

# Chromosome organization in wheat endosperm and embryo

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**Abstract.** We have analysed the chromosome organization in endosperm and embryo of bread wheat (*Triticum aestivum* L.), in order to compare these tissues with developing anthers, in which the centromeres associate, and the developing root xylem vessel cells, in which the chromosomes endoreduplicate to become polytene and associate via their centromeres. Both endosperm and embryo showed a typical Rabl configuration and a degree of non-homologous centromere association and the endosperm also showed extensive telomere association. Wheat endosperm is initially triploid and during its develop-

ment a percentage of the nuclei increase their DNA content to 6C and 12C. 6C nuclei showed twice as many centromeres as 3C nuclei and the centromere number increased further in 12C nuclei. The higher the C-content of a nucleus the more the telomeres associated in endosperm. The vast majority of 12C nuclei showed six rye chromosome arms, although a few showed three associated groups of rye chromosome arms. This means that during endosperm development wheat nuclei show both polyploidization and polytenization.

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Bread wheat (*Triticum aestivum* L.) is a hexaploid species with more than 80% of repetitive sequences, which are homogenized across all chromosomes (Flavell et al., 1977; Heslop-Harrison, 2000). Chromosome-specific in situ paints, which are now used for the analysis of chromosome organization in *Arabidopsis* (Lysak et al., 2003), are therefore still lacking in wheat. However, there are many wheat lines into which chromosomes or parts of chromosomes from other cereals such as rye have been introgressed and labelling of alien chromosomes or chromosome arms has proved a useful tool for the elucidation of chromosome organization in this species. Wheat chromosomes span the width of the nucleus in rod-like structures with the two arms of each chromosome close together and in all

Triticeae analysed so far centromeres and telomeres are located at opposite poles in a Rabl configuration (Abranches et al., 1998; Martínez-Pérez et al., 1999, 2000, 2001). In plants, Rabl configurations have also been found in rye, barley and oats, but not in rice or maize (Dong and Jiang, 1998). Outside the plant world Rabl configurations have been reported in yeast and in *Drosophila* (Hochstrasser et al., 1986; Jin et al., 2000).

Endosperm is derived from the fertilization of the central cell in the megagametophyte of higher plants by a second haploid male gamete, the first one fertilizing the egg cell to form the embryo. The central cell is diploid and the endosperm is therefore triploid, containing three sets of chromosomes, one paternal and two maternal. The first stage in endosperm development is characterized by nuclear divisions without cellularization. Cell wall formation starts from the periphery at day four after fertilization and is completed by day seven (van Lammeren, 1988). Subsequently some nuclei in the central starchy endosperm undergo two or three rounds of DNA replication taking the DNA content up to 12C or 24C (Brunori et al., 1989). An increase in DNA content outside the mitotic cycle can lead to polyteny (through endoreduplication, where the number of chromosomes remains constant while the number of chromatids increases) or polyploidy (an increase in number of chromosome sets) or both.

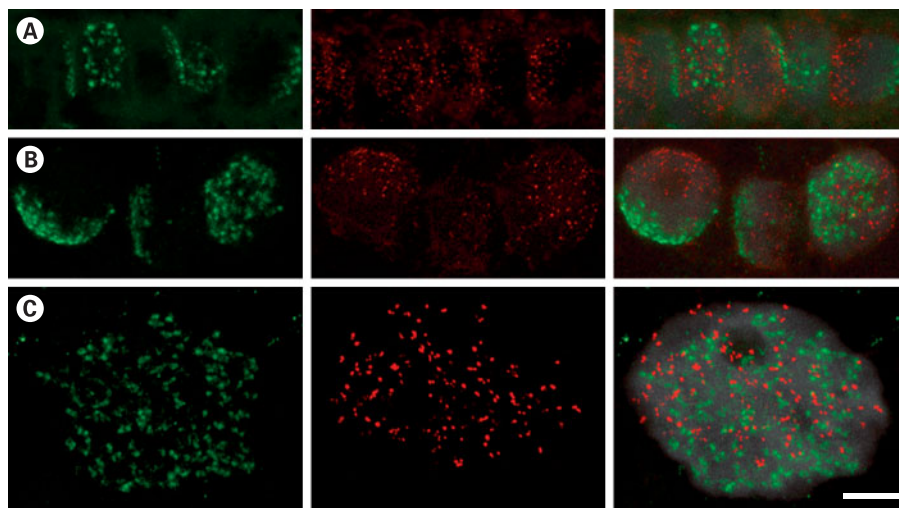
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Fig. 1. Centromeres and telomeres in embryo and endosperm of wheat show a Rabl configuration with telomeres at one pole and centromeres at the other. Confocal stacks spanning the depth of the nuclei. Centromeres are shown in green, telomeres in red. Chromatin (grey) was counterstained with DAPI. (A) Embryo root. All embryo tissues show the same centromere and telomere conformation, but root nuclei are stacked in files and therefore easier to visualise. (B) to (C) endosperm. (B) 3C nuclei at 7 dpa. (C) 16 dpa. Frontal view of an endoreduplicated (12C) nucleus. Note the much greater variation in telomere size compared to (B). Bar, 10  $\mu$ m.



In hexaploid wheat, centromeres are associated in non-homologous pairs early in anther development. The non-homologous centromere associations become homologous pre-meiotically in both meiocytes and tapetal cells and the telomeres form a cluster (bouquet) at the onset of meiosis (Martínez-Pérez et al., 1999). In developing xylem vessel cells in roots chromosomes become polytene and homologues associate by their centromeres (Martínez-Pérez et al., 2001). In this study we describe the chromosome organization in endosperm and compare it to that of the diploid embryo. We show that in contrast to xylem vessel cells polyploidization in endosperm generally occurs through an increase in chromosome number without homologous centromere associations. A proportion of non-homologous centromere associations is found in both endosperm and embryo. Telomere associations in the endosperm increase with an increase in C-content while the percentage of centromere associations remains constant.

## Materials and methods

### Plant material

The following wheat (*Triticum aestivum*) genotype was used: AABBDD,  $2n = 6x = 42$ , cv. Pro INTA Federal with a 1RS (*Secale cereale*) chromosome arm substitution for the 1BS wheat chromosome arm (1B<sup>1</sup>/1R<sup>s</sup>). Plants were grown in a controlled environment room (15 °C, 16-hour photoperiod, 70% humidity).

### Seed sections

Immature seeds were harvested at 7, 16 and 24 dpa (days post-anthesis) and fixed for 6 h in 4% (w/v) formaldehyde, freshly made from paraformaldehyde, in PEM (50 mM PIPES, 5 mM EGTA, 5 mM MgSO<sub>4</sub>, pH 6.9). Endosperm tissue was analysed at 7 and 16 dpa, and embryo tissue (both root and shoot tissue) was analysed at 24 dpa. Fixed seeds were dehydrated through an ethanol:water series (10%, 20%, 40%, 60%, 80% and absolute ethanol for 4 to 12 h per step) and stored in absolute ethanol at 4 °C. 100- $\mu$ m sections were prepared under absolute ethanol using a Vibratome Series 1000plus (TAAB Laboratories Equipment, Aldermarston, UK) and rehydrated in water. They were allowed to dry on polylysine-coated slides (BDH, Poole, UK). Sections were incubated with 1% (w/v) driselase (Sigma), 0.5% (w/v) Onozuka R 10 cellulase (Yakult Pharmaceutical, Tokyo Japan), 0.025%

pectolyase Y23 (Kikkoman, Tokyo, Japan) in PBS (18 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 M NaCl, pH 7.4) for 1 h at room temperature and washed in TBS (10 mM Tris, 140 mM NaCl pH 7.4) for 10 min. They were then treated with RNase A (Sigma, 100  $\mu$ g/ml in 2 $\times$  SSC for 1 h at 37 °C, washed in TBS for 10 min, dehydrated in an ethanol:water series (70% and absolute ethanol) and air-dried.

### Probes and in situ hybridization

Total rye genomic DNA was partly digested with *TaqI* and the fragments were then labelled with biotin-16-dUTP (Roche) by nick translation. Telomeric probes labelled with biotin-16-dUTP (Roche) were prepared according to Cox et al. (1993). Centromeric probes labelled with digoxigenin-11-dUTP (Roche) were prepared according to Aragón-Alcaide et al. (1996) using the following primers for the CCS1 repeat fragment: 5'-CGCAATATCTT-GATTGCATCTATATTC-3' (positions 17 to 43) and 5'-GCTGGTAGT-GAAAAGGTGCCCGATCTT-3' (positions 249 to 223). Sections were first treated with the avidin/biotin blocking kit (Vector Laboratories) according to the manufacturer's instructions using a biotin-blocking step followed by avidin and biotin, respectively, to block biotin-binding sites in the tissue. After a final wash in PBS, FISH was performed in a slightly modified version of Abranches et al. (2000) using 200 ng of each probe in a total volume of 30  $\mu$ l per slide in a hybridization buffer containing 20 $\times$  excess salmon sperm DNA in 50% formamide, 10% dextran sulfate, 2 $\times$  SSC and 0.1% SDS. The probe was denatured in the hybridization mixture for 5 min at 95 °C. Sections were denatured at 75 °C for 8 min in a modified thermocycler (Omnislide, Hybaid, Ashford, UK), and hybridization carried out overnight at 37 °C. Post-hybridization washes were carried out in 0.1 $\times$  SSC, 20% formamide at 42 °C.

### Immunodetection

Biotin-labelled probes were detected with Extravidin-Cy3 (Sigma). Probe labelled with digoxigenin was detected with a mouse anti-digoxin antibody (Sigma) followed by a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 (Molecular Probes). Antibodies were diluted in 4 $\times$  SSC, 0.2% Tween 20 according to the manufacturer's instructions. Antibody incubation was performed in a humid chamber for 1 h at 37 °C followed by 3 $\times$  5-min washes in 4 $\times$  SSC, 0.2% Tween 20 at room temperature. Sections were counterstained in 1  $\mu$ g/ml DAPI for 10 min. Slides were mounted in Vectashield (Vector Laboratories).

### Image acquisition and analysis

Hybridized sections were analysed on a Leica TCS SP2 confocal microscope (Leica Microsystems) equipped with two Argon lasers (351, 363 nm and 457, 488, 514 nm respectively) and two Helium/Neon lasers (543 nm and 633 nm respectively). Section spacing was 0.6  $\mu$ m. The confocal data were then transferred to ImageJ (a public domain program by W. Rasband

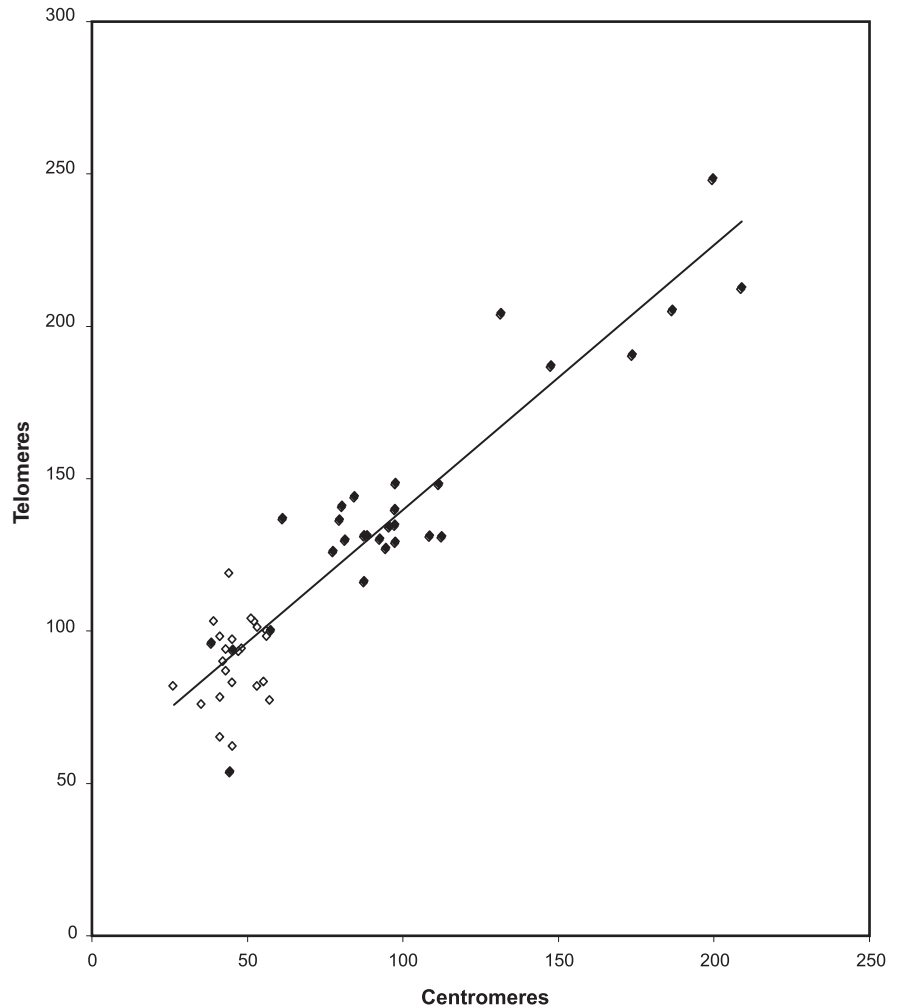


Fig. 2. Correlation between telomere/centromere ratio and C content in endosperm nuclei. Clusters corresponding to 3C and 6C are clearly distinguishable, but the numbers of telomeres and centromeres seen are lower than expected. There is a linear correlation between the number of telomeres and centromeres; the telomere/centromere ratio decreases as the number of centromeres increases. The slope of the trend line is  $y = 0.87x + 51.8$ . Open diamonds: 7 dpa. Black diamonds: 16 dpa. The data from the two different timepoints follow the same overall trend.

available from <http://rsb.info.nih.gov/ij/>). All images were composed using Adobe Photoshop 7.0. C values were estimated for individual nuclei from nuclear volume and DAPI fluorescence intensity.

#### Flow cytometry

For each experiment six to eight isolated embryos from 24-dpa seeds were chopped up with a razor blade and extracted using a two-step disaggregation and DAPI staining kit (CyStain UV Precise P, Partec, Münster, Germany) according to the manufacturer's instructions. The extracts were passed through a 30- $\mu$ m filter and stored on ice until measurement. The DNA content of nuclei (C value) was measured using a Ploidy Analyser PA-II (Partec) with UV excitation by a mercury arc lamp. Six independent experiments were carried out and between 18,000 and 20,000 nuclei were measured each time. Nuclei from young wheat leaves were used as a standard to determine the positions of 2C and 4C.

#### Results

We investigated chromosome territories in endosperm and embryo of wheat. For the endosperm we chose two time points: 7 dpa where 72% of the nuclei are 3C, 27% 6C and the rest greater than 6C and 16 dpa where 40% are 3C, 48% 6C and 12% 12C (as determined by flow cytometry (Wegel and co-investigators, manuscript in preparation)). Both embryo and

endosperm nuclei showed Rab1 configurations with centromeres at one pole of the nucleus and the telomeres at the other (Fig. 1). In the embryo, centromere foci varied greatly in size even within a single nucleus indicating associations among centromeres, whereas telomere foci were more uniform. In the endosperm, centromere foci varied little in size irrespective of the C content of the nucleus. The size of the telomere foci was more variable: small nuclei showed small telomeres, but the telomere signal in larger nuclei ranged from small foci to much bigger ones. Also, in 12C nuclei the number of telomeres did not seem to have increased to the same extent as the number of centromeres.

To quantify our observations we counted the numbers of centromeres and telomeres in endosperm nuclei and centromere numbers in embryo nuclei. Since the density of nuclei in embryo tissue was very high and telomere signal was found on half the nuclear surface it was difficult to distinguish between telomeres from different nuclei and accurate counts could not be made. Centromere association was found in both endosperm and embryo nuclei (Table 1). In the embryo, 2C and 4C nuclei were difficult to tell apart by size and could not be distinguished by centromere counts, which averaged at 27 per

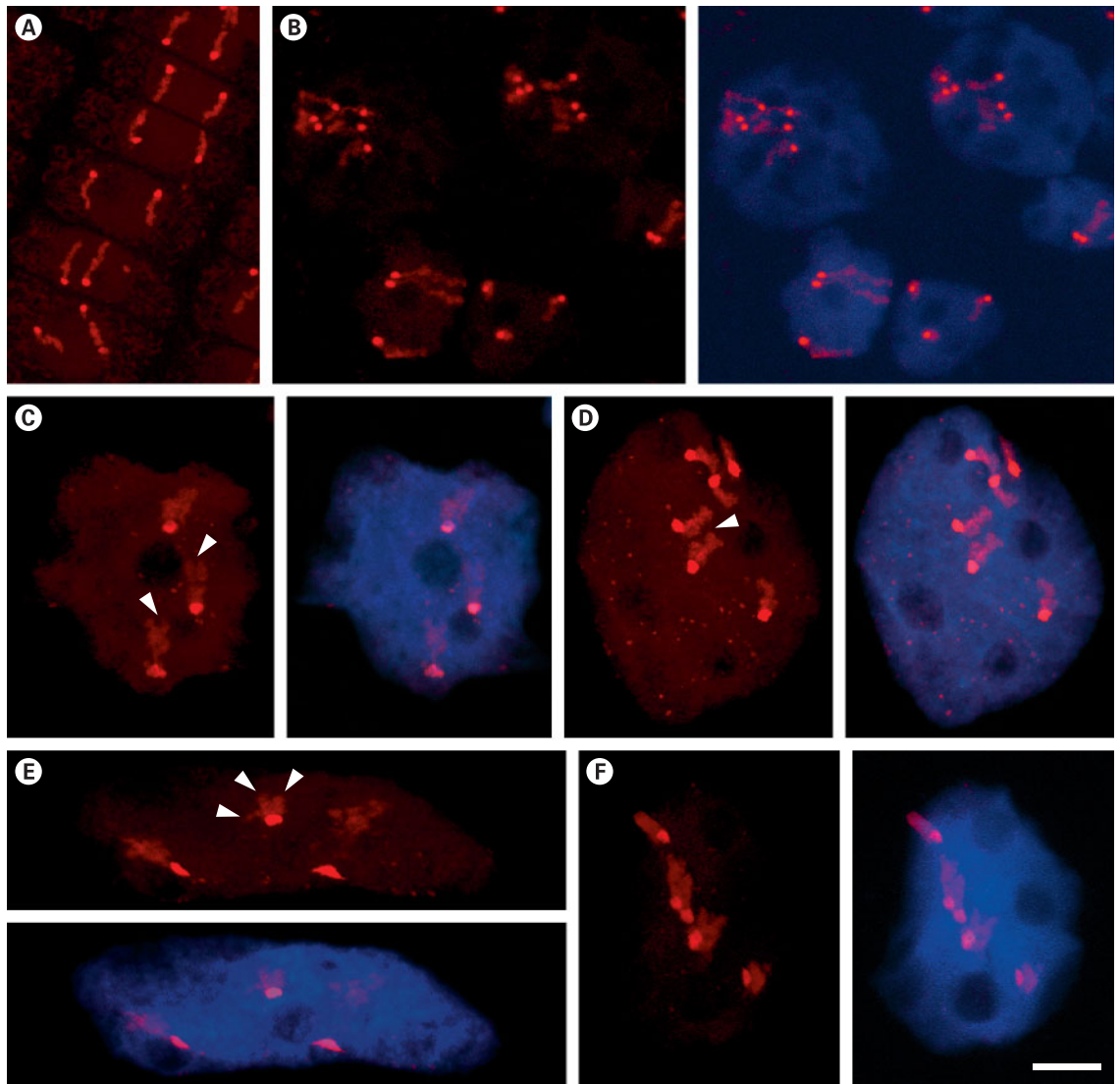


Fig. 3. Rye chromosome arms in a wheat background span the whole of the nucleus in both embryo and endosperm and are not grouped together. Confocal stacks spanning the depth of the nuclei. Rye arms in red with a bright subteleromic heterochromatin knob, DAPI counterstaining of the nuclei in blue. (A) Embryo root showing two rye arms. (B) to (F) endosperm. (B) 7 dpa. The top two nuclei are bigger than other nuclei at this stage, show six separate rye arms and occur only occasionally in central parts of the endosperm. (C) to (F) 16 dpa. (C) 6C nucleus with three rye arms. Both chro-

matids overlap at the telomeres, but separate towards the centromeres (arrow heads). (D) 12C nucleus with six rye arms. Chromatids of two rye chromosome arms seem to separate towards the centromeres (arrow head). (E) 12C nucleus where several chromatids remain associated (arrow heads). (F) 6C nucleus with chromatids at different stages of separation: at the periphery two single chromatids, in the centre two chromatids that are splitting at the centromeres and two chromatids lying next to each other with two discernable telomeres. Bar, 10  $\mu$ m.

nucleus. We determined the C content in embryo nuclei by flow cytometry in six independent experiments and found 85% ( $\pm 3$ ) 2C nuclei and 14% ( $\pm 2$ ) 4C nuclei. In one experiment 5% 8C nuclei were found, while the other samples contained insignificant amounts. The centromere data for the embryo therefore mainly reflected the numbers from 2C nuclei. In endosperm, 6C nuclei showed twice as many centromere signals as 3C ones (92 compared to 48) (Table 1). Based on a haploid chromosome number of 21 we would expect 63 chromatids for 3C nuclei and 126 for 6C nuclei. Thus, an average of 48% of the centromeres in 3C and 54% of the centromeres in

6C nuclei were associated (Table 1). Plotting centromere counts against telomere counts in 53 endosperm nuclei shows that the higher the C-content the more telomeres associate in endosperm: 50 centromeres corresponding to 95 telomeres, 100 centromeres corresponding to 139 telomeres and 200 centromeres to 225 telomeres (Fig. 2).

Labelling of the rye chromosome arms showed that both centromere and telomere associations in embryo and endosperm are non-homologous, since the two or three labelled homologous chromosome arms in embryo or endosperm respectively are clearly separated (Fig. 3). The three rye chro-

Table 1. Centromere numbers in endosperm and embryo

Tissue	Age	Centromeres	Nuclei counted	% centromere association
Embryo	24 dpa	27.2 ± 6.0	41 (2C + 4C <sup>a</sup> )	35 ± 14
Endosperm	7 dpa	47.8 ± 7.5	39 (3C)	48 ± 24
Endosperm	16 dpa	92.3 ± 11.6	19 (6C)	54 ± 18

<sup>a</sup> A DNA content of 2C was measured in 85% of embryo nuclei.

some arms in 6C nuclei were broader than those in 3C nuclei and often separated towards the centromeres (Fig. 3B, C). These observations provide an explanation for the results of the centromere and telomere counts in endosperm: after S-phase, the centromeres begin to dissociate, resulting in a doubling of the number of visible sites, while the telomeres have a strong tendency to remain associated, resulting in a significantly smaller number of visible sites than expected. Some medium sized nuclei, presumably with a DNA content of 6C, showed six separate rye chromosome arms, most notably a few cells at 6 dpa (Fig. 3B). 12C nuclei usually contained six rye chromosome arms (Fig. 3D). Occasionally nuclei with separating chromatids were seen (Fig. 3F), but we found only two 12C nuclei out of 14 examined where the chromosome arms remained associated in three groups (Fig. 3E).

## Discussion

In this study we have shown that the DNA increase in wheat endosperm leads to both polyploidy and polyteny. Evidence for the former comes from the doubling of centromere numbers in 6C nuclei compared to 3C nuclei and the observation of six rye chromosome arms in these cells. Evidence for the latter comes from the frequent occurrence of 12C nuclei with six rye chromosome arms, which must each carry two chromatids and the occasional observation of 12C nuclei with three groups of rye chromosome arms, which must have four chromatids (Fig. 3D, E). In contrast to this, in developing xylem vessel cells in wheat roots, two regions of labelled rye chromatin remained through successive rounds of endoreduplication producing polytene chromosomes (Martínez-Pérez et al., 2001). In the most extensively studied cereal, maize, evidence exists for both polyteny, which seems to play the major part, and polyploidy, which was found in some nuclei in younger, actively dividing endosperm tissue (for an overview see Kowles and Phillips, 1988). The latter corresponds to our findings of nuclei with six separated rye chromosome arms at 7 dpa. Our image data show that chromatids separate along their lengths and later assume widely separated positions within the nucleus (Fig. 3F).

A process leading to the doubling of chromosomes within a nucleus was first described by Geitler (1939): in endomitosis chromosomes contract, the chromatids separate parallel to each other without the formation of a spindle apparatus and decondense again to their interphase conformation while the nuclear envelope remains intact throughout. Endomitosis occurs more frequently in animals and humans and has been

studied intensively in megakaryocytes, which are a special case because the nuclear envelope breaks down in each cycle and a spindle is formed (Italiano and Shivdasani, 2003). In some plants, endomitosis has been found in the cells of the anther tapetum (Oksala and Therman, 1977; D'Amato, 1984). In wheat tapetal cells nuclei divide to form binucleate cells (Bennett et al., 1973). In contrast to the data presented for the tapetum, the separation of chromatids in wheat endosperm does not always happen simultaneously in all chromosomes (Fig. 3F). Cell cycle regulators have been shown to be involved in megakaryocyte endomitosis (Italiano and Shivdasani, 2003) and in endoreduplication in maize endosperm (Grafi and Larkins, 1995). No data are available yet on the regulation of the spindle formation in megakaryocytes. Spindle formation during endomitosis in wheat endosperm is unlikely since chromatid separation is not synchronized, but as the six rye chromosomes are widely distributed, the microtubule network might play a role in the movement of the separated chromatids away from each other.

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