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Relationship between Endopolyploidy and Cell Size in Epidermal Tissue of Arabidopsis

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Relative quantities of DNA in individual nuclei of stem and leaf epidermal cells of Arabidopsis were measured microspectrofluorometrically using epidermal peels. The relative ploidy level in each nucleus was assessed by comparison to root tip mitotic nuclei. A clear pattern of regular endopolyploidy is evident in epidermal cells. Guard cell nuclei contain levels of DNA comparable to dividing root cells, the 2C level (i.e., one unreplicated copy of the nuclear DNA). Leaf trichome nuclei had elevated ploidy levels of 4C, 8C, 16C, 32C, and 64C, and their cytology suggested that the polyploidy represents a form of polyteny. The nuclei of epidermal pavement cells were 2C, 4C, and 8C in stem epidermis, and 2C, 4C, 8C, and 16C in leaf epidermis. Morphometry of epidermal pavement cells revealed a direct proportionality between nuclear DNA level and cell size. A consideration of the development process suggests that the cells of highest ploidy level are developmentally oldest; consequently, the developmental pattern of epidermal tissues can be read from the ploidy pattern of the cells. This observation is relevant to theories of stomate spacing and offers opportunities for genetic analysis of the endopolyploidy/polyteny phenomenon.

INTRODUCTION

Endopolyploidy produces elevated nuclear DNA quantities resulting from consecutive doublings of the original unreplicated, or 2C, ploidy level. This phenomenon is known to be widespread in plants and particularly common among angiosperms (D'Amato, 1977; Nagl, 1978). For example, among the nuclear DNA of curcurbit seedling cells, high percentages have 16C, 32C, and even 64C amounts as determined by flow cytometry (Coleman et al., 1985). Endopolyploidy has been described in specific cell types that are highly specialized and unusually large, such as raphide crystal idioblasts in vanilla (Kausch and Horner, 1984), root hairs in Elodea (Dosier and Riopel, 1978), suspensor cells in Phaseolus (Nagl, 1974), and anther trichomes in Bryonia (Barlow, 1975). Other studies indicate that endopolyploidy may be common in more generalized types of tissues, such as endosperm in maize (Phillips et al., 1983), parenchyma tissue in Vanda seedlings (Alvarez, 1968), cotyledon tissue in peanut (Dhillon and Miksche, 1982), and leaf epidermal tissue in Phaseolus (Kinoshita et al., 1991). A number of these investigators suggest that increases in ploidy level are related to increases in nuclear volume and/or cell size.

Direct measurements of nuclear DNA quantity involve one of two approaches: microspectrophotometric measurement of the quantity of nuclear DNA in selected individual cells of intact tissues, or flow cytometry of nuclei isolated from organs or whole plants. The first approach allows for the maintenance of tissue integrity and identification of specific cell types, but is impractical for large numbers of cells. The second approach can be used to measure large numbers of cell nuclei, but sacrifices the anatomical component and consequently does not isolate specific cell types. To initiate a systematic investigation of the relationship between nuclear ploidy level and cell size in tissues exhibiting a range of cell sizes, the relative quantity of DNA in individual nuclei of epidermal tissue in Arabidopsis was measured microfluorometrically using the DNA-specific fluorochrome 4′, 6-diamidino-2-phenylindole (DAPI).

A recent investigation reported that endopolyploidy up to the 16C level occurs in various vegetative tissues, but not in floral structures of Arabidopsis, as measured by flow cytometry (Galbraith et al., 1991). Dickson et al. (1992), also using flow cytometry, present results from Arabidopsis leaf tissue that show the same range of ploidy levels. We have measured the relative nuclear DNA quantity in three distinct cell types of Arabidopsis leaf and stem epidermal tissue. We report a clear pattern of endopolyploidy and a definite relationship between cell size and ploidy level in this model plant.

RESULTS

Description of Cell Types

Both leaf and stem epidermal tissue of Arabidopsis contain three distinct and easily recognizable cell types examined in

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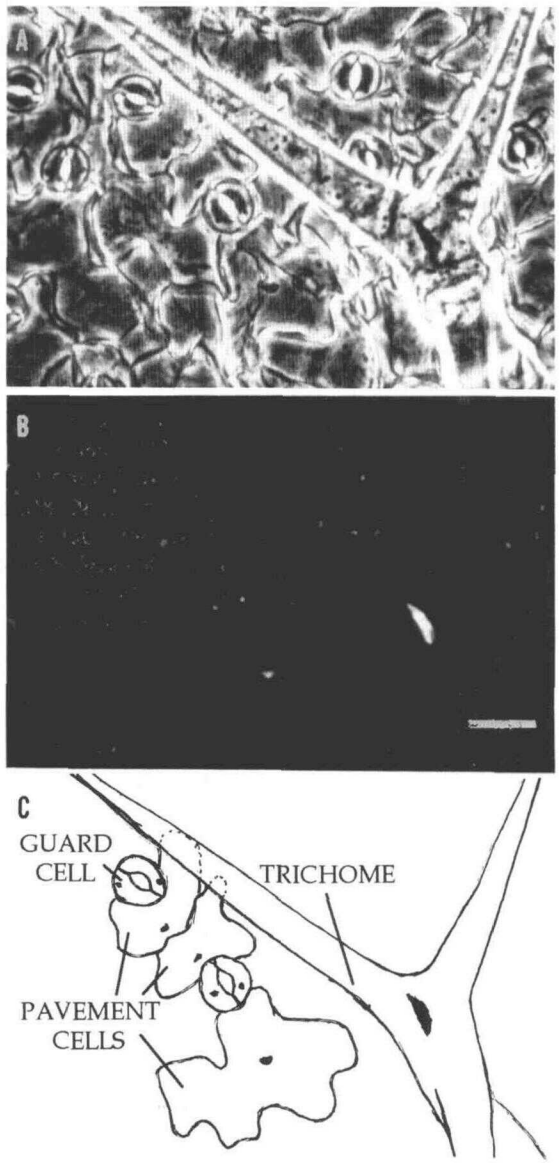

Figure 1. Leaf Epidermal Cell Types of Arabidopsis Illustrated in Three Views of the Same Cells.

(A) Phase micrograph of a portion of an upper leaf epidermal peel.
(B) Fluorescence of DAPI-stained nuclei. Bar = 25 μm.
(C) Sketch identifying cell types and location of nuclei.

this investigation, as shown in Figure 1: guard cells, trichomes, and epidermal pavement cells. Guard cells are highly consistent in size and shape, averaging 20 μm in length and 9 μm in width. Trichomes are unicellular. Their size and shape are somewhat variable, but they are all unusually large cells often exceeding 1 mm in length. Trichome morphology differs with respect to location: stem trichomes are unbranched elongated spike-shaped cells, whereas leaf trichomes generally have three branches (Marks et al., 1991). Epidermal pavement cells exhibit a wide range of sizes and also differ in shape, those of the stem being highly elongated and those of the leaf being generally isodiametric with sinuous outlines. The epidermal pavement cells were selected as the focus of this investigation because they exhibit a wide range of sizes within a small area of the leaf surface.

Nuclear DNA Quantity in Pavement Epidermal Cells

Measurements of the nuclear DNA quantity from a sample of 54 leaf pavement epidermal cells are presented in Figure 2A. The relative fluorescence values, directly proportional to DNA content (Coleman et al., 1981), range from 5.8 to 47.5 and sort out into four distinct subpopulations. The average values for the four subpopulations are given on the graph and indicate successive doubling of the nuclear DNA content. Assignment of specific ploidy levels is based on a comparison of the relative fluorescence measurements of nuclei in leaf epidermal cells with those of mitotic figures of the root tip from the same plant prepared on the same slide. The relative size and shape of each pavement epidermal cell included in this sample can be seen in Figure 2B. Cell dimensions range from 10 to 200 μm.

Figure 3 shows the results obtained for a sample of 92 stem pavement epidermal cells. Although none of the cells observed had a nucleus with the 16C amount of DNA, the stem epidermal sample had a larger overall population of endopolyploid cells. This is especially apparent at the 8C level, with the proportion of 8C cells in the stem more than twice that found in the leaf sample. Table 1 summarizes the results for both leaf and stem pavement epidermal cells. Data from a second leaf epidermal peel in which 56 pavement epidermal cells were measured have been combined with the sample shown in Figure 2 to give a total of 110 leaf cells in the table. Among all pavement epidermal cells sampled, 71% (144 of 202) had undergone endopolyploidy. Several indications of cell size are presented in the table, all of which increase with nuclear ploidy level. Total cell surface area and cell volume calculations utilized an average cell depth value determined by observing representative epidermal cells in cross-section (perpendicular to the plane of the epidermal surface) measured with a calibrated ocular grid.

Nuclear DNA Quantity in Guard Cells

Measurements were also taken of guard cell nuclei in both stem and leaf epidermal peels. In all cases, the range of fluorescence values for guard cell nuclei was small, indicating minimal variation in nuclear DNA quantity. The average values were consistently similar to the average values for the smallest subpopulation of pavement epidermal cells measured in the same sample. Based on this observation and the comparison to mitotic figures in the root tip, the fluorescence readings for the smallest pavement epidermal cells and for the guard cells were

assumed to represent a 2C ploidy level (the absolute values of the fluorescence measurements varied with each different sample preparation).

Nuclear DNA Quantity in Trichomes

Measurements of nuclei of leaf trichomes, the largest cell type, revealed nuclear DNA quantities equivalent to 4C (2 cells), 8C (6 cells), 16C (14 cells), 32C (4 cells), and 64C (1 cell). In addition, there were clusters of relative DNA values at the equivalent of 12C (3 cells), 24C (13 cells), and 48C (3 cells), a phenomenon not encountered in other leaf cell types, either epidermal or mesophyll. Altogether, 75% of the 61 trichome nuclei measured from several leaf peels fell in these peaks, whereas the other 25% (15 cells) were scattered throughout the range of 8C to 64C, between the peaks. The occurrence of these intermediate values may be attributed to the fact that the heavy

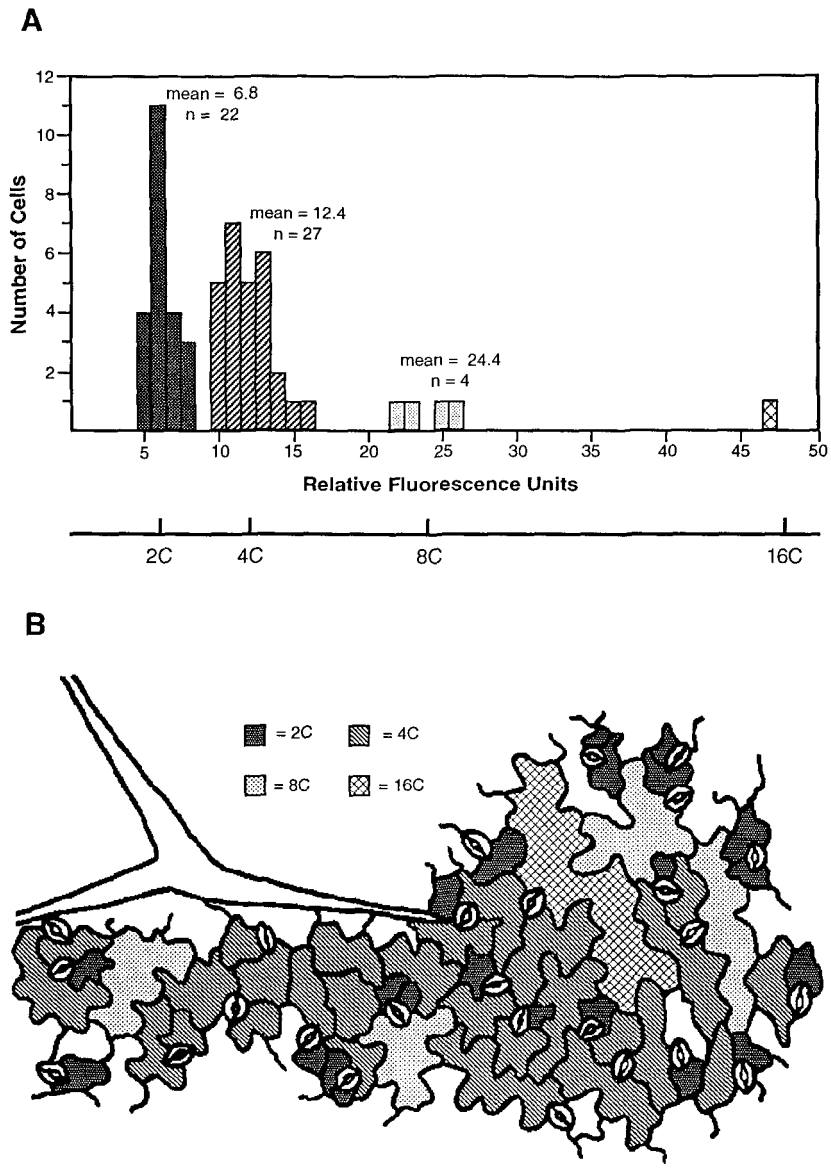

Figure 2. Measurements of Nuclear DNA Quantity in Individual Cells of the Leaf Upper Epidermis.

(A) Distribution of nuclear DNA amounts for 54 pavement cells. The scale indicating theoretical genome copy level is added below the histogram.
(B) Tracing of cell outlines with ploidy level indicated for each pavement cell measured and included in the histogram above.

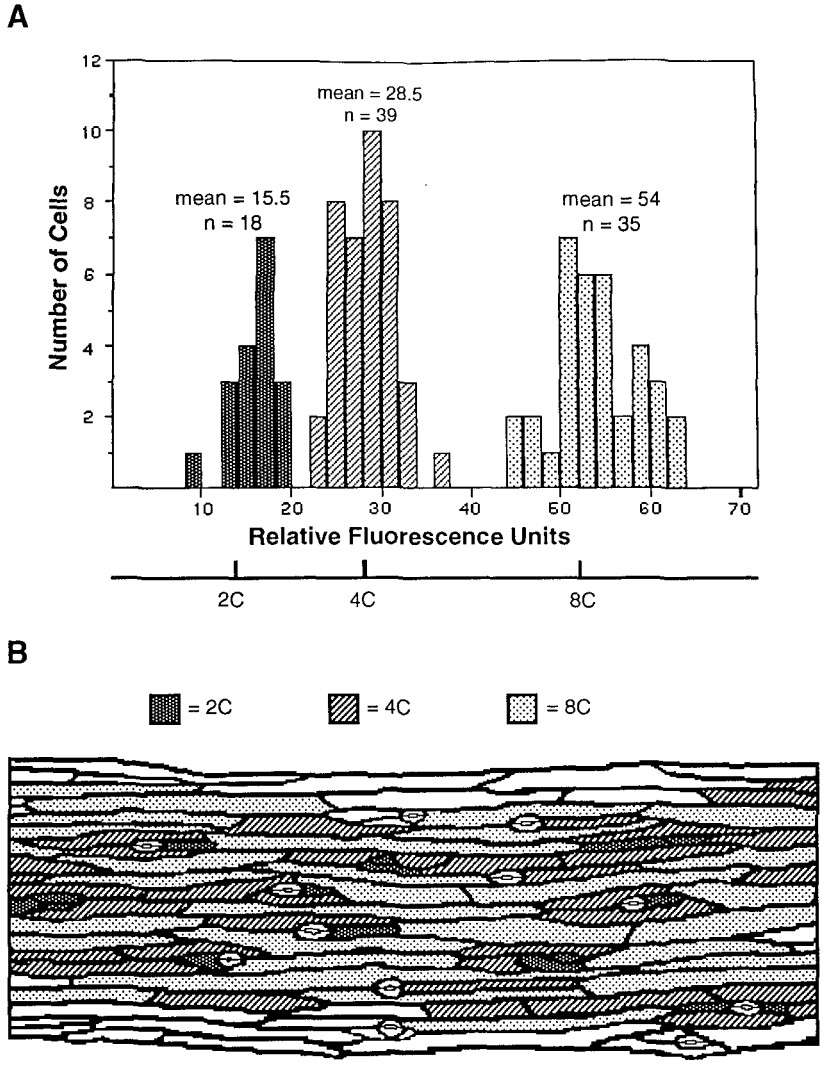

Figure 3. Measurements of Nuclear DNA Quantity in Individual Cells of the Stem Epidermis.

(A) Distribution of nuclear DNA amounts for 92 pavement cells. The scale indicating theoretical genome copy level is added below the histogram.
(B) Tracing of cell outlines with ploidy level indicated for most of the pavement cells measured and included in the histogram above.

walls of trichomes bind some fluorochrome, and that many of the trichome nuclei are elongate, two factors which complicate the determination of appropriate background readings. However, it is also possible that the two chromosome sets in a trichome nucleus may not maintain absolute synchrony in replication.

The cytology of these nuclei offers insight as to the nature of the endomitotic process. A typical trichome of the upper leaf epidermis is shown in Figure 4. The nucleus contains distinct, intensely stained spots, sometimes called chromocenters (Maluszynska and Heslop-Harrison, 1991). The number of these chromocenters (10 to 13) is highly consistent from nucleus to nucleus regardless of the ploidy level, suggesting that even though the quantity of DNA doubles with each endomitotic cycle, the number of chromosomes remains the same and the chromosomes become polytene.

DISCUSSION

Among the epidermal cells measured in this investigation, all the guard cells appeared to have nuclei with the 2C quantity of DNA, whereas all the trichome nuclei appeared to have elevated ploidy levels. The DNA values of trichome nuclei, however, do not all fall into discrete ploidy categories of successive, doubled DNA amounts as is evident in epidermal pavement cells, possibly owing to the technical problem discussed earlier.

Table 1. A Summary of the Relationship between Nuclear DNA Quantity and Cell Size for Epidermal Pavement Cells in Arabidopsis

Cells	Nuclear Genome Size	No. of Cells (% of Total)	Avg. Cell Perimeter in μm (Range)	Avg. Cell Area in μm² (Range)	Avg. Surface Area/ Cell in μm² (Range)[a]	Avg. Cell Volume in μm³ (Range)[b]
Stem cells	2C	18 (20%)	138 (79–193)	614 (244–1070)	2887 (1446–4346)	7368 (2928–12840)
(*n* = 92)	4C	39 (42%)	392 (137–612)	2471 (575–4056)	9650 (2794–15144)	29652 (6900–48672)
	8C	35 (38%)	687 (379–1299)	5254 (2822–9513)	18751 (11416–34614)	63048 (33864–114156)
Leaf cells	2C	40 (36%)	96 (58–200)	480 (154–1331)	1818 (888–4662)	4800 (1540–13310)
(*n* = 110)	4C	53 (48%)	199 (101–350)	1495 (584–3053)	4949 (2178–9260)	14950 (5840–30530)
	8C	16 (15%)	372 (251–451)	3794 (2116–4645)	11306 (6742–13474)	37940 (21160–46450)
	16C	1 (1%)	642	8153	22726	81530

[a] Total cell surface area was calculated for each cell as the sum of two times the measured cell area plus the perimeter times the cell depth (determined from microscopic observation).

[b] Cell volume was calculated for each cell as the cell area times the cell depth.

In Arabidopsis stem and leaf pavement epidermal cells, endopolyploidy is common (Table 1) but not universal. Only 29% (58 of 202 cells) of the leaf and stem pavement epidermal cells measured in this investigation had nuclei with the 2C level of DNA.

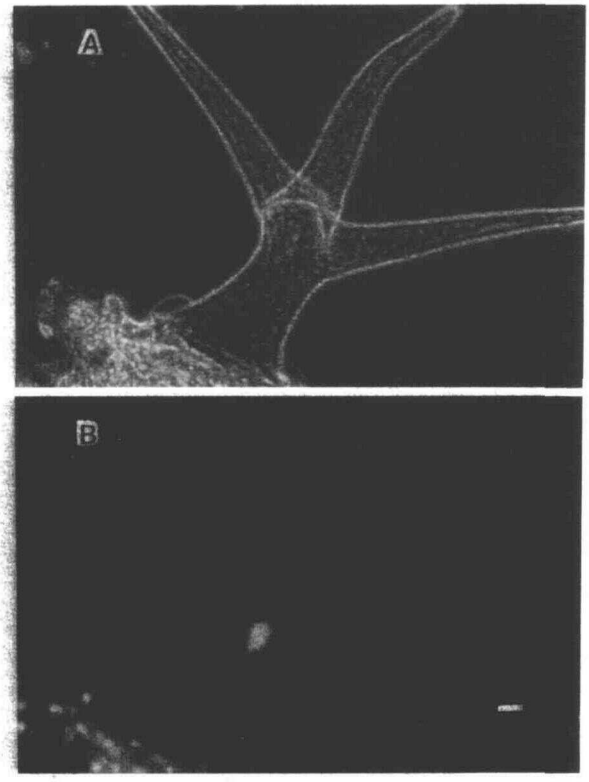

Figure 4. Two Views of the Same DAPI-Stained Leaf Trichome.

(A) Phase contrast.

(B) Fluorescence. Note the large nucleus with 12 distinct chromocenters. Bar = 15 μm.

Although these data are derived from a relatively small sample of cells and are based on a single tissue type, the findings are in general agreement with those of a study using flow cytometry to measure the nuclear ploidy level of thousands of nuclei from a mixture of all Arabidopsis leaf cell types (Galbraith et al., 1991). These investigators reported the approximate proportions of different ploidy levels in cells of a mature rosette leaf to be 2C, 25%; 4C, 28%; 8C, 37%; 16C, 10% (interpreted from their Figure 3D). Because these percentages are skewed toward the higher ploidy levels in comparison with the values reported in Table 1, it would appear that other cell types (predominantly mesophyll cells) may be more highly endopolyploid than pavement epidermal cells. A recent investigation of Arabidopsis leaf anatomy reported that the plane area of mesophyll cells in mature rosette leaves ranges from 800 to 9500 μm² (Pyke et al., 1991). If the relationship between cell area and nuclear genome size presented in Table 1 for leaf epidermal cells holds for leaf mesophyll cells, one would predict that mesophyll cells ranging in area from 800 to 9500 μm² would have ploidy levels of 4C, 8C, and 16C.

The same 2C to 16C range of nuclear ploidy in cells of Arabidopsis leaf tissue has been reported more recently (Dickson et al., 1992), again with the majority of nuclei at the 4C and 8C ploidy levels. These investigators also reported the absence of endopolyploidy in leaf tissue of *Spiraea crenata*; all nuclei in these leaves appeared to be at the 2C ploidy level. Since the absolute 2C value of *S. crenata* is only slightly larger than that of Arabidopsis, the suggestion that endopolyploidy is most prevalent in those plants with small genomes (Nagl, 1978) does not seem to be a universal generalization. It may, however, apply within certain groups. DeRocher et al. (1990) demonstrated the occurrence of endopolyploidy in nine species of succulents with relatively small genomes (<3.5 pg of DNA) and the lack of multiple ploidy levels in two other succulent species with large genomes (>32.0 pg of DNA). Thus, for some groups of plants such as succulents, the occurrence of multiploidy may be related to the absolute size of the genome.

Galbraith et al. (1991) discuss some possibilities with respect to the functional significance of multiploidy, including the need

to coordinate interactions between nuclear and organelle genomes, to accommodate rapid increases in cell volume, and to counter potentially damaging environmental factors. Whether any or all of these suggestions influence the "decision" of any particular cell to enter the endomitotic cycle, it is clear that at least in some tissues, the nuclear ploidy level is proportional to cell size. Yet in Table 1, none of the various cell size parameters is precisely doubled as ploidy level doubles. The cell component most obviously requiring interaction with the nucleus is the cytoplasm (Goff and Coleman, 1987), and we would predict that if cytoplasmic volume could be readily quantified, it would correlate directly with nuclear ploidy. The cell parameter in Table 1 most likely to reflect a change in cytoplasmic volume is average surface area per cell, and this is the column that is the closest to doubling as nuclear DNA quantity doubles, if one omits the 2C category. The latter presumably contains two different subpopulations of cells, those 2C cells that may divide mitotically and other 2C cells determined to exit the standard mitotic cell cycle, as illustrated in Figure 5 and discussed below. The inclusion of both subpopulations in the 2C cell category of Table 1 may partially explain why the means of 2C cell size measurements generally fall below the expectation from the proportionality of 4C, 8C, and 16C categories.

The idea that a cell somehow elects either mitotic or endomitotic cycling may be simply stated in terms of cell cycle options that arise at the end of the S phase. One option is to enter into G_2 and proceed with mitotic cycling, which will result in two new nuclei/cells with the same (original) ploidy level. The other option is to enter into G_1 directly and proceed with endomitotic cycling, resulting in the same (original) nucleus with a doubled ploidy level. A comparison of these two options is presented graphically in Figure 5. Assuming that elevated DNA levels are required to maintain larger cells, perhaps the endomitotic cycle arose as an alternative to the mitotic cycle

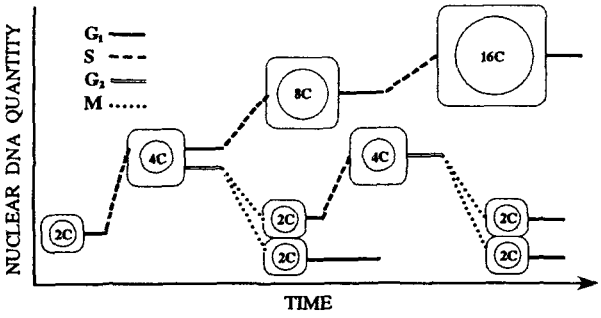

Figure 5. A Graphic Comparison of Endomitotic Cycling and Mitotic Cycling.

The lower sequence represents mitotic cycling in which a nucleus doubles its DNA and divides to form two new daughter nuclei, each of which may enter another mitotic cycle. The upper sequence represents endomitotic cycling in which a nucleus doubles its DNA and, without dividing, may enter another endomitotic cycle.

allowing for more rapid growth. Cell expansion is essential to the growth of any plant organ and in many environmental conditions plants capable of more rapid growth will have a selective advantage.

If endomitotic cycling provides an advantage for those plants (or specific tissues within a plant) adopting that strategy, why don't all the cells become endopolyploid? It seems that some 2C cells are present wherever multiploidy occurs. It is possible that once a cell begins cycling endomitotically, it loses the potential to return to the mitotic cycle. To our knowledge, there are no reports of polytene endopolyploid nuclei undergoing mitosis in plants (Nagl, 1993). If such a relationship between mitotic and endomitotic cycling holds, then an organ with exclusively multiploid cells would be limited in its potential for further growth, wound healing, and/or regeneration. The presence of some 2C cells within the tissue that have not begun cycling endomitotically could obviate any such limitation.

Data presented by Galbraith et al. (1991) comparing Arabidopsis rosette leaves grown under continuous light with those grown under short-day conditions support this suggestion. The short-day conditions prevent bolting and the rosette leaves continue to grow beyond the typical size of leaves under continuous light, increasing their surface area more than fivefold (8 to 45 mm^2). However, they report that the proportions of leaf cell nuclei at the four different ploidy levels remain the same as in leaves grown in continuous light. Because, as we have observed, such aged short-day plants have epidermal cells in the same size ranges as plants beginning to flower, the fivefold leaf surface expansion cannot be due only to expansion of preexisting cells. Instead, existing pavement cells continue to increase in size, whereas further division of 2C pavement cells continually contributes new 2C cells, which either make a stomate (Prabhakaran, 1992) or join, at the lower C values, the parade of polyploidization of pavement cells. Thus, the rough proportions among 2C, 4C, 8C, and 16C cells would remain the same, even as mitosis produces new cells. At least some of the apparently undifferentiated 2C pavement cells obviously constitute a line of "stem" cells in the leaf epidermis.

The ramifications of this combination of observations are intriguing. Cells of highest ploidy level are developmentally oldest, in the sense that they are most distant in time from their last cell division. If the assumption holds that once a cell cycles endomitotically it does not return to the mitotic cycle, then an expanding leaf increases cell number only by replication and division of 2C cells, i.e., particular stem cells among the instantaneous 2C population. Therefore, in a plant continuing to enlarge its leaves, "islands" of polyploid cells (see Figure 2B) can increase in size only by cell expansion.

Because stomates rarely or never neighbor each other, an Arabidopsis 2C epidermal cell must alternate a division that produces one prestomate cell and one stem cell with one or more subsequent divisions producing one prepolyploid cell and one stem cell. The nonrandom but also nonregular spacing of stomates seen in the Arabidopsis leaf (Prabhakaran, 1992), and reported also for other plants (Korn, 1972; Sachs, 1978; Kagan and Sachs, 1991) would be the natural consequence

of this cell lineage pattern. Rather than invoking field effects, stomate spacing may be solely a problem of cell cycling versus differentiation, i.e., a control of cell lineage problem (see also Croxdale et al., 1992), perhaps abetted by a random orientation of unequal cell divisions (Korn, 1972).

The great value of plants like Arabidopsis, in which leaf epidermal cells undergo polyploidization, is that they display overtly the developmental age of each epidermal cell. This character is not obvious in many plants (e.g., wheat) that lack polyploidy in their leaf epidermis (Coleman et al., 1985) and even in sepal and petal epidermis of Arabidopsis in which all cells are 2C (Galbraith et al., 1991). If our explanation has validity, it provides a framework for explaining the spacing of stomates on leaves, and suggests classes of genes affecting the process of nuclear polyploidization for which to look among the many Arabidopsis mutants already available.

METHODS

Plant Materials

Seeds of *Arabidopsis thaliana* ecotype Columbia (generously provided by E. Meyerowitz, California Institute of Technology, Pasadena, California) were surface sterilized and planted in individual 60-mL tubes containing 30 mL of growth media (Estelle and Somerville, 1987) supplemented with 2% sucrose and solidified with 0.8% agar. Plants were grown aseptically in constant light (40 to 50 μE cm^{-2} sec^{-1}) at 25°C.

Specimen Preparation

Mature plants (3 to 4 weeks old) were fixed in a solution of 3 parts 95% ethanol to 1 part glacial acetic acid for 2 hr at room temperature and stored in 70% ethanol at 4°C. Fixed tissue was soaked first in water and then in chloral hydrate solution (25 g in 25 mL) or 0.5 M EDTA before epidermal peels were removed with fine forceps. Peels were placed in a drop of 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 0.005 mg/mL in McIlvaine's buffer, pH 4.1 (Coleman et al., 1981) on a glass slide. Flat portions of a peel were identified and photographed, allowing for the tracing of cell outlines from photographic enlargements prior to data collection.

DNA Measurement

Microspectrofluorometric measurements were made on individual DAPI-stained nuclei using a Zeiss Photomicroscope equipped for epifluorescent excitation as described in Coleman et al. (1981). A combination of round stage and measuring pinholes of appropriate diameter resulted in the isolation of a single nucleus from the remainder of the field before measuring its fluorescence in relative units. A reading from an adjacent area of the specimen without a nucleus was used to correct for background fluorescence for each nucleus analyzed. Appropriate background measurements were more difficult to obtain with trichome nuclei, and in some cases multiple background measurements were averaged before they were subtracted from the nuclear value. Each nuclear fluorescence measurement of epidermal pavement cells was correlated with the specific cell on the tracing of cell outlines as the data were collected. Cell perimeters and surface areas were determined from the tracing of cell outlines using the Microcomp Image Analysis System (Goff and Coleman, 1987).

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