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INTERPHASE STRUCTURE OF ENDOREDUPLICATED NUCLEI IN DIPLOID AND TETRAPLOID *BRASSICA OLERACEA* L.

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Endoreduplication of nuclei is a common phenomenon in plants. Cells with multiplied DNA content are usually termed endopolyploid or polysomatic. The study was aimed at clarifying the chromosomal status of endoreduplicated nuclei. So far it has been generally assumed that endoreduplicated nuclei represent a form of polyploidy. We propose a distinction between true polyploid nuclei, possessing multiple sets of chromosomes, and chromosomes formed within endoreduplicated nuclei. We supposed that chromosomes within endoreduplicated nuclei are not separated, as in true polyploids, but rather are bound together. To clarify these two alternatives, we subjected interphase nuclei exhibiting different degrees of endoreduplication and ploidy to fluorescent in situ hybridization, using diploid and tetraploid cabbage root tips containing nuclei of four different sizes (2C–16C or 4C–32C, respectively) as revealed by flow cytometry. Nuclei were hybridized with an rDNA probe (pTA71), which showed four rDNA hybridization sites on diploid metaphase chromosomes. The number of hybridization sites was constant for both diploid and tetraploid samples (3–6 per diploid and 7–12 per tetraploid nuclei) irrespective of endoreduplicated nuclear size. The results showed more signals only in the tetraploid compared to the diploid genotype, but not between different nuclear sizes within a genotype of the same ploidy. We suggest that these results indicate that chromosomes within endoreduplicated nuclei are actually bundles of sister chromatids. The consequences of this understanding of the bundled chromosomal status of endoreduplicated nuclei are discussed.

Key words: Endopolyploidy, polysomaty, *Brassica oleracea*, fluorescent in situ hybridization.

INTRODUCTION

The terms "endopolyploidy" and "polysomaty" refer to a common, well-studied phenomenon in plants, characterized by multiplication of nuclear DNA amounts in various tissues. In addition to nuclei with 2C and 4C content (corresponding to G0/G1 and G2 cell cycle phases), endopolyploid tissues also contain nuclei with 8C, 16C, 32C, 64C and higher content. Endopolyploidy occurs in a wide range of species and is expressed in tissues at varying developmental stages. Commonly, endopolyploidy is expressed during seed germination (Bino et al., 1993), but it has also been detected in leaf and stem cells (Melaragno et al., 1993; Smulders et al., 1994) and in flowers (Kudo and Kimura, 2001a), as well as in developing fruit or seed tissues such as tomato pericarp (Bergervoet et al., 1996) or maize endosperm (Kowles et al., 1997). Several authors have presented observations of plant endopolyploidy (Geitler, 1940), and have also speculated on the function of this phenomenon in plants in relation to improved cold tolerance in maize seedlings (Wilhelm et al., 1995), the morphology of leaf trichomes in *Arabidopsis* (Traas et al., 1998), senescence phenomena and improvement of assimilative sinks in storage tissues (Bergervoet et al., 1996). The mechanism of endoreduplication has also been investigated at the molecular level (for review: Joubes and Chevalier, 2000). Larkins et al. (2001) reported that the loss of M-phase cyclin-dependent kinase activity and oscillations in S-phase cyclin-dependent kinase are key elements involved in this process.

Our research focused on the following problem: theoretically, increased nuclear DNA amounts caused by endoreduplication cycles can result in two types of chromosomal rearrangement: chromosomes may exhibit polyploidy (in the narrow sense, meaning sets of individual chromosomes) or may actually remain bundled together. In the case of polyploidy, the number of chromosomes is doubled by each endoreduplication cycle and each chromosome is individually separated, while in the case of chromosome nondisjunction the number of chromosome units remains the same because they are bound together. A similar phenomenon in which chromosomes are bundled together is known

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as polyteny, in which giant chromosomes in larval and adult *Diptera* often consist of 1024 or 2048 chromatids for each chromosome (Pardue, 1994). The same was found in plants, where polytene chromosomes with a clear banded organization similar to *Drosophila* were reported by Nagl (1969) in suspensor cells of *Phaseolus vulgaris*. Further studies revealed that giant polytene chromosomes can be found in other plant cells, such as antipodes, tapetal cells of anthers, or endosperm cells of several plant species (Kress, 1996). Shang and Wang (1991) found giant polytene chromosomes formed in embryogenic calli or in root tips regenerated from long-term cell suspension cultures of wheat. The authors observed that in cells with particularly high DNA content (up to 174C), the endoaneupolyploid nuclei exhibit polyteny. Marks and Davies (1979) reported the induction of polyploidy (4x to 32x) and giant polytene chromosomes in cultured cotyledons of *Pisum sativum*; they observed separation of polytene chromosomes into constituent single chromosomes.

From the terminological point of view, endoreduplication, endopolyploidy, polysomaty, mixoploidy and polyteny describe phenomena of increased nuclear DNA levels. Endoreduplication is described by Therman (1995) as a form of somatic polyploidy in which the chromosomes replicate time after time with no chromosome condensation and no mitoses. As reviewed by Levan and Mntzing (1991), the term "endopolyploidy" is associated with polyploid chromosome numbers, thus causing cells with 4x, 8x, 16x, 32x, etc. The term "polyteny" normally describes a special type of giant chromosome, visible in mitosis, that is more or less suborganized into bands and interbands (Rieger et al., 1991). The terms "endoreduplication," "polysomaty" and "mixoploidy" are also related to increased chromosome numbers. These terms are used in most recent papers in which the authors describe the presence of cells or tissues with endoreduplicated nuclear content. Whether the condition of chromosomes within nuclei of increased DNA levels is not polyploidy (in the narrow sense), as generally assumed, but instead represents chromosomes bound together, the use of these terms can lead to misunderstanding. This misunderstanding has consequences related to the response of various biotechnological manipulations which include plant regeneration from cells grown in vitro, for example from intact tissues, calli, cell suspensions, or protoplasts. It is essential to understand the correct status of the chromosomes within such nuclei, in order to predict the success of regeneration and to identify the ploidy of regenerants.

The phenomenon of endomitosis has been studied extensively since the 1930s. Early results were summarized by Geitler (1953), the leading researcher in this field. A particularly interesting scheme of endomitosis was later presented by Tschermak-Woess (1973), who described the occurrence of chromocenters during endomitosis and noted that the number of chromocenters remains more or less constant while their size as well as the nuclear volume increases after each endoreduplication cycle. A few more recently published papers contain partial support for bundled structure of chromosomes within endoreduplicated nuclei. Melaragno et al. (1993) suggested that leaf trichome nuclei with elevated ploidy levels in *Arabidopsis* represent a form of polyteny. This suggestion was based on the constant number of chromocenters (10–13) for all nuclear sizes. Similarly, Bohdanowicz and Dabrowska (1997) observed increased nuclear size, independent of an increase in the number of endochromocenters, in papillar tissues of *Triglochin maritimum* L.

The aim of this study was to present evidence for either independent or bundled chromosomal arrangement within endoreduplicated nuclei, using flow cytometry and rDNA hybridization, and to discuss the importance of a correct understanding of chromosome status within such nuclei.

MATERIALS AND METHODS

PLANT MATERIAL

After screening several potential species using an rDNA probe (data not shown), we chose white cabbage (*Brassica oleracea* var. *capitata*) as an appropriate model plant. Roots and root tips of 3–4-day-old seedlings were used in the study. Autotetraploid cabbage plants were induced spontaneously from microspores obtained in a previous study (Rudolf et al., 1999).

MEASUREMENTS OF NUCLEAR DNA CONTENT

Flow cytometry was used to compare genome sizes in root tip tissue of diploid and tetraploid cabbage and root tissue of diploid cabbage. Seeds were germinated on moistened filter paper for 3–4 days at 25˚C. Nuclear extraction, staining and measurement were done as described previously (Bohanec and Jakše, 1999); briefly, nuclei were released by chopping in 0.1 M citric acid containing 0.5% Tween 20, and the suspension was stained using 5.25 µg/ml 4',6-diamidino–2-phenylindole (DAPI) in 0.4 M disodium hydrogen phosphate. DNA histograms were measured with a Partec PAS IIIi flow cytometer. For correct interpretation, 2C values obtained from leaf tissues showing only basic 2C and 4C values were compared with histograms showing multiple nuclear stages obtained from root and root tip tissues.

SLIDE PREPARATION

Seedling root tips were used to make chromosome preparations. Seeds were germinated on moistened filter paper for 3–4 days in the dark at 25˚C. When the roots were 1.5–2 cm long, seedlings were treated with

a saturated solution of monobromonaphthalene for 24 h at 4˚C and then fixed in 3:1 ethanol-glacial acetic acid fixative. Root tips were washed in distilled water and partially digested with 4% cellulase (Onozuka) and 1% pectolyase (Y–23) in enzyme buffer (75 mM KCl, 7.5 mM EDTA) at 37˚C for 45 min. A few root tips were squashed in a drop of 45% acetic acid. Coverslips were removed after freezing in liquid nitrogen, and the slides were allowed to air-dry. For analysis of isolated nuclei, 3–4-day-old roots of diploid cabbage were chopped in 0.1 M citric acid containing 0.5% Tween 20. The sample was centrifuged at 200 g to increase the concentration of nuclei. After removal of the supernatant, part of the sample was measured by flow cytometry as described before. The other part was put on glass slides and the cover slips were removed after freezing at -80˚C. These slides were also used for in situ hybridization. The quality of the slide preparations was microscopically examined after staining with 2 µg/ml DAPI in distilled water. High-quality preparations were stored at +4˚C until use.

IN SITU HYBRIDIZATION

A 9 kb EcoRI fragment of the rDNA repeat unit from wheat, pTA71 (Gerlach and Bedbrook, 1979), was labelled with biotin–14-dATP by nick translation (Bio-Nick Labelling System kit; GIBCO-BRL) following the manufacturer's instructions. Unincorporated nucleotides were removed from the labelled DNA probe by repeated ethanol precipitation.

Following RNase treatment (100 mg/ml, 1 h, 37˚C), chromosome preparations were washed in 2 × SSC at room temperature for 3×5 min and treated with pepsin (1 mg/ml in 0.01 M HCl) for 10 min at 37˚C. They were then washed 2×5 min at room temperature and treated with 4% paraformaldehyde for 10 min. After another 3 washes for 5 min each in 2 × SSC at room temperature, the preparations were dehydrated in a graded series of 70%, 90% and absolute ethanol. Chromosomal DNA was denatured in 70% formamide solution in 2 × SSC at 70˚C for 2 min. The slides were then dehydrated through a cold ethanol series and air-dried. The hybridization mixture containing 50 ng/slide of labelled DNA probe, 50% formamide, 2 × SSC, dextran sulphate and herring sperm DNA was denatured for 10 min at 80˚C and placed on ice for 5 min before being added to the slides. Hybridization was carried out overnight at 37˚C in a humid chamber.

After hybridization, the slides were washed for 10 min at 42˚C in 50% formamide in 2 × SSC followed by 10 min washes in 2 × SSC at 42˚C and 2 × SSC at room temperature. Detection of hybridization sites was performed by transferring the slides to detection buffer (4 × SSC, 0.1% Tween–20) for 5 min, treating with 3% bovine serum albumin in detection buffer for 30 min, and then incubating with streptavidine conjugated with CY3 (Sigma) for 30 min at 37˚C. The slides were washed in detection buffer at 37˚C for 3 × 5 min. For signal amplification, slides were treated for 5 min with 5% normal goat serum and then incubated with biotinylated anti-streptavidin (Vector lab) at 37˚C for 30 min, before being washed 3×5 min in detection buffer at 37˚C.

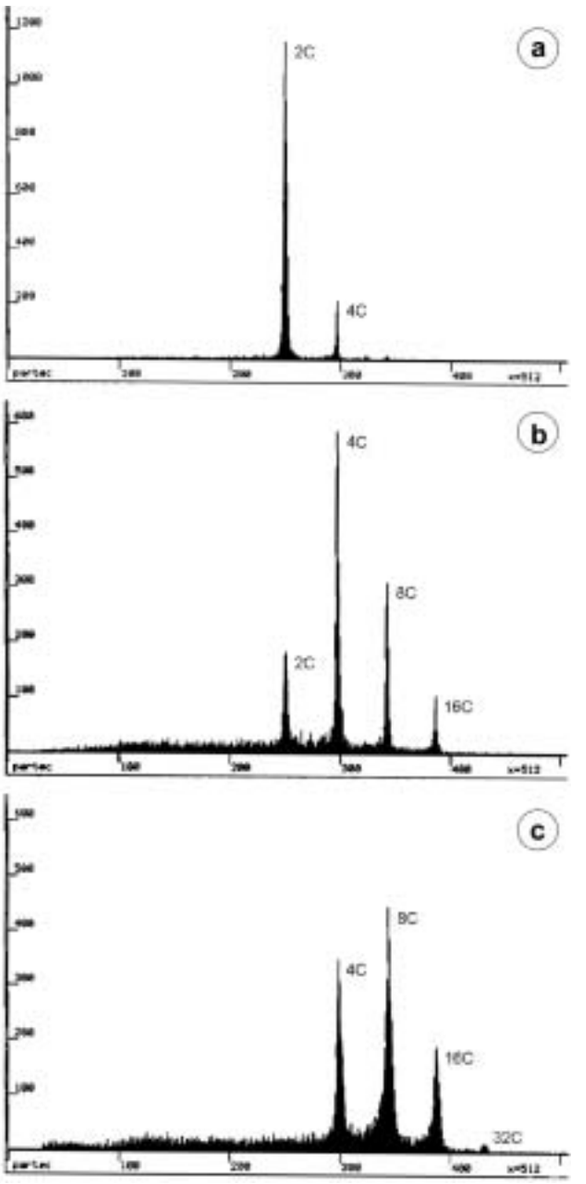

Fig. 1. Flow cytometric analysis showing DNA histograms (log scale) of DAPI-stained nuclear preparations of cabbage tissues: (**a**) Diploid leaf tissue, (**b**) Diploid seedling root tip tissue, (**c**) Tetraploid seedling root tip tissue.

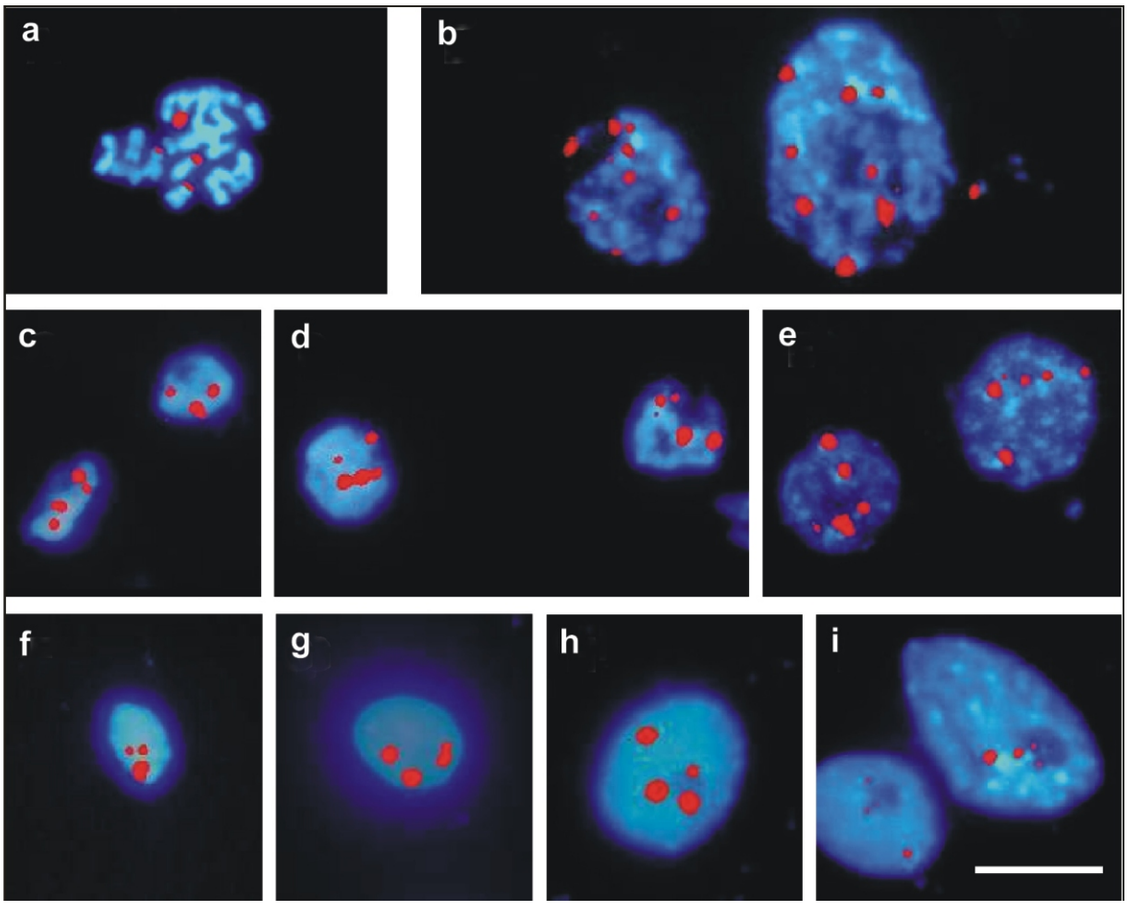

Fig. 2. Fluorescence in situ hybridization of root-tip preparations of *Brassica oleracea*. Blue fluorescence shows DNA counterstaining with DAPI; red CY3 fluorescence indicates in situ hybridization sites of rDNA probe pTA71. (**a**) Metaphase of diploid genotype with two pairs of rDNA loci, (**b**) Interphase nuclei of two different sizes from tetraploid genotype, (**c–e**) Interphase nuclei of different sizes from the same diploid genotype prepared by squash technique, (**f–i**) Diploid interphase nuclei of different sizes from the same sample as analyzed by flow cytometry shown in Fig. 3. Bar = 20 μm for (a–i).

The slides were mounted in antifade solution containing 6 μM DAPI, 0.2 M 1,4 diazobicyclo(2.2.2.)octane (DABCO), and 0.02 M Tris-HCl (pH 8.0) in 90% glycerol. Slides were examined with a Zeiss Jenalumar 250 epifluorescence microscope using appropriate filter sets. Photographs were taken with a Zeiss MC 80 camera on Fujicolor MS 100/1000 ASA color reversal film.

RESULTS AND DISCUSSION

To analyze the chromosomal status within endoreduplicated nuclei, plant tissues with nuclei of endoreduplication stages up to 16C or 32C were subjected to fluorescent in situ hybridization (FISH) using a probe that is present in only a few loci per genome, to produce signals well visible on chromosomes and within interphase nuclei. According to our preliminary research and published data (Górnik et al., 1997; Kudo and Kimura, 2001b), four endomitotic stages are found in young germinated seedlings of *B. oleracea*. Maluszynska and Heslop-Harrison (1993) found that rDNA loci in this species exist only as two major and one minor spot per haploid genome. Snowdon and Köhler (1997), however, reported only two clearly distinguishable pairs on metaphase chromosomes. For these reasons, diploid and tetraploid lines of cabbage were chosen as appropriate plant species for which an available rDNA probe (pTA71) matched the defined criteria.

Flow cytometry of root tip tissues revealed four distinct peaks representing 2C, 4C, 8C and 16C or 4C, 8C, 16C and 32C nuclei in diploid or tetraploid genotypes, respectively, in contrast to leaf tissue (Fig. 1). The root tip cells of both genotypes therefore expressed

an additional two peaks together with two basic nuclear contents, representing nuclei passed through one or two endoreduplication cycle(s).

Examination of 40 slides on which diploid or tetraploid root tips had been squashed showed consistently similar results. Counts of DAPI-stained metaphase chromosomes of the diploid genotype revealed that the somatic chromosome number was 2n = 18; no higher chromosome number was found. Chromosomes subjected to the FISH procedure using a pTA71 probe showed four distinct rDNA hybridization sites present at the terminal locations of four chromosomes (Fig. 2a).

Analysis of slides revealed the presence of nuclei that apparently differ in size and could correspond to the four nuclear classes detected by flow cytometry. The tetraploid nuclei were larger than diploid nuclei. Following the FISH procedure, interphase nuclei of all sizes showed clear red signals, representing sites of probe hybridization. The number of hybridization signals in interphase nuclei of tetraploid genotypes varied from 7 to 12. This variation was present in nuclei of all sizes (Fig. 2b). Analysis of undamaged nuclei of diploid genotypes revealed results similar to those for tetraploid ones. The number of rDNA sites varied from 4 to 6 and was also more or less constant irrespective of nuclear size (Fig. 2c–e). The observed variations in hybridization signals were not associated with nuclear size, since in some cases we found fewer signals in larger nuclei than in smaller ones.

An additional experiment was performed, in which slides were prepared from nuclei of diploid cabbage roots released by chopping in citric buffer (the same method as used for flow cytometry) instead of the standard squash method. This experiment was performed to produce additional information as an indirect indication for quantification of nuclear size. The problem with DAPI-stained nuclei is that nuclear size cannot be quantified exactly, as can be done with nuclei stained by Schiff reagent (Feulgen staining technique) using densitometry or image analysis. An exact distribution into four classes of nuclear sizes is therefore not strictly possible, but the size of nuclei can be assumed on the basis of their appearance. To ensure that the nuclei observed by microscope are comparable to nuclei measured by flow cytometry in this experiment, one part of the same sample was analyzed using flow cytometry and the other part was subjected to FISH analysis. The results of flow cytometry showed that four peaks were present (Fig. 3). The distribution of the four nuclear sizes was not equal, with 2C and 4C representing 9.3% and 38.6%, respectively, and 8C and 16C representing 41.0% and 11.1%, respectively; this means that endoreduplicated nuclei represented 52.1% of all measured nuclei. The other part of the sample, examined by FISH, gave the same result as obtained using the squash technique. The number of hybridization sites

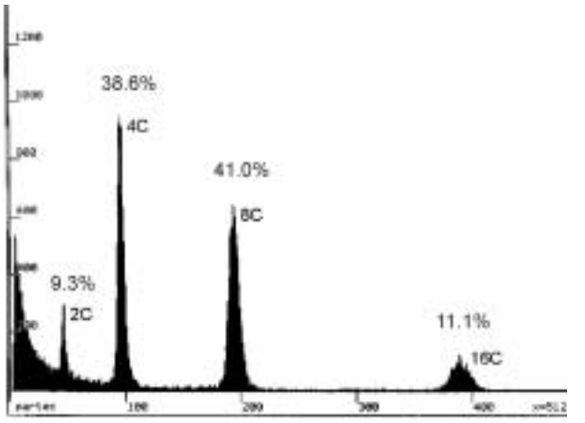

Fig. 3. Flow cytometric analysis showing DNA histogram (linear scale) of DAPI-stained nuclei of the same sample of diploid cabbage root tissue also analyzed by FISH and shown in Fig. 2f–i.

was 3–5 per nucleus and was the same in all four visually determined nuclear classes (Fig. 2f–i).

The observed variation in the number of signals representing rDNA loci has already been reported in decondensed nuclei. In fenugreek, for example, Ahmad et al. (1999) showed an increased number of signals in decondensed interphase nuclei versus those on metaphase chromosomes. Weiss and Maluszinska (1998) reported a decreased number of signals for rDNA loci (1–4 in diploid or 3–8 in tetraploid) in interphase nuclei of *Arabidopsis thaliana*. The increased numbers, which were not observed on condensed metaphase chromosomes, can be attributed to the fact that clusters of rDNA genes are more dispersed in decondensed interphase chromosomes, while the decreased numbers have been explained by possible binding of rDNA sites of homologous chromosomes (H. Weiss, personal communication).

Based on the presented data, we concluded that the chromosomes are organized within endoreduplicated nuclei of cabbage in bundled form. The most convincing evidence was the clear absence of an increased number of signals, even in the largest nuclei. It should be noted that in the case of polyploidy (narrow sense), 32 or 64 signals would be expected in the largest diploid or tetraploid nuclei. Additional confirmation of this hypothesis is provided by the finding that, in contrast to nuclear size, the number of hybridization sites in tetraploid nuclei was double that of diploid nuclei.

Some published data are more understandable if a bundled chromosomal structure is assumed for endoreduplicated nuclei. It is largely assumed that cells

possessing endoreduplicated nuclei represent the end of development. According to Nagl (1981), the endoreduplication cycle irreversibly leads to endopolyploidy. This view is particularly based on study of the endopolyploid status of trichomes in *Arabidopsis* (Melaragno et al., 1993; Traas et al., 1998). This result was questioned by Valente et al. (1998), who observed that nuclei formed by huge endoreduplicated immobilized single cells derived from tobacco mesophyll protoplasts were able to divide amitotically into daughter nuclei. Using confocal microscopy, they saw that after several divisions induced by auxins, the DNA content of the nuclei was reduced (deduplicated) from up to 16C to a basic 2C/4C level. Such cells then turned into normal mitoses and formed microcalli, but during the reduction stages some irregularities in nuclear DNA content were observed. The deduplication process as reported in this study can be explained if a bundled structure of chromosomes is assumed. In the case of polyploid nuclei, amitotic reduction would result in random chromosome sorting; the final cells obviously would be aneuploid and, as such, would hardly survive or regenerate to form normal plants (Stickens et al., 1996).

Other studies of plant regeneration from various tissue culture systems have detected endopolyploidy of initial explants, but the regenerants were mainly at the diploid level (Jacq et al., 1992; Jacq et al., 1993; Colijn-Hooymans et al., 1994; Iantcheva et al., 2001; Ellul et al., 2003). We were unable to find a report of fertile regenerants of higher ploidy such as 8n or 16n originated from endoreduplicated cells. Since the term "ploidy" is often used to describe the status of endoreduplicated nuclei, even the most recent papers tend to explain endoreduplication as polyploidy in the narrow sense. For instance, Sliwinska and Lukaszewska (2005), analyzing the occurrence of polysomaty in various tissues of sugarbeet, suggested that the consequences of culturing organs in vitro exhibiting a high degree of endoreduplication can result in polyploid regenerants. In fact, previously cited papers that reported on a regeneration protocol via cotyledon-derived callus possessing up to 32C peaks (Jacq et al., 1992, 1993) rather demonstrate the opposite.

We suggest that the bundled nature of endoreduplicated nuclei deserves further investigation to confirm or disprove the presented explanation, since an understanding of this phenomenon is obviously of great importance and has implications for several kinds of biotechnology studies.

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REFERENCES

AHMAD F, ACHARYA SN, MIR Z, and MIR PS. 1999. Localization and activity of rRNA genes on fenugreek (*Trigonella foenum-graecum* L.) chromosomes by fluorescent in situ hybridization and silver staining. *Theoretical and Applied Genetics* 98: 179–185.

BERGERVOET JHW, VERHOEVEN HA, GILISSEN LJW, and BINO RJ. 1996. High amounts of nuclear DNA in tomato (*Lycopersicon esculentum* Mill.) pericarp. *Plant Science* 116: 141–145.

BINO RJ, LANTERI S, VERHOEVEN HA, and KRAAK HL. 1993. Flow cytometric determination of nuclear replication stages in seed tissues. *Annals of Botany* 72: 181–187.

BOHANEC B, and JAKŠE M. 1999. Variations in gynogenic response among long-day onion (*Allium cepa* L.) accessions. *Plant Cell Reports* 18: 737–742.

BOHDANOWICZ J, and DABROWSKA D. 1997. Polyploidization of stigmatic papillae in *Triglochin maritimum* L. (Juncaginaceae). *Acta Biologica Cracoviensia Series Botanica* 39: 63–67.

COLIJN-HOOYMANS CM, HAKKERT JC, JANSEN J, and CUSTERS JBM. 1994. Competence for regeneration of cucumber cotyledons is restricted to specific developmental stages. *Plant Cell Tissue and Organ Culture* 39: 211–217.

ELLUL P, GARCIA-SOGO B, PINEDA B, ROS G, ROIG LA, and MORENO V. 2003. The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* L.Mill.) is genotype and procedure dependent. *Theoretical and Applied Genetics* 106: 231–238.

GEITLER L. 1940. Die Polyploidie der Dauergewebe höherer Pflanzen. *Berichte der Deutschen Botanischen Gesellschaft.* 58: 131–142.

GEITLER L. 1953. Endomitose und endomitotische Polyploidisierung. In: Heilbrunn LV and Weber F [eds.], *Protoplasmatologia VI C.* Springer-Verlag, Wien.

GERLACH WL, and BEDBROOK JR. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acids Research* 7: 1869–1885

GÓRNIK K, DE CASTRO RD, LIU Y, BINO RJ, and GROOT SPC. 1997. Inhibition of cell division during cabbage (*Brassica oleracea* L.) seed germination. *Seed Science Research* 7: 333–340.

IANTCHEVA A, VLAHOVA M, HANH TRINH T, BROWN SC, SLATER A, ELLIOT MC, and ATANASSOV A. 2001. Assessment of polysomaty, embryo formation and regeneration in liquid media for various species of diploid annual *Medicago*. *Plant Science* 160: 621–627.

JACQ B, TETU T, SANGWAN RS, DE LAAT A, and SANGWAN-NORREEL BS. 1992. Plant regeneration from sugarbeet (*Beta vulgaris* L.) hypocotyls cultured *in vitro* and flow cytometric nuclear DNA analysis of regenerants. *Plant Cell Reports* 11: 329–333.

JACQ B, TETU T, SANGWAN RS, DE LAAT A, and SANGWAN-NORREEL BS. 1993. Efficient production of uniform plants from cotyledon explants of sugarbeet (*Beta vulgaris* L.). *Plant Breeding* 110: 185–191.

JOUBES J, and CHEVALIER C. 2000. Endoreduplication in higher plants. *Plant Molecular Biology* 43: 735–745.

KOWLES RV, YERK GL, HAAS KM, and PHILLIPS RL. 1997. Maternal effects influencing DNA endoreduplication in developing endosperm of *Zea mays*. *Genome* 40: 798–805.

KRESS H. 1996. Polytene chromosomes: a general model for the eucaryotic interphase state. *International Journal of Insect Morphology and Embryology* 25: 63–91.

KUDO N, and KIMURA Y. 2001a. Flow cytometric evidence for endopoliploidization in cabbage (*Brassica oleracea* L.) flowers. *Sexual Plant Reproduction* 13: 279–283.

KUDO N, and KIMURA Y. 2001b. Flow cytometric evidence for endopolyploidy in seedlings of some *Brassica species*. *Theoretical and Applied Genetics* 102: 104–110.

LARKINS BA, DILKES BP, DANTE RA, COELHO CM, WOO YM, and LIU Y. 2001. Investigating the hows and whys of DNA endoreduplication. *Journal of Experimental Botany* 52: 183–192.

LEVAN A, and MÜNTZING A. 1991. Terminology of chromosome numbers. In: Tsuchiya T. and Gupta PK [eds.], *Chromosome engineering in plants: genetics, breeding, evolution*, 15–26. Elsevier Science Publishers, Amsterdam.

MALUSZYNSKA J, and HESLOP-HARRISON JS. 1993. Physical mapping of rDNA loci in *Brassica* species. *Genome* 36: 774–781.

MARKS GE, and DAVIES DR. 1979. The cytology of cotyledon cells and the induction of giant polytene chromosomes in *Pisum sativum*. *Protoplasma* 101: 73–80.

MELARAGNO JE, MEHROTRA B, and COLEMAN AW. 1993. Relationship between endopolyploidy and cell size in epidermal tissue of *Arabidopsis*. *Plant Cell* 5: 1661–1668.

NAGL W. 1969. Banded polytene chromosomes in the legume *Phaseolus vulgaris*. *Nature* 221: 70–71.

NAGL W. 1981. Polytene chromosomes of plants. *International Review of Cytology* 73: 21–53.

PARDUE ML. 1994. Looking at polytene chromosomes. *Methods in Cell Biology* 44: 333–351.

RIEGER R, MICHAELIS A, and GREEN MM. 1991. *Glossary of genetics: classical and molecular*. Springer Verlag, Berlin, Heidelberg, New York.

RUDOLF K, BOHANEC B, and HANSEN M. 1999. Microspore culture of white cabbage, *Brassica oleracea* var. *capitata* L.: Genetic improvement of non-responsive cultivars and effect of genome doubling agents. *Plant Breeding* 118: 237–241.

SHANG XM, and WANG WC. 1991. DNA amplification, chromatin variations, and polytene chromosomes in differentiating cells of common bread wheat in vitro and roots of regenerated plants in vivo. *Genome* 34: 799–809.

SLIWINSKA E, and LUKASZEWSKA E. 2005. Polysomaty in growing in vitro sugar-beet (*Beta vulgaris* L.) seedlings of different ploidy level. *Plant Science* 168: 1067–1074.

SMULDERS MJM, RUS-KORTEKAAS W, and GILISSEN LJW. 1994. Development of polysomaty during differentiation in diploid and tetraploid tomato (*Lycopersicon esculentum*) plants. *Plant Science Limerick* 97: 53–60.

SNOWDON RJ, and KÖHLER A. 1997. Chromosomal localization and characterization of rDNA loci in the *Brassica* A and C genomes. *Genome* 40: 582–587.

STICKENS D, TAO W, and VERBELEN JP. 1996. A single cell model system to study hormone signal transduction. *Plant Growth Regulation* 18: 149–154.

THERMAN E. 1995. Chromosome behaviour in cell differentiation: a field ripe for exploration? *Genetics* 141: 799–804.

TRAAS J, HÜLSKAMP M, GENDREAU E, and HÖFTE H. 1998. Endoreduplication and development: rule without dividing? *Current Opinion in Plant Biology* 1: 498–503.

VALENTE P, TAO W, and VERBELEN J.-P. 1998. Auxins and cytokinins control DNA endoreduplication and deduplication in single cells of tobacco. *Plant Science* 134: 207–215.

WEISS H, and MALUSZYNSKA J. 1998. Cytogenetic investigations of different types of cells from flower buds of *Arabidopsis thaliana* (L.) Heynh. In: Maluszynska J [ed.], *Plant cytogenetics*, 195–200. Prace Naukowe Uniwersytetu Śląskiego, Katowice.

WILHELM E, BIRADAR DP, BULLOCK DG, and RAYBURN AL. 1995. Endopolyploidization of mesocotyls in Nebraska maize populations selected for cold tolerance. *Crop Science* 35: 958–961.

TSCHERMAK-WOESS E. 1973. Somatische Polyploidie bei Pflanzen. In: Hirsch GH, Ruska H, und Sitte P [eds.], *Grundlagen der Cytologie*, 189–204. VEB Gustav Fischer Verlag, Jena.