor KRP2 controls the onset of the endoreduplication cycle through inhibition of mitotic CDKA;1 kinase complexes (Verkest et al., 2005). A novel class of...
atypical E2F-like proteins has been identified in *Arabidopsis* as DP-E2F-like (DEL) (Vlieghe et al., 2005; Beemster et al., 2006). It was demonstrated that loss of DEL1 function resulted in increased ploidy levels, whereas ectopic expression of DEL1 reduced endoreduplication. On the other hand, Bisbis et al. (2006) isolated CDK inhibitors LeKRP1 and LeKRP2 from developing tomato fruit and showed their transcript expression to be enhanced in the differentiating cells of the gel tissue undergoing endoreduplication.

Although the involvement of various cell cycle-related proteins has been revealed, it is still unclear which proteins play key regulatory roles in endoreduplication which is determined by the genetic programme and growth conditions. Moreover, cell cycle control in single cells depends on the supracellular organization of a tissue or an organ (Weinl et al., 2005).

The functional role of the endoreduplication process also remains elusive. The endoreduplication level of a cell is often inversely correlated with the genome size, which has led to the hypothesis that somatic polyploidy represents an evolutionary strategy to compensate for the lack of phylogenetic increase in nuclear DNA (Nagl, 1976; Sugimoto-Shirasu and Roberts, 2003). Other hypotheses link endoreduplication with metabolic activity, maintenance of the optimal ratio between nuclear and organellar DNA contents, or protection against irradiation (Joubès and Chevalier, 2000; Kondorosi et al., 2000; Larkins et al., 2001). Nevertheless, the endoreduplication level of a cell is most often correlated with the cell size—usually the higher the level of endoreduplication the bigger the cell size (Melaragno et al., 1993; Sugimoto-Shirasu and Roberts, 2003; Ramirez-Parra et al., 2004; Bertin, 2005; Cheniclet et al., 2005; Bisbis et al., 2006; Kladnik et al., 2006). However, the correlation between ploidy level and size is not always tight (see Discussion).

The subject of the present study is determination of the correlation between ploidy level and epidermal cell size during ovary and fruit growth of *O. umbellatum* at the upper and lower parts of these organs, as preliminary observations proved that cell size is different at different sites.

Moreover, it is characteristic of *O. umbellatum* ovary epidermal cells that they contain lipotubuloids, i.e. structures up to now identified only in *O. umbellatum* (Kwiatkowska, 2004). Lipotubuloids are cytoplasmic domains rich in lipid bodies, microtubules (Fig. 3I), and actin filaments, as well as numerous free and endoplasmic reticulum-bound ribosomes. Among these organelles there are a few mitochondria, microbodies, and Golgi structures and—at later developmental stages—autolytic vacuoles (Kwiatkowska, 1971, 1972a, b, 1973, 2004; Kwiatkowska et al., 2005, 2006). Cytocchemical analysis of lipotubuloids revealed unsaturated lipids—as proved by a positive PFAS test and darkening caused by osmium tetroxide—phospholipids identified by Baker’s acid haematein test, free fatty acids detected by Fischler’s reaction, and neutral triacylglycerides detected by means of Nile blue. Positive reactions to acid and alkaline phosphatases, non-specific esterases, and lipase were shown in lipotubuloids by cytoenzymatic investigation (Kwiatkowska, 1966). Periodic acid–Schiff (PAS)-positive polysaccharides which covered lipotubuloid microtubules with a thin layer were also observed (Kwiatkowska, 1973).

Using light and EM-autoradiography with [3H]palmitic acid, it was found that lipotubuloids were the site of intense incorporation of this isotope into the lipids extractable with a lipid solvent. Localization of autoradiographic grains 15 h post-incubation with the isotope-free medium indicated mobilization of lipids and migration of the labelled substances (not extractable with the lipid solvent) from the lipotubuloids to the whole cells (Kwiatkowska, 1972b, 2004). These radioactive substances were localized mainly in the outer layer of the cell wall just beneath the cuticle (Fig. 6 in Kwiatkowska, 1972b). The rotary-progressive motion of lipotubuloids inside the cell probably greatly facilitates both infiltration of the components necessary for lipid synthesis into the cells and exit of the substances distributed inside cells out of them (Kwiatkowska, 1972b; Kwiatkowska et al., 2005, 2006).

On the basis of the above facts, it seems that lipotubuloids are important elements of epidermal cell growth in the *O. umbellatum* ovary. Thus growth rates of lipotubuloids and cells were compared during the development of the upper and lower parts of the ovary and fruit.

It should also be taken into consideration that regulation of the endoreduplication process and growth of a single cell are correlated with supracellular organization of a tissue or an organ and may take place in a non-cell-autonomous pathway (Verkest et al., 2005; Weinl et al., 2005). Growth of epidermal cells, which is well coordinated temporally and spatially, must be in harmony with ovary, seed, and fruit growth (Srivastava and Handa, 2005). In particular, five hormones, namely gibberellins (GAs), auxins, cytokinins, abscisic acid (ABA), and ethylene, are all known to modulate growth and development at various stages of the developing fruit (Ozga and Rienecke, 2003). GAs, auxin, and cytokinins were found at high concentrations during pollination/fertilization and early fruit development. (Srivastava and Handa, 2005). In addition, it was shown that in tobacco, appreciable quantities of hormones start to diffuse from the style to the base of the ovary after pollination (Ozga and Rienecke, 2003).

This being so, in the present studies, the quantities of GA_{3} in the upper and lower parts of the ovary were measured since a gradient of this hormone may play a role in the differential growth of epidermal cells.

The obtained results prove that the sizes of the epidermal cells of *O. umbellatum* ovaries and fruit show a slight correlation with the DNA level. More dynamic growth of the cells in the upper than in the lower part
seems to be provoked by greater participation of lipotubuloids as well as higher levels of GA$_3$ in cell function.

**Materials and methods**

The material for the studies were the ovaries of *O. umbellatum* L (Liliaceae). The plants were growing in the garden of the University of Łódź in natural environmental conditions. In order to assess 2C (telophase) and 4C (prophase) DNA levels, meristematic cells of the 4-d-old root tips of *O. umbellatum* from hydroponic culture were used.

As preliminary observations showed that epidermal cell sizes depended on the localization on the ovary, fragments of epidermis were collected separately from the upper and lower parts of the ovary (shown in Fig. 1 ovary no. 9). Nine stages, determined according to their size and development of the flower, were examined (Fig. 1). Ten ovaries comparable in size were used in each experiment.

**Morphometric analyses**

Fragments of ovary epidermis in a drop of water were analysed morphometrically under a light microscope at a magnification of 400× for cell size and 1000× for lipotubuloids (Jenamed-2 Carl Zeiss, Jena, Germany) at brightfield. A CCD MTV-1801 CB Camera connected to a microscope and computer-aided IMAL-512 system was used for estimation of their surface areas. A hundred randomly chosen epidermal cells and lipotubuloids were measured.

**Cytophotometric analyses**

The material was fixed in an absolute ethanol and glacial acetic acid mixture (3:1, v/v) for 1 h at room temperature, then washed with 96% ethanol and kept in 70% ethanol until further procedures were carried out. The material was hydrolysed for the experimentally established optimal time, i.e. 1 h, in 4 M HCl and next stained in Schiff’s reagent according to the standard method (pararosaniline, Sigma) for 1 h at room temperature. After the Feulgen reaction, the fragments of epidermis were pasted on the object glass while squashed preparations of roots were frozen on dry ice then dried in air at room temperature; subsequently both were embedded in Canada balsam. Three hundred nuclei of the epidermal cells from the upper and lower parts of the ovary as well as the nuclei of meristematic cells from root tips were analysed. The absorbance of Feulgen-stained nuclei was measured at 565 nm using a Jenamed-2 microscope (Carl Zeiss, Jena, Germany) with the computer-aided IMAL-512 system for image analyses, and calibrated in arbitrary units (au). To evaluate the frequency distribution pattern for DNA contents, >300 readings of individual nuclei were taken from the analysed samples.

**Electrophoretical analyses of GA$_3$ content**

Upper and lower parts of *O. umbellatum* ovary walls (see Fig. 1 ovary no. 8) were collected and homogenized (4°C) with 80% methanol to which was added 0.02% BHT (butylated hydroxytoluene). Isolation, purification, and content determination of GA$_3$ were carried out according to Kazmierczak (1999) with modifications. The samples were dissolved in 3 mM sodium borate buffer (pH 8.5). Identification of GA$_3$ and measurement of its content were carried out with a BioFocus 3000 UV scanning detector of the BioFocus 3000 (Bio-Rad) capillary electrophoresis system, using the CZE (capillary zone electrophoresis) method. Separation and detection were carried out in 30 mM sodium borate buffer (pH 8.5) with a silica-coated capillary (24×25 mm) at 260 nm and 5 kV for 20 min. The samples were injected at high pressure (20 psi) and ambient temperature.

Electropherograms were recorded and analysed using BioFocus Integration software 5.0, based on Microsoft Windows, at a detector rise time setting of 1 s and data sampling rate of 5 Hz. The electropherograms with retention times of standard and measured GA$_3$ levels are shown in Fig. 7.

**Results**

Nine stages of ovary development were analysed: the smallest ovaries which were 2.7–3.8 mm long indicated as stages 1, 2, and 3 come from buds; medium ones at stages 4, 5, and 6 come from flowers after anthesis; and the largest ones at stages 7, 8, and 9 are from withered flowers in which ovaries had turned into fruit (Fig. 1).

**Epidermal cell development**

Mitotic figures were not observed in epidermal cells from the stage of cream-coloured 1 mm long ovaries until maturity of fruit. Also the arrangement of epidermal cells and their shape indicated that they did not divide but that
a very intensive elongation of cells takes place. During the present studies, permanent observations of 1 mm long ovaries were not carried out because of difficulties in handling.

The surface areas of the cells from stages 1–9 grew 6-fold and 15-fold in the lower and upper ovary parts, respectively. Figure 2 illustrates the dynamics of growth of the epidermis cells from the upper and lower parts of the ovary. At the beginning, their growth was identical; however, after pistil pollination and during fruit development (stages 7, 8, and 9), the epidermal cells from the upper part of the ovary grew much more quickly and became ~2-fold larger. A significant difference in size could already be observed at stage 7, i.e. at the beginning of fruit development when the cells in the upper part started to grow more quickly while those in the lower part did not enlarge markedly before stages 8–9 (Fig. 2). However, it should be noted that the dynamics of particular epidermis cell growth varied; thus the standard deviation for the last three stages was significant.

**Lipotubuloid development**

Lipotubuloids were in statu nascendi (Fig. 3A) in ovaries ~1 mm long, with lipid bodies forming small groups. Figure 3B–E demonstrates the lipotubuloids which successively increased in their dimensions; as a consequence, the lipid bodies in lipotubuloids grew in number. Spherical lipotubuloids (Fig. 3E, H) might be found in fully developed and pollinated flowers with withering perianths. At that time, the ovary was intensively green. The cells in Fig. 3F came from an ~12 mm fruit which ceased growing and was losing its green colour. The lipotubuloids at this stage were decomposed to single lipid bodies.

During growth of the ovaries from stages 1 to 9 of development and during their turning into fruit, lipotubuloids became ~4-fold larger in the epidermis of both the upper and lower parts of ovaries (Fig. 4). The difference between these two parts appeared at stage 6 in the largest ovaries of full-blown flowers after pistil pollination, which in the opinion of Ozga and Reinecke (2003) was reflected by a significant thickening of the flower peduncle (Fig. 1, stage 6, star). At that stage, lipotubuloids from the upper parts of the ovaries were 2-fold bigger than those from the lower parts (Fig. 4). At the next stage, the lipotubuloids in the cells from the upper parts of fruit diminished and, at the oldest stages (7–9), lipotubuloids with a central vacuole (an area without lipid bodies) appeared, as well as lipotubuloids which were dispersed into single lipid bodies (Fig. 3F).

**Endoreduplication**

At the first three examined stages of development of the ovaries from flower buds, no nuclei with 2C DNA were found. The DNA content was between 2C and 4C in the epidermal cells from both the lower and upper parts of ovaries in comparison with the populations of meristic-matic cells of roots in prophase and telophase. In flowers after anthesis (Fig. 5 stages 4, 5, and 6), the highest DNA levels shifted slightly towards 8C while after perianth withering at stage 8, a clear population of 8C and above appeared; however, at the oldest stage examined (stage 9), this population diminished. It seems that the C DNA value persisting at the middle level at subsequent developmental stages may indicate differential replication of DNA fractions or of single genes (Nagl, 1979). Elucidation of this issue requires further research.

The mean DNA contents and mean epidermal cell areas for each stage of ovary and fruit development (Fig. 6) were estimated; together with the increase in DNA content there was an increase in the rate of cell surface area growth.

**GA₃ content**

Electrophoretical measurements showed that 1 g fresh weight (FW) of the upper part of *O. umbellatum* ovary contained about 62.1 µg of GA₃ while the same amount of the lower part contained about 37.5 µg, which gives a GA₃ concentration of 17.9 nM and 10.4 nM, respectively (Table 1).

**Discussion**

The control of cell size is precisely regulated by genetic, hormonal, and environmental signals. Genetic evidence strongly supports the classical ‘karyoplasmic ratio’ theory that one mechanism to increase the cell size is by increasing the ploidy level within a cell through endoreplication (Nagl, 1976; Sugimoto-Shirasu et al., 2005). A similar type of correlation was observed in *Solanum lycopersicum* (Cheniclet et al., 2005), *Sorgum bicolor* (Kladnik et al., 2006), cabbage (Kudo and Kimura, 2002), mutant *Arabidopsis hyp7* (Sugimoto-Shirasu et al., 2005), during the development of orchid flowers (Lee et al., 2004), and between the volume of shield cells and DNA.
content in antheridia of Chara algae (Kwiatkowska et al., 1990; Maszewski, 1991).

Radically different results were obtained by Leiva-Neto et al. (2004) in experiments with a dominant negative mutant of cyclin-dependent kinase A of Z. mays. It turned out that the endoreduplication level reduced by half did not influence endosperm cell size or gene expression, and only slightly reduced starch and protein synthesis.

Bertin (2005), on the other hand, examining tomatoes developing at different temperatures, concluded that endoreduplication seemed to be more or less loosely correlated with the range of cell size. Thus, rather than being involved in the control of cell growth, endoreduplication may initially determine the potential size of the cell, whereas

Fig. 3. Epidermal cells with lipotubuloids from O. umbellatum ovaries. (A–F) Schematic illustrations: (A) epidermis from 1 mm long ovary with lipid bodies forming small groups; (B, C, D) lipotubuloids increasing in size; (E) cells with spherical lipotubuloids; (F) cells which ceased growing with lipotubuloids at the stage of disintegration; (G, H) microphotographs of small-sized spherical lipotubuloids. Bar=10 μm. n, nucleus; l, lipotubuloid. (I) Fragment of lipotubuloid in EM (for method of fixation, see Kwiatkowska et al., 2005); lb, lipid bodies; mt, microtubules; ER, endoplasmic reticulum. Bar=0.2 μm.

Fig. 4. Increase in the lipotubuloid size at the nine developmental stages of the lower and upper parts of O. umbellatum ovary epidermis. Statistically significant difference between the upper and lower parts of lipotubuloids at \( t_a = 1.98; t_b = 5.87, 1.88, 3.78, 13.14, 9.89, 23.31, 6.99, 2.88, \) and 0.48 at stages 1–9, respectively.
actual cell size would mainly depend on carbon supply to individual cells.

The present results seem to point to a similar conclusion. It is believed that the level of DNA in a nucleus only determines the potential of cell dimensions while their real sizes also seem to depend on other factors. Comparison of the endoreduplication rate and the sizes of *O. umbellatum* ovary epidermis cells leads to the conclusion that a ∼2-fold increase in DNA level in a small population of individual cells during the examined flower and fruit
development period (Fig. 5) corresponded to 6-fold or 15-fold enhancement of average epidermal cell sizes from the lower or upper part of the ovary, respectively. However, the most dynamic cell growth, regardless of their location, was correlated with the period of the highest nuclear DNA content (i.e., stages 7–9) which probably determined this higher potential for cell growth. This potential was manifested over twice as intensively in the epidermis from the upper part of the ovary.

It is of interest to determine what the cause is of such a difference in the intensity of the upper and lower parts of epidermis growth. It can be supposed that there are two main causes: differences in sizes of lipotubuloids and differences in GA3 concentration.

In both the lower and upper parts of the ovary there were characteristic lipotubuloids. The data presented in the Introduction suggest that lipotubuloids are an important element of epidermal cell growth in *O. umbellatum*. Comparison of the growth rate of lipotubuloids from different stages of epidermal development from both the upper and lower parts of the ovary revealed their very similar and limited increase at stages 1–5 and the most intensive increase at stage 9. Moreover, in the epidermis after fruit set (stage 6), a dramatic increase (by 100%) in the size of lipotubuloids appeared which preceded very dynamic enlargement of the epidermal cells in the upper part of the ovary. At the same time, in the lower part of the ovary, the cell and lipotubuloid augmentation was much slower. Thus it seems that lipotubuloids may increase the chance of cell growth during the transformation of an ovary into a fruit, especially in the upper part of the ovary. It should be added that autoradiographic analyses of [3H]palmitic acid incorporation indicated that the fats synthesized in lipotubuloids were metabolized by a cell. That is why the increase in lipotubuloid size did not fully reflect their activity. However, it may be suggested that the larger the lipotubuloids the greater their functional activity. In the upper epidermal cells which stopped growing, lipid bodies of the lipotubuloid became dispersed into single lipid bodies which finally disappeared. This process preceded the appearance of autophagic vacuoles containing lipase and acid phosphatase in the lipotubuloids (Kwiatkowska, 1971). It may be hypothesized that the substances coming from degradation of lipid bodies are used by intensively growing cells for the synthesis of cell components.

Moreover, since there are phospholipids in lipotubuloids (Kwiatkowska, 1966) they may directly participate in the regulation of the processes within a cell. In recent years, the role of phospholipids in plant growth and development and in the response of plants to biotic and abiotic stress has been postulated (Cowan, 2006).

The results obtained with the capillary electrophoresis system showed that the upper part of the ovary wall contained 65% more GA3 than the lower part. This fact suggests that a higher concentration of GA3 intensified epidermal cell growth of the upper part of the ovary. Similarly, a higher concentration of GA3 accompanied the period of the most intensive growth during tomato fruit development (Srivastava and Handa, 2005). Therefore, it seems that the role of DNA endoreduplication in *O. umbellatum* epidermal cell growth was modified by the function of lipotubuloids in lipid synthesis and the different content of gibberellin.
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