MAPPING AND CYTOLOGICAL CHARACTERIZATION OF MAIZE MEIOTIC MUTANTS *SEGII* AND *DSYCS*

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ABSTRACT

Homologous pairing during meiosis is an important process for the fidelity of recombination and chromosome segregation. The activity of homologous pairing has been linked to events that occur during the early stages of recombination, namely double strand break formation and single end invasion. The exact activities that regulate the genetic network of homologous pairing are not completely understood. The first chapter of this dissertation provides a review of research regarding homologous pairing. To understand more about the activity, a forward genetics approach was used to identify two mutants of maize, *segII* and *dsyCS*. Cytological characterization of these mutants and mapping the genetic lesion underlying each of their phenotypes comprise the second and third chapters.

Combining FISH and immunolocalization studies, the *segII* mutant displays abnormalities beginning at the leptotene stage of meiosis, which result in reduced installation of DSB repair proteins, non-homologous pairing, reduced chiasmata, and crossovers between non-homologous chromosomes. Application of cisplatin to *segII* mutants to induce double strand breaks at leptotene partially rescues repair protein installation, suggesting that a lack of DSBs underlies the abnormal meiotic phenotype *of segII*. Analysis of the *segII* mutant also indicates that maize displays crossover homeostasis through its reduced single end invasion events. The number of chiasmata is disproportionally reduced when compared to the number of SEI: 26% versus 2% respectively.

The *dsyCS* mutant was also analyzed using FISH and immunolocazliation studies. It displays a similar repair protein abnormality to *segII*, a more severe pairing phenotype than *segII* and, most notably, a high rate of anaphase bridges. A FISH probe to the

telomeres reveals interchromosomal connections at diakinesis, which suggests that improper telomere repair is at least partially responsible for the bridges. *dsyCS* displays non-homologous crossovers, and together with *segII* and the previously characterized *zmRad51* mutant, suggests that maize has at least three genetic paths to create non-homologous chiasmata.

BIOGRAPHICAL SKETCH

Christopher G. Bozza graduated from Montville Township High School in 2000 where he placed first in the state for the earth science league and fifth in the state for the chemistry science league. He then attended Cook College of Rutgers University where he graduated from in 2004 with a bachelor's degree in Biotechnology and a minor in Biochemistry. In the fall of 2004 he enrolled in the Plant Cell and Molecular Biology program of the Plant Biology field at Cornell University. In 2005 he joined Wojtek Pawlowski's lab where he conducted his thesis research.

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LIST OF ABBREVIATIONS

APW = Artificial Pond Water

CE = Central Element of the Synaptonemal Complex

CH = Crossover Homeostasis

Cisplatin = *cis*-diamminedichloroplatinum(II)

CO = Crossover

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

DABCO = 1,4-diazabicyclo[2.2.2]octane

DSB = Double Strand Breaks

DUF = Domain of Unknown Function

FISH = Fluorescent *In Situ* Hybridization

IDP = Insertion / Deletion Polymorphisms

NCO = Non-Crossover

Ph1 = *Pairing homeologous 1*, Mutant of Wheat

SC = Synaptonemal Complex

SEI = Single End Invasion

SIC = Synapsis Initiation Complex

TIR = Terminal Inverted Repeat of the *Mutator* Transposable Element

TUNEL = Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

VDE = Endonuclease of *S. cerevisiae*, Produced by *VMA1* gene

Wt = Wild Type

CHAPTER 1

Cytogenetics of Homologous Chromosome Pairing in Plant Meiosis

Abstract

Three activities serve to hallmark meiotic cell division: homologous chromosome pairing, synapsis, and recombination. Recombination and synapsis are well-studied but the activity of homologous pairing still has not been fully elucidated. Many studies in plants have yielded insights into the mechanisms of chromosome pairing interactions. Research in several plant species showed the importance of clustering of telomeres on the nuclear envelope (telomere bouquet formation) in facilitating alignment of homologous chromosomes. Homologous pairing was also shown to be tied to the early stages of recombination by mutant analyses in Arabidopsis and maize. In contrast, little is known about the mechanisms that guide homolog interactions after their rough alignment by the bouquet and before the close-range recombination-dependent homology search. The relatively large and complex genomes of plants may require additional mechanisms that are not needed in small genome eukaryotes, to distinguish between local homology of duplicated genes or transposable elements and global chromosomal homology. Plants provide an excellent large genome model for the study of homologous pairing and dissection of this system.

Introduction

Homologous chromosome pairing, which encompasses interactions between chromosomes that lead to juxtaposition of the homologs, is one of the least understood meiotic processes. Chromosome pairing takes place during the early stages of meiotic

prophase I and coincides with two other major meiotic processes: recombination and synapsis. Recombination starts with formation of double-strand breaks (DSBs) in chromosomal DNA, which are later repaired, leading to crossovers between a single sister chromatid of each homologous chromosome. Synapsis involves the installation of a protein that bridges the gap between homologs to hold them together. Pairing, synapsis, and recombination not only occur concurrently but there is also a great deal of coordination between the three processes (Pawlowski and Cande, 2005).

Plants have been at the forefront of homologous pairing research for several decades (Maguire 1967, Burnham et al. 1972, Maguire 1984, Maguire 1994, Franklin et al. 1999, Pawlowski et al. 2003). Good genetic tools, particularly in *Arabidopsis thaliana* and *Zea mays*, facilitate identifying genes regulating pairing and elucidation of their functions. Additionally, plants, unlike many other taxa, do not have meiotic checkpoints that arrest the progression of meiosis upon signs of recombination or synapsis abnormalities. This enhances the dissection of the functions of the mutated gene(s) by allowing examination of downstream effects of the mutations.

To understand the complexity of homologous pairing, one needs to consider the obstacles that homologs must overcome to pair. (i) Homologous loci may be spatially separated by relatively large distances in the nucleus at the start of meiosis. (ii) Compact heterochromatin may create difficulties in accessing an orthologous locus for homology recognition. (iii) Repetitive DNA sequences, such as transposable elements and large gene families, may obscure proper homology and lead to ectopic pairing interactions. Consequently, homologous chromosome pairing must include several distinct stages. First, homologous chromosomes must be co-aligned and brought into

close proximity. The existence of homology between the partners must then be established via a sequence-based homology search. Finally, ectopic pairing interactions involving members of gene families, transposable elements, and other repetitive DNA sequences must be eliminated so that true homology can be established along the entire chromosome.

Pre-meiotic chromosome pairing

In plants, homologous pairing of entire chromosomes is uniquely tied to meiosis. However, evidence exists that specific chromosome regions can, in some cases, form homology-based pairing associations in pre-meiotic nuclei. Specifically, studies of Arabidopsis interphase nuclei show that high copy number repeats tend to cluster together, even though the arrangement of chromosome arms is largely random (Schubert et al., 2007). There is also evidence that heterochromatic regions of homologous chromosomes associate closely before meiosis in maize (Maguire, 1967). In polyploid wheat containing the *Ph1* gene, somatic association of centromeres has been reported (Martinez-Perez et al., 2001). Thus there may be some activities prior to meiosis that favor homologous pairing. However, the intimate association of meiotic chromosomes along their entire length in plants, as well as in most other taxa, is always formed *de novo* during early meiotic prophase I.

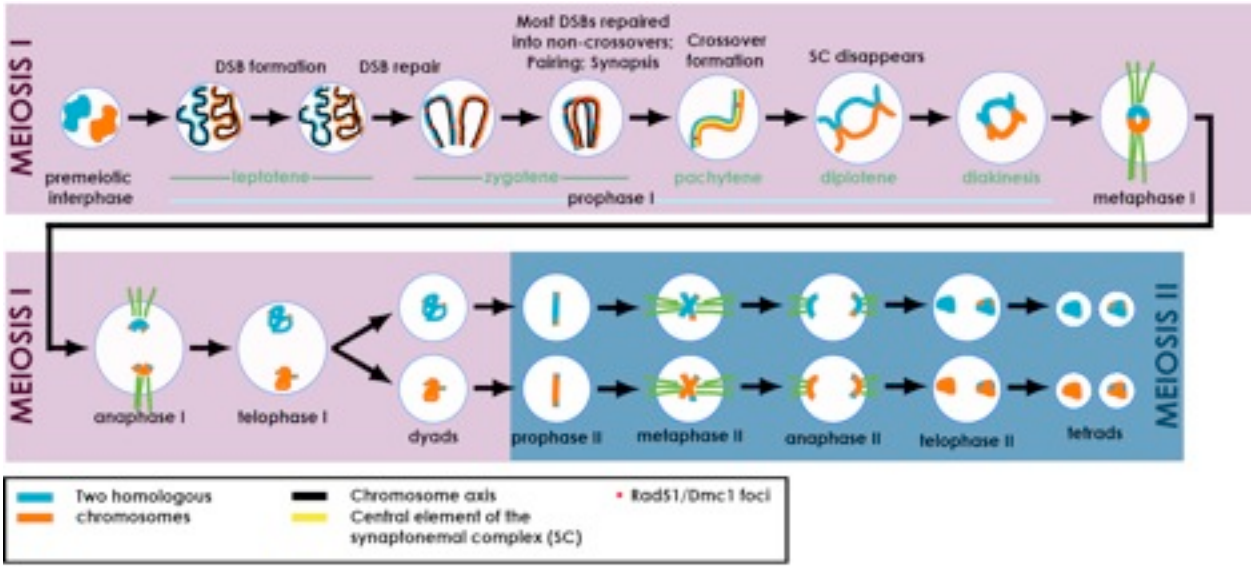
The telomere bouquet and co-alignment of chromosomes

Homologous chromosome pairing in early meiotic prophase is accompanied by dynamic repositioning of chromosomes in the nucleus and formation of a cytological

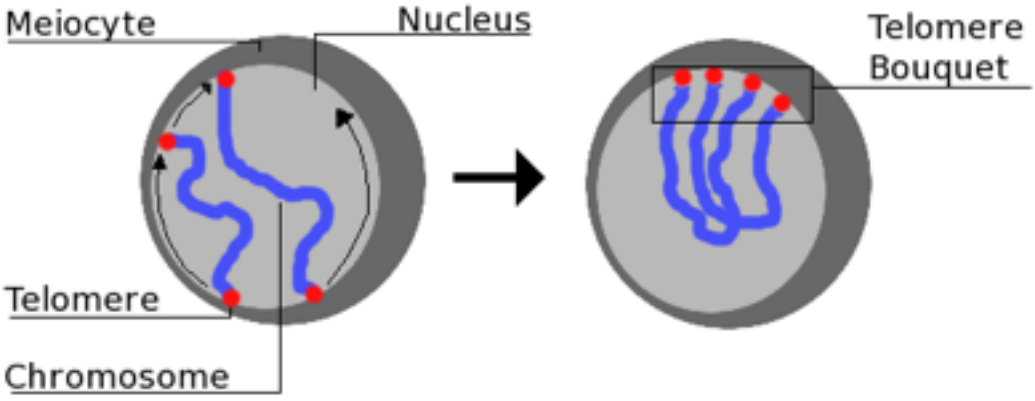
structure called the telomere bouquet. The bouquet consists of telomeres from all chromosomes clustered on the nuclear envelope. In most species, including maize, wheat, and rye, the bouquet forms in late leptotene and persists until early pachytene (Golubovskaya et al., 2002; Harper et al., 2004). Bouquet formation has been observed in many plant species, as well as animals and fungi, with a notable exception being Arabidopsis (however, see below). The timing of telomere bouquet formation just before the onset of chromosome pairing suggests that the bouquet may play a role in pairing. Indeed mutants defective in bouquet formation show slowed and inefficient chromosome pairing (Golubovskaya et al., 2002; Harper et al., 2004; Niwa et al., 2000).

The formation of the telomere bouquet proceeds in two distinct stages. First, the telomeres attach to the nuclear envelope in late leptotene (Figure 1.1b). They then slide to one location, close to the nucleolus, which in late leptotene migrates from the center of the nucleus to its periphery (Golubovskaya et al., 2002; Harper et al., 2004). Clustering of the telomeres is thought to be an active and sudden process (Bass et al., 1997).

Figure 1.1. Meiosis overview and telomere bouquet formation. a. Diagram illustrating meiosis with important processes noted at each stage. Green represents spindle fibres. b. cartoon depicting attachment and movement of telomeres along the nuclear periphery to produce the telomere bouquet in c.



b. c.



Bouquet formation, which juxtaposes chromosomes, and chromosome pairing appear to be linked, however the molecular nature of this link is far from certain. It has been proposed that the bouquet affects pairing by (i) generating a telomere – centromere polarization, which leads to a rough co-alignment of homologs, and (ii) confining chromosomes to a limited nuclear space, which shortens the distances between chromosomes across which the homology search must operate (Scherthan, 2001). This model implies that chromosome pairing starts at telomeres, which seems to be the case, at least in plants (Bass et al., 2000). An alternative model for the bouquet function has recently been proposed in budding yeast and suggests that the bouquet affects early stages of meiotic recombination, which are known to be directly linked to the progression of homologous chromosome pairing (Wu and Burgess, 2006).

Little is known about factors that affect bouquet formation. The physical end of the chromosome is, interestingly, not required for attachment to the nuclear envelope because ring chromosomes in maize participate in bouquet formation (Carlton et al., 2003). However, studies in yeast suggest that the protein complex normally present at the telomeres is required for telomere attachment to the nuclear envelope (Harper et al., 2004). Species-specific factors also affect the bouquet; Bass et al. showed that maize chromosomes in oat-maize addition lines (42 oat chromosomes plus 2 maize chromosomes) exhibit the bouquet dynamics typical for oat rather than maize (Bass et al., 2000). Research using yeast (*S. cerevisiae*) and mouse models showed that progression through early stages of recombination also affects telomere clustering (Liebe et al., 2006; Pandita et al., 1999).

At the mechanistic level, the bouquet formation is best understood in yeast *Schizosaccharomyces pombe*. A number of genes encoding structural bouquet proteins have been identified in this species, and mutants in these genes allowed the study of specific functions of these proteins (Chikashige et al., 2006; Harper et al., 2004). However, very few of these genes have apparent sequence homologs in other groups of eukaryotes, including plants, suggesting that even though the overall bouquet structure is conserved, specific aspects of telomere clustering evolve more quickly. In plants, several mutants showing bouquet defects are known, including *pam1*, *dy1*, *dsy1*, *afd1*, and *phs1* in maize, and *sy1* and *sy9* in rye (Bass et al., 2003; Golubovskaya et al., 2006; Golubovskaya et al., 2002; Pawlowski et al., 2004; Sosnikhina et al., 2005). However, since the causes underlying nearly all these mutations are unknown, it is not clear which of these mutants represent specific bouquet defects and which are primarily defective in other meiotic processes, such as recombination, but also affect telomere clustering. A *bona fide* telomere clustering mutation and the best studied of these is the *pam1* mutation in maize (Golubovskaya et al., 2002). In the *pam1* mutant, telomeres attach to the nuclear envelope but fail to cluster. This leads to defects in many downstream meiotic processes, such as chromosome pairing and synapsis. On the other hand, the initiation and early progression of meiotic recombination are normal. Eventually, the defects in the *pam1* mutant make meiosis progression slow and inefficient. However, some cells complete meiosis, suggesting that telomere clustering is not absolutely required for successful completion of meiosis.

Although the vast majority of eukaryotes show the presence of a telomere bouquet, *Arabidopsis thaliana* is one of the few exceptions. However, it has been

observed that, instead, Arabidopsis telomeres cluster around the nucleolus in the pre-meiotic interphase, which may serve a similar function to that of the bouquet in other species (Armstrong et al., 2001).

Meiotic recombination and chromosome pairing

Meiotic recombination serves two purposes: to create genetic diversity and to provide mechanical stability for the paired chromosomes after the synaptonemal complex (SC) disintegrates and until chromosomes segregate in anaphase I. Extensive data suggest that a subset of meiotic recombination activities is also essential to promote pairing of homologous chromosomes in plants, as well as fungi and mammals, but, interestingly, not in *Caenorhabditis elegans* or *Drosophila* (Dernburg et al., 1998; McKim et al., 1998).

Meiotic recombination

The earliest recombination step is formation of double-strand breaks (DSBs) in the chromosomal DNA. The DSBs are resected from 5' to 3' to leave 3' single-stranded DNA (ssDNA) overhangs. ssDNA is then bound by proteins that promote homologous recombination. The meiotic recombination pathway eventually leads to formation of crossover and non-crossover (gene conversion) events.

Meiotic DSBs in plants, as in all species examined thus far, are created by SPO11, a member of the type II topoisomerase protein family (Keeney et al., 1997). Unique from other taxa, plants possess multiple copies of SPO11: Arabidopsis and maize have three, while the rice genome contains four (Grelon et al., 2001) (Pawlowski

et al., unpublished). Of the three Arabidopsis homologs, only SPO11-1 and SPO11-2 have a meiotic function (Grelon et al., 2001; Stacey et al., 2006) and the phenotypes of Arabidopsis *spo11-1* and *spo11-2* mutants appear to overlap considerably. Neither are able to create crossovers to physically connect chromosomes, nor are they able to synapse chromosomes, which causes metaphase I to have entirely univalent chromosomes.

Once the DSBs are created, they are acted upon by the MRN protein complex, consisting of MRE11, RAD50, and NBS1 (Bleuyard et al., 2004; Bundock and Hooykaas, 2002; Puizina et al., 2004; Waterworth et al., 2007). Arabidopsis meiocytes deficient in MRE11 and RAD50 show chromosome breakage and meiotic sterility as a result of being unable to repair SPO11-induced DSBs (Bleuyard et al., 2004; Bundock and Hooykaas, 2002; Puizina et al., 2004).

Following the resection of the DSBs, a 3' ssDNA overhang is generated. This overhang is bound by two recombination proteins, RAD51 and DMC1, which promote homologous recombination through single end invasion (SEI) of homologous double-stranded DNA. RAD51 exhibits both vegetative DNA repair and meiotic function while DMC1 is meiosis-specific (Doutriaux et al., 1998; Klimyuk and Jones, 1997). *rad51* mutants in Arabidopsis show univalent chromosomes instead of bivalents at metaphase I caused by chromosome pairing defects and absence of chiasmata (Li et al., 2004). In addition, they also exhibit chromosome breakage as a result of DSBs being unrepaired. In maize, plants deficient in RAD51 activity also exhibit chromosome breakage, as well as non-homologous synapsis and, most strikingly, chiasmata between non-homologous chromosomes (Li et al., 2007). In contrast to the *rad51* mutant, DMC1-defective

Arabidopsis plants do not show chromosome fragmentation (Siaud et al., 2004). These observations indicate that DSB repair using the homologous chromosome as template is chiefly the responsibility of DMC1. RAD51 predominantly repairs meiotic DSBs using a sister chromatid as template, instead of the homologous chromosome.

Studies of yeast and mice show that, following SEI, the meiotic recombination pathway splits into two parallel branches: one leading to crossovers (COs) and one to non-crossovers (NCOs) (Allers and Lichten, 2001; Guillon et al., 2005; Hunter and Kleckner, 2001). Crossovers are reciprocal recombination events that lead to the exchanges of chromosome arms. Non-crossovers (gene conversions) are generated through a non-reciprocal repair of DSBs, without a double Holliday junction intermediate. The presence of separate CO and NCO pathways may be universal in all meiotic species, including plants, although this has not been confirmed in all model species yet.

Steps of meiotic recombination that affect chromosome pairing

A number of studies in a variety of species, including maize and *Arabidopsis thaliana*, indicate that homologous chromosome pairing is tightly linked to the progression of meiotic recombination. A strong connection between pairing and recombination exists also in mammals and fungi (Pawlowski and Cande, 2005). In contrast, chromosome pairing does not depend on recombination in *C. elegans* and *Drosophila* (Dernburg et al., 1998; McKim et al., 1998).

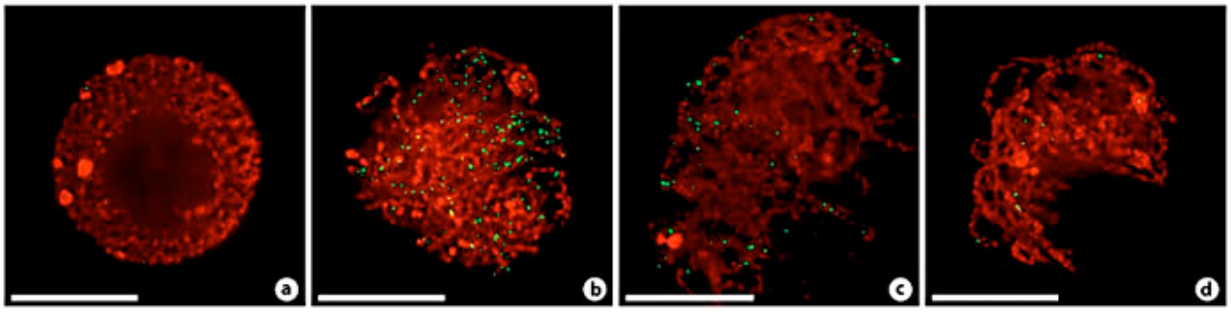
Numerous plant mutants in early recombination genes show defects in chromosome pairing in addition to their recombination defects, indicating that pairing

requires initiation of meiotic recombination and progression though early steps of the recombination pathway (Hamant et al., 2006; Pawlowski and Cande, 2005). In contrast, mutant studies show that late recombination steps, such as crossover formation, are not required for homologous pairing (Higgins et al., 2004; Jackson et al., 2006). Even though the pairing-recombination link has been well established, the nature of this interaction has not yet been resolved. It is possible that chromosome pairing utilizes the recombination pathway DNA intermediates. Alternatively, some recombination proteins may have dual functions, affecting both pairing and recombination. Homologous pairing in plants, as in mammals and fungi, requires recombination initiation. Arabidopsis mutants in the *SPO11-1* and *SPO11-2* genes, which fail to create DSBs, do not pair or synapse (Grelon et al., 2001; Stacey et al., 2006). This is also true for the DSB resection step: *atmre11* mutants are unable to pair homologs in about 90% of meioses (Puizina et al., 2004).

The strongest evidence linking recombination and pairing, however, is derived from the SEI step of meiotic recombination, which is facilitated by a protein complex that includes RAD51 and DMC1. In most meiotic species, these proteins form numerous foci on meiotic chromosomes in early meiotic prophase I (Figure 1.2) (De Muyt et al., 2007; Franklin et al., 1999; Pawlowski et al., 2003; Terasawa et al., 1995). Franklin et al. suggested, based on the observations in maize, that the number of RAD51 foci vastly exceeds what is required for formation of COs and proposed that the extra foci are utilized for the chromosome homology search (Franklin et al., 1999). *In vitro* studies show that RAD51 and DMC1 coat the ssDNA overhangs, forming nucleoprotein filaments. In order for this short range mechanism of DMC1/RAD51 mediated

homology search to be effective, the homologs must be in a close alignment. Evidence from yeast studies indicates that a resected ssDNA may extend to approximately 2 kb (Lee et al., 1998). Each nucleotide contributes about 0.6 nm to the length of a DNA strand (Murphy et al., 2004). Assuming that binding of RAD51 and DMC1 discourages DNA secondary structure, a linear nucleoprotein filament of 2 kb will extend to about 1,200 nm. This is sufficient to bridge the roughly 400 nm distance between chromosomes brought together by an initial “rough” alignment prior to pairing (Tesse et al., 2003). In the small genome budding yeast, the length of a RAD51/DNA filament would even be adequate to span the ~ 1μm-wide telomere cluster (Trelles-Sticken et al., 1999). However, this mechanism would not be effective in bringing the homologs into the pre-pairing alignment in large genome species, such as maize, where the bouquet cluster is approximately 7 μm in diameter (Carlton et al., 2003).

Figure 1.2. Distribution of RAD51 foci in wild-type maize meiocytes. Dynamic changes of RAD51 localization in meiotic prophase I, coincide with homologous chromosome pairing, which supports the proposed role of RAD51 in homology recognition. **(a)** Leptotene. **(b)** Mid-zygotene. **(c)** Late zygotene. **(d)** Pachytene. Red: chromatin, green: RAD51. Images are flat projections from several consecutive optical sections through 3-dimensional nuclei. Bar = 10 µm. Reprinted from Pawlowski et al.: Altered nuclear distribution of recombination protein RAD51 in maize mutants suggests involvement of RAD51 in the meiotic homology recognition. Plant Cell 8:1807-1816 (2003).

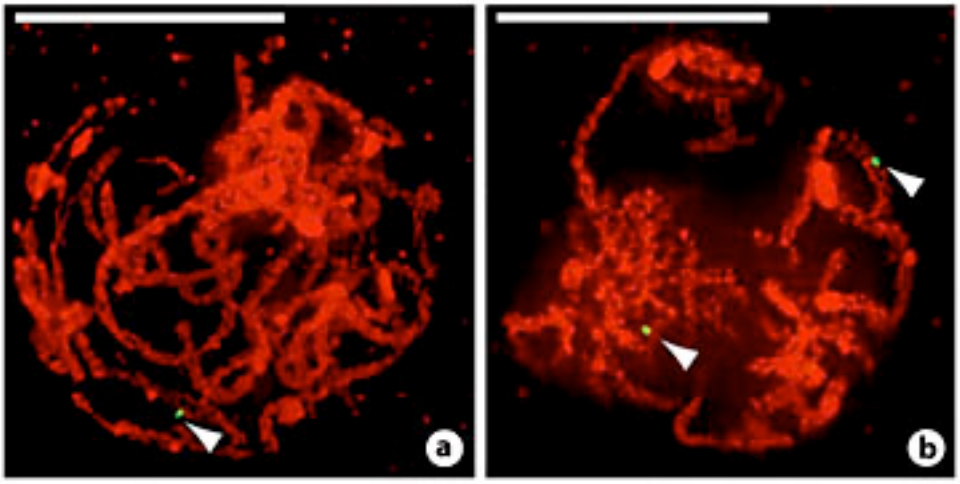


Coordination of pairing and recombination

Mutational analyses have not, thus far, uncovered any plant genes participating in the chromosome homology search that act completely independently of recombination. However, a small group of genes has been identified, which encode proteins that are not primarily involved in DSB repair, but instead coordinate pairing and recombination. Mutants in these genes show a phenotype where homologous chromosome pairing is replaced by associations between non-homologous partners (Figure 1.3). In plants, this gene group contains *Phs1* described in maize (Pawlowski et al., 2004), and *HOP2* and *MND1*, which were first identified in yeast (Leu et al., 1998; Tsubouchi and Roeder, 2002) but recently also shown to have homologs in several other species, including plants (Domenichini et al., 2006; Kerzendorfer et al., 2006; Panoli et al., 2006; Schommer et al., 2003). Studies in Arabidopsis, yeast and mouse showed that HOP2 and MND1 form a heterodimer that is able to interact with DMC1 to stimulate its homology search activity (Petukhova et al., 2005; Pezza et al., 2006; Vignard et al., 2007). In Arabidopsis, HOP2/MND1 localize to chromatin from leptotene through pachytene (Vignard et al., 2007). This localization is not dependent on the SPO11-generated DSBs and does not significantly overlap with the DMC1 foci. Mutants lacking HOP2 or MND1 in Arabidopsis, yeast, and mouse are unable to proceed beyond DMC1/RAD51 loading and exhibit univalents at metaphase I (Domenichini et al., 2006; Kerzendorfer et al., 2006; Leu et al., 1998; Petukhova et al., 2003; Tsubouchi and Roeder, 2002). In the *hop2* mutant in yeast approximately 60% of synapsis takes place between non-homologous chromosomes (Leu et al., 1998). The molecular mechanism of action of the HOP2/MND1 complex is not yet clear. While several authors proposed

that its primary role is to directly facilitate the SEI invasion process (Chen et al., 2004; Tsubouchi and Roeder, 2002), Zierhut et al. suggested that it may have a more general function in affecting chromatin and/or higher order chromosome structures of the homologous target (Zierhut et al., 2004).

Figure 1.3. Homologous pairing in a wild-type maize meiocyte in pachytene **(a)** and a pairing-like association of non-homologous chromosome association in a maize meiotic mutant *segII* at the same stage of meiosis **(b)**. Red: chromatin, green and marked with white arrows: 5S ribosomal RNA loci on maize chromosome 2. Images are flat projections from several consecutive optical sections through 3-dimensional nuclei. Bar = 10 µm.



The maize *phs1* mutant also exhibits a severe pairing phenotype with 95% of chromosome pairing-like associations forming between non-homologous partners (Pawlowski et al., 2004). However, in this mutant, RAD51 fails to load on the sites of meiotic DSBs, suggesting that PHS1 acts earlier in meiosis than HOP2/MND1.

Synapsis and homologous pairing

Synapsis is the process of installing the central element (CE) between two paired chromosomes. Although synapsis normally takes place between homologs, aberrant meiosis can produce associations between non-homologous chromosomes, which become synapsed together. Such abnormal synapsis has been observed in haploids (De Jong et al., 1991) and in meiotic mutants defective on homology recognition (Leu et al., 1998; Pawlowski et al., 2004). This would suggest that synapsis is more of a "default" process that does not take into account homology and will take place between non-homologous chromosomes if homologs are not available. However, unlike other models, Arabidopsis mutants lacking the CE demonstrate the ability to associate non-homologous chromosomes as bivalents at metaphase I (Higgins et al., 2005), suggesting that synapsis may be important for homology recognition.

Chromosome pairing in polyploids

The complexity of chromosome pairing increases dramatically in polyploid species harboring genomes with similar (homeologous) chromosomes. To deal with this issue, polyploid species have evolved genetic systems controlling recognition of homologous *versus* homeologous chromosomes. The best known such system exists

in hexaploid bread wheat (*Triticum aestivum*) which possesses three highly similar genomes, A, B, and D. Proper homology recognition, which ensures that, for example, chromosomes from the A genome pair with their A genome homologs and not with homeologs from the B or D genome, is controlled by the *Pairing homeologous* (*Ph1*) locus (Riley and Chapman, 1958). The *Ph1* locus is likely to exert its influence by regulating the chromatin structure (Prieto et al., 2004) but the exact mechanism of its action remains unknown. It has been proposed that the action of *Ph1* results in disruption of pre-meiotic associations of centromeres of chromosomes from different genomes (Martinez-Perez et al., 2001). However, a counter-argument stating that the *Ph1* acts during meiosis has also been made (Corredor et al., 2007).

Cloning of the *Ph1* locus has proved difficult due to limited allelic diversity. The only *Ph1* mutations are deletions and generating new alleles through EMS mutagenesis has not been possible (Wall et al., 1971). Recent work has narrowed *Ph1* to a 2.5 Mb region containing a structure consisting of a segment of subtelomeric heterochromatin that inserted into a cluster of *cdc2*-related genes after polyploidization (Griffiths et al., 2006).

Outlook

Even though elucidating the chromosome behavior and interactions that lead to homologous pairing has been a research goal for many decades, there are still many unanswered questions. The area of most progress in understanding pairing in plants is the link between the homology search step and the progression of meiotic recombination. This research is facilitated by the fact that recombination proteins are

some of the most evolutionary conserved proteins involved in meiosis and it has been possible to utilize knowledge from small genome meiotic model systems, such as yeast, to understand the processes in plants. However, SEI mediated by the RAD51/DMC1 protein complex is unlikely to fully explain the process of pairing of homologous chromosomes, especially in complex genome species such as most plants. Such genomes most certainly require mechanisms that would prevent ectopic pairing interactions between repetitive DNA sequences. Consequently, it is likely that some mechanisms regulating pairing in plants are different from those in small genome eukaryotes. This underscores the need for original gene discovery to identify plant-specific pairing regulators. An example of validity of this approach is the identification and cloning of *Phs1* in maize, which does not have obvious sequence homologs in species outside of the plant kingdom (Pawlowski et al., 2004). Another process, where finding plant-specific genes may be expected is telomere bouquet formation, since it appears that very few of the known yeast bouquet genes have sequence homologs in plants.

In addition to the plant-specific, or complex genome-specific aspects of pairing, there are remaining questions concerning the chromosome pairing mechanisms that are shared by all, or most, species. The first is the mechanism of alignment of homologous chromosomes within the effective range for SEI, part of which is undoubtedly the function of the telomere bouquet. The second is the coordination of SEI with other prophase I processes. In elucidating both these processes, research using plants can certainly play a major role.

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Chapter 2

Mapping and Cytological Characterization of the Maize *segII* Mutant

Abstract

Pairing of homologous chromosomes during prophase of meiosis is essential for accurate segregation of genetic material and successful gamete production. While other mechanisms of meiosis, such as recombination, are well explored, homologous pairing remains the least understood meiotic activity.

A forward genetic approach was used to find genes affecting homologous pairing and to define their roles within the genetic network regulating pairing. Screening a collection of meiotic mutants in maize revealed a novel homologous pairing-defective mutant, *segII*, which was found in a *Mu*-tagging population. Initially, the mutant presented univalent chromosomes at metaphase I, which implies deficient crossover formation. To understand the nature of the defect, *segII* was examined by fluorescent *in situ* hybridization (FiSH) for pairing at the 5S rRNA locus and found to have significant pairing defects. The overall level of mispairing, 72%, is distinct from previous mutants which generally voided pairing altogether or, in the case of the maize mutant, *phs1*, showed 5% of normal levels.

In order to further characterize the activity of *segII* and to define its position in the genetic network regulating pairing, I examined the initiation of meiotic recombination (formation of double-strand breaks), the installation of proteins thought to be primarily involved in the homology search and DNA repair, the number and nature of chromosomes at metaphase I, and positionally mapped the gene underlying the

phenotype. Based on the results of the characterization, I also pursued complementing the phenotype by chemically inducing DSBs and looking for the mutant's response through immunolocalizing repair proteins.

The *segII* mutant mispairs chromosomes 72% of the time at the 5S locus and regularly creates non-homologous crossovers. *segII*'s earliest observed phenotype appears to be s deficiency in DSB formation based on γ-H2AX phosphorylation and failure to install large amounts of DSB repair proteins. A consequence of the *segII* mutation in pachytene is that synapsis is delayed. Chemical induction of DSBs at least partially restores the installation of one of these proteins, RAD51. The number of chiasmata in *segII* was reduced to 26% as opposed to the reduction of repair proteins which was 2%. This aspect of *segII*'s phenotype demonstrates that maize possesses a crossover homeostasis mechanism, though co-localization analysis indicates it is not achieved by increased RAD51 and DMC1 localization. Observation of the *segII* phenotype inspires a model linking mispairing in DSB mutants to chromosome movements in meiosis.

Introduction

Homologous pairing during prophase I of meiosis is one of the least studied aspects of reproduction. While the biochemical activities of recombination are relatively well analyzed and conserved recombination proteins have been identified as also playing a role in homologous pairing, the interplay between chromosome movements, recombination proteins, and the ultimate result of homologous chromosome juxtaposition are not well understood (Sheehan and Pawlowski, 2008).

Identifying new mutations that affect homologous pairing is an important method to completely define all the elements that interact to create the pairing process The prevalent system controlling pairing in organisms from yeast to higher eukaryotes involves intermediates of the recombination pathway. Recombination defects starting as early as double strand break (DSB) production are known to significantly affect homologous pairing (Romanienko et al. 2000, Grelon et al. 2001, Stacey et al. 2006). The identification of mutations that negatively affect DSB formation and repair has shown that pairing in recombination-dependent pathway organisms is significantly reliant on the efficient formation of a 5-20x excess of DSBs as compared to crossover events (Franklin et al. 1999, Plug et al. 1996, Bishop et al. 1994).

Due to meiotic DSBs being the result of small deletions, their repair necessitates at least a short synthesis from a homologous template in order to restore genome integrity (Neale et al. 2005). Due to a temporary block to sister chromatid repair, shown to be mediated by components of the axial element, chromosomes must seek their homolog for repair prior to the release of this block (Xu et al. 2005). This requires chromosomes to align within repair distance of their homolog in order for the nucleoprotein filament formed by RAD51/DMC1 to physically interact with the homologous template. It is hypothesized that the search for a homologous template at the DSB site controls homologous pairing of entire chromosomes. Based on an analysis of RAD51 foci dynamics in zygotene and pachytene, Franklin et al. (1999) proposed that the RAD51 nucleoprotein filament is an important biochemical mechanism driving homologous pairing.

Examining full knockouts for DSBs has greatly informed homologous pairing research by indicating that the absence of proper DSB formation and repair leads to chromosome pairing defects. Thus, DSBs are the launching point to investigating homologous pairing. In *spo11* knockouts of Arabidopsis and mouse the restoration of pairing, repair protein installation, and synapsis has been accomplished by complementing the phenotype through chemical DSB induction (Libby et al. 2003, Romanienko et al. 2000, Sanchez-Moran et al. 2008). One study induced a single DSB through usage of a VDE endonuclease in a *spo11* knockout of yeast. The single DSB proved insufficient to stimulate pairing and synapsis (Neale et al. 2002).

Previous reports investigating hypomorphic DSB phenotypes have only been conducted in yeast. For example, Henderson et al. (2004) assembled a collection of yeast mutants representing a DSB gradient from wild type down to null phenotypes. However, the analysis was limited to synaptic initiation without regard to pairing fidelity or crossover analysis. Martini et al. (2006) assembled a set of hypomorphic DSB mutants to examine crossover homeostasis and the results indicated that viability of progeny spores in yeast is affected below 80% of wild type DSB activity, although cytology of meiosis I was not examined.

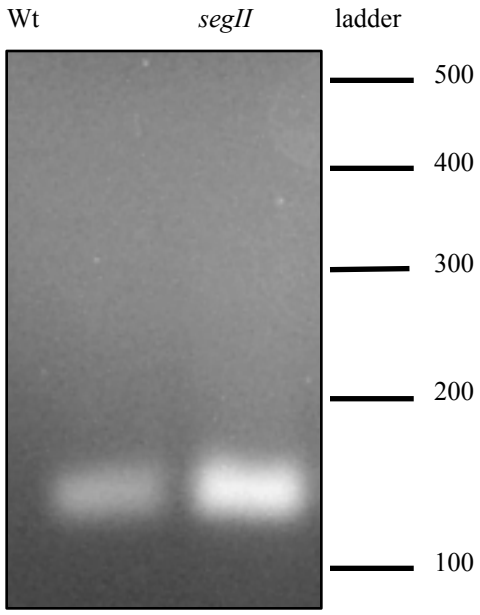
Results

Identification of the *segII* mutant and mapping the *segII* gene

segII was one of several mutants isolated from the maize meiotic mutant collection initially characterized as male and female sterile. It was selected for analysis by a subsequent screen for univalents at metaphase I, and RAD51 foci abnormalities at the zygotene stage (Pawlowski et al. 2003). RAD51 is a homolog of the bacterial recombinase RecA and, along with meiosis specific DMC1, works to provide double strand break repair from homologous chromosome templates during meiosis. Abnormal foci numbers for RAD51 indicate a problem in recombination and homologous pairing.

The phenotype initially appeared similar to a previously identified mutant of maize, *phs1* (Pawlowski et al. 2004). To understand this similarity, I conducted a semi-quantitative RT-PCR examining *Phs1* expression to determine whether *segII* is a hypomorphic allele of *phs1*. Analysis using the intensity function of ImageJ of the RT-PCR shown in Figure 2.1 indicates that the *segII* mutant transcribes Phs1 at 90% of the wt level. Thus, the *segII* mutation does not represent a hypomorphic allele of the *Phs1* gene that could cause the observed phenotype.

Figure 2.1. Semiquantitative RT-PCR analysis of *ZmPhs1* expression in the *segII* mutant. ImageJ analysis of band intensity confirms that *segII* is not a hypomorphic allele of *Phs1*. Ladder sizes are given in bp.



Initial cloning efforts for *segII* were based on a hypothesis underlying the generation of the mutant, which originated in a *Mutator* transposon population, ergo *Mu* activity is likely the cause for the mutation. This topic is reviewed in Lisch and Jiang (2009) Accordingly, I employed a strategy attempting to use *Mu* to identify the gene underlying the *segII* mutation. This strategy was to digest genomic DNA with different combinations of restriction enzymes and perform a Southern blot analysis with ^{32}P labeled oligonucleotide probes to internal elements of the highest probability *Mu* elements responsible. This would cause *Mu* elements to segregate at different sizes, and any bands representing *Mutator* elements that are exclusively present in mutants could be identified. Since *segII* is a recessive mutation and segregates in a 3:1 ratio, all mutants must be homozygous for any causative *Mu* insertion. Comparing *Mu* elements of mutants against *Mu* elements of genotyped-by-progeny wild-type and heterozygous plants would point to any *Mutator* elements that underly the *segII* phenotype. Using multiple combinations of restriction enzymes allows resolution of different regions of the genome. This was important since the resolution of the gels was limited to fragments ≤10kb.

I created initial blots for each *Mu* element with different enzyme digestions using a small test population before moving to a larger sample. This was done by digesting genomic DNA from 3 homozygous mutants, 3 homozygous wild-types, and 3 heterozygotes with 11 unique double enzyme digests. These blots were probed for the most commonly found *Mutator* elements; 1, 6, 8, and *MuDR*, and then analyzed for bands segregating only with heterozygotes and homozygous mutants (Lisch and Jiang 2009). Two *Mu1*

bands segregated on the tester population using a *SalI*/*EcoR1* restriction enzyme combination and were analyzed on a larger population of 18. These *Mu1* elements were both found in homozygous wild-type plants in the larger population, eliminating them as candidates for the mutation. The lack of a *Mutator* element responsible has two plausible explanations. The *segII* mutation could be the result of a *Mu* deletion rather than an insertion, thus the putative *Mu1* element from the tester population may have been a spurious correlation. A second possibility is that there is a *Mu* element which migrated at the same size generated in the background of the wild-type plants that were used to reject the potential *Mu* elements.

Following the Southern blot approach, a map based cloning approach was used. As the maize genome sequence became publicly available, a map-based approach became feasible (Schnable et al. 2009). Previous mapping, using markers from across the genome, indicated linkage of the *segII* phenotype to markers on the short arm of chromosome 5 or the short arm of chromosome 3. DNA-based markers were selected in both regions from the maize genetic map and were evaluated to determine which region exhibited *bona fide* linkage.

The *segII* mutation had previously been crossed as a heterozygote to the B73 and the Mo17 inbred maize backgrounds. Progeny were selfed to produce segregating lines for mapping. These two inbred backgrounds display a high rate of DNA marker polymorphisms and were advantageous when searching for polymorphisms between the *segII* background and either of these diverse parents. Two markers of those

selected on chromosome 5 (5.1.2 and 5.0.2) were found to display polymorphism in the Mo17 crossed mutants and showed linkage (8cM) in a test population (n=12). Further markers were selected on chromosome 5 in the region.

Using the most recent version of the maize sequence (release 5b.60: http://maizesequence.org), I defined the *segII* region proximally by marker IDP3110 at a physical location of 9,951,826 and distally by marker IDP3664 at a physical location of 8,698,384. Table 1 shows individuals used in the tester population at polymorphic markers in the region. It is notable that the distal portion of the shown genotypes are predominantly homozygous wild type. This is representative of the proximity to the telomere and the observation that a significant amount of recombination happens at the distal-most portion of chromosomes.

The *ZmSpo11-1* gene is located near the mapping interval and was a logical candidate to examine based on its known function in other species and specific aspects of the phenotype. Sequencing of the full length *Spo11-1* genomic sequence, including several hundred bases beyond the 5' and 3' UTRs, revealed no detectable sequence abnormalities that would explain the phenotype. Screens for *Mu* insertions were conducted using a primer to the terminal inverted repeat (TIR) of *Mu* combined with primers spaced regularly throughout *Spo11*-1 exons. These screens revealed no *Mu* elements in *Spo11*-1. Quantitative real time PCR analyzed using Bio-Rad iQ5 Version 1.0 software was also used to confirm that there were no abnormalities in *Spo11*-1 expression due to any disrupted distant promoters in *segII* mutants.

The mapping interval on the most recent maize sequence draft contains 30 candidates (Table 2). None of these candidates represents a homolog of a known meiotic gene (Table 3).

Table 2.1. The *segII* mapping interval. +/+ denotes a homozygous wild type allele pattern at the marker, +/- denotes heterozygous allele pattern, -/- denotes homozygous mutant allele pattern. Grey boxes denote that the genotype for that individual at that marker was indeterminable.

	Physical Location (in Mb)	**6.15**	**6.86**	**8.70**	**9.95**	**11.40**	**13.05**	**28.45**
	Marker Name	IDP4720	IDP9016	**IDP3664**	**IDP3110**	IDP8393	IDP9255	IDP2139
individuals								
943a		**+/+**	**+/+**	**-/-**	**-/-**	**+/-**	**-/-**	**+/-**
943b		**+/+**	**+/+**	**-/-**	**-/-**	**-/-**	**+/-**	**+/-**
943d		**+/+**	**+/-**		**-/-**	**-/-**	**-/-**	**-/-**
943e		**+/+**	**+/+**	**-/-**	**-/-**	**-/-**	**+/-**	**-/-**
943f		**+/+**	**+/+**		**-/-**	**-/-**	**-/-**	**-/-**
943g		**+/+**	**+/+**	**-/-**	**-/-**	**-/-**	**-/-**	**-/-**
943h		**+/+**	**+/+**		**-/-**	**-/-**	**+/-**	**-/-**
943i		**+/+**			**+/-**	**+/-**	**+/-**	**+/-**
943j		**+/+**			**-/-**	**-/-**	**-/-**	**-/-**
943k		**+/+**			**-/-**	**-/-**	**-/-**	**-/-**
943l		**+/+**			**-/-**	**+/-**	**-/-**	**+/-**
944a		**+/+**	**+/+**	**+/-**	**-/-**	**-/-**		**-/-**
944b		**+/-**	**+/+**	**-/-**	**-/-**	**-/-**		**-/-**
944c		**+/+**	**+/+**	**-/-**	**-/-**	**+/-**		**+/-**
944f		**+/+**	**+/-**	**-/-**	**+/-**	**+/-**		**+/-**
944g		**+/+**	**+/-**	**-/-**	**-/-**	**-/-**		**-/-**
944h		**+/+**	**+/-**	**-/-**	**-/-**	**-/-**		**+/-**
944i		**+/+**	**+/-**	**+/-**	**-/-**	**-/-**		**+/-**

Table 2.2. Candidate genes in the *segII* mapping interval on chromosome 5.

Physical Distance covered	Genes in Physical distance	Identified domains
8698384-8800000	No genes	
8800000-8900000	GRMZM2G019958	Uncharacterized protein
	GRMZM2G019945	A domain for membrane and nuclear functions.
8900001-9000001	No genes	
9000002-9100002		
	GRMZM2G701333	S-cleavage site
9100003-9200003	GRMZM2G385543	Helix-Loop-Helix DNA binding domain
	GRMZM2G129554	DNA/RNA Helicase
	GRMZM2G129681	Helix Hairpin Helix DNA binding Domain & POA allergen C
	GRMZM2G129700	DNA-J protein targetting to mitochondria
9200004-9300004	GRMZM2G005365	Fanconi Anemia WD repeats & Zn finger RING
9300005-9400005	GRMZM2G122863	Uncharacterized protein
	GRMZM2G164470	Putative membrane function (DUF domain)
9400006-9500006	GRMZM2G152466	Tubulin/alpha/beta/delta Tub & Tub FtsZ GTPase
9500007-9600007	No genes	
9600008-9700008	GRMZM2G082293	DUF
	GRMZM2G082343	Helix-Loop-Helix DNA binding domain
	GRMZM2G160664	DNA dependant ATPase Mini chromosome maintenance
	GRMZM2G461145	Metallo-dependent phosphatase
	GRMZM2G160619	Leucine rich repeat and kinase domain
	GRMZM2G160611	Uncharacterized protein
	GRMZM2G160606	ITP-ase like domain
	GRMZM2G461119	Molybdenum binding / E2 Binding
	GRMZM2G145935	Short domain similar to a pre-mRNA interacting protein
9700009-9800009	GRMZM2G415791	Uncharacterized protein
	GRMZM2G380291	Uncharacterized protein

Physical Distance covered	Genes in Physical distance	Identified domains
	GRMZM2G396565	Nucleotide diphospho sugar transferase
9800010-9900010	GRMZM2G104316	Uncharacterized protein
	GRMZM2G104332	Uncharacterized protein
9800010-9900010	GRMZM2G104342	GRAS transcription factor
	GRMZM2G543920	Uncharacterized protein
9900011-9950000	GRMZM2G096596	Adenylate cyclase associated CAP-C
	GRMZM2G096591	Glucoside hydrolase
	GRMZM5G878347	Uncharacterized protein

Table 2.3. Locations of maize homologs of previously identified meiotic genes in plants. All genes listed on chromosome 5 are outside the mapping interval.

Protein Name	Chromosome	Homolog Name	
DSB creation			
PRD1	9	GRMZM2G308884_P01	
PRD2 (MPS1)	4	GRMZM2G133969_P02	
PRD3	9	GRMZM2G055899_P06	
SPO11-1	5	GRMZM2G129913_P04	
SPO11-2	1	GRMZM2G052581_P01	
SPO11-3	1	GRMZM2G052581_P01	
DSB Detection & Resection			
MRE11A	2	GRMZM2G106056_P02	
MRE11B	4	GRMZM2G309109	
RAD50	4	GRMZM2G030128_P01	
NBS1	5	GRMZM2G006246_P01	
ATM	3	GRMZM2G464523_P01	
ATR	5	GRMZM2G128938_P01	
RPA1	4	GRMZM2G115013_P01	
RPA2	6	GRMZM2G076329_P01	
DSB protein loading and repair			
BRCA1-1	6	GRMZM2G080314_P01	
BRCA1-2	8	GRMZM2G052688_P01	
BRCA2	10	GRMZM2G134694_P01	
RAD51A1	7	GRMZM2G121543_P02	
RAD51A2	3	GRMZM2G084762_P01	
RAD51C	3	GRMZM2G123089_P02	
DMC1	3	GRMZM2G109618_P01	
Inter Homolog Repair Bias			
RAD54	5	GRMZM2G083138_P01	
HOP2	5	GRMZM2G177942_P01	
MND1	2	GRMZM2G102242_P02	
Axial element/ Lateral element/SC			
AFD1	6	GRMZM2G059037_P04	
ASY1	2	GRMZM2G035996_P01	
MER3	4	GRMZM2G346278_P01	
ZYP1	10	GRMZM2G324390_P01	

Cytological characterization of the *segII* mutant

Homologous Pairing

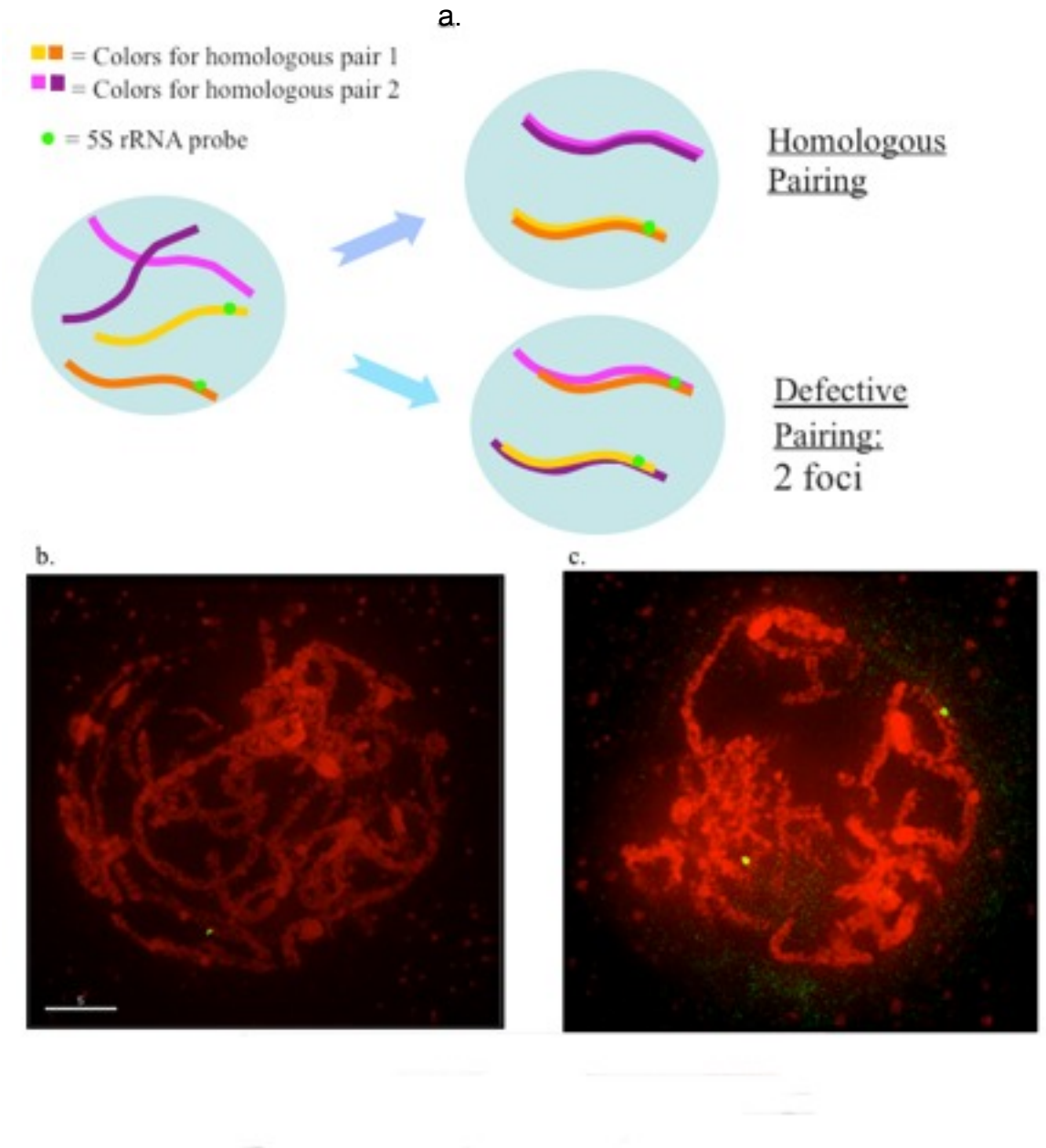
Initial characterization of the *segII* mutant was aimed at determining the extent of any homologous pairing deficiency. In order to establish whether homologous portions of the genome are aligning, I used a fluorophore conjugated DNA probe targeting the 5S rRNA locus. This locus contains a highly repetitive sequence allowing for a robust signal and clear visualization using fluorescent *in situ* hybridization (FISH). The 5S rRNA locus is located on the long arm of chromosome 2 and is easily visualized in the early sub-stages of prophase I.

In prophase I, the pachytene stage represents the ideal time to assay pairing since all chromosomes are normally synapsed with a partner to form bivalent chromosomes. The cytology at this stage in maize is excellent and bivalent chromosomes are distinguishable from univalents by thickness. In some situations where the chromosome twist causes the 5S locus to lie perpendicular to the plane of view, the 5S probe on each member of the bivalent is visible.

In the leptotene stage, chromosomes appear as unpaired univalents and the 5S signals generally appear well separated from each other in the nucleus. Later, in zygotene and pachytene, if pairing occurs properly, the paternal and maternal 5S loci are juxtaposed and appear as one FISH signal on a bivalent chromosome. Mispairing in pachytene would result in two foci appearing on separate bivalents (Figure 2.2a).

segII chromosomes display a 72% rate of mispairing at pachytene (n=32). This result confirms that the *segII* mutation is affecting homologous pairing and that further characterization is required to determine the meiotic mechanisms through which *segII* influences pairing.

Figure 2.2. Chromosome pairing. a. A cartoon depicting homologous pairing in a nucleus with two chromosome pairs. b. Flat projection of a 3-dimensional image showing pairing of the 5S rRNA loci in pachytene nuclei of wild-type and c. *segII* meiocytes. Red: DAPI stained chromosomes, Green: 5S rRNA probe. Scale bar represents 5 µM.



Telomere bouquet

The telomere bouquet is a cytological structure observed in many organisms that is thought to play a role in pairing. The telomere bouquet occurs early in zygotene at the time when chromosomes begin to pair. Observations of the *pam1* mutant of maize indicate that the primary defect of this mutant is the formation of the bouquet (Golubovskaya et al. 2002). The result is desynchronization of meiocyte development and meiosis progression in *pam1* anthers, and reduction of homologous pairing to 41%. This study has determined that bouquet formation is critical for successful homologous pairing of maize. Observations of *segII's* telomere bouquet, through FISH probes specific to the telomeres, indicate no perturbations in the progression or timing of bouquet formation.

γ-H2AX phosphorylation

Knowing that homologous pairing is highly impaired, but not abolished, I asked whether DSB formation might be a cause of the phenotype. In mutants where DSBs are completely abolished, homologous pairing is also defective (Romanienko 2000, Grelon et al. 2001, Stacey et al. 2006). I wanted to determine whether significantly decreased production of DSBs might be responsible for the observed significant reduction in homologous pairing.

DSBs in DNA are identified by phosphorylation of the histone subunit H2AX, at residue S139 (for review see Fillingham, 2006). Examination of γ-H2AX phosphorylation has been used previously to determine whether meiotic DSBs are created during meiosis in

Arabidopsis, human, and mouse (Uanschou et al. 2007, Chicheportiche et al. 2007, Sanchez-Moran et al. 2008, Paull et al. 2000, Mahadevaiah et al. 2001, Kumar et al. 2010). To investigate DSB formation in the *segII* mutant I conducted immunolocalization of the phosphorylated γ-H2AX variant histone during meiosis. A commercial antibody to γ-H2AX was used to stain prophase 1 chromosomes of wild type and *segII* meiocytes (figure 2.3). The staining displayed two classes of γ-H2AX foci: less intense ‘s’ foci and more intense, larger ‘l’ foci.

Figure 2.3. γ-H2AX staining in *segII* and wild-type meiocytes. a,b and c are images of wild-type meiocytes in leptotene, early, and late zygotene, respectively. d, e, and f are images of *segII* meiocytes in leptotene, early, and late zygotene, respectively. Scale bars represent 5 μM.

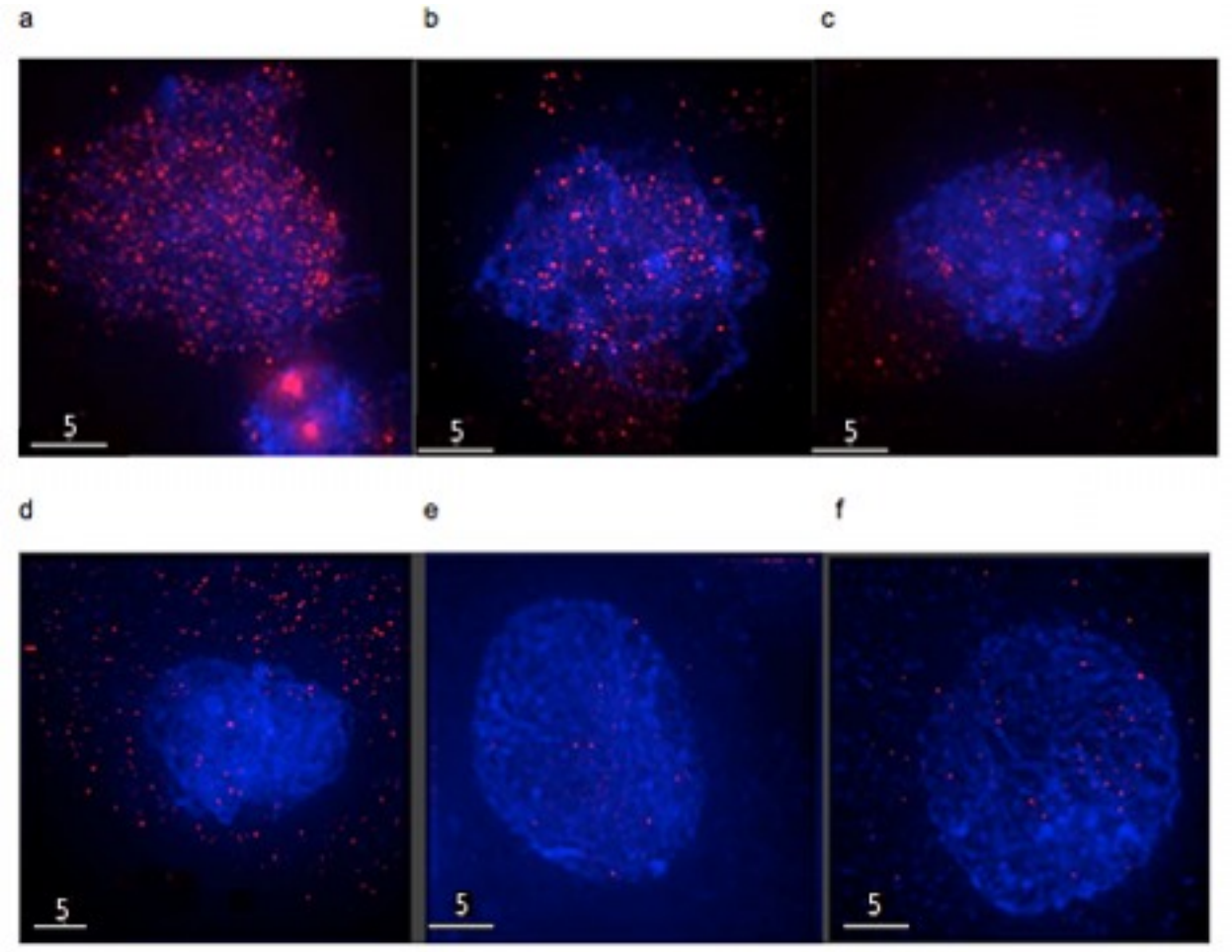


Table 2.4. Numbers of γ-H2AX foci in wild-type and *segII* meiocytes.

s-foci	Leptotene	Early Zygotene	Late Zygotene
Wt	**299** (n=13, SD=236)	**483** (n=19, SD=238)	**549** (n=22, SD=208)
segII	**88.2** (n=5, SD=37.5)	**146** (n=16, SD=119)	**205** (n=10, SD=52)
l-foci	**Leptotene**	**Early Zygotene**	**Late Zygotene**
Wt	**82** (n=15, SD=62)	**220** (n=18, SD=142)	**120** (n=21, SD=67)
segII	**17.6** (n=5, SD=16.7)	**11** (n=16, SD=12)	**68** (n=12, SD=32)

As shown in Table 3, *segII* exhibits a smaller number both small and large γ-H2AX foci during all stages observed compared to wild type meiocytes. Despite an overall lower foci count, the *segII* mutant displays a relative increase in both foci in late zygotene as compared to early zygotene. Previous studies have reported presence of γ-H2AX foci in *spo11*$^{-/-}$ meiocytes and suggested that it may be an uncharacterized chromatin remodeling, late replicating DNA, or chromosome damage unrelated to recombination (Chicheportiche et al. 2007, Libby et al. 2003). Chicheportiche et al. (2007) were able to draw conclusions for repaired/repairing status of small and large γ-H2AX foci in mouse, however their findings do not match well with what is observed in maize. This indicates that γ-H2AX follows different dynamics in maize after installation.

ASY1 installation

To further characterize the *segII* mutant, I looked at the installation of the ASY1 protein involved in chromosome axis formation in early prophase. ASY1 is a homolog of yeast HOP1, which is a component of the axial element (Caryl et al. 2000). Aberrant chromosome structure would indicate that the gene underlying the *segII* mutation acts prior to DSB formation. In wild-type, ASY1 installs along all chromosome axes prior to DSB formation and is visualized as bright continuous stretches along unsynapsed chromosomes (Golubovskaya et al. 2006). In *segII*, the appearance of ASY1 does not differ significantly from wild type siblings during leptotene.

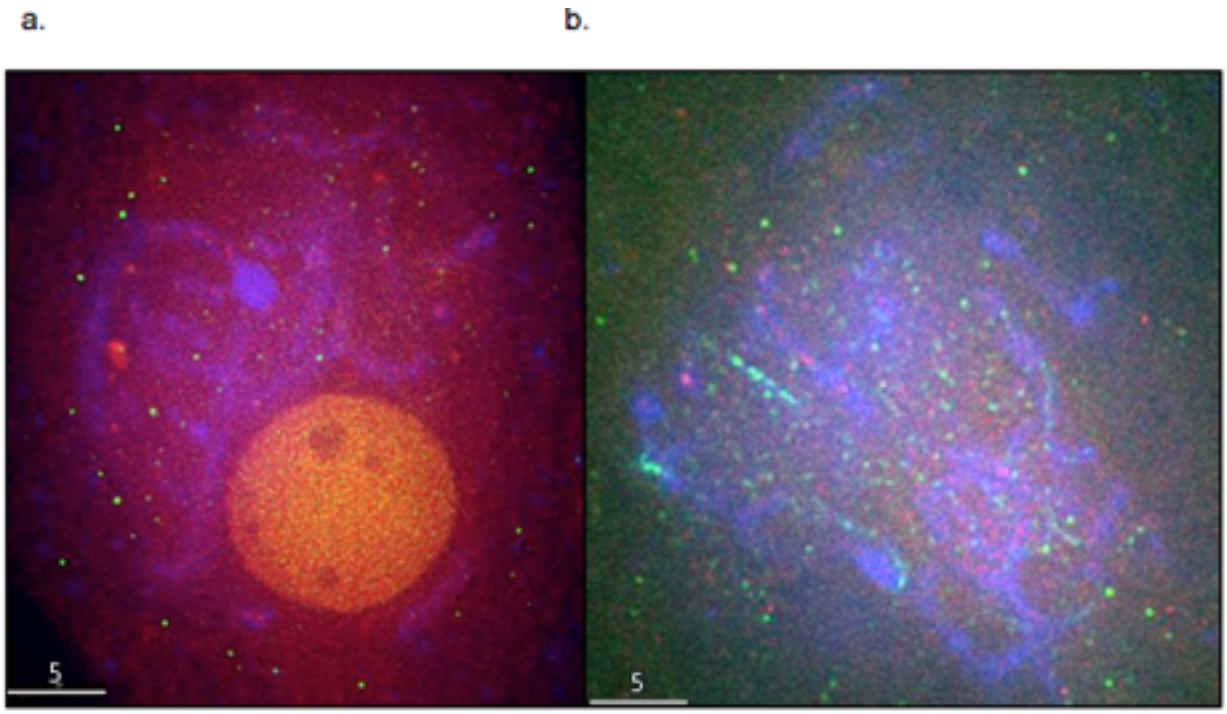
ZYP1 installation

Following DSB repair, chromosomes become synapsed together. The synapsis of chromosomes is accomplished by the recruitment and installation of ZYP1 to bridge the gap between members of what will be a bivalent by interacting with the axial element on both members. In yeast this occurs at the sites of DSB repair and crossover formation, based on work associating ZYP1 at synapsis initiation complexes (SICs) (Fung et al. 2004). Studies of the relative positions of synapsis initiation and crossover distributions have previously suggested ZYP1 installs at DSB repair sites in maize. (Burnham et al. 1972, Anderson et al. 2003, Maguire 1972, Maguire 1994) Maize and other large chromosome plants have been shown to initiate synapsis at more sites than there are crossovers (Gillies 1985). This could reflect a requirement for efficient synapsis set by the chromosome size increase between higher plants and yeast. The installation of ZYP1 at sites of SEI repair events is an intuitive mechanism of the pairing process; repair events normally only occur from a homologous template.

My immunolocalization experiments in maize indicate that staining of ZYP1, the central element, and ASY1, a component of the axial element, are generally mutually exclusive. Since ZYP1 interacts with the axial element to form the synaptonemal complex, it is possible that ZYP1 occludes the antigenic site of ASY1. At pachytene, wild-type cells are at or near complete synapsis, showing ZYP1 staining along all chromosomes with ASY1 staining occasionally seen in small segments.

At pachytene in the *segII* mutant, a delay in ZYP1 staining is seen along with persistence of long stretches of ASY1 staining, and late pachytene meiocytes eventually install ZYP1 along the full length of bivalents (Figure 2.4). This delayed ZYP1 installation indicates that *segII* chromosomes install ZYP1 at a slower rate than in wild type. This could be due to *segII* having a defect in chromosome structure, resulting in inefficient polymerization of ZYP1. However, the existence of a structural phenotype that displays pleiotropic effects from leptotene to pachytene and yet does not otherwise alter the articulated chromosome structure is unlikely. More simply, if there are fewer DSBs as γ- H2AX staining suggests, and there are fewer crossovers, then a delay in synapsis would be a logical consequence of fewer SICs.

Figure 2.4. ASY1 and ZIP1 installation. a. wild type meiocyte at pachytene, no ASY1 signal (green) is visible along chromosome axes. b. *segII* meiocyte at pachytene displays stretches of ASY1 staining along chromosome axes. Blue: DAPI staining of chromosomes, Red: ZYP1, Green: ASY1

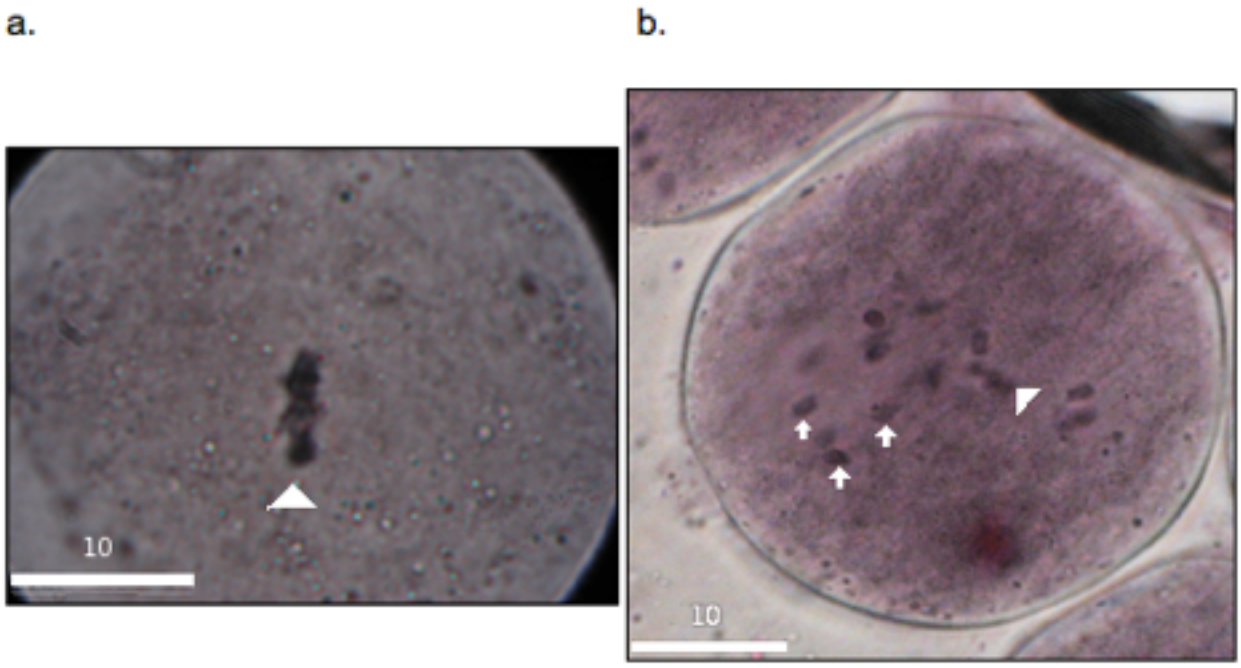


Univalent and bivalent counts

In order to determine whether crossovers are affected in the *segII* mutant, I looked at the downstream effects of the mutation. Crossovers are important structurally in meiosis for the fidelity of chromosome segregation since without crossovers to preserve bivalent partners following synaptonemal complex dissolution, chromosomes segregate randomly at metaphase I. Since presence of bivalents in late prophase I requires crossovers, distinguishing univalents and bivalents at metaphase I allows an estimate of the number of crossovers that are generated in *segII*.

Using acetocarmine staining of metaphase I meiocytes, (Figure 2.5) I observed that wild type cells show all chromosomes aligning on the metaphase plate, indicating that every chromosome pair possesses at least one crossover. *segII* displays between 14 and 15 chromosomes at metaphase I (n=24, SE=2.0). Counting this many chromosomes indicates that there are 5 bivalents and so between 5 and 10 crossovers form in *segII* meiocytes, assuming one crossover per chromosome arm.

Figure 2.5. Presence of univalents and bivalents in *segII* mutant meiocytes at metaphase I. a. Wt meiocyte stained with acetocarmine at metaphase I. b. *segII* meiocyte stained with acetocarmine at metaphase I. Arrow heads represent the metaphase plate, arrows represent univalents. Chromosomes (dark staining bodies) that are lined up at the middle of the cell represent bivalents, while those that are off-center are univalents.



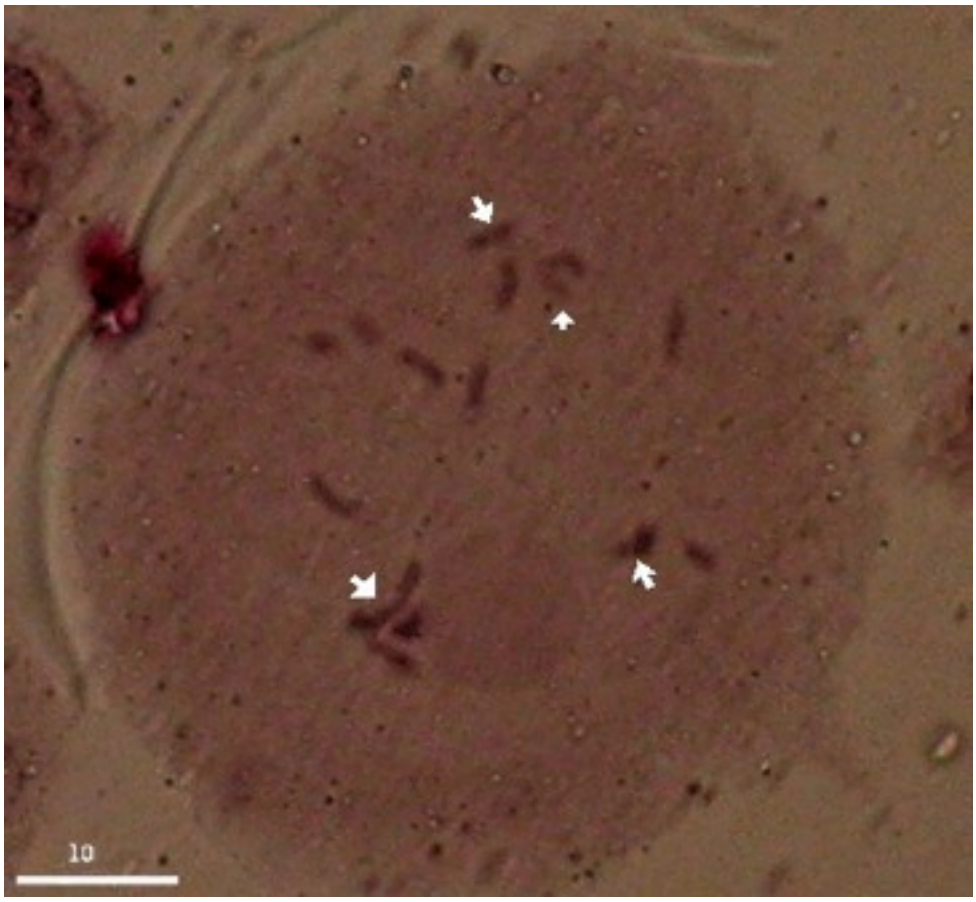
In wild-type cells, the number of crossovers can be determined by counting chiasmata using a chromosome spreading technique. Chromosomes with two chiasmata appear as ring structures, while chromosomes with one chiasma appear as V or Y shaped structures. Using this method, 16.8 (n=9, SE=0.5) chiasmata were observed in wild type maize. In 30% of cells chromosome 10, the shortest chromosome of maize, was seen to have no chiasmata as it was observed separated from its homolog. Interestingly, in hundreds of wild type meiocytes visualized using acetocarmine, none displayed obviously univalent chromosomes failing to line up at metaphase I. This indicates that the spread technique results in an underestimatation (~0.3 fewer) of the number of crossovers.

In *segII* meiocytes, I used chromosome squashes to count chiasmata, as the spreading technique did not provide sufficient chromosome separation (Figure 2.6). Crossover counts using this method at diakinesis yielded 4.3 (n=9, SE=0.47) crossovers per meiocyte. The acetocarmine method likely underestimates the number of crossovers since only cells which contain well separated chromosomes can be counted. This would explain the disparity between the observed 4.3 crossovers than the bivalent/ univalent estimate of 5 crossovers.

While slightly lower than expected, the crossover counts at diplotene/diakinesis confirm that the *segII* mutation results in many fewer crossovers than wild type (a 74% decrease). Bivalents are the result of the obligate crossover rather than the norm of

one crossover per arm. This result suggests that while not all chromosomes show a crossover, all chromosomes are synapse.

Figure 2.6. Presence of univalents and bivalents in diakinesis. Meiocytes of *segII* stained with acetocarmine and visualized using a bright light microscope. Arrows indicate bivalents that are held together by chiasmata.

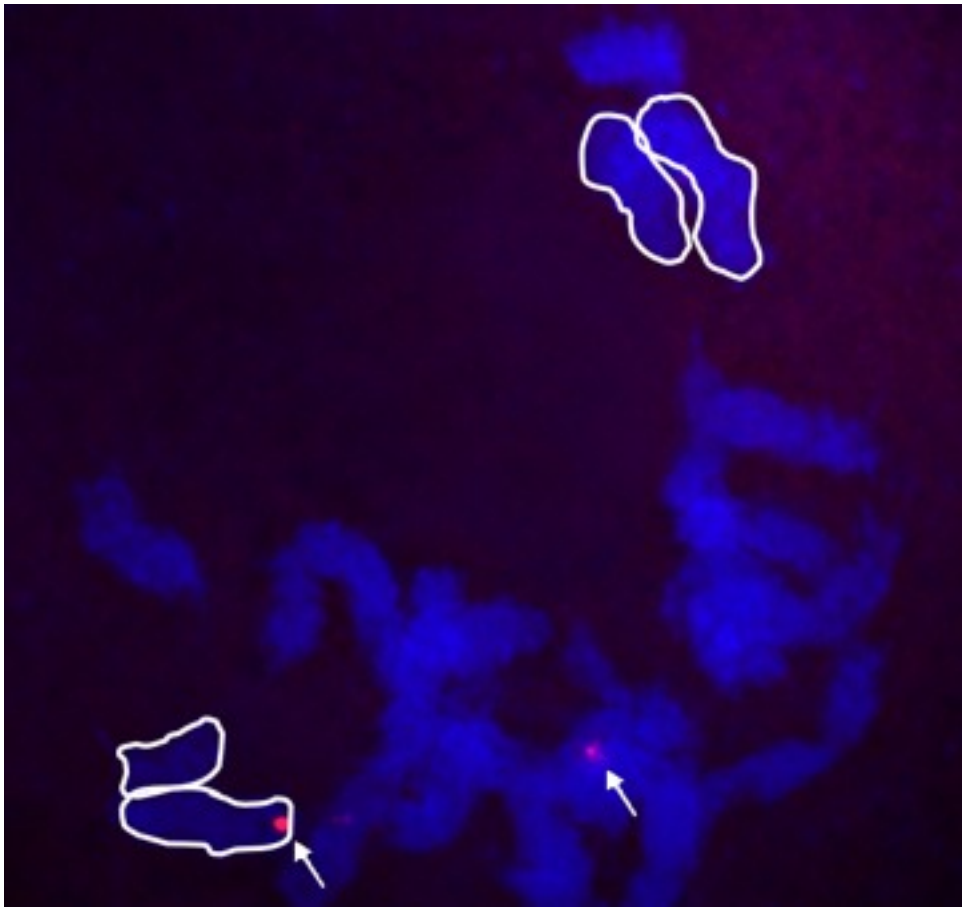


Crossovers between non-homologous chromosomes

Since I observed non-homologous pairing at pachytene, I wanted to investigate whether these mispaired chromosomes could generate non-homologous crossovers in the *segII* mutant. In addition to 4',6-diamidino-2-phenylindole (DAPI) to visualize chromosome structure at diplotene/diakinesis, probes to 5S rRNA and centromere 4 were used to assess whether crossovers were exclusively between homologous chromosomes (Figure 2.7). Any bivalents at these stages must be held together by crossovers, ergo any bivalents containing only one of the 4 signals (two pairs) must have a non-homologous crossover.

In many spreads of *segII* at this stage, all four probe signals were present on entangled chromosomes and as such, homologous/non-homologous status was indeterminable. Nevertheless, in 4 of 42 spreads, bivalent chromosomes carrying the FISH signals were separated from the chromosome amalgam. In each of these four cases, the bivalants consisted of non-homologous chromosomes. This was determined through possession of one signal from the two probe pairs, or was determined to be non-homologous by the size differences between the partner chromosomes (heteromorphic bivalents).

Figure 2.7. Non-homologous crossovers in the *segII* mutant. The bottom bivalent contains chromosome 2 and an unidentified smaller chromosome, while the top bivalent is heteromorphic. Blue - DAPI stained chromosomes. Red - 5S probe. Non-homologous pairs are outlined in white. Arrows indicate the position of the 5S probe.



RAD51 and DMC1 localization

To investigate the non-homologous pairing and crossovers in *segII*, I looked to the installation of two proteins known to be crucial to the fidelity of repair, pairing, and crossovers: RAD51 and DMC1. These proteins are both homologs of the bacterial recombinase RecA. *Rad51* is expressed in somatic and meiotic cells for homologous template based DNA repair, while *Dmc1* is specifically expressed during prophase I of meiosis. During meiosis, RAD51 and DMC1 are installed in numbers several fold greater than crossovers, which reflects the number of DSBs created (Terasawa et al. 1995).

The initial characterization of *segII* by Pawlowski et al. (2003) demonstrated that RAD51 is installed at ~2% of wildtype; however, the association of DMC1 with RAD51 was not examined at that time. In order to look at the installation of DMC1, I raised an antibody against a peptide synthesized to match the most distinct site of DMC1 as compared to RAD51.

Using this antibody in conjunction with the anti-RAD51 antibody, I found that RAD51 and DMC1 recruitment was significantly decreased in *segII* at early zygotene (Figure 2.8b). In early zygotene, the time when the numbers of RAD51 and DMC1 foci are at their peak, *segII* mutant meiocytes showed installation of ~2% for both RAD51 and DMC1 foci.

Figure 2.8. RAD51 and DMC1 localization. Flattened projections of 3D images. a. Wt nucleus at early zygotene displays many foci of both RAD51 and DMC1 b. *segII* nucleus at early zygotene displays few foci, indicated by arrows. Autofluorescence from the nucleolus appears bright due to *segII*'s weaker staining and the Deltavision RT's auto-adjustment. Blue: DAPI staining of chromosomes, Red: DMC1, Green: RAD51

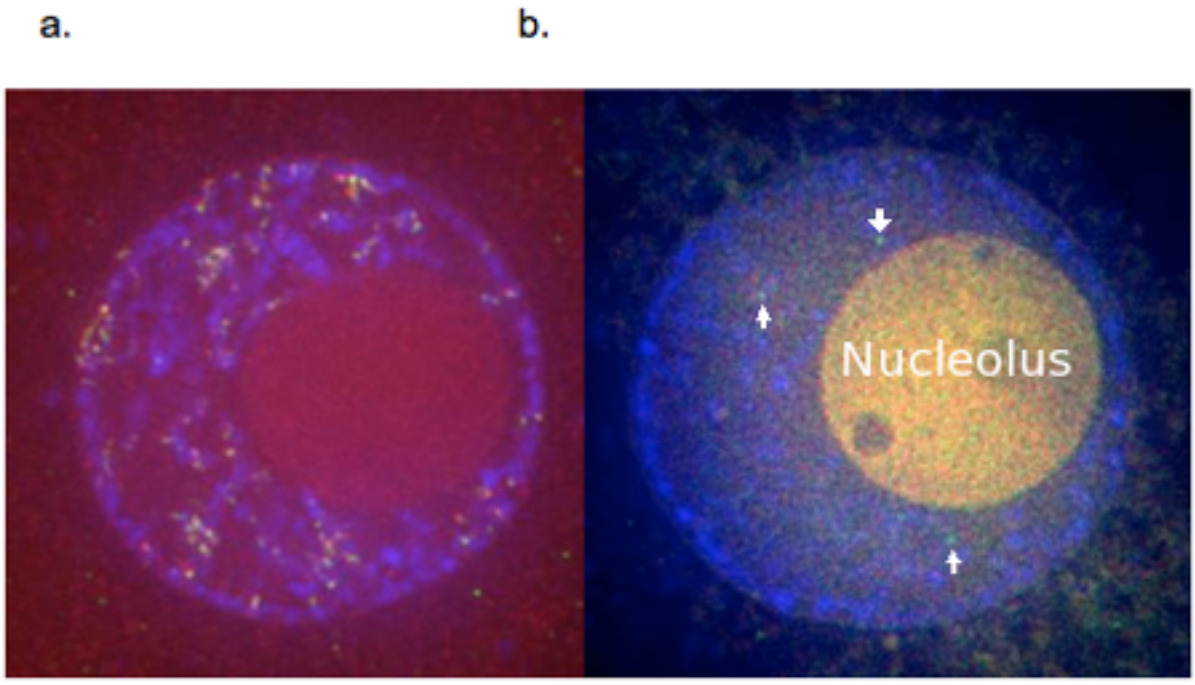


Table 2.5. RAD51 and DMC1 foci numbers in *segII* and wild-type meiocytes.

Early Zygotene	RAD51	DMC1	% DMC1 Co-Localized with RAD51
Wt	466 (n=12, SE=35)	203 (n=11, SE=47)	57% (n=11, SE=10)
segII	10.9 (n=31, SE=1.8)	5 (n=31, SE=0.8)	69% (n=28, SE=7)
% of Wt	2.3%	2.4%	
Late Zygotene			
Wt	86.5 (n=30, SE=15)	24.2 (n=30, SE=6)	32% (n=21, SE=7)
segII	4.5 (n=20, SE=1.0)	2.05 (n=20, (0.8)	34% (n=9, SE=15)
% of Wt	5.2%	8.4%	

Cisplatin treatment

The phenotype with regards to γ-H2AX foci and RAD51/DMC1 installation suggests that the primary defect in the *segII* mutant could be DSB formation. To examine this hypothesis, I attempted to complement the *segII* mutant phenotype by chemically inducing double strand breaks.

cis-diamminedichloroplatinum(II) (cisplatin) is a chemical that is capable of forming adducts with DNA bases. These chemical bonds are removed by excision of the nucleotides to which cisplatin is bonded, and repaired through RecA activity (Kartalou & Essigmann 2001). The excision of bases from dsDNA is very similar to the DSBs generated by SPO11, and these DSBs have been shown to undergo meiotic repair and recombination (Hanneman et al. 1997).

Several studies have reported that cisplatin successfully complements DSB deficient mutants in mouse and Arabidopsis. Treatment with cisplatin has been shown to cause increased synapsis, RAD51 and DMC1 foci restoration, and progression past meiotic checkpoints. (Romanienko et al. 2000, Libby et al. 2003, Sanchez-Moran et al. 2008)

Rad51 and *Dmc1* are the hallmarks of single end invasion (SEI) based homologous repair in meiosis. Following cisplatin treatment, an increase in their installation indicates that the cisplatin is successfully creating DSBs, and that DSBs in the *segII* mutant are able to be resected. Cisplatin was dissolved in Artificial Pond Water (APW) and used to treat cut tassels. The treatment is stressful, and due to the limited amount of control

over viability of specific portions of maize tassels treated, and the developmental gradient of the tassel, not all anthers harvested are able to yield cells that are viable for immunolocalization (see Methods and Materials). Wild-type cells treated with APW in early zygotene and *segII* cells treated with APW in late zygotene were not available for data collection.

Table 2.6. RAD51 and DMC1 foci numbers in wild-type and *segII* meiocytes following cisplatin treatment.

	Early Zygotene	
	RAD51	**DMC1**
Wt-untreated	466 (n=12, SE=35)	203 (n=11, SE=47)
Wt-APW	n/a	n/a
***segII*-untreated**	10.9 (n=31, SE=1.8)	5 (n=31, SE=0.8)
***segII*-APW**	1.87 (n=39, SD=0.4)	0 (n=39)
***segII*-Cisplatin**	99.5 (n=6, SE=19)	2 (n=6, SE=0.9)
	Late Zygotene	
	RAD51	**DMC1**
Wt-untreated	86.5 (n=30, SE=15)	24.2 (n=30, SE=6)
Wt-APW	39 (n=17, SE=8)	0.35 (n=17, SE=0.2)
***segII*-untreated**	4.5 (n=20, SE=1.0)	2.05 (n=20, (0.8)
***segII*-APW**	n/a	n/a
***segII*-Cisplatin**	36.5 (n=11, SE=9)	0 (n=11)

Data from these experiments are presented in Table 5. DMC1 localization is restored to a lower degree than RAD51 by the treatment. DSBs caused by cisplatin in *segII* meiocytes are unable to recruit DMC1. It is presumed that this reflects the stressful nature of cisplatin treatment, as the observed meiocytes were administered cisplatin at the time when DSBs normally occur. Despite the greater number of potential sites to install DMC1, DMC1 foci decrease in the *segII* mutant with cisplatin treatment (2) *versus* untreated *segII* cells (5) at the same stage of prophase I. Additionally, based on the Wt-APW (lacking cisplatin) control versus Wt-untreated at late zygotene, DMC1 is much more heavily affected by experimental conditions than RAD51, as its installation is reduced to 1.4% of normal vs. RAD51's 45% installation. However, the data indicate that that the cisplatin treatment is able to induce DSBs. At early zygotene, cisplatin treated *segII* meiocytes have at least 10 fold more DSBs compared to untreated meiocytes, based on induced RAD51 foci. The recruitment of RAD51 to cisplatin generated DSBs indicates that resection likely occurs in *segII*.

Discussion

The *segII* phenotype is unlike other meiotic mutants identified to date in that it displays a strong deficient pairing phenotype: 72% of pairing interactions are between non-homologous chromosomes. Only the *dsy2* mutant of maize has been shown to have a similar level of non-homologous pairing (75%), but *dsy2* differs significantly from *segII* in that it displays 10 fold more RAD51 foci, the majority of which form thread-like structures, and displays limited synapsis indicating a problem with ZYP1 recruitment or polymerization while *segII* can fully synapse, albeit slowly (Franklin et al. 2003,

Golubovskaya et al. 2011). Curiously, *segII* has synapsis across all ten bivalents even though only five SEI containing DMC1 occur throughout the genome.

I attempted to discern the nature of the *segII* mutation through cytological examination of events in prophase I. No published study has described a DSB hypomorph with regards to the cytology of repair proteins or crossovers (Neale et al. 2002, Henderson et al. 2004, Martini et al. 2006). In particular, those authors assumed chiasmata to be exclusively between homologous chromosomes in *spo11* hypomorphs. Because of this recombination was only assayed with molecular, rather than cytological, methods and so non-homologous crossovers were not detectable. This is the first report of non-homologous crossovers in a DSB hypomorph. Although I do report evidence that a reduced number of DSBs is directly responsible for the presence of non-homologous chromosome associations and crossovers, I propose an explanation regarding pseudo-homology.

The earliest observable meiotic phenotype in *segII* is the strong decrease in γ-H2AX phosphorylation. While small and large foci are observed in the *segII* mutant, they are both significantly reduced in overall number at each stage of prophase examined. S-foci are only at 30% by the normal time of DSB induction. L-foci vary much more strongly; from as low as 20% of wt levels in leptotene to over 50% at late zygotene. γ-H2AX foci persist throughout the repair stages, indicating that they are not removed synchronously with repair and/or that there may be a small amount of ongoing DNA damage during meiosis. The latter claim is supported by *spo11, mei1*, and *mei4*

mutants of mouse, which despite being deficient in DSB production, display some γ-H2AX foci at zygotene and pachytene (Chicheportiche et al. 2007, Libby et al. 2003, Kumar et al. 2010).

Aside from the overall reduction of γ-H2AX foci in the *segII* mutant, the mutant displays a significant increase in the γ-H2AX foci number at late zygotene. This observation could indicate that there is a delay in creation DSBs or their phosphorylation in the *segII* mutant, or that the rate of repair of the few existing DSBs is slow and so they accumulate.

To examine the late γ-H2AX foci and determine whether they are the result of delayed DSBs, I immunolocalized RAD51 at pachytene in the *segII* mutant to see if this DNA damage was being repaired by homologous repair processes. The observations for *segII* are interesting in that there appear to be two different types of meiocytes at pachytene. The first type, a minority of observed cells, displays a 10-20 fold increase over wild-type (and the second type) in RAD51 foci at pachytene; average 229 (n=4, SE=68). The second type displays a wild-type number of RAD51 foci averaging at 11 (n=16, SE= 3.4), Previous characterization of wild-type maize at pachytene indicates that 7-22 foci are normal at this stage (Franklin et al. 1999). The presence of the first type of meiocytes suggests that RAD51 could experience delayed installation in a subset of *segII* meiocytes, or that a subset of cells experienced a late wave of DSBs. However, if *segII* generates DSBs at late zygotene, chromosomes are already paired as bivalents and synapsing at this time. The late timing of these breaks precludes any potential

DSBs formed or phosphorylated at pachytene from contributing to homologous pairing. Consequently, the late forming DSBs are unable to rescue *segII* meiocytes, as evidenced by the lack of viable pollen. This raises the question of whether the *segII* γ-H2AX foci at the leptotene and early zygotene stages are the result lack of DSBs or a delayed installation of RAD51. The RAD51 localization at cisplatin induced DSBs addresses this issue. Cisplatin is able to restore RAD51 protein installation at early zygotene in *segII* meiocytes, which means that there is not a delay in phosphorylation or processing of DSBs. The subset of cells generating late-forming breaks are an unexpected feature of *segII*, and could represent late SPO11 complex activity, or possibly apoptosis.

DSB deficient mutants, which create no SPO11-complex-induced DSBs, are expected to install undetectable amounts of RAD51 and DMC1 (Chicheportiche et al. 2007, Libby et al. 2003, Kumar et al. 2010). However, in *segII* meiocytes, RAD51 and DMC1 are both installed at ~2% of wild type, indicating that there is a very low level of DSB creation. It is interesting that γ-H2AX signals 20% of wt activity in *segII*, but that repair proteins are installed at a significantly lower level. The disparity between γ-H2AX and repair protein levels could indicate a problem with resection, although unlike *segII*, resection mutants display chromosome fragmentation. Additionally, cisplatin treatment of *segII* meiocytes induces significant numbers of foci for RAD51. The ability to restore RAD51 foci through DSB induction also indicates that resection is not defective

Crossover homeostasis is exhibited in *segII*

Crossover homeostasis is an important phenomenon demonstrated in yeast and mouse, which is defined as the ability to sacrifice non-crossovers in favor of crossovers under limiting SEI conditions, and is associated with crossover assurance (Yanowitz 2010). Prior to these results, crossover homeostasis has not been demonstrated in maize. Without homeostasis, based on the number of RAD51 and DMC1 foci observed in the *segII* mutant one should expect only about 2% of the normal number of crossovers: less than 1 per meiocyte. However, I observed substantially more crossovers: 26% of the wild-type number. These data substantiate the idea that crossover homeostasis occurs in maize.

The *segII* mutant is an excellent tool to study crossover homeostasis since the number of DSBs and subsequent crossover-competent SEI events (containing both RAD51 and DMC1) are significantly fewer than the number of bivalents. DSB mutants in higher eukaryotes (*spo11, mei1, and atprd1, atprd2, atprd3*) examined prior to this have been null mutants or have had significantly more SEI than *segII* (de Muyt et al. 2007, de Muyt et al. 2009).

Since there appear to be a limited number of DSBs in *segII*, I hypothesized that one mechanism for crossover homeostasis (CH) could involve a higher co-localization of RAD51 and DMC1 at DSBs. However, while I observed that the DMC1 co-localization level in the mutants appears increased compared with wt (69% vs. 57% respectively), it is not statistically significant as determined by Welch's unpaired t test ($p > .05$). RAD51

co-localization was also tested and found not to be significant). Crossover homeostasis in *C. elegans* functions through the RTEL-1 protein, such that all SEI are able to form crossovers until RTEL-1 forces dissolution of "extra" SEI events (Youds et al. 2010). The data gathered here from maize is compatible with the RTEL-1 mechanism of *C. elegans* and an RTEL-1 type mechanism may be the method that maize mediates crossover homeostasis.

A biological imperative for a multitude of SEI

The *rad51* and *dmc1* mutants from multiple model species demonstrate that either protein alone is insufficient to create SEI that are competent to complete homologous pairing and crossing-over genome wide (Li et al. 2007, Siaud et al. 2004, Shinohara et al. 1997). A question previously raised regarding DSBs, is whether one repair event per chromosome successfully fills the minimal requirement for pairing and the obligate crossover, or whether many repair events, both crossovers and non-crossovers, from a consistent partner are required to successfully complete meiosis. Though the minimum number of SEI events required to satisfy the requirements of the homology search cannot be determined, a model can be proposed as to why the requirement is greater than 1.

The maize genome contains a high number of repetitive elements in addition to many duplicated genes, due to an ancient genome duplication. (Schnable et al. 2009, Blanc and Wolfe 2004) This means that the genome contains a number of sites that have multiple matches for homologous repair (pseudo-homology). SEI repair for any site,

homologous or ectopic, requires physical connection with another chromosome. Maize chromosomes have been observed to undergo significant movement in early meiosis. These movements would allow pseudo-homologous regions to interact and it is proposed that this is the case and continued movement is a mechanism that prevents ectopic recombination (Sheehan and Pawlowski 2009). This model implies that there is either a method to slow down chromosome movements to allow homologous recombination, or that homologous recombination (as compared to ectopic) is stronger than chromosome arm movements.

Observations of *segII* led to consideration of how a hypomorphic DSB mutant would affect chromosome movement. I propose that a multitude of SEI events are responsible for slowing down chromosome movement. That is, many SEI occur between a homologous pair of chromosomes, which slows down movement of both partners of the future-bivalent. This would stabilize juxtaposition of homologous chromosomes versus non-homologous chromosomes for pairing and synapsis. Single ectopic recombination sites would not have a sufficiently strong effect to slow down both members of the ectopic pair during zygotene. As meiosis proceeds to pachytene, chromosomes are observed to slow down (Sheehan and Pawlowski 2009). In this model, the pre-pachytene period would accommodate a DSB hypomorphic mutant, granting the ability for the few SEI to overcome the weaker movement forces, thus allowing for pairing and synapsis of chromosomes. However, the few SEI created by a hypomorph would be able to secure both homologous and ectopic recombination interactions, which undermines the fidelity of the process.

Future directions

Cloning the *segII* gene.

In order to complete the work and identify the gene underlying the *segII* mutation several steps are necessary. The first would be to continue to create markers in the interval on chromosome 5 while identifying more mutants that are recombinant in the region, allowing the candidates and region to be narrowed. Second, the genes remaining could be prioritized for analysis by examining the characterized expression of homologs in Arabidopsis (Libeau et al. 2011). At this point, genes would be sequenced in order from highest meiotic expression to lowest to identify any sequence alterations that could give rise to the mutation. Identified sequence aberrations would then be corroborated by examining all available *segII* mutant plants (~400) for the mutation while an allele is pursued for complementation testing.

In order to confirm the identified gene through allelism testing, crosses made to an *Ac/Ds* line available through the Brutnell lab could be screened (Kolkman et al. 2005). In 2010, 3 *Ac/Ds* lines became available that had insertions at 7.26, 7.69, and 7.98 Mb into chromosome 5 (I.S06.0320, I.S08.1100, and B.W06.0457 respectively). Based on data at the time, these were all inside the mapping interval. These lines were used to cross with phenotypically wild type plants in families segregating for the *segII* phenotype, which results in crosses to both heterozygotes and homozygous wt plants. Crosses specifically to heterozygotes (as identified by interval markers) can be screened for complementation of the *segII* phenotype, though the likelihood is low since *Ac/D*s works

best within 2 cM. However, all crosses could be submitted to a PCR screen to identify transpositions closer or inside the mapping interval for future crosses.

Cytological investigations

Investigating the late forming DSBs in the subset of *segII* meiocytes using an alternative to γ-H2AX, annexin-V, for apoptosis recognition may clarify the excess DSBs in these meiocytes (O'Brien et al. 1997). Production of a triple mutant of *segII* and *rad51* (*Rad51A* and *Rad51B*) in maize would also help to clarify the *segII* phenotype. Alleviation of the fragmentation seen in the *rad51A* and *rad51B* double mutant would provide support for *segII* as a hypomorphic DSB mutant.

Experiments to generate information from other maize mutants can be conducted using the DMC1 antibody generated here. Immunolocalizing DMC1 in the *rad51* mutant of maize would provide information about the similarities of maize and Arabidopsis DMC1 localization, the latter of which is reduced to 20% installation in *atRad51* mutants (Vignard et al. 2007). Correlating DMC1 foci in this mutant could provide insights into the contribution of DMC1 to crossover homeostasis in the absence of RAD51. Localization of γ-H2AX and DMC1 in the *phs1* maize mutant would address questions raised about *Phs1* function in pairing and recombination. (Edlinger et al. 2011, Kumar et al. 2010)

Materials and methods

anti-DMC1 antibody generation

The zmDMC1 antibody was created by Custom Polyclonal Antibody production at Rockland Immunochemicals (Gilbertsville, PA). Antibodies raised against full length DMC1 or RAD51 have the potential to crossreact with the other RecA homolog (Neyton et al. 2004). To avoid this, the sequence of a DMC1-unique 18 aa peptide located at the N terminus, MAPTRHADEGGQLQLIDA was selected. Guinea pigs were given a primary injection and three booster shots to foster antibodies in their system. After harvesting the guinea pigs, the antibody was affinity-purified using the ZmDMC1 peptide.

Immunolocalization

Immunolocalization experiments were performed and foci counted as previously described (Golubovskaya et al. 2006, Pawlowski et al. 2003). Co-localization was determined by signals overlapping or appearing within 3 pixels of each other at the Deltavision RT workstation's most powerful resolution (200 nanometers). Immunolocalization was conducted using the following antibodies: rabbit anti-AtASY1 (Armstrong et al. 2002) diluted 1:500, guinea pig anti-ZmZYP1, diluted 1:500, rabbit anti-hsRAD51 (Terasawa et al. 1995), diluted 1:500, guinea pig anti-DMC1 used at 1:50, mouse anti-H2AX (commercial) used at 1:400.

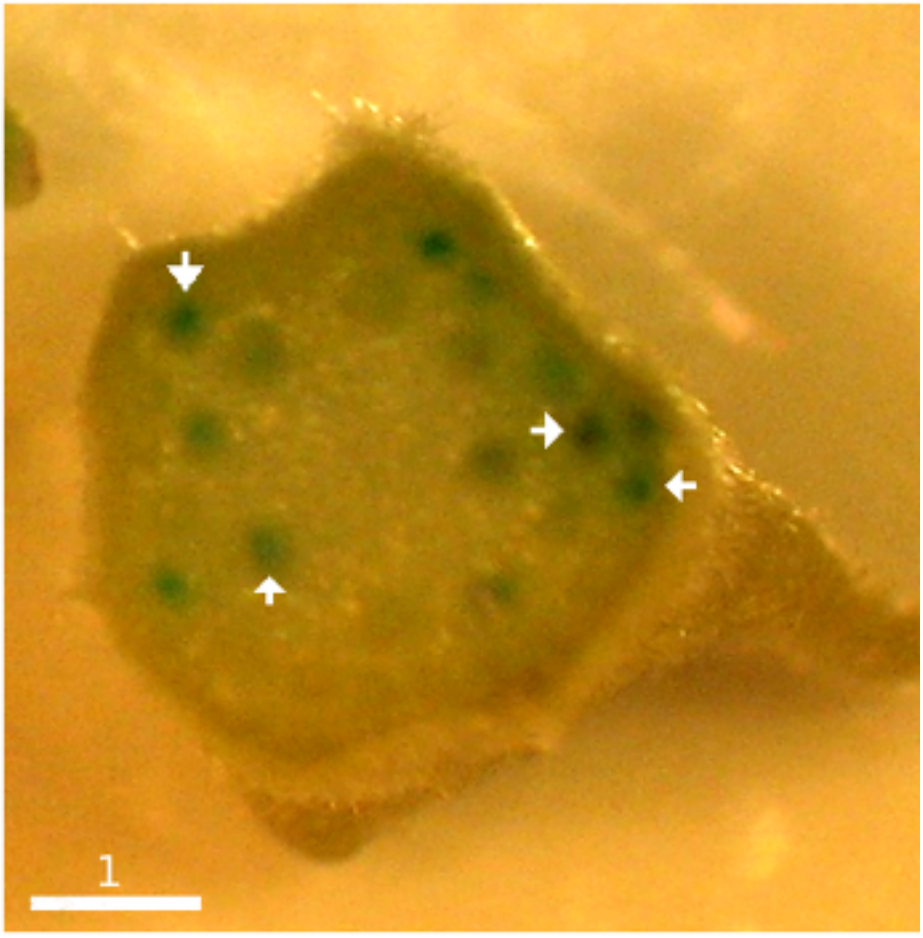
Cisplatin treatment

This is the first report of cisplatin in maize and several methods of cisplatin delivery were attempted to minimize stress to the meiocytes. The successful method involved harvesting the tassel by severing the fourth visible node from the rest of the plant under water with a sharp blade. Successive portions of stalk, sheath, and attached leaves were removed under water until only 2-3 leaves surrounded the immature tassel. The remaining tassel was then placed in a 50mL falcon with 5-10 mL of cisplatin solution (see below) so that the base of the stalk remained in solution. The portion of the tassel that emerged from the falcon tube was wrapped in parafilm to prevent evaporation of the solution and desiccation of the tassel. A preliminary time course indicated that a minimum of 10 hours was required for uptake of the cisplatin solution to reach the top of the tassel. Cisplatin treatment ran for approximately 15 hours at which point tassels were moved into an APW 50mL falcon for another 10-20 hours. Tassels and meiocytes became visibly stressed if soaked in cisplatin for the entire time course. The tassel becomes limp and discolored and any meiocytes extruded from anthers appear shriveled. The chromosomes in shriveled meiocytes do not display meiotic structure when stained and imaged with either acetocarmine or DAPI. Time courses were intended to allow anthers to receive cisplatin treatment during leptotene and allow harvest in zygotene. This was based on duration of meiotic stages described in Hsu et al. 1988. (leptotene: 43 hours, zygotene: 31 hours)

Cisplatin solution

Based on the previous report of cisplatin complementation in Arabidopsis, we used 2.5 mg/mL cisplatin (Sanchez-Moran et al. 2007). The cisplatin solution was made up in APW. APW was used previously to maintain maize anthers for chromosome movement studies (Sheehan and Pawlowski 2009) and contains salts, which assist in maintaining cisplatin in solution. Two to three drops of blue food coloring were added so that delivery of the solution could be tracked through vascular elements. Vascular elements displayed differing affinities for transport of the cisplatin solution as determined by the intensity of blue stain (Figure 2.9). As a result some flowers received amounts of cisplatin that induced apoptosis, while others received amounts that complemented the phenotype. It is possible that some anthers received little to no cisplatin, and that the exact amount of foci restored is an underestimate since anthers from multiple flowers were used to create pads for analysis.

Figure 2.9. Monitoring of cisplatin uptake into tassels using a food coloring dye. Cross-section of the tassel stalk. Dark blue spots, as indicated by white arrows, are vascular elements stained with the food-coloring dye following cisplatin treatment. scale bar is 1mM.



FISH

Maize 5s rRNA and centromere 4 (Cent4) repeats were labeled with fluorescein isothiocyanate (FITC) –dUTP using nick translation (Roche) to create FISH probes. A cyanine-3 labeled oligonucleotide (CCCTAAA) was used to detect telomeres for bouquet analysis. FISH and 3-D microscopic imaging were carried out as previously described (Golubovskaya et al. 2002, Pawlowski et al. 2003)

Acetocarmine staining

Anthers were fixed as in Li et al. (2007) for staging. They were squashed and stained with acetocarmine following the procedure of Dempsey (1994).

Chromosome spreads

Whole anthers were harvested fixed and stored in 3:1 ethanol to acetic acid. To initiate the spread procedure they were washed in 1x citric buffer on ice. Anthers were then added to a structural weakening buffer at 37C for 40 minutes. The reaction was stopped by adding cold 1x TE followed by 3 rinses in 100% EtOH. EtOH was allowed to evaporate and 35 uL of 3:1 acetic acid: methanol (95:5 in the most aggressive case) was added to the anthers on a microscope slide. Anthers were then cut and the meiocytes extruded into the solution as quickly and carefully as possible. The solution containing the meiocytes was dropped onto glass slides in 8uL aliquots from a height of no less than 8 inches (additional height did not provide better spreading). Slides were allowed to dry in a humid chamber (standing water in the bottom of a container containing a platform for slides). Chromatin was crosslinked to slides by exposure to 120 mJ per cm^2.

Probe hybridization of chromosome spreads

Slides were either stored at -20C or used immediately following UV crosslinking. Upon use the slides were hybridized with probe as in Birchler et al. (2007) with two exceptions: spreads were marked with permanent marker on the opposite side from the spread so the breathing procedure was unnecessary and I used a DABCO solution that also contained DAPI to mount the slides.

Semiquantitative RT-PCR

Rt-PCR was conducted using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen Cat. No. 12574-018). 1 ug of total RNA was used as the template. Conditions were as described in Pawlowski et al. 2004.

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Chapter 3

Mapping and Cytological Characterization of the Maize *dsyCS* Mutant

Introduction

To study the role of RAD51 in homologous pairing and chromosome recombination, Pawlowski et al. (2003) examined a number of maize meiotic mutants for localization of the RAD51 recombination protein. One of the mutants that was introduced in this study was *dsyCS*, which was found to install RAD51 at 2% of wild-type levels. This indicated that the the gene underlying *dsyCS* acts at or prior to double strand break repair. There are several notable events affecting meiotic recombination prior to RAD51 installation, including chromosome axis element installation, DSB creation, DSB signaling, DSB resection, and the traffic of the proteins to and from the sites of breaks at each of these steps. Investigation into the RAD51 localization pattern of *dsyCS* led Pawlowski et al. (2003) to propose potential mechanisms responsible for the meiotic defect in the *dsyCS* mutant. The first was that this mutant may be impaired in DSB formation, the second was that it may be impaired in loading of RAD51.

In the first scenario, mutants that abolish DSB formation exhibit univalents at metaphase I, and an absence of synapsis and crossovers (deMuyt et al. 2009, Libby et al. 2003, Romanienko et al. 2000, Sanchez-Moran et al. 2008, Stacey et al. 2006). In the second case, mutants of the MRE11-RAD50-NBS1 (MRN) complex have been shown to display different phenotypes depending on the member affected in Arabidopsis. *rad50* and *mre11* mutants of Arabidopsis display genome fragmentation in the presence of SPO11-induced DSBs (Puizina 2004, Bleuyard et al. 2004). The maize

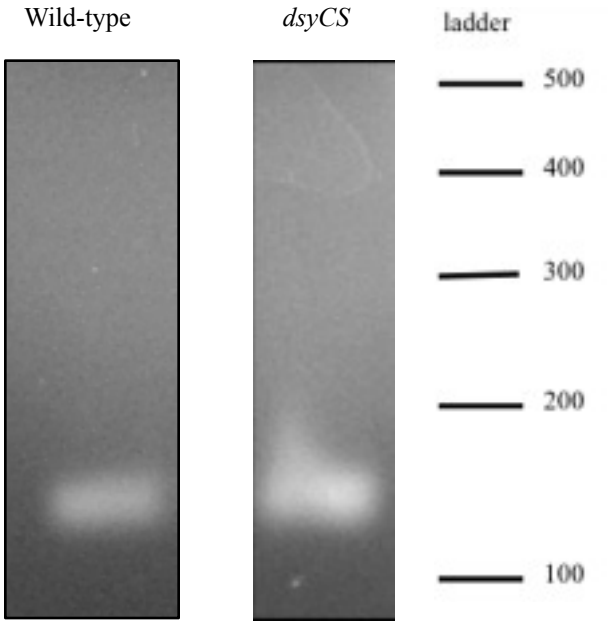
genome has two copies of *Mre11* and at the time of writing, mutants in only one of the copies have been examined, leaving open the question of the phenotype of a double mutant (Altun 2008). The *nbs1* mutant of Arabidopsis does not display a meiotic phenotype, suggesting that it is not conserved as a required complex member for resection in Arabidopsis (Waterworth et al. 2007).

Results

Identification and mapping of *dsyCS*

dsyCS was one of several mutants initially isolated from the maize meiotic mutant collection as male and female sterile. It was selected for analysis by a subsequent screen for univalents at metaphase I, and RAD51 foci abnormalities at zygotene (Pawlowski et al. 2003). This phenotype initially appeared similar to a previously identified mutant of maize, *phs1* (Pawlowski et al. 2004). Consequently, I conducted a semi-quantitative RT-PCR assay of *Phs1* expression to determine whether *dsyCS* was a hypomorphic allele of *phs1*. Analysis of the image in Figure 3.1, using the intensity function of ImageJ software, reports that *dsyCS* expresses Phs1 at 90% of wild type. This result indicates that the *dsyCS* mutation is not a severe hypomorphic allele of *Phs1*.

Figure 3.1. Semiquantitative RT-PCR analysis of *zmPhs1* expression in the *dsyCS* mutant. At far left is wild type, the second lane is *dsyCS*. The ladder bands in lane four, are, from top: 500, 400, 300, 200, and 100 (faintly visible). ImageJ analysis of band intensity confirms that *dsyCS* is not a hypomorphic allele of *Phs1*.



dsyCS originated in a *Mutator* transposon population and I used the same strategy to create Southern blots as in chapter 2 of this thesis. Though some *Mu* elements showed appropriate linkage on the small tester population, when taken to a larger population of 34, no *Mu* element consistently segregated with the mutant phenotype.

The *dsyCS* mutation had previously been introduced into the B73 and the Mo17 inbred backgrounds. These two inbred backgrounds display a high rate of DNA marker polymorphisms and were advantageous when looking for PCR marker polymorphisms between the *dsyCS* background and either of these diverse parents. Previous mapping efforts by Pawlowski et al. (2003, unpublished) showed linkage of *dsyCS* to chromosome 4.

The Maize Assembled Genomic Island (MAGI) In-del polymorphism (IDP) map (Iowa State University (ISU) IBM map 7: http://magi.plantgenomics.iastate.edu/cgi-bin/cmap/) was used to choose markers on chromosome 4. Eventually, I was able to delineate the *dsyCS* region with two flanking markers, IDP7265 at a physical location of 71,065,974 (according to maize sequence release 5b.60: http://maizesequence.org) and IDP803 at physical location 136,058,931. This ~55 Mb region contains 701 genes in the Filtered Gene set. When compared to the list of maize meiotic gene homologs in Table 3 (Chapter 2), two notable candidates present themselves. Maize homologs *RAD50* and *PRD2* are both found inside the mapping interval and both of these genes would be consistent with the onset of the first meiotic phenotype (RAD51 foci).

zmPrd2 was identified in an earlier version of the maize sequence map in this region and two screens for genetic abnormalities were conducted. The first was a PCR screen looking for *Mu* elements, which was conducted using primers anchored in exons and the *Mu* TIR primer. The second was a PCR screen using primers anchored in exons, at the intron/exon borders, looking for large deletions or insertions visible on a gel. Neither screen revealed any sequence abnormalities.

Cytological characterization of the *dsyCS* mutant

Chromosome pairing

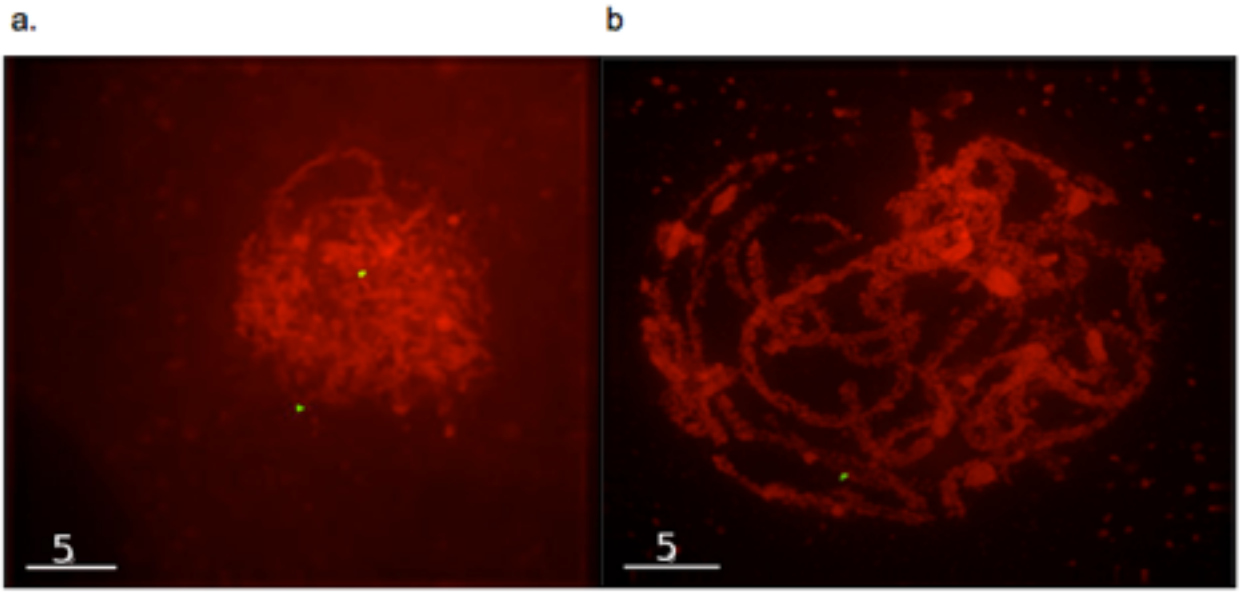
Initial characterization of the *dsyCS* mutant was aimed at determining the extent of any homologous pairing deficiency indicated by RAD51 foci abnormalities. In order to determine whether homologous portions of the genome align, I used a fluorophore conjugated DNA probe targeting the 5S rRNA locus. This locus contains a highly repetitive sequence allowing for a robust signal and clear visualization for *in situ* hybridization (FISH). The 5S rRNA locus is located on the long arm of chromosome 2 and is easily visualized in the early substages of prophase I.

In prophase I, the pachytene stage represents the ideal time to assay pairing since all chromosomes are normally synapsed with a partner to form bivalent chromosomes. The cytology at this stage in maize is excellent and bivalent chromosomes are distinguishable from univalents by thickness. In early leptotene, chromosomes appear as unpaired univalents and the 5S signals generally appear well separated from each other in the nucleus. In zygotene and pachytene, if pairing occurs properly, the paternal

and maternal 5S loci are juxtaposed and appear as one FISH signal on a bivalent chromosome. Mispairing in pachytene would result in two foci appearing on separate bivalents (Figure 3.2).

dsyCS presents a very strong level of non-homologous pairing, 92% (n=91). This is a level of severity that places *dsyCS* together with the *phs1* mutant of maize (95% non-homologous pairing) (Pawlowski et al. 2004).

Figure 3.2. Chromosome pairing analysis in the *dsyCS* mutant. a. Flat projection of a 3-dimensional image showing pairing of the 5S rRNA loci in pachytene nuclei in *dsyCS* (a) and wild type (b) meiocytes. Red: DAPI stained chromosomes, Green: 5s rRNA probe. scale bar is 5 μM.



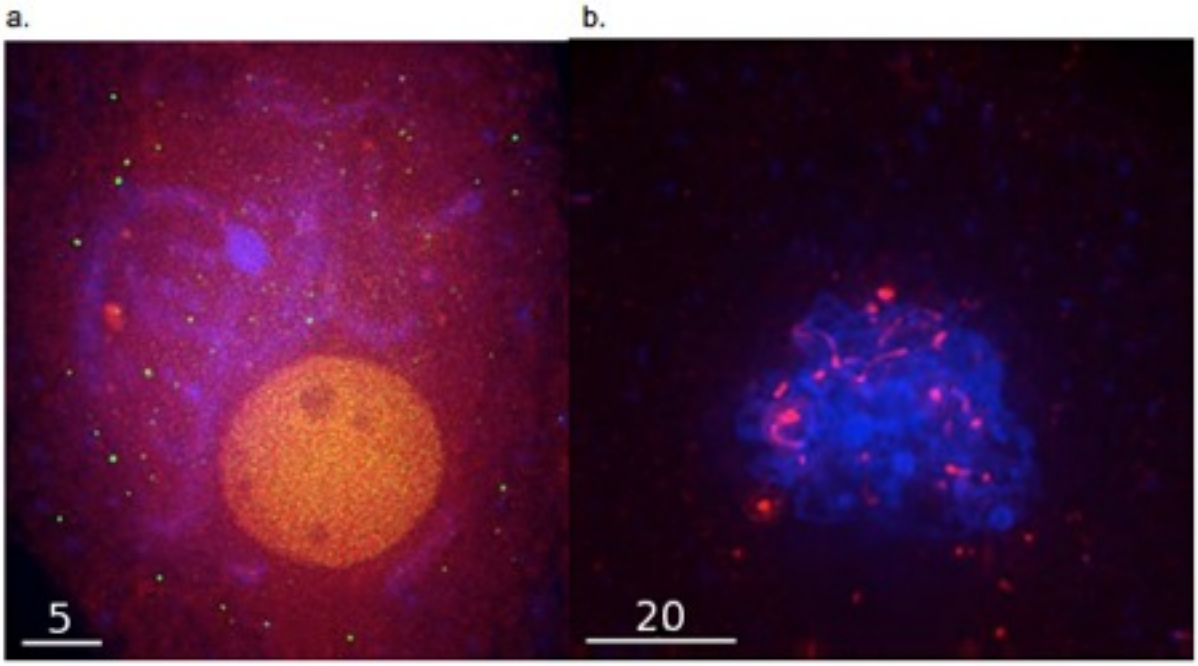
Telomere bouquet

The telomere bouquet is a cytological structure observed in many organisms and thought to play a role in pairing. The telomere bouquet occurs early in zygotene at the time when chromosomes begin to pair. Observations of *dsyCS*' telomere bouquet through FISH probes targeting the telomere repeat indicate no perturbations in bouquet formation.

ZYP1 installation

Following DSB repair and pairing, chromosomes become synapsed together, which is accomplished by the recruitment and installation of the ZYP1 protein between the chromosome in a bivalent. *dsyCS* displays installation of long stretches of ZYP1, similar to the wild type (Figure 3.3). ZYP1 initially appears as short stretches in zygotene and proceeds to long stretches in pachytene. However, it is clear that not all pachytene bivalents have ZYP1 installed between them.

Figure 3.3. ZYP1 installation in wild type (a) and *dsyCS* (b.) meiocytes at pachytene. Blue: DAPI staining of chromosomes, Red: ZYP1. Green at left is ASY1 staining. scale bars are 5 μM and 20 μM respectively.

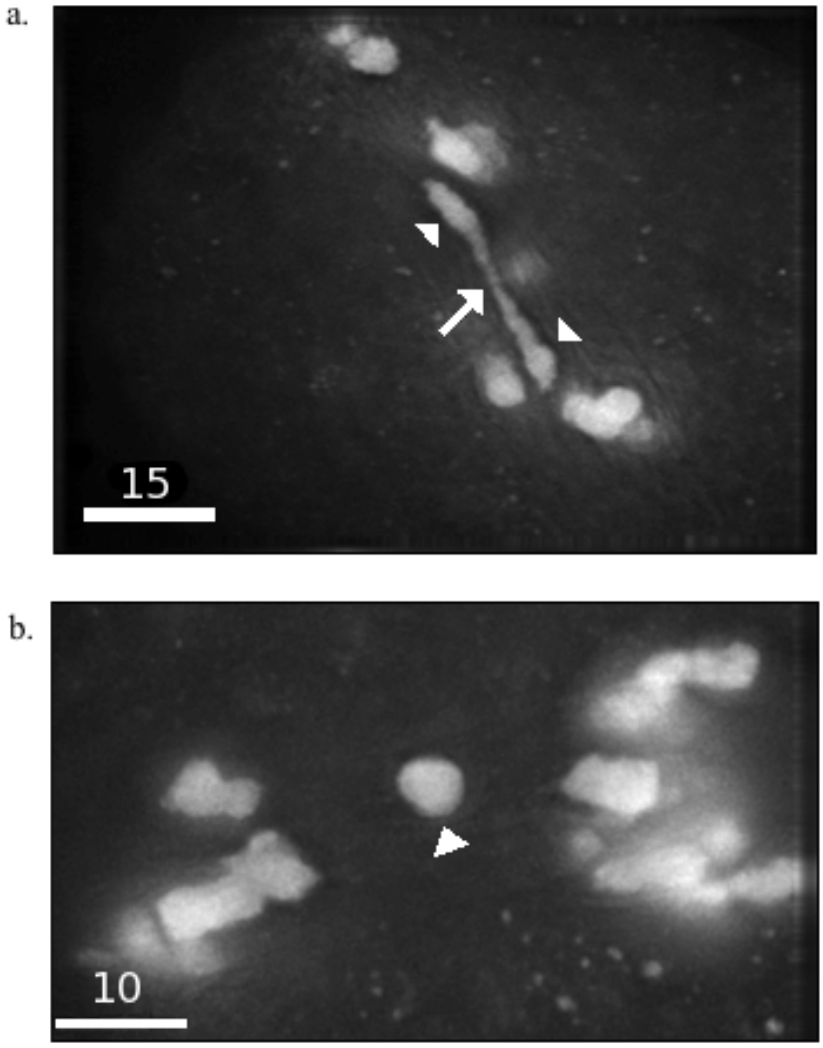


Bivalents and univalents counts at metaphase I

To determine whether crossover formation is affected in the *dsyCS* mutant, I examined the numbers of univalents and bivalents at metaphase I to estimate the number of crossovers. Using chromosome spread, I observed that wild-type cells show all chromosomes aligning on the metaphase plate, indicating that each chromosome pair possesses at least one crossover. *dsyCS* displays ~15 (14.9) chromosomes at metaphase I (n=12, SE=3.6). Counting this many chromosomes indicates that there are 5 bivalents and 10 univalents, and so between 5 and 10 crossovers formed in *dsyCS* meiocytes,

During the univalent/bivalent analysis, I observed anaphase I bridges between chromosomes (figure 3.4). Anaphase I bridges are the result of two centromeres being possessed by a single chromosome (Schwartz 1953, McClintock 1938). The presence of anaphase bridges in meiosis suggests the occurrence of recombination between non-homologous chromosomes. This was a regular event in *dsyCS* meiocytes, as it was observed in 7 of the 11 meiocytes examined.

Figure 3.4. Chromosomes in anaphase I meiocytes in the *dsyCS* mutant. white bodies are DAPI stained chromosomes. a. An anaphase bridge is indicated by the arrow, the two chromosomes making up the bridge are indicated by arrowheads . b. A lagging univalent chromosome is indicated by the arrowhead and an overall uneven chromosome distribution is seen at left and right sides. scale bars are 15 and 10 μM respectively.

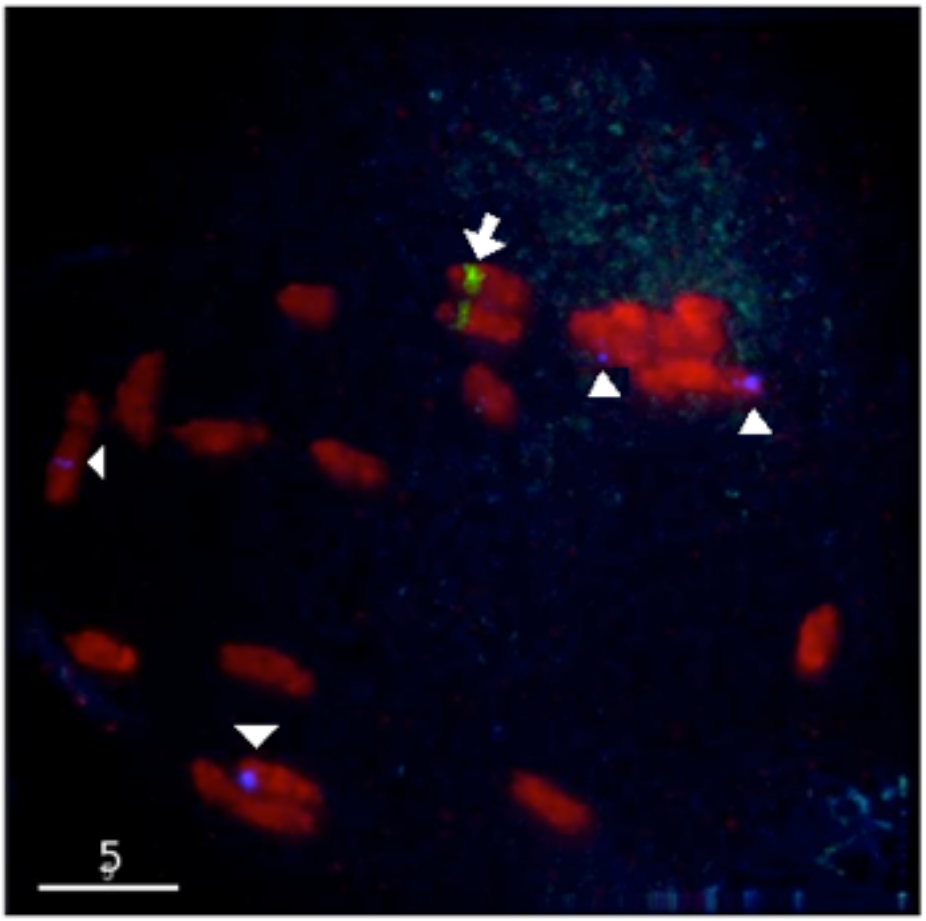


Crossover formation between non-homologous chromosomes

Since I observed the formation of non-homologous bivalents in *dsyCS*, I decided to investigate whether there are also non-homologous crossovers in this mutant, using the FISH technique to label maize chromosomes. Since chromosomes appeared entangled in spreads of *dsyCS*, only probes to identify specific chromosomes were used (Figure 3.5).

Due to the nature of the *dsyCS* mutation, chromosomes were often clumped together, even with the most aggressive spreading strategy. However, in five of twenty-eight spreads, a bivalent pair containing a probe was separated away from the main clump of chromosomes, and was clearly seen to have formed a crossover with a non-homologous chromosome. These data suggest that *dsyCS* often forms non-homologous crossovers between bivalents.

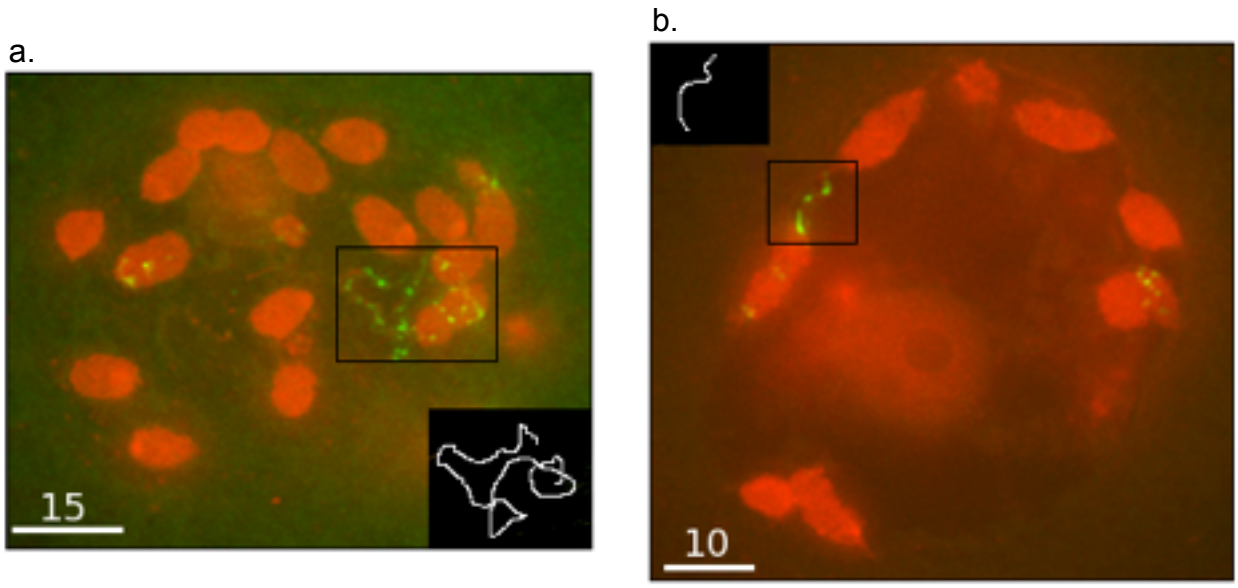
Figure 3.5. Non-homologous bivalents in the *dsyCS* mutant. DAPI stained chromosomes are in red, the 5S probe is in green, TR-1 FISH probe is in blue. Chromosomes 2, white arrow, form a homologous bivalent. However the TR-1, white arrow heads, which marks chromosomes 4 and 6 confirms that the heterologous bivalent at the bottom contains an incorrect partner. scale bar is 5 μM.



Telomere bridges

I also examined chromosome spreads in the *dsyCS* mutant using the telomere repeat probe. I found that in six of twenty-six late diakinesis meiocytes, chromosomes of *dsyCS*, which are nearly at metaphase I compaction levels, display long thin connections between telomeres of different chromosomes (Figure 3.6). The signal originating from the stretched chromatin provides a robust signal, compared to the chromosomes, which could be due to probe accessibility to compacted chromatin. I believe that the anaphase I bridges could be attributed, at least in part, to this phenomenon.

Figure 3.6. Telomere bridges in the *dsyCS* mutant. A flat projection of a 3D image of *dsyCS* diakinesis nuclei. Inset boxes are a trace of the telomere probe pattern inside the black lined boxes. a. The telomere loops around for several um between the chromosomes. b. The telomere is clearly seen stretched between two chromosomes at left. red: DAPI stained chromosomes, green: Telomere repeat probe. scale bars are 15 and 10 μM respectively.



RAD51 and DMC1 localization

To investigate the recombination pathway in the *dsyCS* mutant, I examined installation of two proteins known to be crucial for meiotic DNA repair: RAD51 and DMC1. These proteins are both homologs of the bacterial recombinase RecA. RAD51 is expressed in somatic and meiotic cells, while DMC1 is specifically expressed during prophase I of meiosis. Both proteins form distinct foci on chromosomes, which may be used as an indicator of the number of DSBs created (Li et al. 2007, Couteau et al. 1999, Siaud et al. 2004).

The initial characterization of *dsyCS* by Pawlowski et al. (2003) demonstrated that the mutant shows numbers of RAD51 foci at ~2% of the wild-type foci number. Using a combination of the anti-DMC1 and anti-RAD51 antibodies, I found that there was a significant decrease in the numbers of both RAD51 and DMC1 foci in *dsyCS* meiocytes at early zygotene (Table 3.1). In addition, their co-localization was reduced to less than 1% of wildtype.

Table 3.1. RAD51 and DMC1 foci in *dsyCS* and wild-type meiocytes.

Zygotene	RAD51	DMC1	% DMC1 Co-Localized
Wt	466 (n=12, SE=35)	203 (n=11, SE=47)	57% (n=11, SE=10)
dsyCS	22 (n=6, SE=4.5)	7.8 (n=6, SE=3.2)	10% (n=6, SE=6.5)
% of Wt	4.7%	3.8%	<1%

Discussion

The *dsyCS* mutant exhibits a unique meiotic phenotype with regards to homologous pairing and crossing over, with over 90% non-homologous pairing as well as crossovers between non-homologous chromosomes. Crossovers regularly generate dicentric chromosomes and telomeres of different chromosomes can be seen physically connected. Chromosomal foci of DNA repair proteins RAD51 and DMC1 are both reduced to less than 5% of wild-type installation and their co-localization is particularly impaired, at less than 1% of wild type. A study of the *rad51* maize mutant demonstrated that the recombinase activity of DMC1 is able to create crossovers, albeit non-homologously and at 40% efficiency (Li et al. 2007). It is, therefore possible that *dsyCS*' non-homologous crossover activity results from the DMC1-only repair events.

Two candidate genes were found in the *dsyCS* mapping interval that are homologs of previously identified meiotic genes in Arabidopsis: *PRD2* and *RAD50*. *PRD2* was identified in Arabidopsis as being a protein required for the creation of SPO11 induced DSBs (DeMuyt et al. 2009). *Atprd2* mutants exhibit short siliques and in four of five mutant alleles exhibited zero crossovers, the remaining allele showed a total of two crossovers from 51 cells examined. Without DSB formation, as in the other Arabidopsis DSB mutants identified by DeMuyt et al. (2009), crossovers and synapsis are unable to occur.

The *rad50* mutant of Arabidopsis displays chromosome fragmentation at metaphase I, with structural abnormalities as early as pachytene (Bleuyard et al. 2004). However, the

rad50 mutant appears to have a limited number of chromosomes aligning on the metaphase plate, indicating that crossovers took place. Synapsis was not investigated in the Arabidopsis *rad50* study.

There is not a clear case supporting either of these candidates. The low numbers of RAD51 and DMC1 foci could be the result of impaired DSB creation (*Prd2*) or a lack of DNA resection (*Rad50*) allowing these proteins to bind. While synapsis does not occur in DSB mutants, *dsyCS* could be a DSB hypomorph, which would allow synapsis to be seeded. The number of chiasmata in *dsyCS* is not compatible with the *atPRD2* mutant phenotype, but again could be the result of hypomorphic DSB activity rather than a complete knockout. In the maize *dsyCS* mutant, fragmentation of chromosomes, which is expected from *rad50* mutants of other plant species, is not seen. If either of these candidates is the source of the *dsyCS* phenotype, it will provide unique insights into the differences in plant meiosis between maize and Arabidopsis as well as help define the role for the excess of DSBs in prophase I.

The bridges seen between telomeres of chromosomes in *dsyCS* are a striking phenotype not seen as the result of meiotic activity in any previously identified meiotic mutant that I am aware of. The structural basis of these bridges is unknown at present, though studies in mouse DNA repair indicate that anaphase bridges and these telomere connections, are the result of NHEJ or homologous DNA repair (Acilan et al. 2007). The latter would represent a new class of meiotic mutants, as telomeres and their

immediately adjacent regions have been reported as suppressed for DSB formation in yeast (Borde et al. 2004, Gerton et al. 2000).

It is notable that the *segII* and *dsyCS* mutants both display non-homologous pairing and crossovers, despite the availability of RAD51 and DMC1 proteins. Studies of these mutants indicate that there are multiple ways to affect the fidelity of chiasmata in maize, beyond directly affecting the integrity of repair events themselves, shown by the *zmrad51* mutant. Two possibilities are that there could be a minimum requirement for the number of SEI events, as discussed in chapter 2 regarding pseudo-homology, or that the formation of DSBs is directed by a complex of proteins, each member of which contributes a biochemically unique activity. In the case of the *dsyCS* mutant, perhaps the mutation is in a gene responsible for blocking DSB formation in telomeres.

dsyCS exhibits crossover homeostasis

Crossover homeostasis is the ability to sacrifice non-crossovers in favor of crossovers under limiting SEI conditions, and is associated with crossover assurance (Yanowitz 2010). Without homeostasis in *dsyCS*, the amount of SEI events (<5%) would yield <5% of crossovers: ~1 per *dsyCS* meiocyte. The 5% of SEI events present in *dsyCS* yield significantly more crossovers: at least 25% of wild type. The results presented here provide the second case for crossover homeostasis in maize (the *segII* mutant being the first).

Future directions

The mapping of *dsyCS* remains an important goal. Further markers need to be selected on chromosome 4 to narrow the mapping interval. Simultaneously, sequencing analysis of *RAD50* could be conducted to investigate this high profile candidate. Though the interval for *dsyCS* is still too large for *Ac/Ds* tagging to reliably generate an allele with currently available transpositions, there is an *Ac/Ds* insertion (I.W06.0870R) less than 2 Mb from the *zmPrd2* candidate, which could be utilized to generate *zmPrd2* mutants for future studies.

During the course of my research, I generated a mutant population to search for new alleles of *dsyCS* by crossing a population segregating for the unique mutation with EMS-treated pollen from B73 inbred plants. This population can be screened for sterile individuals to look for complementation. Crossing meiotic mutants as females can provide a small amount of seed in some cases, and this could be done with any sterile individuals to recover the allele.

dsyCS has several phenotypic aspects that could be further clarified. The first would be to define the earliest manifestation of the mutation. To do this, ASY1, AFD1, and γ-H2AX could be immunolocalized and examined. Further, the chromosome connections at telomeres and reduced RAD51/DMC1 foci indicate that it would be very interesting to examine the nature of DSB formation in this mutant. Recent examination of DSBs has been done through hybridization to comprehensive genomic arrays, which exclude repetitive elements such as telomeres (Mancera et al. 2008, Chen et al. 2008).

Examining the relative abundance of DSBs formed at telomeres could provide insight into the nature of this defect. Additionally, the telomere connections could be examined through *in vivo* studies. Telomere movement could be tracked *in vivo* as described by Sheehan and Pawlowski (2009). *dsyCS* heterozygotes were previously crossed to *segII* heterozygotes. To further examine the nature of DSBs, *dsyCS/dsyCS*; *segII/segII* progeny could be evaluated. Establishing whether the phenotype is additive or one gene is epistatic to the other would yield insight to the *dsyCS* mechanism of action.

Methods & Materials

anti-DMC1 antibody generation

The zmDMC1 antibody was created by Custom Polyclonal Antibody production at Rockland Immunochemicals (Gilbertsville, PA). Antibodies raised against full length DMC1 or RAD51 have the potential to crossreact with the other RecA homolog (Neyton et al. 2004). To avoid this, the sequence of a DMC1-unique 18 aa peptide located at the N terminus, MAPTRHADEGGQLQLIDA was selected. Guinea pigs were given a primary injection and three booster shots to foster antibodies in their system. After harvesting the guinea pigs, the antibody was affinity-purified using the ZmDMC1 peptide.

Immunolocalization

Immunolocalization experiments were performed and foci counted as previously described (Golubovskaya et al. 2006, Pawlowski et al. 2003). Co-localization was determined by signals overlapping or appearing within 3 pixels of each other at the Deltavision RT workstation's most powerful resolution (200 nanometers). Immunolocalization was conducted using the following antibodies: rabbit anti-AtASY1 (Armstrong et al. 2002) diluted 1:500, guinea pig anti-ZmZYP1, diluted 1:500, rabbit anti-hsRAD51 (Terasawa et al. 1995), diluted 1:500, guinea pig anti-DMC1 used at 1:50, mouse anti-H2AX (commercial) used at 1:400.

FISH

Maize 5s rRNA and centromere 4 (Cent4) repeats were labeled with fluorescein isothiocyanate (FITC) –dUTP using nick translation (Roche) to create FISH probes. A cyanine-3 labeled oligonucleotide (CCCTAAA) was used to detect telomeres for bouquet analysis. FISH and 3-D microscopic imaging were carried out as previously described (Golubovskaya et al. 2002, Pawlowski et al. 2003)

Acetocarmine staining

Anthers were fixed as in Li et al. (2007) for staging. They were squashed and stained with acetocarmine following the procedure of Dempsey (1994).

Chromosome spreads

Whole anthers were harvested fixed and stored in 3:1 ethanol to acetic acid. To initiate the spread procedure they were washed in 1x citric buffer on ice. Anthers were then added to a structural weakening buffer at 37C for 40 minutes. The reaction was stopped by adding cold 1x TE followed by 3 rinses in 100% EtOH. EtOH was allowed to evaporate and 35 uL of 3:1 acetic acid: methanol (95:5 in the most aggressive case) was added to the anthers on a microscope slide. Anthers were then cut and the meiocytes extruded into the solution as quickly and carefully as possible. The solution containing the meiocytes was dropped onto glass slides in 8uL aliquots from a height of no less than 8 inches (additional height did not provide better spreading). Slides were allowed to dry in a humid chamber (standing water in the bottom of a container

containing a platform for slides). Chromatin was crosslinked to slides by exposure to 120 mJ per cm^2.

Probe hybridization of chromosome spreads

Slides were either stored at -20C or used immediately following UV crosslinking. Upon use the slides were hybridized with probe as in Birchler et al. (2007) with two exceptions: spreads were marked with permanent marker on the opposite side from the spread so the breathing procedure was unnecessary and I used a DABCO solution that also contained DAPI to mount the slides.

Semiquantitative RT-PCR

RT-PCR was conducted using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen Cat. No. 12574-018). 1 ug of total RNA was used as the template. Conditions were as described in Pawlowski et al. 2004.

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