THE INTERPLOIDY HYBRIDIZATION BARRIER IN *ZEA MAYS* L.

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by,

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……………………Couldn't do it without Michael and Diana Bauer, or as I like to call them, Dad and Mom. This is for you two and the whole Bauer family. Thank you all!

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List of Abbreviations

2x	Diploid maize plant
3x	Triploid maize plant
4x	Tetraploid maize plant
5x	Pentaploid maize plant
Ab10	Abnormal maize chromosome 10
acc 1	*acetyl-coA carboxylase 1*
acc 2	*acetyl-coA carboxylase 2*
BETL	Basal endosperm transfer layer
B-A	Translocation of a maize chromosome arm onto the maize B chromosome
CenH3	Centromeric histone 3
CentC	A DNA sequence repeat located in all centromeres on maize chromosomes
Cent4	A DNA sequence repeat located in the centromere on maize chromosome 4
CRM	centromere-specific retrotransposons
DAP	Days after pollination
DAPI	4'-6-Diamidino-2-phenylindole
dek1	*defective kernel 1*
DME	*DEMETER*
EBN	Endosperm balance number
ESR	Embryo surrounding region
FIE	*FERTILIZATION INDEPENDENT ENDOSPERM*

FIS2	*FERTILIZATION INDEPENDENT SEED DEVELOPMENT 2*
FISH	Fluorescence *in situ* hybridization
GFP	Green fluorescent protein
HAP	Hours after pollination
kPa	KiloPascals
m	Maternally inherited genome
MEA	*MEDEA*
MET1	*METHYLTRANSFERASE1*
MET1a/s	*METHYLTRANSFERASE1 ANTI-SENSE TRANSGENE*
myo1	*myosin heavy chain*
NOR	Nucleolar organizer region; contains rDNA genes
p	Paternally inherited genome
RNAi	RNA interference
rp1	*resistance to Puccinia sorghi 1*
rp3	*resistance to Puccinia sorghi 3*
RT-PCR	Reverse transcriptase PCR
TR-1	Tandemly repeated DNA sequences 1

Nomenclature: All maize crosses, the female plant is listed first and the male is listed second

Abstract

The development of kernels in maize (*Zea mays* ssp *mays*,) can be disrupted with interploidy crosses. An interploidy cross occurs when two different ploidies of the same species, i.e. a diploid and a tetraploid, cross fertilize. When this occurs, the development of the resulting kernels are highly abnormal causing a failure in their ability to acquire and store nutrients, thus ultimately resulting is small defective kernels that will often abort the accompanying maize embryo.

The bulk of the maize kernel is taken up by the endosperm, which is one of the products of double fertilization. First, two maternally derived haploid cells fuse together forming the diploid central cell (2m), which is then fertilized by the paternally derived haploid sperm (1p) creating the triploid endosperm nucleus (2m:1p). The maternal genomes, having been inherited by every endosperm on an ear, have an interest in the survival of all. Many endosperms can be fertilized by different pollen donors, thus the paternal genome has only an interest in the survival of its own endosperm. Due to the different developmental interest, the parental conflict hypothesis suggests that the inherited paternal genome in the endosperm expresses genes that aid in nutrient acquisition in an effort to take nutrients away from competing siblings, and also suppress or imprint genes that would aid in equal nutrient distribument between all endosperms. The inherited maternal genome, having an interest in equal development of all kernels, expresses genes that aid in equal nutrient distribument, and suppresses or imprints genes that aid in nutrient acquisition. For normal development to occur, a ratio of imprinted loci of 2m:1p has evolved, in which interploidy crosses

disrupts resulting in the formation of defective kernels. A second theory predicts that disruption occurs due to an abnormal dosage of regulators in the central prior to fertilization. The diploid central cell contains a certain dosage of regulators that are set to activate the correct set of loci in the post-fertilized triploid endosperm nucleus. Interploidy crosses create an abnormal dosage of regulators relative to their target loci after fertilization, and this abnormal dosage leads to the development of a defective endosperm.

Until now, these two theories have not been separately examined. With the use of nitrous oxide, this dissertation has been able to test the regulator dosage hypothesis without disrupting the ratio of inherited imprinted loci. When this occurs, development becomes abnormal and results in the formation of a defective kernel. This dissertation also provides evidence that, not only are the dosage of developmental regulators important in setting the correct developmental program, but the dosage of regulators are only needed immediately after fertilization. When the dosage of developmental regulators is disrupted in later stages, normal development was not affected. These results also give further insight into the endosperm balance number hypothesis, the effect of B-A translocations on endosperm development, and also an additional pressure that could have aided in the evolution of genomic imprinting.

This dissertation also shows that 17% of kernels develop abnormally when pollinated by a high haploid producing line of maize called RWS. Because RWS creates a higher than normal amount of spontaneously developing egg cells without fertilization, it was hypothesized that the defective kernels could be due

to spontaneous development of the diploid central cell, and would be predicted by the regulator dosage hypothesis to develop abnormally. Even though RWS creates a higher than normal amount of haploids, this dissertation describes evidence that the defective kernels are not due to spontaneous development of the central cell.

Late in endosperm development, the cells cease mitosis, but DNA synthesis continues, resulting in cells greatly increasing their genomic size. To better understand how the nucleus is organized, a cytogenetic experiment was performed using fluorescent *in situ* hybridization (FISH) using many of the molecular markers known in maize including a few associated with the maize B-chromosome. Data is presented that even though cell division has stopped, the endoreduplicated chromosomes remain associated. The chromosome stays loosely associated along its length, but remains tightly associated at both the centromeric regions and knob regions. Evidence is also presented that interploidy crosses can change the molecular structure of the endoreduplicated chromatin.

Chapter 1:

Introduction and Background

Introduction

Domesticated corn, or *Zea mays* ssp *mays*, has been an important agricultural food crop for thousands of years and is in such high demand today that it takes roughly 25 maize plants per person per day to be able to maintain the current standard of life in America (WILKES 2004). Even though maize is an important agricultural and economical food crop, it has also been an invaluable model organism for understanding biology and evolution (DOEBLEY 2004; JONES 2005). Maize is considered a hermaphroditic plant in that it has both but separate male and female reproductive organs. Both reproductive organs produce spores, which then go through several rounds of mitosis to generate the gamete producing gametophytes. The female or megagametophyte of maize contains many cells: the egg cell, two synergid cells, multiple antipodal cells, and two polar cells that fuse to create a diploid central cell (Figure 1.1A). The male or microgametophyte is the end result of two mitotic events, producing the vegetative or pollen nucleus and two sperm nuclei. The vegetative nucleus drives the production of the pollen tube, which grows down the silk tissue, and penetrates the female ovule in which the two sperm are deposited (MARTON *et al.* 2005). The production of seeds in flowering plants is the end result of double fertilization. One sperm fertilizes the egg, which will develop into the diploid

embryo; the other sperm fertilizes the diploid central cell, which will develop into the triploid endosperm.

Shortly after fertilization, the primary endosperm nucleus will begin mitosis without cell wall formation creating a syncytium (endosperm development as reviewed in: BERGER 1999; KIESSELBACH 1949; OLSEN 2001). In the syncytium, the nuclei migrate and position themselves around a large vacuole (Figure 1.1B). Approximately three to four days after pollination (DAP), cell walls form and encapsulate the nuclei (Figure 1.1C). Mitosis continues until 10-12 DAP after which mitotic activity decreases. A cell age gradient is formed, with older cells in the middle of the endosperm, and the younger daughter cells on the edge of the endosperm (SLOCOMBE *et al.* 1999). The outer cells maintain a low mitotic potential to expand the endosperm while starch is being created and stored. Storage of starches and sugars continues until seed maturity (SLOCOMBE *et al.* 1999). By this time, the endosperm cells have differentiated to four distinct cell types: embryo surrounding region, aleurone layer, basal transfer layer, and the starchy endosperm tissue.

The embryo surrounding region (ESR) is located around the embryo and is distinguished by its dense cytoplasm (OPSAHL-FERSTAD *et al.* 1997). Its function is currently under investigation, but it has been hypothesized to be necessary for the transfer of nutrients (BONELLO *et al.* 2002; OPSAHL-FERSTAD *et al.* 1997; VAN LAMMEREN 1986). The basal endosperm transfer layer (BETL) has thick cell walls that connect the endosperm to the maternal plant. The main function of the BETL is to transfer nutrients from the maternal phloem into the

starchy endosperm, but also may aid in fungal resistance due to sequence similarity of exclusively expressed fungal resistance genes (HUEROS *et al.* 1999; SERNA *et al.* 2001). The aleurone layer is a single cell thick layer that, except for the BETL region, completely surrounds the endosperm. The aleurone expresses many genes, but its primary role is to aid in digestion of starch during embryo germination (BECRAFT and ASUNCION-CRABB 2000; OLSEN 2001). The starchy endosperm is the largest segment of the maize kernel and is involved in creating and storing starch that will be used later by the germinating embryo (OLSEN 2001; SLOCOMBE *et al.* 1999).

Starting around 10 DAP, cells cease dividing mitotically and instead enter into an endoreduplication phase, in which DNA synthesis continues without accompanying cell division (EDGAR and ORR-WEAVER 2001; KOWLES and PHILLIPS 1985; KOWLES and PHILLIPS 1988; KOWLES *et al.* 1990; KOWLES *et al.* 1992; LARKINS *et al.* 2001). The cells located in the center of the nucleus begins endoreduplicating first, followed by the cells next to them, and then the cells next to them, etc. This results in a gradient of cells in which the innermost cells have gone through the most rounds of endoreduplication, and the cells on the outer edge have gone through the least. Because of the repeated rounds of DNA synthesis without cell division, the nucleus enlarges, as does the total cell volume to accommodate the increase in ploidy (KOWLES and PHILLIPS 1988). The largest mean ploidy of the endosperm is reached around 16 DAP, in which some nuclei can reach a ploidy of 200x or more. Different inbred lines of maize have different levels of endoreduplication. Crossing together two different inbred lines, the

hybrid maize endosperm proceeds through a similar endoreduplication pattern of the maternal inbred line indicating that the endoreduplication pattern is maternally influenced (DILKES *et al.* 2002; KOWLES *et al.* 1997). After 16 DAP, there is a remarkable decline in overall mean ploidy of the endosperms tissue. This is due to an increase in cell death. Just as in endoreduplication, the cells in the center-most portion of the endosperm proceed to cell death first, followed by a wave of cell death emanating from that center location to the edges (YOUNG and GALLIE 2000). Cell death will continue until seed maturity (~ 45 DAP), and at maturity, all cells in the endosperm have disintegrated, except for the aleurone layer (BERGER 1999; OLSEN 2001).

Spontaneous development

In some plant species such as citrus and dandelion, development of the embryo and endosperm can proceed asexually in a process called apomixis (SPIELMAN *et al.* 2003). To understand the necessity for seed development upon fertilization, a search was conducted looking for mutants that would cause a non-apomictic plant, *Arabidopsis thaliana*, to develop without fertilization. Three genes were identified: *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*), *FERTILIZATION INDEPENDENT SEED DEVELOPMENT 2* (*FIS2*), and *MEDEA* (*MEA*)(GROSSNIKLAUS *et al.* 2001; GROSSNIKLAUS *et al.* 1998; MA 1999). All three mutations cause the diploid central cell to start developing soon after polar nuclei fusion, but development arrests early. The stage of developmental arrest falls into two classes. The *FIE* mutants develop to the syncytium stage and then

stops, while the *FIS2* and *MEA* mutants develop past the cellularization stage, and then cease as a multicellular tissue (MA 1999). The *FIE, FIS2* and *MEA* genes are homologous to the *Drosophila* Polycomb gene repression complex of proteins. The current hypothesis is that in normal development, *FIE*, *FIS2*, and *MEA* work together along with other unknown plant Polycomb-like proteins to repress cell proliferation in the central cell and this repression is relieved after fertilization.

Interploidy hybridization barrier

Little is known about the molecular aspects of maize endosperm development. One method in studying developmental processes is to create mutants or conditions that disrupt the normal developmental program, and investigate for molecular changes. It has been observed that interploidy cross hybridizations succeed in disrupting normal maize endosperm development (Figure 2.1) (COOPER 1951; RANDOLPH 1936). Interploidy crosses result in mature kernels that are much smaller when compared to normal, and have a near zero germination rate (COOPER 1951). Even though interploidy crosses create defective kernels, the growth and differentiation of the endosperms are phenotypically different depending on the direction of the interploidy cross (Figure 2.1).

Crossing a maternal diploid (2x) with a paternal tetraploid (4x) creates a tetraploid endosperm (4x), and development begins with an increased rate of mitosis, especially in the ESR. This increased mitotic activity creates many

endosperm cellular layers that eventually embed and distort the embryo. The syncytium of nuclei are cellularized earlier than 4 DAP. Starch and nutrient accumulation also begin early, but the BETL does not differentiate properly, and is virtually absent (COOPER 1951). Consequently there is very little nutrient transfer from the maternal sporophyte, leading to approximately 1/3 of the central endosperm being filled with starch. In normal kernels, mitosis ceases around 12 DAP, but in 2x x 4x crosses, the number of mitotic cells begins to slow around 12 DAP, but does not completely stop (LEBLANC *et al.* 2002). Endoreduplication can be detected after that time, but proceeds at a very slow rate and on average does not reach the high ploidy of the normal 2x x 2x crosses. Overall, in the 2x x 4x crosses, the cells, especially surrounding the embryo, become highly meristematic, which embeds and misshapes the embryo (COOPER 1951). The meristematic activity, combined with the low nutrient availability in the endosperm, leads to a very low germination rate.

In the reciprocal interploidy cross, the maternal 4x is crossed with the paternal 2x, forming a pentaploid (5x) endosperm. Development appears to match that of the normal 2x x 2x crosses, except that mitosis is much slower and cellularization occurs late, around 5-6 DAP (COOPER 1951). The slow mitotic index results in the differentiation rate of the endosperm tissue being abnormal. A layer of thin poorly formed BETL tissue is produced. Nutrient acquisition begins earlier, and by 6-7 DAP the endosperm is small, but entirely filled with starch. Mitosis ends around 8 DAP, and is followed by an early induction of the endoreduplication phase. Endoreduplication proceeds normally except that the

overall mean DNA amplification does not reach that of the 2x x 2x crosses (LEBLANC *et al.* 2002). Seeds from 4x x 2x crosses are capable of germination, but growth of the seedling is retarded and either development failure ensues, or the adult plant is sterile.

Endosperm balance number

Interploidy crosses have been observed in multiple plant species (COOPER and BRINK 1945), but in some cases it does not always induce defective offspring. An interesting example of this is found in the genus *Solanum* (JOHNSTON and HANNEMAN 1982; JOHNSTON *et al.* 1980), which has many closely related species in with varying ploidy. Crosses of the diploid *Solanum chacoense* with the tetraploid species of *Solanum acaule* yielded plump seeds, but crosses with another tetraploid *Solanum tuberosum* did not. In an attempt to force the latter fertilization to succeed, Johnston (1980, 1982) doubled the genomic content of *S. chacoense* from a diploid to tetraploid. The newly formed tetraploid *S. chacoense* could now successfully fertilize the tetraploid *S. tuberosum*, but could no longer cross with the tetraploid *S. acaule*. These observations led to the hypothesis that for successful interploidy hybridizations to be achieved, ploidy itself was not the important determinant, but rather each species must have the same endosperm balance number (EBN) (JOHNSTON *et al.* 1980). The fact that *S chacoense* could successfully cross with *S. acaule* to produce plump offspring indicates that they must have the same EBN even though they are of different ploidy. By contrast, *S. tuberosum* must have a

different EBN, such that interploidy crosses fail. When the *S. chacoense* genome was doubled to the tetraploid level, so was the EBN, allowing it to cross successfully with *S. tuberosum*, but not with *S. acaule*. This series of observations suggested that there is a gene, or group of genes that must be expressing appropriately before and/or after fertilization for the proper developmental pathway to be initiated. Even though EBN hypothesis has proven useful in plant breeding, the molecular explanation under lying intercross barriers remains a mystery.

Why do interploidy crosses fail?

The molecular cause for interploidy cross failure has been explored for decades. Müntzig (MÜNTZIG 1933) thought that the ploidy ratio among maternal plant:embryo:endosperm were important, such that the normal ratio must be 2:2:3; interploidy crosses perturb that ratio resulting in developmental failure. Watkins (WATKINS 1932) disproved that relationship by observing kernels that developed normally when the maternal plant:embryo:endosperm ploidy ratio was 2:4:6. He concluded that the maternal plant is not involved; rather, an embryo:endosperm ratio of 2:3 is needed for proper development. This hypothesis was generally accepted until it was shown that the embryo also could be dispensed with, and the endosperm would still develop normally indicating that the endosperm has to be triploid, or any multiples of three (SARKAR and COE 1971). The issue of triploidy was addressed by using the *indeterminate gametophyte* (*ig*) of maize (LIN 1984). The *ig* mutation causes the formation of

central cells with different ploidies, either because only a single polar nucleus is formed or because more than two nuclei fuse (KERMICLE 1971). As a result, an ear may contain different maternal gametophytes with different nuclear ploidies formed in the central cell. Using this mutation in interploidy crosses (Lin 1984), endosperms were created that not only had varying ploidy, but also varying in their ratio of inherited parental genomic ratios. Although he could create various endosperms that were 6x in by different combinations of maternal and paternal genomes, it was only the endosperm that received four maternal genomes and two paternal genomes that developed normally. By examining several different ploidies, he concluded normal endosperm development depends, not on triploid or multiples thereof, but on a genomic ratio of maternal (m) to paternal (p) genomes of 2m:1p needs (LIN 1984). It was this experiment that later inspired the hypothesis that the cause of endosperm developmental failure from an interploidy cross was due to improper balance of inherited imprinted loci (HAIG and WESTOBY 1989; HAIG and WESTOBY 1991; WILKINS and HAIG 2003).

Genomic imprinting

Genomic imprinting occurs when a gene's expression is influenced by its parental origin (ALLEMAN and DOCTOR 2000; CONSTANCIA *et al.* 2004; GEHRING *et al.* 2004). The most extreme case is when the gene from one parent is completely silent, while the gene from the other parent is active, even though the DNA sequence of the two parental alleles is identical. In plants, genomic imprinting is endosperm specific (ALLEMAN and DOCTOR 2000). A classic

example of an imprinted locus in *Arabidopsis thaliana* is *FWA* (KINOSHITA *et al.* 2003). *FWA* is expressed in the central cell, but the expression is restricted to only the maternally inherited alleles. The paternally inherited allele remains silent.

Imprinting doesn't have be defined as a completely on/off state. Many genes are expressed from both parental origins, but the extent of expression can differ (DILKES and COMAI 2004; GUO *et al.* 2003). For many genes in plants, but not all, there is a correlation between a gene's expression level and its corresponding DNA methylation (LI *et al.* 1993). DNA methylation is an epigenetic mark in which a methyl-group is covalently attached to a cytosine nucleotide by the DNA methyltransferase class of enzymes. A silent gene also contains a high amount of DNA methylation at its promoter sequence, while an active gene contains very little (BENDER 2004).

The parental conflict hypothesis

To explain the development of defective kernels resulting from interploidy crosses, Haig and Westoby proposed the parental conflict hypothesis (HAIG and WESTOBY 1989; HAIG and WESTOBY 1991). An ear of maize contains hundreds of female ovules, all of which contain two maternal genomes in the central cell. Since all kernels have the same maternal genetic investment, the maternal plant has equal interest in the survival of every seed. This results in gene expression that discourages a bias in nutrient storage that favors one ovule over another, which favors equal distribution of nutrients to all seeds. Even though all kernels

on the maize ear contain the same maternal contribution, they can differ in their, paternal makeup because the maize ear does not discriminate among different maize pollen donors. This can result in an ear with kernels that have been pollinated by many different pollen parents. Consequently, the genomic contribution from the pollen parent only has an interest in the survival of itself. This results in selection for paternal genes that favor maximal nutrient acquisition. The conflict of interest between the inherited maternal genomes and the inherited paternal genomes represents an "arms race" which has driven an evolutionary process, resulting in a parent specific silencing of certain genomic loci (WILKINS and HAIG 2003). In the maternal genome, loci that favor nutrient acquisition are silenced by imprinting, while loci that discourage nutrient acquisition are highly expressed. In the paternal genome, loci that discourage nutrient acquisition are silenced by imprinting, while the loci that favor nutrient acquisition and storage are highly expressed.

Based on this theory and because imprinting is only found in the endosperm, Haig and Westoby argue that endosperm developmental failure from interploidy crosses occurs because the ratio of inherited imprinted loci has changed, and for development to proceed normally, the 2m:1p ratio must be maintained (HAIG and WESTOBY 1989; HAIG and WESTOBY 1991; WILKINS and HAIG 2003). For example, a diploid maternal plant when crossed with the tetraploid paternal plant results in a tetraploid endosperm, in which the ratio of imprinted loci is now 2m:2p (Figure 2.1). In the reciprocal, maternal tetraploid and paternal diploid, interploidy cross results in a pentaploid endosperm in which

the ratio of imprinted loci is now 4m:1p (Figure 2.1). In all cases of interploidy crosses, the 2m:1p ratio is disrupted, and because the disrupted ratio is different depending on the direction of the cross, the abnormal developmental phenotype of the endosperm is also different (COOPER 1951; RANDOLPH 1936).

Genomic imprinting and the parental conflict hypothesis

Because of the correlation of imprinting and DNA methylation, Adams *et al* (ADAMS *et al.* 2000), examine the effect of reducing methylation before fertilization. To reduce the methylation in *Arabidopsis thaliana*, they made transgenic plants expressing anti-sense transcripts to the *METHYLTRANSFERASE1* (*MET1*) gene to reduce *MET1* activity in the female gametophyte. Without *MET1*, the methylation distribution would be disrupted, as would the imprinting marks, allowing genes that would normally be silenced to now be expressed (FINNEGAN *et al.* 1996; GENGER *et al.* 1999). When a diploid *MET1* anti-sense (*met1a/s*) mutant was crossed as female with a normal diploid male, the resulting kernels resembles that of the 4x x 2x interploidy cross. The interpretation was that the *met1a/s* plant is now expressing endosperm-promoting genes that are normally silenced in the pollen. The same argument was used for the reciprocal cross. When the normal diploid was a female and the *MET1a/s* was used as the male, the resulting defective kernels resembled that of the 2x x 4x interploidy cross, indicating that the genes, normally silenced in the pollen, are now expressed. It is worth noting that the *MET1a/s* transgene would result in demethylation of large areas of the genome, but the

demethylation would likely not be complete because of the presence of redundant methyltransferase genes (BENDER 2004). In addition, the anti-sense technique may not be fully penetrant, as the RNAi machinery could silence the *met1a/s* transgene. Thus, both interploidy cross failure and the *met1a/s* phenocopy can also be explained by other hypotheses.

Endosperm size factors

Another examination of the role of inherited imprinted loci utilized maize B-A chromosomes containing endosperm size factors (BIRCHLER and HART 1987). Some maize lines contain a small, subtelocentric, and dispensable chromosome known as the B chromosome. No known genes exist on this chromosome, but the B chromosome has some unique properties. During formation of the maize pollen grain, at the second mitotic division, the B chromosome centromere will non-disjoin (CARLSON and CHOU 1981; ROMAN 1947). This results in a pollen grain with two non-identical sperm: one sperm with two B chromosomes, and the other sperm with none. When this pollen grain fertilizes a female gametophyte, the sperm with the non-disjoined B chromosomes will preferentially fertilize the embryo, leaving the endosperm with no B chromosomes (CARLSON 1969).

By using various techniques, translocations between different chromosomes of maize (called the A chromosomes) and the B chromosomes were produced (BIRCHLER and ALFENITO 1993; ROMAN 1947; ROMAN and ULLSTRUP 1951). Currently, a large collection B chromosome centromeres attached to different A chromosomal arms at various break points is available

(BECKETT 1994). When a particular B-A chromosome is passed through the pollen, it can non-disjoin creating sperm in which one will have the additional chromosomal arm, and the other will have none (BIRCHLER 1980; BIRCHLER and ALFENITO 1993; ROMAN and ULLSTRUP 1951). When this pollen fertilizes the female gametophyte, the egg will receive an extra copy of the A chromosomal arm, while the endosperm will be deficient. When some paternal chromosomal arms are missing in the endosperm, the mature kernels are smaller than normal, but when other paternal chromosomal arms are lost, the kernels appeared normal (BIRCHLER 1980; BIRCHLER 1993; BIRCHLER and HART 1987). Apparently, the missing chromosomal arms resulting in the small kernels must contain loci that are important for endosperm development, while the missing arms resulting in the normal endosperms do not. These genetic loci were termed endosperm size factors, and were postulated to the loci that are needed in a 2m:1p ratio for normal development.

Endosperm size factors are hypothesized to be located on chromosome 1L (long arm), 1S (short arm), 2L, 4S, 5S, 7S, 7L, 10L (BIRCHLER 1993; BIRCHLER and HART 1987). If a small kernel develops when the chromosomal arm is not inherited from the father, it was logical to test if inheriting an extra but same chromosomal arm from the maternal plant could rescue the small kernel phenotype. For some chromosomal arms, this was not the case and the small kernel phenotype persisted, consistent with the parental conflict hypothesis. The missing paternal alleles must be imprinted (silenced) when inherited maternally such that no change in kernel size is noticed. However, for some kernels that

inherited the extra maternal chromosomal arm, the small kernel phenotype appeared to be worse, which is not consistent with the parental conflict hypothesis.

It has also been observed that when crossing together different maize lines, the resulting kernels resemble in morphology to that of the maternal parent. Because of the this observation, along with the endosperm size factors, the results from interploidy cross failure, and additional observations that do not fit into predictions of the parental conflict hypothsis (BIRCHLER 1993; DILKES and COMAI 2004), the interploidy hybridization barrier can be explained by an additional hypothesis.

Regulator dosage hypothesis

Birchler (1993) documented that there is a maternal control over the size of the female central cell. A tetraploid maize central cell is bigger than a diploid, for example. This observation will lead to an additional complication with interploidy cross failure. During development of the female gametophyte, the primary endosperm nucleus is composed of a fusion between two polar nuclei. The regulator dosage hypothesis suggests that the diploid central cell contains many proteins and RNA's (which will be addressed as "regulators") that are needed in a certain dosage to activate the correct developmental pathway of the triploid endosperm nuclei that forms after fertilization (BIRCHLER 1993; DILKES and COMAI 2004). A putative "regulator" in maize might be *baseless1* (GUTIERREZ-MARCOS *et al.* 2006), which is needed to be expressed in the central cell prior to

fertilization for proper endosperm development. Another putative "regulator" in *Arabidopsis* is *DEMETER* (CHOI *et al.* 2002).

A tetraploid maize plant contains a fusion of two diploid polar nuclei in the female gametophyte forming a tetraploid central cell, which is a larger cell with more cytoplasm than when the female parental plant is diploid. The tetraploid central cell is readied for development so that, as soon as it is fertilized and creates the hexaploid endosperm nuclei, the correct development pathway can be initiated. The regulator dosage hypothesis posits that an interploidy cross disrupts the dosage of those regulators relative to their post-fertilized target loci, activating a developmental pathway leading to a defective kernel. To address this more precisely, a diploid maternal plant forms a diploid central cell that is "programmed" to activate the post-fertilized triploid nucleus towards the correct developmental pathway (Figure 2.2, a diagram of the regulator dosage hypothesis). When the diploid central cell is fertilized by pollen from a tetraploid plant, the endosperm is tetraploid rather than the normal triploid. The dosage of regulators to target loci has been changed, and this creates an inappropriate gene activity that ultimately triggers a developmental pathway leading towards a defective endosperm. The reciprocal cross follows a similar trend. The tetraploid maternal plant develops in the female gametophyte and tetraploid central cell, which is "programmed" to activate the post-fertilized hexaploid endosperm target loci and begin endosperm development. When the tetraploid central cell is fertilized by the haploid pollen from a diploid paternal plant, a pentaploid endosperm nucleus forms, creating a differential dosage of regulators to their

target loci. This theory, similar to the parental imprinting theory, predicts that because the dosage of regulators to target loci is disrupted differently depending on the direction of the interploidy cross, so will the developmental program initiated giving the different defective developmental phenotype observed.

Apomixis

An interesting caveat to both hypotheses is apomixis. Apomictic plants are able to have embryo development without fertilization (BICKNELL and KOLTUNOW 2004; SPIELMAN *et al.* 2003). In most apomictic plants, the embryo will not be fertilized, but the endosperm will. For example, in the Citrus genus, a sexually derived embryo develops in the same ovule sac as a sporophytic derived embryo. Both the meiotically derived embryo and endosperm must be pollinated for the sporophytic diploid embryo to develop. In this case, the endosperm maintains balance. In gametophytic apomixis, the embryo sac develops unreduced, and the diploid embryo will not develop until the endosperm is fertilized. Because of the unreduced nature of the gametophyte, the central cell is tetraploid and will contain a differential dosage of regulators to target loci after fertilization. An explanation that would correct the dosage in some species has been suggested; because the central cell from this type of apomictic development may contain only one diploid polar nucleus, fertilization by a haploid sperm would restore the required dosage. It has also been suggested that in *Dichanthium annulatum*, if there are two diploid polar nuclei, one disintegrates restoring the dosage of regulators. If the embryo develops without fertilization,

what happens to the second sperm? Some observations from *Ranunculus auricomus* suggest that the two diploid polar nuclei are both fertilized, and this double fertilization of the central cell would put the regulators back in the correct dosage.

Even though many theories explain how plants can “fix” the dosage and or imprinting ratio (Spielman *et al.* 2003), there are many other apomictic species that seem to develop a normal endosperm despite the disruption. In *Tripsacum dactyloides*, a single sperm fertilizes the unreduced, tetraploid central cell. Even though there is an improper dosage of regulators to target loci, development appears to be normal. The same is also true for autonomous apomictic plants that develop both embryo and endosperm without fertilization. In this case, the endosperm only has a maternal genomic contribution and develops normally. In both apomictic situations, it seems logical that for development to occur, the methylation patterns that are coincident with gene activity must be differentially distributed, or possibly absent, to adjust gene activity and allow the correct dosage of regulators so that development can proceed normally.

Summary

The failure of endosperm development due to interploidy crosses could be due to two reasons, neither of which has been studied on its own. 1) There is an imbalance of inherited imprinted loci in the endosperm (Haig and Westoby 1989; Haig and Westoby 1991). 2) There is an altered dosage of gene regulators to their target loci in the endosperm cell after fertilization (Birchler 1993). This

dissertation presents results that address three topics dealing with the molecular genetics of endosperm development: 1) dosage of regulators to target loci is important for normal endosperm development; 2) evaluation of spontaneous central cell development as a cause of defective kernels in lines that induce high frequencies of haploid embryos; 3) examination of endoreduplicated chromosomes in endosperm and impact of interploidy crosses on chromosomal organization.

Chapter 2:

Nitrous Oxide Treatment of Endosperm Early in Development Creates Polyploidy and Defective Kernels

Introduction

The bulk of the maize kernel is composed of endosperm tissue. The maize endosperm functions as a nutrient and starch storage tissue, which will be consumed by the germinating embryo. The mechanisms controlling growth and development of the endosperm remains largely unknown. One method for studying development is to examine mutations that disrupt the developmental pathway. This is an unfavorable method for studying endosperm development because mutations often result in defective kernels, causing the accompanying embryo to abort and making it difficult to carry the mutation to the next generation. A better method would be to genetically create an environmental situation that results in defective kernel development. An effective method for performing this is by utilizing the interploidy hybridization barrier (Figure 2.1) (COOPER 1951; RANDOLPH 1936; SARKAR and COE 1971; WOODELL and VALENTINE 1960). Interploidy hybridization, or interploidy crossing, occurs when plants of different ploidies cross-fertilize.

Maize has been shown to be an ancient allotetraploid (GAUT and DOEBLEY 1997). Approximately 4-5 million years ago, the genome of the maize progenitor plant doubled from 5 to 10 chromosomes (SWIGONOVA *et al.* 2004). Since that time, the progenitor has differentiated into many different species and subspecies of maize (DOEBLEY 2004). Theses subspecies have similar chromosomal makeup, but cross-fertilizations are not highly successful, and can often resemble that of an interploidy cross. The fertilization barrier, if overcome, could have benefits in crop improvement due to the opportunity to introduce new beneficial alleles. Thus, understanding how interploidy crosses fail to develop properly would give a greater insight into how maize breeders could use closely related crop species to improve overall crop yield.

The maize female gametophyte contains many cells including the haploid egg and the diploid central cell (Figure 1.1A). The endosperm is the one of the two products of double fertilization in which the haploid egg is fertilized by a haploid sperm to form the diploid embryo, while a second haploid sperm fertilizes the diploid central cell forming the triploid endosperm. Soon after fertilization, the endosperm goes through many rounds of mitosis without cytokinesis. This results in a single cell that contains many nuclei surrounding a large central vacuole (Figure 1.1B). Around 4 DAP, the cell walls form around the nuclei (Figure 1.1C), and from 5-12 DAP go through a high rate of mitosis. The kernel reaches maturity around 40-45 DAP.

It is generally accepted that the cause of interploidy cross failure is due to the inheritance of an irregular ratio of imprinted loci (BAROUX *et al.* 2002b; HAIG and WESTOBY 1989; HAIG and WESTOBY 1991). Normally, the developing endosperm receives two maternal genomic sets and one paternal (2m:1p). Interploidy crosses disrupt that ratio, resulting in defective kernels (Figure 2.1). In addition to the ratio of imprinted loci, interploidy crosses could also disrupt the dosage of regulators to their target loci (BIRCHLER 1993). Before fertilization, the central cell contains proteins and RNA's (which will be referred to as "regulators") that activate appropriate loci after fertilization for proper development to begin. Interploidy crosses create differential dosage of those regulators to target loci, triggering the developmental pathway that results in defective kernels (see introduction and Figure 2.2A).

To test the regulator dosage theory, one must be able to alter the dosage of regulators without altering the ratio of maternal to paternal genomes (Figure 2.2B). Recent advances in tetraploid production using nitrous oxide gas give us that opportunity (KATO and BIRCHLER 2006). Nitrous oxide has been shown to disrupt microtubule nucleation, arresting cells in metaphase (KATO 1999). Exposing mazie plants to a high pressure of nitrous oxide has been successful in creating autotetraploids of maize. Taking the same approach, applying nitrous oxide immediately after central cell fertilization can be used to create autohexaploids of the maize endosperm (Figure 2.2B). This will not only create an entirely polyploid endosperm, but will also successfully alter the dosage of regulators to target loci without disrupting the 2m:1p ratio. If defective kernels

Figure 2.1. Effect of interploidy crosses on maize endosperm development.

Effect of Interploidy cross on endosperm development. 2x x 2x column) the effect of crossing a maternal diploid with a paternal diploid. 2x x 4x column) the effect of crossing a maternal diploid with a paternal tetraploid. 4x x 2x column) the effect of crossing a maternal tetraploid with a paternal diploid. 4x x 4x column) the effect of crossing a maternal tetraploid with a paternal tetraploid. 10, 12, and 14 DAP endosperm tissue is the result of crossing diploid and tetraploid maize line Oh43. The mature kernels are from crossing diploid and tetraploid maize line N6.

Figure 2.1. Effect of interploidy crosses on maize endosperm development.

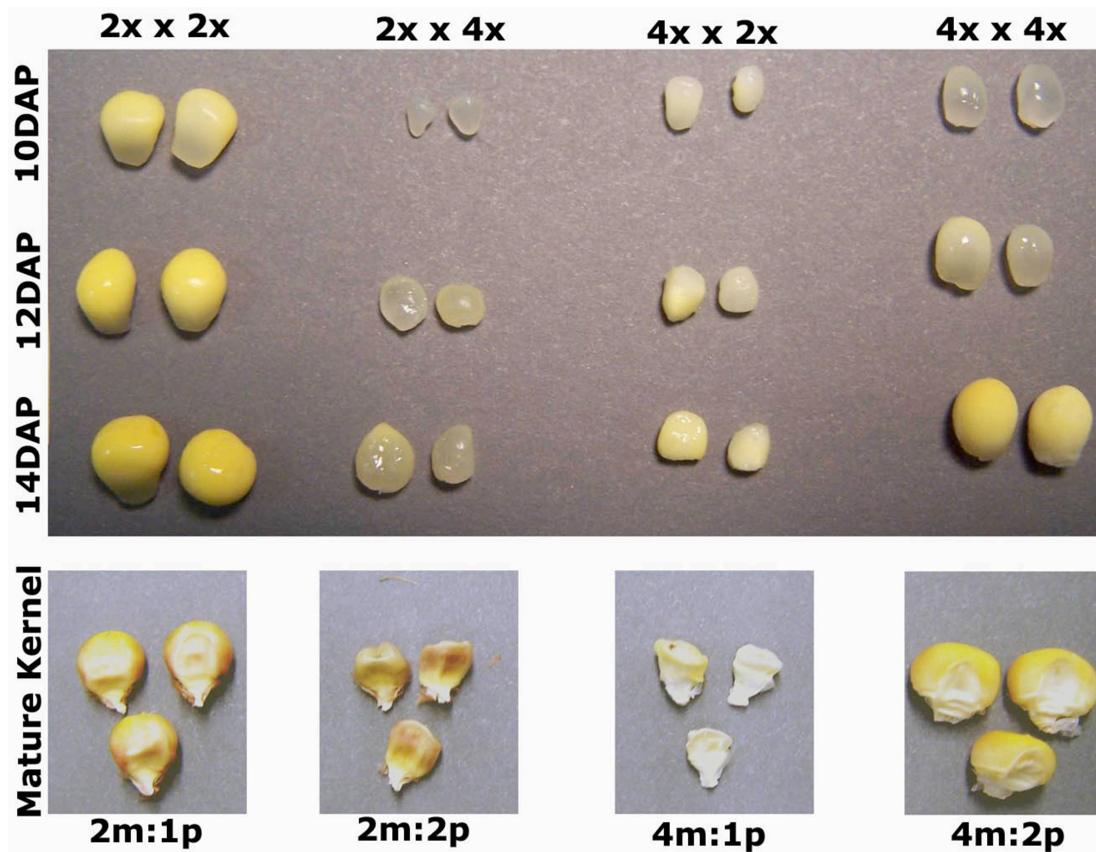
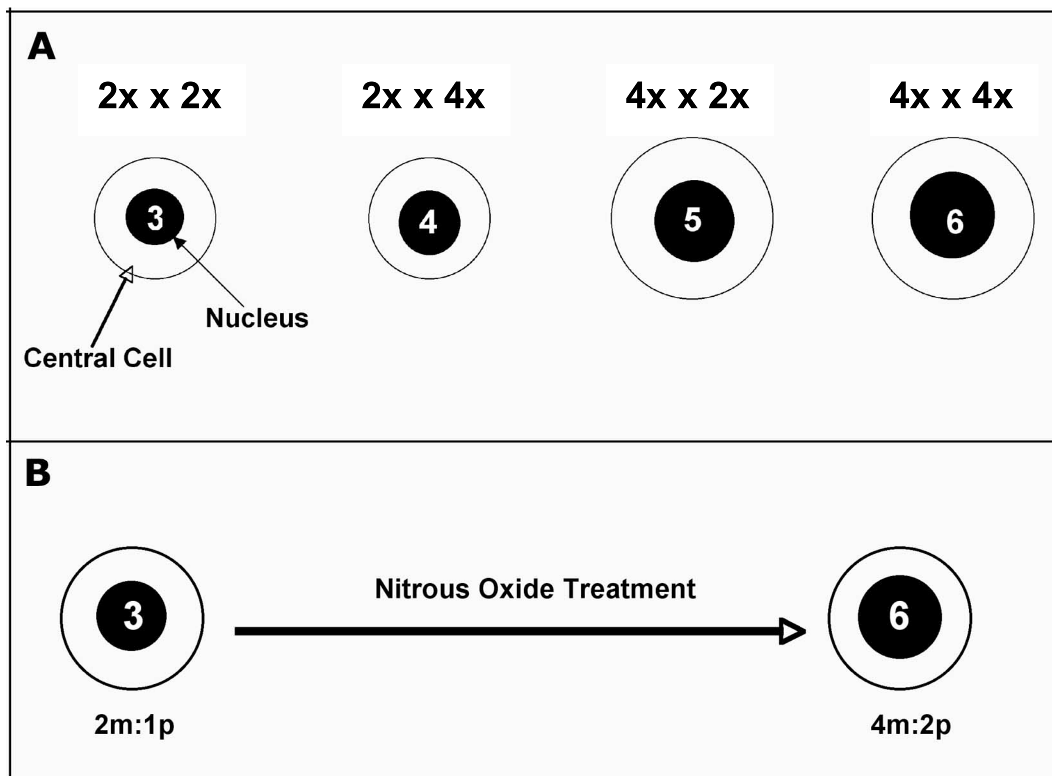


Figure 2.2. Diagram of the regulator dosage hypothesis.

The white circle represents the central cell before fertilization. When the maternal plant is diploid (2x) the central cell is diploid and contains the correct dosage of "regulators" that will trigger the appropriate development of the post-fertilized triploid nucleus. In 2x x 4x crosses, the post-fertilized nucleus is tetraploid, not triploid, and this altered dosage of regulators to target loci triggers a defective kernel developmental pathway. When the maternal plant is 4x, the central cell is 4x, and contains the correct dosage of regulators that will direct the correct development of the post-fertilized hexaploid (6x) nucleus. In 4x x 2x crosses, the post-fertilized nucleus is pentaploid (5x) and this altered dosage of regulators to target loci triggers a defective kernel developmental pathway. B) To test the Regulator Dosage Hypothesis, the maize triploid nucleus will have the genomic content doubled with nitrous oxide, which will not alter the ratio of inherited parental loci. Maternal genomes (m) and paternal genomes (p).

Figure 2.2. Diagram of the regulator dosage hypothesis.



are produced, they should hexaploid, while any kernels that develop normally would be predicted to escape genome doubling and remained triploid.

Materials and Methods

Maize Stocks, crosses, and nitrous oxide treatment

In spring of 2004, the Oh43 line of maize was grown in the Sears Green house, located on the campus of the University of Missouri, Columbia, MO. The greenhouse is on a 16 hour day and 8 hour night cycle. The temperature reached 77°F during the day, and dropped to 68°F during the night. When the maize plants reached maturity, they were self-pollinated, using controlled pollinations (http://www.maizegdb.org/IMP/WEB/pollen.htm). At 12, 14, 16, 18, 20, 22, and 24, and 120 hours after pollinations, the plants were placed inside a nitrous oxide treatment device, which was subsequently filled with the gas until a pressure of 600 KPa was reached. The plants were treated for 5 or 10 hours. After exposure, the plants were removed from the nitrous oxide treatment device, placed back into the greenhouse.

Nuclei spreads

Around 10-14 DAP, both normal and defective kernels, were harvested. For maize ears harvested at 12 DAP, the ear was placed into a smaller nitrous oxide treatment device, which was filled with the gas until 10 KPa was reached.

This was done to arrest mitotically dividing cells at metaphase, which increased the efficiency of chromosome spreading and counting (KATO 1999). After 3 hours of treatment, the maize kernels were harvested and placed in 90% acetic acid for 10 min, followed by transfer to a 70% ethanol solution. Tissue can be stored in 70% ethanol at -20°C for an extended period of time. Kernels harvested at time points later than 12 DAP were not gassed the second time, but instead was subjected directly to the acetic acid and ethanol treatment.

From the center of the endosperm tissue, a section roughly the size of 1mm^3 tissue was removed and washed in ice cold 1X citric acid buffer (10mM sodium citrate, 10mM EDTA, pH 5.5) for 10 minutes. The block of tissue was then incubated at 37°C for 30 min in 20 μl of digestion buffer (2% cellulase, 1% pectolyase, 10 mM EDTA). The digestion buffer was replaced using 1xTE (10 mM Tris-HCl, 1 mM EDTA, pH 7) and incubated on ice for 5 min. After removal of TE, 100% ethanol was added. The tissue was broken up by gently flicking the tube or by moderate shaking. The sample was then pelleted by centrifugation at 6000xg for 30 seconds. Ethanol was removed, and one to two drops of spreading solution was added (27:3, acetic acid:methanol). The endosperm sample was gently mixed, and then, using a pipette, the sample was dropped onto a glass slide. The slides were incubated at room temperature in a cardboard box lined with wet paper towels for 2 hours (hrs) or until slides were dry. For some endosperm samples, taken between 10-12 DAP, the DNA stain, acetic orcein, was added to visualize the maize chromosomes using light

microscopy (KATO 1997; KATO 1999). All other slides were subjected to florescence *in situ* hybridization (FISH).

Fluorescence *in situ* hybridization

A clone of a highly repetitive element found only near the centromere on chromosome 4 (Cent4; PAGE *et al.* 2001), was amplified using M13 primers. Preparation of directly labeled probes for fluorescence *in situ* hybridization (FISH) was performed as described previously (KATO *et al.* 2004).

Nuclei spreads that were prepared as described above, were UV-crosslinked for 2m (total energy, 120 mJ/cm^2). At the center of the cell spreads, 5 µl of 2x SSC solution containing autoclaved salmon-sperm DNA (100 ng/µl) were applied. A plastic cover slip was added, and the chromosomes on the slide were denatured by placing the slide on a wet paper towel in an aluminum tray floating in boiling water (100°C) for 5 min. The slides were then rapidly cooled by placing them on an ice-cold metal tray. The probe mixture (5 µl, in 2X SSC/1 mM EDTA) was denatured as well (100°C, 5 min) and rapidly cooled on ice. After removal of the coverslip from the denatured slide, the denatured probe was applied to the middle of the nuclei spread. The plastic coverslip was reapplied, and the slides were incubated at 55°C overnight in a humidity chamber containing 2X SSC soaked paper towels. Slides were washed in 2X SSC for 20m at 55°C. After a brief wash with PI buffer (0.2 M NaH_2PO_4, pH 7.8, 0.1% Igepal CA-630 from Sigma-Aldrich, Inc), the slides were mounted with Vectashield mounting medium (Vector Laboratories, Inc) containing DAPI (1.5 μg/ml of 4'-6-

Diamidino-2-phenylindole) counter stain. Image capture and data processing of FISH images were performed as described by Kato et al. (2004).

Results

Nitrous oxide treatment

Diploid maize plants were self-pollinated. Previous studies suggested that the first mitotic event post-fertilization in the endosperm occurs roughly 14-15 hours after pollination (MOL *et al.* 1994). To ensure doubling of the triploid nucleus, at 14 hours after pollination the maize plants were subject to nitrous oxide for 10 hours (Figure 2.3A), then relieved of the gas and grown to 10-14 days after pollination. Nitrous oxide treatment of the maize ear generated a high degree of defective kernels (Figure 2.3B). The defective kernels and a large portion of the normal kernels were harvested and examined for their genomic ploidy. First, acidic orcein stain were used (Figure 2.4), which showed that the defective kernels not only doubled (Figure 2.4B), but in some cases doubled twice (Figure 2.4C) or three times (Figure 2.4D).

Due to the defective nature of the endosperm, obtaining well-spread chromosome preparations proved to be difficult. This was overcome by using a FISH based approach (Figure 2.5). A molecular marker located near the centromere on chromosome 4 (Cent4) was used. The presence of three hybridization signals was interpreted to mean that the endosperm had three chromosomes 4, thus leading to the conclusion that the endosperm was triploid

(Figure 2.5A). If 6, 12, or 24 hybridization signals were seen, than the endosperm was concluded to be hexaploid (Figure 2.5B), dodecaploid (Figure 2.5C), or 24-ploid respectively (Figure 2.5D). The observation of 24 hybridization signals was rare, and more than 24 were never seen.

Using a combination of both orcein staining and FISH, 60 defective kernels were examined, 43 with a ploidy of hexaploid or greater (polyploidy), and three triploid (Table 2.1). Eleven defective kernels were mosaic, in that nuclei exhibited two ploidies of hexaploid or greater. Orcein staining revealed four aneuploid defective kernels with around 30 chromosomes or less. For comparison, full kernels, were also examined. Of 54 kernels, 22 were triploid and 32 were hexaploid (Table 2.1).

Figure 2.3. Nitrous oxide treatment of maize plants.

A) Nitrous oxide treatment chamber. Plants were pollinated and placed in this chamber with nitrous oxide gas for 5 or 10 HAP at 600KPa. B) Maize ear treated for 10 HAP with nitrous oxide gas, then removed and grown until 14 DAP. Black arrows point to defective kernels resulting from this treatment. HAP, hours after pollination. DAP, days after pollination.

Figure 2.3. Nitrous oxide treatment of maize plants.

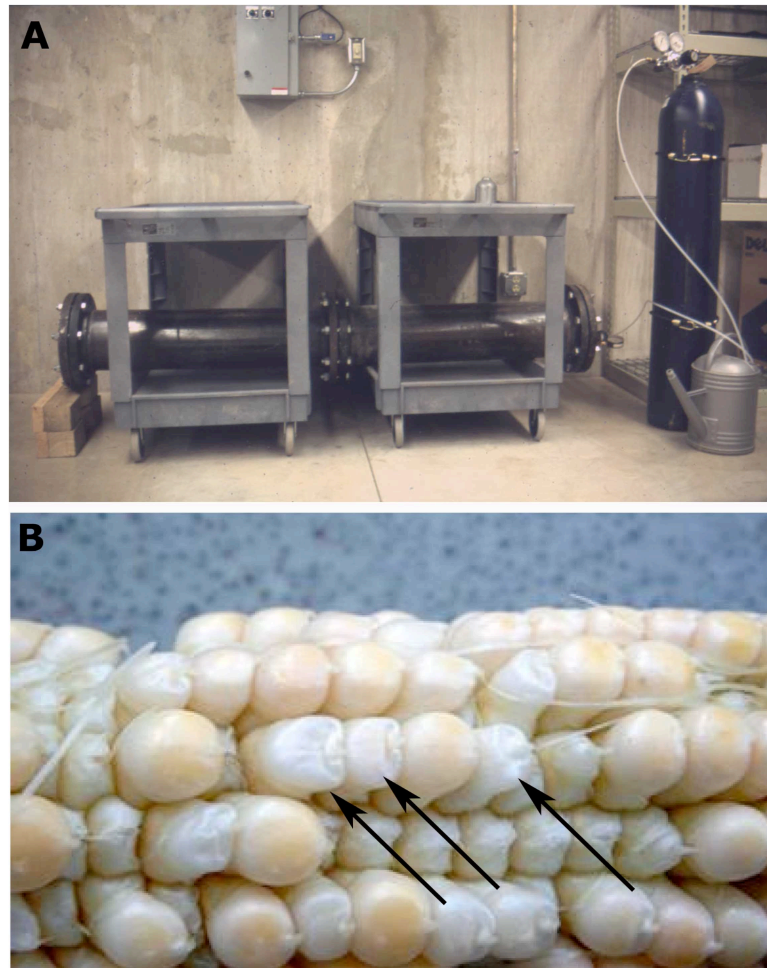


Figure 2.4. Chromosome spreads from maize endosperm tissue.

Maize endosperm tissue was harvest between 10-12 DAP. A) A chromosome spread from an endosperm tissue that was not treated with nitrous oxide. Chromosome spreads from defective kernels resulting from nitrous oxide treatment: B) 60 chromosomes, C) ~120 chromosomes. D) ~240 chromosomes. DAP, days after pollination.

Figure 2.4. Chromosome spreads from maize endosperm tissue.

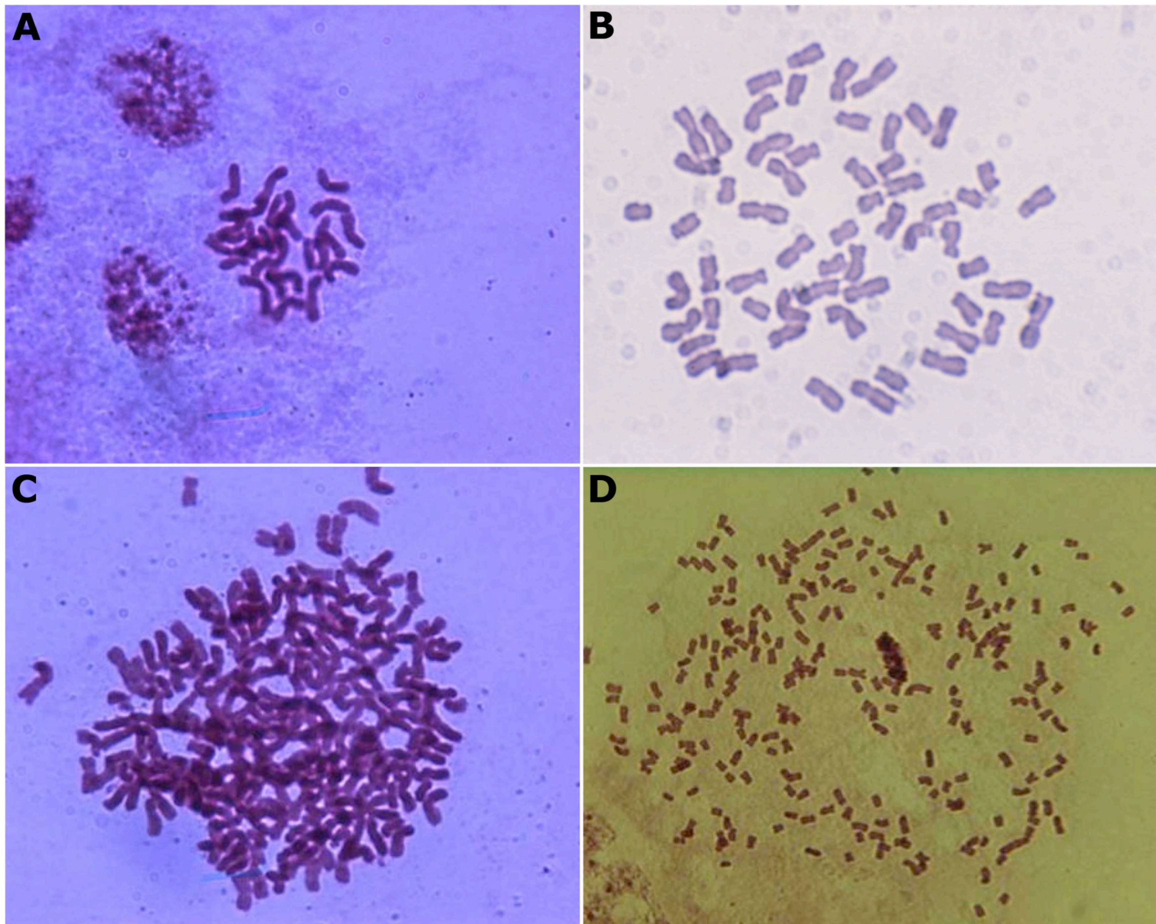


Figure 2.5. FISH for ploidy analysis

Cent4 was used as a FISH probe to determine ploidy. Cent4 was directly labeled with Oregon Green and appears green in all panels. A) Three Cent4 signals in a untreated kernel. FISH on defective kernels: B) Six Cent4 signals indicating hexaploidy C) Twelve Cent4 signals indicating dodecaploidy. D) Twenty four Cent4 signals indicating 24-ploidy. DAPI = blue, bar = 10μm.

Figure 2.5. FISH for ploidy analysis

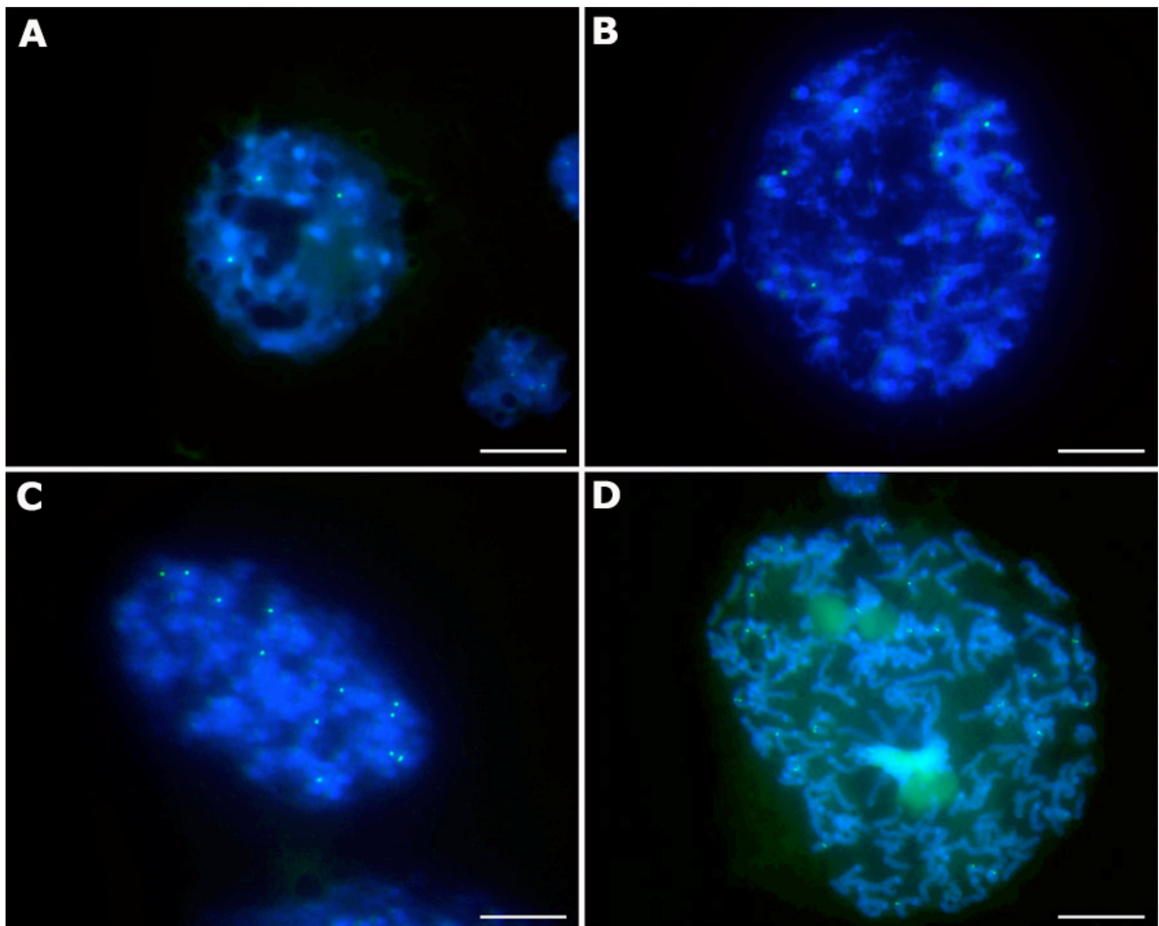


Table 2.1. Nitrous oxide creates polyploidy endosperms

Defective Kernels			Full Kernels		
Polyploid	=	43	Polyploid	=	32
Triploid	=	3	Triploid	=	22
Aneuploid	=	4			
Mosaic*	=	11			
N	=	60	N	=	54
*(both different polyploid)					

Differential nitrous oxide treatment time

Two types of endosperms have been recovered: the types that were defective, and the types that were normal. It is possible that the normal endosperms that were also polyploid could have arisen from successfully doubling of the syncytial endosperm, in which it contained two or more nuclei (Figure 2.6). This would indicate that there could be a developmental window in which doubling during that time would cause endosperm failure. To examine this issue, a nitrous oxide time course treatment was performed. Maize plants were treated with nitrous oxide at different time points after fertilization: 12, 14, 16, 20, 24 HAP. Figure 2.7 shows that when plants are treated early in development, there is a small amount of defective endosperms. When we apply the treatment closer to 16 and then to 20 HAP, the percentage of defective endosperms increased (Figure 2.7). When nitrous oxide treatment is applied at 24 HAP, the percentage of defective endosperms decreases (Figure 2.7 & Figure 2.8).

Thus, creating polyploid endosperms at later stages of development does not produce defective kernels. This could be explained by a failure of the nitrous oxide gas to create polyploid endosperms. However, this is not the case because we observe an increase in hexaploids in the normal endosperms (Figure 2.7). The increase in polyploid endosperms in the normal kernels then, in turn, decreases the total number of tripoid endosperms (Figure 2.7). This suggests that the nitrous oxide treatment creates polyploid endosperms at all stages that it was applied, but when the treatment was at a time point in which there was a high degree of syncytial endosperms at or beyond the two nuclei

stage, there was also a decrease in defective endosperms even though ploidy was increased (Figure 2.7). Treatment at the later stage of 120 HAP produced no defective kernels at all (Figure 2.8), which is consistent with a developmental window of susceptibility.

In the normal endosperms examined, a small portion seemed to fit into a category other than triploid or hexaploid or any multiple of three that could be explained by multiple doublings (Figure 2.7). Some endosperms were diploid, mosaic, or tetraploid. This number also seemed to slightly increase as the treatment time was applied at later stages of development. This suggests that, not only is the endosperm genome more tolerant of ploidy changes, but more tolerant of other genomic abnormalities.

Figure 2.6. Doubling at different stage of development and their hypothetical results.

A) Doubling at first mitotic division would result in an abnormal dosage of genes to target gene loci. This would cause the induction of an unfavorable developmental pathway resulting in a defective kernel. B) Doubling at the two nuclei stage (or beyond) is predicted to not adversely affect endosperm development.

Figure 2.6. Doubling at different stage of development and their hypothetical results.

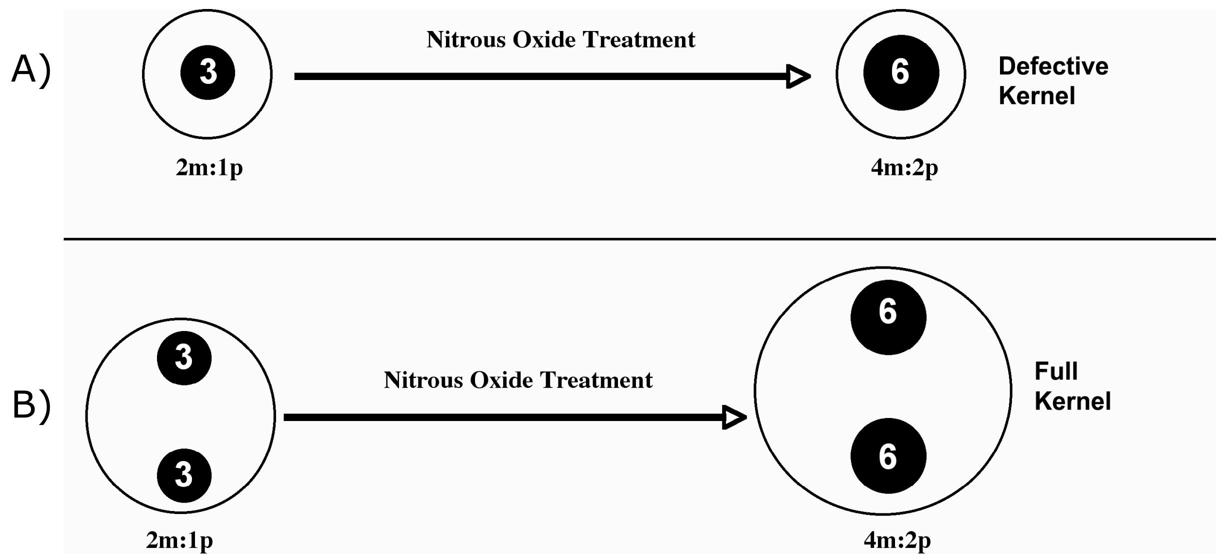


Figure 2.7. Effect of Nitrous Oxide on Maize Kernels. The genetic makeup of kernels harvested from both defective and normal kernels**.** As the nitrous oxide treatment is applied at time points before 16 HAP and after 20 HAP, the percentage of Defective Kernels (—◆—) decreased. When the nitrous oxide treatment deviate to later HAP, the percentage of hexaploid endosperms in the phenotypically normal kernels increase (▲) . As the percentage of hexaploid endosperm increase in the phenotypically normal kernels, the percentage of triploid endosperms decrease (■). In addition to an increase in hexaploid endosperms in phenotypically normal kernels, there is also a slight increase in endosperm that have a chromosomal makeup that is either mosaic, or a ploidy other than triploid and hexaploid (●). Trend lines were generated using the polynomial function in Excel (Microsoft, inc). HAP = hours after pollination.

Figure 2.7. Effect of Nitrous Oxide on Maize Kernels

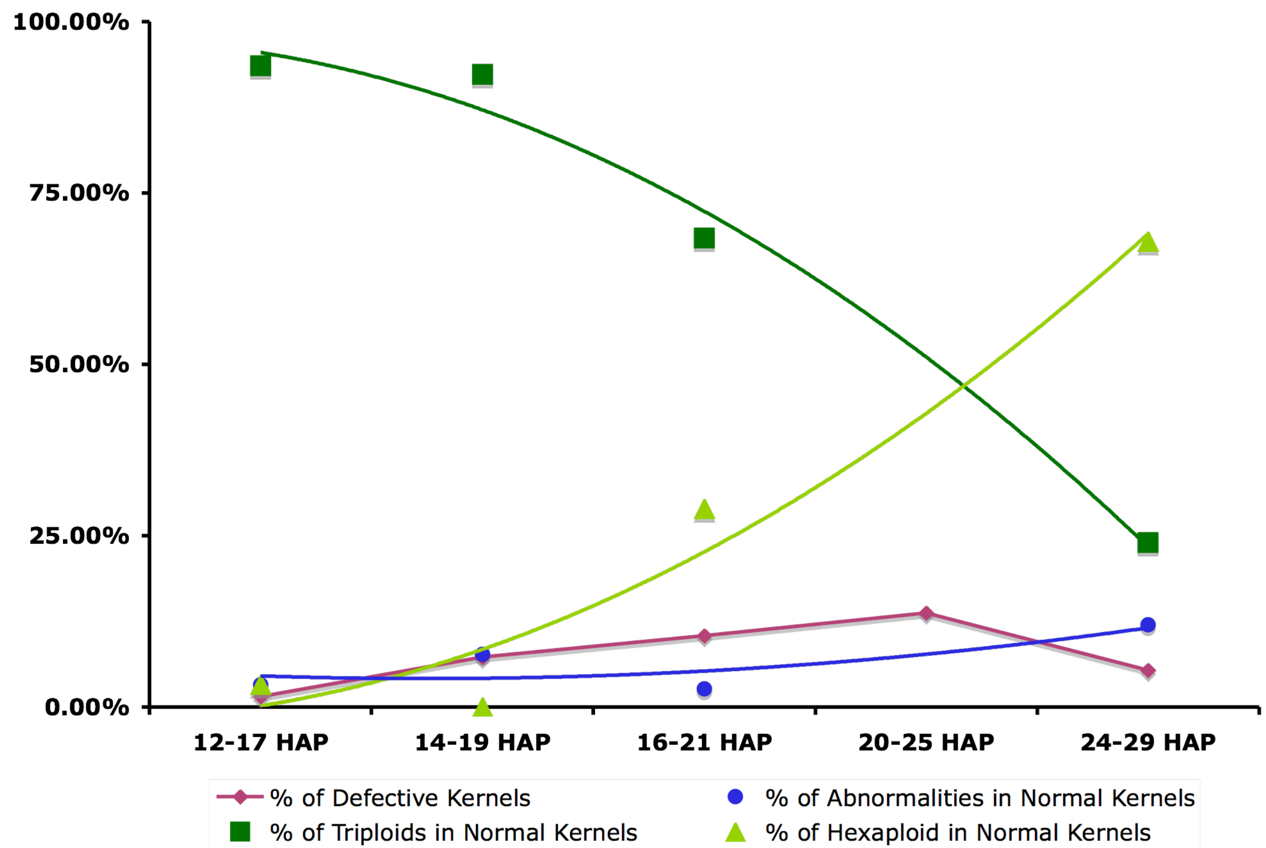


Figure 2.8. Nitrous oxide treatment of maize ears at 120 HAP

The left maize ear was not treated with nitrous oxide. The right maize ear was treated from 120-140 HAP with 600 KPa of nitrous oxide. After treatment, the ear was grown to maturity. Comparing the right maize ear with the left control maize ear, no defective kernels could be detected. Hours after pollination, HAP. KiloPascals, kPa.

Figure 2.8. Nitrous oxide treatment of maize ears at 120 HAP

Discussion

Defective kernels, due to interploidy crosses, have been thought to occur by an abnormal ratio of imprinted loci (HAIG and WESTOBY 1989; HAIG and WESTOBY 1991; WILKINS and HAIG 2003). Another explanation is that, in interploidy crosses, there is a differential dosage of regulators in the central cell relative to their target loci after fertilization (BIRCHLER 1993; DILKES and COMAI 2004). During interploidy crosses, both the ratio of inherited imprinted loci and the dosage of regulators to target loci are changed, and no known study has been done to test each one independently. To adequately test the regulator dosage hypothesis, nitrous oxide was used to double the chromosomal content of the endosperm before the first mitotic event post-fertilization. This would successfully test the regulator dosage hypothesis without disrupting the imprinted loci ratio. When plants were treated with nitrous oxide at 14-24 hours after pollinations, a significant increase in defective kernels was noticed (Figure 2.3B). Examination of the defective kernels indicated that the majority of them were not only hexaploid, but some were also dodecaploid and 24-ploid (Table 2.1). If the defective kernels were due other variables outside of polyploidy, we should observe a nearly equal number of triploid and polyploidy endosperms in our total number of defective endosperms harvested. Out of 60 endosperms harvested, the majority were polyploid indicating that creating polyploid endosperm soon after fertilization severely affected the developmental pathway. Using the regulatory dosage hypothesis, it would be predicted that the normal kernels would be triploid, successfully escaping the polyploid process. This was not

observed, but instead about a relatively equal ratio of triploid and polyploid endosperms was formed (Table 2.1).

To understand why polyploid endosperms create defective kernels in some instances, but normal kernels in others, a time course examination was performed. This was done because the maize ear contains hundreds of female ovules, in which all can be fertilized at a different time after pollination. At any given time point, there exist central cells that are not pollinated, endosperms that are at the one triploid nucleus stage, and endosperms that are at the two nuclei stage or beyond. At different time points after fertilization, the ratio of these changes. As time deviates away from pollination, there is first an increase in the number of fertilized central cells, which will soon be followed by a decrease of those endosperms that contain a single triploid nucleus, and an increase of endosperms at or beyond the two nuclei stage. The introduction of nitrous oxide gas at different time points after pollination would give different results in both the amount of defective endosperms and amount of polyploid normal endosperms, only if the timing of polyploidization were important. The results support this hypothesis because when nitrous oxide treatment was at the early time points (10 or 12 HAP), in which there is a high degree of non-fertilized ovules, it was observed that a low number of defective kernels formed. As the nitrous oxide treatments were moved to a later time point after pollination (14, 16, 18 HAP), we see an increase in defective endosperms because (Figure 2.7), more endosperms would be fertilized and thus be at the one nuclei stage. The amount of defective kernels decrease when the treatment of nitrous oxide gas was

moved to even later time points after pollinations (20 HAP and beyond) (Figure 2.7). This can be explained by the decrease of total number of single nucleus endosperm cells on an ear, and an increase of syncytial endosperm cells at the 2 or 4 nuclei stage. This suggests that the dosage of regulators is important at a set time period after fertilization to initiate the correct set of genes to begin normal endosperm development, but dosage of those regulators are not as important once the correct developmental pathway is underway.

Because treating endosperms at later time points decreases the production of defective kernels, we should observe an increase in polyploid endosperms in the normal kernels and consequently a decrease in triploid endosperms. The results shown in Figure 2.7 fit that prediction. It was also observed that nitrous oxide could produce normal endosperms that are tetraploid or mosaic (containing nuclei of two different ploidy at or above the hexaploid level). The endosperms containing those chromosomal contents also seem to increase slightly in phenotypically normal kernels (Figure 2.7). This suggests that not only are developing endosperms more tolerant of changes in the dosage of regulators to their respective target loci later in development, but also to other chromosomal abnormalities.

By using nitrous oxide, we can successfully test the regulator dosage theory without changing the ratio of imprinted loci. The results suggest that, soon after fertilization, there exist a set time period in which the dosage of regulators to target loci seems highly important. This dosage regulators triggers the correct suite of genes to be turned on, or remain silent, starting the correct

developmental pathway. After that time period, when the developmental pathway is set, creating polyploid nuclei does not disrupt it. This observation supports previous work investigating endosperm size factors, and is consistent with a maternal influence on endosperm development.

Endosperm size factors are possibly involved in dosage regulation

The endosperm size factors were uncovered using B-A translocation chromosomes in maize (BIRCHLER and HART 1987). Using B-A chromosomes, it is possible to create endosperms that are missing or gaining an extra chromosomal arm (BIRCHLER and ALFENITO 1993; ROMAN 1947). When some arms are missing, the endosperm was smaller than normal, and it was concluded that the missing chromosomal arm contained factors important for endosperm growth (endosperm size factors). B-A chromosomes can also, at a low frequency, be lost during mitosis. It was observed that if the B-A translocated chromosome was lost during early mitotic events after endosperm fertilization, the growth appeared undeterred and no discernible size difference compared to the normal kernels was noticed (BIRCHLER 1980; BIRCHLER 1993; BIRCHLER and HART 1987). This indicated that the endosperm size factors are needed during the early phases of development, most likely before the first mitotic division of the newly formed triploid nucleus. After that time period, the dosage of the endosperm size factors seemed to be of less importance to overall growth of the kernel. Because of the similarity in phenotype of the endosperm size factors and the results presented here, this suggests that endosperm size factors could be

involved in maintaining proper dosage of regulators in the central cell and/or post-fertilized endosperm.

Dosage regulation and the endosperm balance number

The dosage regulator hypothesis could also explain the observation of the endosperm balance number in potatoes (JOHNSTON *et al.* 1980). The diploid *S. chacoense* can successfully hybridize to the tetraploid *S. acaule*, but when tetraploid varieties of *S. chacoense* were created, they could no longer fertilize the *S. acaule* tetraploid (JOHNSTON *et al.* 1980). Before fertilization, the regulators located in the pre-fertilized cell of *S. chacoense* are in the correct dosage to trigger target loci and activate a favorable developmental pathway after fertilization with haploid sperm from *S. acaule*. When *S. chacoense* genome was doubled to a tetraploid, this changed the ploidy and size of the pre-fertilized cell, and also the dosage of regulators. When the *S. chacoense* tetraploid was crossed with *S. acaule*, there was a differential dosage of regulators to target loci and the developmental pathway initiated leads to a defective seed. This indicates that overall ploidy is not important, as long as the regulators are in the correct dosage compared to their target loci, and that all the necessary genes for later stages of development are present.

Evolution of genomic imprinting

The results also suggest an additional selective pressure for the evolution of imprinting in the maize endosperm. The parental conflict hypothesis argues that imprinting arose because of the different developmental interest of the inherited genomes in the maize endosperm (HAIG and WESTOBY 1989; HAIG and WESTOBY 1991). The male inherited genomic content is interested in acquiring as many resources as possible, in an effort to detour resources from other developing endosperms that could have been fertilized by a competing male. The maternally inherited genomes have a developmental interest in every endosperm on the ear and express genes that suppress nutrient acquisition, which encourages equal distribution of resources to all seeds. This will ensure equal survival of the kernels. According to the parental conflict hypothesis, genomic imprinting could have evolved because of the competing interest of the inherited genomes. Inherited male genomes would need to imprint or silence genes that would encourage resource sharing, and express genes that would encourage resource acquisition. The inherited maternal genomes, having an interest in survival of all kernels, would need to imprint or silence genes involved in nutrient acquisition and express genes that encourage equal resource distribution. This also has been further extended to suggest why endosperms inherit multiple maternal genomes.

The nitrous oxide treatment results suggest that imprinting could have also evolved to maintain the correct dosage of developmental regulators in the pre-fertilized central cell by controlling gene expression. The evolution of the maize

endosperm remains an enigma. The evolutionary pressure that would cause addition of the second maternal genome is unknown, but it is logical that, when this occurred, a differential dosage of regulators to genetic target loci would arise. To ensure proper endosperm development, gene expression would have to be adjusted so that the regulators are back to the proper dosage. Imprinting could have arisen as a means to modify that expression (BEAUDET and JIANG 2002). Dosage regulation as a selective pressure for genomic imprinting evolution adequately explains the observation of imprinting being limited to that of the endosperm and not the embryo (ALLEMAN and DOCTOR 2000). It would be interesting to examine the presence of genomic imprinting in genus's like *Nymphaea* that have a diploid endosperm (BAROUX *et al.* 2002a).

Chapter 3:

Defective Kernels Due to a High Haploid Inducing Line of *Zea mays*

Introduction

In most organisms, division of an egg cell is repressed until fertilization. In plants, an extremely small portion of pollinations result in cell division and embryo development without fertilization. This has been documented to occur in multiple ways: 1) a gamete that does not reduce its chromosome number, forming a diploid egg, which proceeds directly to development; 2) a diploid somatic cell that dedifferentiates and proceeds to develop into an embryo 3) the spontaneous development of a male gamete (after pollen tube penetration), egg or any other cell of the embryo sac (BYLICH and CHALYK 1996; CHALYK 1994; LASHERMES and BECKERT 1988).

The production of haploid maize plants has great potential in maize breeding and genetics (CHALYK 1994). One useful tool is to double the haploid to produce a completely homozygous diploid line. Typically, homozygous lines are generated by self-fertilizing a plant up to 7 generations, minimum. By utilizing doubled haploids, the time required for the generation of inbred lines can be reduced by about half. Doubled haploids also allow the expression of recessive mutants and/or other genes that have a favorable effect on growth and

reproduction. They also have the capacity to completely remove lethal or harmful alleles from the genetic background (CHALYK 1994).

Spontaneous maternal haploid production has been reported for many species (KIMBER and RILEY 1963; MAGOON and KHANNA 1963). The first haploid maize was reported by Stadler and Randolph (unpublished 1929, reported in (RANDOLPH 1932). The cause of spontaneous haploids is unknown, but for them to occur, there must be a breakdown in the molecular repression of development prior to fertilization. An excellent method of examining the breakdown of this repression is by exploiting plants that have a dramatic increase in spontaneous haploid production. Such a maize line, Stock 6, was found by Coe (COE 1959; COE and SARKAR 1964). For comparison, an examination of 29 different American and European maize germplasm resulted in a haploid frequency of .07% [16 haploids/23,300 fertilization events (LASHERMES and BECKERT 1988)]. Stock 6 increased that frequency over ten-fold, averaging a maternal haploid production of 3.2%, but only when used as a male or pollen parent.

Stock 6's high haploid production ability has many hypothetical causes. One possibility is that both sperm fertilize the polar nuclei, creating a tetraploid endosperm while triggering development of the haploid egg. Currently, endosperms associated with a haploid embryo have all been shown to be triploid (SARKAR and COE 1966). Another presented hypothesis is that the haploid embryo arises from one of the sperm fertilizing the central cell while the second sperm fertilizes a different cell in the embryo sac. The initiation of haploid egg development, in addition to the development of a second fertilized diploid cell

would result in a kernel having twin embryos: one diploid and one haploid (COOPER 1943), but there were not any observable twin diploid embryos associated with the haploid embryos (SARKAR and COE 1966).

It has also been postulated that the developmental timing of the two sperm were different, resulting in some pollen tubes containing one mature sperm and another immature sperm, in which the immature sperm lost its capacity to fertilize the egg, but maintained the ability to trigger haploid egg development (BYLICH and CHALYK 1996). A cytogenetic study was done to study the sperm nuclei, and any significant structural differences could not be established, indicating that all of the sperm in Stock 6 were morphologically normal (SARKAR and MAHENDRU 2000).

To understand the genetics of haploid induction, several different high haploid producing lines of maize were crossed together to test if different loci could affect the haploid induction rate. When several high haploid producing lines of maize were crossed together, higher haploid producing lines were generated. One particular line, RWS, could produce up to 10% haploids (LASHERMES and BECKERT 1988). Under further examination of RWS, it was found that, not only were haploids produced at a much higher frequency, but there was also a high rate of defective kernel production (Figure 3.1, Table 3.1). We hypothesized that since RWS has a high rate of spontaneous embryo development, it also has a high rate of spontaneous development of the endosperm as well. Spontaneous endosperm development would be diploid which would disrupt the dosage of regulators to there target loci in the central cell

prior to development, resulting in a spontaneous and erroneous developmental pathway leading to defective kernels.

Materials and Methods

Genetic stocks and crosses

RWS, RWS-Green Fluorescent Protein (RWS-GFP), and Oh43 diploid and tetraploid plants were grown in the Sears Plant Growth Facility on the University of Missouri campus. The GFP transgene was expressed by the 35S constitutive promoter. RWS was self pollinated, and RWS-GFP was crossed to Oh43.

Nuclei spreads and fluorescent *in situ* hybridization

Kernels were harvested and fixed in 90% acetic acid for 10 minutes, and than stored in 70% ethanol. Subsequent nuclei spreading and Fluorescent *in situ* hybridization were performed as described on page 29-31 (KATO *et al.* 2006).

Maize diploid Oh43 maize plants, along with a diploid *c1* tester line, were pollinated with RWS-GFP were harvested, and pictures were taken on the Leica stereomicroscope (Leica Microsystems) using the Leica DFC290 digital camera. The GFP and FISH images were adjusted using Adobe photoshop 7.0.

Results

Defective endosperms

Maize high haploid inducing line, when used as the male or pollen parent, produced around 18% defective kernels (Figure 3.1, Table 3.1). This is in contrast to other maize plants, which produce less than 0.1% defective kernels. Thirty-five defective kernels were planted, but zero germinated.

RWS does not produce a higher than normal amount of diploid pollen

The defective kernels at 14 DAP showed a high degree of variability, but on average were much smaller than the normal kernels (Figure 3.2A). The morphology of the defective kernels resembled that of the defective kernels from an interploidy cross (Figure 3.2A). This could be explained by an increased production of diploid pollen. If RWS produced a higher than normal amount of diploid pollen, than a larger then normal number of phenotypically normal kernels would occur when RWS is crossed to a tetraploid. When the female tetraploid was fertilized by the male RWS, all defective kernels resulted (Figure 3.2B), indicating that there is not a higher number of diploid pollen produced.

Figure 3.1. RWS when used as a pollen parent causes defective kernels

A) RWS when used as male causes severely defective kernels as indicated by the black arrows. B) Isolated kernels of the two types with normal kernels on the left and defective kernels on the right.

Figure 3.1. RWS when used as a pollen parent causes defective kernels.

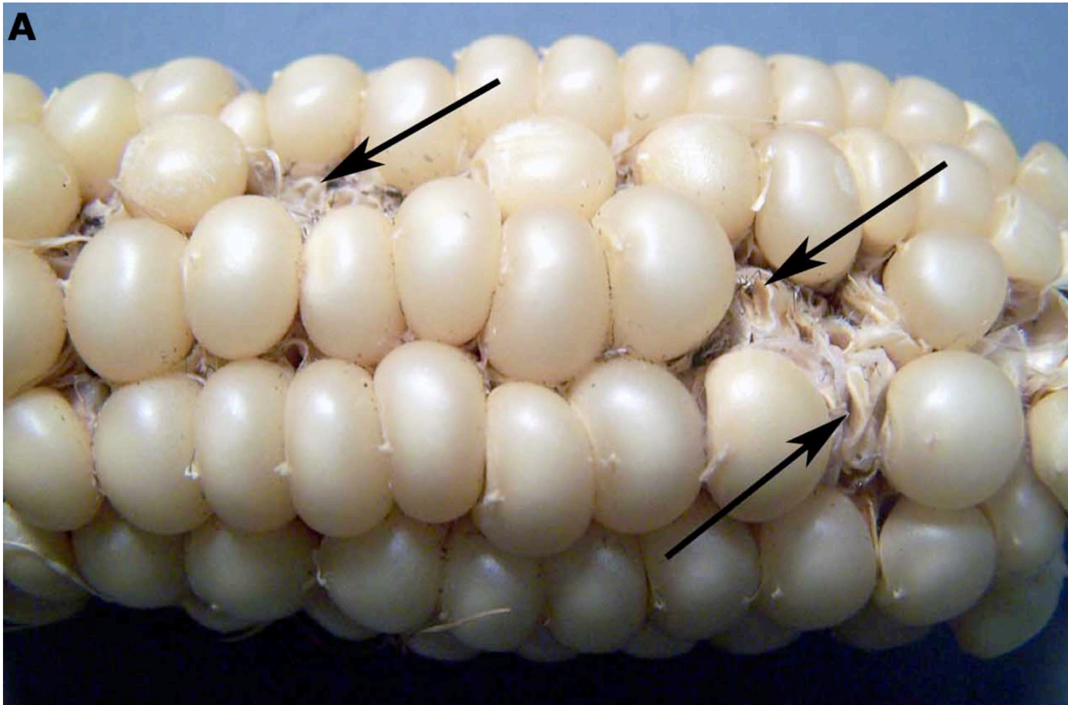


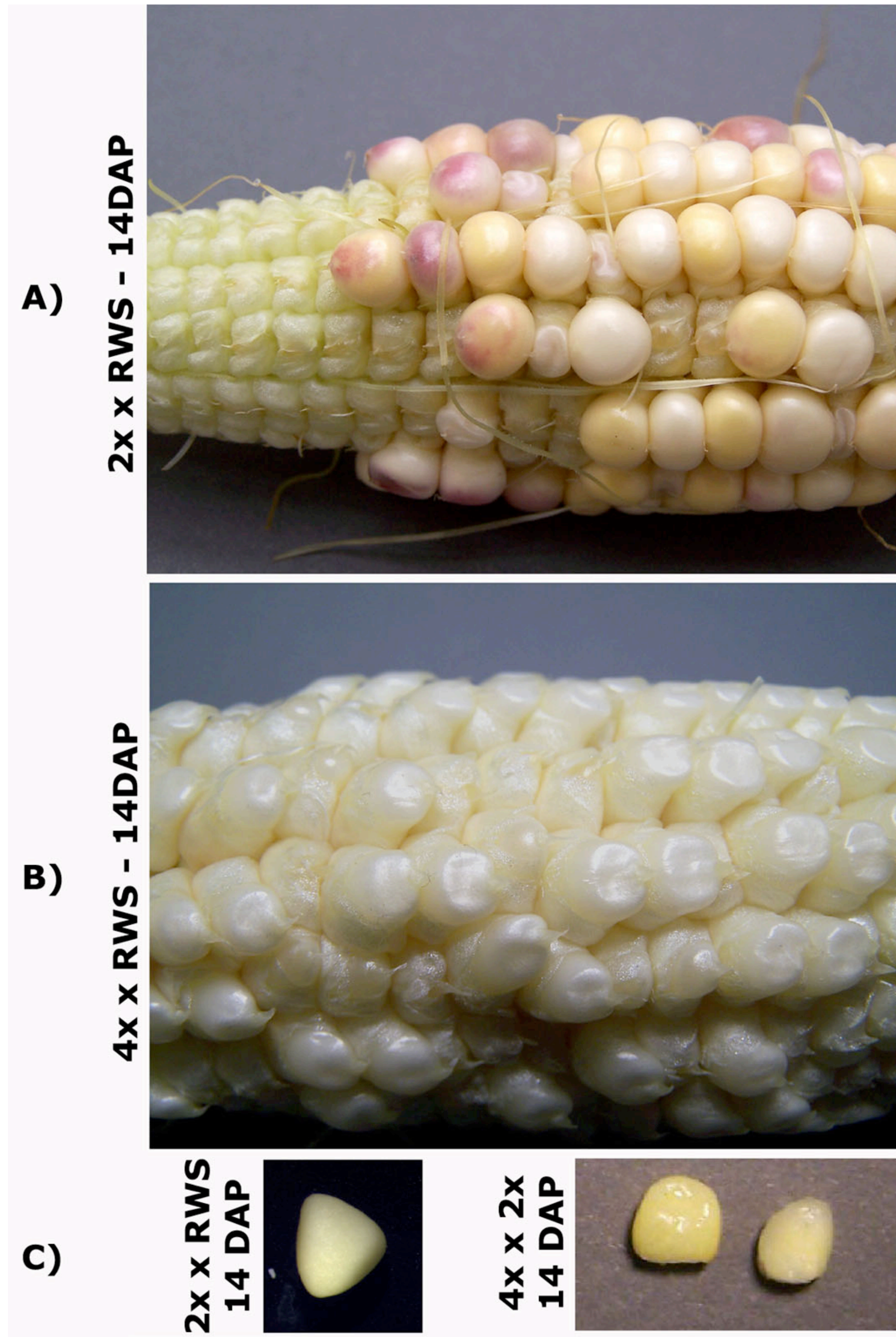
Table 3.1. RWS when used as a pollen parent produces a high percentage of defective kernels.

		Normal	Defective	%Defective
Ear 1	171	138	33	19.30%
Ear 2	163	137	26	15.95%
Ear 3	118	96	22	18.64%
Total Kernels	452	371	81	17.92%

Figure 3.2. Defective kernels morphologically resemble those of a 4x x 2x interploidy cross.

A) A 2x maize plant crossed with RWS at 14 DAP. B) A maternal tetraploid crossed by RWS. No normal or more filled kernels are formed indicating that RWS does not form diploid pollen. C) When the endosperm of a defective kernel from the 2x x RWS cross was isolated, the morphology resembled that of a 4x x 2x interploidy cross in both size and starch accumulation. DAP = days after pollination. 2x = diploid. 4x = tetraploid.

Figure 3.2. Defective kernels morphologically resemble those of a 4x x 2x interploidy cross.



Defective kernels are not due to spontaneous development of the endosperm

When the endosperms of a defective kernel was isolated, it resembled those of a 4x x 2x interploidy cross defective endosperm in both biomass and starch accumulation (Figure 3.2C). Both the parental conflict hypothesis and regulator dosage hypothesis would predict that defective endosperms would occur if the central cell spontaneously developed without fertilization, resulting in diploid endosperms with no paternal contribution. Along with the fact that RWS has a higher than normal rate of haploid embryo development, it was examined if there is a high rate of spontaneous development of unfertilized central cells. To test this, RWS containing a GFP transgene (RWS-GFP) expressed by the 35S constitutive promoter was crossed to a diploid maize plant. Spontaneous developing endosperms, not receiving the parental genomic contribution, would not be expected to express GFP. Of the 22 of defective kernels examined, all but two expressed GFP (Figure 3.3).

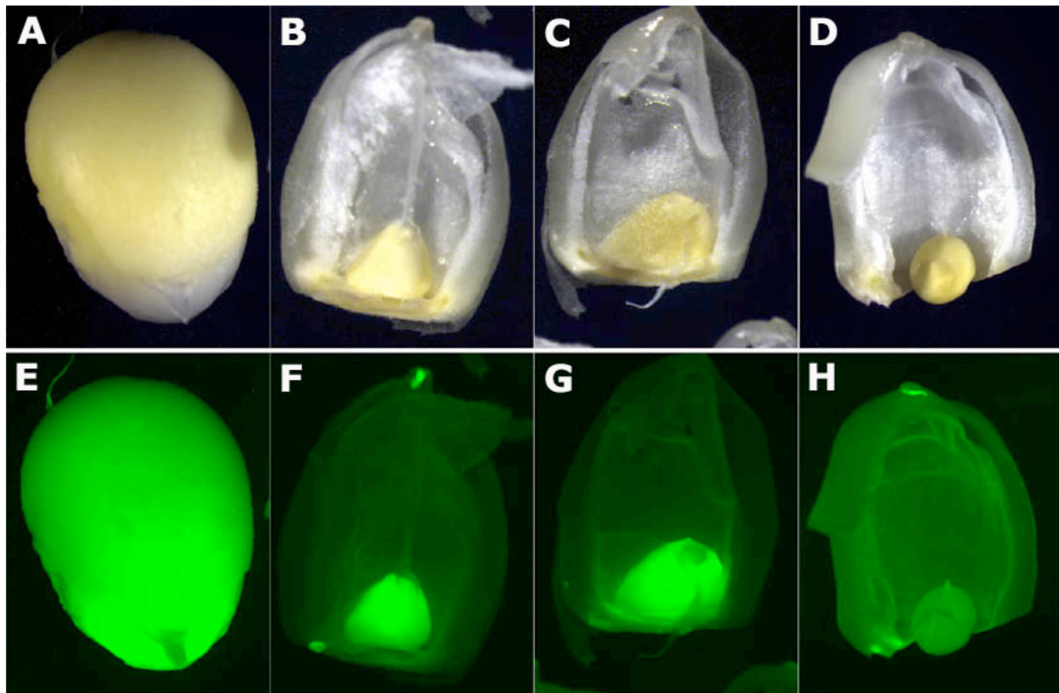
Defective kernels are mostly, but not always, triploid

To further examine the ploidy of the defective kernels, fluorescent *in situ* hybridizations were conducted using Cent4. Cent4 is a molecular marker located near the centromere of chromosome 4, thus three Cent4 fluorescent signals would be observed in a triploid nucleus. Two Cent4 fluorescent signals would be observed in a spontaneously developing diploid endosperm, and four Cent4 fluorescent signals would be an endosperm pollinated by a diploid pollen. Of 19

Figure 3.3. Defective kernels are not due to spontaneous development of the central cell

Kernels harvested at 14 DAP from a 2x x RWS ear. A) A normal kernel. B-D) Defective kernels, which are small and do not fill the pericarp. E) Normal kernel from A, expressing GFP indicating it was fertilized by sperm from RWS-GFP maize plant. F & G) kernels from B & C respectively expressing GFP indicating they were pollinated by sperm from the RWS-GFP maize plant. This occurred for 20/22 examined defective kernels. H) Kernel from D that is not expressing GFP, which occurred 2/22 examined defective kernels.

Figure 3.3. Defective kernels are not due to spontaneous development of the central cell



defective kernels screened, 12 showed three Cent4 fluorescent signals indicating they were triploid (Figure 3.4A, Table 3.2). In the other five kernels, two were tetraploid, one was hexaploid, and one had a mixture of tetraploid and octoploid nuclei (Table 3.2). The tetraploid and tetraploid/octoploid endosperms also expressed GFP ruling out contamination as a possible explanation. Two other defective endosperms showed abnormalities in their ploidy in which both had a mixture of nuclei that had two or three Cent4 signals (Figure 3.5).

RWS and aneuploid sperm

It has been hypothesized that pollen from a high haploid producing line of maize contains a low percentage of aneuploid pollen (CHALYK 2003). An attempt was made to address this hypothesis by examining the FISH hybridization pattern of other molecular markers: the maize 5S rDNA gene family is located on chromosome 2, the 16S rDNA gene family on chromosome 6, and the *rp1* gene family on chromosome 10. The distribution of these FISH markers confirmed that 12 of the collapsed kernels were triploid (Figure 3.4), two were tetraploid, one was hexaploid, one had a mixture of tetraploid/octoploid nuclei (Table 3.2). It was also confirmed that two endosperms seemed to have abnormalities in their chromosome ploidy. One endosperm contained a mixture of nuclei in that some contained two signals of NOR, while others had three (Figure 3.5C-D). The other endosperm contained a mixture of nuclei in that some contained two cent4 signals and three *rp1* signals, while other nuclei contained three Cent4 and four *rp1* signals (Figure 3.5E-F). If the last two endosperms were defective due to

Figure 3.4. FISH analysis of defective kernels resulting from 2x x RWS pollinations

A defective kernel that is triploid for Cent4, *Rp1* (A), 5S rDNA loci (B), and NOR region. DNA was stained with DAPI = blue, bar = 10μ.

Figure 3.4. FISH analysis of nuclei from defective kernels resulting from 2x x RWS pollinations

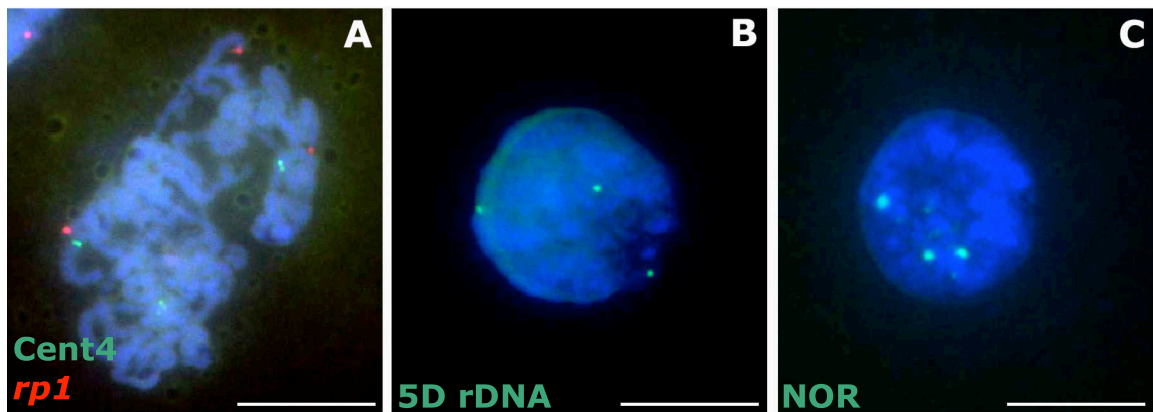


Table 3.2. Majority of defective kernels appear to be triploid.

	Triploid	Tetraploid	Hexaploid	Mosaic	Abnormalities	Total
Ploidy	13	2	1	1[+]	1[*],1[^]	= 19

+ Mosaic of tetraploid and octoploid nuclei.

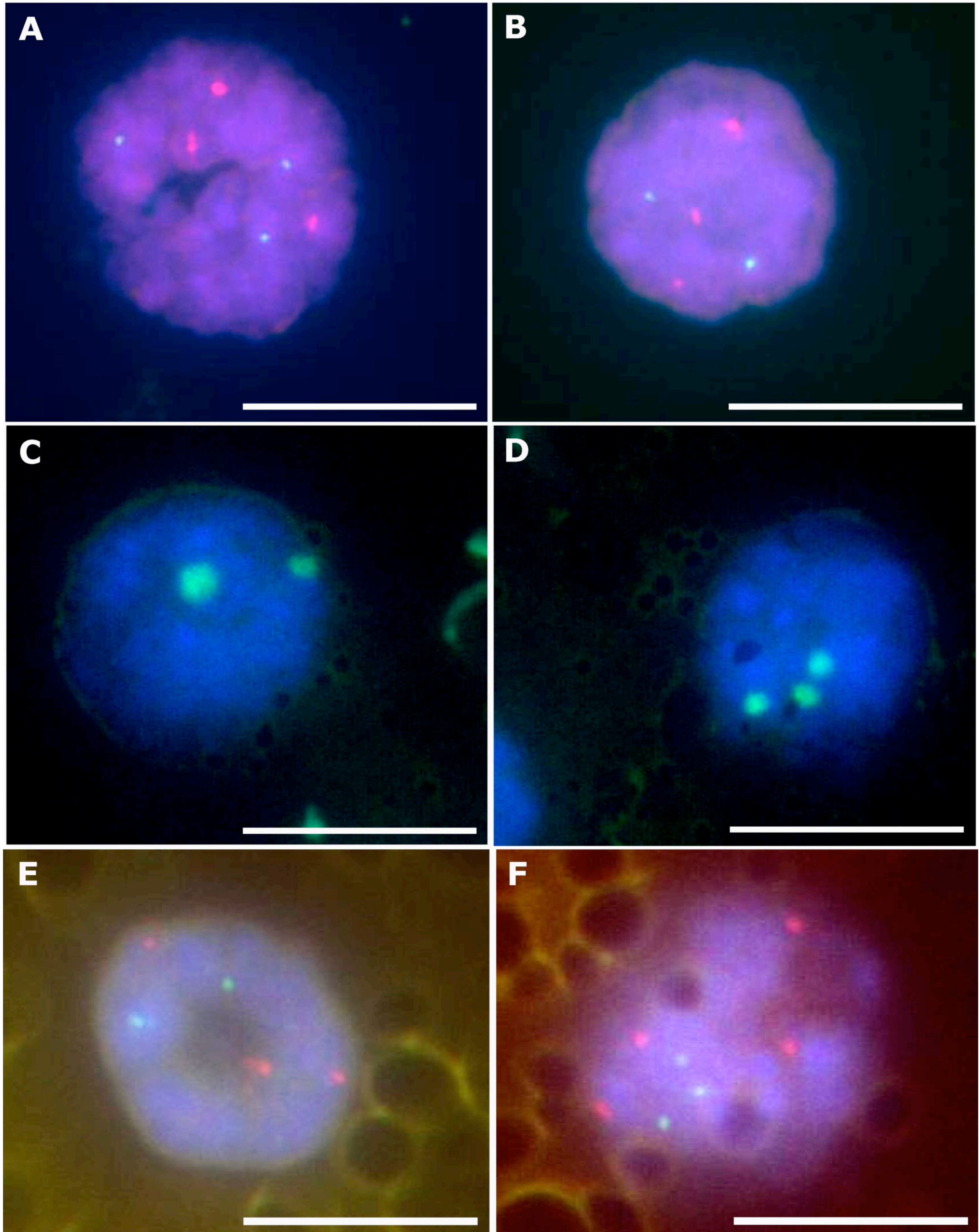
[*]Nuclei were a mosaic of diploid and tripoid for NOR and Cent4. Triploid for all other markers.

[^]Nuclei were a mosaic of triploid and tetraploid for *rp1.* Triploid for all other markers.

Figure 3.5. 2x x RWS defective kernels with chromosomal abnormalities

A-D) A maize endosperm that contains a mixture of nuclei that varies from three Cent4 signals (A) and two Cent4 signals (B). This same endosperm in A & B also has a mixture of nuclei that varies from two NOR signals (C) or three (D). E-F) A different maize endosperm tissue that contains a mixture of nuclei that when there were two Cent4 signals, there were also three *Rp1* signals (E) and when there were three Cent4 signals, there were also four *Rp1* signals (F). DNA was stained with DAPI = Blue. Bar = 10μm.

Figure 3.5. 2x x RWS defective kernels with chromosomal abnormalities.



aneuploid pollen, in would be expected that the endosperms, being derived from one endosperm nucleus, would all contain the same aneuploid morphology. This suggests that the defective kernels are not due to aneuploid sperm. It should be noted that the endoreduplicated nature of the endosperm tissue, could give false FISH signals, so this type of analysis may be need further verification.

Discussion

When RWS is used as a pollen parent, approximately 17% of the resulting kernels are defective (Table 3.1). Phenotypically, the resulting morphology of the defective kernel was similar to that of the defective kernels created from interploidy crosses (Figure 3.2A&C). Crossing RWS onto a tetraploid should reveal the presence of diploid pollen by developing phenotypically normal kernels. When this was attempted, all defective kernels were formed on the maternally derived tetraploid ear, indicating the near absence of diploid sperm formation (Figure 3.2B). This result supports previous observations by Mahendru and Sarker (2000). Tetraploids often contain enlarged nuclei and cells. In tetraploids, the sperm would be diploid and should consequently form larger sperm nuclei than haploid sperm produced by diploid plants. In the sperm examined from a high haploid producing line, all appeared cytologically identical (SARKAR and MAHENDRU 2000). Because the majority of the kernels formed from using a paternal RWS maize plant contain diploid embryos and triploid endosperms, this indicates that most of the sperm nuclei are haploid. Since all

the sperm examined looked essentially identical, this indicates that they all were likely haploid (SARKAR and MAHENDRU 2000).

The morphology of the defective endosperm being similar to that of a 4x x 2x interploidy cross (Figure 3.2.C) and also because of the high rate of spontaneous embryo formation of RWS, it is reasonable to suspect that the defective kernels maybe due to spontaneous development of the central cell. The spontaneously developing central cell would be diploid and lacking the paternally inherited genome. This would disrupt both the ratio of imprinted loci predicted by the parental conflict hypothesis and the dosage of regulators relative to their target loci predicted from the regulator dosage hypothesis. To test the spontaneously developing central cell hypothesis, RWS paternal plants carrying the green fluorescence protein transgene was crossed to a maternal Oh43 diploid maize plant. In 22 defective kernels resulting from this cross, all but two were expressing GFP (Figure 3.3). To verify the GFP expression results, fluorescent *in situ* hybridization using Cent4 was carried out. The majority of kernels contained three Cent4 flourescence signals indicating that they were triploid. Interestingly, two kernels were tetraploid, one was hexaploid, and one was a mosaic of tetraploid/octoploid nuclei, and two contain abnormalities in their ploidy. In those latter 7 cases, all were expressing GFP indicating that the pollen parent was the RWS-GFP. The tetraploid endosperms can be adequately explained by the formation of diploid pollen, which has been observed to occur normally at a low frequency (RANDOLPH 1935; SARKAR and MAHENDRU 2000). It has also been previously observed that, at a very low frequency, the female

ovule will be unreduced (RANDOLPH 1935). Combining that with the low rate of diploid pollen even though extremely rare, could explain the hexaploid kernels.

Defective hexaploid endosperm

The parental conflict hypothesis and the regulator dosage hypothesis would predict the development of defective tetraploid endosperms. It is interesting that the hexaploid endosperm fails to develop normally. As predicted by the parental conflict theory, if the ratio of inherited parenal loci is two maternal and one paternal, then development should proceed normally. The hexaploid kernel did not, indicating one of the following: 1) either the correct ratio of 4m:2p imprinted loci was not enough for proper development; 2) genomic methylation pattern was disrupted, changing the inherited imprinted loci ratio; 3) fertilization of a tetraploid sperm and a diploid central cell; or 4) environmental conditions caused the formation of the defective kernel. The abnormal development of a hexaploid kernel could be predicted from the regulator dosage hypothesis. Evidence suggests that the genotype of the maternal plant has an influence upon the development of the gametophyte (BIRCHLER 1993). Even if the central cell is tetraploid, an incorrect dosage of regulators relative to their target loci could possibly occur if the diploid sperm from RWS has loci expressing differently than normal. A test by Watkins (1932) indicated that the maternal plant does not greatly affect the overall development of the maize endosperm, but he used two closely related lines of maize, in which slight differences in dosage may be tolerated. These slight changes may not be tolerated if Oh43 and RWS are more

evolutionarily distantly related. Overall, because of the extremely low hexaploid sample size, further tests need to be conducted.

Aneuploid sperm hypothesis

For defective kernels to also arise from the fertilization from aneuploid sperm, there must be an extensive amount of aneuploid pollen produced. Usually missing one chromosome from the paternal parent does not create defective kernels to the extent that that has been observed with paternal RWS (BIRCHLER 1980; BIRCHLER 1993; BIRCHLER and ALFENITO 1993; BIRCHLER *et al.* 1990). If aneuploidy is the cause of the high amount of defective kernels when RWS is used as the pollen parent, then RWS must produce a higher than normal amount of singular chromosomal duplicated or deficient sperm. A single chromosome difference would not be expected to be highly noticeable on a cytological level and could escape the observations of Mahendru and Sarker (2000). Preliminary work, examining the male floral meiotic chromosomes during diakinesis of prophase, suggests that aneuploid pollen could be produced at a high level (CHALYK 2003), but an examination should be done to assess the rate of pollen abortion, which would test this idea as the cause of defective kernels. Aneuploidy is an interesting hypothesis and should be considered. To address this issue, FISH probes for other chromosomal locations were used to expand on the Cent4 ploidy analysis. All kernels analyzed confirmed the Cent4 study (Table 3.2; Figure 3.4). Two endosperms showed abnormalities in their chromosomal ploidy (Figure 3.5). One endosperm contained a mixture of nuclei

in which some showed two signals of Cent4 or NOR, while others showed three signals of Cent4 or NOR (Figure 3.5A-D). The other endosperm showed a mixture as well, in which some nuclei had two signals of Cent4 and three signals of *rp1*, while other nuclei contain three signals of Cent4 and four signals of *Rp1* (Figure 3.5E-F). If the last two endosperms were pollinated by aneuploid sperm, the mosaic nature of the endosperm would not be expected because all nuclei are derived from a single fertilization event. One explanation of the abnormal FISH signals would be due to the endoreduplicated nature of the maize endosperm.

Endoreduplication is when the cell cycle continues through multiple rounds of DNA synthesis without cytokinesis. This results in an increase in ploidy of the maize endosperm nuclei. It is possible that extra signals could not be due to aneuploidy, but due to endoreduplicated chromosome strands. Also, because of the 3-D nature of the nucleus, two signals could be close together or on top of each other resembling one signal. This would result in the observation of only two signals in some nuclei but not others. Even though aneuploidy may be unlikely, it has not been appropriately disproven and further examinations need to be completed.

Regulator dosage hypothesis and defective kernels from RWS

Since the paternal genome is haploid, creating a triploid endosperm after fertilization, but developmental failure occurs regardless, can be adequately explained by the regulator dosage hypothesis. According to the regulator dosage

theory, the central cell contains the correct dosage of regulators to trigger the appropriate developmental pathway. There is a correlation between the expressed or silent state of a gene and its level of DNA methylation. An expressing gene has very little DNA methylation, while a silent gene has a large amount of DNA methylation (BENDER 2004). Defective kernels could arise if the amount and/or distribution of DNA methylation has changed in some of the inherited paternal genomes, changing the expression potential of the central cell regulators target loci.

This hypothesis could also explain why the defective endosperms have a morphologically similar phenotype to that of a maternal tetraploid and a paternal diploid (4x x 2x) interploidy cross. If the methylation patterns have changed, it would be expected that some of the target loci could have a reduced or silent expression. This change would mimic the previously mentioned interploidy cross because of the relatively elevated regulators to the gene expression output of the target loci. The altered methylation patterns could potentially also explain the abnormalities observed. If the methylation patterns are altered at the centromere, one could hypothesize that it would disrupt its function and cause some chromosomes to non-disjoin or be lost during mitosis, but any connection between methylation and centomere function needs further examination.

Chapter 4:

Organization of Endoreduplicated Chromosomes in the Endosperm of *Zea mays* L.

Introduction

The endosperm results from one of the two products of double fertilization in flowering plants. When the pollen tube enters the ovule, two sperm nuclei are released. One sperm will fertilize the haploid egg cell, which will develop into the diploid embryo. The other sperm will fertilize the diploid central cell, which will form a triploid endosperm (OLSEN 2001). After fertilization, the endosperm proceeds through several rounds of nuclear division without cytokinesis to form a multi-nucleated syncytium (Figure 1.1B). By the fourth day after pollination (DAP), the endosperm cellularizes the nuclei (Figure 1.1C), and mitosis then follows with the highest mitotic index occurring between 10-12 DAP. The endosperm cells begin to differentiate into four types: basal endosperm transfer layer, embryo surrounding region, aleurone layer, and the starchy endosperm. The basal endosperm transfer layer forms at the base and is thought to be involved in nutrient transfer between the maternal plant and the endosperm (HUEROS *et al.* 1999). The embryo surrounding region is named after its morphology and appears to help regulate nutrient flow between the endosperm and the growing embryo (OPSAHL-FERSTAD *et al.* 1997). The aleurone layer

surrounds the endosperm and is active during seed germination; it breaks down the nutrients stored in the starchy endosperm into simpler compounds that can be easily absorbed by the embryo.

The starchy endosperm, starting as early as 10 DAP, will forgo the common cell cycle, and begin an endoreduplication phase (DILKES *et al.* 2002; KOWLES and PHILLIPS 1985). The classical cell cycle occurs in four distinct stages. A rest phase (G1) is followed by the DNA replication phase (S). After the S-phase, the cell will rest again (G2) before entering the mitotic phase (M). Endoreduplication is a variant of the cell cycle, in which the DNA replicates but skips most if not all of the M-phase (EDGAR and ORR-WEAVER 2001). This process results in an increase in nuclei ploidy and in cell size. The increase in cell size occurs coincidentally with the accumulation of nutrients (LOPES and LARKINS 1993). The cells in the center most portion of the endosperm enter endoreduplication first, followed by the most adjacent cells and so forth, resulting in cells proceeding through different rounds of endoreduplication (DILKES *et al.* 2002; KOWLES and PHILLIPS 1985; LARKINS *et al.* 2001). Beginning around 20 DAP, the cells in the starchy endosperm start to disintegrate, and by 40-45 DAP all cells in the endosperm, excluding the aleurone layer, have degraded.

The amount of endoreduplication a nucleus can achieve depends on the environment and genotype (BAROW and MEISTER 2003; DILKES *et al.* 2002; KOWLES and PHILLIPS 1985; SETTER and FLANNIGAN 2001). Roughly 90% of all angiosperms have endoreduplicating tissues (D'AMATO 1984). Endoreduplication outside of the endosperm has been observed in maize roots, embryos (BIRADAR

et al. 1993), and leaves (CAVALLINI *et al.* 1997). Across the plant kingdom, endoreduplication has been observed in various types of vegetative, flowering, embryonic, and fruit tissues (BAROW and MEISTER 2003; JOUBES and CHEVALIER 2000; KUDO and KIMURA 2002; LEMONTEY *et al.* 2000; NAGL 1974; TRAAS *et al.* 1998). In addition, many animals including *Drosophila*, grasshoppers, and mice also experience this change in cell cycle (Bower, 1987; Edgar and Orr-Weaver, 2001; Kiknadze and Istomina, 1980). Endoreduplication can be triggered using various hormone treatments (JOUBES and CHEVALIER 2000) and chemical agents (CORTES *et al.* 2004). It has also been observed that some cells respond to stress and anticancer drugs by inducing endoreduplication (MINGO-SION *et al.* 2004).

Even though there have been many studies that examine the timing, epigenetic control, and the role of endoreduplication in endosperm development, there have been relatively few studies that examine the organization of endoreduplicated chromosomes. Previous studies in maize have demonstrated that chromatin fibers in interphase nuclei increase dramatically in girth at the higher rounds of endoreduplication. This finding has led to the idea that endoreduplicated chromosomes in the maize endosperm have a polytene-like structure (KOWLES and PHILLIPS 1985). It has also been previously noted that as more rounds of endoreduplication occur, the number of deeply staining heterochromatic areas such as knobs and nuclear organizing regions (NOR) does not change (DUNCAN and ROSS 1950). This observation indicates that even though endoreduplication creates higher ploidy endosperm, the number of

chromosomes does not change, but the increase in ploidy is the result of the chromatids endoreduplicating, giving the wide chromatid appearance.

Endoreduplication can be disrupted by performing interploidy crosses (Figure 2.1) (LEBLANC *et al.* 2002). When a diploid (2x) maize plant is crossed by a tetraploid (4x), endoreduplication is not readily detected until 14-16 DAP, and then proceeds slowly until 24 DAP without any noticeable DNA degradation or cell death as detected by flow cytometry. In the reciprocal cross of a 4x maternal plant crossed by a paternal 2x plant, the mitotic phase is shortened, resulting in an early start to the endoreduplication phase beginning around 8 DAP. After 8 DAP, the endoreduplication phase remains similar to a normal 2x by 2x cross, including the largest number of endoreduplication rounds peaking around 16 DAP and cell death starting around 20 DAP.

By using fluorescent *in situ* hybridization with a variety of molecular markers, we studied the structure of the endoreduplicated maize chromosomes. We find that the whole chromosome replicates during endoreduplication, and the newly replicated chromosomes stay associated throughout their entire length. We also discovered that the endoreduplicated chromosomes stay tightly coalesced at the centromeric and knob regions giving a cytological appearance of unreplicated sites. When examining the structure of endoreduplicated chromosomes from interploidy endosperm tissue, the majority of the chromosomes appear to be less condensed especially at heterochromatic sites.

Materials and Methods

Nuclei spreads

Maize inbred lines, 2x Oh43, 4x Oh43, and HiIIB (with B chromosomes) were grown in the Sears Greenhouse at the University of Missouri, Columbia. Maize endosperm tissue was harvested between 14 and 16 DAP. The tissue was fixed in ice cold 90% acetic acid for a minimum of ten minutes (m), then transferred to 70% ethanol, after which the method preceded the same sequence of events as in the methods described on page 33. This technique, designed to maximize chromosome spreading, will sometimes cause the intact nuclei to become misshaped and irregular. The nuclei preparations from interploidy crossed endosperms were from tissue harvested and frozen at -80°C. The interploidy, normal 3x, and normal 6x control endosperms were thawed at room temperature in TE for 5m; then the procedure was performed as described as on page 29.

PCR probes

The following heterochromatic PCR clones were described by Kato and colleagues (KATO *et al.* 2004): 180bp knob repeat (PEACOCK *et al.* 1981), centromeric repeat C (CentC; ANANIEV *et al.* 1998a), centromeric repeat on chromosome 4 (Cent4; (PAGE *et al.* 2001), 350bp TR-1 repeat (ANANIEV *et al.* 1998b), 5'-TAG-3' microsatellite repeat, 2-3-3 5S rDNA repeat, NOR-173, 4-12-1 subtelomere repeat, and telomere repeat (5'-CCCTAAA-3'; RICHARDS and

AUSUBEL 1988). Additional euchromatic PCR clones for the gene families *zein*, *rp1*, and *rp3* were used as described previously (KATO *et al.* 2006). PCR clones were also obtained for *CRM2* (NAGAKI *et al.* 2003), the B specific chromosome repeat (ALFENITO and BIRCHLER 1993), and the "CL" B chromosome repeat (CHENG and LIN 2004). All clones were amplified using M13 primers with the exception of TR-1, which used MR77-specific primers (CHEN *et al.* 2000; KATO *et al.* 2004). Preparation of directly labeled probes for fluorescence *in situ* hybridization (FISH) was performed as described previously (KATO *et al.* 2004). The directly labeled probes for *zein*, *rp1*, and *rp3* were generated with an increase in DNA polymerase concentration during nick translation (KATO *et al.* 2006). Fluorescence *In Situ* Hybridization (FISH) was performed as described on page 31.

Slot blot hybridizations

Endosperm tissue was harvested from Oh43, and quickly frozen in liquid nitrogen at 8, 10, 12, 14, and 16 DAP. DNA was isolated using a Dneasy® Plant Mini Kit (Qiagen, cat# 69104). An aliquot of 100, 250, or 500 ng of total DNA was added in replicate to the DNA slot blot (BioRad Laboratories). After the DNA was adhered to the membrane (BioRad Laboratories, cat# 162-0159) via vacuum filtration, the DNA was crosslinked to the membrane by exposure to UV light. The membrane was subjected to prehybridization for 4h in Perfecthyb Plus (Sigma Chemicals). PCR products for each probe (CentC, Cent4, Knob, NOR, and TR-1) or leaf genomic DNA were radiolabeled with dCTP^{32} (Perkin-Elmer,

Inc) using random priming with the DECAprime™ II kit (Ambion). After labeling, 125 μl of 1x TE were added to the reaction. The total reaction was added to a G-50 Sephadex column to remove any unincorporated nucleotides. Radionucleotide incorporation was evaluated using a Beckman LS6001C scintillation counter (Beckman Coulter, Inc). Radiolabeled PCR products were applied to the hybridization solution to a final concentration of 1.5 million counts/ml. The membrane was hybridized overnight (18-20h) at 65°C, and then washed three times (0.5xSSC, 0.1%SDS) at 65°C. The membranes were exposed on phosphorimager plates.

The hybridizations were carried out consecutively with three probes, beginning with Cent4. Removal of radiolabeled Cent4 probes was accomplished by incubating the membrane with 0.4M NaOH for 30m at 42°C, and then washing the membrane with neutralizing buffer (0.1x SSC. 0.1% SDS, 0.2M Tris-HCl pH 7.6) for at least 30m at 42°C. Overnight phosphorimage exposure confirmed the removal of probe. The second hybridization used radiolabeled CentC, knob, NOR, or TR-1, which was subsequently removed as described above. The last hybridization probe, a loading control, was generated as described using random hexaprimers against leaf genomic DNA.

Slot blot analysis was performed by quantifying the band intensity (obtained from phosphorimage plates) using the Image Gauge software (version 3.3, FujiFilm). A band intensity number (or band signal) was obtained for all DAP's. A ratio was calculated by dividing the band signal obtained for Cent4, CentC, knob, NOR, or TR-1 by the band signal obtained from the same slot with

the control leaf DNA probe. The leaf probe served as a DNA loading control and was used to normalize the blot. A ratio was also calculated for the band signal for CentC, knob, NOR, or TR-1 with that of the band signal obtained from the same slot for Cent4. We averaged the mean of the ratios and plotted them in line graphs setting the mean of the ratios for 8 DAP to 1. Line graphs were created and standard errors were calculated using Microsoft Excel (Microsoft, inc). At 8 DAP very little to no endoreduplication has occurred (KOWLES *et al.* 1990); thus over- or under-endoreduplication is determined by comparing the mean of the ratios of 10, 12, 14, and 16 DAP to the ratio at 8 DAP.

Antibody labeling of CenH3

Antibody labeling procedure for centromeric histone 3 (CenH3; ZHONG *et al.* 2002) was derived from Lamb et al. (LAMB *et al.* 2005). CenH3 antibody was provided by Kelly Dawe (University of Georgia). Tissue was harvested in the same manner as described for the nuclei spread. Endosperms were incubated in ice cold 4% paraformaldehyde in Phosphate Buffered Saline (PBS) for 2h. After the paraformaldehyde was replaced with 100% methanol, the tissue can be stored at -20°C for several months. After the endosperm was washed with PBS for one hour, a 1 mm^3 block of tissue was dissected from the middle portion of the starchy endosperm and incubated in a digestion buffer (2% cellulase, 1% pectolyase, 10 mM EDTA) for 15m at 37°C. The tissue was then washed with PBS for 5m. After the removal of the initial PBS wash, 50 ul of fresh PBS were added. The tissue was then broken apart by flicking the tube. The mixture was

added to the center of a poly lysine coated slide, and spun in a swinging bucket centrifuge for 3m at 150xg. The slides were incubated in PBS with 0.5% Triton for 30m followed by a second wash with1X PI (0.4M NaH_2PO_4, NaOH, 0.5% Igepal CA-630, pH 7.8) for 2h. CenH3 antibody was added to 50 ul of PI+3% BSA (Bovine Serum Albumin), and the mixture applied to the center of the nuclei spread. The slide was incubated in a plastic container lined with wet paper towels overnight at room temperature. The slides were washed three times with PBS for 10m each and subsequently incubated in 1X PI at room temp for 2 hours. The secondary antibody conjugated with fluorescein (Upstate Biotechnology) was added to the slide in the same manner as the primary antibody. Slides were again incubated in a plastic container with wet paper towels at 37°C for 3h; then three washes were performed with PBS in the dark. The slides were fixed in 10% formaldehyde for 10m, washed again three times with PBS, and allowed to dry completely in the dark. DAPI deeply stains the highly heterochromatic knob region (as seen in Figure 4.6); consequently FISH with the knob repeat was not necessary, but FISH for CentC and Cent4 was performed as described on page 31.

Results

FISH pattern for the centromeric and knob regions

As the cell proceeds through endoreduplication, the nucleus grows dramatically in size. At later stages, thick fibrous chromatin strands can be

observed (Figure 4.1). To investigate the organization of the chromatin strands, the FISH patterns from probes that hybridized to either heterochromatin or euchromatin (see materials and methods) were examined. Even though it was difficult to obtain all signals in the same focal plane, FISH with Cent4 shows a hybridization pattern that identified the three copies of chromosome 4 and showed that each chromosome had proceeded through multiple rounds of endoreduplication (Figure 4.2). The Cent4 signal pattern was not random throughout the nucleus, but confined to three small clusters. This result indicates that the endoreduplicated strands stay associated after DNA synthesis. The FISH pattern for CentC shows only a single signal at each chromosome, even though Cent4 shows that the chromosomes have proceeded through many rounds of endoreduplication (Figure 4.2A). The signal intensity of CentC varies for each centromere (ANANIEV *et al.* 1998a); thus some centromeres were difficult to detect. A single signal was also seen for another centromeric repeat, *CRM*, as well for the knob and TR-1 repeats (Figure 4.2B-D). The difference in organization of signals between Cent4 and CentC is unlikely to be due to intensity differences because even weak CentC and knob hybridizations are single sites.

Figure 4.1. Endoreduplicated nucleus. The nucleus on the right is prior to endoreduplication. The nucleus on the left is highly endoreduplicated. DNA was stained with DAPI. Inset shows the increase in the girth of the chromatin fibers.

Figure 4.1. Endoreduplicated nucleus.

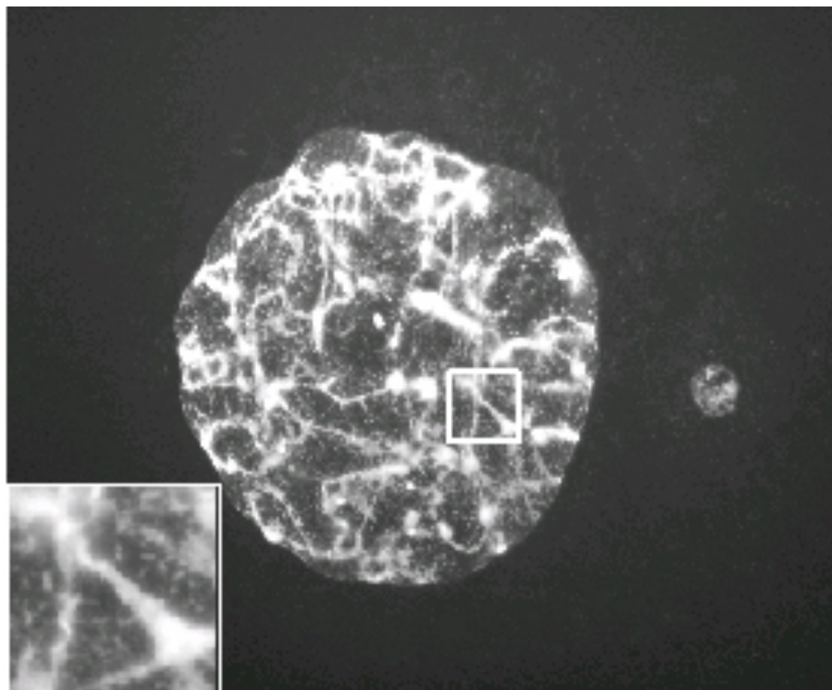
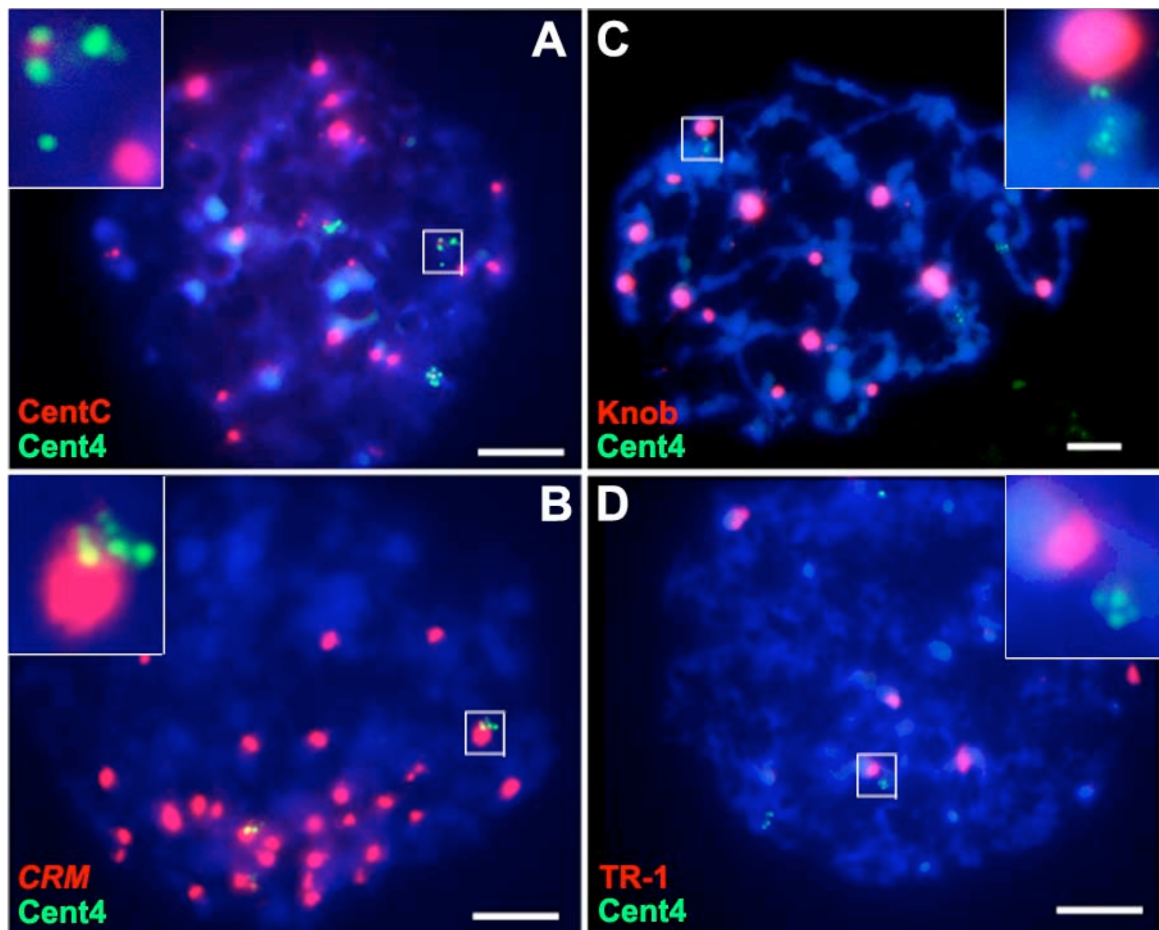


Figure 4.2. FISH analysis of centromeric and knob regions of the chromosomes. FISH signals for Cent4 (A-D, green) form a small cluster, while the FISH signal for CentC (A, red), CRM (B, red), Knob (C, red), and TR-1 (D, red) repeats all show one large signal. Insets show magnification of corresponding FISH. DNA staining with DAPI is blue in all merged images. Bar=10 mm

Figure 4.2. FISH analysis of centromeric and knob regions of the chromosomes.



FISH pattern of genic regions

Next, we examined three gene clusters: *Rp1*, *Rp3* and *Zein*. These loci all show three single hybridization signals on non-endoreduplicated endosperm nuclei (Figure 4.3A-C). On endoreduplicated nuclei, *Rp1* and *Zein* have a very similar hybridization pattern as Cent4 (Figure 4.3D-I), showing a cluster of multiple signals slightly more spread. The *Rp3* hybridization pattern, on the other hand, varied from a small cluster to a single signal (data not shown). Genetic localization indicates that *Rp3* is very close to the centromere on chromosome 3 (Collins et al., 1998).

Chromosome endoreduplication

To visualize a whole endoreduplicated chromosome, we took advantage of the B chromosome. The B chromosome is a supernumerary chromosome that has no known genes, contains many common and specific molecular markers and is small in size (Carlson, 1978; Lamb et al., 2005). It is the latter two traits that make it ideal for endoreduplication study. The B-repeat is a sequence specific to this chromosome, which is located at and around the centromere as well as in the subtelomeric region near the tip of the long arm (ALFENITO and BIRCHLER 1993; LAMB *et al.* 2005). FISH using the B repeat on endoreduplicated chromosomes showed one signal at the centromere, but a cluster pattern at the distal end of the long arm (Figure 4.4A-E). DAPI staining also showed that the endoreduplicated chromatids remain associated throughout their entire length (Figure 4.4). Other FISH markers, such as the microsatellite (Figure 4.4C), and

“CL”-repeat (Figure 4.4D), showed large clusters of signal painting the heterochromatic portions of the chromosome. When examined during endoreduplication, CentC, which is spread throughout the B chromosome (LAMB *et al.* 2005), showed clusters of signals (Figure 4.4B). The telomere probe also revealed a small single cluster surrounding a stronger, brighter signal area (Figure 4.4E). While the B-repeat sequence has some similarity with the telomeric sequence (ALFENITO and BIRCHLER 1993), most of the B-repeat signals did not co-localize with the telomere signals.

FISH analysis for the centromere and knob regions exhibited a single signal. This observation suggests that either the centromeric and knob regions do not participate in endoreduplication, or that the endoreduplicated fibers are held more tightly together and appear as one signal. To examine this issue, we isolated endosperm genomic DNA at 8, 10, 12, 14, and 16 days after pollination (DAP) and performed slot blot analysis (see materials and methods). While at 8 DAP very little to no endoreduplication has taken place (KOWLES *et al.* 1990), an increase in the number of rounds of endoreduplication occurs through 10, 12, 14, and 16 DAP. Samples were not taken past 16 DAP due to the onset of DNA degradation (KOWLES and PHILLIPS 1985).

If the centromeric and knob regions are not endoreduplicating, but other regions are, we expect the ratio of centromeric or knob repeats to the total amount of DNA to decrease in later DAP. If endoreduplication occurs uniformly, the ratio of centromeric and knob repeats to the total amount of DNA loaded should remain constant (Figure 4.5A-B). We find that the ratio of centromeric

Figure 4.3. Endoreduplication of the *rp1* and *zein* loci. *rp1* and *zein* signal patterns are not randomly distributed in the nuclei, but form a small cluster slightly larger than the cluster signal observed for Cent4 (green), indicating that the chromosomes stay associated after endoreduplication. In non-endoreduplicated nuclei (A-C), *rp1*, *rp3*, and *zein* exhibit a single hybridization signal; showing that the cluster's observed in endoreduplicating nuclei (D-I) are due to endoreduplication. A) *rp1*, B) *rp3*; C) *zein*; D) *rp1*; E) Cent4; F) merged image of D (red) and E (green); G) *zein*; H) Cent4; I) merged image of G (red) and H (green). Insets show magnification of corresponding FISH. DNA staining with DAPI is blue in all merged images. Bar=10 μm

Figure 4.3. Endoreduplication of the *rp1* and *zein* loci.

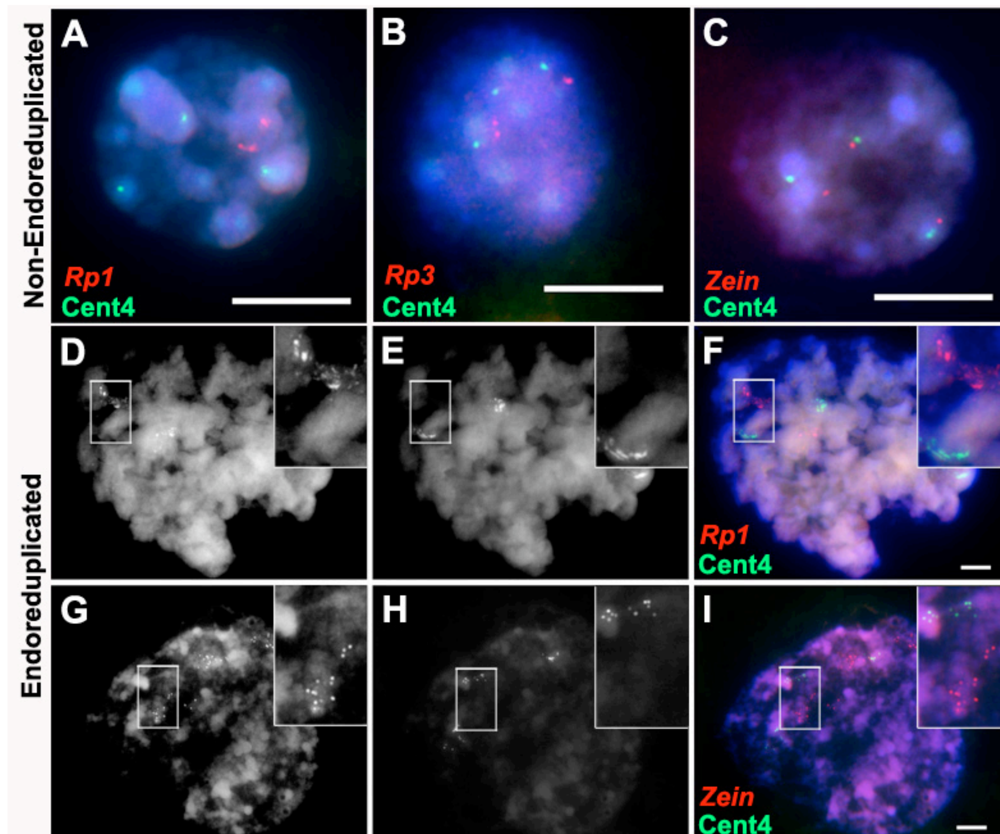
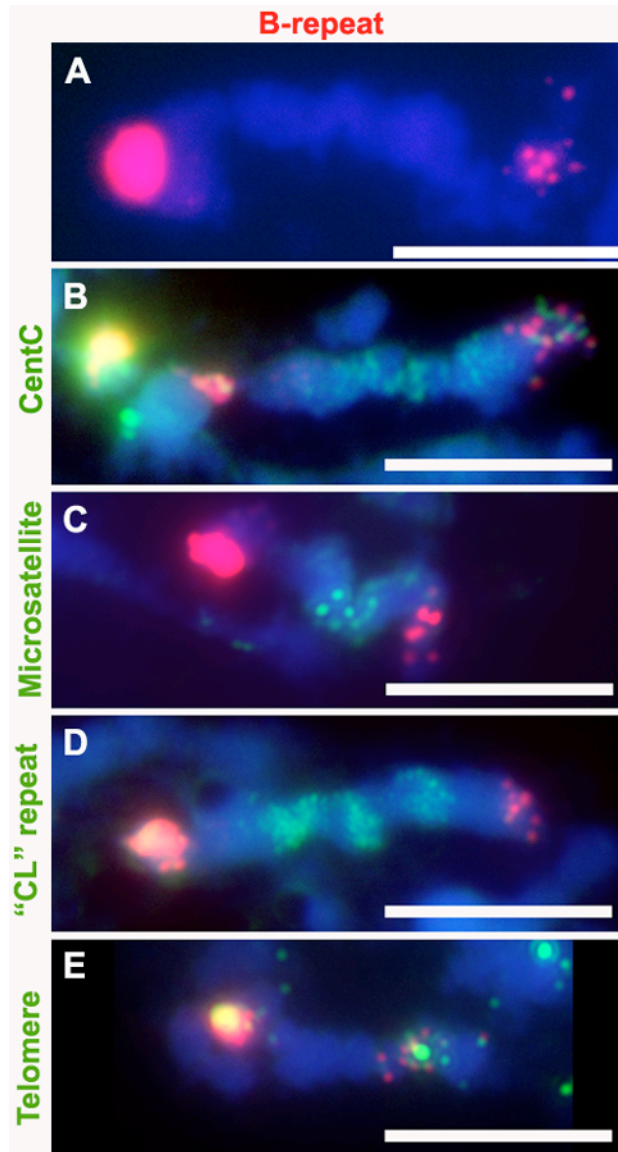


Fig 4.4. FISH analysis using markers on the B-chromosome. The B-repeat pattern on the tip of the long arm shows a cluster signal pattern (A-E, red) indicating that the B chromosome has been endoreduplicated. The B-repeat (A-E, red) and the CentC signal (B, green) at the centromere have one signal similar to centromeres and knobs on the A-chromosomes. FISH pattern along the long arm for CentC (B, green), TAG-microsatellite (C, green), "CL"-repeat (D, green), and telomere (E, green), show a cluster signal pattern along the long arm of the B-chromosome. DNA staining with DAPI is blue in A-E. Bar=10 μm

Fig 4.4. FISH analysis using markers on the B-chromosome.



and knob related repeats behave in the latter manner. The percentage of CentC signal is consistent through all time points examined (Figure 4.5A-B). To investigate further, we compared the CentC signal to Cent4, a marker that is observed by FISH to undergo endoreduplication. The CentC:Cent4 ratio was constant in later stages of endosperm development (Figure 4.5.B). Together the CentC:DNA and CentC:Cent4 data strongly indicate that the centromeric regions do proceed through multiple rounds of endoreduplication. Thus, the single signal observed by FISH is due to the tight cohesion of the centromeres so that only one hybridization signal is observed.

The ratio of knob repeat signal to DNA was consistently equal throughout all time points examined (Figure 4.5A). An identical pattern was observed when comparing the knob repeat signal to Cent4 (Figure 4.5B). The slot blot data for the NOR region showed a similar trend. Like the knob repeat and CentC, it was also endoreduplicated; however, it appeared to be moderately over-endoreduplicated at 12, 14, and 16 DAP (Figure 4.5A). The same was true for the NOR ratio when compared to Cent4 (Figure 4.5B).

The data for the TR-1 repeats indicated that it was being replicated during endoreduplication as well. There were also slight changes in endoreduplication with a small increase at 10 DAP, which was maintained during subsequent rounds of DNA replication at 12, 14, and 16 DAP (Figure 4.5A). The TR-1:Cent4 ratio also shows endoreduplication and remained fairly constant with the predicted endoreduplication ratio (Figure 4.5B). All repeats examined showed a

fairly equal replication rate compared to Cent4 whose replication was confirmed by the scatter hybridization pattern observed in FISH images.

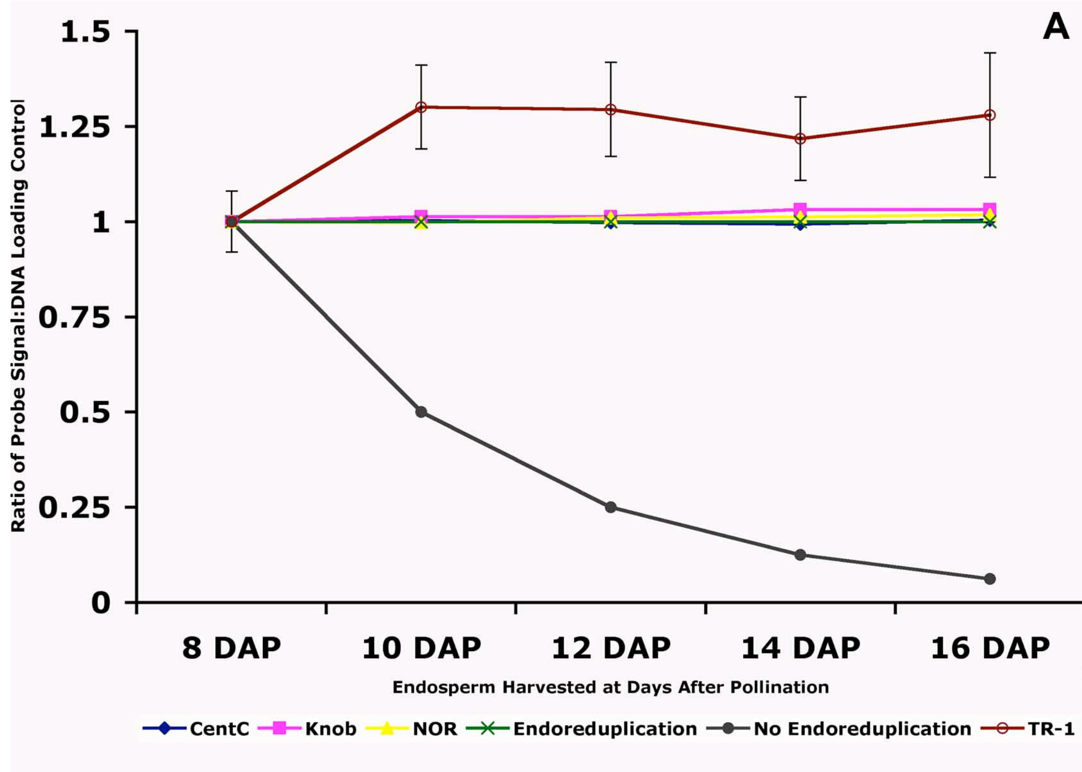
Even though TR-1 showed a slightly different replication pattern, its amount relative to Cent4 remains nearly constant. We also examined the ratio of Cent4 repeats to the total amount of DNA loaded. The slot blot technique also served as a control to show that Cent4 endoreduplicated with the rest of the genome. The ratio of Cent4 repeats did not change significantly through all DAP verifying the observations with FISH (data not shown).

Does cenH3 deposit at the knobs?

The FISH hybridization pattern and slot blot analysis suggested that endoreduplicated chromosomes stay tightly associated at both the centromere and the knob regions. The tight association of chromatin at the centromere occurs during the normal cell cycle. This behavior for the knobs was unexpected. To examine the possibility that knobs and centromeres acquire similar molecular properties in endoreduplicated chromosomes, we tested whether the knob regions acquired centromeric histone 3 (CenH3), which has been shown to incorporate at kinetochore sites (ZHONG *et al.* 2002). Immunofluorescence showed CenH3 present at the centromeres, but it was not similarly accumulated at the knob regions (Figure 4.6, white arrows). This result suggests that the knobs do not acquire centromeric characteristics and the basis of cohesion cannot be ascribed to this type of chromatin configuration.

Fig 4.5. Slot blot analysis of hetrochromatic regions in endoreduplicating tissue. A) The ratio of the signal intensity of CentC, Knob, NOR, and TR-1 to the loading control. B) The ratio of the signal intensity of CentC, Knob, NOR, and TR-1 signal to Cent4 signal taken from the same slot. Because very little to no endoreduplication has taken place by 8 DAP, all signals are normalized so that 8 DAP is equal to one. n=6 for all times points for all examined chromosomal sites except for TR-1:Cent4 where n=2 for all time points.

Fig 4.5. Slot blot analysis of hetrochromatic regions in endoreduplicating tissue.



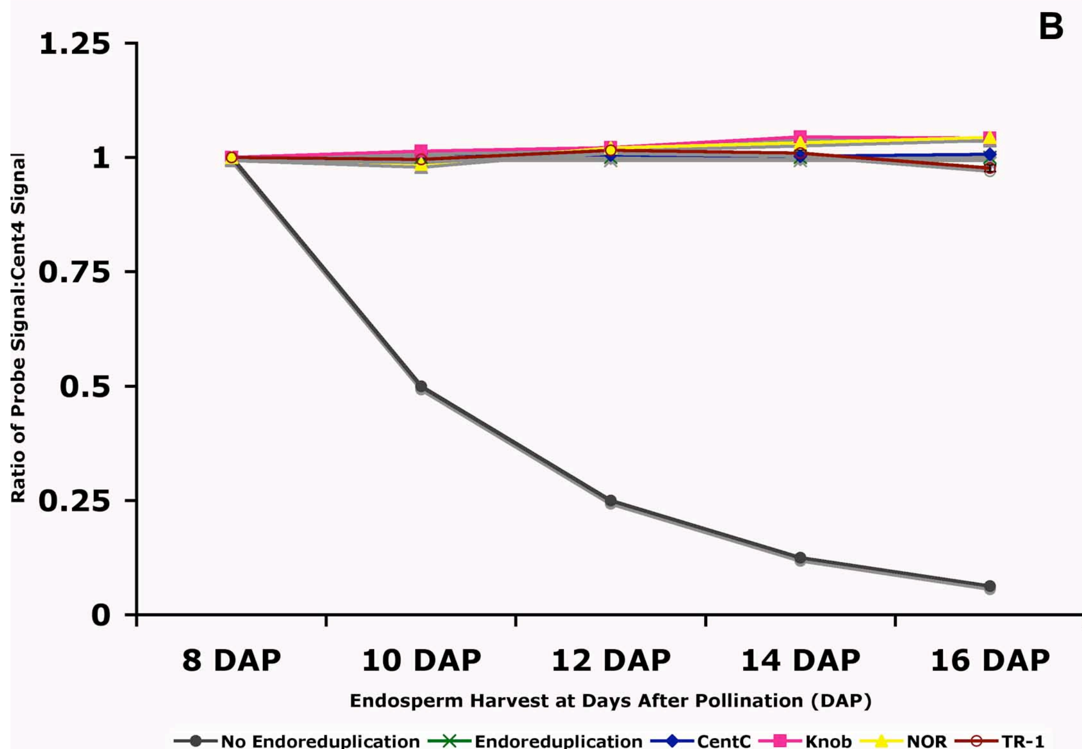
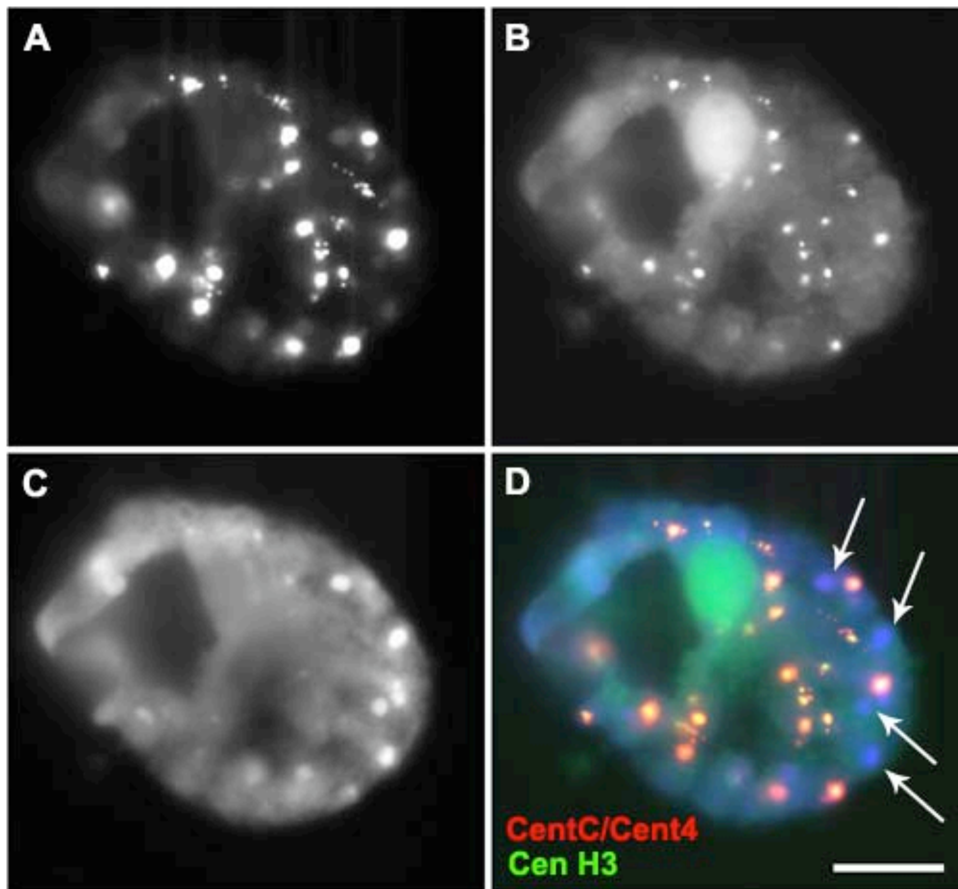


Fig 4.6. CenH3 immunostaining. Antibody directed against centromeric histone 3 (CenH3, A & D green) shows that CenH3 only incorporates at the centromeric sites (CentC/Cent4, B & D red). The knob regions (white arrows), which are deeply stained with DAPI (C & D blue), show no localization of CenH3. DNA staining with DAPI is blue. Bar=10 μm

Fig 4.6. CenH3 immunostaining



FISH pattern of the centromeric and knob regions in interploidy cross endosperms

Endosperm development is dramatically disrupted in interploidy crosses in which the resulting endosperm is defective and the kernels will most often not germinate (COOPER 1951; RANDOLPH 1935; SARKAR and COE 1971). With regard to endoreduplication, interploidy crosses disrupt not only the time in development at which endoreduplication begins, but also affect the number of rounds of endoreduplication (LEBLANC *et al.* 2002). Due to the severe effect interploidy crosses have on these aspects of endosperm biology, we examined whether interploidy crosses would also severely affect the organization of the endoreduplicated chromatin.

The knob and centromeric regions stay highly compact after many rounds of endoreduplication in both 2x plants (Figure 4.2A&C) and 4x plants (Figure 4.7G-H; Figure 4.8G-H). When a maternal 2x plant is crossed by a paternal 4x plant, or vice versa, the centromeric regions appear less associated (Figure 4.7C-D). The CentC signal indicated that adherence of the centromeric regions is still occurring, but the association is reduced in the interploidy cross endosperms. We observed the same hybridization pattern for the knob repeat. In the 2x and 4x plants, there is a tight coalescence of endoreduplicated fibers (Figure 4.8A-B&G-H, respectively), but in interploidy crosses, the adherence is less causing some decondensation of the chromatin (Figure 4.8C-D, 4.9 & 4.10).

The Cent4 hybridization pattern in the interploidy crosses does not seem to differ significantly compared to the 2x and 4x plants (Figure 4.7 - green). The NOR region and the 5S rDNA gene cluster follow a similar pattern except that it is slightly more spread in interploidy endosperms (Figure 4.11 & Figure 4.12, respectively), but the difference from the 2x and 4x controls is not as severe as observed in the centromeric and knob heterochromatic regions. A slightly more dispersed phenotype was also observed with rp3 regions as well (Figure 4.13). These results suggest that there is a generalized decondensation of chromatin in interploidy endosperms but centromere and knob sites are most affected.

4.7. FISH hybridization pattern of CentC repeats on endosperms derived from Interploidy crosses. Endosperm nuclei were harvested from 2x, x 2x (A & B) , maternal 2x crossed by paternal 4x (C & D), maternal 4x crossed by paternal 2x (E & F) and from 4x x 4x (G & H) plants. FISH with CentC repeats (in red with the corresponding gray value in right column) indicates that this heterochromatic region in interploidy crosses (C-F) are more dispersed when compared to the controls (A, D, E, H). Cent4 hybridization is green, and DAPI staining is blue. Bar=10 μm

Fig 4.7. FISH hybridization pattern of CentC repeats on endosperms derived from interploidy crosses

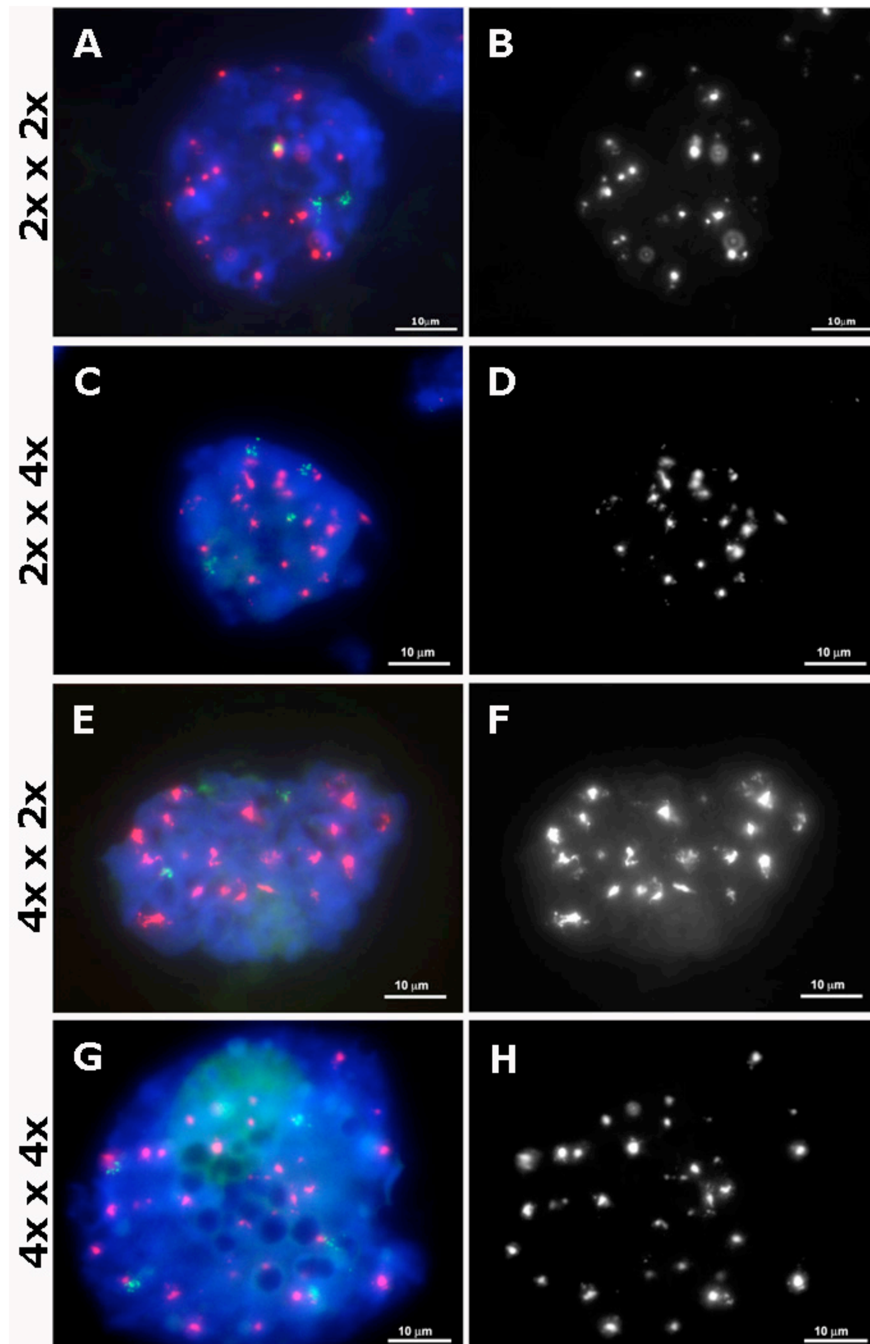


Fig 4.8. FISH hybridization pattern of knob repeats on endosperms derived from interploidy crosses. Endosperm nuclei were harvested from 2x, x 2x (A & B) , maternal 2x crossed by paternal 4x (C & D), maternal 4x crossed by paternal 2x (E & F) and from 4x x 4x (G & H) plants. FISH with Knob repeats (in red with the corresponding gray value in right column) indicates that heterochromatic regions in interploidy crosses (C-F) are more dispersed when compared to the controls (A, D, E, H). Cent4 hybridization is green, and DAPI staining is blue. Bar=10 μm.

Fig 4.8. FISH hybridization pattern of knob repeats on endosperms derived from interploidy crosses

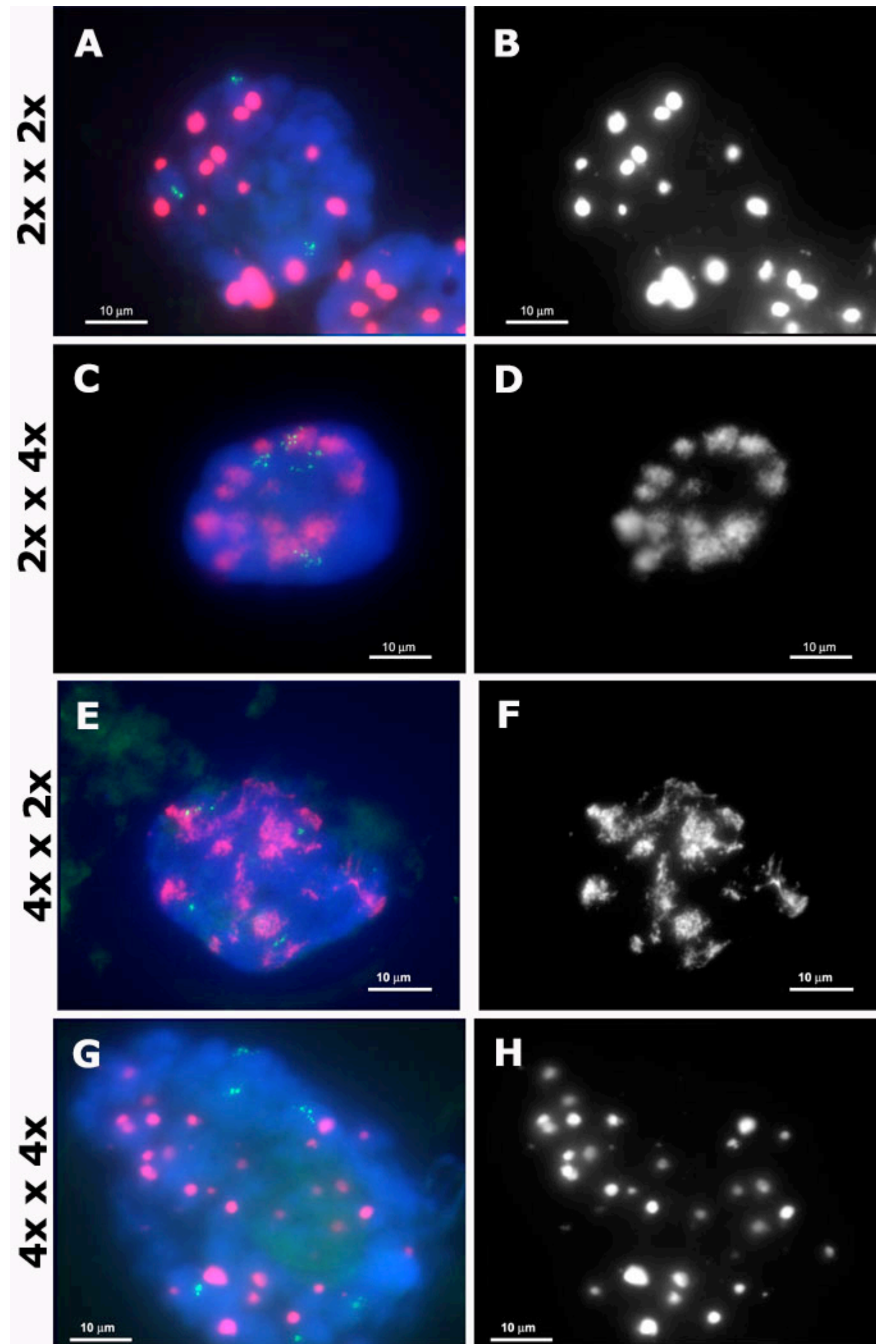


Fig 4.9. Various distributions of FISH hybridization pattern of knob repeats on endosperms derived from the 2x x 4x interploidy cross. All endosperm nuclei were harvested from maternal 2x crossed by paternal 4x (A-H) plants. FISH with Knob repeats (in red with the corresponding gray value in right column) indicates that heterochromatic regions in interploidy crosses have different distribution patterns. Some seem to be more normal (A-B) while the majority (C-H) have a more dispersed pattern when compared to the controls (Figure 4.8A-B & G-H). Cent4 hybridization is green, and DAPI staining is blue. Bar=10 μm

Fig 4.9. Various distributions of FISH hybridization pattern of knob repeats on endosperms derived from the 2x x 4x interploidy cross

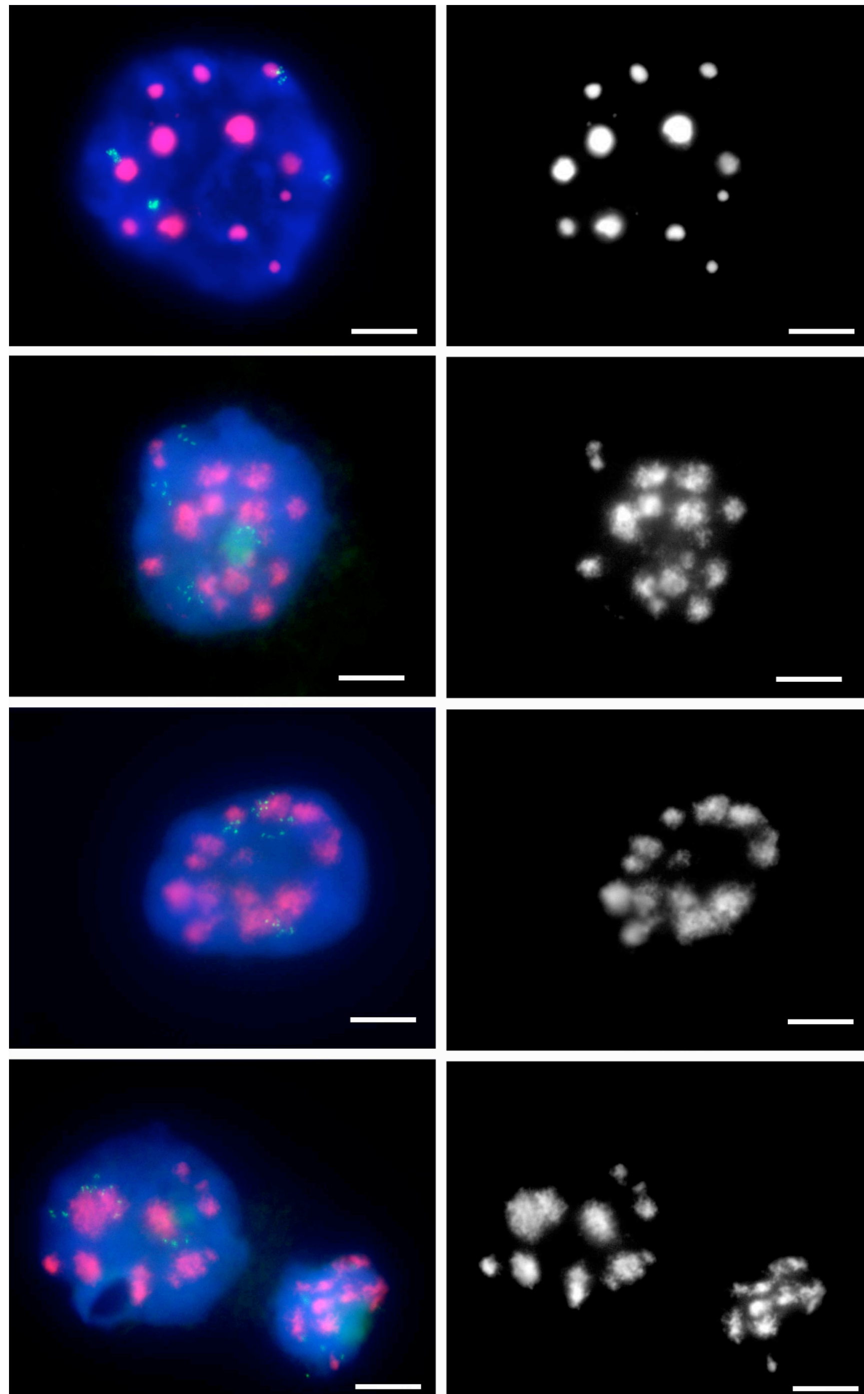


Fig 4.10. Various distributions of FISH hybridization pattern of knob repeats on endosperms derived from the 4x x 2x interploidy cross. All endosperm nuclei were harvested from maternal 4x crossed by paternal 2x (A-H) plants. FISH with Knob repeats (in red with the corresponding gray value in right column) indicates that heterochromatic regions in interploidy crosses have different distribution patterns. Although none seem to be completely normal, some seem to be more normal (A-B) while the majority (C-H) has a severely diffused pattern when compared to the controls (Figure 4.8A-B & G-H). Cent4 hybridization is green, and DAPI staining is blue. Bar=10 μm

Fig 4.10. Various distributions of FISH hybridization pattern of knob repeats on endosperms derived from the 4x x 2x interploidy cross.

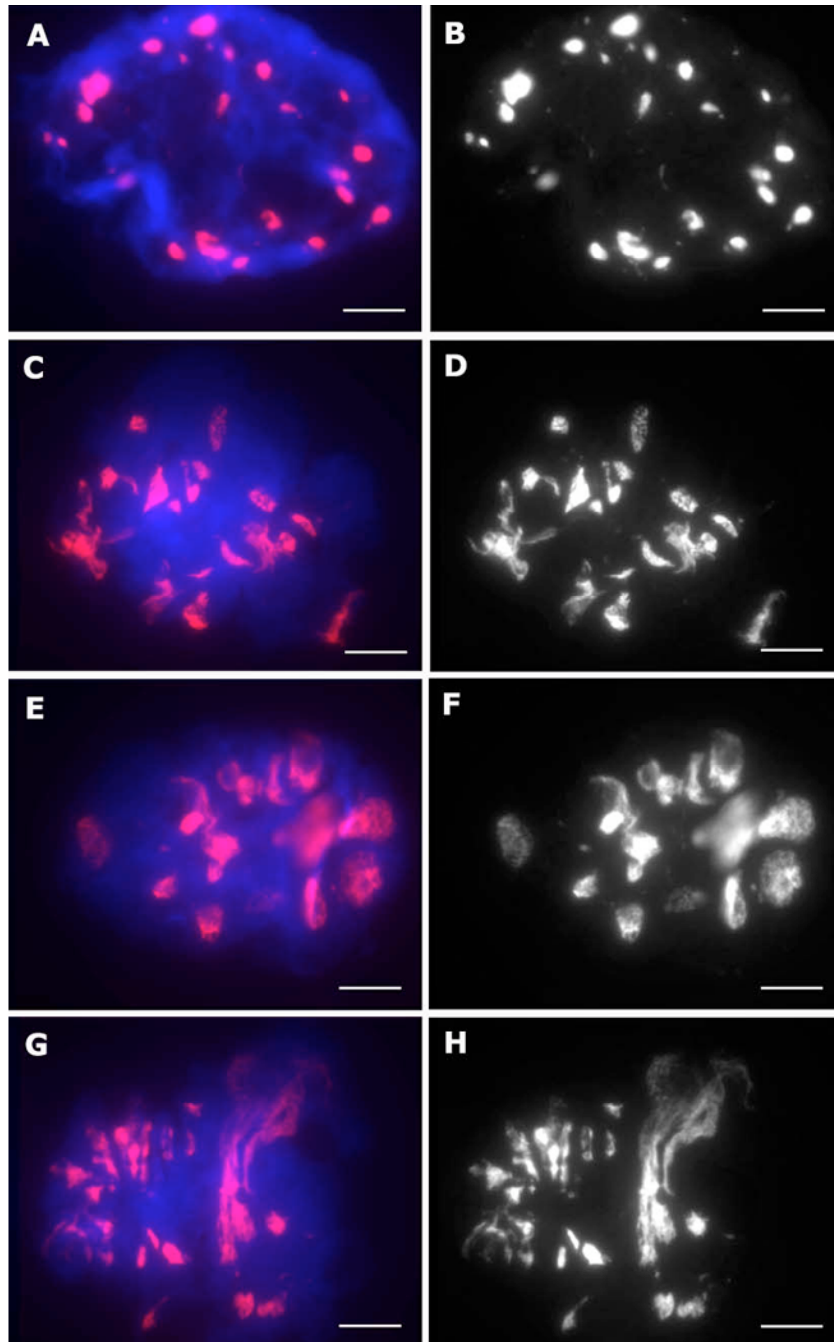


Fig 4.11. FISH hybridization pattern of NOR repeats on endosperms derived from interploidy crosses. Endosperm nuclei were harvested from 2x, x 2x (A & B) , maternal 2x crossed by paternal 4x (C & D), maternal 4x crossed by paternal 2x (E & F) and from 4x x 4x (G & H) plants. FISH with NOR repeats (in red with the corresponding gray value in right column) indicate that those regions in interploidy crosses (C-F) appear to be slightly more diffused when compared to the controls (A, B, E, H). Cent4 hybridization is green, and DAPI staining is blue. Bar=10 µm

Fig 4.11. FISH hybridization pattern of NOR repeats on endosperms derived from interploidy crosses.

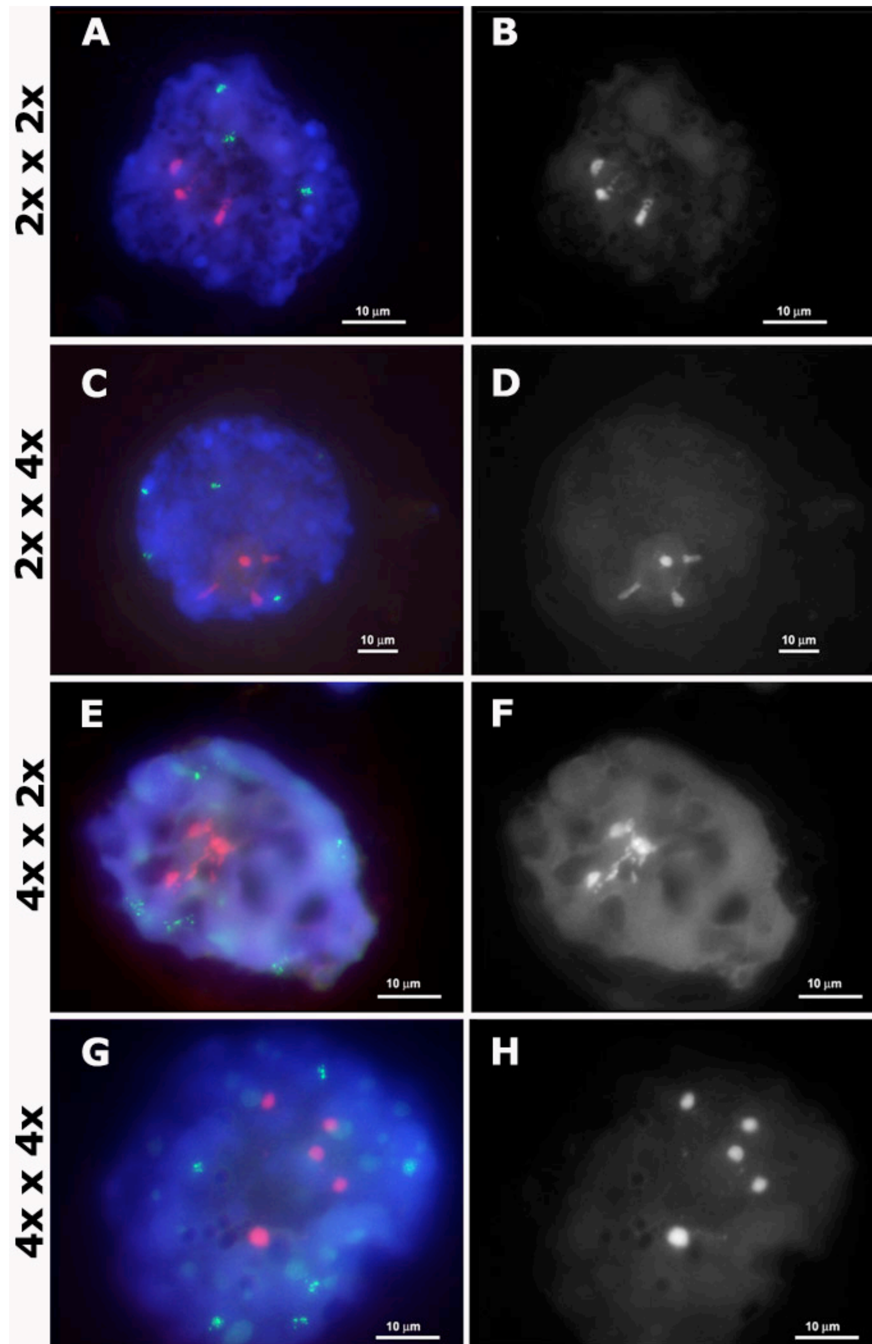


Fig 4.12. FISH hybridization pattern of 5S rDNA repeats on endosperms derived from interploidy crosses. Endosperm nuclei were harvested from 2x, x 2x (A & B), maternal 2x crossed by paternal 4x (C & D), maternal 4x crossed by paternal 2x (E & F) and from 4x x 4x (G & H) plants. FISH with the 5S rDNA repeats (in red with the corresponding gray value in right column) indicate that those regions in interploidy crosses (C-F) appear to be slightly more diffused when compared to the controls (A, B, E, H). Cent4 hybridization is green, and DAPI staining is blue. Bar=10 μm

Fig 4.12. FISH hybridization pattern of 5S rDNA repeats on endosperms derived from interploidy crosses.

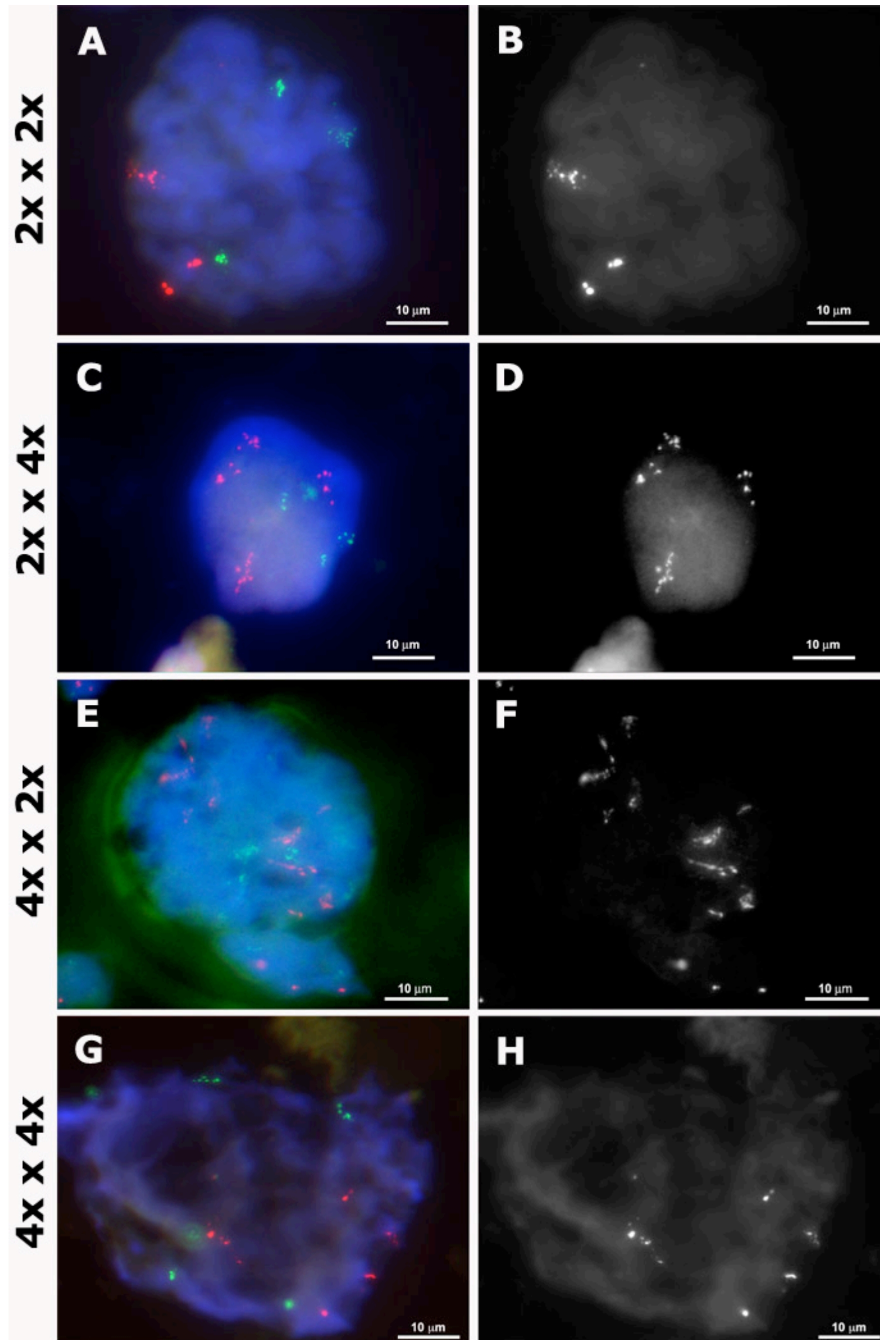
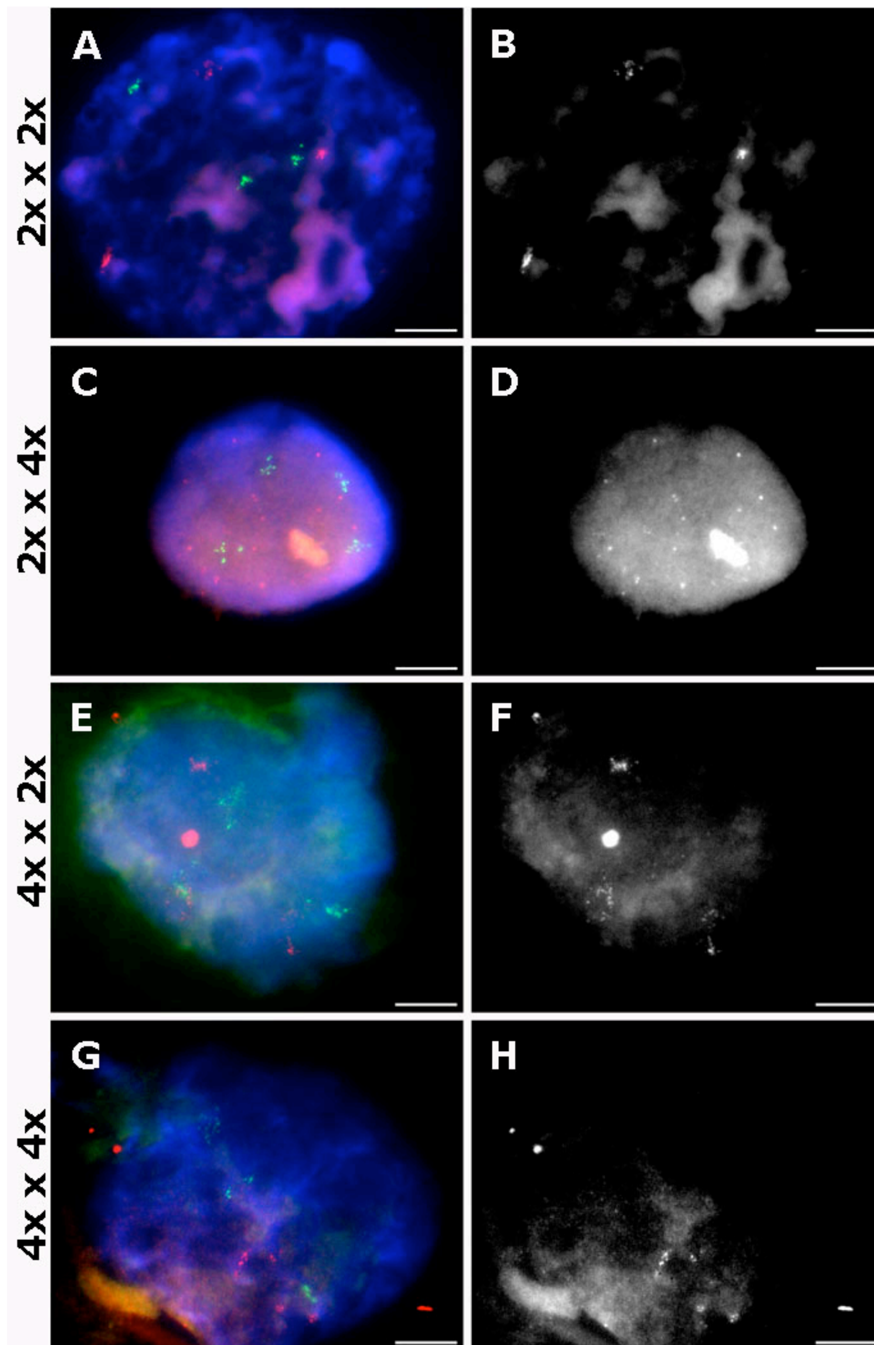


Fig 4.13. FISH hybridization pattern of *rp3* gene family in endoreduplicating endosperms derived from interploidy crosses. Endosperm nuclei were harvested from 2x, x 2x (A & B) , maternal 2x crossed by paternal 4x (C & D), maternal 4x crossed by paternal 2x (E & F) and from 4x x 4x (G & H) plants. FISH with the *rp3* repeats (in red with the corresponding gray value in right column) indicate that those regions in 2x x 4x interploidy crosses (C-D) appear to be greatly more distributed when compared to the controls (A, B, G, H). In the 4x x 2x interploidy crosses, the distribution is only slightly more difuse than the controls (A, B, G, H). Cent4 hybridization is green, and DAPI staining is blue. Bar=10 µm

Fig 4.13. FISH hybridization pattern of *rp3* gene family in endoreduplicating endosperms derived from interploidy crosses.



Discussion

Endoreduplicated chromosomes have a unique structure. As the process of endoreduplication proceeds, the newly replicated strands remain associated (Figure 4.2 – 4.4). This chromosome configuration can be seen with fluorescently labeled Cent4, *rp1*, and *zein* signals as well as B-chromosome specific probes. During endoreduplication, observation of Cent4, *rp1*, and *zein* regions show that the signals form a cluster. This pattern was verified by FISH analysis of the B-chromosome with the B-repeat, “CL”-repeat, microsatellite, and telomeric repeat (Figure 4.4). The telomere signal also consistently displayed a pattern intermediate between a centromere/knob pattern and cluster pattern typical for Cent4; we generally found a bright single signal surrounded by a cluster of weaker signals for this probe. This finding could be explained by the telomeres associating with each other more weakly than what is observed at the centromere and knob sites but stronger than the remainder of the chromosome.

The finding that the centromeric and knob regions stay more closely associated than the remainder of the chromosome during endoreduplication is intriguing. Such an association would be predicted for the centromeres because they remain attached in S-phase during the mitotic cell cycle. Association of endoreduplicated chromosomes at the knob was somewhat unexpected. Knobs were first described by Barbara McClintock (McClintock 1929) as highly heterochromatic regions in the maize genome. All knobs consist of an 180bp repeat (Peacock *et al.* 1981) and/or a 350bp repeat (TR-1; Ananiev *et al.* 1998b). Due to the tight binding of knobs in endoreduplicating tissue, we wished to

determine whether knobs might be acquiring centromeric properties under endoreduplicating conditions. We explored this issue by examining CenH3, which deposits only at regions of the chromosomes in which the kinetochore will form (ZHONG *et al.* 2002). Figure 4.6 shows CenH3 is only localized at the centromere and not at the knob regions.

Knobs have no known function except in lines of maize carrying Abnormal Chromosome 10 (Ab10; HIATT *et al.* 2002; LONGLEY 1937; RHOADES and VILKOMERSON 1942; YU *et al.* 1997). In these lines, chromosome 10 contains extra segments and rearrangement of DNA that are not part of the normal version. In the presence of Ab10, knobs are converted into neocentromeres during meiosis (HIATT *et al.* 2002; RHOADES and VILKOMERSON 1942). In Ab10, spindle fibers attach to the centromere normally, but at the knobs the arms will bend toward the spindle fiber and attach laterally (YU *et al.* 1997). The binding of knobs to spindle fibers creates a neocentromere. The binding of spindle fibers to neocentromeres is not direct and centromeric histone 3 is not incorporated in these sites. Our results indicate that the knob organization in endoreduplicated chromosomes is not due to the normal acquisition of centromeric properties.

Endoreduplication is a variant of the normal mitotic cycle in which a repetition of the S-phase and a G-phase occurs. The part of the cell cycle that "skipped" is different depending on either the species or the tissue that the endoreduplication is occurring (EDGAR and ORR-WEAVER 2001). For example, in drosophila salivary glands, the cell cycle is normal until about half way through the S-phase, in which the cell cycle switches back to the G_1 phase and continues

through S-phase again. In S-phase in Drosophila, the euchromatic regions replicate first followed by heterochromatic regions. Because the endoreduplication phase skips the later half of the S-phase, and begins the cell cycle over, it creates highly endreduplicated euchromatic regions, and underendoreduplicated heterochromatic regions. Figure 4.5, indicates that the heterochromatic regions in maize complete endoreduplication suggesting that the part of the cell cycle that is skipped starts in the G_2 phase. In older, more endoreduplicated tissue, the polytene-like strands become more apparent. This could be due to changes in the endoreduplication cell cycle, in that early in endoreduplication, the cell cycle may “jump” back to the G_1 phase at an earlier time point than in older more endoreduplicated tissues. This hypothesis suggests that older tissue may reach the very early stages of prophase. Because the endosperm is a heterogenous mix of tissue at different stages of development makes this type of investigation difficult.

A plausible hypothesis for the structure of endoreduplicated chromosomes is that there might be chromatin binding proteins adhering to the chromosomes, with higher concentrations at the centromere and knob regions. An attractive candidate might be cohesins (MICHAELIS *et al.* 1997). In yeast and animal cells, cohesins form complexes that are deposited along the chromosomes during S-phase and are used to hold the chromatids together. In late prophase, the majority of cohesins are removed leaving small but detectable amounts at the centromeric regions until metaphase (NASMYTH 2005; WAIZENEGGER *et al.* 2000). Examining the relative amounts of cohesins deposited along the maize chromatin

would be integral to uncovering the mechanism that holds endoreduplicated fibers together, but this test cannot presently be conducted because no antibodies have been produced for the maize proteins.

Interploidy crosses dramatically disrupt development of the maize caryopsis (Figure 2.1). In both the diploid and tetraploid parents, the centromeric and knob regions adhere after many rounds of endoreduplication (Figure 4.7 – 4.8). The endosperms derived from interploidy crosses display a range of phenotypes. When examining the distribution of CentC , Knobs, and NOR, a small number of nuclei appear to resemble the phenotype of the wildtype parents, but in a majority of the interploidy nuclei, the FISH pattern indicates that there is moderate disassociation of the centromere and knob regions after many rounds of endoreduplication (Figure 4.7 - 4.11). The FISH pattern is not scattered throughout the nucleus, but is instead in large conglomerates indicating that the endoreduplicated fibers are still associating, but in a more diffuse formation. When comparing loci that displayed a clustered signal pattern in the wildtype plants, a similar phenotype was observed in the interploidy derived endosperms (Figure 4.7 – 4.13). It appears that the cluster pattern could be slightly more dispersed, especially in maternal 4x crossed by paternal 2x derived tissue. Overall, both types of interploidy crosses produce a disruption of the chromatin organization.

Chapter 5:

Global Gene Expression Changes In the Endosperm of *Zea mays* Due To Interploidy Crosses

Introduction

The molecular events that ensure the proper development of the endosperm have not been well studied. The classical method of dissecting the genes involved in development is to uncover mutations that result in defective kernels, and determine what aspect of the developmental process has been disrupted. A very effective way to disrupt endosperm development is by crossing plants of two different ploidies, or an interploidy cross (COOPER 1951; RANDOLPH 1935; SARKAR and COE 1971).

Crossing a maternal diploid (2x) with the paternal tetraploid (4x) begins with a faster rate of mitosis, especially in the embryo surrounding region (ESR) in comparison to a normal 2x x 2x cross (interploidy crosses as reviewed in: (COOPER 1951). Cellularization of the nuclei occurs earlier than 4 DAP, and the high degree of mitosis, normally between 5-12 DAP, ceases around 8-10 DAP followed by an induction of endoreduplication at an earlier time point, which is coincident with the early starch formation and storage. Even though starch formation and storage begins early, the BETL does not differentiate properly and

is virtually absent, resulting in very little nutrients being transferred. This leads to approximately 1/3 of the starchy endosperm filled with starch.

In the reciprocal interploidy cross, the maternal 4x is crossed with the paternal 2x, forming a pentalploid (5x) endosperm. Mitosis is slower than in the normal diploid by diploid cross, and cellularization occurs around 5-6 DAP. The BETL form small and thin and is capable of some nutrient transfer, which begins early and by 6-7 DAP the endosperm is small and entirely filled with starch.

Theories as to why interploidy crosses develop defective kernels focus on either 1) the expression or silence of imprinted alleles (HAIG and WESTOBY 1989; HAIG and WESTOBY 1991), or 2) the molecular interaction of regulators and genes immediately after central cell fertilization (BIRCHLER 1993; BIRCHLER *et al.* 2001). Both theories suggest that development is disrupted by triggering a differential expression of important genes, steering development down an incorrect path. By initiating gene expression erroneously, the resulting genes that are expressed down stream in the developmental pathway would also be disrupted. Gene expression changes at various time points were examined using microarrays on Oh43 and B73 maize inbred lines. Together with known morphological changes (COOPER 1951; LEBLANC *et al.* 2002; RANDOLPH 1935; SCOTT *et al.* 1998), a better understanding can be made about how interploidy crosses disrupt development.

Materials and Methods

Maize stocks and crosses

Maize inbred lines of Oh43 and B73, along with their autotetraploid derivatives (KATO and BIRCHLER 2006), were grown in the Sears plant growth facility on the campus of the University of Missouri. In crosses with maize, the maternal plant is listed first, and were performed for each maize line as follows: 1) diploid x diploid, 2) diploid x tetraploid, 3) tetraploid x diploid, and 4) tetraploid x tetraploid.

Maize endosperm harvest

Maize endosperm tissue was harvested for all crosses at four time points after pollination: 8, 10, 12, and 14 days after pollination. Between 40 - 80 endosperms were harvested from each ear, and four ears were harvested for each time point and for each cross. The tissue was immediately frozen in liquid nitrogen and stored at -80°C. When all time points were collected, the endosperm tissue was sent to Ceres, Inc for microarray hybridization and data analysis.

Microarray

Affymetrix Maize Genome Array chips were hybridized with endosperm RNA at Ceres, Inc (Figure 5.1). It contains 17,555 probe sets representing 14,850 transcripts from ~ 12,113 UniGene clusters (Affymetrix catalog# 900616).

One hundred sixty two coding sequences from the mitochondrial genome (AY506529.1) and 111 coding sequences from the chloroplast genome (X86563.2) are also included on the array. The sequence data for this array was collected from public data sources including *Zea mays* UniGene Build #42 (July 23, 2004) and GenBank (through September 29, 2004). The GeneChip Maize Genome Array contains 15 probe pairs per probe set. Affymetrix corn chip representing 17k features was used for hybridization and analysis. After hybridization chip images were checked visually for uniformity and anomaly.

Microarry data analysis

Ratios used for correlation calculations:

1) For 2x x 2x crosses: the ratio=signal intensity of other DAP/signal intensity of 8 DAP of 2x x 2x.

2) For all other crosses: Ratio=Signal intensity of the cross/signal intensity of 2x x 2x in the same DAP.

There are more single reps for B73 than Oh43. At the technical level, this may contribute to larger number of differentially expressed genes in the B73 dataset than in Oh43 dataset when using Benjamini and Hochberg correction for one-way ANOVA (BENJAMINI and HOCHBERG 1995). The average signal intensity across all chips per gene was used as the control. Unless indicated otherwise, we used average signal intensity per gene across all chips as a control (for example, the control for gene X in Oh43 was the average of signal intensity of gene X for 30 chips) instead of using 2x x 2x signal intensity as a control.

Therefore, ratio=signal-intensity/control. We were able to compare intensities across all chips including 2x x 2x chips in this way. For data analysis, we screened signal intensity with cutoff > 50; this resulted in ~ 12K genes out of 17K on the chip.

Statistical analysis of using Benjamini-Hochberg and Bonferroni methods

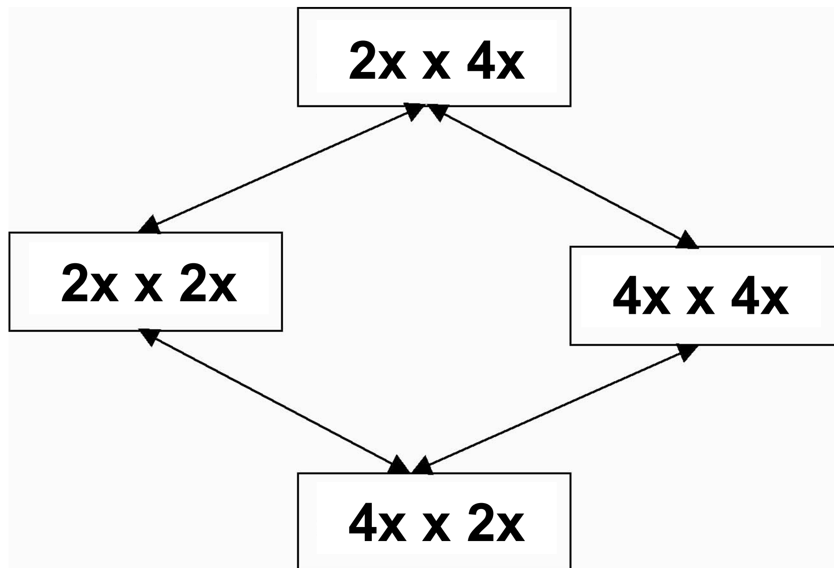
Data were analyzed using GeneSpring software (Agilent Technologies, Santa Clara, CA) for statistical analysis and clustering. There was more of an interest in differential expression patterns between crosses rather than between different time-points, so one-way ANOVA tests were used on all crosses for all genes with signal > 50 (~ 12,000 genes). The Benjamini and Hochberg method (BENJAMINI and HOCHBERG 1995) and Bonferroni method (BONFERRONI 1936) were used for multiple testing corrections. The Benjamini and Hochberg gave a larger set of 3,420 genes with statistical significance for Oh43 and 4,012 for B73, both at 1% false discovery rate (FDR). The Bonferroni method contains a much higher stringency and gave only ~ 600 differentially expressed genes for Oh43 and B73.

Hierarchal analysis

A principle component analysis and K-means clustering was done within GeneSpring to achieve Benjamini and Hochberg and, separately, Bonferroni, hierarchal trees.

Figure 5.1. Microarray schematic for interploidy comparisons for 8, 10, 12, and 14 DAP harvest RNA. Maize tissue was harvested at 8, 10, 12, and 14 DAP. Each arrow represents a double comparison that was done for all time points. 2x x 2x was compared to 2x x 4x and 4x x 2x and vice versa. The 4x x 4x was compared to the interploidy crosses as well.

Figure 5.1. Microarray schematic for interploidy comparisons for 8, 10, 12, and 14 DAP harvest RNA.



Results

Four different types of maize crosses were done using Oh43 and B73 maize inbred lines: 2x x 2x, 2x x 4x, 4x x 2x, and 4x x 4x. Oh43 and B73 maize lines were crossed with their respected tetraploid inbred lines. Cross hybridizations of the two inbred lines did not occur. Endosperm tissue was harvested at 8, 10, 12, and 14 DAP followed by RNA extraction and microarray hybridization. Using Genespring software and ANOVA analysis, expression patterns that were above equal expression was analyzed. Interploidy crosses resulted in 3,420 genes in Oh43 and 4,012 genes in B73 to be differentially expressed. Of those genes that are differentially expressed, 1,783 genes were common to both inbred lines (Figure 5.2).

To better understand the relationship of gene expression changes, hierarchial condition trees, using the 3,420 Oh43 and 4,012 B73 differentially expressed genes, were made (Figure 5.3 & 5.4). The hierarchial trees were compared to the morphological phenotype observed in normal and interploidy crosses (Figure 2.1). Expression patterns of the interploidy crosses which are more similar to the expression pattern of the normal crosses, also had a endosperm phenotype that is more similar regardless of which DAP it was harvested (Figure 5.4).

Figure 5.2. Number of differentially expressed genes in Oh43 and B73 interploidy crosses. Oh43 uncovered, combing both directions of the interploidy cross, 3,420 genes that are differentially regulated. In B73, combining both directions of the interploidy cross, 4,012 genes are missexpressed. 1,783 missexpressed genes correlated to both genotypes.

Figure 5.2. Number of differentially expressed gene is Oh43 and B73 interploidy crosses.

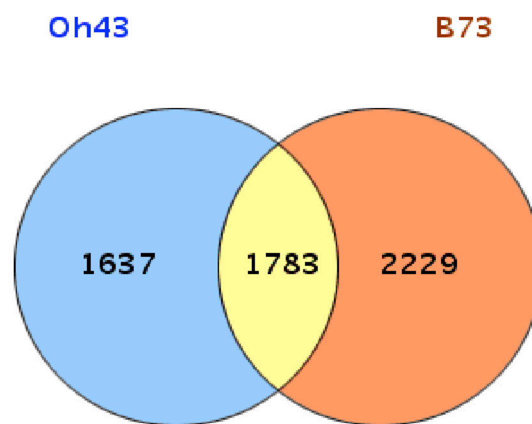


Figure 5.3. Hierarchial tree from Oh43. The 3,420 differently expressed genes were compared to each other to examine overall gene expression similarities among different time points after pollination. DAP=days after pollination

Figure 5.3. Hierarchial tree from Oh43.

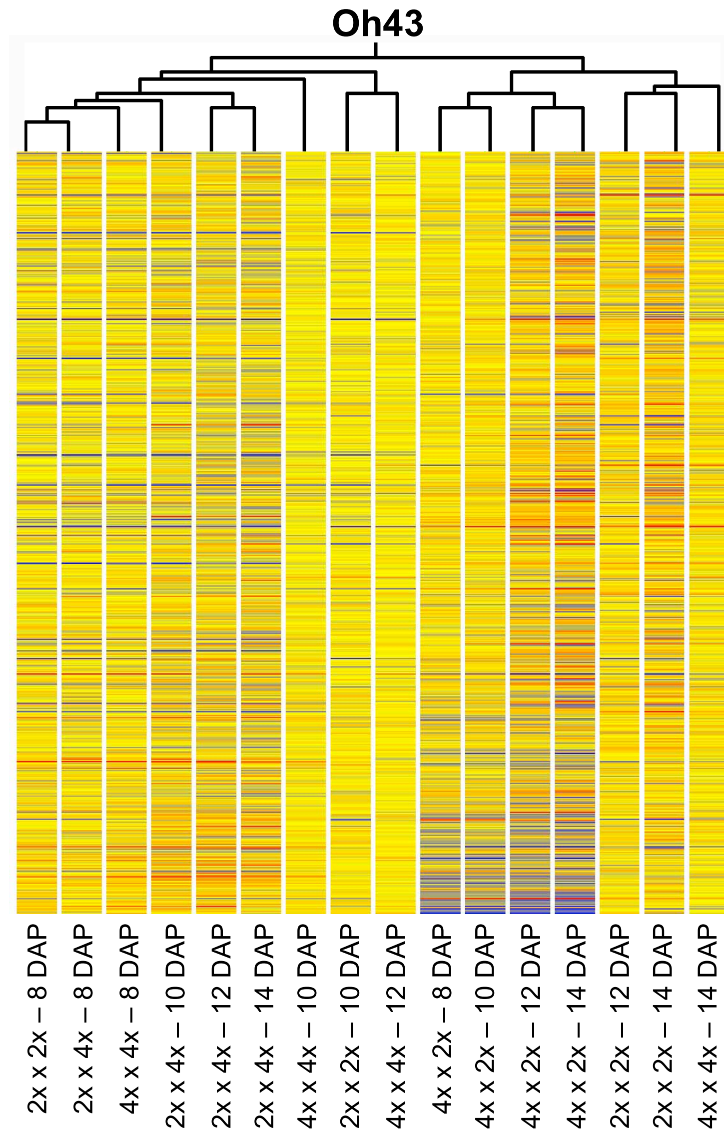
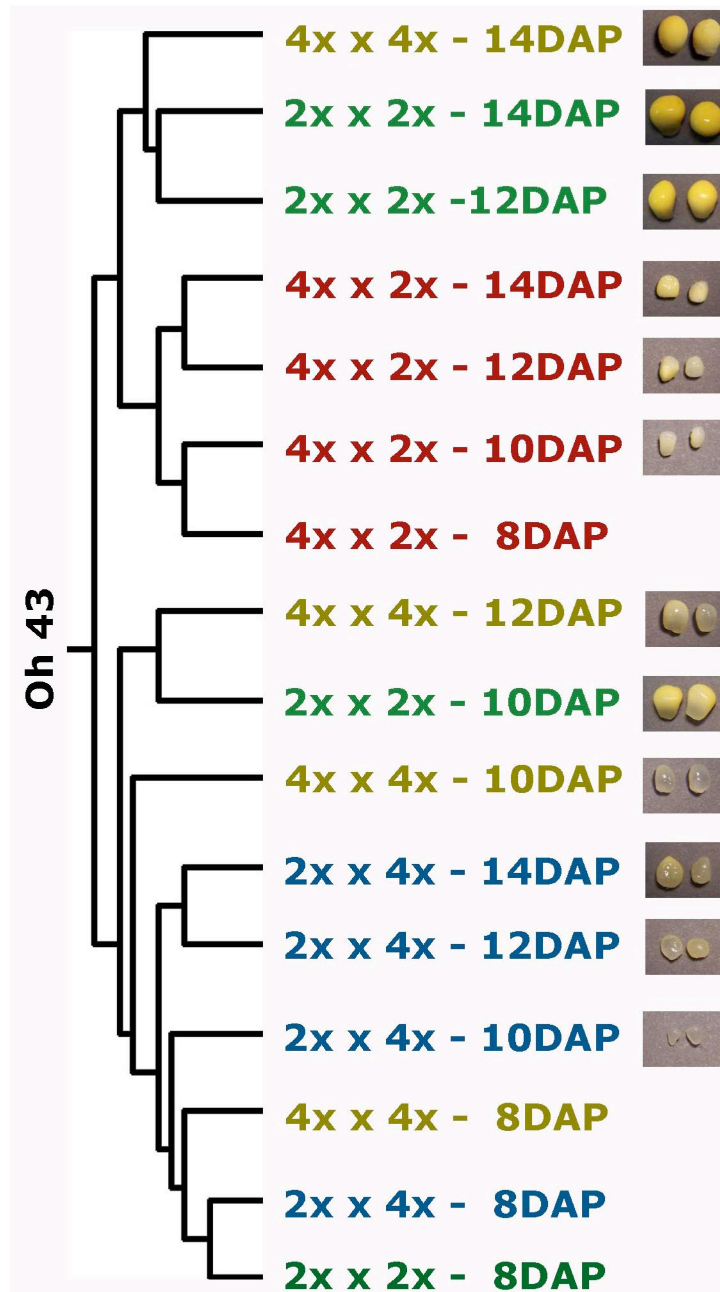


Figure 5.4. Comparing gene expression profiles with morphological characteristics. Morphologies that are most closely related by appearance also seem to correlate with related gene expression profiles.

Figure 5.4. Comparing gene expression profiles with morphological characteristics.



Discussion

Differential genomic expression changes in interploidy crosses

Interploidy crosses cause the collective genome to change expression between 8 and 14 days after pollination relative to their normal cross counterparts. When interploidy crosses were performed between diploid and tetraploid varieties of Oh43, 3,420 genes showed altered expression. When the same interploidy cross was performed between B73 diploid and tetraploids, 1,783 genes were differentially expressed (Figure 5.1). A theoretical reason for the difference in genomic changes in gene expression could be because of the different allelic content of the Oh43 and B73 genomes. Even though interploidy crosses in both lines of maize creates defective kernels, the different allelic content of each may trigger a slightly different defective development pathway which would result in a somewhat different down stream genomic expression pattern. It would be interesting to perform hybrid interploidy crosses diploid or tetraploid Oh43 interploidy crossed onto diploid or tetraploid B73 lines, and examine the downstream gene expression changes. By comparing hybrid interploidy crosses with inbred interploidy crosses could give a better insight into the maternal control of early gene activity.

Hierarchial trees

Diploid female x tetraploid male

Comparing differentially expressed genes using hierarchial trees matches well with phenotypic observation (Figure 5.4) (COOPER 1951). When the maternal 2x is crossed with a paternal 4x, regardless of which DAP, the gene expression pattern is more similar to the gene expression pattern of 2x x 2x-8 DAP and the 4x x 4x-8 DAP endosperm tissue (Figure 5.3 – 5.4). The gene expression data also suggest that development proceeds very slowly, so that at 14 DAP the genomic expression pattern has diverged slightly from the 2x x 2x 8 DAP and 4x x 4x-8 DAP, but is still more closely related to those control time points than that of the slightly later control time points of 2x x 2x and 4x x 4x 10 DAP (Figure 5.3-5.4).

Tetraploid female x diploid male

At 4x x 2x 8 DAP, the genomic expression pattern is more similar to that of the later stages 12 and 14 DAP of the 2x x 2x and the 14DAP of the 4x x 4x control endosperms expression patterns (Figure 5.3 - 5.4). The data also suggest that this rapid development must slow down remarkably. While the 4x x 2x 8 DAP and 10 DAP expression pattern is similar to that of the 12 and 14 DAP control crosses, the 4x x 2x - 12 and 14 DAP expression pattern is more similar to that of the 4x x 2x - 8 and 10 DAP expression (Figure 5.3 – 5.4). This further supports the idea that there is rapid development, at least to the 8 DAP stage of

4x x 2x interploidy cross, but the development then slows down and begins to stall.

It is also observed that the 4x x 2x interploidy crosses occupy a hierarchal branch all to themselves (Figure 5.3 – 5.4). This suggests that once endoreduplication begins, there is very little change in gene expression pattern, which would be consistent with the end of cellular differentiation of the endosperm. This also seems to be the case for 2x x 2x and 4x x 4x control endosperms because there is very little difference between the 12 and 14 DAP endosperm (except the 4x x 4x -12DAP), in which both should be in the endoreduplication phase. This suggests that the difference in genomic expression pattern between the 4x x 2x - 14DAP and both control 2x x 2x and 4x x 4x - 14 DAP samples are due to downstream gene expression changes resulting from a differential gene activation/repression immediately after fertilization. It would be logical to hypothesize that the genomic expression pattern may change dramatically after 16 DAP when the cell death begins, and it would be interesting to examine that pattern to assess its similarity to the interploidy cross endosperm.

Comparing microarray data with morphology

It is interesting that in 2x x 4x interploidy crosses, previous observations suggest that a higher than normal amount of mitosis occurs (COOPER 1951; SCOTT *et al.* 1998). Combining that observation with the gene expression pattern results, suggests that the majority of cells do not exit mitosis, and very few enter

the stage of development in which differentiation takes place, which would explain the near disappearance of the BETL layer (COOPER 1951; HUEROS *et al.* 1999).

The reciprocal 4x x 2x interploidy crosses, even though it may have an increase in developmental rate, has been previously noted that there is a decrease in the amount of mitotic cells (COOPER 1951). This suggests that most cells may rapidly proceed through the mitosis phase and possibly the differentiation phase, which would explain why some endosperm tissues, like the BETL, develop exceptionally thin and small in interploidy crosses. This may additionally explain some observations such as early starch accumulation (COOPER 1951), early endoreduplication (LEBLANC *et al.* 2002), and due to a very shortened mitotic phase there is a marked decrease in overall biomass (Figure 2.2 & 5.4).

Gene expression changes could also be due to the initiation of an incorrect developmental pathway, and the changes we observe are not necessarily due to changes in the speed of the developmental progress. This can be addressed by comparing morphological phenotype with the corresponding DAP genomic expression profile (Figure 5.4). When this is done, there appears that similar morphological phenotype separate together on the hierarchial tree, suggesting that changes in the rate of developmental progress contributes to the defective kernel phenotype.

Chapter 6:

Fluorescent *in situ* Hybridization using large cDNA's

Introduction

The maize genome contains many genes that remain unmapped. It would be advantageous to quickly map loci to chromosome arms, or to more specific molecular regions on chromosomes. Due to recent improvements to FISH techniques, the detection of very small maize genomic targets is possible (KATO *et al.* 2006). This also has the potential to be used to develop a karyotyping system, which is based on features such as genes and not highly repetitive elements, which require prior identification.

Materials and Methods

A search algorithm that identifies mRNAs of a given size from sequences deposited in GenBank was used to find candidate sequences for FISH probe development. It filters GenBank entries by species, type and size. Maize sequences that are annotated as cDNAs or mRNAs and are larger than 6000 bp were manually examined for those that were previously mapped. The *dek1* mRNA (GeneBank AY061806; 7110 bp; (LID *et al.* 2002) was chosen for a chromosome 1 specific probe development. Two additional long mRNAs,

including one whose genomic position was unknown, were selected for use as FISH probes: the unconventional myosin heavy chain (*myo1*; GeneBank AF104924; 5375 bp) and acetyl-coenzyme A carboxylase (*acc1*; GeneBank AY312171; 7324 bp). PCR primers were designed for reverse transcriptase-PCR reaction (Table 6.1).

For reverse transcriptase PCR, template RNA was extracted from leaves or immature endosperm tissue using Trizol reagent (Invitrogen, Cat. 15596-018) and long cDNAs were amplified using Invitrogen's SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity polymerase (Cat. 12574-030). To eliminate NOR hybridization by cDNA based FISH probes, the RNA template was enriched for poly-A containing mRNA using Promega's PolyAtract mRNA isolation system IV (Cat. Z5310). The cDNA was directly used for nick translation as described on page 31.

Results

About eleven percent of maize genes contain repetitive sequences in their introns (HABERER *et al.* 2005) and will not be suitable as FISH probes without removal of the repeats. Because fully processed mRNAs do not contain introns and are therefore likely to be free of repetitive DNA, a database search was conducted for large maize cDNA sequences to use as FISH probes. Thirty-six candidate mRNA sequences larger than 4000 bp were identified including both mapped and unmapped genes. Of these, several with cDNA sequences greater than 6000 bp were selected for further analysis, including *Zea mays* B73 calpain-

Table 6.1. PCR probe production.

Chromosome arm	Probe name	Selected PCR product length, bp	Primers 5'-3'	Template	Sequence accession #
1S	*dek1*	6861	GGA CAC CAC GGA GTT GTT TT TGC GTT AAC AGG AAC GAC AG	Whole RNA and mRNA from leaves, Oh43	AY061806
2S/10L	*acc1/acc2*	7090	CTC AAA GGC CTT GCC ACT AC GCA CCA GGT CCA AAA GAA AA	mRNA from leaves, Oh43	AY312171
3L	*myo1*	2674	ACC ATT TCT GCC CTC CGT CCC CTC TCA ACT TAT TTT CAC TGC TGC TGC TTC CTG CCT CAT	mRNA from leaves, Oh43	AF104924
		2489	TGA GGC AGG AAG CAG CAG CAG TGA AAA TAC GCA TCC GGC CCC TTT TCA CTA CAG		

like protein (*Dek1*; 7110 bp), which has been mapped to chromosome 1S (LID *et al.* 2002), the unconventional myosin heavy chain (*Myo1*; 5375 bp) and the acetyl-coenzyme A carboxylase (*Acc1*; 7324 bp) genes. The *Myo1* gene had not previously been localized, and the maize genome contains two highly similar *acc* genes (ASHTON *et al.* 1994) present on chromosome arms 2L (*Acc2*) and 10L (*Acc1*) (www.maizegdb.org). The resulting PCR products were used as FISH probes (Figure 6.1). Probe *Dek1* labels the expected interstitial position on chromosome 1S and additional signal was seen at the nucleolus organizer region (NOR) due to contaminating cDNA from the rDNA genes. By using RNA that is enriched for poly-A containing mRNA as the RT-PCR template, the NOR hybridization signal was eliminated (Figure 6.1A) and purified mRNA was used as the template for subsequent RT-PCR reactions. The *Myo1* probe produced a signal on the distal end of chromosome 3L (Figure 6.1C). The *Acc1*/*Acc2* probe hybridized near the centromere on chromosome 2L and at an interstitial position on chromosome 10L (Figure 6.1B). Thus, known positions of the genes *Dek1*, *Acc1* and *Acc2* are verified, and the position of *Myo1* gene has been discovered.

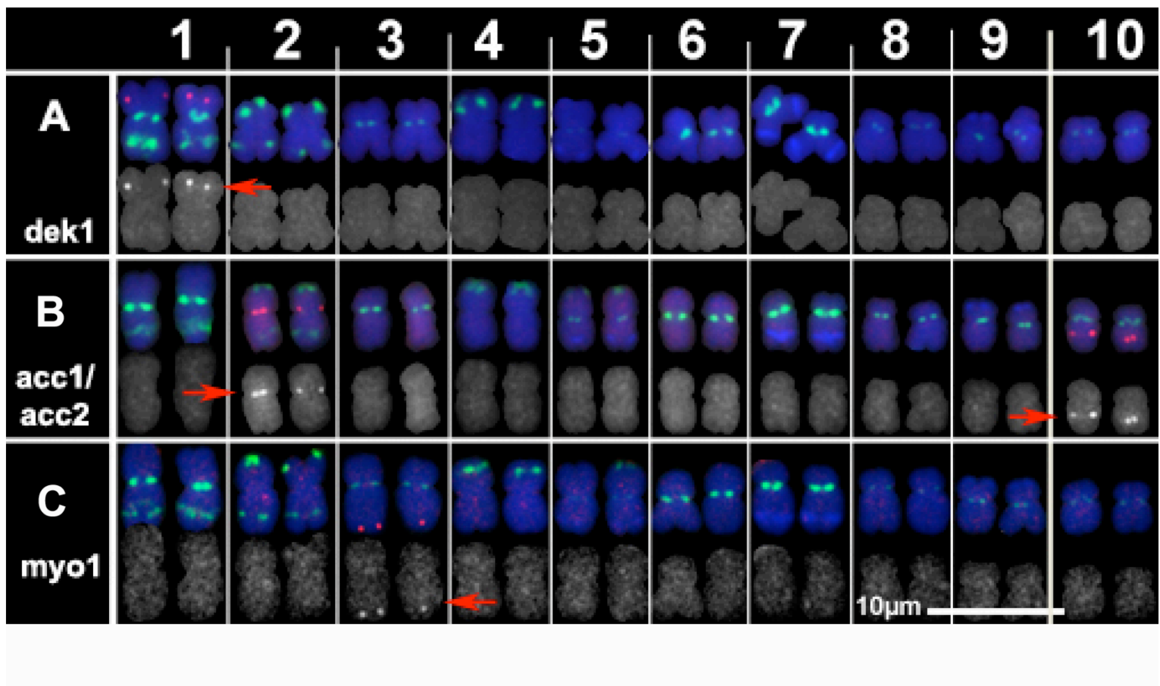
Discussion

By selecting the unique regions, any sequence of sufficient size can be used to design FISH probes (Figure 6.1). This approach can be applied for any species for which sequence data is available. In particular, for species with extensive cDNA sequences available, the identification of large cDNAs provides an effective method to generate a collection of useful FISH probes. Numerous

sequences of unmapped maize genomic DNAs, mRNAs and cDNAs are currently available and this technique could be applied to determine their cytological position. Other probes may be included in different colors to identify each chromosome in addition to the sequence of unknown position. For the common inbred lines B73, Oh43, and KYS all chromosomes can be identified with the inclusion of the CentC and TAG microsatellite probes in one channel and DAPI counterstaining. As an alternative, a probe collection consisting of 5S, *rp3*, Cent4, *expB11*, BAC8L, *expB9* and *rp1* all labeled in one color will allow each chromosome to be identified based on signal intensity and position as well as chromosome size. Either approach uses only two fluorescent channels for karyotyping, leaving others free for experimental probes. The utility of such an approach has been demonstrated by determining the chromosomal location of numerous transgene insertions (YU *et al.* 2006), and of the *myo1* gene (Figure 6.1C).

Figure 6.1. cDNA FISH on somatic chromosomes from maize inbred line B73. Somatic chromosomes from inbred line B73 were hybridized with small target probes (red) and with repetitive element probes (CentC, TAG microsatellite, and the 180 bp knob repeat) which in combination with size and arm length ratios allow each chromosome to be identified. CentC and TAG microsatellite signals are green and the 180 bp knob repeat signals are blue. Chromosomes from individual preparations were cut out and arranged in rows. In each row the merged image is presented to show the chromosomal position of each small target probe. Below the merged image, the gray values are displayed for the small target signals as follows: (**A**) *dek1*, (**B**) *acc1*/*acc2* and (**C**) *myo1*. The red arrow indicates the position of the signals.

Figure 6.1. cDNA FISH on somatic chromosomes from maize inbred line B73.



Chapter 7:

Conclusions and Future Directions

Nitrous oxide treatment of endosperm early in development creates polyploidy and defective kernels

At least since the discovery of genetics (MENDEL 1865), a general curiosity among scientists is, how does an organism develops from a single fertilized cell. This becomes even more difficult in plants because the female gametophyte is embedded in the maternal sporophyte. To examine how genes can coordinate the correct cellular differentiation and developmental program, mutations can be created, followed by various experiments that determine what was mutated and how that disrupts development. In the plant endosperm, this becomes difficult because disruption in endosperm development often aborts the embryo making it difficult to carry the mutation to the next generation. Another method in generating defective kernels is to set up an environment that disrupts development, and follow that with experiments to determine how the new environmental condition did that.

The interploidy hybridization barrier is a natural phenomenon that creates defective kernels due to disrupting the cellular environment in the newly fertilized endosperm. How is the cellular environment disrupted? The data presented in this dissertation support the hypothesis that interploidy crosses disrupt the

dosage of developmental regulators to their respective target loci in the newly fertilized central cell. Such disruption of the dosage of developmental regulators activates or represses the incorrect set of genes, resulting in a developmental pathway ending in a defective kernel. This was shown, not by changing the level of regulators in the central cell, but by changing the number of target loci, while maintaining the ratio of inherited parental loci, immediately after fertilization. This was accomplished by using nitrous oxide to create polyploid endosperm. The data not only showed that the dosage of regulators is important, but also they are important only immediately after fertilization. Once the developmental program has started, chromosomal changes do not observably affect it, suggesting a cellular or genetic compensation mechanism is present. The data presented in this dissertation, along with other published results gives further insight into the effect of the endosperm balance number, apomixis, and an additional mechanism on the evolution of genomic imprinting in the maize endosperm.

To further examine regulator dosage hypothesis, it would be necessary to examine how the dosage of regulators is set in the central cell. This can be explored by examining a large population of enhancer trap lines. An enhancer trap is when a transgenic reporter is inserted into a chromosome, which can be used to identify tissue-specific enhancers. In such a construct, a promoter sensitive to enhancer regulation is fused to a reporter gene, such that expression patterns of the reporter gene will identify nearby enhancers. This can be difficult in the maize central cell due its embedment into maternal tissue. The reported

gene would have to contain a promoter that is specific to the central cell (the *Demeter* promoter for example) and also a sequence (an epitope tag), that would be novel as to enable it to be identified in a RT-PCR reaction. This could be an effective method to identify regions in the maize central cell that are active in gene expression, in which some of those genes must be developmental regulators. Examining for central cell specific genes and varying the dosage of those genes, further inferences can be made into which loci are important for endosperm development.

Regardless of the experiment that can be done, it would be imperative to attempt to identify those regulators. It has recently been shown, with much difficulty, that isolation of central cells is possible (KRANZ *et al.* 1998). By doing this, it was shown that *in vitro* fertilization of the central cell development into a relatively normal appearing endosperm occurs, indicating all the necessary regulators are in the correct dosage in the central cell before fertilization. With much work, it could be possible to collect enough central cells to isolate RNA and attempt a cDNA library. Once the cDNA library is generated, this can be applied to a tiling array which should indicated which genes are expressed (BERTONE *et al.* 2005).

Defective kernels due to a high haploid inducing line of maize

RWS, when used as a pollen parent, creates a high percentage of defective kernels. The defective kernels are not due to polyploid sperm, nor are they due to spontaneous development of the diploid central cell. It is unlikely that

RWS develops aneuploid sperm because this was not readily detected with the probes we used, but still remains a possibility. If the sperm is normal, then a logical explanation is that there is a change in either the distribution or level of DNA methylation of the sperm nucleus, which coincidently alters the activity of the genetic loci contributed. This fits in well with the regulator dosage hypothesis because the paternally inherited loci could be changed, most likely silencing important paternally derived loci. This could explain why the morphology of the defective kernels resembles that of a 4x x 2x interploidy cross. This is supported as well by the endosperm balance number, which shows that overall ploidy is irrelevant as long the dosage of regulators to the target loci is correct. Overall, the paternally contributed genome, even though haploid, creates an abnormal dosage of regulators to target genetic loci in the post-fertilized endosperm cell, starting an incorrect developmental pathway.

Evidence indicates that there are genetic factors involved with high haploid producing lines. It is reasonable to suggest that genetic factors are also involved in the production of defective kernels. Crossing RWS onto a non-haploid producing line and generating recombinant inbred lines from the progeny followed by uncovering genetic marker association could map genetic factors. This would give a better insight into the genetics involved in both high haploid and defective kernel production.

Organization of endoreduplicated chromosomes in the endosperm of Zea mays L.

Beginning around 10 DAP, the nuclei in the maize endosperm stop mitosis and begin endoreduplication. Endoreduplication occurs when the cells continue DNA synthesis, but skips mitosis and cell division. When this occurs, the ploidy increases, as does the volume of both the nucleus and the cell. With the use of FISH, immunocytochemistry, and slot blot analysis, it has been discovered that, when the cells synthesize the chromosomes, they stay attached to each other at both the centromeres and at the knobs. The attachment of the chromatin at the knobs is not due to deposition of centromeric histone H3, which suggests that the attachment is not a direct cause of kinetochore or centromere like functions. Interploidy crosses are very successful at creating defective kernels. This dissertation provides evidence that interploidy crosses severely disrupt the adhesion of the chromatin at the knob, and also slightly less severely at the centromere. As with the morphological phenotype of the endosperm, it also appears that the direction of the interploidy crosses also gives a different degree of disruption. This disruption could be due to downstream changes in gene expression and chromatin configuration due to the initiation of incorrect development genes soon after fertilization.

To gain a better understanding of how endoreduplicated chromosomes stay attached, an examination of the distribution of cohesins should be done. Cohesins form complexes that are attached along the chromosome during S-phase, which is used to hold the chromatids together. The majority of the

cohesins are removed in prophase, but some stay at the centromere until late metaphase. Because endoreduplication occurs due to multiple rounds of S-phase with no mitosis, it is logical to hypothesize that cohesins are deposited along the arms holding the endoreduplicated chromatin fibers together, but are highly concentrated at the centromeres and knob in as much as the endoreduplicated fibers do not separate after replication. How cohesins are deposited along the chromatin is relatively unknown, but interploidy cross could disrupt that causing the knobs to relax and separate.

Because of the diffuse appearance of the knobs, it also could be due to relaxing of the chromatin. Many DNA and histone modifications are known to exist in knobs and other heterochromatic sites. Chromatin immunoprecipitations (Ch.I.P.) assays, using modified histone antibodies could provide insight in how the chromatin has changed due to interploidy crosses.

Global gene expression changes due to interploidy crosses

Many experiments have been done that examine the morphological changes that occur due to interploidy crosses. A question that has not been examined thoroughly is gene expression changes, and to examine if those changes correlate with similar morphological changes. The data presented in this dissertation suggest this is the case. Previous observations suggest that 2x x 4x interploidy crosses have an increased mitotic rate. It was observed that in 2x x 4x interploidy cross at 14 DAP, the gene expression profile does not differ much from that of the 2x x 2x or 4x x 4x - 8 DAP. This suggests that endosperm

development proceeds at a slower pace in the 2x x 4x cross. In the 4x x 2x interploidy cross, the data indicate that development is proceeding rapidly, shortening the differentiation phase of the endosperm life cycle. This would explain both why the BETL region is underdeveloped and why the 4x x 2x - 8 DAP gene expression profile mostly resembles the 2x x 2x and 4x x 2x -14 DAP.

It is important to mention that interploidy crosses presumably initiate an erroneous developmental pathway, and a lot of gene expression changes are due to downstream consequences of this and not due to changes in development rates. When comparing gene expression changes through hierarchial trees and the morphological phenotype at the corresponding DAP, they look very similar, suggesting that changes in developmental timing are a consequence of interploidy crosses.

The microarrays performed were done using the GeneChip® arrays produced by Affymetrix. These chips were constructed using predominantly sequence information from the maize inbred line W23, B73, Oh43, W22, W23, W64A, and Black Mexican Sweet, and only contain roughly 1/3 of the predicted maize genes (STUPAR and SPRINGER 2006). Because Affymetrix array hybridizations only allow one sample hybridizations at one time, some of the gene expression changes could be due allelic difference between our samples and the non-Oh43 and B73 oligos on the array. Also, due to the complex statistical analysis, the information generated from this study needs to be both reproduced and verified. One method of reproduction would be to use the cDNA arrays generated by the maize gene discovery project

(http://www.maizegdb.org/documentation/mgdp/), which utilize RNA hybridization competition between the samples so that a comparison can be made, plus it contains more genes than Affymetrix. Even further verification would follow using either RT-PCR or northern analysis of any significant genes in which gene expression changes have been uncovered.

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Vita

Matthew John Bauer was born in Centralia, IL on April 21, 1976. While living in Shobonier, IL he graduate from Patoka High School in 1994, after which he spent two years at Kaskaskia College. While there, he became the captain of the college bowl team, the vice-president of the student council, and was a member of Phi Theta Kappa, and national honor society. After receiving his Associate of Sciences degree in 1996, he went to the Southern Illinois University in Edwardsville where he was awarded a Bachelor of Science degree in 1998. Matthew then started as a lab assistant at Genome Systems, Inc in October of that year. In 2000, he began graduate school at the University of Missouri in Columbia. While as a student, he was involved with the interdisciplinary plant group, help revive the biology graduate student association, and was a member the graduate professional council. In 2005, he was given the Superior Graduate Student award for Biological Sciences and was also inducted into the Rollins Society, an honor society for Graduate and Professional Students. In May of 2006, he married April Orsborn and subsequently earned a Ph.D. in Biological Sciences in November of that year. After completion of his degree, he plans on continuing doing research in the field of molecular genetics and cell biology as a post-doctoral associate.