Heritable Loss of Replication Control of a Minichromosome Derived From the B Chromosome of Maize

Rick E. Masonbrink¹, Shulan Fu², Fangpu Han², & James A. Birchler¹*
¹Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA;
²State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese National Academy of Sciences, Beijing China

*Corresponding Author
Running Title: **Minichromosome Amplification**

Keywords: replication, minichromosome, B chromosome

Corresponding Author: James Birchler, 311 Tucker Hall, University of Missouri, Columbia, MO 65211, USA

Tel: (573) 882-4905; Fax: (573) 882-0123

E-mail address: birchlerj@missouri.edu
ABSTRACT

During an accumulation regime of a small telomere-truncated B chromosome, a derivative with large variations in size and multiple punctate centromere loci exhibiting amplified copy numbers was discovered. Multiple centromere satellite loci or transgene signals were documented in amplified chromosomes suggesting over-replication. Immunolocalization studies revealed multiple foci of biochemical markers characteristic of active centromeres such as CENP-C and phosphorylation of histones H3S10 and H2AThr133. The amplified chromosomes exhibit an absence of chromosome disjunction in meiosis I and an infrequent chromosome disjunction in meiosis II. Despite their unusual structure and behavior these chromosomes were observed in the lineage for seven generations during the course of this study. While severely truncated relative to a normal B chromosome, the progenitor minichromosome is estimated to be at least several megabases in size. Given that the centromere and transgene signals at opposite ends of the chromosome generally match in copy number, the replication control is apparently lost over several megabases.
Endoreduplication is essentially the replication of a genome without cell division, and is typically found in terminally differentiated cells in specialized tissues. This process is thought to increase the availability of DNA templates to amplify gene expression, because the transcriptional and translational activities of the cell increase proportionally with each genome doubling (D’AMATO 1984; LARKINS et al. 2001). Studies in plants and animals show that endoreduplication is correlated with the lowering of M-phase cyclins, responsible for the G2 to M transition, and with a higher abundance of S-phase cyclins, responsible for the G1 to S transition (EVANS et al. 1983). The oscillation of cyclins and a regulatory protein degraded by the anaphase promoting complex (Dbf4) regulate the activity of cyclin dependent kinases (CDK) and Dbf4 dependent kinases (DDK), respectively. They phosphorylate the mini-chromosome maintenance proteins 2-7 (MCM), which form the pre-replication complex necessary for replication licensing (TUTEJA et al. 2011). While all previous descriptions of endoreduplication involve whole genome duplication, (e.g. BAUER and BIRCHLER 2006), we describe a single, supernumerary chromosome that routinely amplifies copy number without separation while the remainder of the genome replicates normally.

The amplified chromosome originated from the B chromosome (B) of maize, a supernumerary chromosome that persists in the genome with a selfish inheritance resulting in higher-than-expected transmission (LONGLEY 1927). The B can accumulate in the genome by surviving meiosis as a univalent, nondisjunction at the second pollen mitosis, and preferential fertilization of the egg rather than the central cell by the B-
containing sperm, (Carlson 1969; Carlson and Roseman 1992; Roman 1948).

Preferential fertilization is associated with a single gene in the normal karyotype (A chromosomes), but nondisjunction and univalent survival are conditioned by the B chromosome (Carlson 1969; Carlson and Roseman 1992; Chiavarino et al. 2001).

Three regions of the B chromosome are essential for nondisjunction at the second pollen mitosis; one cis-acting site in the proximal heterochromatin and two trans-acting sites in the proximal and distal euchromatin (Carlson 1973; Carlson 1978; Lin 1978; RhoaDES and Dempsey 1972; RhoaDES et al. 1967; Roman 1949; Ward 1973).

Nondisjunction of the B chromosome has also been observed in the tapetum, endosperm, and roots, although these properties do not appear to contribute to its selfish inheritance (Alfenito and Birchler 1990; Chiavarino et al. 2000; Masonbrink and Birchler 2010).

Previously, maize embryos with B chromosomes were bombarded with telomere repeats resulting in chromosomal truncations of A and B chromosomes (Yu et al. 2007). One of these truncations (86-B23) had most of the B chromosome long arm removed, resulting in a chromosome slightly larger than a B chromosome centromere. This chromosome was accumulated to multiple numbers when a normal B chromosome was in the genome. During this program of accumulation, we fortuitously discovered a lineage in which this chromosome replicated multiple times, but remained a single entity.

MATERIALS AND METHODS
**Mitotic in-situ Hybridization:** Root tip FISH was performed as described with a slight modification (MASONBRINK and BIRCHLER 2010). To make cell wall digestion times more predictable, distilled water was substituted in washes for ethanol. Fluorescently labeled oligonucleotide probes consisted of a telomere repeat that strongly cross hybridizes to the B repeat; and CentC, which hybridizes to the centromere of all chromosomes and in the B chromosome long arm (ALFENITO and BIRCHLER 1993; ANANIEV et al. 1998; LAMB et al. 2005). The telomere oligonucleotide will label the B chromosome centromere because of the partial homology of the B centromere specific repeat (ALFENITO and BIRCHLER 1993) and produces such a strong signal that the exposure time for the A chromosome telomeres is seldom long enough for visualization. Nick translated PCR products consisted of the Stark repeat (LAMB et al. 2007), the centromeric retrotransposon of maize (CRM) (NAGAKI et al. 2003), the 180bp knob repeat (PEACOCK et al. 1981), and the cassette from the telomere-truncation experiments without the telomere array (WY96) (Yu et al. 2006).

**Meiotic Fluorescence in-situ Hybridization:** Meiotic FISH was performed as described previously (MASONBRINK et al. 2012). FISH probes consisted of fluorescein labeled telomere oligonucleotide (green) and a Texas Red labeled CentC oligonucleotide.

**Immunocytochemistry:** Immunolocalization for mitosis and meiosis were performed as described (HAN et al. 2009). The polyclonal antibody against maize CENP-C was raised in rabbit [GL Biochem (Shanghai) Ltd.]. A monoclonal rabbit antibody (04-817) raised against histone H3 phosphorylated at Ser-10 was obtained from Upstate. The phosphorylated H2a antibody was previously described (DONG and
The images were taken as a confocal z-stack (Zeiss LSM 710 NLO), and a flat projection of the three-dimensional image was created with the ZEN 2009 Light Edition (Zeiss), and processed with Photoshop CS 3.0.

RESULTS

A small B chromosome derived-minichromosome (86-B23) (Yu et al. 2007) (Figure 1a) was accumulated to multiple copies using the maize B chromosome accumulation mechanism. In two generations this minichromosome was accumulated to five copies before finding an unusually large chromosome with multiple distinct centromere loci. In the next generation we observed many of these minichromosomes changing in size and quantity between cells of the same root. Smaller sized chromosomes typical of the progenitor were usually found in every cell, while the larger sized chromosomes were restricted to fewer cells as if they were present only in certain developmental lineages. These chromosomes had multiple distinct CentC signals per minichromosome (Figure 1b). In addition, we probed the chromosome with CRM, the centromeric retrotransposon of maize (Figure 1c); Stark repeat (Lamb et al. 2007), which is found in the centromere and the distal heterochromatin of the B chromosome (Figure 1d); WY96, the truncation transgene cassette without the telomere array (Figure 1e); and the 180 bp knob repeat, a constituent of maize heterochromatin (Figure 1f) (Peacock et al. 1981). Each probe hybridized to multiple sites on the endoreduplicated chromosomes and the quantity of these signals was correlated with the size of the chromosome. For the medium sized chromosome in Figure 1e, the transgene, which exhibits the most discrete signals, hybridized to between 16-20 sites. In a single cell of one individual, the chromosome deviated from the typical spherical appearance and
was observed to form a large circle (Supplemental Figure 1). While we attempted to increase the copy number of the amplifying chromosome, we were not able to observe a cell with more than eight. As a sample of the frequency of their occurrence, the numbers were recorded from three different root tips. 29 of 48 cells (60%) of the cells showed a large chromosome, whereas the normal appearing minichromosome was observed in the remaining cells.

Immunocytochemistry was used to characterize the chromatin of the amplified chromosomes, and to determine if multiple centromere loci on a single chromosome could condition multiple active centromeres. CENPC is an inner kinetochore protein that is a biochemical marker of centromere activity. An antibody against CENPC was found to localize to multiple loci on the large amplified chromosomes (Figure 2). The larger of such chromosomes were found to exhibit up to three or more detectable foci. In contrast, the smaller versions and the A chromosomes never had more than one binding site for each chromosome. Sister chromatid cohesion is indicated by phosphorylation of serine 10 of histone H3 (KASZAS and CANDE 2000). The amplified chromosomes had multiple sites of sister chromatid cohesion indicated by bound phosphorylated H3S10 antibody (Figure 3). An antibody to phosphorylated H2AThr133, a histone modification associated with centromere function (DONG and HAN 2012), also had multiple foci (Figure 4).

Throughout meiosis the amplified chromosome continued to vary in size and frequency between cells of the same tassel and anther. In one anther nearly all the stages of meiosis were observed, excluding pachynema, from the same plant that had multiple amplified chromosomes. In pachynema these chromosomes appeared small.
and compact, a deviation from the typical string-like morphology of pachytene chromosomes (Figure 5a). In diplonema two spherical copies of the chromosome had "sticky" behavior, a characteristic of B chromosomes (Figure 5b) (McClintock 1933). In diakinesis we observed three smaller sized chromosomes of various sizes, and a noticeable division of B repeat to opposite ends of a chromosome (Figure 5c). At metaphase I, the amplified chromosomes appeared to nondisjoin as demonstrated by being closer to one pole than all other chromosomes (Figure 5d). This was also seen in somatic cells of the anther (Supplemental Figure 2). All amplified chromosomes that did not nondisjoin lagged at the metaphase plate. They apparently eventually moved to one pole as evidenced by the lack of micronuclei in subsequent stages. Chromosome disjunction was never observed with this chromosome at meiosis I (Figure 5e). Unequal numbers of amplified chromosomes were observed in prometaphase II, probably the result of precocious monopolar movement at metaphase I and nondisjunction at anaphase I (Figure 5f). At anaphase II some sister separation was apparent, but many times the chromosome nondisjoined to one pole (Figure 5g). In telophase II, the large spherical shaped chromosomes were still present, as well as a higher quantity of small versions (Figure 5h).

While screening the amplifying chromosome line, a small version was found that resembled the progenitor of the amplifying chromosome with two CentC FISH signals and a characteristic small size. It retained these characteristics and remained at one copy in every cell examined in the root. Because this minichromosome was stable, we self-pollinated and outcrossed the plant to see if amplification would reinitiate. In screening the next generation we observed enlarged chromosomes in root tips from ten
of eighteen seedlings, demonstrating that the amplifying phenotype could reinitiate. We also screened forty seeds by FISH from the original transformant seed stocks to determine if amplification was a property of this chromosome but were unable to find an amplifying minichromosome. While the earliest seed stocks did not contain an amplified chromosome, we did find one plant in which the minichromosome frequently nondisjoined between cells of the same root resulting in quantity shifts from zero to four minis and increased chromosome size (Supplemental Figure 3), but the amplifying phenotype was not recapitulated.

**DISCUSSION**

Here we describe a chromosome that changes size and structure among cells of the same plant. Its behavior is similar to a univalent chromosome during meiosis and mitosis, frequently nondisjoining even in the absence of a normal B chromosome. The fact that the normal karyotype replicates and separates normally indicates the presence of a cis-acting modification in this amplifying chromosome.

A potential explanation for this behavior is that there is an enhancement of nondisjunction without the subsequent sister separation. The sister chromatids of the normal B chromosome eventually separate, but the replicated sisters may remain adhered through successive replication cycles, thus doubling the size of the chromosome. For this hypothesis to explain the observed structure, the chromosome's sister chromatids would have to remain adhered through multiple mitoses and thus would drastically lower its presence among monitored cells. This possibility would halve the number of cells in the lineage receiving such a chromosome for each instance of
nondisjunction. Considering that we found an instance of eight such chromosomes in one cell with an approximate ten to sixteen centromere sites (Figure 1b), the odds of finding this would be extremely low. In this scenario, nondisjunction could explain the number of centromere loci present, but the observed frequency of amplified chromosomes would be much lower than that observed. Moreover, nondisjunction of the B chromosome has never been observed to produce such a conglomerate (MASONBRINK and BIRCHLER 2010).

Another potential consideration might be that the minichromosome formed a ring structure. Then with sister chromatid exchange within the ring, multiple centromeres would be present. This scenario also seems unlikely because the sister centromeres of rings typically separate at anaphase and rupture the chromosome. On this hypothesis an atypical behavior of rings would need to occur to form larger and larger structures due to repeated failure of sister chromatid separation. Also, this scenario would not predict the finding of rare cells with many different sized chromosomes spanning ten to twenty copies (Figure 1) nor is it consistent with the observation that when they are not present, the normal minichromosome was observed. Lastly, McClintock studied small ring chromosomes in maize (MCCLINTOCK 1938) and their structure and behavior are not concordant with the chromosomes described here.

A more likely scenario might be that an initial modification occurred that circumvents replication licensing, thus initiating multiple rounds of replication per cell cycle in select cells (NISHITANI and LYGEROU 2002). The minichromosome could then increase to various sizes between cells and permit the accumulation of multiple copies. This scenario would also group the larger amplified chromosomes in selected cells,
while the smaller versions would be present in most cells. A combination of over-replication and nondisjunction could also allow multiple chromosome copies of varying sizes to accumulate, while also limiting the number of cells having large variants.

The latter mechanism would double the number of centromere signals at each replication cycle, which would create chromosomes with 2, 4, 8, 16, 32, etc. centromere signals. The number of centromere signals is directly proportional to the number of single copy transgenes, which have distinct condensed FISH signals (Fig 1e inset). In counting the number of transgene signals, we noticed that the number deviates from the strict doubling predicted. This deviation might result from the splitting of some of the larger conglomerates in some mitoses, not all copies of the chromosome continuing to replicate in synchrony, or limitations on the detection of all copies.

The number of centromeric FISH signals (Figure 1) and antibody binding sites associated with sister chromatid cohesion and centromeric activity (Figures 2-4) were often at unequal frequencies. Inactivated centromeres, i.e., centromeres that do not recruit a kinetochore (HAN et al. 2006), within the amplified structure may account for this signal discrepancy, possibly explaining the relative stability of these chromosomes. The precocious monopolar movement and nondisjunction observed in metaphase I and II and anaphase I and II were more frequent with larger versions of the amplified chromosome. In fact, the only sister separation observed in meiosis occurred with small versions of the chromosome in anaphase II. Early monopolar movement is frequent with univalent B chromosomes (CARLSON and ROSEMAN 1992), but is at much lower levels than what was seen with the amplified chromosome.
This unusual chromosome was found in a lineage derived from a truncated B chromosome. This amplifying behavior acts in cis, namely only the minichromosome is affected, thus suggesting some change, be that mutational or epigenetic, that was perpetuated over the seven generations that the chromosome was under scrutiny. Nevertheless, a progenitor- sized chromosome was still observed in this lineage suggesting that single copies were possible and that the amplification could repeatedly occur from a single copy. Many small B chromosome derivatives have been studied in our laboratory over many years (Han et al. 2007) without any similar cases being observed.

This minichromosome contains the knob heterochromatin that is adjacent to the B centromere indicating that the knob is proximal to the site of truncation and that the B centromere is intact. The core of the B centromere is approximately 700 kilobases and the B repeat cluster spans a minimum estimate of 3.7 megabases (Jin et al. 2005). Thus, this example provides insight into the size range of replication control. Replication of this chromosome is regularly unrestricted and apparently spans megabases given that the transgene signals at the chromosome terminus generally match the number of centromere signals. The discovery of this chromosome will provide a model system for future analyses of replication control.

Acknowledgements

This work was supported by National Science Foundation grant DBI0701297 and DBI 0922703.
LITERATURE CITED


CARLSON, W. R., 1978 Identification of genetic factors controlling centromeric function in maize in Maize Breeding and Genetics, edited by D. B. WALDEN.


LAMB, J. C., A. KATO and J. A. BIRCHLER, 2005 Sequences associated with A chromosome centromeres are present throughout the maize B chromosome. Chromosoma 113: 337-349.


**Figure Legends**

**FIGURE 1.** Centromeric sequences in the amplified chromosome. Telomere probes, which cross-hybridize to the B repeat and were used for detection of the B repeat, are green. The inset pictures are the red channel of an amplified chromosome. Red arrows denote amplified chromosomes and white arrows denote unmodified full-sized maize B chromosomes. (a) CentC (red), a centromeric satellite repeat, localizes to a single locus on the progenitor chromosome of the amplified chromosome. (b) CentC (red) had multiple distinct signals on the amplified chromosome. (c) Multiple CRM (red) signals are on the larger amplified chromosome, while the smaller versions have only two. (d) Stark repeat (red), which is found in the distal heterochromatin of the long arm and in the centromere of the B chromosome, hybridized throughout the amplified chromosome. (e) WY96 (red), the single copy truncation cassette probe, hybridized to multiple distinct loci on the amplified chromosome. (f) Knob (red) is found in the proximal heterochromatin of the B chromosome, and also in the amplified chromosome. Scale bars are 10 microns.

**FIGURE 2.** Multiple sites of bound CENPC on larger amplified chromosomes. The yellow arrows denote amplified chromosomes with multiple sites of bound CENPC, which is a marker for centromere activity. The red arrows denote small amplified chromosomes with one site of CENPC binding. The top inset is the red CENPC antibody channel showing multiple signals on each large amplified chromosome. The top left box within the inset shows multiple CENPC signals on the amplified
chromosome. The middle inset is B repeat, which is found on the B chromosome and throughout the amplified chromosomes. The bottom inset is the DAPI channel. The white arrows denote three B chromosomes. B repeat is green and CENPC is red. Note that A chromosomes have one distinct signal per sister chromatid. Scale bar is 10 microns.

**FIGURE 3.** Active sites of sister chromatid cohesion (H3S10) in the amplified chromosome. The yellow arrow denotes an amplified chromosome with multiple sites of bound H3S10 antibody, which is a marker for sister chromatid cohesion. The red arrows denote smaller amplified chromosomes. The white arrows denote B chromosomes. Notice all A chromosomes have one distinct signal per sister chromatid. The red channel is the H3S10 antibody. Scale bar is 10 microns.

**FIGURE 4.** Sites of functional centromeres (phosphorylated H2A) in the amplified chromosome. The yellow arrow denotes an amplified chromosome with multiple sites of bound phosphorylated H2AThr133 antibody, which is a marker for centromere function. The red arrow denotes a small amplified chromosome. The top inset is the red H2A antibody channel showing multiple signals per large amplified chromosome. The middle inset is the B repeat channel, which is found on both B chromosome arms at distal positions and throughout the amplified chromosome. The bottom inset is the DAPI channel. The white arrow denotes a B chromosome. B-repeat probes are green. Note that A chromosomes have one distinct H2A signal per sister chromatid. Scale bar is 10 microns.
**FIGURE 5.** The meiotic behavior of an amplified chromosome. All meiocytes were obtained from a single anther, except the pachytene cell. Yellow arrows denote amplified chromosomes, and white arrows denote B chromosomes. CentC probes are red, and telomere probes, which strongly cross-hybridized with the B repeat, are green. (a) The amplified chromosome appears small at pachynema with diffuse B repeat chromatin. (b) Two amplified chromosomes in diplonema. (c) Three amplified chromosomes in diakinesis. (d) Two amplified chromosomes, both with early monopolar movement typical of univalent B chromosomes at metaphase I. (e) Two amplified chromosomes, the larger with persistent early monopolar movement and the smaller with late monopolar movement at anaphase I. A univalent B chromosome lagging and separating sister chromatids is present (white arrow). (f) Prometaphase II with the top cell having two amplified chromosomes, while the bottom cell has one larger amplified chromosome and a univalent B chromosome. (g) Anaphase II with the left cell segregating sister chromatids of three amplified chromosomes and one large amplified chromosome nondisjoining to one pole without sister chromatid separation. The right cell has one amplified chromosome lagging at the metaphase plate while a larger amplified chromosome has nondisjoined to one pole. (h) Telophase II with seven amplified chromosomes. The larger amplified chromosomes are in distinct spherical shapes. Scale bars are 10 microns.