An Investigation of Maize B Chromosome-Derived Minichromosomes

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The undersigned, appointed by the dean of the Graduate School,

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A candidate for the degree of

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LIST OF ABBREVIATIONS

FISH - Fluorescence in-situ hybridization

B - B chromosome

Mini - Minichromosome

BFB - Breakage fusion bridge cycle

bar - bialophos resistance gene

GUS - β-glucuronidase gene

CDK - Cyclin dependent kinase

atm - atmosphere

EtOH - Ethanol

DAPI - 4'-6-diamidino-2-phenylindole

BAC - Bacterial artificial chromosome

PCR - Polymerase chain reaction

NOR - 45s rDNA

TE - Tris-EDTA

SSC - Sodium Chloride-Sodium Citrate Buffer

DDK - Dbf4 dependent kinase

MCM - Minichromosome maintenance proteins

CRM - Centromeric retrotransposon of maize

ABSTRACT

The maize (*Zea mays*) B chromosome is a supernumerary chromosome with a selfish inheritance characterized by nondisjunction at the second pollen mitosis, univalent survival in meiosis, and preferential fertilization of the egg. The apparent lack of known genes allows this inheritance to accumulate the B chromosome to multiple copies in the genome with relatively little effect. By bombarding telomere-containing constructs with selection markers into maize embryos with B chromosomes, multiple B chromosome truncations of varying sizes were found. Using these B-derived minichromosomes, we were able to address minichromosome pairing and accumulation limits, which led to the observation of dosage dependent nondisjunction of the B chromosome and the discovery of an asynchronous endoreduplicating chromosome. Because the stacking of multiple genes in crop plants is becoming more prevalent, establishing a system of multiple minichromosomes could ease the development and increase the capabilities of transgenic plants.

Fluorescence *in-situ* hybridization (FISH) was used to undertake a quantitative study of maize plants with different numbers of B chromosomes to observe if instability increases by increasing B dosage in root tip tissue. B chromosome nondisjunction was chiefly absent at low copy number, but increased at higher B numbers indicating that nondisjunction rates are dependent on the dosage of B's in the sporophyte. Differences

in nondisjunction were also documented between odd and even doses of the B. In plants that have inherited odd numbered doses of the B chromosome, B loss is nearly twice as likely as B gain in a somatic division. When comparing plants with even doses of B's to plants with odd doses of B's, plants with even numbers had a significantly higher rate of increase in number.

The maize B chromosome's accumulation mechanism was used to accumulate four different sized minichromosomes, derived from the B chromosome, to test the chromosome limits of the cell. The accumulation of high numbers of B chromosomes is associated with multiple phenotypes including reductions in fecundity and vigor, but when minichromosomes are accumulated these symptoms are absent. We also found that multiple B chromosome derived minichromosomes can coexist with A chromosome derived minichromosomes. During the years that experiments were conducted, we found many B chromosome rearrangements and fragments, two recoverable A chromosome fragments, and observed a minichromosome breakage-fusion-bridge cycle in the root. The meiotic behavior of four different sized minichromosomes was examined at multiple copies in an otherwise diploid background. While the multiple copies were associated in multivalent configurations in prophase I, they often dissociated into univalents or bivalents prior to metaphase I. The largest mini's behavior closely resembled the progenitor B chromosome, but all smaller chromosomes showed a failure of sister chromatid cohesion. In addition to the meiotic behavior, we observed many anomalies of univalent behavior and possible heterochromatic fusions of B repeat associated heterochromatin.

The smallest telomere truncated B chromosome was increased to multiple copies using the B chromosome's accumulation mechanism. During the process of the accumulation regime, instances were found in which this chromosome had amplified in copy number without separation, resembling endoreduplication. These unusual chromosomes contained multiple sites of centromere satellite DNA. Despite their unusual structure they were inherited over many generations. Immunocytochemistry was used to identify sites of sister chromatid cohesion with phosphorylated H2A and phosphorylated H3S10, and centromere activity with CENPC, an inner kinetochore protein. We characterized the mitotic and meiotic behavior and observed an increasing likelihood of nondisjunction that correlates with the chromosome's size.

CHAPTER 1: INTRODUCTION

Genetically modified organisms with stacked genes are becoming more prevalent on the market, as they increasingly fulfill the needs of agriculture. According to a USDA report, in 2000 1% of corn and 20% of cotton were stacked with herbicide tolerance and Bt genes, but by 2011 these numbers increased to 49% and 58%. Minichromosomes offer a gene expression platform that is independent of the genome, thereby facilitating the rapid development of transgenic crops. This study describes the nondisjunction of B chromosomes in roots, the meiosis of multiple minichromosomes, the accumulation of minichromosomes, and a heritable loss of replication control in a single chromosome.

B chromosomes are differentiated from the standard karyotype (A chromosomes) by three criteria: they are dispensable, they do not pair or recombine with A chromosomes, and they have a meiotic or mitotic drive that results in higher-than-expected transmission to the next generation (Jones et al., 2003). B chromosomes come in many shapes and sizes, and have many species-specific effects on the genome that can be classified as parasitic, beneficial, or benign. They are widespread in nature and their origin is an enigma. Even so, B chromosomes are the subject of intense study and have proven useful for genetic mapping, chromosome engineering, and gene dosage studies.

The parasitic B chromosome hypothesis was first proposed by Ostergren in 1947 by observing more B chromosomes in the progeny of *Anthoxanthum aristatum* (annual vernalgrass) than was found in parents (ÖStergren, 1947). Most B chromosomes have a meiotic or mitotic drive that allows multiple numbers of B chromosomes to accumulate in the organism. There are three categories of drive: premeiotic, meiotic, and

postmeiotic. Premeiotic drive usually involves directed mitotic nondisjunction to gamete progenitor cells prior to spermatogenesis, which is the drive mechanism for B chromosomes in the grasshopper *Calliptamus pellucida* (Nur, 1969) and locust *Locusta migratoria* (Kayano, 1971). Directed nondisjunction during meiosis usually occurs through the female and may be the result of a differential size between the meiotic poles (Jones, 1991). In *Lillium callosum* (small field lily) B's are frequently incorporated in the egg rather than the central cell, because the univalent B chromosomes are frequently found outside the metaphase plate on the broader micropylar pole in 80% of observed cells (Kimura et al., 1961). In *Myrmeleotettix maculatus* (mottled grasshopper) the B chromosome was observed in positions nearer to the pointed egg pole in 90% of cells, although the drive is somewhat balanced by drag through the male with a B chromosome transmission of 30% (Hewitt, 1973). Postmeiotic drive is the most common form of drive, and is characterized by directed nondisjunction in the first or second gametophyte mitosis (*Secales cereale (rye), Z. mays*) (Camacho, 2004).

The longevity of a B chromosome is dependent on the A genome's response and acclimation to B chromosome invasion. The B can evolve a pathway to increase its lifespan by integrating into the A genome, by contributing an advantageous trait, or by becoming parasitic (Camacho, 2004). The initial invasion of B chromosomes can dramatically increase the mean number of B's in population that are naive to B chromosomes,. This has been observed in populations of *Eyprepocnemis plorans* (grasshopper), *Phrochilodus lineatus* (fish), and *Trypoxylon albiarse* (wasp) (Camacho, 2004).

Certain genotypes are more susceptible to B chromosome drive, and resistance usually originates as suppression of drive and/or tolerance of B chromosomes. Specific genetic polymorphisms in the A genome are associated with high and low B chromosome transmission rates and have been identified in the following species: *Festuca pratensis(meadow fescue)*, *Z. mays*, *M. maculatus*, *Pseudococcus affinis* (mealybug), and *Hypochoeris maculata* (Spotted Hawkweed) (Camacho, 2004). In *P. affinis* the paternal set of chromosomes become heterochromatic and inactive during spermatogenesis, while the maternal set remains euchromatic. During meiotic chromosome segregation the euchromatic set are the only set to form sperm and the B chromosome decondenses, ensuring its inclusion in the sperm. A genetic polymorphism related defense of the *P. affinis* A genome affects B chromosome decondensation and prevents its inclusion in the sperm (Nur et al., 1988).

The maize B chromosome drive is characterized by nondisjunction at the second pollen mitosis, survival of a univalent in meiosis, and preferential fertilization of the egg, rather than the central cell (Carlson, 1969, Roman, 1948, Carlson et al., 1992). While nondisjunction has been associated with regions on the B chromosome, preferential fertilization and univalent survival are associated with the A genome. Preferential fertilization was first suggested by Carlson in 1969, when discovering a maize inbred line that blocked preferential fertilization through the female (Carlson, 1969). Later experiments were done that artificially selected lines for high and low transmission, and demonstrated that a single gene expressed by the female is responsible for preferential fertilization (Chiavarino et al., 2001). A dominant gene was subsequently identified that

favors elimination of univalent B chromosome transmission through the female (Gonzalez-Sanchez et al., 2003).

An alternative A genome defense to B chromosomes lies with the development of tolerance. Varying levels of tolerance exist among individuals of the same species, which is characterized by the response of the A genome to the presence of multiple B's. Tolerance can range from complete intolerance, where fitness is directly proportional to the number of B's, to complete tolerance, where fitness is unaffected by the presence of B's (*F. pratensis*) (Bosemark, 1956). Incomplete tolerance, where fitness is unaffected at low numbers but lowers fitness at high numbers of B's, is the most common type (*Z. mays*) (Camacho, 2004). In rare cases B chromosomes can have a positive effect on the A genome, as straw weight increases with *S. cereale* plants that have two B chromosomes (Jones, 1991).

Odd and even numbers of B chromosomes seem to affect organisms differently. This was first observed in maize, when odd numbers of B chromosomes were found to have increased numbers of chiasmata than even numbers (Darlington et al., 1941), and was subsequently described in rye (Jones et al., 1969). In rye the odd and even numbers of B's have a direct effect on dry nuclear mass, protein, RNA, plant height, and number of tillers (Jones et al., 1969). The odd-even effect is widespread among plants and animals, and odd numbers typically show more detrimental effects than evens (Jones, 1995). This phenomenon seems to be related to the type of drive mechanisms that involve nondisjunction during mitosis rather than meiosis (Camacho et al., 2004). The mechanism of this phenomenon remains a mystery, but may have a relationship with the somatic associations of paired and unpaired chromosomes (Jones et al., 1969).

At low copy numbers the phenotypes that B chromosomes cause are hard to distinguish, but are exacerbated by their accumulation in the genome. The cell size and duration of DNA synthesis are increased with increasing numbers of B's, which would be expected with additional DNA (Hof et al., 1963). Morphological phenotypes in plants are usually associated with reductions in vigor (healthy balanced growth), fertility (low seed set), flowering time, and germination, although some plants have improvements in these characteristics (Jones et al., 1982). The effects of B chromosomes are sometimes benign, as in *Haplopappus gracilis* the color of the achene changes from brownish red to dark purple in the presence of B chromosomes and as B chromosomes increase in maize, white stripes appear on the leaves (Jackson et al., 1960, Staub, 1987). Beneficial effects are even present in some species; the fungus *Nectria haematococca* gains resistance to antibiotics from its host *Pisum sativum* (garden pea) (Miao et al., 1991).

The most ubiquitous effect that B chromosomes have on the genome are alterations of recombination. In most animals and plants, B chromosomes increase chiasmata frequencies in A chromosomes, which is the prominent argument for their adaptive evolution in the genome. Darlington proposed that increased recombination will augment genetic variation and promote rapid evolution in populations (Darlington, 1958). Another hypothesis suggests that increased chiasmata frequencies may be a defense for the development of resistance to B chromosome accumulation (Bell et al., 1990).

B chromosomes are widespread in wild populations in Fungi, Animalia, and Plantae, but some common characteristics of their host species have become evident. Population studies have shown that B chromosomes are mostly found in outbreeding species, which may be why they are never found in superior agricultural inbred species

(Jones, 1995). The presence of B chromosomes is also directly correlated with genome size and inversely correlated with chromosome number (Levin et al., 2005). The presence of B chromosomes is equally distributed between different ploidies (Jones, 1995), but there appears to be a large disparity between monocots (8%) and dicots (3%) (Levin et al., 2005). They are found in 14% of orthopteran insects studied (Camacho 2004) and in 10-15% of angiosperms (Jones, 1995), although B chromosomes may be overlooked in some species caused by their absence from some tissues (Jones et al., 1982). For example, in *Aegillops mutica* (Mochizuki, 1957) and in *Aegilops speltoides* (Mendelson et al., 1972), B chromosomes are restricted to aerial tissues.

Since B chromosomes are categorized by three characteristics -- drive, dispensability, and lack of recombination with the A genome -- they tend to be rather plastic in shape and size (Jones et al., 2003). Therefore the structure of B chromosomes can vary greatly among the same species. A survey in 1995 indicated that at least 65 plant species have two or more known B chromosome forms (Jones, 1995). Most B chromosomes are heterochromatic stemming from their high content of repetitive DNA, including, satellite repeats, ribosomal repeats, and transposable elements (Camacho, 2004). Many species' B chromosomes have exclusive repeats not found in the A genome, but are also concentrated with repeats found on A chromosomes, which may find their way to the B chromosome through transpositions of mobile elements (*Z. mays*, *Brachyscome dichromosomatica*) (Alfenito et al., 1993, Houben et al., 2001, Cheng et al., 2003, Houben et al., 2012, Lamb et al., 2005, Lamb et al., 2007).

Although most established B chromosomes cannot be linked back to a specific progenitor chromosome, an apparent B chromosome in *Plantago lagopus* was observed

throughout development. It started as a trisomic ring chromosome 2S, which produced a plant with an isochromosome showing the characteristics of a B chromosome (Dhar et al., 2002). Another novel B chromosome derived from a triploid rice plant was analyzed with 72 chromosome arm-specific markers and found that the B did not originate from a single A chromosome fragment (Cheng et al., 2000).

Despite the detrimental effects of B chromosomes, its dispensable nature and genetic inactivity has been utilized for many purposes. B-A translocations have been used to map recessive genes as well as provide the capability to manipulate gene dosage in the A genome (Birchler, 1991, Beckett, 1991). B-A translocations have also been used to dissect the functions of the maize B chromosome. Roman in 1949 identified a trans-acting factor responsible for nondisjunction on the B, and Ward[40] in 1973 localized this trans-acting factor for nondisjunction to the distal euchromatin on the long arm (Fig 1.1a) [41]. Lin in 1978 found that the proximal euchromatin on the long arm was essential for nondisjunction and could act in trans (Fig 1.1b), while a deletion of the distal long arm heterochromatin had no effect. In 1979 Lin found a B-A translocation with the minute short arm of the B joined to the long arm of chromosome ten (B-10L), along with the reciprocal translocation (10-B). Because the B-10L chromosome was capable of nondisjunction, but the presence of the 10-B chromosome increased the frequency, an enhancer of nondisjunction may be present on the B chromosome short arm (Fig 1.1d) [42]. With the use of a B-9 isochromosome the function of the proximal knob was dissected and found to be essential for nondisjunction of the B centromere (Fig 1.1c) (Carlson et al., 1981).[44]

Figure 1.1 The regions that control nondisjunction of the maize B chromosome. Region (a) is the trans-acting distal euchromatin. Region (b) is the proximal long arm euchromatin. Region (c) is the proximal knob near the centromere. Region (d) is the B chromosome short arm that enhances nondisjunction. Regions (b), (c), and (d) are cis-acting (Carlson et al., 1981)

Breakage Fusion Bridge Cycle

McClintock described two types of the breakage fusion bridge cycle: the chromatid type and the chromosome type. The chromatid type breakage fusion bridge cycle (BFB) was initiated by recombination between a normal chromosome 9 and a chromosome 9 with an x-ray induced paracentric inversion. The crossover resulted in an acentric and a dicentric chromosome (Dp9), which could undergo breakage in the subsequent anaphase. McClintock tracked the progression of the breakage fusion bridge cycle by observing the changes in phenotype caused by the loss or gain of three dominant endosperm genes and one dominant plant gene. She found that these broken chromosomes would replicate and sisters would fuse before the chromosomes could heal, resulting in the formation of a new dicentric chromosome. These successive chromosome breaks and fusions continued throughout the plant and when fertilizing the endosperm, but when fertilizing the embryo the cycle was interrupted. Apparently these broken chromosomes healed prior to fusion, and remained stable in future nuclear and plant generations (McClintock, 1939).

Mcclintock then investigated if the broken chromosomes remained unsaturated in the gametophyte by contributing one broken chromosome from each gamete to form the zygote. In sporophytic mitoses, two anaphase bridges were present and led to breaks and fusions in telophase nuclei. The dominant plant and endosperm genes were variegated throughout the plant, which was expected with the duplications and deficiencies that occurred with uneven breaks between the two centromeres. This was named the chromosome-type breakage fusion bridge cycle, as both chromatids of the whole chromosome were involved in this BFB cycle (McClintock, 1942).

To further characterize the process of chromosome healing, a new study was of the BFB cycle was initiated. A translocation between the short arm of chromosome 9 and the B chromosome centromere (TB-9Sb) was crossed to Dp9 to obtain a recombination event resulting in a inverted duplication fused to the B chromosome centromere (B-9-Dp9) (Fig 1.2a). A foldback interchromatid exchange occurred in B-9-Dp9 and resulted in a dicentric chromosome (Fig 1.2 B & C). The chromatid-type BFB cycle between the two B chromosome centromeres was active in the sporophyte. Nondisjunction of the B centromere at the second pollen mitosis converted the chromatid type BFB to the chromosome type BFB (Fig 1.2I). In the following cell cycles, anaphase bridges would form when centromeres of the dicentric would go to opposite poles, but remained whole when both centromeres cosegregated to a pole (Fig 1.2 j-q). Over a ten week course of study, variegated plants with double bridges gradually declined to 7.1%, showing that healing did not occur at a specific time. Many chromosomes of varying sizes were found from healing of the B-9-Dp9 dicentric chromosome. The smallest of these derivatives were approximately the size of a centromere, and were designated minichromosomes (Zheng et al., 1999). A mechanism for stabilization of the dicentric was elucidated with the observation of a stable dicentric chromosome with a smaller inactivated centromere and a large active centromere (Han et al., 2006). In a later study, this inactivated B centromere was reactivated in breakage and fusion events that placed the two smaller inactive centromeres on one chromosome, thereby inducing the recovery of centromere function (Han et al., 2009).

The pairing, disjunction, and sister chromatid cohesion of twenty-two minichromosomes derived from the B-9-Dp9 BFB cycle were studied at one and two

copies. Despite the origin of these minichromosomes, none would pair with chromosome 9 nor the B chromosome when at one copy. Eight of these minichromosomes, when at one copy, progressed to one pole at metaphase I and separated sister chromatids at anaphase II. The other fourteen minis separated sister chromatids at anaphase I. The larger minichromosomes frequently paired when at two copies, unlike the smaller minichromosomes. One very small sized minichromosome was observed as a bivalent in all of the cells examined, while one larger sized minichromosome was incapable of such pairing (Han et al., 2007).

Telomere Truncation

Another method for forming minichromosomes was discovered with the electroporation of human chromosome terminal repeats into human/hamster cell lines. Farr and colleagues successfully transformed six lines and found the introduced sequences at the ends of chromosomes. In one of the six lines they found cytogenetic evidence of chromosome breakage and healing. They inferred that the terminal locations of these integrated telomere sequences were the result of chromosomal truncations, and thus telomere truncation was founded (Farr et al., 1991).

In 2006, Yu and colleagues successfully truncated maize chromosomes with telomeres. They used Agrobacterium-mediated transformation on Hi-II embryos to introduce two constructs containing 2.6kb Arabidopsis thaliana telomere repeats and one control without telomere repeats. FISH was used to karyotype bialophos (bar) resistant calli to determine if truncations were present. The constructs with telomeres yielded 176

transgenic events, 67% of the transgenes were at distal chromosome locations, while the control construct was distal in only 25% of 44 events. Although not all distal transgenic loci were correlated with truncations every truncation observed was correlated with a distal transgene (Yu et al., 2006). In human cell lines telomere truncated

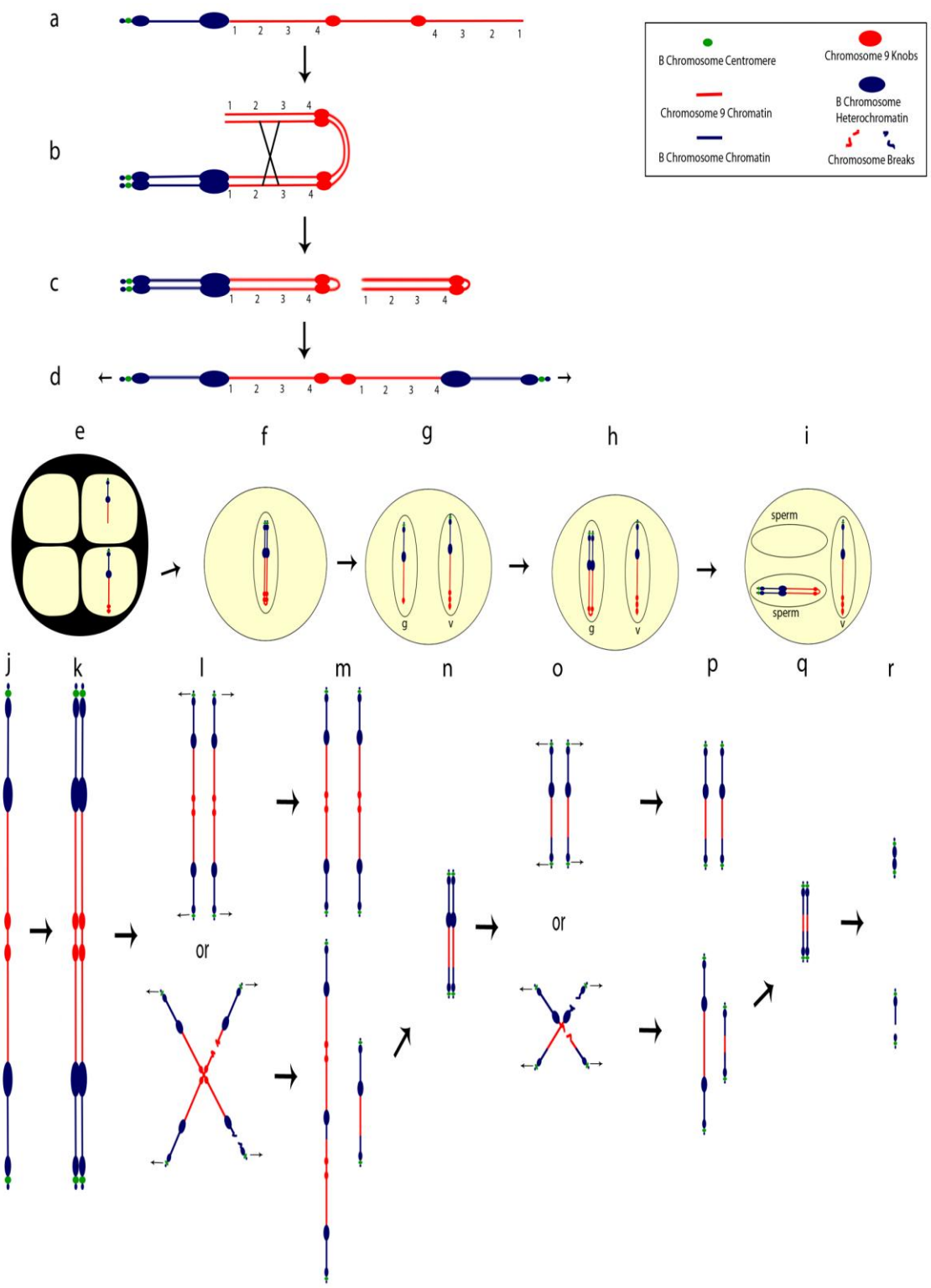

Figure 1.2 Formation of minichromosomes by the breakage-fusion-bridge cycle. (a) TB-9Sb-Dp9, an inverted duplication of the short arm of chromosome 9 translocated to a B

chromosome. Numbers represent duplicated regions. (b) In prophase I, TB-9Sb-Dp9 pairs in duplicated regions, resulting in a foldback exchange. (c) In anaphase I, the dicentric chromosome will proceed to one pole, while the acentric fragment will eventually be lost. (d) In anaphase II, the sister centromeres separate to opposite poles and the chromosome breaks. Small black arrows indicate poleward movement. (e) Products of meiosis in telophase II are shown. (f) Following the bottom right cell, DNA replication and fusion of chromatids occurs. In anaphase, the centromeres go to opposite poles and break anywhere between the two centromeres. (g) Broken chromosomes are depicted in the generative nucleus (G) and vegetative nucleus (V). (h) The chromosome in the generative nucleus replicates and the sister chromatids fuse, while the vegetative nucleus decondenses. (i) The fused chromosome nondisjoins to one pole at the second pollen mitosis and avoids breakage. (j) The dicentric chromosome linearizes in the zygote. (k) Replication of the dicentric occurs. (l) In anaphase, both centromeres can proceed to the same pole (top) and avoid breakage or proceed to opposite poles and break anywhere between the two centromeres (bottom). (m) The two broken chromosomes fuse (bottom). (n) The smaller chromosome proceeds through the next replication cycle. (o) In anaphase, both centromeres can proceed to the same pole and avoid breakage (top) or proceed to opposite poles and break anywhere between the two centromeres (bottom). (p) The two broken chromosomes fuse (bottom). (q) The smaller chromosome proceeds through the next replication cycle. The breakage and fusion of the dicentric continues until a stabilization event occurs, which may be explained by one of two hypotheses: (r) (Top) One centromere becomes inactive. (Bottom) Breakage can occur with subsequent

telomeric healing prior to replication, preventing fusion of broken chromosome ends (Gaeta et al., 2012)

fragments were capable of developing neocentromeric activity, but this phenomenon was not observed in maize (Saffery et al., 2001). Most minichromosomes were lost prior to fertilization, due to the production of deficiency gametes (Yu et al., 2006).

Engineered Minichromosomes

The properties of the maize B chromosome make it a favorable prospect for the development of engineered minichromosomes. First, the maize B chromosome does not have any genes that would be detrimental to the organism and thus is a viable candidate for truncation. Secondly the maize B chromosome can be accumulated to multiple numbers without affecting the phenotype, and can reach double digits before hindering vigor and fecundity. In fact, the highest number of B chromosomes ever accumulated was 34, although this plant was sterile (Randolph, 1941). B derived minichromosomes provide the capability to transfer selected traits to superior inbred lines without the linkage drag of adjacent genes that would occur with an A chromosome derived minichromosome or genome insertion.

In a successive publication, Yu and colleagues used telomere truncation with biolistics to develop minichromosomes that could be transmitted. They cobombarded the same telomere containing cassettes and the pAHC25 plasmid into maize HiII embryos with B chromosomes, to see if telomeres could truncate B chromosomes. The pAHC25 plasmid included a β-glucuronidase (GUS) gene along with an extra bar gene to ensure herbicide resistance was obtained if the truncation cassette was lost on a truncated, acentric fragment. Out of 281 screened bar resistant events, they obtained seven A chromosome truncations and 55 B chromosome truncations. The minichromosomes

derived from the B chromosome were transmissible, unlike their previous experiment's A chromosome truncations. Both constructs (pAHC25 and pWY76 or pWY86) colocalized in 84% of events screened, and GUS expression was present in nine of seventeen events.

Most minichromosomes retained normal sized centromeres throughout the study, but two exceptions were observed. Event 86B136 was an A chromosome truncation in the centromere of chromosome 6. Another event, 86-B23, was derived from a B chromosome and had the B repeat array in the centromere reduced (Yu et al., 2007). Although a previous study indicates that centromere size is strongly correlated with transmission levels (Phelps-Durr et al., 2004), these minichromosomes were capable of high transmission frequencies (Yu et al., 2007).

Endoreduplication in Plants

Endoreduplication is a common process in specialized tissues of plants and animals and results from multiple cycles of DNA replication without cell division. This unique nuclear cycle is usually present in terminally differentiated cells that are highly metabolically active, and may serve as a source of extra templates for increased gene expression. Multiple studies have shown that endoreduplication may simply be controlled by reductions in M-phase cyclin dependent kinases (CDK) and oscillations of S-phase CDKs, thereby manipulating the cell cycle (Larkins et al., 2001).

Cells normally cycle between states of synthesis and cell division interceded by gap phases. The cell cycle appears to be regulated by CDKs, which are regulated through oscillations in cyclin levels. There are two different families of CDKs in plants, but their regulation roles are unclear in the maize cell cycle. Both maize p34 CDK's are related to the two p34 CDKs in *A. thaliana* (Colasanti et al., 1991). One of the *A.*

thaliana's CDKs controls the cell's competence to divide, and the other is regulated by the cell cycle (Shaul et al., 1996). It appears that the regulation of CDKs occurs primarily through the phosphorylation and dephosphorylation of the kinase component and through interactions with CDK inhibitors. Two *A. thaliana* CDK inhibitors (ICK1 and ICK2) are related to mammalian p21 and p27 CDK inhibitors that regulate specific CDKs, which suggests a role in cell cycle regulation (Larkins et al., 2001).

The foremost endoreduplicating tissue known in maize is the endosperm, which is a seed storage organ developed from fertilization of two polar nuclei by the sperm. The most rapid period of endosperm growth is from 8-12 days after pollination, after which cell division and elongation cease. At 12 days after pollination the endosperm usually endoreduplicates through four to five cycles, which is correlated with the accumulation of a CDK inhibitor (Kowles et al., 1985). The levels of endoreduplication seem to follow a gradient from highly endoreduplicated cells in the central endosperm to lower levels in the aleurone periphery (Larkins et al., 2001).

CHAPTER 2: SPOROPHYTIC NONDISJUNCTION OF THE MAIZE B CHROMOSOME AT HIGH COPY NUMBERS

Introduction

B chromosomes are supernumerary chromosomes that persist with the standard karyotype through a distinctive inheritance. Generally, B's are comprised of noncoding, repetitive DNA, thereby preventing genic conflicts, but also have a species-specific meiotic and/or mitotic drive that resists their removal from the genome (Jones, 1995). This abnormal mode of inheritance and neutral effect on fitness permits B's to accumulate multiple copies in subsequent generations, although high numbers can be detrimental (Jones et al., 2008). Their unknown origins are only obscured by their diversity, as their presence is sporadic within populations of many species, and they have a species-specific drive and morphology (Jones et al., 2003).

The maize B chromosome capitalizes on several steps of the life cycle to achieve its mitotic drive as the basis of its accumulation mechanism. Beginning in meiosis, univalent B chromosomes can survive in the absence of a homologue, although more stability is obtained when B's are paired. However, the major aspect of its drive mechanism occurs at the second pollen mitosis, in which the B chromatids fail to separate and nondisjoin to either pole in 50% to 100% of cases (Carlson, 1988). The B containing sperm gains an advantage by preferentially fertilizing the egg in 60% to 70% of cases.

With the combination of these properties the B can surpass levels of Mendelian inheritance and proliferate in future generations (Roman, 1948).

Other effects caused by the maize B chromosome have been documented. Nondisjunction of the B also occurs in somatic nuclei of the tapetum, where it decreases the stability of A chromosomes and increases micronuclei formation (Chiavarino et al., 2000). In the presence of B's, high knobbed races of maize undergo elimination of knobbed chromosome arms at the second pollen mitosis (Rhoades et al., 1967). Even numbers of B's can marginally increase the frequency of recombination in proximal regions of A chromosomes, while odd numbers of B's show an even greater increase in recombination (Chang et al., 1974). Knob and B heterochromatin replicates late relative to euchromatin (Pryor et al., 1980a).

Initially we observed B's nondisjoining in somatic cells with high copy numbers and investigated the details of this newly discovered property. Our hypothesis was that higher doses of B's in the genome would increase the frequency of nondisjunction and increase the range of variability. FISH analysis allowed us to screen root tips and elucidate any relationships that were present in a collection of spreads.

Material and Methods

Plant materials and Cell Preparation

Root tip chromosome analysis was performed similarly to previously described procedures (Kato et al., 2004). Three inbred B73+B ears were obtained and seeds were germinated in a 30°C incubator for 2–3 days on moist paper towels. Roots were cut at a length of 2–3 cm and subjected to nitrous oxide treatment at 10 atm for 2–3 h. The roots

were then fixed on ice with 90% acetic acid and either immediately used or stored at –20°C overnight. Roots were transferred and soaked in distilled water on ice for 30 min and 1 mm was cut from the end of each root tip. Then they were immediately moved to a digestion buffer (1% cellulase, 2% pectolyase, in 1 × citric buffer) and incubated in a water bath for 38–42 min at 37°C. Digestions were then rinsed thoroughly with 70% EtOH, which was immediately replaced with 24 μL of 100% acetic acid. The tips were then crushed with a metal dissecting probe and 6 μL were dropped on slides in a humid chamber (cardboard box lined with wet paper towels).

Fluorescence in-situ hybridization

The slides were then hybridized with 10 μL of a 2 ng/μL 5' end labeled (Fluorescein or Texas Red) CentC oligonucleotide and/or a telomere oligonucleotide. CentC hybridized with centromeres in all chromosomes, including the long arm of the B chromosome, and the telomere probe cohybridized with the B repeat (produces a much stronger signal on the B-specific repeat than on telomeres). A plastic coverslip was placed on top of the probe mixture and then slides were denatured in a boiling water bath and hybridized at 55°C for at least 1 hour. Slides were then dipped in room temperature 2 × SSC and soaked in 2 × SSC at 55°C for 5 min. The slides were dried and mounted with Vectashield with 4'-6-diamidino-2-phenylindole (DAPI). A glass coverslip was applied and the slides were examined on an Olympus BX61 fluorescence microscope.

Results

We screened 83 plants and nearly 750 cells and then identified B number changes in root tip cells of single plants (Fig 2.1). To minimize the risk of counting errors, the

chromosomes of each spread were counted to ensure the presence of 20 A chromosomes. Multiple cells were screened per plant and the median B number was used for the classification. In this process we identified chromosomal abnormalities, including one aneuploid plant, a plant with 2 B isochromosomes, a plant with a B-A translocation, many B centromere fragments, and a plant mosaic for a B isochromosome (Fig 2.2).

Nondisjunction occurred in plants with five or more B's, as B frequencies rarely changed in plants with lower numbers. There was an obvious bias in the frequency of nondisjunction, because rates of nondisjunction varied tremendously in the population. In fact, because one plant had such an inconsistent number of B's, it was rendered obsolete for the analysis, as the progenitor zygotic number could not be established. In other plants in this same genetic background, nondisjunction was not observed (Fig 2.3). Two regression analyses were performed; one compared the range of variability in B chromosomes to the median B number by counting the B chromosomes that changed, and the other to elucidate the relationship between nondisjunction rates and median B number by examining the number of cells with altered B chromosome numbers. Although the first analysis was insignificant, nondisjunction rate increases were directly proportional to increases in median B number (Fig 2.4, $P < 0.01$).

Profiling odd and even plants separately showed an interesting relationship when comparing B chromosome loss to gain. *G*-tests followed by *Chi* squared tests were performed to display these associations. In plants with odd numbers of B's, mitotic nondisjunction was biased towards loss at a rate of more than 2:1 to gain ($P < 0.05$). A comparison between plants with even and odd numbers of B's showed significantly higher rates of gain in plants with even numbers ($P < 0.01$). Comparisons among

different numbers of evens and among different numbers of odds for gains and losses did not produce any significant differences (Table 2.1).

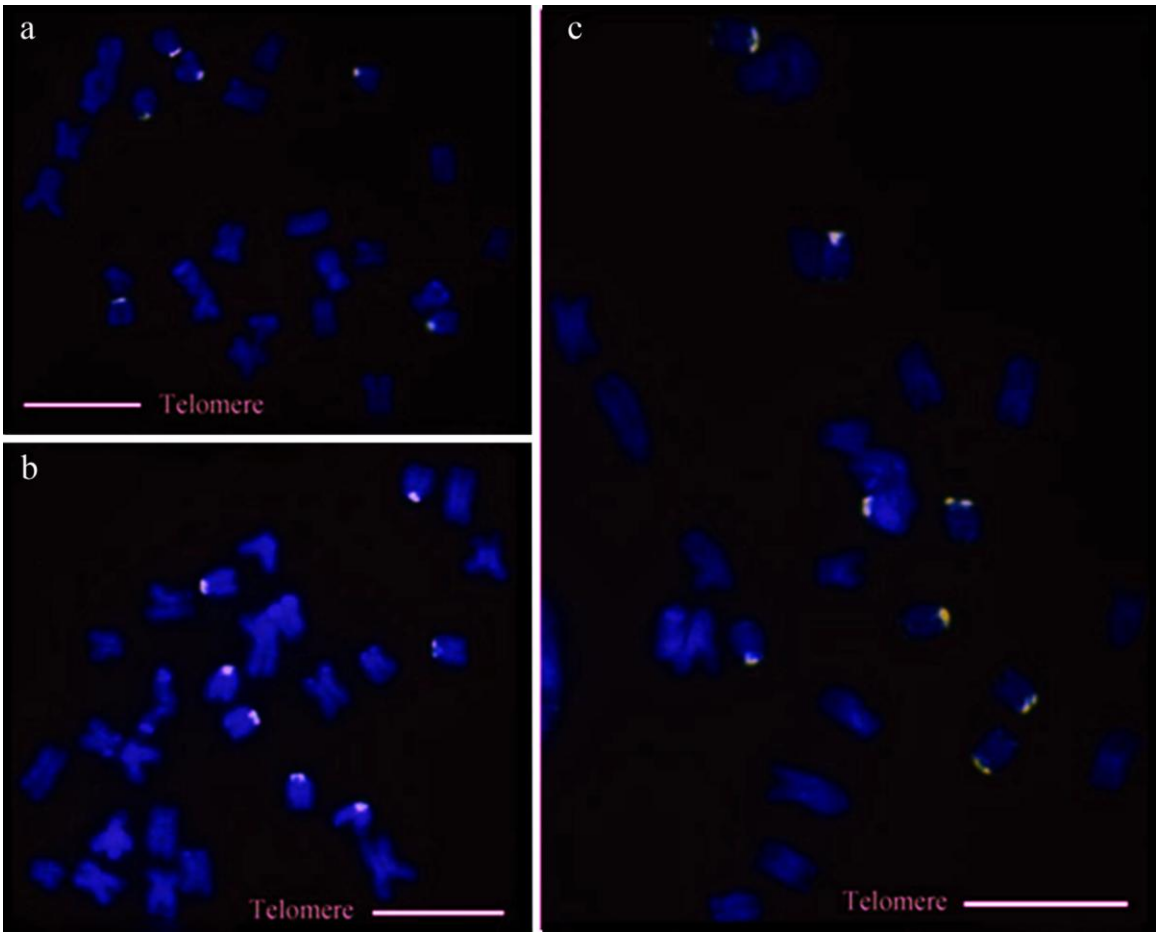

Figure 2.1 Nondisjunction of B chromosomes in a plant with a progenitor zygotic number of seven B's. (a) A chromosome spread showing a reduction in B number to 6 B's. (b) A chromosome spread showing the median number of B's. (c) A chromosome spread showing an increase in B number to 8 B'S. Green is 5' Alexafluor labeled telomere repeat that cross-hybridizes to the B specific repeat. Due to the large B repeat signal, exposure times that were used do not detect the telomere signals at the ends all other chromosomes. Scale bar is 10 microns

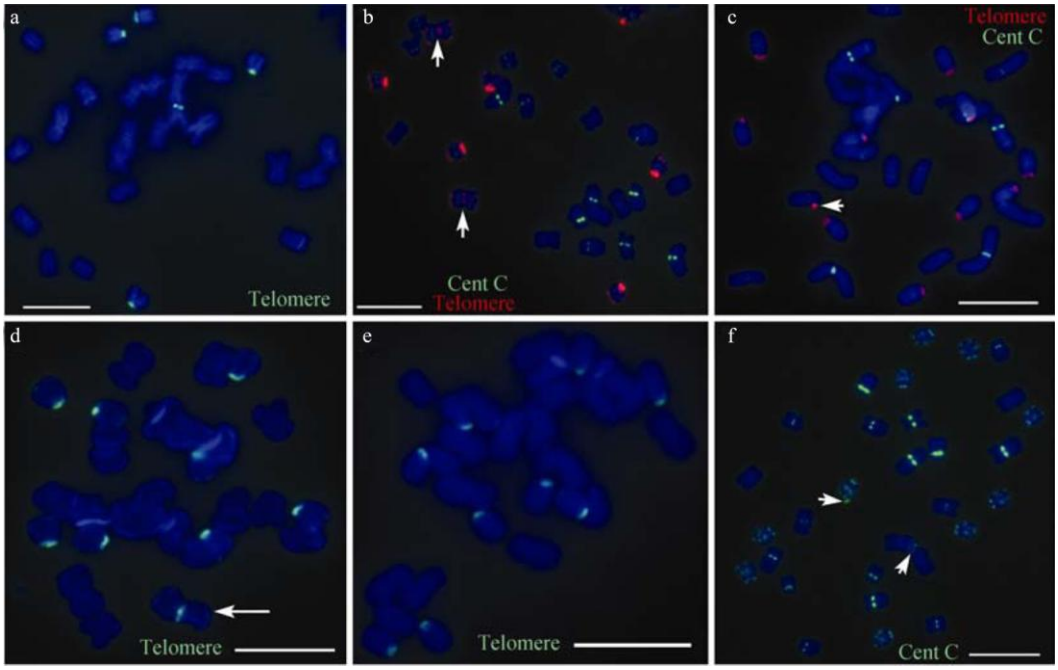

Figure 2.2 Chromosomal abnormalities. (a) An aneuploid with 21 A chromosomes and 5 B's. (b) Two B isochromosomes indicated by arrows in a plant with 5 B's. At the top right is an interphase nucleus of a nearby cell. (c) A B centromeric fragment in a plant with 8 B's. (d) A plant with 8 B chromosomes and a mitoticly unstable B isochromosome. (e) A normal cell missing the mitoticly unstable B isochromosome and gaining an additional B chromosome. (f) Arrows denote a B-A translocation and a B centromeric fragment in a plant with 10 B's. Scale bar is 10 microns

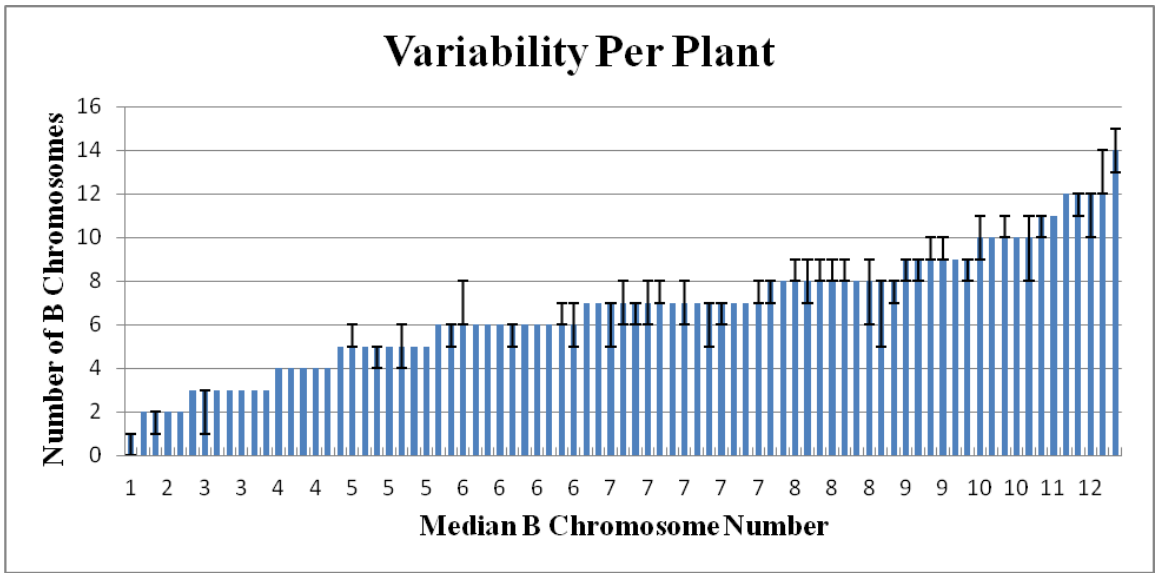

Figure 2.3 A comparison of variability of B chromosome numbers in individual plants. On the X axis each individual root tip from a single is represented with the projected zygotic number of B chromosomes. The Y axis represents the variation in the number of B chromosomes in the root of each individual.

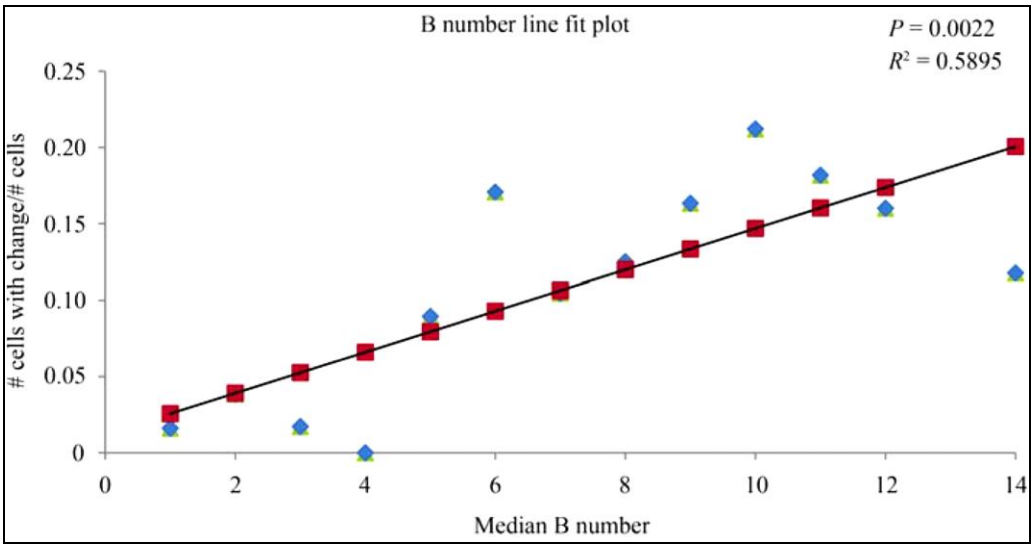

Figure 2.4 Nondisjunction increases proportionally with an increase in B number. A regression analysis indicating an increase in variation is directly correlated with an increase in B number with a *P* =0.0022 and *R2* = 0.5895. Blue is actual data and red is the predicted rate of increase

Table 1
G-tests showing the significance of odd and even numbers.

	Observed	Expected ratio	Expected frequency	Ratio	ln ratio	P value *Chi* square
Even more	22	311	19.5	1.12821	2.6538	
Even less	17	311	19.5	0.87179	–2.3324	
Total	39	622			0.6428	0.4227
Odd more	12	438	19	0.6316	–5.5144	
Odd less	26	438	19	1.3684	8.1551	
Total	38	876			5.2814	0.0216
Even gain	22	311	14.1175	1.5584	9.7598	
Odd gain	12	438	19.8825	0.6035	–6.0592	
Total	34	749			7.4012	0.0065
Even loss	17	311	17.8545	0.9521	–0.8337	
Odd loss	26	438	25.1455	1.0340	0.8688	
Total	43	749			0.0703	0.7909
Even total	39	311	31.9720	1.2198	7.7494	
Odd total	38	438	45.0280	0.8439	–6.4486	
Total	77	749			2.6016	0.1068

Table 2.1 G-tests showing the significance of odd and even numbers of B chromosomes. Plants with odd numbers of B's were more likely to experience a decline in numbers of B's. Plants with even numbers of B's were more likely to experience an incline in numbers of B's

Discussion

Our results indicate that at higher copy numbers, nondisjunction and odd-even effects are present in root tip tissue and may be a trend for the maize B chromosome in the whole organism. As B's increase in number, a proportional increase is seen in nondisjunction rates, although nondisjunction was virtually absent in plants with four or fewer B's. This observation could imply that nondisjunction is either repressed at low numbers or so infrequent that it was not observed. In the microgametophyte, low numbers of B's nondisjoin regularly, which may indicate that the microgametophytic environment is more conducive to nondisjunction to foster the accumulation mechanism of the B chromosome.

The mechanism for the odd-even effect is obscure. Because the loss to gain ratio in plants with even numbers of B's is nearly 1:1, stability seems to be conferred when chromosomes have a homologue present, although there is no evidence for somatic homologue association. Somatic nondisjunction itself could complicate the odd-even effect as well as an estimation of its magnitude, because different numbers of B chromosomes can be present in different developmental lineages assayed in any one root tip. Odd-even effects have been observed for both the maize and rye B chromosomes despite a lack of evidence for somatic pairing, leaving the basis for the effects obscure (Morais-Cecílio et al., 1996).

Recent advances in techniques for mitotic arrest (Kato, 1999) and for chromosome painting (Kato et al., 2004) have allowed a more in depth investigation into the maize B chromosome during the life cycle and have revealed new B phenomena, although not entirely unrelated to previously established traits. B nondisjunction in the

sporophyte and gametophyte are probably related, but the male gametophyte may be a more tolerant environment for the nondisjunction property that contributes to the B accumulation mechanism.

CHAPTER 3: THE ACCUMULATION OF MULTIPLE COPIES OF MAIZE MINICHROMOSOMES

Introduction

B chromosomes are supernumerary chromosomes found in many species and proliferate using species-specific methods of meiotic or mitotic drive. The maize B chromosome accumulates by surviving as a univalent in meiosis, nondisjunction at the second pollen mitosis, and by the B-containing sperm preferentially fertilizing the egg (Longley, 1927, Roman, 1948, Carlson, 1969, Carlson et al., 1992). Although no identified genes have been localized to the maize B chromosome, it can increase recombination in the normal karyotype (A's) and in certain cultivars, can trigger breaks in knobbed chromosomes at the second pollen mitosis (Nel, 1973, Randolph, 1941, Hanson, 1969, Rhoades et al., 1967).

The survival mechanism of the B chromosome can be manipulated to accumulate multiple B's in the genome. Although vigor and fertility are affected when numbers are significantly increased, by selecting for plants with the highest number of B's in successive generations, 34 B chromosomes have been observed (Randolph, 1941). The first phenotype of maize plants with multiple B chromosomes is a longitudinal white striping on leaves, which appear sporadically when five copies are present, and are increasingly frequent as B chromosomes are accumulated (Staub, 1987). Obtaining plants with more than 22 B's was increasingly difficult, as reductions in vigor and fertility hindered further accumulation (Randolph, 1941).

Successive studies have been undertaken to dissect properties of the maize B chromosome using breakage derivatives and translocations of the B. There are several essential regions of the B chromosome necessary for nondisjunction at the second pollen mitosis. The distal euchromatin and a segment in the proximal euchromatin of the B are trans-acting and must be present in the cell for the two cis-acting sites in the proximal euchromatin and proximal heterochromatin to initiate nondisjunction (Roman, 1949, Ward, 1973, Carlson, 1973, Lin, 1978, Rhoades et al., 1972, Rhoades et al., 1967, Carlson et al., 1981). The short arm of the B chromosome can also act in trans to increase the frequency of B chromosome nondisjunction, but is not essential (Carlson, 1978a, Lin, 1979).

In this study we used four different sized minichromosomes to test the accumulation limits of B chromosome derivatives. The first two minichromosomes 86-74 and 76-15a are derived from the bombardment of telomere repeats into maize embryos resulting in truncations of the B chromosome (Yu et al., 2007). The 76-15a minichromosome has approximately half of the distal heterochromatin of the B intact, resulting in a minichromosome half the size of a normal B chromosome (Fig 3.1d). The 86-74 minichromosome is a truncation of the B that removed all of the distal heterochromatin, but appears to have all of the proximal euchromatin intact. (Fig 3.1c, Fig 1.1a-d) (Yu et al., 2007). The smallest minichromosomes are mini 9 and 20, which (Fig 3.1a and Fig 3.1b) are derived from a breakage fusion bridge cycle (BFB). Minichromosome 20 is missing all chromatin distal to the proximal knob of the B, and

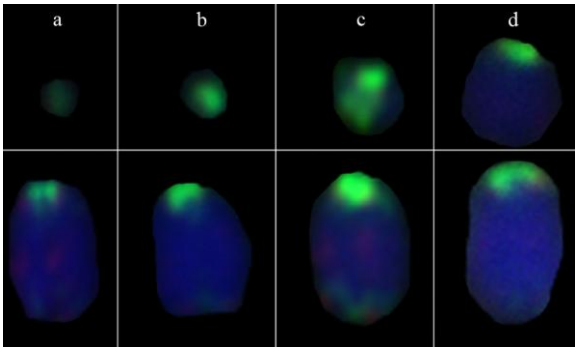

Figure 3.1 The different relative sizes of B-derived minichromosomes (top) to the B chromosome (bottom). Each cell has differential levels of condensation and thus requires a standard B chromosome for a comparison between minichromosomes. (a) Minichromosome 9, is the smallest chromosome derived from the breakage-fusion-bridge (BFB) cycle. (b) Minichromosome 20 is the second smallest chromosome derived from the BFB cycle. (c) Minichromosome 86-74 is the second largest minichromosome derived from telomere truncation. (d) Minichromosome 76-15a is the largest minichromosome derived from telomere truncation

minichromosome 9 appears to be missing this knob that is a prerequisite for nondisjunction at the second pollen mitosis. Both mini 9 and mini 20 have the same origin, which started with an inter-chromatid exchange on TB-9SbDp9, a B centromere translocated to an inverted duplication of chromosome arm 9S. Crossovers in this orientation result in the formation of a dicentric chromosome transmitted to the next generation. A series of breaks and fusions removed the intervening chromatin, and a small chromosome, equivalent in size to a B chromosome centromere, eventually stabilized. The trans-acting factor of a normal B chromosome in the genome enabled the accumulation of these minichromosomes using the mitotic drive of the B chromosome (Han et al., 2007, Zheng et al., 1999).We will examine whether the accumulation limits are controlled by the mass of chromatin, or possibly the number of centromeres.

Methods

Plant Materials

Minichromosome 9 and minichromosome 20 were both derived from the breakage fusion bridge cycle in a mixed background (Han et al., 2007, Kato et al., 2005). 86-74 and 76-15a minichromosomes were obtained by bombarding telomere containing constructs into HI-II maize embryos containing B chromosomes (Yu et al., 2007). The breeding program for accumulation kept each minichromosome line isolated with constant self and sib pollinations. To obtain higher frequencies of minichromosomes in the next generation a male was selected that had the highest number of minichromosomes, while keeping at least one B chromosome in the background to supply the trans-acting factors for nondisjunction.

Mitotic in-situ Hybridization

Root tip chromosome screens were performed as previously described in detail (Masonbrink et al., 2010) with a slight modification. Distilled water was substituted for all ethanol washes, which kept cell wall enzymatic digestion times predictable. The fluorescently labeled probes consisted of a microsatellite TAG repeat, the nucleolar organizer repeat (45S rDNA), a telomere repeat oligonucleotide that strongly cross hybridizes to the B repeat, and a CentC oligonucleotide that hybridizes to the centromere of all chromosomes as well as the B chromosome long arm (Alfenito et al., 1993, Lamb et al., 2005).

Probe Development

Probes were designed as previously described (Danilova et al., 2008). Table 3.1 lists the details to single gene probe development.

PCR Conditions

As a template for PCR reactions, BAC DNA was used (Table 3.1). The reactions included JumpStart REDTaq ReadyMix (Sigma, cat. P0982), 5 μM of each primer, and 0.5–4 ng/μl of template DNA. PCR cycles consisted of 95°C, 5 min; initial denaturation, 35 cycles; 95°C at 30 s, annealing temperature 57°C at 30 s, 72°C; extension time, 1 min for each 1,000 bp; and final extension, 3× extension time.

Results

The B chromosome was accumulated to 21 copies in a single plant of the B73 inbred line, which was sterile (Fig 3.2). The two largest minichromosomes, 76-15a and 86-74, were accumulated to 19 copies, while mini 9 and 20 were accumulated to 9 and 17 copies,

Gene Name	Primers	PCR product length	Source BAC	Accession Number
Elongation factor1-gamma 3	AAATTTATGGCGTGTATAAAACAAAA GCAACCCTAGATTTCGCTTC	3.3kb	240f20	AC204632
Auxin independent growth promoter	GCGTCGGTGACTGGCATA TGCCCTGATGCAACATATATCT	3.6kb	272b9	AC195262
Protein phosphatase type-2c	TCGATAGAGAAGGTGTCATCTATATCT CTGCTACGGTTTAACGATTGC	4.7kb	114p3	AC207260

Table 3.1 The three gene specific probes used to identify a proximal site on 6L.

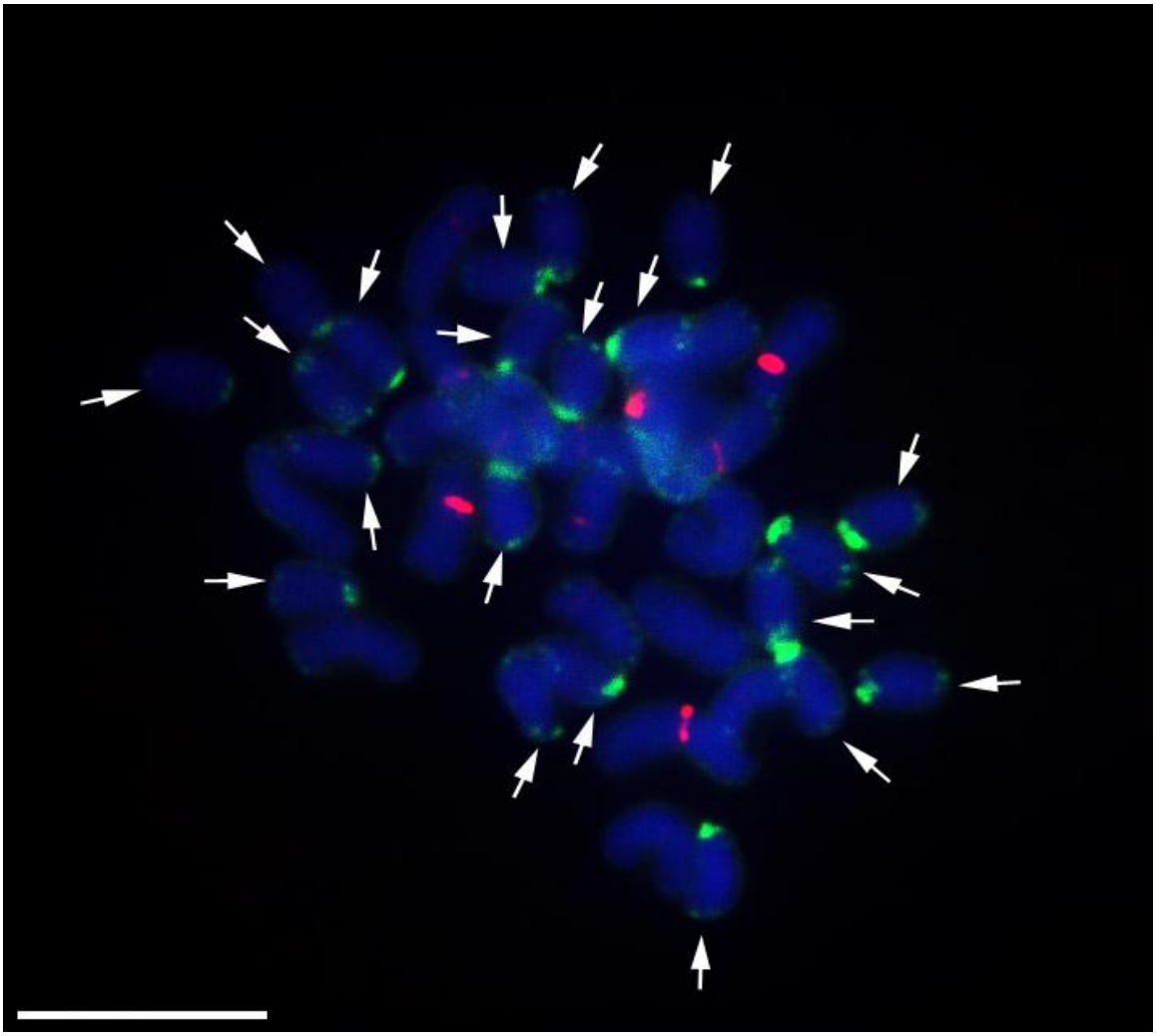

Figure 3.2 A maize root cell with 21 B chromosomes accumulated in the B73 inbred line. Each white arrow points to a B chromosome. Typically the average number of B chromosomes per cell is a slightly lower number among cells of the same root, due to the somatic nondisjunction of the B chromosome in roots (Masonbrink et al., 2010). Differential signal strength on the B chromosomes is a result of background signal reduction in Adobe Photoshop 5.0. Telomere probes, which cross-hybridize to the B repeat, are green. CentC probes are red. The scale bar is 10 microns

respectively, all of which also contain one to five B chromosomes (Fig 3.3). All plants with the highest number of minichromosomes failed to produce seeds, although these plants did produce silks and some pollen. From generation to generation the increase in the number of minichromosomes was progressively less, while plants with lower numbers were frequently observed (Table 3.2). In nearly every generation a new maximum number of minichromosomes was found, while a generational increase in the average number of minichromosomes per plant was much less pronounced. The nondisjunction of minichromosomes between cells of the same root tip may have contributed to the maximal chromosome counts. This was apparent when the maximal number in a cell was not the most frequently found number of minichromosomes in the root squash. In addition, B repeat chromatin was commonly diffuse and outstretched between multiple mini and B chromosomes that frequently clumped together in metaphase spreads (Fig 3.4).

Plants with at least four B chromosomes displayed white longitudinal leaf stripes, while plants that had more than ten copies had additional severe phenotypes including: reduced fertility, reduced vigor, a zigzag pattern of internode growth, and asymmetric leaf blades (Fig 3.5 and Fig 3.6). Other than reduced fertility, plants with minichromosomes did not show B chromosome accumulation-related phenotypes, even at high copy numbers.

An A derived minichromosome in combination with B derived minichromosomes

The origin of a chromosome (86B-136) carrying a nucleolar organizer region (NOR) derived from telomere truncation experiments was clarified by combining three

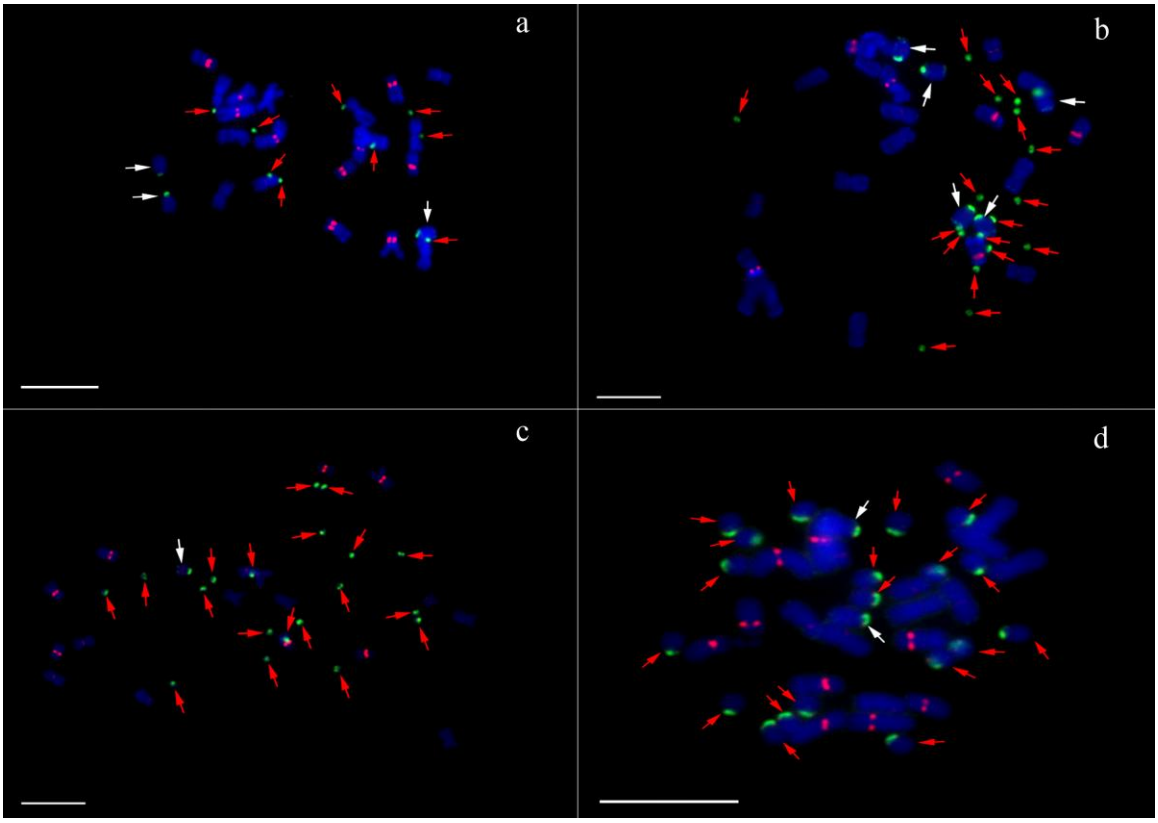

Figure 3.3 Cells from each minichromosome line showing the maximum number of minichromosomes. Red arrows denote minichromosomes, and white arrows denote normal B's. (a) A cell showing the maximum number of mini 9's at 9 copies and 3 B chromosomes. (b) A cell showing the maximum number of mini 20's at 17 copies and 5 B chromosomes. (c) A cell showing the maximum number of the 86-74 minichromosome at 19 copies and 1 B chromosome. (d) A cell showing the maximum number of the 76-15a minichromosome at 19 copies and 2 B chromosomes. Typically the average number of minichromosomes and B chromosomes per cell is a slightly lower number among cells of the same root, due to somatic nondisjunction of B chromosomes and minichromosomes in roots. Telomere probes, which cross-hybridize to the B repeat, are green, and CentC probes are red. The scale bar is 10 microns

Type	Gen	Number of Minichromosomes in a Plant																			n=	Avg
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
Mini 76-15a	1	3	3	9	9	14	16	7	2	2	1	0	0	0	0	0	0	0	0	0	0	66	4.1
	2	0	0	1	2	4	2	11	10	6	5	2	0	0	0	0	0	0	0	0	0	43	6.6
	3	0	0	0	1	1	0	4	4	7	7	1	1	1	2	0	1	0	0	0	0	30	8.4
	4	0	0	1	1	3	4	4	1	4	2	2	1	0	0	0	0	0	0	0	0	23	6.5
	5	0	0	0	3	3	1	13	7	13	3	4	2	3	2	0	1	2	0	0	1	58	8.2
	6	0	0	0	2	3	4	3	3	7	6	1	2	1	0	0	0	0	0	0	0	32	7.2
Mini 86-74	1	0	10	10	19	16	5	2	1	0	0	0	0	0	0	0	0	0	0	0	0	63	3.1
	2	1	1	4	4	9	9	10	13	6	4	2	2	1	0	0	0	0	0	0	0	66	5.9
	3	0	4	10	14	13	25	16	13	7	1	2	3	0	1	0	0	0	0	0	0	109	5.1
	4	0	0	2	1	2	6	8	9	5	14	9	6	5	7	4	5	3	3	0	2	91	9.9
Mini 20	1	3	8	4	11	9	6	4	2	8	0	0	0	0	0	0	0	0	0	0	0	55	4.0
	2	3	10	18	14	19	12	10	8	2	0	2	1	0	0	0	0	0	0	0	0	99	3.9
	3	3	2	4	0	3	4	3	0	2	3	1	1	1	0	0	0	0	1	0	0	28	5.6
	4	0	2	1	2	4	7	3	3	2	2	2	1	5	4	1	0	1	1	0	0	41	7.9
	5	2	2	4	5	5	4	10	6	13	6	7	8	4	1	1	0	0	1	0	0	79	7.3
Mini 9	1	17	33	25	6	5	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	89	1.7
	2	9	12	13	13	11	4	3	1	0	1	0	0	0	0	0	0	0	0	0	0	67	2.7
	3	7	17	18	16	5	3	3	2	2	2	0	0	0	0	0	0	0	0	0	0	75	2.8
	4	19	13	24	12	10	9	5	3	1	0	0	0	0	0	0	0	0	0	0	0	96	2.7
	5	13	22	18	9	3	6	2	3	1	0	0	0	0	0	0	0	0	0	0	0	77	2.4

Table 3.2 The accumulation of each minichromosome type in the last 4-6 generations. Yellow boxes highlight the frequency of plants with the average number of B's per plant in each generation. Blue boxes highlight the highest minichromosome number found in each generation. The "n=" category shows the number of plants screened in each generation out of 48-96 germinated seeds. This discrepancy is related to the number of unviable seeds

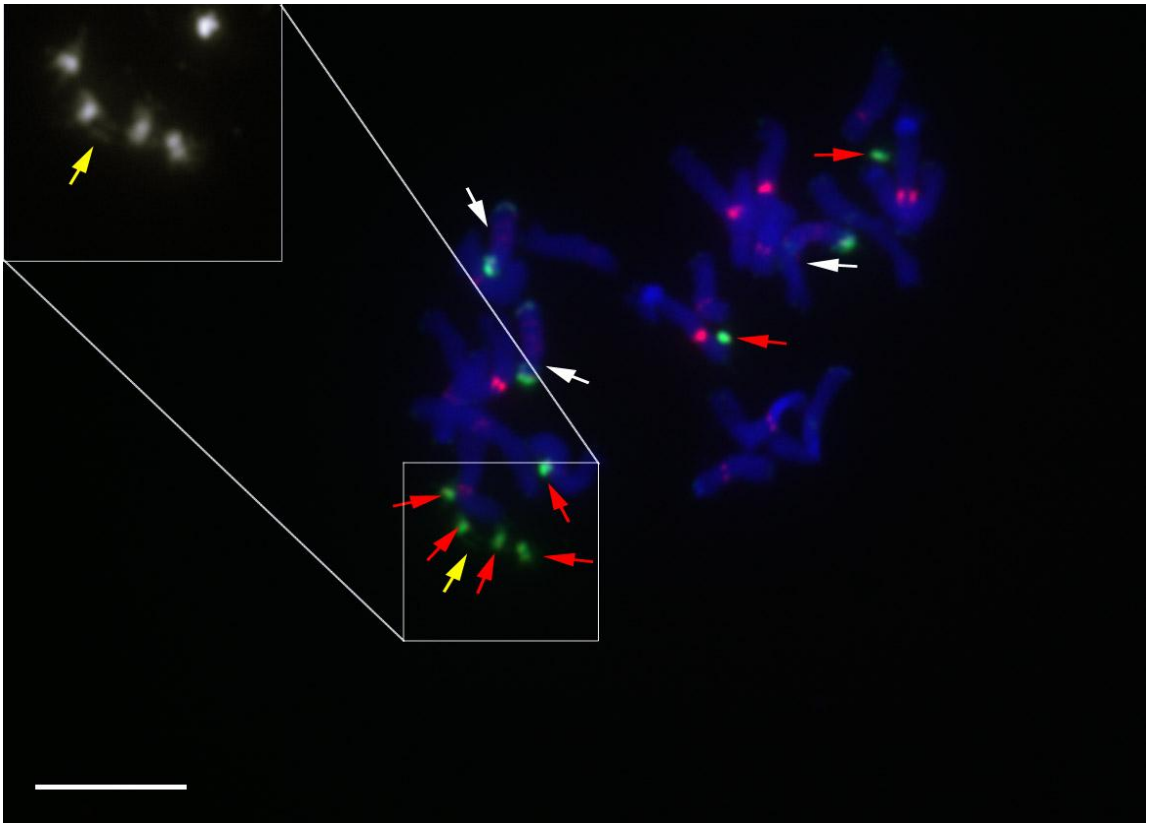

Figure 3.4 A cell from a maize plant showing diffuse B repeat chromatin. White arrows denote B chromosomes, red arrows denote minichromosomes, and the yellow arrow denotes diffuse B repeat chromatin. Inset shows diffuse B repeat chromatin in the green channel (B repeat). Telomere probes, which cross-hybridize to the B repeat, are green and CentC probes are red. The scale bar is 10 microns

Figure 3.5 A plant with thirteen full-sized B chromosomes showing reduced vigor, a zigzag pattern of growth at internodes, white longitudinal leaf striping, and branching

Figure 3.6 Leaf of a plant with 12 B's showing asymmetric leaves and lengthwise white stripes

gene specific FISH probes to the proximal end of the long arm of chromosome 6 as well as the NOR (45S rDNA) (Fig 3.7a & Table 3.1) (Yu et al., 2007). This truncation was thought to be a fission of the centromere, but as the gene-specific probes indicate, the proximal end of 6L is still present on the 86B-136 minichromosome (Fig 3.7a). During its characterization, another stably inherited fragment was found (Fig 3.7a). We were able to accumulate two copies of this fragment (Fig 3.7b), but could not clarify its origin. B chromosomes were added to the genome with these minichromosomes, but neither A-derived minichromosome could increase beyond two copies, (Fig 3.7b and Fig 3.7c). A 86B136 containing plant was crossed to the 86-74 minichromosome line and multiple numbers of the 86-74 mini as well as two 86B136 minichromosomes were found in the next generation (Fig 3.7c).

Other abnormalities observed

Aneuploidy of the normal karyotype (A chromosomes) was seen occasionally, although most screens did not involve a count of the A chromosomes. The progeny of one 76-15a minichromosome plant had high levels of aneuploidy (53% of seeds) to varying degrees (21A's - 25A's). In addition, two B-A translocations were found. The first B/A translocation is a complex rearrangement between one chromosome 4 homologue, an unidentifiable A chromosome, and two minichromosomes (Fig 3.8a). The second translocation involved chromosome 4, identified by the telomere probe cross-hybridization to Cent4 (Fig 3.8b). Two A chromosome fragments of an unknown origin were observed, one with a CentC signal (Fig 3.8d) and one without (Fig 3.8c). The fragment without a centromere signal was differentiated from the commonly distended NOR by having two sets of telomere signals. B chromosome derived abnormalities were

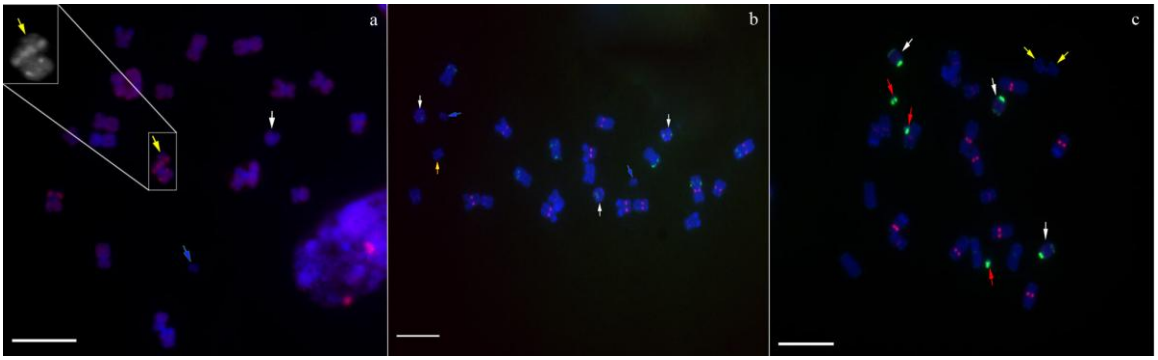

Figure 3.7 Characterization of minichromosome 86B136 and another unknown fragment. White arrows denote B chromosomes and blue arrows denote the fragment of unknown origin. Yellow arrows denote the 86B136 minichromosomes and red arrows the 86-74 minichromosomes. (a) A maize plant with a 86B136 minichromosome, a B chromosome, and a minichromosome of unknown origin probed with three gene-specific red probes on chromosome arm 6L, and an additional red NOR probe. Inset shows red channel only with normal chromosome six (lower) adjacent to the 86B136 minichromosome (upper). (b) A maize plant with a 86B136 minichromosome, two minichromosomes of unknown origin, and 3 B chromosomes. CentC probes are red and TAG probes are green. (c) A maize plant with two 86B136 minichromosomes, three 86-74 minichromosomes, and three B chromosomes showing two types of minichromosomes with different inheritance mechanisms. Telomere probes, which cross-hybridize to the B repeat, are green and CentC probes are red. All scale bars are 10 microns

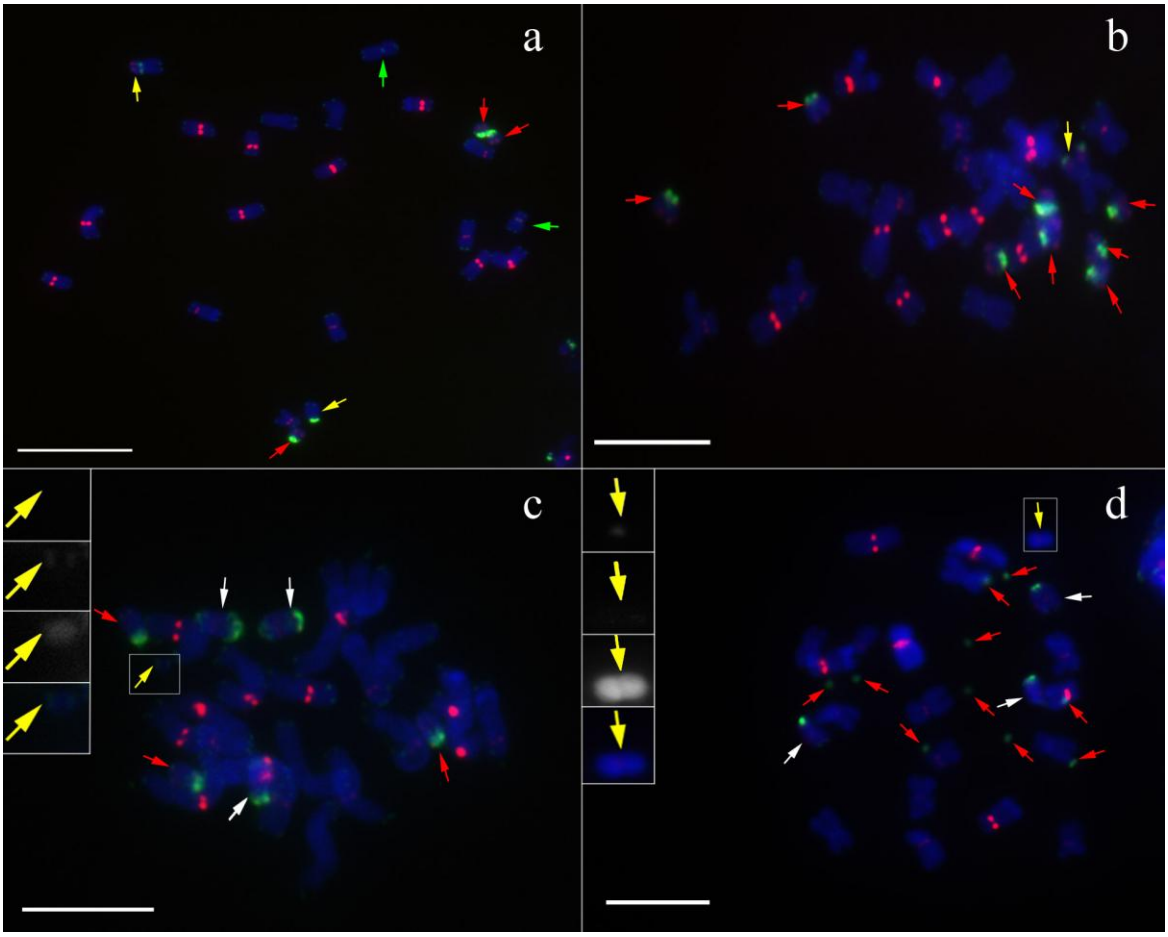

Figure 3.8 B-A translocations and A chromosome fragments. White arrows denote normal B chromosomes, red arrows denote minichromosomes, and yellow arrows denote chromosomes of interest. (a) A reciprocal translocation of an unidentified A chromosome (yellow arrows), a B chromosome, and chromosome 4. A B chromosome centromere-sized B repeat signal is internal on the A-B chromosome, and one chromosome 4 homologue is significantly reduced in size (green arrows), indicating a complex rearrangement. (b) A B-A translocation (yellow arrow) of chromosome 4 without the reciprocal exchange present. (c) An A chromosome fragment that is missing the CentC centromere repeat and doesn't appear to have a constriction (yellow). The two sets of telomere signals distinguish it from the NOR, which commonly becomes appears

distended. (d) An A chromosome fragment (yellow) with two sets of telomere signals and a CentC signal in the primary constriction. Telomere probes, which cross-hybridize to the B repeat, are green and CentC probes are red. The scale bars are all 10 microns

more commonly observed, which were derived from chromosome breaks in the 86-74 mini, the 76-15a mini, or from normal B chromosomes. One frequently observed derivative in the 86-74 and 76-15a minichromosome lines was a small chromosome consisting of only the B centromere, which was still capable of nondisjunction (Fig 3.9a). Such a derivative was indistinguishable from mini 9 and 20 and therefore could not be scored if they formed in these two lineages. Another derivative appeared to be missing distal portions of the B chromosome long arm (Fig 3.9b). In addition, two derivatives from the B and 86-74 mini were found missing most of the B repeat at the centromere (Fig 3.9c & 3.9d).

Multiple rearranged minichromosomes were found with additional B repeat signals on both ends of the chromosome (Fig 3.10). Although multiple B repeat signals could indicate the presence of a dicentric chromosome, these rearranged minichromosomes were stable, possibly the result of centromere inactivation. Another indication of their stability was the constriction that was only seen at one of these B repeat signals. Even so, one of these derivatives was increased to two copies in a subsequent generation and remained stable (Fig 3.10C).

During the accumulation of the 76-15a minichromosomes, a plant was found that had chromosomes with B repeat at the distal ends of both arms, indicative of fusion between two broken chromosomes (Fig 3.11). The chromatin between these distal B repeat signals constantly increased and decreased in size, and frequently chromosomes undergoing the BFB would appear at multiple different sizes with one B repeat signal. The frequent structural change in these chromosomes indicates the presence of two

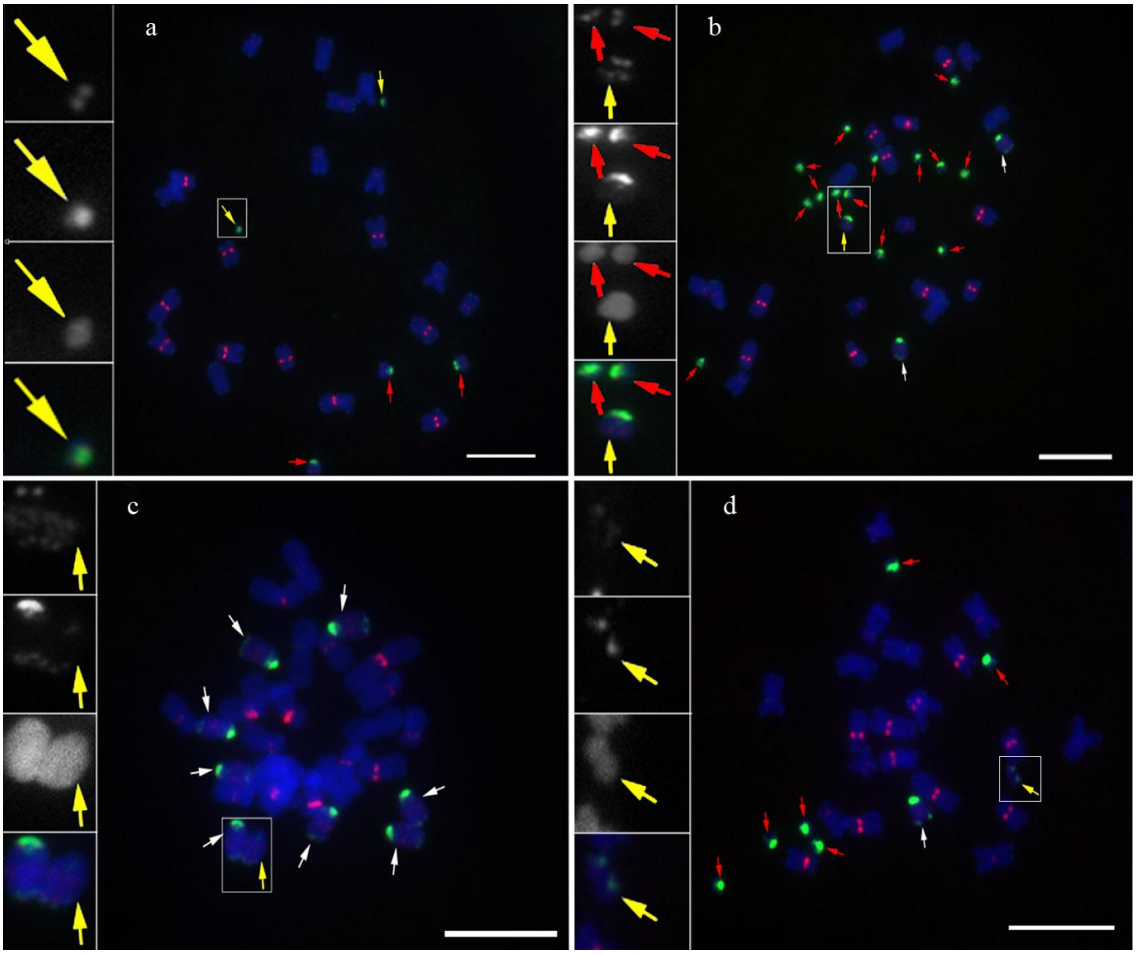

Figure 3.9 Breakage derivatives of the B chromosome and /or minichromosomes. White arrows denote B chromosomes, red arrows denote minichromosomes, and yellow arrows denote the broken derivatives. Insets in descending order, show the red (CentC), the green channel (B repeat), the blue channel (DAPI), and all channels. (a) A cell from a maize plant with three 86-74 minichromosomes and two centromere-only derivatives derived from a B chromosome or a minichromosome. (b) A cell from a maize plant with 1B chromosome and fifteen 86-74 minichromosomes including a broken B derivative that is missing half of the long arm. (c) A cell from a maize plant with 9 B chromosomes including a B chromosome with reduced B repeat. (d) A cell from a plant with 1 B chromosome and six 86-74 minichromosomes including the 86-74 minichromosome with

reduced B repeat signal at the centromere. CentC probes are red and telomere probes, which cross-hybridize to the B repeat, are green. The scale bars are 10 microns

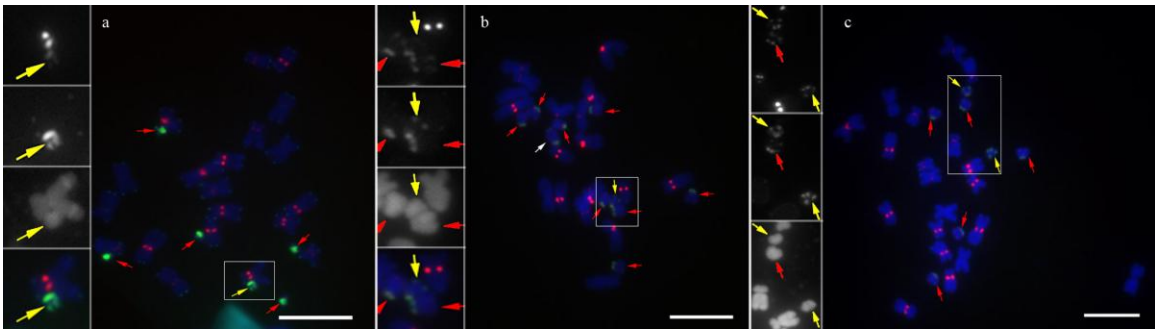

Figure 3.10 Three B chromosome derivatives with B repeat on both chromosome arms. CentC probes are red, and telomere probes are green. Insets in descending order, show the red (CentC), the green channel (B repeat), the blue channel (DAPI), and all channels. (a) A cell from a plant with five 86-74 minichromosomes and 1 rearranged minichromosome with unequal distal B repeat signals on both arms (b) A cell from a plant with eight 76-15a minichromosomes, one rearranged minichromosome with B repeat in distal positions on both arms (yellow arrow), and one B chromosome. (c) A cell from a plant with five 76-15a minichromosomes (red arrows) and two rearranged minichromosomes with B repeat signals in distal positions on both arms. Scale bars are 10 microns

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Figure 3.11 A chromosome type breakage-fusion-bridge cycle. Six cells from the same root tip showing one B chromosome and one 76-15a minichromosome that remains stable in all cells, while various numbers of rearranged minichromosomes participate in a BFB cycle. Telomere probes are green, CentC probes are red, and DAPI is blue. White arrows denote B chromosomes, red arrows denote minichromosomes that appear monocentric, blue arrows denote chromosome fragments, and yellow arrows denote possible dicentric chromosomes. (a) A cell with a large dicentric chromosome, two monocentric minichromosomes, and a B chromosome. (b) The red channel (CentC) of "a" showing an internal section of the dicentric missing CentC. (c) The green channel (B repeat) of "a". (d) The blue channel (DAPI) of "a" showing a metacentric constriction on the large dicentric (e) A cell with a large dicentric chromosome, two monocentric minichromosomes, a B chromosome, and a chromosome fragment. (f) Red channel of "e" showing the similar structure to the dicentric in "a" and a lack of signal to the chromosome fragment. (g) The green channel of "e". (h) The blue channel of "e" showing the chromosome fragment. (i) A cell with two medium sized dicentric chromosomes, three monocentric minichromosomes, and one B chromosome. This may be a descendent cell of the large dicentric showing that broken ends may heal as shown by a similar pattern of CentC. (j) The red channel to "i" showing both dicentrics have similar structure to half of the dicentric in "a" and "e". (k) The green channel of "i". (l) The blue channel of "i". (m) A cell with a small dicentric, a monocentric minichromosome, and a B chromosome. (n) The red channel to "m" showing two separate sets of CentC signals to the small dicentric chromosome. (o) The green channel of "m" showing a separation of the signal in the dicentric chromosome. (p) The blue channel of "m". (q) A cell with one

very small dicentric chromosome, two small dicentrics, two monocentric minichromosomes, and one B chromosome. (r) The red channel of "q" showing the distal centromere locations on the three dicentrics. (s) The green channel of "q". (t) The blue channel of "q". (u) A cell with two medium sized dicentrics, three monocentric minichromosomes, and one B chromosome. (v) The red channel of "u" showing one dicentric with internal CentC and one without the internal signal, indicating a similar lineage to the dicentrics in "i". (w) The green channel of "u". (x) The blue channel of "u". All scale bars are 10 microns

functional centromeres actively breaking and fusing among sister cells of the same root, suggesting the presence of a breakage-fusion-bridge cycle.

Discussion

The highest reported number of B chromosomes ever accumulated in a maize plant was 34 (Randolph, 1941), and in our study the maximum number obtained was 21 (Fig 3.2). The discrepancy between the numbers could be from breeding plan differences. The previously reported number was obtained by maintaining hybrid vigor, while our maximum was achieved through consistent inbreeding.

The minichromosome maxima were obtained in the same manner as the B's accumulation, although the rate was somewhat slowed by the necessity of keeping 1-2 normal B's in the genome to provide the trans-acting nondisjunction factors. Accumulation of shortened B derivatives was also investigated in a previous study, although sterility was seen before more than 16 copies were found (Randolph, 1941). The B chromosome is capable of somatic nondisjunction in roots, which also occurs for minichromosomes. Somatic nondisjunction played a role in achieving the maximum number, as it was the peak quantity seen in a range of cells. Because the minichromosomes could not be accumulated to higher numbers than B chromosomes, the limit is more likely based on B centromere quantities, rather than a chromatin mass limit.

Multiple phenotypes have been associated with increased numbers of B chromosomes in the genome including: reduced fertility, decreased vigor, defective seeds, scarred endosperm, pollen abortion, and white longitudinal stripes on leaves (Fig 3.5 and Fig 3.6) (Staub, 1987, Randolph, 1941). The only phenotype connected to minichromosome accumulation was reduced fertility, which was seen previously with the

accumulation of the shortened B derivatives (Randolph, 1941). This suggests that all previously described B chromosome accumulation-associated symptoms may be caused by multiple copies of the long arm of the B chromosome.

The cross of the 86-B136 A chromosome truncation and 86-74B truncation proves that two types of minichromosomes can coexist with one remaining a stable number while another increases in quantity. Previous studies with telotrisomics have shown that significant reductions in vigor and fertility occur with increased doses of genes linked to the additional chromosome (Rhoades, 1940). The same issues were apparent with the 6S minichromosome, although it is possible that the smaller A-derived minichromosome, which lacked a transgene, may be a better candidate for dual gene expression platforms.

The F type B chromosome derivatives described by Randolph in 1941 as "essentially naked centromeres" are likely the same as our centromere-only B derivatives. The derivatives with reduced centromeres are probably the complement to a centromere-only break, since most of the B repeat is missing. A less common break site may be present at the proximal end of the distal heterochromatin on the long arm of the B (Fig 3.9b). Randolph describes the frequencies of certain breakage sites along the B that are commonly recovered, indicating that certain locations along the B chromosome are more fragile (Randolph, 1941).

The various chromosomal rearrangements with B repeat at distal regions on both arms likely arise from breaks in the long arm of minichromosomes or B chromosomes followed by chromosome fusion. When the two centromeres go to opposite poles in a subsequent anaphase, the broken chromosomes would fuse in the telophase nucleus

initiating a BFB cycle. This would account for the changes in size of the chromatin between the two centromeres. The chromosomes with B repeat at distal locations on both arms in Fig 3.8 probably arose from a BFB cycle, but were stabilized with centromere inactivation, as previously described (Han, Lamb et al. 2006). Two of these stable rearranged chromosomes have differing amounts of B repeat (Fig 3.10a and Fig 3.10b), and the constriction associated with centromere activity seems to follow the larger signal, which is in agreement with previous studies (Han et al., 2009).

The present study indicates that engineered minichromosomes derived from the B chromosome can be increased in copy number to amplify the output of the added genes. The amplification requires the presence of a full sized B chromosome. The total number of extra chromosomes for the various cases was about 20. Based upon Randolph's result of achieving 34 B chromosomes in a hybrid, this might be an approach to surpass the limits we observed, but this would require, first introgression into different inbred lines, and their subsequent amplification.

CHAPTER 4: THE BEHAVIOR OF MULTIPLE MAIZE MINICHROMOSOMES IN MEIOSIS

Introduction

B chromosomes are supernumerary chromosomes that are found in many species despite an unconventional inheritance and few if any genes. They proliferate using species-specific mechanisms of meiotic or mitotic drive resulting in a higher-than-expected transmission (Longley, 1927, Houben et al., 2011). The maize B chromosome escapes elimination from the genome and proliferates using three advantages: survival of a univalent in meiosis, nondisjunction at the second pollen mitosis, and preferential fertilization of the egg by the B-containing sperm. While genetic control of preferential fertilization has been associated with chromosomes of the normal karyotype (A's), univalent survival and the ability to selectively nondisjoin is linked to the B (Chiavarino et al., 2001, Carlson et al., 1992, Carlson, 1969). One region that enables nondisjunction of the B chromosome is localized to the distal tip of the long arm, and it must be in the same cell for nondisjunction of the B centromere to occur (Roman, 1947). Using this process, B chromosomes can be accumulated to multiple copies with little discernible effect on the plant; however, a phenotype becomes apparent as their numbers increase (Randolph, 1941, Staub, 1987).

Because the maize B chromosome is the progenitor to the minichromosomes described in this study, it is essential to understand the pairing dynamics of B's. The behavior of B chromosomes is dependent on the genetic background, but typically B

chromosomes pair as bivalents when two copies are present. A univalent B chromosome can also precociously migrate or behave as a laggard in anaphase I, which can lead to their elimination from the nucleus (Ricci et al., 2007). Maize B chromosomes stimulate recombination in areas proximal to the centromere of A chromosomes, and in rye the total chiasma frequency of B's in a cell is directly proportional to the number of B's in a plant (Nel, 1973, Jones et al., 1967). Rye B chromosomes do not increase the frequency of chiasmata in A chromosomes, but they do affect the distribution of chiasma between chromosome arms of A bivalents with increasing quantities of B's. When number of B's was increased in rye, it was observed that the frequency of univalent B and A chromosomes increased, which was attributed to the increased variation of chiasma frequencies (Jones et al., 1967).

The meiotic behavior of minichromosomes at one and two copies was described previously using 22 B chromosome-derived minis of varying sizes (Han et al., 2007). In one copy, all of the minis exhibited precocious sister chromatid separation at anaphase I. When two copies of a mini were in the genome, they observed bivalent formation in fourteen of the twenty-two minis at a frequency of 25-100%. Smaller chromosomes were frequently univalent at the metaphase I plate, and showed precocious sister chromatid separation at anaphase I (Han et al., 2007). While this study addressed the pairing of minis at two copies, a study of higher numbers would provide information useful for the utility of engineered minichromosomes in circumstances in which an increased copy number is desired for maximizing gene output and provide new insight into chromosome pairing behavior.

The pairing of four different B-derived minichromosomes was compared in this study: two telomere truncated (half & fifth) (Yu et al., 2007) and two derived from the breakage fusion bridge cycle (20 & 9) (Zheng et al., 1999). The smallest chromosomes, mini 9 and mini 20, were the product of an interchromatid crossover on TB-9SbDp9, an inverted duplication of chromosome arm 9S translocated to a B chromosome. A crossover in the inverted region forms a dicentric chromosome which initiates a series of breaks and fusions that continue into the next generation and eventually remove the intervening chromatin of the dicentric until only a small chromosome remains (Han et al., 2007, Zheng et al., 1999).

Methods

Mitotic in-situ Hybridization

Root tip chromosome analyses were performed as previously described (Masonbrink et al., 2010) with a slight modification. Distilled water was substituted for all ethanol washes, as cell wall digestion times were more predictable. Fluorescently labeled telomere repeat oligonucleotides strongly cross-hybridize with the B-specific repeat and were used to detect B chromosomes. The telomere signals are underexposed and they usually cannot be seen (Alfenito et al., 1993). Fluorescently labeled CentC oligonucleotides hybridize to the centromeres of all chromosomes and also the B chromosome long arm (Lamb et al., 2005).

Meiotic Fluorescent in-situ Hybridization

Tassels were fixed when the anthers on the main branch began to turn yellow, while the lowest part of the secondary branches were still small and white. They were fixed in 3:1 ethanol to acetic acid at 4°C overnight. Then the tassels were transferred to 70% ethanol

and stored at -20°C. Anthers were extracted from the glumes and acetocarmine squashes were used to approximate the meiotic stages. Once staged, the anthers were transferred to distilled water for 15 minutes. They were cut open and the entire anther was placed in a digestion buffer (1% cellulase, 2% pectolyase in 1X citric buffer pH 5.5) and incubated at 37°C for 45-60 minutes. Once digestion was complete the tubes were transferred to ice, where the digestion buffer was aspirated and replaced with 20µl of 90% acetic acid 10% methanol. The anthers were then crushed with a metal dissecting probe and the entire volume was pippetted onto a slide in a humid chamber (a cardboard box lined with wet paper towels). Then a 20µl mixture of telomere oligonucleotide (2 ng/µl) and CentC, centromeric repeat oligonucleotide (2 ng/µl) (in 1X TE/2X SSC) was applied, and a coverslip added to the top. The slides were placed in a metal tray lined with moist paper towels and boiled in a water bath for 4 minutes. They were then incubated at 55°C overnight in a sealed container lined with wet paper towels. DAPI in Vectashield was applied to each slide followed by a coverslip, and the slides were then viewed with an Olympus BX61 fluorescence microscope and analyzed with Metamorph software.

Statistics

Statistics were performed using Microsoft Excel. Data for multivalents, B repeat strands, monopolar attachments, and lagging chromosomes was coded using a binary format of 0 for absence and 1 for presence. Typically multiple events were present in each cell, but the variation in mini and B numbers and the distinction between bivalents, univalents, and sisters would affect accuracy if all events were counted for each cell. Therefore cells were treated as technical replicates and each plant was considered a bioreplicate. It is possible that because the bioreplicates for each plant were not equivalent in the numbers

of B's and minis that comparisons between minis may be skewed. We differentiated the presence of diffuse B repeat from the structural damage that occurs with overexposure to acetic acid by only recording a positive observation when CentC and DAPI fluorescence were distinct condensed signals (Dash, 1994).

Results

Prophase

B chromosomes and all minichromosomes could pair as bivalents, but also associated in multivalent configurations via heterochromatic fusions throughout prophase I (Fig 4.1 & 4.2a-l). The highest number of multivalent fusions were observed during pachytene for all minis, but declined by approximately 20% in diakinesis (Fig 4.3, Fig 4.4). There was not an obvious difference in the rate of multivalent formation among different sizes of minis despite the increase in heterochromatin with increasing chromosome size (Fig 4.3). As the total number of minis and B's increased, we observed an increase in multivalent associations (Fig 4.5). A general decline in the frequency of multivalents left most chromosomes in bivalent or univalent configurations at metaphase I (Fig 4.6a-d).

B chromosomes and minis were frequently associated in pachytene (72%, n=6), but only 10.7% of metaphase I and anaphase I cells from the fifth mini indicated that B's and minis paired, which was observed only when a univalent mini and a univalent B would otherwise be present. While the half sized mini associated with B's in prophase, in metaphase I and anaphase I cells, pairing was absent. Pairing among B's and minis was only measured for the fifth and half sized minis, because it was difficult to determine for

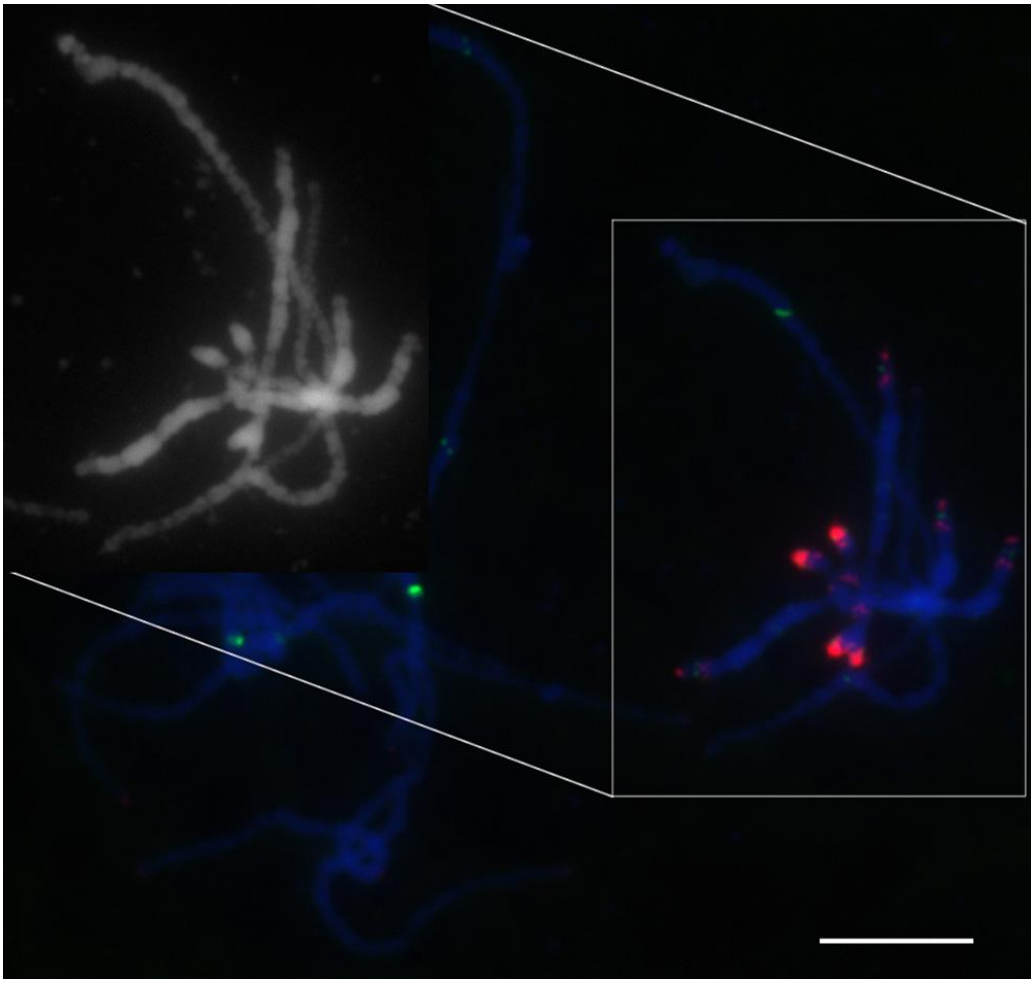

Figure 4.1 Heterochromatic fusion among eight B chromosomes. Inset shows DAPI channel of nonhomologous associations of B. Telomere probes were red; CentC probes were labeled green. Scale bar is 10 microns

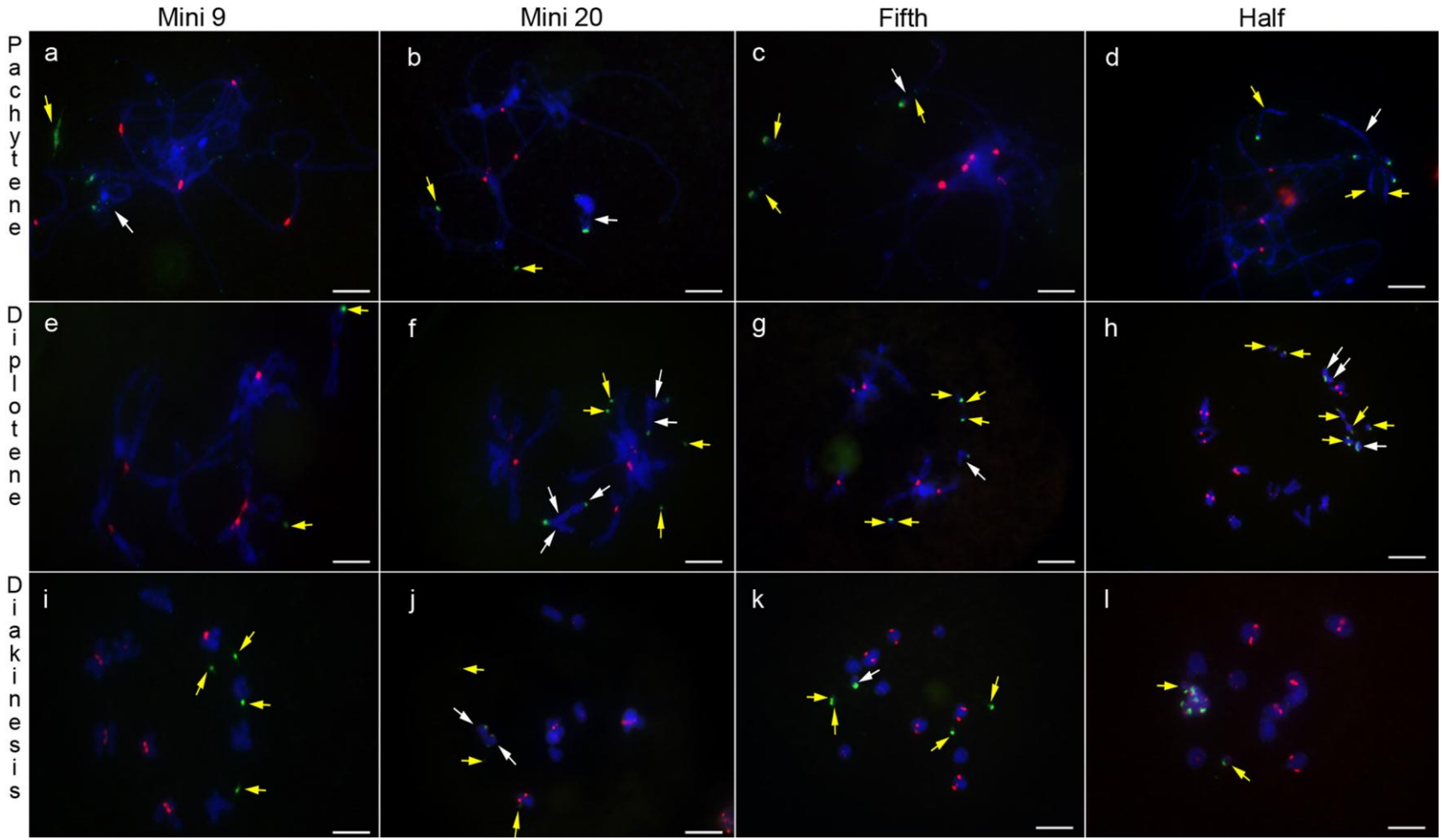
Mini 9
Mini 20
Fifth
Half
Pachytene
Diplotene
Diakinesis
a
b
c
d
e
f
g
h
i
j
k
l

Figure 4.2 All four minis in the stages of pachynema, diplonema, and diakinesis. Yellow arrows denote minis; white arrows denote B chromosomes. (a) Mini 9 with 0 B's and 4 minis in pachytene (b) Mini 20 with 4 B's and 6 minis in pachytene, (c) Mini fifth with 1 B and 5 minis in pachytene (d) Mini half with 2 B's and 6 minis in pachytene (e) Mini 9 0 B's and 4 minis in diplotene (f) Mini 20 with 2 B's and 4 minis in diplotene (g) Fifth mini with 1 B and 5 minis in diplotene (h) Half mini with 3 B's and 6 minis in late diplotene/early diakinesis (i) Mini 9 with 0 B's and 4 minis in diakinesis (j) Mini 20 with 3 B's and 4 minis in diakinesis (k) Fifth mini with 1 B and 5 minis in diakinesis (l) Half mini with 4 B and 7 minis in diakinesis. Scale bar is 10 micron

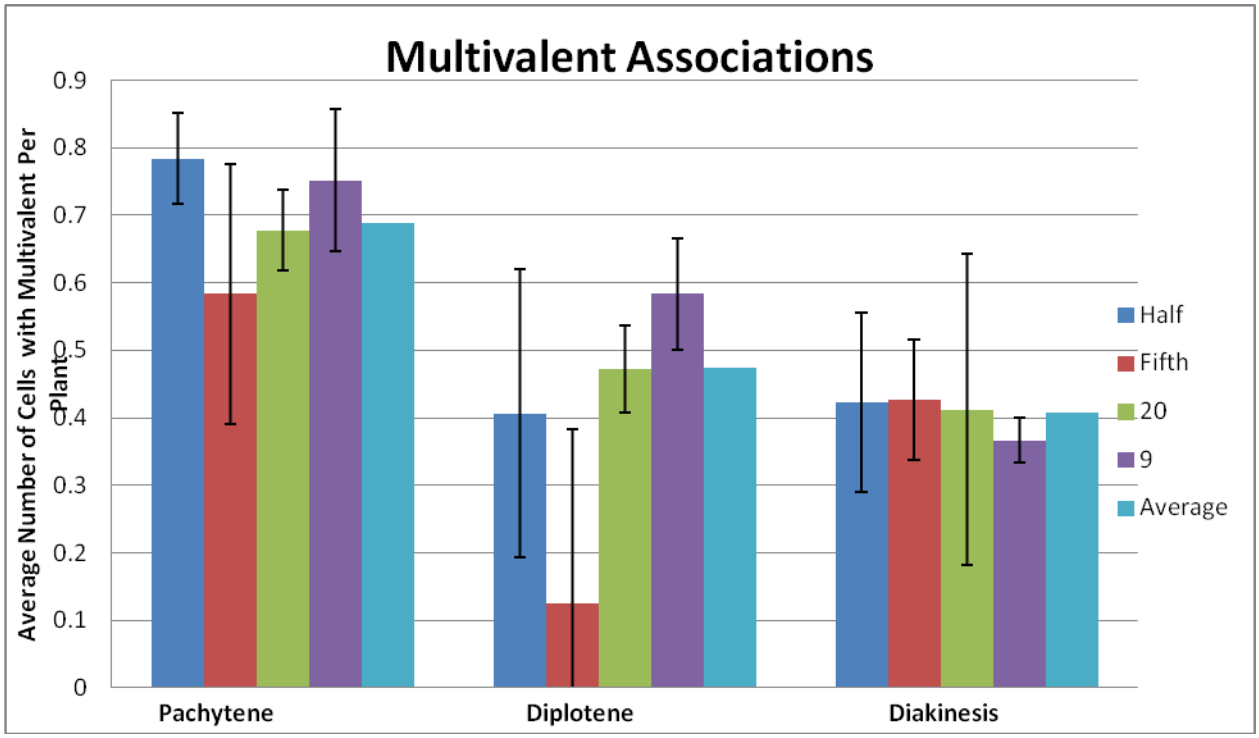

Figure 4.3 Average multivalent frequencies of each mini in prophase. The number of B chromosomes and minichromosomes varied among the replicate plants in this analysis. In pachytene the average number of B's and minis in each plant for mini 9 was 8.5, mini 20 was 7.4, fifth-sized was 6, half-sized was 6.8. In diplotene the average number of B's and minis in each plant for mini 9 was 8.5, mini 20 was 7.3, fifth-sized was 5.7, half-sized was 6.5. In diakinesis the average number of B's and minis in each plant for mini 9 was 8.5, mini 20 was 5.4, fifth-sized was 6.8, half-sized was 6.5

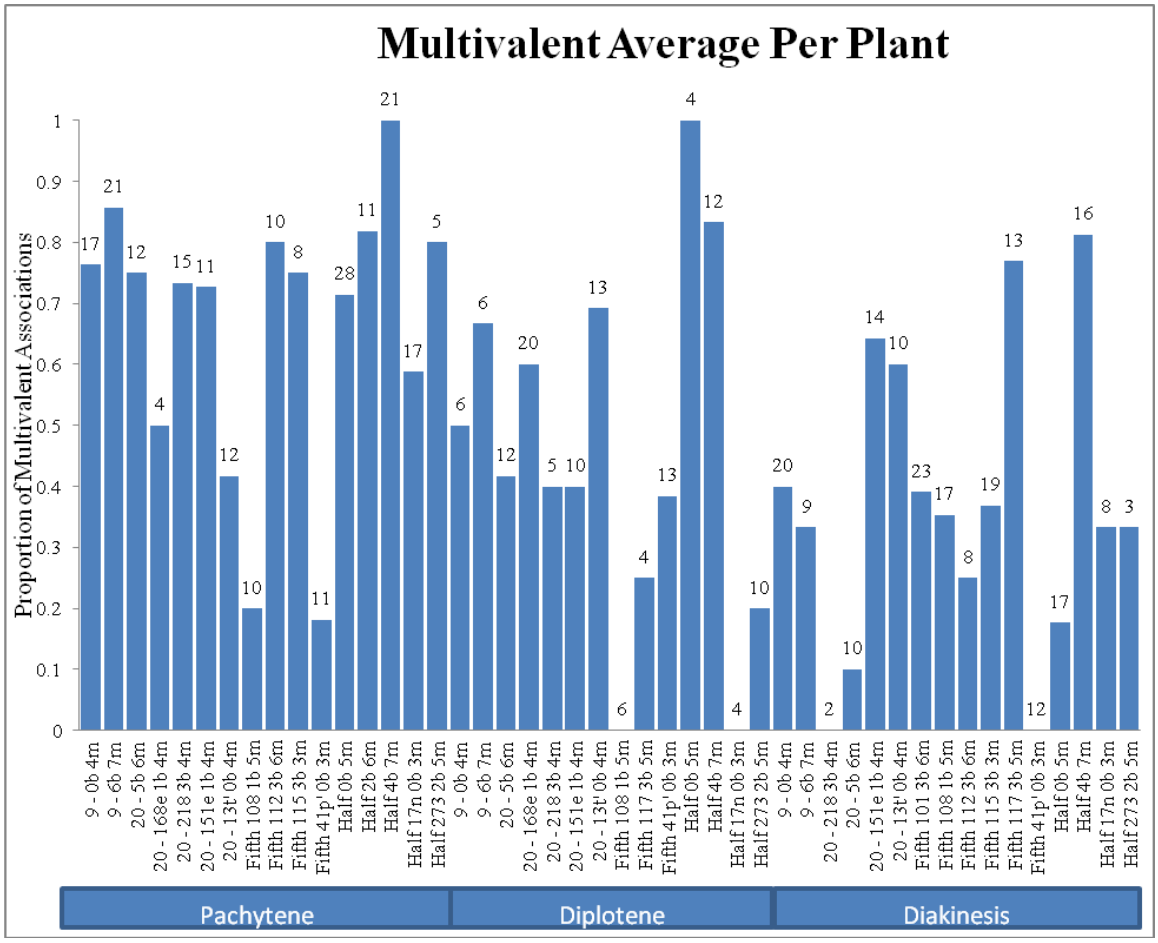

Figure 4.4 The average frequency of multivalents in prophase for each plant. Each plant was highly variable among mini types and bioreplicates. Each bar represents one plant in either pachytene, diplotene, or diakinesis, although some plants could not be examined at all stages. The name of each plant and the corresponding number of minis and B's is below each bar. The numbers at the top of columns are the number of cells examined

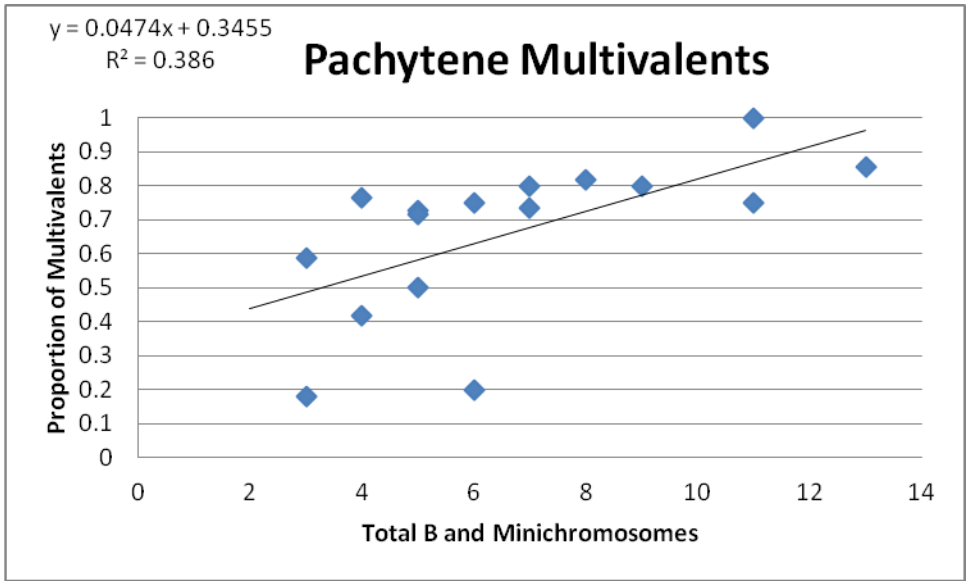

Figure 4.5 The average frequency of multivalents increased with increases in the total number of B's and minis. The average percentage of meiotic cells showing multivalents was compared to the total number of B's and minis

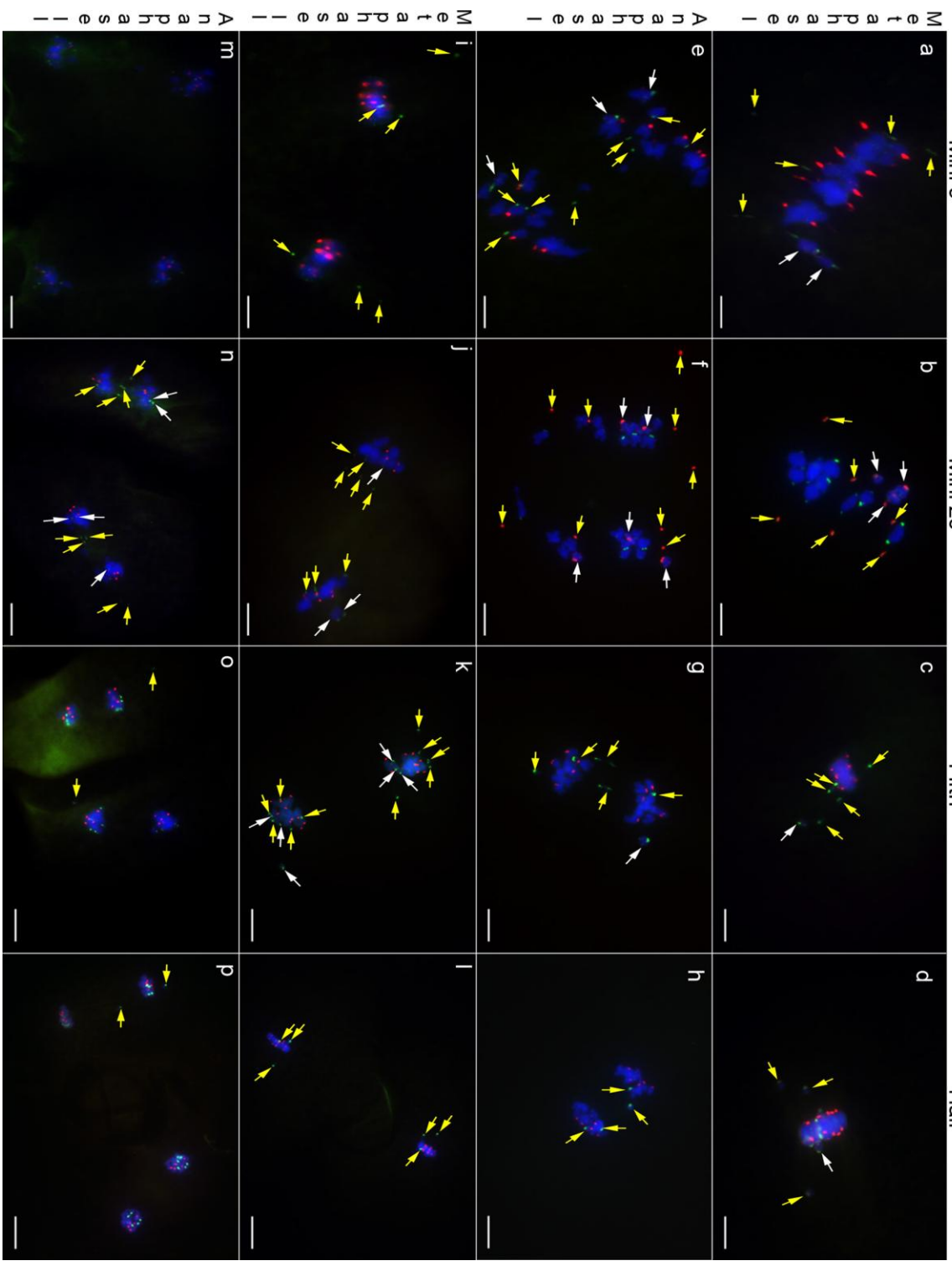

Figure 4.6 All four minis at metaphase I, anaphase I, metaphase II, and anaphase II. Yellow arrows denote minis and white arrows denote B chromosomes. (a) Mini 9 with 0 B's and 4 minis in metaphase I (b) Mini 20 with 2B's and 4 minis in metaphase I (c) Mini

fifth with 1B and 5 minis in metaphase (d) Mini half with 4 B's and 7 minis in metaphase with only monopolar attachments identified (e) Mini 9 with 6 B's and 7 minis showing fewer than expected minis and B's in anaphase I (f) Mini 20 with 5 B's and 6 minis in anaphase I (g) Mini fifth with 1 B and 5 minis in anaphase I (h) Mini half with 0 B's and 3 minis in anaphase (i) Mini 9 with 0 B's and 4 minis showing fewer than expected minis in metaphase II (j) Mini 20 with 4 B's and 6 minis in metaphase II (k) Mini fifth with 4 B's and 6 minis in metaphase II (l) Mini half with 0 B's and 3 minis in metaphase II (m) Mini 9 with 6 B's and 7 minis in anaphase II (n) Mini 20 with 4 B's and 6 minis in anaphase II (o) Mini fifth with 3B's and 6 minis in anaphase II (p) Mini half with 4 B's and 7 minis in anaphase II. All scale bars are 10 microns

the small minis. The half-sized mini paired with B's at a higher rate than the fifth minis during prophase (Fig 4.7). The larger (half and fifth-sized) minis and B's demonstrated intrachromosomal fold-back pairing in pachytene. These univalent fold-back chromosomes appeared to be half the length of bivalents and B repeat FISH signals of the centromere and distal tip associated (Fig 4.8).

B-specific repeat sequences were frequently decondensed and outstretched chromatin fibers were visible during prophase I (Fig 4.2a & i, Fig 4.9). These fibers were detectable with B repeat FISH probes, but did not typically stain with DAPI, similarly to the uncondensed NOR regions in chromosome 6 (Phillips et al., 1971). The average frequency of these outstretched chromatin fibers was greater among the smaller minis (20 & 9) (Fig 4.9 and Fig 4.10). The chromatin fibers frequently stretched to touch the heterochromatic areas of other chromosomes, especially other B's and telomeres of A chromosomes (Fig 4.11a, Fig 4.2a). This diffuse chromatin was not observed in metaphase I, anaphase I, or telophase I cells, but was visible again in prometaphase II (Fig 4.11b).

Metaphase I

The half-sized mini had the highest rate of bivalent formation among all minis studied at 40% (n=15 cells), while the fifth-sized mini was more frequently seen as a univalent at 72% (n=88 cells). The smaller minis, 9 and 20, rarely formed bivalents. Mini 9 paired as bivalents in 22% of cells and univalents in 65% of cells (n=46 cells). Mini 20 demonstrated 16% bivalent pairing, but 56% were univalent and 24% were dissociated sister chromatids (n=99 cells). Most minis seldom paired as bivalents in metaphase I, but bioreplicates were highly variable. While mini 9 and 20 typically had low rates of

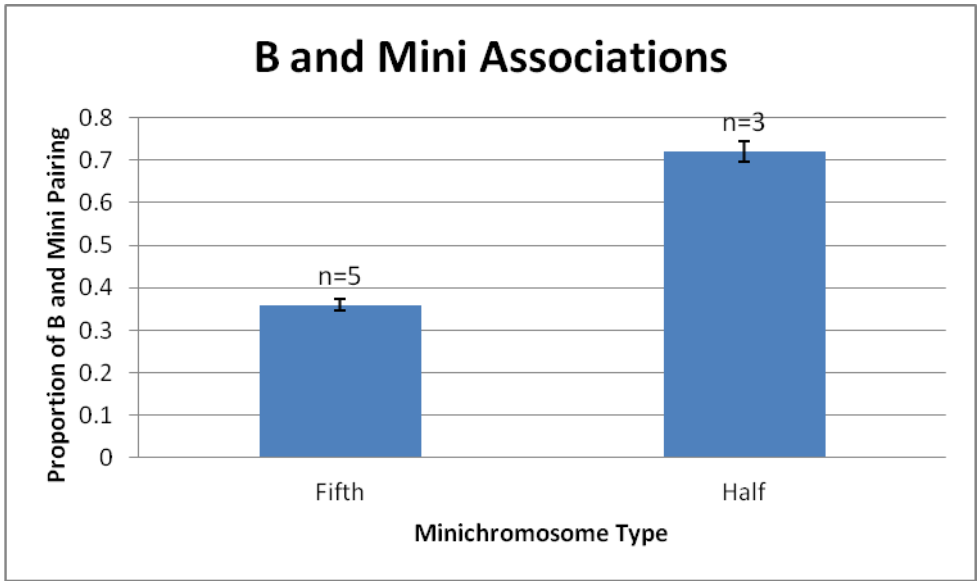

Figure 4.7 The percentage of cells with mini and B bivalents in prophase I. Error bars show the standard error between plants

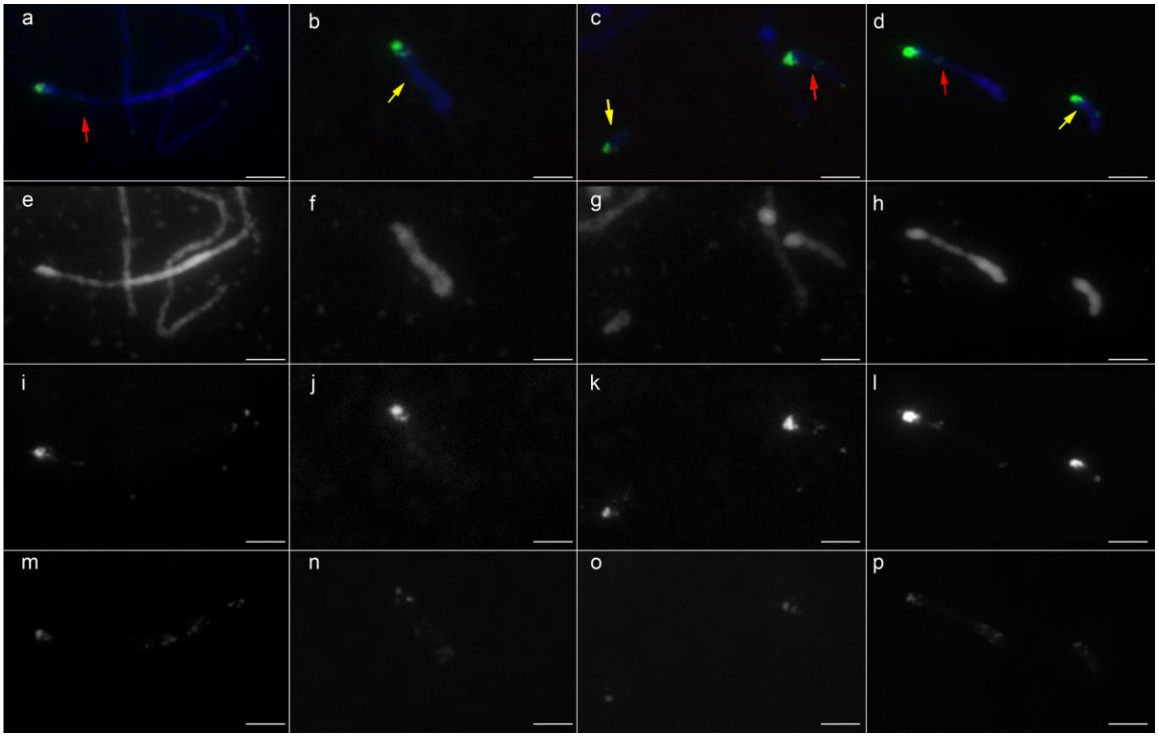

Figure 4.8 Univalent foldback B chromosomes and minichromosomes. B chromosomes and minichromosomes in nonhomologous foldback associations during pachynema. B repeat sequences are labeled green, cent c sequences are labeled red, and blue is the DAPI chromatin stain. Red arrows point to full length bivalent B's and minichromosomes. Yellow arrows point to the univalent self foldback chromosomes. (a) A bivalent B chromosome showing the full length of a pachytene B chromosome. (b) A univalent B chromosome in a self foldback configuration. (c) A univalent fifth-sized minichromosome in a self foldback configuration, and a full length bivalent fifth-sized minichromosome. (d) A univalent half sized minichromosome in a self foldback configuration and a full length bivalent half-sized minichromosome. (e, f, g, h) The DAPI channel of the top figures. (i, j, k, l) The green B repeat channel of the top figures. (m, n, o, p) The red cent c channel of the top figures. Scale bars are 10 microns

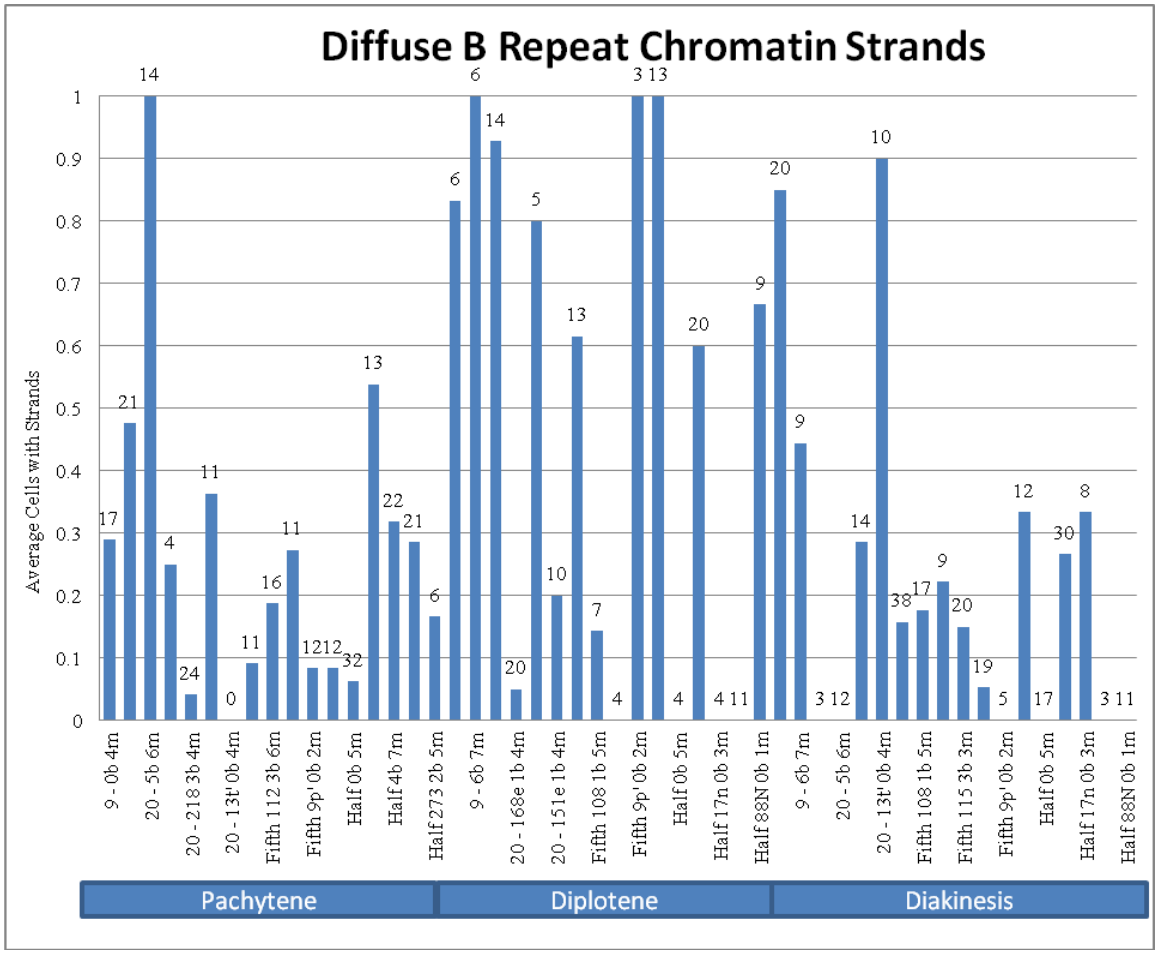

Figure 4.9 The average diffuse B repeat chromatin in prophase for each plant, showing the variability among mini types and bioreplicates. Each bar represents one plant in either pachytene, diplotene, or diakinesis, although some plants did not have all stages. The name of each plant and the corresponding number of minis and B's is below each bar. The numbers at the top of columns are the number of cells examined

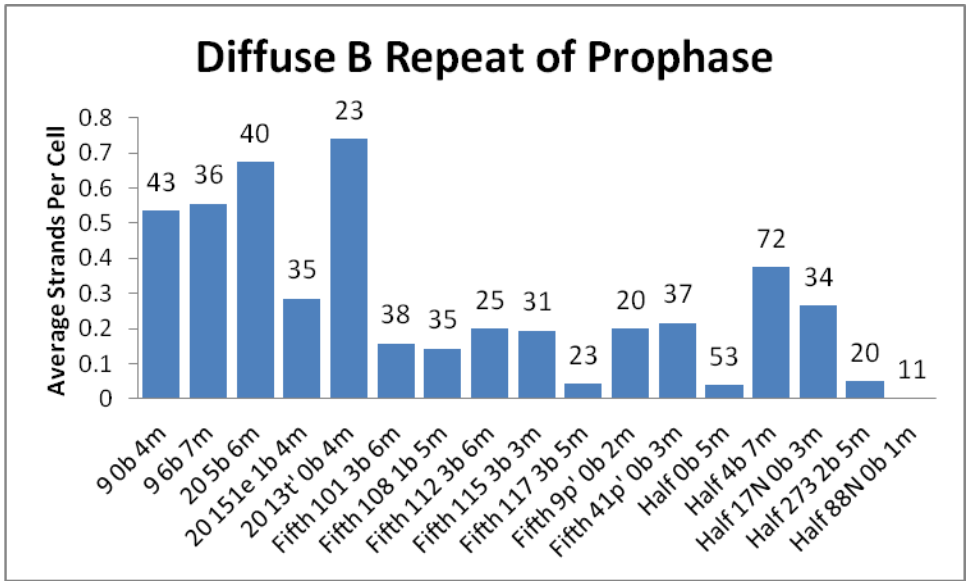

Figure 4.10 Average frequency of diffuse chromatin strands for each plant throughout all stages of prophase. The name of each plant and the corresponding number of minis and B's is below each bar. The numbers at the top of columns are the number of cells examined

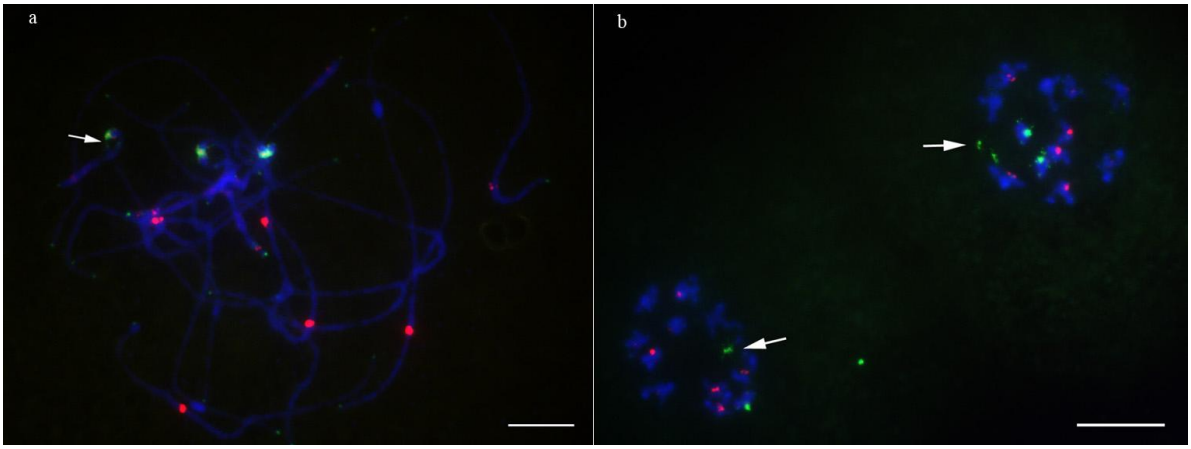

Figure 4.11 The sticky behavior of minichromosomes. (a) Half sized mini associated with a telomere of an A chromosome in pachytene (b). Mini 9 with 6 B's and 7 minis, arrows show diffuse chromatin strands. Scale bar is 10 microns

bivalent pairing, one plant from mini 9 and 20 with zero B's and four minis had the highest frequencies of bivalent pairing among all minis in this study.

Seven of nineteen plants had B's and minis with different copy numbers between the root tip cells and the meiocytes from the same plant showing nondisjunction occurred in a progenitor cell. Copy number changes reflect both the gain and the loss of B's and minis resulting in higher and lower copy numbers in the anther (Fig 4.12).

Frequently mini and B chromosomes prematurely moved to a single pole (Fig 4.6a-d). In plants that had B's, the monopolar migration of B's occurred in 45% of cells (n=74), while all minis together averaged 58% (n=114 cells). This was also observed with A chromosomes in 5.3% of cells in four of thirteen plants (n=114 cells) (Fig 4.13).

Anaphase I and Telophase I & II

Minis, B chromosomes, and A chromosomes retained monopolar attachment in 27%, 23%, and 1.5% of cells, respectively (n=194) (Figure 4.6e-h). Unpaired chromosomes that did not progress to a pole early usually remained at the metaphase plate and had early sister chromatid separation in anaphase I, while all other chromosomes progressed to their respective poles. Minis were seen lagging in 37.5% of cells (n=173), while B chromosomes and A chromosomes were more stable at 19.6% (n=92) and 12.1% of cells, respectively (n=173). While many of the lagging A chromosomes were slowly progressing to a pole, occasionally anaphase bridges and broken fragments were observed. No significant difference in monopolar attachment or lagging was seen when comparing the different mini types (Fig 4.13 and Fig 4.14).

Meiosis II

Mini 20								
Root	1B 4Mini	3B 4Mini	3B 4Mini	0B 4Mini	3B 4Mini			
Anther	1B 4Mini	3B 4Mini	4B 6Mini	0B 3Mini	3B 4Mini			
Fifth								
Root	0B 4Mini	3B 6Mini	3B 4Mini	3B 5Mini	0B 2Mini	0B 3Mini	3B 4Mini	1B 4Mini
Anther	1B 5Mini	3B 6Mini	3B 3Mini	3B 5Mini	0B 2Mini	0B 3Mini	4B 6Mini	1B 4Mini
Half								
Root	0B 5Mini	2B 6Mini	3B 7Mini	0B 4Mini	0B 1Mini	2B 5Mini		
Anther	0B 5Mini	2B 6Mini	4B 7Mini	0B 3Mini	0B 1Mini	2B 5Mini		

Figure 4.12 The number of B's and minis found in each plant, showing a difference in the number of B's and minis between the root karyotype and the tassel karyotype.

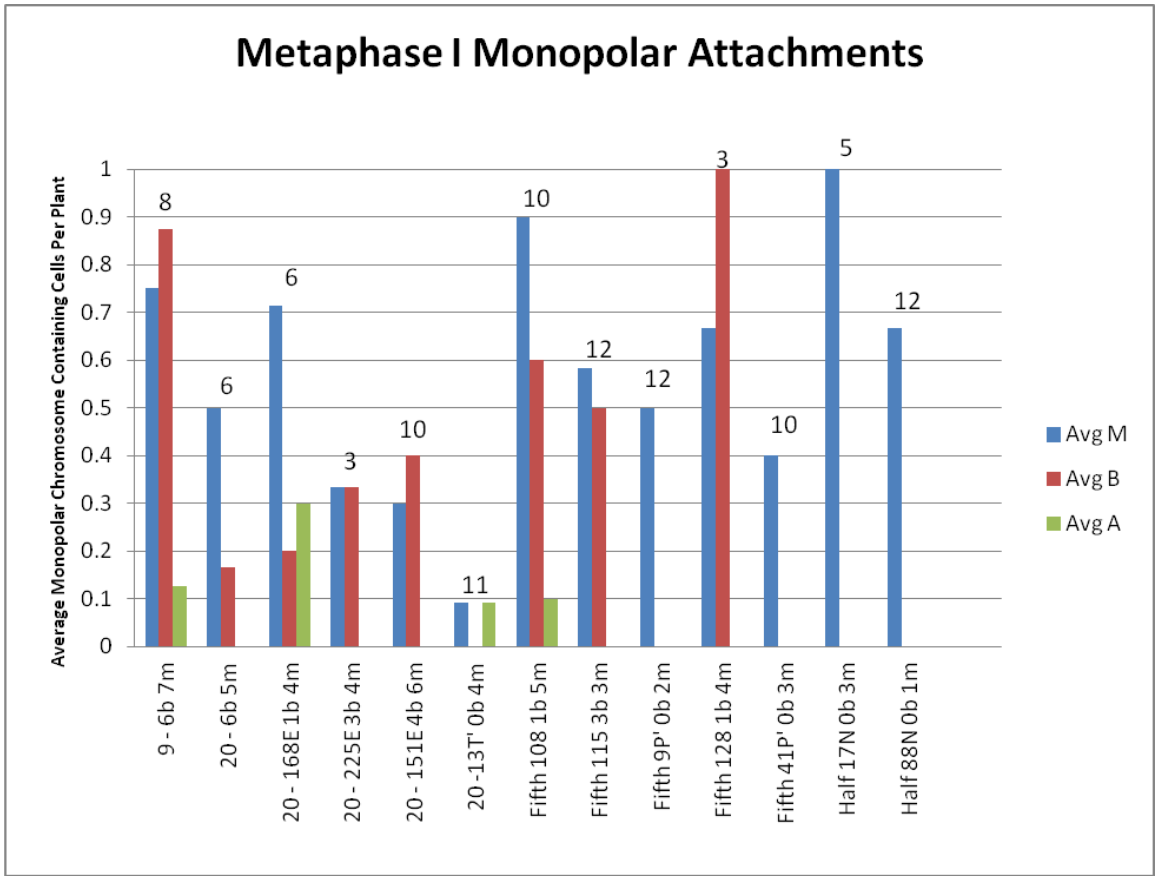

Figure 4.13 Percentage of cells with precocious migration for each plant at metaphase I for each plant. Some plants did not have B's and thus could not be scored for B precocious migration. The name of each plant and the corresponding number of minis and B's is below each bar. Numbers on top of columns denote the number of cells examined

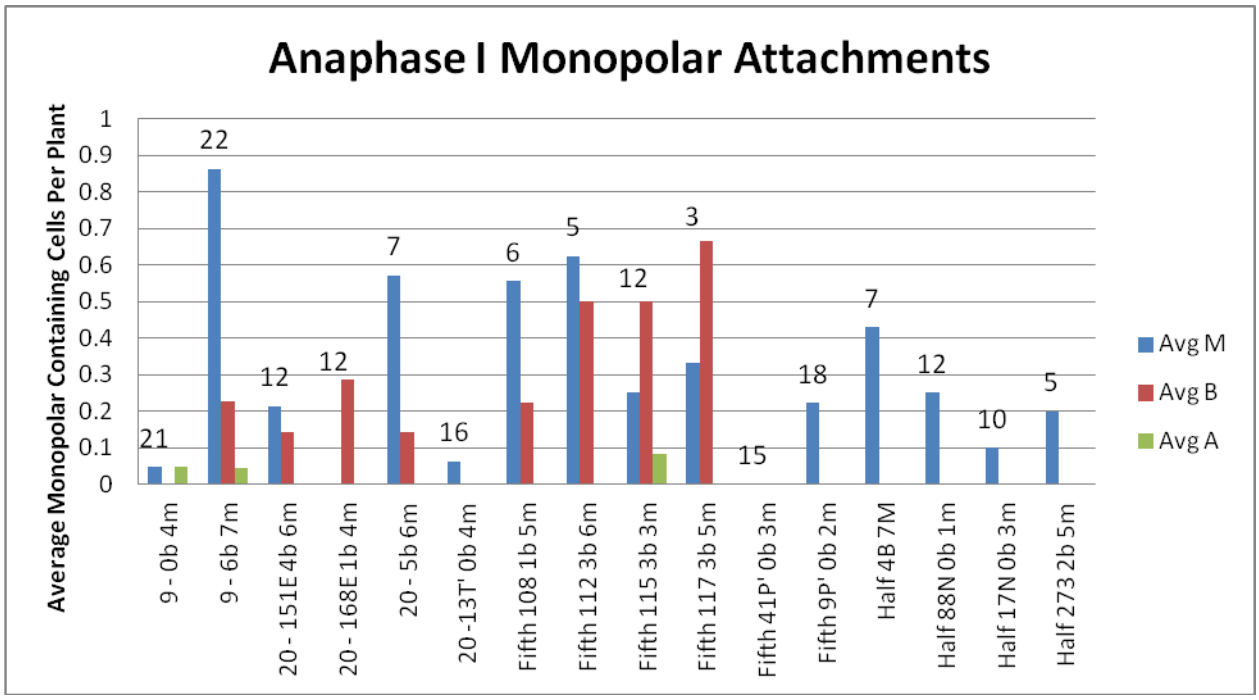

Figure 4.14 Percentage of cells for each plant with persistent monopolar migration of at anaphase I. Some plants did not have B's and thus could not be scored for B persistent monopolar attachment. The name of each plant and the corresponding number of minis and B's is below each bar. Numbers at the top of columns denote the number of cells examined

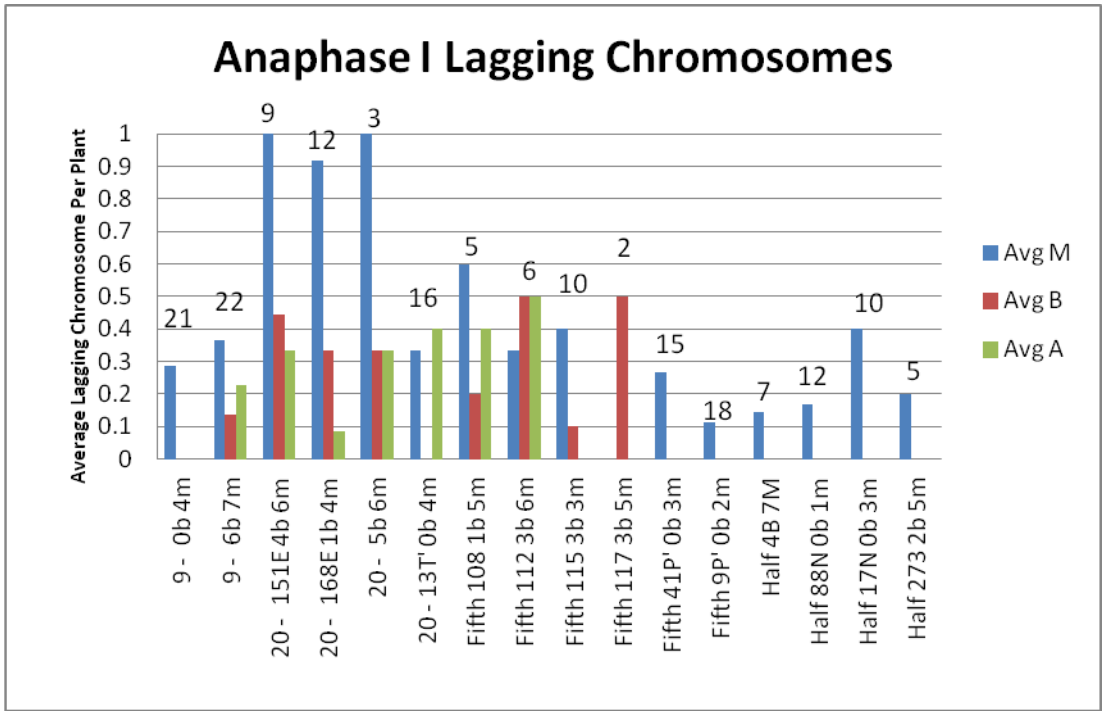

Figure 4.15 Percentage of cells with lagging chromosomes at anaphase I, for each plant. Some plants did not have B's and thus could not be scored for B laggards. The name of each plant and the corresponding number of minis and B's is below each bar. Numbers at the top of columns are the number of cells examined

In metaphase II and anaphase II cells, minis and B's were frequently seen lagging at the metaphase plate, and early monopolar movement was still evident (Fig 4.6i-p). FISH signals were the only reference during telophase II, because most of the chromatin was uncondensed. By observing the number of centromere signals associated with the B repeat, we resolved the number of minis present in each cell. In a comparison of the expected number of sisters in telophase II and the observed signals, we determined that some minis were lost, although few micronuclei were observed. Cell division timing was also asynchronous, shown by multiple meiotic stages in a single anther, and meiosis II sister cells in different states of division.

Discussion

Centromeres and knob heterochromatin from nonhomologous chromosomes are often found "fused" in pachytene, but these associations disappear by diakinesis (McClintock, 1933). This phenomena is likely the basis of the multiple minichromosome associations observed here (Fig 4.1), as well as the B heterochromatin associations previously described (McClintock, 1933). Fusions also occur in minichromosomes contributing to the association of multiple minis, which were very similar to previous descriptions of the B chromosome (McClintock, 1933). Pairing with synapsis in diakinesis never involved more than two chromosomes, although the heterochromatic associations persisted until metaphase I. Increasing the chromosome copy number did not affect the occurrence of persistent bivalents, but did increase the appearance of multivalent associations in pachytene (Fig 4.5).

The B repeat associated diffuse chromatin appears to be related to a diffuse stage of meiosis that occurs between pachytene and diplotene. Typically this occurs in all chromosomes of the genome, especially in plant hybrids and polyploids (Klasterska, 1976), but also occurs specifically with the B repeat. This diffuse stage has been linked as a source of meiotic defects such as: desynchronization of meiotic stages in the anther, stainability of chromatin, nonhomologous multivalent interactions or stickiness of chromosomes, anaphase bridges, and chromosome breaks (Klasterska, 1976). Many tassels that were collected for this study had pachytene and tetrad stages only, and within an anther multiple stages of meiosis were observed. The diffuse B repeat chromatin was rarely DAPI positive, which may indicate that it was below the limit of detection. The B repeat chromatin was frequently observed associating with regions of nonhomologous chromosomes, and B's are relatively frequently recovered in broken and rearranged forms.

Bivalent pairing was somewhat infrequent, leaving many minichromosomes univalent at the metaphase plate. Increasing the abundance of homologues is expected to increase the frequency of bivalent formation. Instead our observations are similar to rye B chromosome observations that show univalents increasing with increasing numbers of B's (Lukaszewski 2010). Our data support previous work that univalents can have a monopolar attachment in metaphase I and anaphase I, and they will either lag or progress to one pole (Ricci et al., 2007, McClintock, 1933). The twenty A chromosomes in the genome were the background control and demonstrated monopolar attachment in 5.3% (n=114) of cells at metaphase I and 1.5% (n=194) of cells at anaphase I. Considering that these numbers are for all 20 chromosomes, these rates are relatively low. Because this

behavior was mostly seen with univalent minis and B's, the sharp decline in A chromosome monopolar attachment may reflect the high fidelity pairing of A chromosomes.

In the absence of a pairing partner, the B, as well as any univalent minis, were observed in a self fold-back configuration of heterochromatic fusion. This event is not specific to the B chromosome, but has been described for univalent monosomic chromosomes of the normal karyotype (McClintock, 1933). Evidence was also presented to indicate that translocations may result from these nonhomologous associations (McClintock, 1933). It would be interesting to understand the evolutionary significance behind these associations, and if they affect chromosome structure.

Lagging chromosomes of all three chromosome types were quite frequent, but could be from a variety of causes. The most frequent lagging chromosomes, were univalent minis and B's that had early sister chromatid separation. The lagging chromosome's early sister separation may be from an increased duration of exposure to separase which may be concentrated at the metaphase plate, and would cause the cohesion of sister centromeres to dissolve early (Lukaszewski, 2010). The fact that very few micronuclei were seen shows that they eventually progressed to a pole. Altogether minis behaved more normally in meiosis when an even number of homologues was present and B's were absent from the genome. The higher percentage of lagging A chromosomes may be the result of B chromosome action on the pairing of A chromosomes. It is known that B's cause delayed replication in the knobs of A chromosomes and can trigger their elimination at the second pollen mitosis, which could

explain the increased frequency of bridges and fragments observed at anaphase I (Pryor et al., 1980b).

Both lagging chromosomes and monopolar attachments were present in meiosis II, probably from the univalents remaining from the separation of bivalents in meiosis I. The number of signals in telophase II cells often did not equal the expected quantity of chromosomes, and micronuclei could not account for the discrepancy. Either minichromosomes were being lost, some sister chromatids did not separate in telophase II, or minichromosomes re-associated through heterochromatic fusions in telophase II cells, thus merging the CentC and B repeat signals.

The results described here illustrate that the copy number of engineered minichromosomes will not be stable for applications involving multiple minichromosomes, only a range of copy numbers will be possible to obtain. However, for applications involving vegetative propagation, the chromosome number changes will be fixed. For applications requiring a stable transmission, truncated B chromosomes near the size of the normal B will pair and segregate normally and have lost the nondisjunction property (Kato et al., 2005).

Summary

1. Minichromosomes behave much like their B chromosome progenitors in respect to their nonhomologous associations and univalent behavior.
2. The pairing of all minichromosomes at metaphase I is dramatically reduced when compared to their B counterparts.

3. The diffuse behavior of the B repeat seems to be related to the diffuse stage observed between pachytene and diplotene, and may be responsible for the sticky behavior of B chromosomes.

CHAPTER 5: HERITABLE LOSS OF REPLICATION CONTROL OF A MINICHROMOSOME DERIVED FROM THE B CHROMOSOME OF MAIZE

Introduction

Endoreduplication is essentially the replication of chromosomes without cell division, and is typically associated with 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 Dbf4, a regulatory protein degraded by the anaphase promoting complex, regulates the activity of cyclin dependent kinases (CDK) and Dbf4 dependent kinases (DDK). These kinases then activate the mini-chromosome maintenance proteins 2-7 (MCM), which form the pre-replication complex necessary for replication licensing (Tuteja et al., 2011). In the process of studying engineered minichromosomes, a single chromosome was discovered that was routinely present in an over-replicated state.

The progenitor of the endoreduplicated chromosome was a B chromosome, which is a supernumerary chromosome that persists in the genome with a selfish inheritance that

enables a higher-than-expected transmission to the next generation (Longley, 1927). The maize B chromosome accumulates in the genome by nondisjoining to one pole at the second pollen mitosis, surviving meiosis as a univalent, and conferring a preference for the B-containing sperm to fertilize the egg, rather than the central cell (Carlson, 1969, Roman, 1948, Carlson et al., 1992). 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 (Chiavarino et al., 2001, Carlson, 1969, Carlson et al., 1992). Three essential regions of the B chromosome must be present for nondisjunction to occur; two cis-acting sites in the proximal euchromatin and heterochromatin, and a trans-acting site in the distal euchromatin (Roman, 1949, Ward, 1973, Carlson, 1973, Carlson, 1978b, Lin, 1978, Rhoades et al., 1972, Rhoades et al., 1967). 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 (Chiavarino et al., 2000, Alfenito et al., 1990, Masonbrink et al., 2010).

In 2007, 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 had most of the B chromosome long arm removed, resulting in a chromosome slightly larger than a B chromosome centromere. This chromosome was capable of accumulating 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 changed copy number, but remained a single entity.

Methods

Mitotic in-situ Hybridization

Root tip FISH were performed as previously described with a slight modification (Masonbrink et al., 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 a CentC that hybridizes to the centromere of all chromosomes and the B chromosome long arm (Ananiev et al., 1998, Alfenito et al., 1993, Lamb et al., 2005). Nick translated PCR products consisted of the Stark repeat, the centromeric retrotransposon of maize (CRM), and the cassette from the telomere truncation experiments, without the telomere (WY96) (Nagaki et al., 2003, Lamb et al., 2007, Yu et al., 2007).

Meiotic Fluorescence in-situ Hybridization

Tassels were fixed when the anthers on the main branch began to turn yellow, while the lowest part of the secondary branches were still small and white. They were fixed in 3:1 ethanol to acetic acid at 4°C overnight. Then the tassels were transferred to 70% ethanol and stored at -20°C. Anthers were dissected from the glumes and acetocarmine squashes were used to determine the appropriate meiotic stages. Once staged, the anthers were transferred to distilled water for 15 minutes. They were cut open and placed in a digestion buffer (1% cellulase, 2% pectolyase in 1X citric buffer pH 5.5) and incubated at 37°C for 45-60 minutes. Once digestion was complete the tubes were transferred to ice, where the digestion buffer was aspirated and replaced with 20µl of 90% acetic acid 10% methanol. The anthers were then crushed with a metal dissecting probe and the entire volume was pippetted onto a slide in a humid chamber (a cardboard box lined with wet paper towels). Then a 20µl mixture of telomere oligonucleotide (2 ng/µl) and CentC, centromeric repeat oligonucleotide (2 ng/µl) (in 1X TE/2X SSC) was

applied, and a coverslip added to the top. The slides were placed in a metal tray lined with moist paper towels and boiled in a water bath for 4 minutes. They were then incubated at 55°C overnight in a sealed container lined with wet paper towels. DAPI in Vectashield was applied to each slide followed by a coverslip, and were then viewed with an Olympus BX61 fluorescence microscope and analyzed with Metamorph software.

Immunocytochemistry

Immunolocalization for mitosis and meiosis were performed as described (Han et al., 2009). The polyclonal antibody of maize CENH3 was raised against rabbit with a peptide as described (Zhong et al., 2002). A monoclonal rabbit antibody (04-817) raised against histone H3 phosphorylated at Ser-10 was obtained from Upstate. 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), processed with Photoshop CS 3.0.

Results

A small B chromosome derived-minichromosome (86-B23) was accumulated to multiple copies using the maize B chromosome's accumulation mechanism (Yu et al., 2007). This minichromosome was frequently lost in root mitoses and occasionally multiple numbers were found in a single cell. Over two generations this minichromosome was accumulated to five copies before finding an unusually large chromosome with multiple centromere FISH signals (CentC). In the next generation we observed many of these minichromosomes changing in size and quantity between cells of the same root with multiple distinct CentC signals per minichromosome (Fig 5.1c). In addition, we probed the chromosome with CRM, the centromeric retrotransposon of maize (Fig 5.1a);

Stark repeat, which is found in the centromere and the distal heterochromatin of the B chromosome (Fig 5.1b); and WY96, the truncation cassette without the telomere (Fig 5.1d). Each probe hybridized to multiple distinct sites on the endoreduplicated chromosome and the quantity of these signals was correlated with the size of this chromosome. The larger sized minichromosomes were found locally in a few cells, while the smaller variants were found in nearly every cell. Intermittently the chromosome deviated from the typical spherical appearance and formed a large circle (Fig 5.2). While we attempted to increase the copy number of this endoreduplicated chromosome, we were not able to observe a cell with more than eight endoreduplicated chromosomes. Immunocytochemistry was used to determine if multiple centromere loci on a single chromosome could condition multiple active centromeres. The endoreduplicated chromosome had multiple sites of sister chromatid cohesion indicated by bound H3S10 (Fig 5.3) (Kaszas et al., 2000). Another antibody specific to the maintenance of sister chromatid cohesion, phosphorylated histone H2A, bound to multiple loci on a large version of the endoreduplicated chromosome (Fig 5.4). An antibody against CENPC, an inner kinetochore protein, also localized to multiple loci on large versions (Fig 5.5). In contrast, the smaller versions with less endoreduplication always had one binding site for each sister centromere.

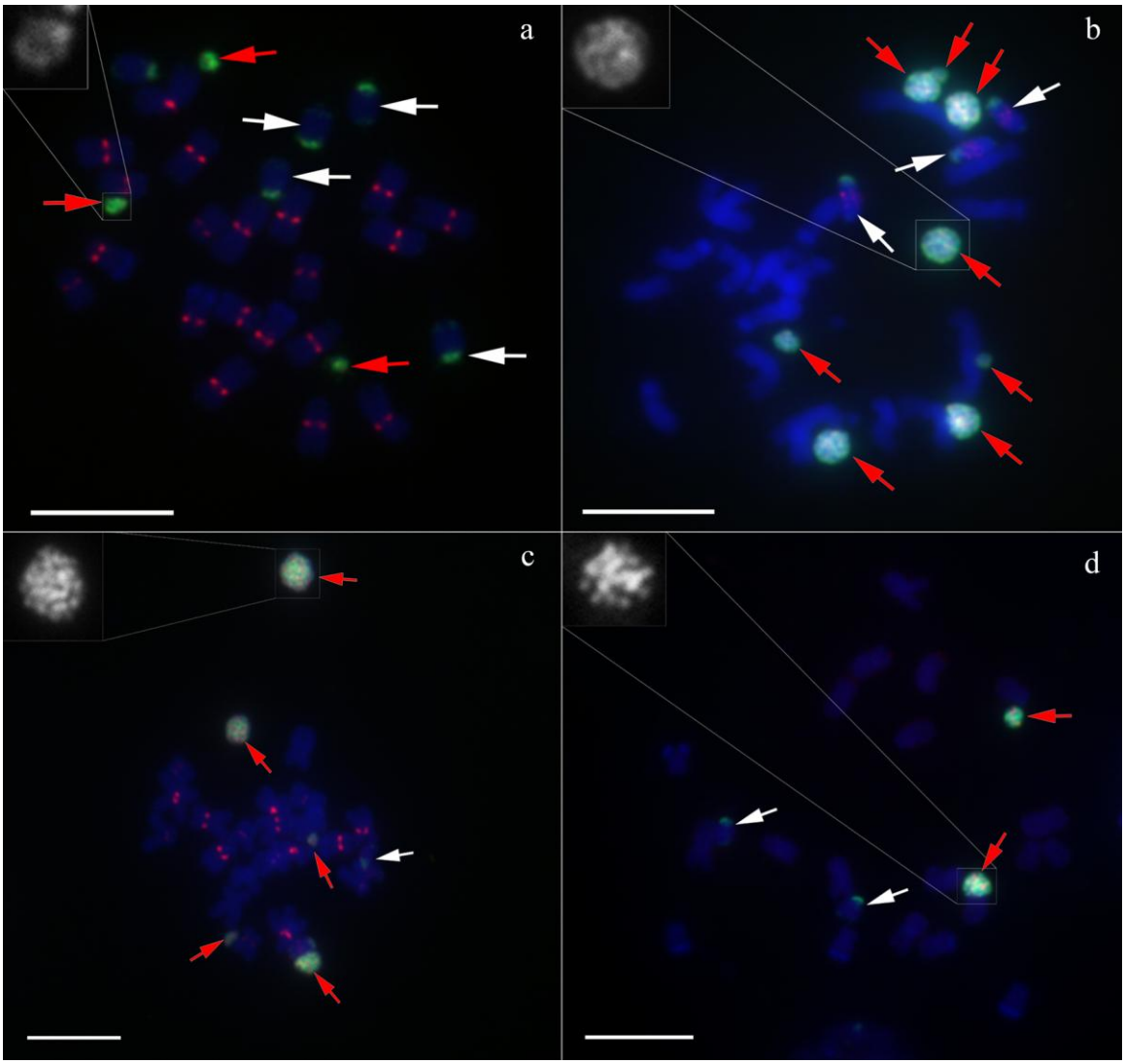

Figure 5.1 Centromeric sequences in the endoreduplicated chromosome. Telomere probes, which cross-hybridize to the B repeat, are green. (a) Multiple CRM (red) signals are on the larger endoreduplicated chromosome, while the smaller versions have only two. (b) Stark repeat (red), which is found in the distal heterochromatin of the long arm and in the centromere of the B chromosome, hybridized all throughout the endoreduplicated chromosome. (c) CentC (red), a centromeric satellite repeat, had multiple distinct signals on the endoreduplicated chromosome. (d) WY96 (red), the truncation cassette without the telomere, hybridized to multiple distinct loci on the

endoreduplicated chromosome. The inset pictures are the red channel to an endoreduplicated chromosome. Red arrows denote endoreduplicated chromosomes, and white arrows denote normal sized B chromosomes. Scale bars are 10 microns

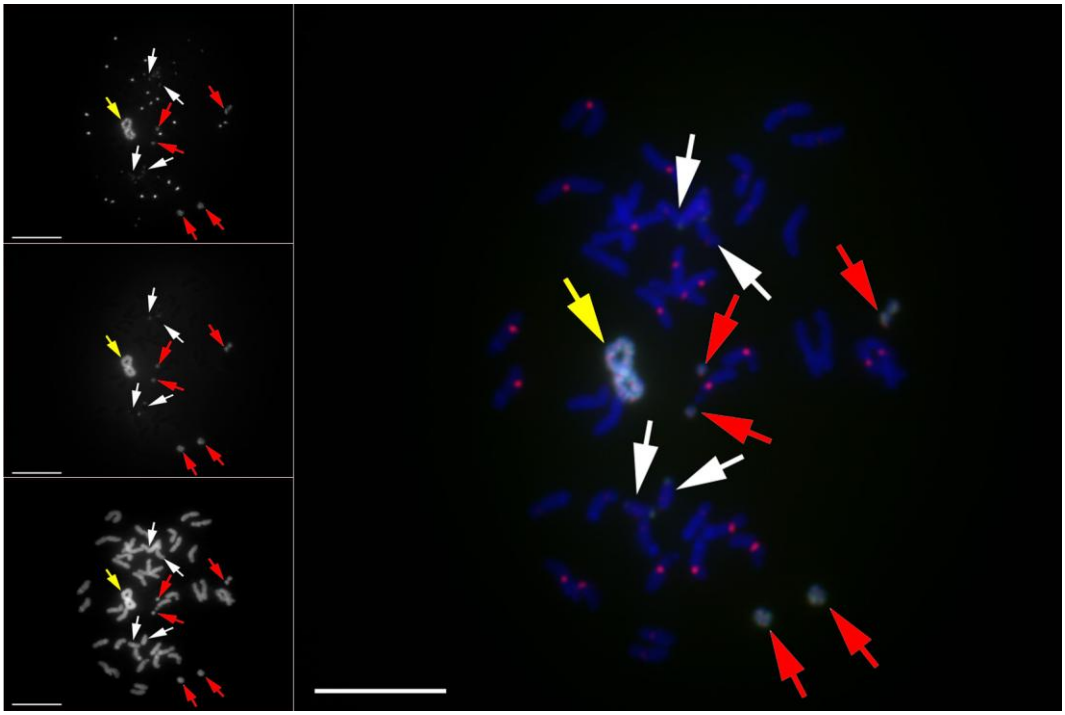

Figure 5.2 A circle shaped endoreduplicated chromosome twisted into a figure eight when sister chromatids were separating in the rest of the cell. The top inset is CentC in the red channel showing multiple distinct signals. The middle inset is B repeat (telomere) in the green channel. The bottom inset is DAPI in the blue channel. Yellow arrows denote the endoreduplicated chromosome in a figure eight. Red arrows denote smaller endoreduplicated chromosomes. White arrows denote B chromosomes. Telomere probes cross-hybridize to the B repeat. Scale bar is 10 microns

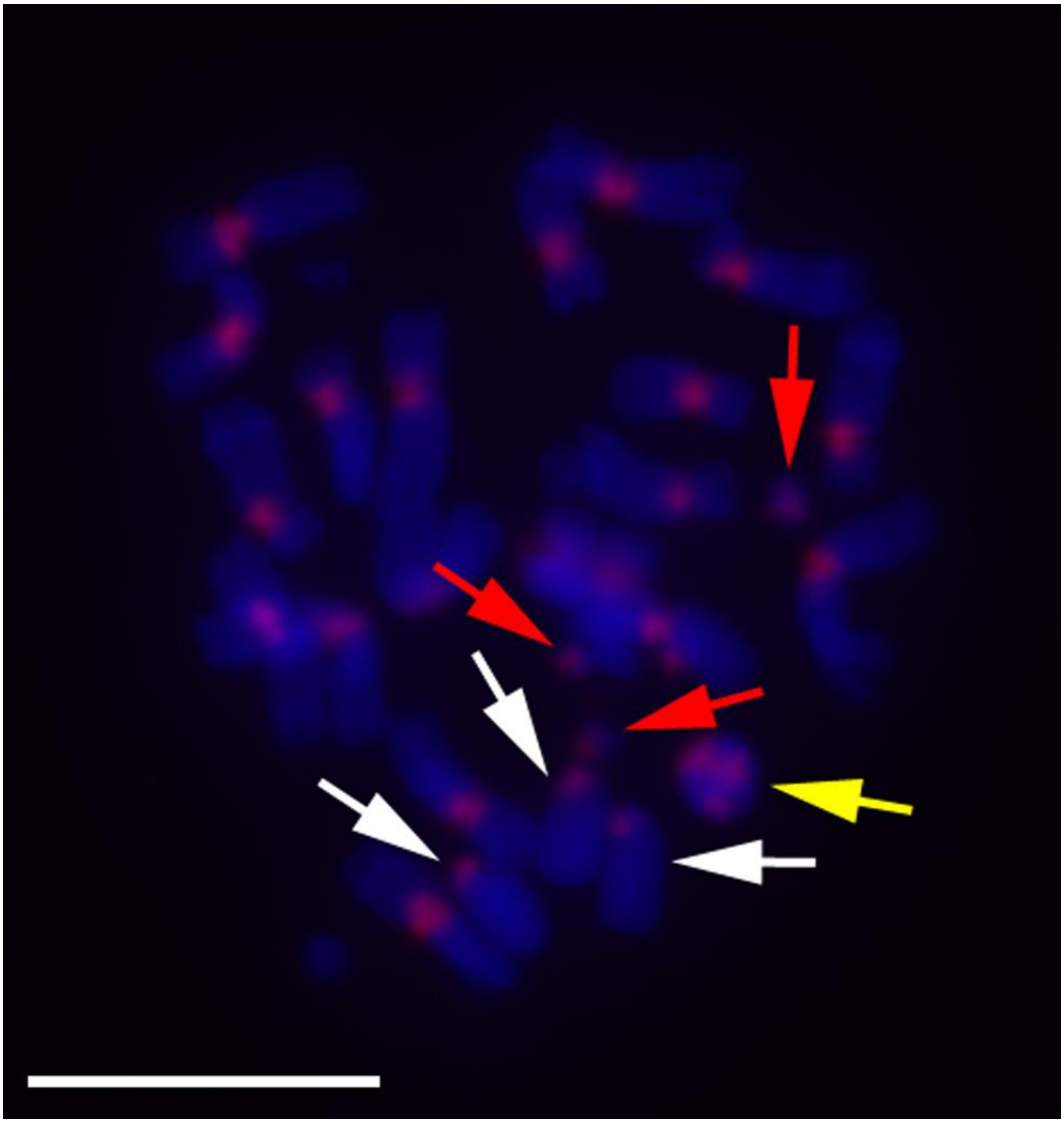

Figure 5.3 Active sites of sister chromatid cohesion (H3S10) in the endoreduplicated chromosome. The yellow arrow points to an endoreduplicated chromosome with at least three sites of bound H3S10 antibody, which is a marker for sister chromatid cohesion. The red arrows denote smaller endoreduplicated chromosomes with one site of sister chromatid cohesion. The white arrows denote B chromosomes. The blue arrow points to a potential endoreduplicated chromosome without a H3S10 signal. Notice all A chromosomes have one distinct signal per sister chromatid. The red channel is the H3S10 antibody. Scale bar is 10 microns

Throughout meiosis the endoreduplicated chromosome continued to change size and frequency between cells of the same tassel and anther. All stages of meiosis were observed in one anther, excluding pachynema, which came from the same plant. In pachynema these chromosomes appeared small and compact, a deviation from the typical string-like morphology of pachytene chromosomes (Fig 5.6a). In diplonema two copies of the chromosome were found in the spherical shape, and had the characteristic "sticky" behavior of B chromosomes (Fig 5.6b) (McClintock, 1933). In diakinesis we observed three smaller sized minis of various sizes, and a noticeable division of B repeat to opposite ends of one chromosome (Fig 5.6c). At metaphase I, the endoreduplicated chromosomes frequently had early monopolar movement. The chromosomes with early monopolar movement were closer to the pole than all other chromosomes in anaphase I (Fig 5.6d). All endoreduplicated chromosomes without monopolar movement lagged at the metaphase plate and eventually moved to one pole evidenced by the lack of micronuclei in subsequent stages. Endoreduplicated chromosome disjunction was never observed at anaphase I. (Fig 5.6e). Unequal numbers of endoreduplicated chromosomes were observed in the prometaphase II, probably the result of monopolar movement at metaphase I (Fig 5.6f). At anaphase II some sister separation was apparent, but many times the chromosome had monopolar movement without sister separation (Fig 5.6g). In telophase II large

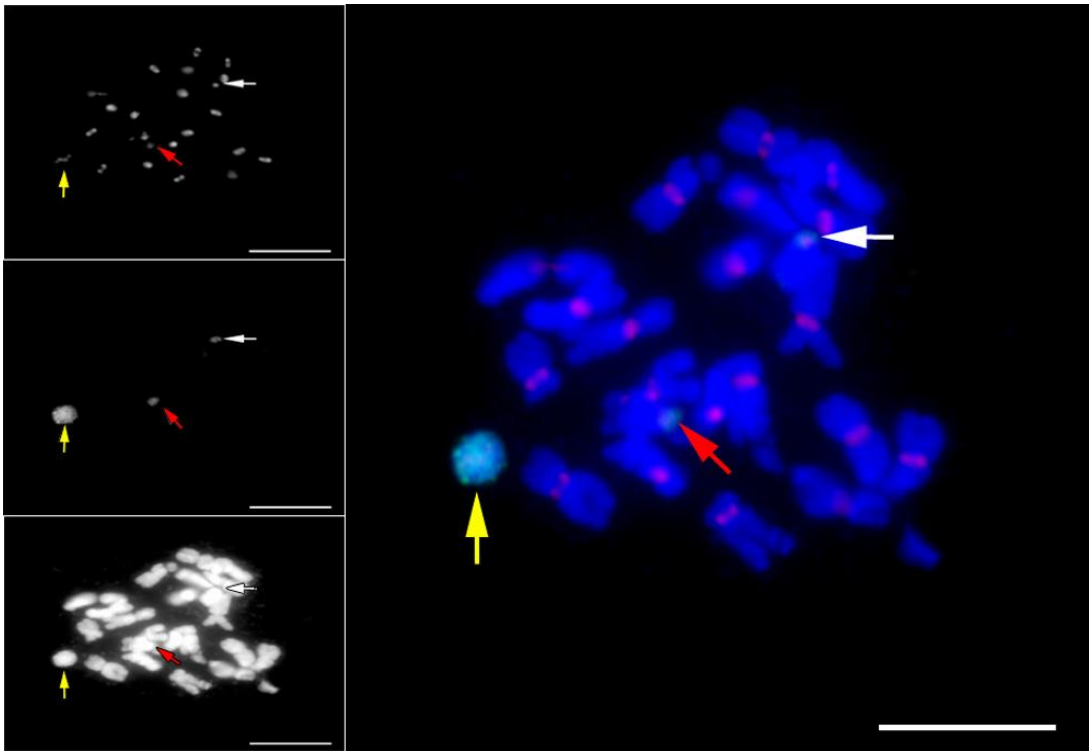

Figure 5.4 Active sites of sister chromatid cohesion (phosphorylated H2A) in the endoreduplicated chromosome. The yellow arrow denotes an endoreduplicated chromosome with at least two sites of bound phosphorylated H2A antibody, which is a marker for sister chromatid cohesion. The red arrow denotes a small endoreduplicated chromosome with one site of antibody binding. The top inset is the red H2A antibody channel showing multiple signals per large endoreduplicated chromosome. The middle inset is the B repeat channel, which is found on both B chromosome arms at distal positions and throughout the endoreduplicated chromosome. The bottom inset is the DAPI channel. The white arrow is a B chromosome. B-repeat probes are green. Notice all A chromosomes have one distinct signal per sister chromatid. Scale bar is 10 microns

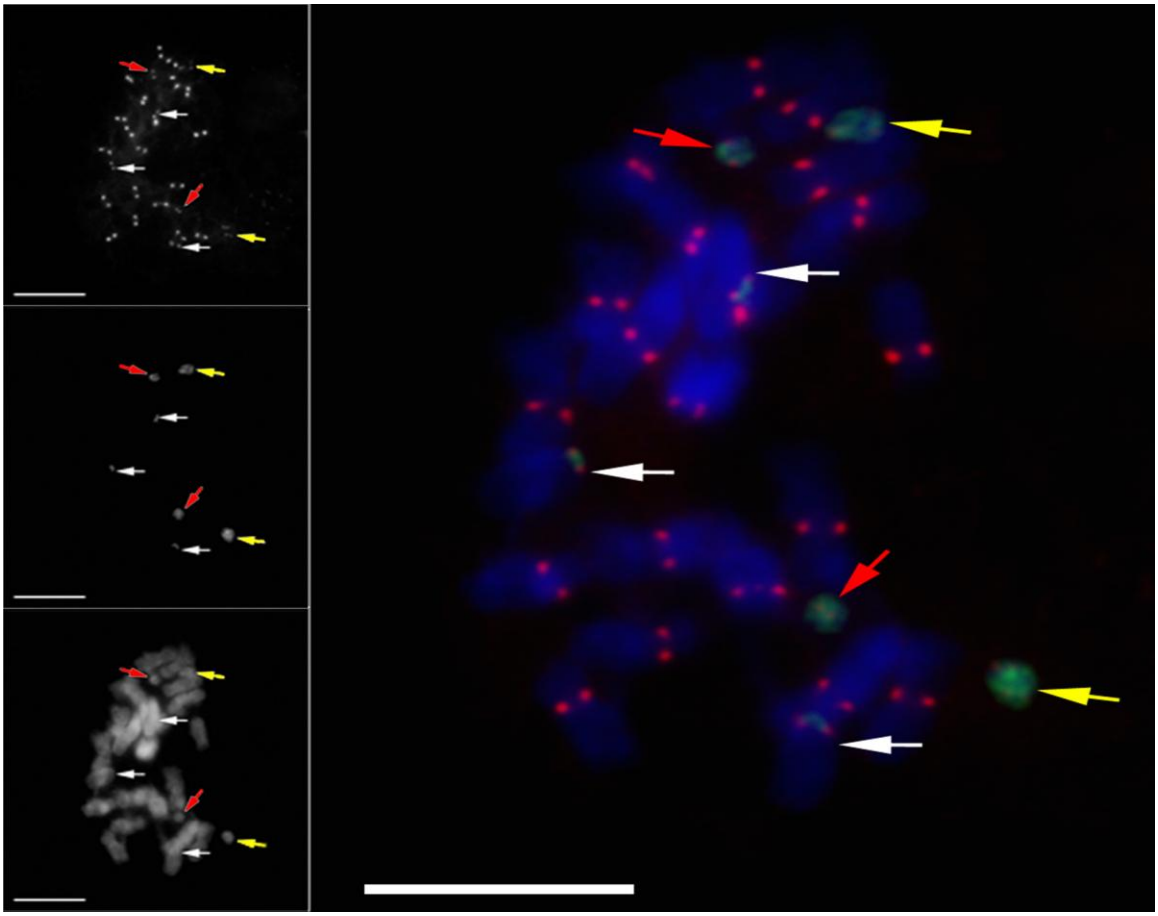

Figure 5.5 Multiple sites of bound CENPC on larger endoreduplicated chromosomes. The yellow arrows denote two endoreduplicated chromosomes with at least two sites of bound CENPC, which is a marker for centromere activity. The red arrows denote small endoreduplicated chromosomes with one site of antibody binding. The top inset is the red CENPC antibody channel showing multiple signals on each large endoreduplicated chromosome. The middle inset is B repeat, which is found on the B chromosome and throughout the endoreduplicated chromosomes. The bottom inset is the DAPI channel. The white arrows denote three B chromosomes. B repeat is green and CENPC is red. Notice all A chromosomes have one distinct signal per sister chromatid. Scale bar is 10 microns

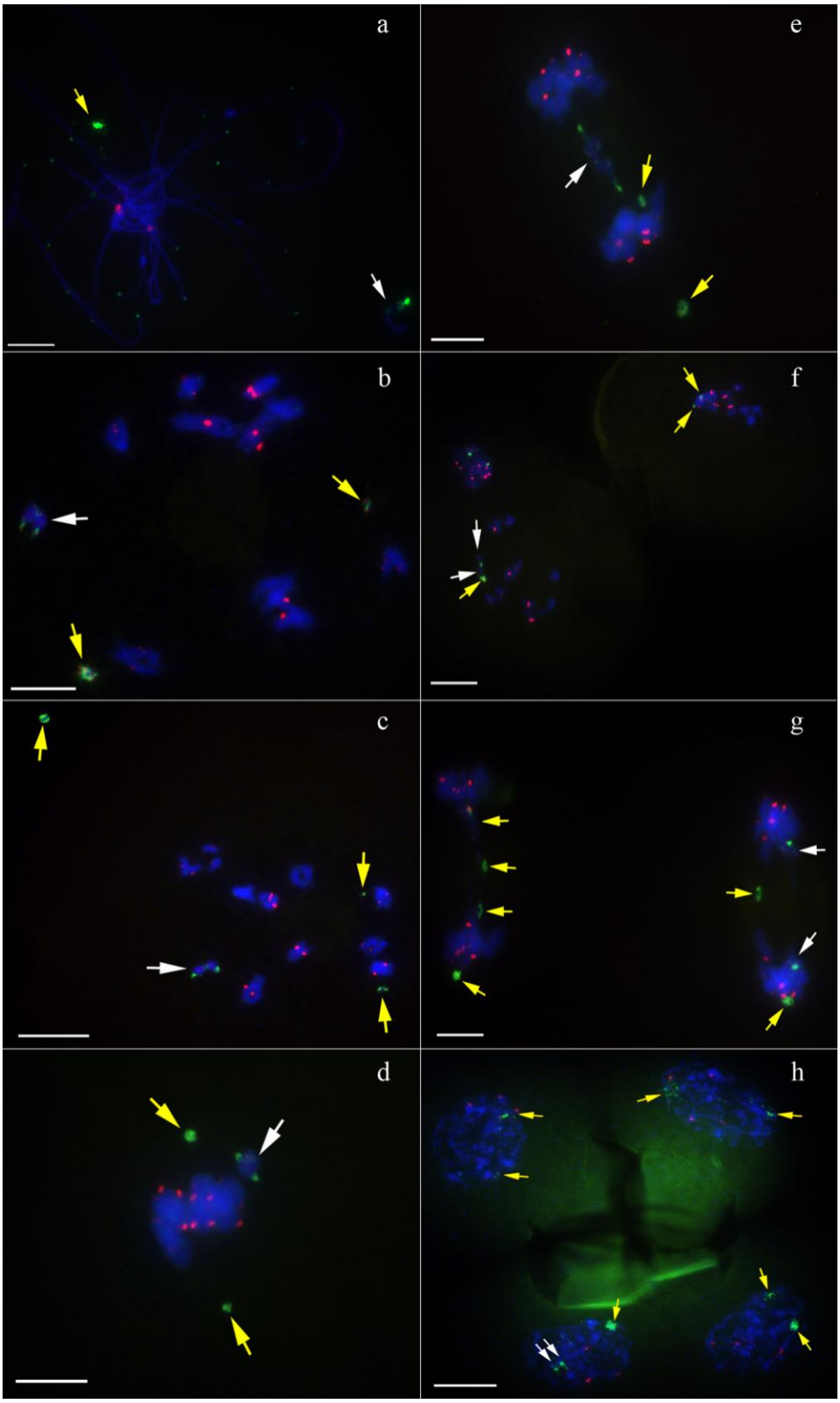
a
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Figure 5.6 The meiotic behavior of an endoreduplicated chromosome. All meiocytes were obtained from a single anther, except the pachytene cell. (a) The endoreduplicated chromosome appears small at pachynema with diffuse B repeat chromatin. (b) Two endoreduplicated chromosomes in diplonema. (c) Three endoreduplicated chromosomes in diakinesis. (d) Two endoreduplicated chromosomes, both with early monopolar movement typical of univalent B chromosomes at metaphase I. (e) Two endoreduplicated 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. (f) Prometaphase II with the top cell having two endoreduplicated chromosomes, while the bottom cell has one larger endoreduplicated chromosome and a univalent B chromosome. (g) Anaphase II with the left cell segregating three endoreduplicated chromosome's sister chromatids and one large endoreduplicated chromosome nondisjoining to one pole without sister chromatid separation. The right cell has one endoreduplicated chromosome lagging at the metaphase plate while a larger endoreduplicated chromosome has nondisjoined to one pole. The larger endoreduplicated chromosomes are in distinct spherical shapes. The univalent B chromosome must have nondisjoined in both meiotic divisions to be in one cell. Yellow arrows denote endoreduplicated chromosomes, and white arrows denote B chromosomes. Telomere probes, which cross-hybridize to the B repeat, are green. CentC probes are red. Scale bars are 10 microns

spherical shaped chromosomes were still present, as well as a higher quantity of small versions (Fig 5.6h). Somatic cells of the anther also showed early monopolar movement (Fig 5.7).

While screening the endoreduplicated chromosome line, a minichromosome was found that resembled the progenitor of the endoreduplicated chromosome with two CentC FISH signals and the characteristic small size. It retained these characteristics and remained at one copy in every cell examined in the root (Fig 5.8a). Because this minichromosome was stable, we self pollinated the plant to see if endoreduplication would reinitiate. In screening the next generation we observed endoreduplicated chromosomes in root tips from ten of eighteen kernels (Fig 5.8b). We also FISH screened forty seeds from the original transformant seed stocks to approximate the onset of endoreduplication and were unable to find an endoreduplicating minichromosome. While the earliest seed stocks did not contain an endoreduplicated chromosome, we did find one plant in which the mini frequently nondisjoined between cells of the same root resulting in quantity shifts from zero to four minis and increased chromosome size (Fig 5.9).

The plants with an endoreduplicated chromosome had severe reductions in vigor and fertility, although some recovery was observed in an outcross. These problems were not encountered for the first two generations, until this chromosome began endoreduplicating in the subsequent five generations. Other than these symptoms, B chromosome accumulation-related phenotypes were absent.

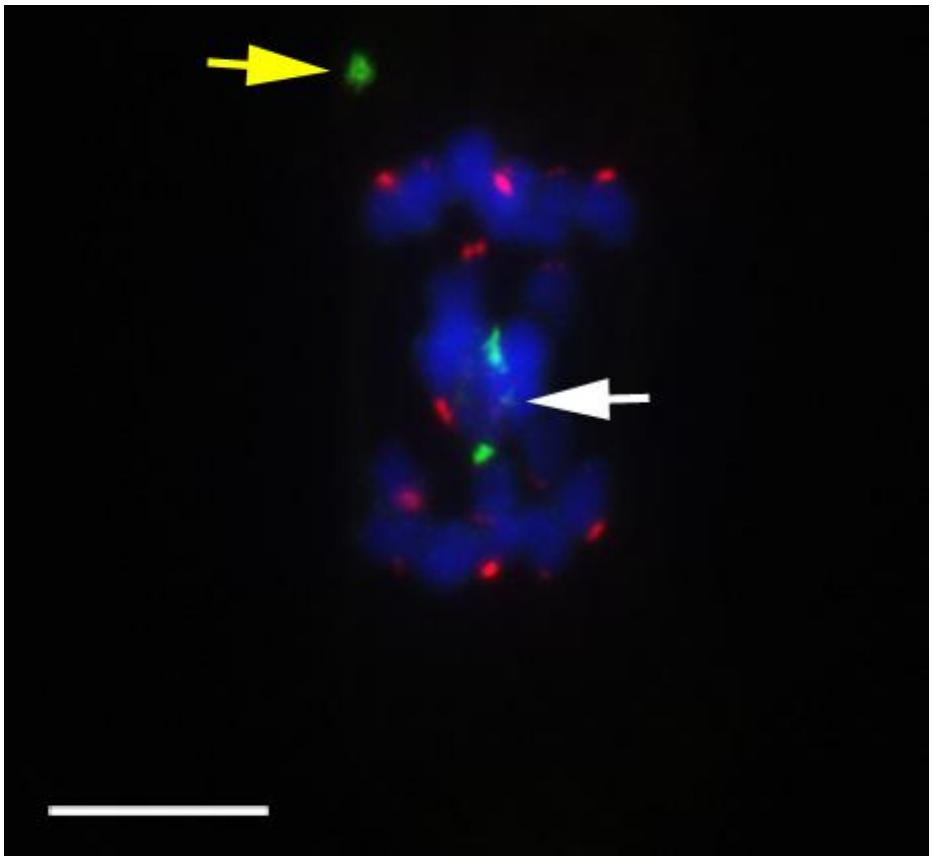

Figure 5.7 Somatic cells of the anther showing early monopolar movement occurring in mitosis. While screening for meiocytes, somatic cells of the anther were found in anaphase. The endoreduplicated chromosome nondisjoined with early monopolar movement (yellow arrow). The white arrow denotes to a B chromosome. Telomere probes, which cross-hybridize to the B repeat, are green. CentC probes are red. Scale bar is 10 microns

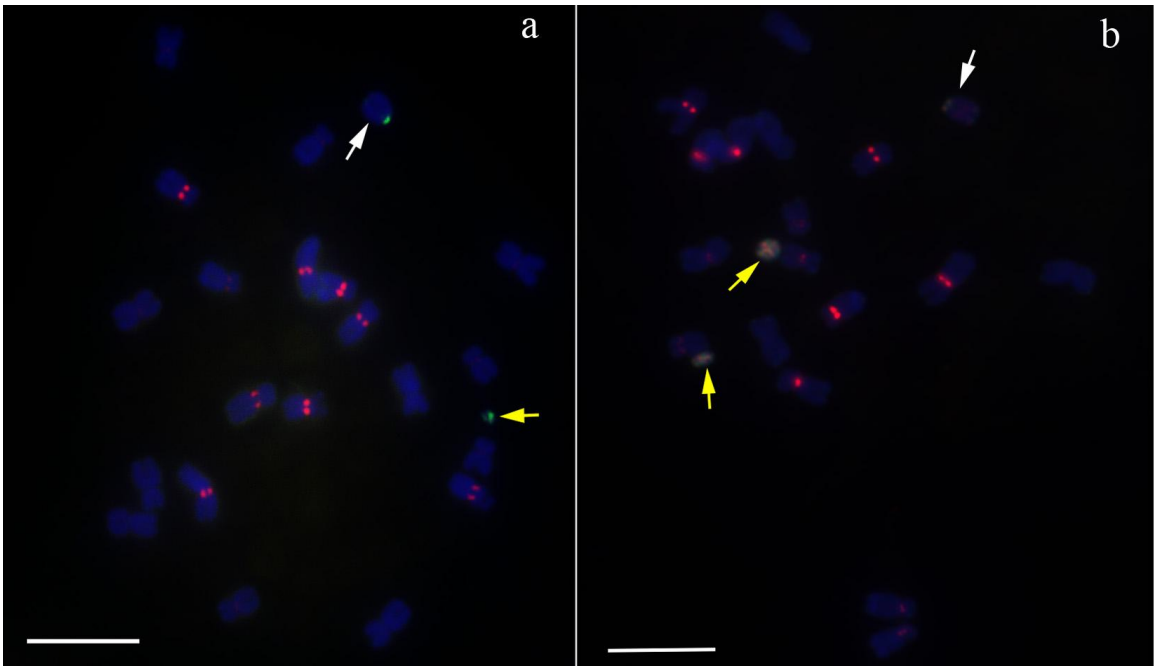

Figure 5.8 A study of a stable, small variant of the endoreduplicated chromosome. (a) A root cell showing the typical shape and size of the small variant (yellow arrow). This chromosome was found at one copy and had one centromere signal per sister chromatid in every cell observed. (b) A root cell from kernels of the next generation showing two endoreduplicated chromosomes (yellow arrows). White arrows denote B chromosomes. Telomere probes, which cross-hybridize to the B repeat, are green. CentC probes are red. Scale bar is 10 microns

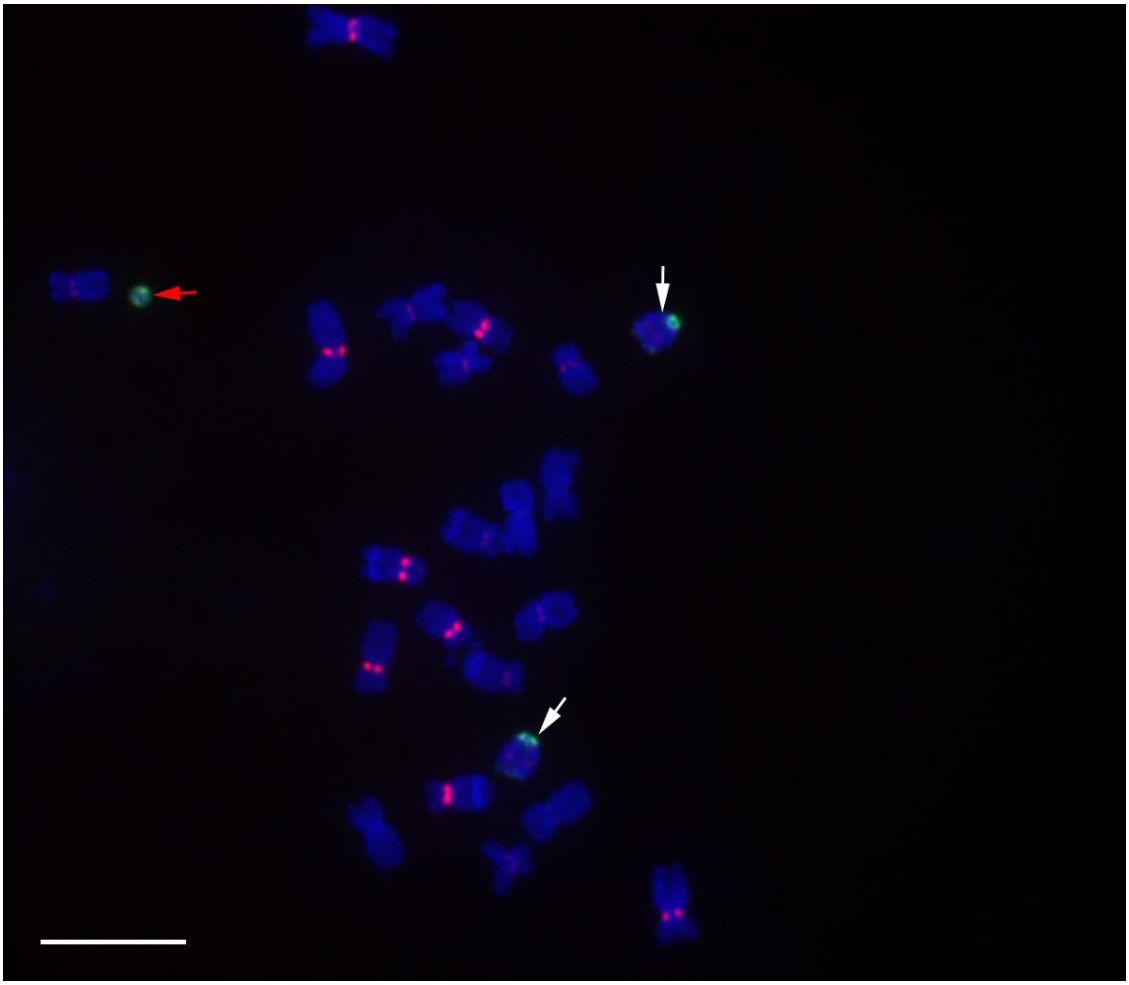

Figure 5.9 A minichromosome from the earliest transformation generation that is increased in size, but still retains one centromere signal per sister chromatid. The insets show from top to bottom: the CentC (red) channel, the telomere (green) channel, and the DAPI (blue) channel. The yellow arrow denotes the minichromosome. The white arrow denotes a B chromosome. Telomere probes, which cross-hybridize to the B repeat, are green. CentC probes are red. Scale bar is 10 microns

Discussion

Here we describe a chromosome that changes size and structure among cells of the same plant. The fact that the normal karyotype replicates and separates normally indicates the presence of a cis-acting modification in the endoreduplicating chromosome.

A potential explanation is that there is an enhancement of nondisjunction without the subsequent sister separation. The normal B chromosome's sisters eventually separate, but the endoreduplicated sisters may remain adhered through successive replication cycles, thus doubling the size of the chromosome (Longley, 1927). Likewise diplochromosomes are produced with colchine and separase knockouts have four fused chromatids, although they dissociate in the following mitosis (Kumada et al., 2006, Barber, 1940). To explain the observed structure, the endoreduplicated chromosome's sister chromatids must remain adhered through multiple mitoses, which would drastically lower its presence among monitored cells. This possibility would halve the number of cells in the lineage receiving this endoreduplicated chromosome for each nondisjunction event. Considering that we found six chromosomes with an approximate ten to sixteen centromere sites (Fig 5.1b), the odds of finding this would be extremely low ($1/2^6$). In this scenario nondisjunction could explain the number of centromere loci present, but the observed frequency of endoreduplicated chromosomes would be much lower than that observed.

A more likely scenario begins with an initial modification that circumvents replication licensing, thus initiating multiple rounds of replication per cell cycle (Nishitani et al., 2002). The minichromosome could then increase to various sizes between cells and permit the accumulation of multiple copies. This would also group the

larger endoreduplicated 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. Both scenarios require a modification of replication that is potentially epigenetic, but we cannot rule out a mutational basis.

Both mechanisms would double the number of centromere signals per extra replication cycle or lack of sister chromatid separation, which would create endoreduplicated chromosomes with 2, 4, 8, 16, 32, etc. centromere signals. The number of centromere signals is directly proportional to the number of transgenes, which have distinct condensed FISH signals (Fig 5.1d inset). In counting the number of transgene signals, we noticed that the number deviates from the strict doubling predicted. This deviation might result from separation of some of the copies in some mitoses.

The monopolar attachments that were observed in metaphase I and II and anaphase I and II were more frequent with larger versions of the endoreduplicated 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 et al., 1992), but is at much lower levels than what was seen with the endoreduplicated chromosome.

The source of initiation of endoreduplication was investigated by screening the earliest transformation stocks and studying a stable minichromosome derived from the endoreduplicated lineage. In this screen a minichromosome was observed with larger amounts of DAPI staining chromatin, but always had a single centromere site. The stable minichromosome behaved like a typical chromosome and had a complete absence of

nondisjunction in the root cells. Endoreduplication occurred in the following generation, showing that this condition is irreversible.

The maize B chromosome can cause a delay in the development as the B's frequency increases in the genome (Kato, 1970). This may be related to the diversity of the meiotic stages in the anther, as the endoreduplicating chromosome frequently changes size and centromere copy number between root tip cells and meiocytes. This differential abundance between adjacent cells could promote drastic changes in cell division times and possibly be a cause for the low, but consistent fertility over seven generations.

The B chromosomal origin of this endoreduplicating chromosome probably played a role in the initial genetic/epigenetic architecture necessary for the endoreduplication of a single chromosome in an otherwise stable genome. Further study may elucidate the genetic or epigenetic changes that enable independent endoreduplication of a single chromosome. Since this chromosome lacks vital genes and isn't limited to a specific lineage of cells, significant studies can be carried out to understand the mechanism of endoreduplication.

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis describes the behavior of B chromosomes and minichromosomes at multiple copy numbers in mitosis and meiosis, including an investigation of a single endoreduplicating chromosome derived from a small B-derived minichromosome. Early studies on the B chromosome at multiple numbers provided an excellent foundation for our study of minichromosomes. With the pairing of multiple B's and the accumulation limit for B chromosomes already established, we could examine previous findings in more detail with FISH and address new phenomena specific to minichromosomes.

The sporophytic nondisjunction of the B chromosome in roots has been observed previously (Randolph, 1941), but related phenomena, such as the differential gain and loss of B chromosomes in plants with odd and even numbers of B's has not been examined. Plants with even numbers of B's were more likely to gain a B chromosome than plants with odd numbers of B's ($P = 0.0065$). Plants with odd numbers of B's were more than twice as likely to lose a B rather than gain one ($P = 0.0216$). We also discovered that as B chromosomes increase in numbers in the genome, nondisjunction increases proportionally. These results add to the numerous odd-even effects that B chromosomes exhibit in many species (Jones et al., 1982).

Four types of minichromosomes derived from the maize B chromosome were accumulated to identify whether the number of centromeres or the amount of chromatin

set the cell's supernumerary chromosome tolerance. Three minichromosomes that varied in size from half the size of the B chromosome to essentially just a B centromere were accumulated to similar levels. Thus the chromosomal limit cannot be based on the amount of chromatin, but the number of centromeres is a more likely explanation. The smallest minichromosome, mini 9, was missing the proximal knob to the B chromosome, which may have contributed to its lower accumulation limit. Future attempts at establishing the accumulation limits of minichromosomes should include outcrosses to maintain hybrid vigor which may increase the cell's tolerance for supernumerary chromosomes.

Another phenomena observed during this program of accumulation was the absence of B chromosome accumulation-related leaf stripes and leaf asymmetry in plants with minichromosomes. This phenomenon links these phenotypes to regions distal to the breakpoint in the half sized minichromosome, which includes half of the distal heterochromatin and the euchromatic distal tip.

Minichromosomes with differing origins and inheritance mechanisms, 6S (86B136) and the fifth-sized mini (86-74) can coexist in the same plant. The B chromosome could not induce nondisjunction in the 6S chromosome, which limits this minichromosome from attaining higher than two copies. The 86-74 minichromosome can be accumulated in the presence of normal B chromosomes, while the 6S minichromosome can remain at stable numbers in the genome.

Future directions for this study will address the effect that multiple minichromosomes can have on the expression of transgenes. Will transgene expression increase proportionally with the number of minichromosomes, or will silencing be observed? In

addition, examining the differential expression of an A-derived minichromosome and B-derived minichromosome in the same plant may be interesting.

The behavior of multiple minichromosomes in meiosis led to the description of many B chromosome specific phenomena including diffuse B repeat and nonhomologous associations in meiotic prophase. We detailed the characteristic diffuse state of B repeat chromatin that was found throughout the prophase of meiosis I and II, which occurs at a higher rate in the smaller minichromosomes, 9 and 20. The closest related research describes diffuse chromatin in a short temporal stage of diplonema in *Rosa* (Klasterska, 1976). In *Rosa*, this stage has been correlated with numerous chromosomal abnormalities including translocations and chromosome breaks. The nonhomologous multivalent associations of the B chromosome were present with all sizes of minichromosomes, indicating that the amount of chromatin is not related to this phenomenon.

The pairing of different sized B-derived minichromosomes has been detailed previously, and our study mirrors the previous results that larger chromosomes have a higher rate of pairing (Han et al., 2007). We also frequently saw a large number of minichromosomes that behaved as univalents by exhibiting early monopolar movement at the metaphase plate or separating sister chromatids in anaphase.

Future directions for this study will incorporate a larger number of bioreplicates with the same number of minichromosomes to further elucidate statistical relationships between the size, diffuse B repeat, nonhomologous associations, pairing, and segregation. Having two different data sets examining the effect of B chromosomes on the aforementioned variables should be informative, as in some species B's directly affect the pairing of the normal karyotype (Murray, 1978).

The discovery of an endoreduplicating chromosome coexisting with the normal diploid karyotype, initiated a study of its mitotic and meiotic behavior and the characterization of its structure. The chromosome frequently changed size and centromere copy number between sister cells resembling endoreduplication. Antibodies to phosphorylated H2A and H3S10, histones associated with the maintenance of sister chromatid cohesion, bound to multiple loci on the larger versions of this chromosome. An antibody to CENPC, an inner kinetochore protein, showed similar results by binding to multiple loci in larger versions of the chromosome, further confirming the presence of multiple active centromeres. The meiotic behavior of this chromosome exhibited behavior similar to univalent B chromosomes, with early monopolar movement off of the metaphase plate at metaphase I and II, including an absence of sister chromatid separation in anaphase I.

The characterization of this chromosome led us to investigate its origin. In the last few generations we observed small minichromosome with similar to the endoreduplication chromosome's progenitor including: the size, the centromere signals, and behavior in root mitoses. This minichromosome was stable in every cell, but when the progeny of this plant were examined, endoreduplicated chromosomes were observed. Because we could not reverse the endoreduplicating behavior, we tried to determine the onset of this behavior starting in the earliest transformation generations. Although we were unable to observe a minichromosome with more than two centromere signals, we did observe a minichromosome that was larger than its progenitor.

The mechanism for the formation of this chromosome is still under investigation, but two hypotheses may explain its unique behavior. A chromatin configuration that allows

nondisjunction with infrequent sister chromatid separation could explain its formation, but for each nondisjunction event, cells in the lineage would be reduced by half. Under these circumstances, one chromosome with 32 centromeres would be found in one out of sixteen cells, which is lower than what we observed. A more likely hypothesis lies with the over-replication of DNA. Over-replication would allow for many cells to have the chromosome with multiple centromere signals. This would also group the larger endoreduplicated chromosomes in a specific lineage of cells, which was observed. To account for the variation in numbers between sister cells, these chromosomes must separate when a certain threshold of centromeric DNA is reached.

Future directions to study this endoreduplicated chromosome are numerous. Our hypothesis of over-replication can be tested with immunocytochemistry for parts of the pre-replication complex and for particular chromatin modifications that promote the over-replication of DNA, such as H3K27me1. The study of this chromosome should also shed some light on the cis effects necessary to trigger endoreduplication. Because this chromosome is dispensable and therefore can avoid the typical caveats of gene imbalances that occur with endoreduplication of the normal karyotype, many studies can be done to elucidate the mechanism of endoreduplication.

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