The Plant Cell, Vol. 17, 2431–2438, September 2005, www.plantcell.org © 2005 American Society of Plant Biologists

RESEARCH ARTICLES

Uniparental Chromosome Elimination at Mitosis and Interphase in Wheat and Pearl Millet Crosses Involves Micronucleus Formation, Progressive Heterochromatinization, and DNA Fragmentation

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Complete uniparental chromosome elimination occurs in several interspecific hybrids of plants. We studied the mechanisms underlying selective elimination of the paternal chromosomes during the development of wheat (*Triticum aestivum*) × pearl millet (*Pennisetum glaucum*) hybrid embryos. All pearl millet chromosomes were eliminated in a random sequence between 6 and 23 d after pollination. Parental genomes were spatially separated within the hybrid nucleus, and pearl millet chromatin destined for elimination occupied peripheral interphase positions. Structural reorganization of the paternal chromosomes occurred, and mitotic behavior differed between the parental chromosomes. We provide evidence for a novel chromosome elimination pathway that involves the formation of nuclear extrusions during interphase in addition to postmitotically formed micronuclei. The chromatin structure of nuclei and micronuclei is different, and heterochromatinization and DNA fragmentation of micronucleated pearl millet chromatin is the final step during haploidization.

INTRODUCTION

After interspecific fertilization, two different parental genomes are combined within one nucleus, which, in most cases, is embedded within the maternal cytoplasm. Such a novel genomic constitution may result in intergenomic conflicts leading to genetic and epigenetic reorganization (Riddle and Birchler, 2003). Even if in most cases the parental genomes remain combined after a successful fertilization, an elimination of specific DNA sequences frequently follows in the early stages of allopolyploidization (Liu et al., 1996; Feldman et al., 1997). In grasses, a partial somatic elimination of chromosomes from one parental species may occur, for example, in wide crosses of *Hordeum lechleri* × *H. vulgare* (Linde-Laursen and von Bothmer, 1999), *Avena sativa* × *Zea mays* (Riera Lizarazu et al., 1996), or *Triticum aestivum* × *H. vulgare* (Barclay, 1975). Complete uniparental chromosome elimination also occurs in some interspecific hybrids between closely related species (as *H. vulgare* or *H. parodii* × *H. bulbosum* and *H. marinum* × *H. vulgare*; Kasha and Kao, 1970; Subrahmanyam, 1977; Finch, 1983) as well as between remotely related parental species (*Aegilops* spp, ryegrass [*Lolium multiflorum*], barley, oat, rye [*Secale cereale*], or wheat × *Pennisetum glaucum*, *Sorghum bicolor*, *Tripsacum dactyloides*, or *Z. mays*; Zenkteler and Nitzsche, 1984; Laurie and Bennett, 1986, 1988; Rines and Dahleen, 1990; Chen and Hayes, 1991; Matzk and Mahn, 1994; Matzk, 1996; Matzk et al., 1997).

Crosses between wheat and maize and between *H. vulgare* × *H. bulbosum* are used for generating homozygous doubled haploid wheat and barley plants, respectively, from heterozygous maternal plants. The elimination rate of *H. bulbosum* chromosomes in *H. vulgare* × *H. bulbosum* hybrid embryos is affected by temperature (Pickering, 1985; Pickering and Morgan, 1985) and by the ploidy level of the *H. bulbosum* genome (Ho and Kasha, 1975). A tissue-specific elimination of alternative whole parental genomes was observed in the embryo and endosperm of *H. marinum* × *H. vulgare* crosses (Finch, 1983). Elimination of parental chromosomes has also been observed in somatically produced wide hybrids. In these cases, the elimination tends to be irregular and incomplete, leading to asymmetric hybrids or cybrids (Liu et al., 2005). In several metazoa, such as nematodes, copepods, sciarid flies (Goday and Ruiz, 2002), hagfish, and marsupials, chromatin/chromosome elimination is part of normal cell differentiation and/or sex determination (Kloc and Zagrodzinska, 2001).

Several hypotheses have been presented to explain uniparental chromosome elimination during hybrid embryo development in plants, for example, differences in timing of essential mitotic processes due to asynchronous cell cycles (Gupta, 1969) or asynchrony in nucleoprotein synthesis leading to a loss of the most retarded chromosomes (Bennett et al., 1976; Laurie and Bennett, 1989). Other hypotheses propose the formation of multipolar spindles (Subrahmanyam and Kasha, 1973), spatial

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.105.034249.

separation of genomes during interphase (Finch and Bennett, 1983; Linde-Laursen and von Bothmer, 1999) and metaphase (Schwarzacher-Robinson et al., 1987), parent-specific inactivation of centromeres (Finch, 1983; Kim et al., 2002; Jin et al., 2004; Mochida et al., 2004), and by analogy with the host-restriction and modification systems of bacteria (Boyer, 1971), degradation of alien chromosomes by host-specific nuclease activity (Davies, 1974).

Initial cytological studies revealed a rapid preferential uniparental chromosome loss by formation of micronuclei during mitosis in early hybrid embryos (Kasha and Kao, 1970). Chromosomes destined for elimination often did not congregate properly at metaphase and lagged behind other chromosomes at anaphase (Laurie and Bennett, 1989). These observations are consistent with the classical mechanism of micronucleus formation, which involves the enclosure of lagging chromosome fragments during reformation of nuclear membranes at the end of mitosis (Heddle and Carrano, 1977; Schubert and Oud, 1997). It is not yet clear how the micronucleated paternal genome is finally eliminated.

This work provides a more detailed insight into the processes of selective elimination of paternal chromosomes during the development of wheat × pearl millet hybrid embryos. The selective elimination of pearl millet chromosomes was found to consist of consecutive steps: parental interphase chromatin separation, micronucleus formation, heterochromatinization, and DNA fragmentation of micronucleated chromatin. In addition to mitotic micronucleus formation by nonsegregating chromatids, pearl millet chromatin-containing micronuclei are extruded directly from interphase nuclei.

RESULTS

The Elimination of Pearl Millet Chromatin in Developing Hybrid Embryos Is Sequential

First, we studied the distribution of pearl millet chromatin in morphologically well preserved 6-d-old wheat × pearl millet embryos by whole-mount genomic in situ hybridization (GISH) (Figures 1A and 1B). Wheat line S6, the female parent, carries a translocation (1B/1R) with the short arm of the rye chromosome 1R (Matzk et al., 1997). Therefore, as an internal control, GISH with differently labeled genomic DNA probes of pearl millet and rye yielded a rye-specific hybridization signal in almost all nuclei of the embryos independent of the developmental stage (Figures 1E and 1F). By contrast, the percentage of cells with a pearl millet–specific signal varied between embryos at different stages as well as between embryos at the same stage and between

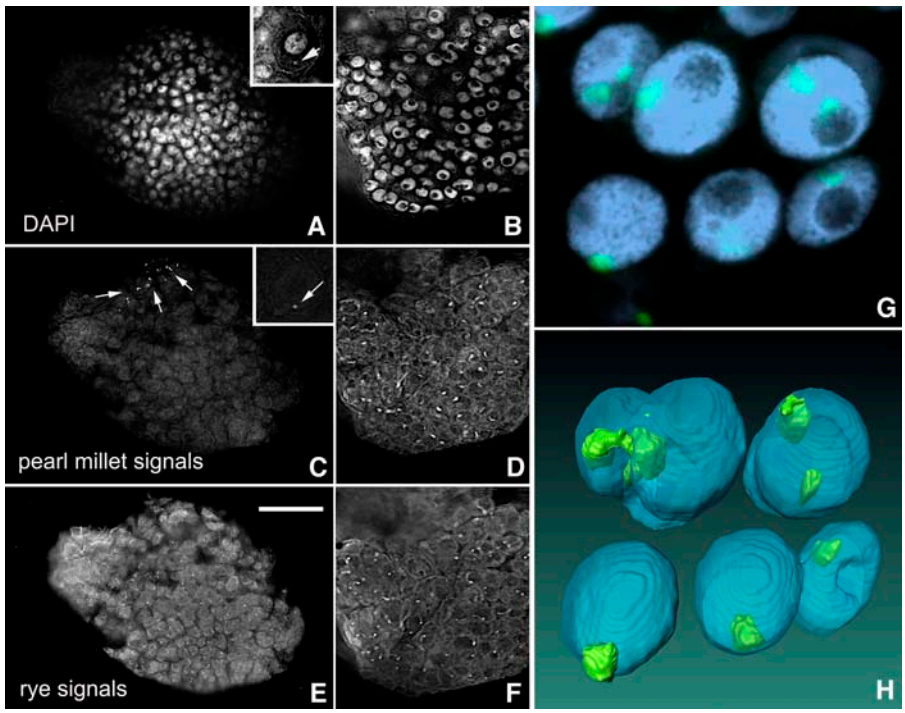

Figure 1. Distribution of Pearl Millet Chromatin in Wheat × Pearl Millet Embryos.

(A) to **(F)** Whole-mount GISH on two 4′,6-diamidino-2-phenylindole (DAPI)-stained 6-d-old wheat × pearl hybrid embryos (**[A]** and **[B]**) with pearl millet DNA (**[C]** and **[D]**) and rye DNA (**[E]** and **[F]**). Note that the proportion of cells with pearl millet chromatin varies between embryos of the same age. Pearl millet chromatin was within small (**[C]**, arrows) or large **(D)** cell clusters. Further enlarged cells with a pearl millet DNA-positive micronucleus (arrows) are shown in the insets in **(A)** and **(C)**. Rye-specific hybridization signals are clearly visible in almost all embryonic nuclei (**[E]** and **[F]**). Bar in **(E)** = 50 μm. **(G)** and **(H)** Selected interphase nuclei after whole-mount GISH with labeled pearl millet DNA before **(G)** and after **(H)** three-dimensional modeling. Pearl millet chromatin (in green) occupies a predominantly peripheral position. The DAPI-stained wheat chromatin is indicated in blue.

different regions of individual embryos. Irrespective of the region within the embryo, the pearl millet–specific signals were detected in small (Figure 1C) or large (Figure 1D) cell clusters. Pearl millet–positive chromatin was observed inside and/or outside the major nucleus during interphase. The external hybridization signals coincided with the positions of one or more additional micronuclei (Figures 1A and 1C, insets). Inside the nucleus, the pearl millet chromatin usually occupied one or two spherical or spindle shaped territories (Figures 1G and 1H). Three-dimensional reconstruction of interphase nuclei clearly demonstrated that the parental genomes were spatially separated and tended to occupy distinct domains within the interphase nuclei. Pearl millet chromatin destined for elimination was found to occupy peripheral positions (Figure 1H). In contrast with the more condensed pearl millet chromatin, the rye chromatin revealed a partly decondensed string-like appearance.

To analyze the temporal progression of chromosome elimination during embryo development, GISH was performed on 83 squash preparations made from embryos 6 to 23 d after pollination (DAP). The number of pearl millet chromatin-containing nuclei decreased during embryo development in a manner that varied among embryos of the same stage (Figure 2). The highest percentage (30%) of cells containing pearl millet–positive micronuclei was observed in embryos 6 to 8 DAP. In embryos 17 to 23 DAP, micronuclei were only occasionally observed. Pearl millet–specific signals were detected only in a few cell clusters of embryos older than 19 d, suggesting that a minority of pearl millet chromatin undergoes a slow rate of elimination that allows it to be retained for a long period. To test whether or not elimination was completed, DNA gel blot hybridization with a pearl millet centromere-specific repeat as a probe was performed on DNA from potted plants. Eight out of 178 young plantlets still revealed weak pearl millet–specific signals. When the same plants were reanalyzed at the mature stage, no signals remained.

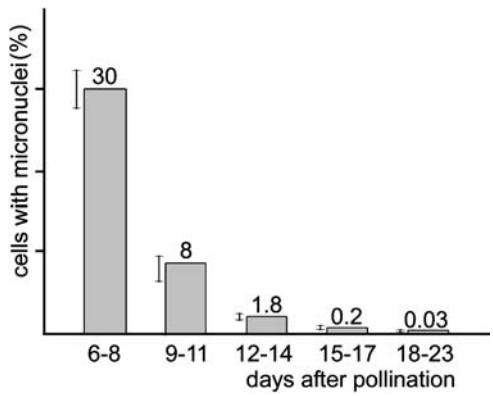

Figure 2. The Percentage of Cells Containing Micronuclei in Wheat × Pearl Millet Hybrid Embryos at Different Developmental Stages.

The histogram is based on the analysis of 1337 cells from 19 embryos 6 to 8 DAP, of 1573 cells from 10 embryos 9 to 11 DAP, of 4691 cells from 16 embryos 12 to 14 DAP, of 11,349 cells from 19 embryos 15 to 17 DAP, and of 12,858 cells from 19 embryos 18 to 23 DAP. The 95% confidence intervals are indicated as a bar to the left of each column.

Pearl Millet Chromosomes Are Structurally Rearranged and Become Reduced in Size in Hybrid Embryos

At mitosis, besides the standard type of metaphase chromosomes, dicentric pearl millet chromosomes of unusual size were identified after simultaneous hybridization with labeled genomic DNA and the pearl millet centromere-specific probe (Figures 3A and 3B). The additional centromere could result from a centric translocation or a chromosome fusion event. In interphase nuclei older than 17 DAP, most of the pearl millet chromosomes were reduced in size and displayed centromere-specific signals with no, or only minor, traces of chromosome arm-specific signals (Figures 3C and 3D). Pearl millet–specific chromatin without centromeric signals was observed in <1% of embryos. These observations indicate that pearl millet chromosomes are eliminated in portions with the centromere region remaining until last.

Pearl Millet Chromosomes Form Micronuclei during Cell Division as well as during Interphase

The mitotic behavior of pearl millet chromosomes was analyzed to determine whether micronuclei are formed exclusively by nonsegregating chromosomes as is usually assumed (Ford and Correll, 1992). The segregation behavior differed between pearl millet and wheat chromosomes. At anaphase, some pearl millet chromosomes lagged behind wheat chromosomes, and the sister chromatids segregated asymmetrically (Figure 3F). The level of chromosome condensation also partially differed between the parental genomes, with chromosomes of pearl millet often less condensed (Figure 3F, arrows). This observation is consistent with a loss of paternal chromosomes during cell division via lagging chromosomes that form micronuclei (Laurie, 1989; Mochida et al., 2004). In addition, pearl millet chromatin bodies similar to micronuclei in shape and size were found attached to the main interphase nuclei (Figures 3G and 3H), suggesting that they are extruded directly at interphase. Hence, budding of pearl millet chromatin seems to be another pathway of micronucleus formation and specific genome exclusion.

Alternatively, buds of pearl millet chromatin might represent micronuclei fusing with the main nucleus, but this would reverse the process of elimination. The size of micronuclei containing chromatin of pearl millet varied considerably (Figures 3I to 3L). To determine the number of pearl millet chromosomes per micronucleus, pearl millet centromere-specific probes were hybridized in situ together with labeled pearl millet genomic DNA. In >80% of micronuclei, one to three pearl millet centromeres were counted (Figure 3I), and in the early stages of embryo development (6 DAP), large micronuclei with up to seven pearl millet centromeres occurred (Figure 3J). Hence, the entire haploid pearl millet genome can either be eliminated concomitantly, either as individual chromosomes, or fused together prior to exclusion.

Micronuclei without centromeric sequences of pearl millet were rarely observed (0.5% of micronuclei; Figure 3L). Only 5% of the micronuclei also contained traces of maternal chromatin (Figure 3K), and those containing wheat chromatin alone were extremely rare (0.2% of micronuclei; data not shown). This indicates that the majority of micronuclei contained at least one pearl millet chromosome and possibly acentric chromosome fragments.

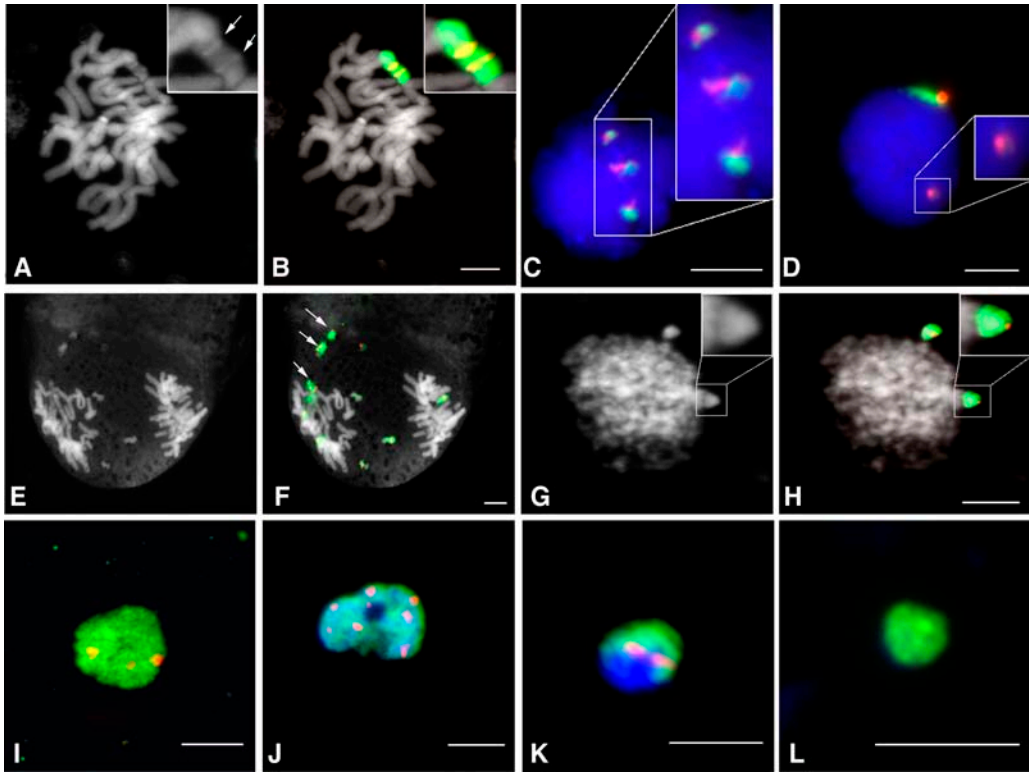

Figure 3. Elimination Process of Pearl Millet Chromosomes during Cell Division as well as during Interphase.

Dividing cells (**[A]**, **[E]**, and **[F]**), interphase nuclei (**[C]**, **[D]**, **[G]**, and **[H]**), and micronuclei (**[I]** to **[L]**) of wheat × pearl millet embryos after in situ hybridization with pearl millet genomic DNA (green) and pearl millet centromeric sequences (red). Note dicentric pearl millet chromosomes (**[A]** and **[B]**, arrows), exclusively labeled pearl millet centromeric signals **(D)**, and centromeric signals associated with very small amounts of pearl millet chromatin **(C)**. Anaphase with lagging asymmetric pearl millet chromosomes (**[E]** and **[F]**). Interphase nuclei with budding pearl millet chromatin (**[G]** and **[H]**). Centromere-containing micronuclei (**[I]** to **[K]**); centromere-free micronucleus **(L)**. The micronucleus in **(K)** contains traces of unlabeled wheat chromatin. Bars = 5 μm.

Chromatin Structure Differs between Nuclei and Micronuclei, and Degradation of Micronucleated DNA Is the Final Step in Chromosome Elimination

Ultrastructural studies showed that micronuclei are surrounded by a double membrane with nuclear pores like normal nuclei (Figures 4C and 4D, arrowheads). However, the different staining intensities of the heterochromatin between nuclei and micronuclei indicate a different degree of chromatin condensation. Micronuclei contained either exclusively heterochromatin (Figures 4A and 4C) or a mixture of euchromatin and heterochromatin (Figures 4B and 4D). The latter micronuclei resembled the normal nucleus of the same cell except that the heterochromatin was more dense (Figure 4B). These micronuclei were significantly larger than those that were predominantly heterochromatic (cf. Figures 4C and 4D).

To analyze the final step of elimination, the integrity of pearl millet DNA in micronuclei was tested by combining GISH with terminal dUTP nick end-labeling (TUNEL) assays. Some micronuclei with pearl millet chromatin displayed strong TUNEL signals (Figure 5), indicating that their DNA was strongly fragmented. No DNA cleavage was found in wheat chromatin-containing major nuclei. Apparently, pearl millet chromatin initially undergoes extensive fragmentation immediately prior to haploidization of the maternal genome. Together, the experimental results suggested a multistep model for pearl millet chromatin elimination from hybrid embryos (Figure 6).

DISCUSSION

The combined analyses of wheat × pearl millet crosses indicate that uniparental chromosome elimination in developing hybrid embryos occurs in a complex stepwise manner. We confirmed that mitotic chromosome elimination starts immediately after fertilization (Laurie and Bennett, 1989; Mochida et al., 2004), but contrary to previous reports, we found chromatin of both parental species still present in mature embryos. We show that heterochromatinization and DNA fragmentation in micronuclei formed by extrusion of paternal chromatin from interphase nuclei is involved in the pathway of haploidization, in addition to the formation of micronuclei after nonsegregation of paternal chromosomes or fragments during karyokinesis (Subrahmanyam and Kasha, 1973; Bennett et al., 1976; Thomas, 1988; Mochida and Tsujimoto, 2001; Mochida et al., 2004). On the one hand, the

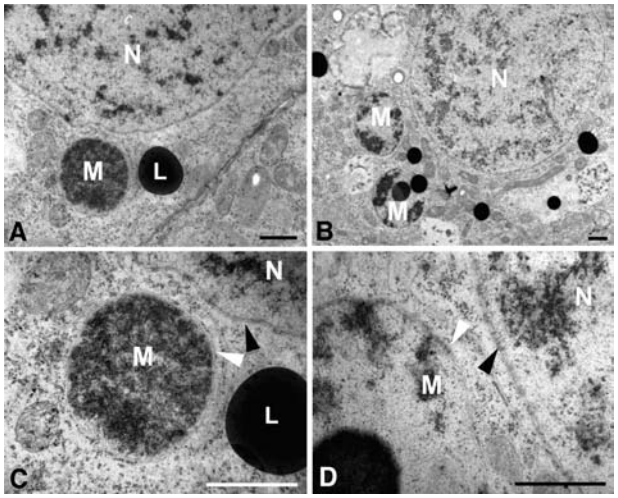

Figure 4. The Morphology of Nuclei and Micronuclei in Wheat × Pearl Millet Hybrid Embryos.

(A) The electron density of the small micronucleus (M) indicates a high content of heterochromatin compared with the nucleus (N). L, lipid droplet.
(B) Cell with two large micronuclei each containing a substantial amount of euchromatin.
(C) Higher magnification reveals double membranes surrounding the nucleus (black arrowhead) and the micronucleus (white arrowhead).
(D) Double membranes of nucleus and micronucleus with nuclear pores (black and white arrowheads); note that the heterochromatin of the micronucleus is more electron dense than that of the nucleus.
Bars = 1 μm.

elimination of pearl millet chromosomes was complete in adult plants, which is an important prerequisite for the generation of homozygous doubled haploid wheat plants. On the other hand, the observed late completion of elimination might increase the potential for chromatin introgression from pearl millet into wheat.

The distinct peripheral localization of the pearl millet chromatin during interphase may indicate that the interphase arrangement of both parental genomes differs. While wheat chromosomes follow the Rabl-orientation with centromeres clustered at one pole and telomeres at the opposite one (Dong and Jiang, 1998), chromosomes of pearl millet do not (our unpublished data). However, this difference cannot be a general reason for uniparental elimination of chromosomes in hybrids since chromosomes of both *H. vulgare* and *H. bulbosum* show Rabl-orientation, albeit that *H. bulbosum* chromosomes disposed at the periphery are often excluded from the daughter cells (Finch, 1983; Kim et al., 2002).

The selective degradation of pearl millet chromosomes could be triggered by asynchronous DNA replication of the two parental genomes. Inhibition of DNA replication induces DNA double strand breaks and genome rearrangements (Michel, 2000). Assuming that the timing of DNA replication differs between wheat and pearl millet as reported for the parental genomes of *Nicotiana tabacum* hybrids (Gupta, 1969), asynchrony of DNA replication may lead to breakages of pearl millet chromosomes. Subsequent rearrangements of the paternal genome might result in the observed dicentric or shortened pearl millet chromosomes. Alternatively, a hybridization-mediated genomic shock (McClintock, 1984) might trigger a genome-specific activation of mobile elements and thus cause structural chromosome aberrations as reported for artificial allopolyploids of *Arabidopsis thaliana* (Comai et al., 2000) and wheat (Kashkush et al., 2002) as well as for mammalian hybrids (O'Neill et al., 1998).

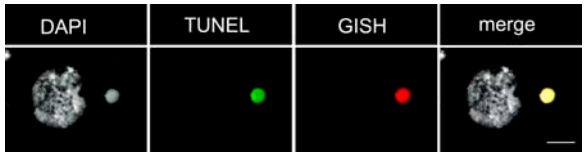

Figure 5. Interphase Nucleus of a Wheat × Pearl Millet Hybrid Embryo with a Micronucleus-Containing Pearl Millet Chromatin after TUNEL (Green) and GISH (Red) Experiments.

The TUNEL signal indicates fragmentation of micronucleated pearl millet DNA. Bar = 5 μm.

The centromere regions of pearl millet chromosomes are eliminated last. This might be due to its mobile and heterochromatic nature or to the absolute requirement for mitotic competence of surviving chromatin. If such a centric fragment is retained rather than lost during elimination of parental chromosomes, a subsequent spontaneous chromosome doubling could provide an ideal prerequisite for the de novo formation of supernumerary chromosomes, a scenario similar to that described in *Coix* (Sapre and Deshpande, 1987), where a B chromosome was generated spontaneously as a result of the crossing of two species.

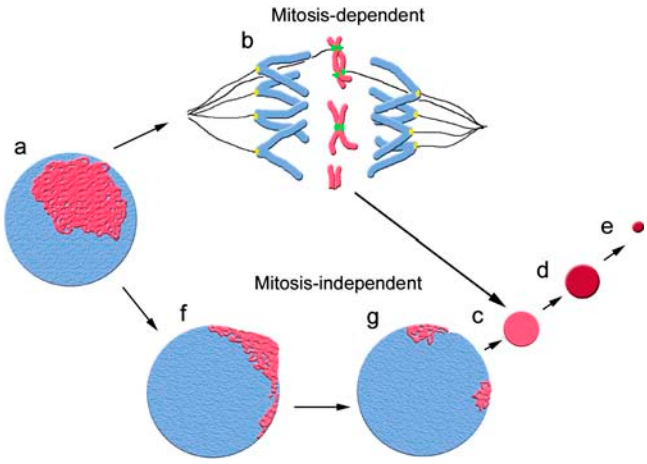

Figure 6. Schematic for the Mitotic and Interphase Elimination of Pearl Millet Chromosomes from Wheat × Pearl Millet Hybrid Embryos.

Mitotic elimination: (a) spatial separation of parental genomes; (b) imperfect segregation of pearl millet chromosomes caused by (1) faulty kinetochore/spindle fiber interaction, (2) the presence of an additional centromere, or (3) absence of a centromere; (c) formation of micronucleus; (d) heterochromatinization and DNA fragmentation of micronucleus; (e) disintegration of micronucleus. Interphase elimination: (a) spatial separation of parental genomes; (f) budding of pearl millet chromatin; (g) release of pearl millet chromatin-containing micronucleus; (c) formation of micronucleus; (d) heterochromatinization and DNA fragmentation of micronucleus; (e) disintegration of micronucleus.

Our finding of a selective elimination of pearl millet chromatin through extrusion from hybrid nuclei during interphase and subsequent micronucleation is consistent with the interpretation that nuclear buds containing extrachromosomal elements, such as double minutes, may form micronuclei in mammalian tumor cells during interphase (Shimizu et al., 1998; Tanaka and Shimizu, 2000). The striking similarities to a mechanism for formation of micronuclei in mammals may indicate an evolutionarily conserved process that allows intact maternal chromatin/DNA and alien chromatin/DNA to be distinguished and the latter to be removed from the nucleus. The detailed mechanism by which pearl millet chromatin is extruded through the nuclear double membrane remains unclear. In *Sciara* flies, the selective extrusion of chromosomes during interphase is accompanied by a local accumulation of rough endoplasmatic reticulum and mitochondria at the potential site of chromosome elimination. This is compatible with the idea of a localized membrane synthesis necessary to produce a nuclear bulge (Perondini and Ribeiro, 1997).

Our ultrastructural data indicate an increased condensation level of micronucleated chromatin and reveal a correlation between the size of micronuclei and the ratio of euchromatin and heterochromatin. Large micronuclei always contain a large amount of euchromatin, whereas small micronuclei are almost completely heterochromatic. It is tempting to speculate that these variations in ultrastructure reflect the gradual degradation of micronuclei from relatively large, euchromatin-containing structures into small strongly heterochromatic ones.

The process of elimination of micronuclei is similar, and partly analogous, to the events observed during programmed cell death. Nuclei undergoing an apoptosis-like death (Fukuda, 2000) and micronuclei with pearl millet chromatin exhibit similar features, such as chromatin condensation, nuclear shrinkage, and DNA fragmentation. The recognition and consecutive elimination of pearl millet DNA via micronuclei seem to be regulated processes. A specific chromatin topology possibly dictates endonuclease activation and genome-specific fragmentation. The drastic changes in the integrity of DNA and chromatin compaction during uniparental genome elimination suggest that posttranslational histone modification might play a role in promoting and directing these changes. Heterochromatinization and compaction of chromatin is associated with developmentally determined chromosome elimination in *Sciara*. Differential acetylation of histones H3 and H4 and methylation of histone H3 are candidate drivers of chromosome elimination in sciarid flies (Goday and Ruiz, 2002) and in the programmed DNA elimination process that accompanies macronuclear development in *Tetrahymena* (Taverna et al., 2002). For wheat × pearl millet embryos, it remains to be seen whether modification of histones or other chromatin proteins differs between the parental genomes.

METHODS

Plant Material, in Vitro Culture of Hybrid Zygotes, and Preparation of Embryos

The Salmon line (S6) of hexaploid wheat (*Triticum aestivum*, $2n = 6x = 42$) was used as female parent (Matzk et al., 1997). S6 carries a translocation of the short arm of rye (*Secale cereale*) chromosome 1R into wheat chromosome 1B (1B/1R). The accession PEN 5/78 (Institute of Plant Genetics and Crop Plant Research) of pearl millet (*Pennisetum glaucum*, $2n = 2x = 14$) was used as pollen donor. Wheat spikes were emasculated 1 to 2 d before anthesis and pollinated 1 d later with fresh pearl millet pollen or with dehiscing anthers (Kumlehn et al., 1997). In order to minimize the complexity of steps involved in the process of chromosome elimination, we kept the plant growing conditions constant at 16 h light with ~20°C and 8 h dark with ~14°C.

For isolation of hybrid zygotes, 2 to 4 h after hand-pollination, spikes were cut off and surface-sterilized for 10 min in 2.5% NaOCl solution supplemented with 0.01% Tween 20. Zygote isolation was performed as described (Kumlehn et al., 1997). Immature wheat pistils used for conditioning zygote cultures were isolated under sterile conditions from spikes that had emerged 2 to 4 cm from the flag leaf sheath. Zygotes were cultured in 12-mm Millicell inserts (Millipore); each zygote was placed into a 35-mm Petri dish containing 3 mL of N6Z medium (Kumlehn et al., 1998). Half of the medium used had been preconditioned for 2 weeks by culturing four immature wheat pistils per milliliter. Prior to zygote transfer, 200 μL of the culture medium was transferred from the Petri dish to the Millicell insert. Zygotes were taken up with a glass capillary with an opening diameter of 100 μm. The capillary was interfaced to a Cell Tram Vario (Eppendorf) by Teflon tubing filled with 0.55 M mannitol. Liquid uptake and release were regulated by manually operating the Cell Tram. The zygotes were released onto the semipermeable membrane of a Millicell insert. For further conditioning, six precultured wheat pistils were added per Petri dish outside the Millicell insert. All cultures were incubated in the dark at 24°C for 2 to 4 d.

Embryo development was stimulated by dipping the spikes into an aqueous solution of 50 ppm Dicamba (Sigma-Aldrich) 2 DAP. Embryo rescue was necessary to generate plants from mature embryos. Therefore, embryos were excised ~18 DAP and placed on Kruse medium under sterile conditions (Matzk and Mahn, 1994).

For in situ hybridization of in vivo–grown embryos, ovaries were dissected 6 to 23 DAP, fixed in ethanol:acetic acid (3:1), and stored at 4°C. To isolate the embryos, ovaries were stained with acetocarmine. Embryos were dissected in distilled water with fine needles under a stereomicroscope. For preparation of plant specimens, isolated embryos were squashed in 45% acetic acid between slide and cover slip.

Fluorescence in Situ Hybridization on Squashed and Whole-Mount Embryos and Construction of a Three-Dimensional Image

A member of the pearl millet centromere-specific sequence repeat family, pPgKB1 (Kamm et al., 1994), and total genomic DNA of pearl millet and rye were used as probes for fluorescence in situ hybridization (FISH) after labeling by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP.

FISH on squashed embryos was performed as previously described (Houben et al., 2001). Briefly, 80 ng biotin/digoxigenin-labeled genomic DNA, 50 ng digoxigenin-labeled pearl millet centromere-specific sequence, and 800 ng of sonicated, unlabeled total wheat genomic DNA (used as competitor to suppress nonspecific hybridization) were applied per slide. Hybridization sites of digoxigenin- or biotin-labeled probes were detected using sheep antidigoxigenin-rhodamine, rhodamine anti-sheep antibody, or the streptavidin Alexa 488 system, respectively. Epifluorescence signals were recorded with a cooled CCD camera (ORCA-ER; Hamamatsu). The images were optimized for contrast and brightness with Adobe Photoshop 7.0.

For whole-mount GISH (Caperta et al., 2002; Santos et al., 2002), embryos were isolated and fixed in 4% (w/v) formaldehyde freshly prepared from paraformaldehyde (PFA) in MTSB buffer (50 mM PIPES, 5 mM $MgSO_4$, and 5 mM EGTA, pH 6.9) under vacuum for 20 min at room temperature prior to washing in MTSB for 10 min. Tissues were made permeable by incubating with 2% (w/v) cellulase Onozuka R10 (Serva)

and 2% (w/v) pectinase (Sigma-Aldrich) in citrate buffer for 70 min at 37°C. Embryos were subsequently washed in 2 × SSC (standard saline citrate) for 10 min and allowed to dry on multiwell slides. GISH was performed as described for squashed embryos.

A three-dimensional model of in situ–hybridized interphase nuclei was reconstructed using high-level image processing techniques. First, optical section stacks were collected with a Zeiss 510 meta confocal laser scanning microscope. Automatic procedures for the segmentation of the nuclei and the paternal DNA have been implemented with MATLAB (The MathWorks). This was mainly achieved by combining a priori knowledge, principal component analysis, three-dimensional watershed segmentation, and thresholding. Finally, the resulting two-dimensional gray-tone image stack containing the segmentation information was transformed into a three-dimensional model with the visualization and modeling software AMIRA (TGS Europe).

TUNEL Assay Combined with GISH

The TUNEL assay was conducted according to the manufacturer's instructions of the ApopTag apoptosis detection kit manual (S7110; Serological Corporation) with some modifications. Freshly dissected embryos were fixed for 20 min in ice-cold 4% PFA in PBS. After washing in cold PBS, embryos were made permeable by incubating with 2.5% cellulase Onozuka R10 (w/v) Serva, 2.5% (w/v) pectinase (Sigma-Aldrich), and 2.5% pectolyase Y-23 (Sigma-Aldrich) in PBS for 30 min at 37°C. The embryos were squashed in PBS between glass slide and cover slip. The cover slips were removed after freezing in liquid nitrogen. Air-dried specimens were postfixed in 4% PFA for 10 min at room temperature and then incubated in TUNEL-equilibration buffer for 5 min at room temperature. The end-labeling reaction was done by incubating the slides in terminal deoxynucleotidyl transferase in reaction buffer for 60 min at 37°C. Slides were then incubated in stop/wash buffer for 30 min at 37°C, washed three times for 3 min in PBS at room temperature, and stored in 70% ethanol at −20°C. After the TUNEL reaction, GISH was performed as described above. The transferase-incorporated digoxigenin-dUTP was detected with fluorescein isothiocyanate–conjugated sheep antidigoxigenin antibodies amplified by fluorescein-5-isothiocyanate–conjugated rabbit anti-sheep antibodies. In situ–hybridized pearl millet DNA labeled with biotin was detected with avidin-conjugated Texas red. Finally, slides were mounted with DAPI/Vectashield and analyzed under the conditions used for FISH. TUNEL-negative controls were performed without terminal deoxynucleotidyl transferase enzyme and positive controls with additional DNase I treatment as recommended by the manufacturers.

Transmission Electron Microscopy

Three-day-old embryos originated from in vitro–cultivated zygotes were fixed with 2% glutaraldehyde and 2% formaldehyde in cacodylate buffer (50 mM, pH 7.0) for 2 h. After three 15-min washes with the same buffer, the embryos were postfixed with 1% OsO_4 for 2 h and then washed again with buffer and distilled water before embedding in 1.5% low melting point agarose. Small blocks of 1 × 1 mm were cut, each containing a single embryo. These blocks were dehydrated in a graded ethanol series followed by embedding in Spurr's low viscosity resin. After thin sectioning, samples were stained with 4% uranyl acetate and lead citrate. Digital recordings were made on a Zeiss 902 electron microscope at 80 kV.

ACKNOWLEDGMENTS

We thank Ingo Schubert (Institute of Plant Genetics and Crop Plant Research), Richard Pickering (New Zealand Institute for Crop and Food Research, Christchurch, New Zealand), and Jeremy Timmis (University of Adelaide, Australia) for critical reading of the manuscript and anonymous reviewers for helpful comments. We are grateful to Katrin Kumke, Heike Büchner, Mechthild Pürschel, and Bernhard Claus (Institute of Plant Genetics and Crop Plant Research) for excellent technical assistance. This work was supported by the Bundesministerium für Bildung und Forschung (Berlin, Germany) and Icon Genetics GmbH (Halle, Germany).

Received May 12, 2005; revised July 1, 2005; accepted July 1, 2005; published July 29, 2005.

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Uniparental Chromosome Elimination at Mitosis and Interphase in Wheat and Pearl Millet Crosses Involves Micronucleus Formation, Progressive Heterochromatinization, and DNA Fragmentation

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Plant Cell 2005;17;2431-2438; originally published online July 29, 2005;
DOI 10.1105/tpc.105.034249