Microscopic characterization of a transposon-induced male-sterile, female-sterile mutant in *Glycine max* L.

by

Katherine A. Thilges

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Plant Biology

Program of Study Committee:
Harry T. (Jack) Horner, Major Professor
Madan K. Bhattacharyya
Silvia R. Cianzio

Iowa State University

Ames, Iowa

2016

DEDICATION

I would like to dedicate this thesis to my wonderful family: Paul and Terri Thilges; Sarah Thilges and Phil Tetley; Maggie and Ryan Harm and Jessica Thilges. Their unwavering support and encouragement made this road much easier to navigate. And to everyone who has helped along the way.

TABLE OF CONTENTS

LIST OF FIGURES

TABLE

NOMENCLATURE

MS	Male-sterile
FS	Female-sterile
MSFS	Male-sterile, female-sterile
WT	Wild-type
Het	Heterozygous
MT	Mutant

ACKNOWLEDGMENTS

I would like to thank my committee chair and major professor, Dr. Harry T. (Jack) Horner (ISU GDCB), and my committee members, Dr. Madan K. Bhattacharyya (ISU Agronomy) and Dr. Silvia R. Cianzio (ISU Agronomy), and ex officio members Dr. Marc Albertsen (DuPont Pioneer), Dr. Mark Chamberlin (DuPont Pioneer), and Dr. Devinder Sandhu (University of Wisconsin at Stevens Point), for their guidance and support throughout the course of this research. I would also like to acknowledge the memory of Dr. Reid G. Palmer (USDA, ISU Agronomy) who identified the male-sterile, female-sterile mutant used in this study.

In addition, I would also like to thank my friends, colleagues, and the department faculty and staff for making my time at Iowa State University a wonderful experience. I would also like to thank DuPont Pioneer for their support of my research. Also, thanks to the Iowa State University Microscope and Nanoimaging Facility for their expertise and help.

ABSTRACT

A male-sterile, female-sterile mutant was identified in a transposon-tagging study in soybean (*Glycine max* L.). This mutant displayed abnormalities in both micro- and mega-sporogenesis, as well as gametogenesis. Vegetatively, the mutant showed no visible differences from the wild-type phenotype. Analyses of male meiotic chromosomes were done to better understand any issues that could occur to cause sterility. Wild-type and mutant anthers and ovules were cleared throughout their development, followed by confocal scanning laser microscopy to look for any abnormalities, and to determine the timing of abortion in both the male and female organs in the mutant. Additionally, scanning electron microscopy was done to observe the differences between wild-type and mutant anthers and male cells near maturity and mutant abortion. Results indicate there are chromosomal segregation abnormalities in the mutant male meiosis. Mutant anther development proceeds through meiosis to form abnormal triads, tetrads and pentads to the released microspore stage, after which the resulting male cells become irregularly shaped and eventually abort. Mutant megasporogenesis proceeds through meiosis from megaspore mother cell through two meiotic divisions resulting in the tetrad stage which then progresses to the single megaspore and to the early formation of a megagametophyte which ceases further development These first-time cytological and anatomical results were compared with an already-published molecular study of this sterile soy mutant and to provide insights into this dual sterility in soybean.

CHAPTER I

INTRODUCTION

The purpose of this Introduction is to provide an overview of sexual reproduction in higher plants, specifically soybean (*Glycine max* L.), focusing on the processes during male and female meiosis and abnormalities that can occur to cause sterility, and finally to touch upon the mechanism that I believe causes the double sterility in this soybean mutant that is the subject of this thesis as presented in Chapter 2.

Glycine max L.

The soybean, *Glycine max* (L.) Merr., is one of many plants that was domesticated and is used for human consumption. The soybean genus *Glycine* Willd. is a part of the family Fabaceae/Leguminosae and thus is in the subfamily Papilionoideae, and the tribe Phaseoleae. The genus *Glycine*, has two subgenera, *Glycine* (perennials) and *Soja* (annuals) (Orf 2010). The mutant studied in this reasearch is in the *Glycine* sub-genus. Soybean has a chromosome number of $2n = 40$, so it is considered a diploidized tetraploid. This is the result of a diploid ancestor (n=11), which underwent aneuploid loss (n=10); followed by polypodization (2n=20) and eventual diploidization (n=20). Two rounds of genome duplications may have occurred (Johnson 1962).

Soybean is an annual plant that ranges in height from 75 to 125 cm. The vegetative branching can be sparse or dense based on the cultivar or the growing conditions. The most common of these branching patterns seen in soybean is first-order branching, in which branches extend off of the main apical meristem. The soybean root system does have a taproot, but it is often undistinguishable from other roots and from the large mass of secondary roots. Besides these roots, the soybean plant does have multi-

branched adventitious roots that emerge from the lower portion of the hypocotyl. Soybean is a legume and fixes nitrogen through symbiosis with *Rhizobium.* In the vegetative progression of soybean there are four different types of soybean leaves. The very first pair of leaves is represented by cotyledons, followed by a second pair of primary leaves, trifoliate foliage leaves, and the prophylls (Lersten & Carlson 2004). All of these primary leaves are ovate in shape and are oppositely arranged in pairs at the stem. The previously described leaves occur at the first node above the cotyledons. All subsequent leaves are trifoliolate and distichously arranged (Lersten & Carlson 2004).

The *Glycine max* flower is papilionaceous with a characteristic irregular and butterfly-like corolla. This flower is composed of a tubular calyx of five sepal lobes that are unequal, and a corolla comprised of five parts. These parts are the posterior banner petal, two lateral wing petals, and two anterior keel petals (Lersten & Carlson 2004). None of these five parts of the corolla are fused. The male floral organ, consisting of the ten stamens is called the androecium, occurs in two groups; nine stamens fused together as a single structure, and a single posterior stamen; this arrangement is called a diadelphous pattern. The female floral anatomy is comprised of a single pistil that is unicarpellate and has one to four campylotropous ovules aligned along the posterior suture. The style is about half the length of the ovary (gynoecium) and curves backward toward the free posterior stamen, then terminating in a capitate stigma (Lersten & Carlson 2004).

A closer look at the development of the female organ of soy reveals that in the gynoecium the typically two to four ovule primodia are produced and developed simultaneously. As development proceeds each ovule becomes campylotropous, with its

micropylar end directed upward toward the stigma. Covered by a single-layered protoderm, the ovule primordia are all about the same size. After ovule initiation the hypodermal archesporial cells can be distinguished. These archesporial cells are larger than the surrounding cells and contain more densely staining cytoplasm (Lersten & Carlson 2004). Each archesporial cell become a functional megasporocyte (megaspore mother cell). Periclinal divisions in the hypodermal region produce two parietal layers of nucellus between the megasporocyte and the epidermis of the ovule. Meiosis of the megasporocyte results in a linear tetrad of haploid megaspores. Three of the four megaspores abort resulting in a functional megaspore at the chalazal end and three aborted micropylar megaspores. The first mitotic division of the functional megaspore produces a two-nucleate megagametophyte(Lersten & Carlson 2004). These nuclei are displaced to opposite ends of the cell by the formation of a large central vacuole. This is followed by a second mitosis that produces a total of four nuclei. A progression of two more mitotic divisions produces an eight-nucleate megagametophyte with four nuclei located at the chalazal end and four at the micropylar end (Lersten & Carlson 2004). These divisions all occur with karyokinesis, but no cytokinesis. One nucleus migrates from each end toward the center of the cell forming two polar nuclei that eventually fuse. This eight-celled stucture is now the mature megagametophyte consisting of three chalazal antipodals and an egg and two synergids at the micropylar end (egg apparatus), all contained within the large central cell with its two polar nuclei. At this point in development cytokinesis has occurred to form the egg, synergids, and antipodals (Lersten & Carlson 2004). The egg and synergids can be differenitated by vacuole orientation. The egg nucleus is displaced toward the chalazal end of the cell by a vacuole. Both synergids

have a vacuole at the chalazal end of the cell, which means the nuclei are positioned toward the micropylar end. Subsequent maturation of the megagametophyte results in the breakdown of the three antipodal cells (Lersten & Carlson 2004).

At pollination the diadelphous stamens are elevated so the anthers are in close proximity to the stigma. This allows the pollen to shed directly onto the stigma. This floral morphology results in a high percentage of self-fertilization. Natural cross pollination varies from <0.5% to about 1% in annual soybean varieties (Palmer et al. 2011). It has been noted that pollination may occur the day before full opening of the flower (Lersten & Carlson 2004). Both female and male development and maturation occur in parallel. During double fertilization, one sperm nucleus fertilizes the egg cell and the other sperm nucleus combines with the polar nuclei. The fertilization results in a diploid zygote and a triploid endosperm. The megagamtophyte becomes an embryo sac and the ovule is now termed a seed. As the seed develops following fertilization, the nucellus ruptures at the micropylar end, exposing the embryo sac so that the suspensor of the embryo is now in direct contact with the epidermis of the outer integument. As development proceeds each seed becomes campylotropous, with its micropylar end directed upward toward the stigma (Lersten & Carlson 2004).

Each stamen primordium contains a homogenous mass of cells surrounded by a protoderm layer. As the development continues the apical portion forms a four-lobed anther and a short filament. Each one of these four lobes (microsporangium) encloses a mass of archesporial cells that is surrounded by four to six layers of cells derived from periclinal divisions of the protoderm. These layers of cells will mature into the epidermis, endothecium, parietal layers, and tapetum. These archesporial cells then give rise to a

sporogenous mass that further develops into twenty-five to fifty microspore mother cells in each microsporangium. Each one of these mother cells secretes a callose wall around itself. The surrounding tapetal cells are binucleate. As meiosis initiates in the mother cells, the tapetal cells will enlarge and at the completion of microsporogenesis only the outer cell walls will remain. As meiosis occurs in the mother cells a two-nucleate dyad forms with no partitioning cell wall. A second nuclear meiotic division follows, which reults in four microspore nuclei that share a common cytoplasm. Cytokinesis proceeds shortly after, separating the microspore nuclei into four microspores each surrounded by callose. Each of these microspores contains small vacuoles, mitochondria, large plastids, and a single large nucleus (Lersten & Carlson 2004). The young pollen wall is initiated around each microspore while still in the callose tetrad, and at pollen maturity the outer wall or exine is composed of the tectum, columellae, and endexine; three colpi with pores are present. The mature pollen grain exine has sculptured and smooth areas. Pollen grains are nourished by the tapetum cell layer. The late vacuolated pollen grains prior to germination and pollen tube formation undergo mitosis and cytokinesis which produce a vegetative cell and a generative cell. The generative cell will then divide prior to release from the anther. At the time of maturation and release from the anther (dehiscence) the pollen grains range from 21-30 μm in diameter and are tricolporate (Lersten & Carlson 2004).

Soybean Genetics

A forward genetic screen is the science of using a population of randomly induced mutants and identifying a specific mutant phenotype of interest. This process can be quite difficult in soybean, so more often reverse genetic strategies are used. This is where the

soybean contains a mutation of a target gene and this mutant phenotype is characterized to understand the function of the target gene (Bilyeu 2008). Another aspect of genetic work in soybean is the use of transposon insertion, where populations of mutants can be induced. These populations can then be used to target soybean genes (Cui et al. 2013, Hancock et al. 2011, Mathieu et al. 2009)

Meiosis

The division and replication of cells are the basis for both vegetative and sexual reproduction in most organisms. The evolution of meiotic division was significant in development of sexual reproduction in plants. This division provides a mechanism that reduces the chromosome number by half from the diploid to the haploid. The meiotic cycle also provides reorganization events of the genetic material through crossing over that enhances genetic variability for the next generation (Cohn 1969). The four products of meiosis have half as many chromosomes as the parent cell. Meiosis consists of two nuclear divisions in which the chromosomes duplicate only once, but the nucleus and cytoplasm divide twice to produce four daughter cells (Cohn 1969). Prophase I of meiosis is of an extremely long duration in comparison with the other three stages. The volume of the nucleus begins to increase in early prophase I, which is called leptotene. By the beginning of leptotene chromosomes are and become very long. The chromosomes then develop a number of small coils, which can vary in their degree of condensation. These coils are called major coils, which will only continue to grow in diameter as prophase I proceeds. Since the chromosomes remain rather elongate, individual chromosomes are hard to identify (Ross et al. 1996). Also, chromatids remain associated since the kinetochore is still functionally single in each chromosome The nucleolus is apparent

during leptotene but in some cells is relatively small at first, increasing in size during leptotene and zygotene (Cohn 1969). Following leptotene, the next substage in prophase I is zygotene. The most significant event of zygotene is the beginning of synapsis of homologous chromosomes. Since each chromosome already consists of two chromatids, each pair of homologous chromosomes consists of four chromatids and is referred to as a tetrad (Ross et al. 1996). This pairing may begin at the ends of the chromosomes, proterminal including the telomeres, and progress toward the kinetochore region. The pairing may also begin near the kinetochore, procentric, and progress to the ends or it may be random (Cohn 1969). Incomplete pairing of the segments may occur when chromosomes are very long. In the majority of meiotic cells most of the chromosomes will become almost entirely synapsed by the end of the zygotene substage. The coiling of the chromosomes continues during zygotene. The chromosomes become shorter and thicker as the major coils increase in diameter. These coils are considerably larger than their mitotic counterparts, the somatic coils. Pachytene begins when the pairing of the chromosomes is complete in zygotene (Cohn 1969). Pachytene is one of the longer sub-stages of prophase I and is characterized by several important events. The chromosomes will grow shorter and thicker as a result of the increase in coil diameter and the appearance of another level of coiling. Within the major coils and at right angles, minor coils arise. The relational coiling of the homologues persists, and the direction of this coiling is usually opposite that of the internal major coiling. Individual pairs of homologous chromosomes (bivalents, composed of four strands) can now be identified, and the staining properties of the chromosomes permit recognition of heterochromatic and euchromatic regions (Cohn 1969). It is possible to detect areas within each bivalent

where there is a physical exchange between adjacent chromatids. This is called a chiasma, and it is due to an exchange of genetic material, or crossing over. The number of chiasmata in a bivalent can depend on the lengths of the chromosomes (Cohn 1969). The presence of more than one chiasma is less likely in a short segment of a chromosome than it is in a long segment. There is usually one chiasma observed in each arm of a chromosome. The chiasmata are very easily discernible in diplotene the next substage. In diplotene there is a continued condensation of the chromosomes through increased coiling. As the chromosomes repel each other, the bivalent assumes a configuration that is consistent with the number and positions of its chiasmata. These factors govern the configuration throughout the next prophase I stage, diakinesis, and metaphase I. The sites of the chiasmata are the only places at which the homologs are associated (Cohn 1969). The chiasmata are most distinct during diplotene, and at the end of this sub-stage chiasmata begin to move toward the ends of the chromosomes in the process of terminalization. The increase in coiling and the resulting shortening of the chromosomes, causes the chiasmata to appear to slide off the ends of the chromosomes through diplotene and the next stages of prophase I (Ross et al. 1996). This shortening produces a kind of unraveling of the paired chromosomes. During diakinesis the bivalents migrate to the periphery of the nucleus and are separated from each other. Although the coils tighten within the chromosomes, making them shorter still, and terminalization continues, the shapes or configurations of the bivalents are very similar to those in diplotene. Often the chromosomes in this last sub-stage of prophase I exhibit a higher degree of staining than before, as a function of the increased density of the coils. The nucleolus begins to disappear and is no longer visible at the end of diakinesis (Cohn 1969). The last event of

prophase I is the disruption and dispersal of the nuclear envelope, releasing the chromosomes into the cytoplasm of the cell. By this time the spindle fibers have organized to establish the poles of the cell, and they will determine the axis of orientation of the chromosomes in metaphase I (Ross et al. 1996). In meiosis, as in mitosis, the chromosomes move to the equatorial region of the cell after the disruption of the nuclear envelope. The orientation of the chromosomes in meiotic metaphase is quite different from that in mitotic metaphase. In metaphase I the chromosomes lie near the equator with their kinetochores toward the poles and their arms toward the equator. The kinetochore regions of the homologues appear to repel each other to create this arrangement (Cohn 1969). The kinetochore of each chromosome behaves as an individual unit. The position of each member of a bivalent with respect to the poles is random. The areas of association between the chromosomes of each bivalent are determined by the number and positions of the chiasmata. The effective difference between meiotic and mitotic orientations is that the chromosomes are not lined up along the equator in meiosis (Cohn 1969). Therefore, the number of chromosomes dividing in anaphase I will not be the same as the number of chromosomes present in the original cell. Although the kinetochore of each chromosome remains functionally single in metaphase, it is structurally double; but not divided. Spindle fibers extend between the poles and are attached only to the kinetochores of the chromosomes as chromosome fibers. Meiotic coils are apparent. The two homologs comprising a bivalent move to opposite poles of the cell during anaphase leading to segregation of chromosomes carrying chromatids from both parents due to crossing over (Cohn 1969). The kinetochores move first, carrying with them the arms of the chromosomes and effecting the complete

terminalization of the chiasmata. In contrast to mitotic chromosomes, the arms of the chromatids in each chromosome then diverge, apparently repelling each other. The consequence of this stage of meiosis is a reduction of the chromosome number by one half, from the diploid to the haploid number, but is still 2C in soybean considering it is a diploidized tetraploid. This is the essential difference between the first meiotic division and mitosis. The events of telophase are practically the same in meiosis and mitosis. The chromosomes elongate through a loosening of their coils, the nucleolus reappears, and a nuclear envelope forms around each polar group of chromosomes. Cytokinesis may or may not occur, and so the products of the first meiotic division may be two cells or two nuclei with a common cytoplasm. In the latter case the nuclei will be separated by a plasma membrane at the end of the second meiotic division (Cohn 1969).

Gametogenesis or the development of gametes has as its basis both meiotic and mitotic cell divisions. The divisions of upmost importance are meiotic, for they reduce the chromosome count to the haploid number. When fertilization occurs, the diploid number is restored (Cohn 1969). Throughout meiosis there is a wide range of genes that are necessary for the normal completion of meiosis. A disruption in any of these genes can cause a range of effects from sterility to polyploidy. Since many of these genes involved in meiosis are not well understood, research is ongoing to better understand them (Mercier et al. 2008).

Soybean Sterility

Pollen sterility is when functional pollen grains cannot be produced by the plant. The development of functional pollen involves a series of sequential events starting from the initation of anthers and culminating in anther dehiscence, as has been described

in the previous section. In pollen sterile plants, functional pollen grains cannot form due to a range of issues (Buntman & Horner 1983). There are three issues that can cause sterility, one of them is meiotic abnormalities.These result from polyploidy or hybridzation. This can often cause univalent and mutlivalent chromosome formations. Another issue is one that has choromosomal deficiencies and rearrangements that can result in the formation of DNA unbalanced gametes. The third class that can cause sterility is the presence of lethal genes. These genes are usually recessive and are only sterile under the homozygous condition (Shivanna 2003). Pollen grains can be produced, but fail to dehisce. Pollen grains can also fail to separate from each other in some mutants.

In soybean there are mutations that affect microsporogenesis and/or microgametogenesis (Graybosch et al. 1988). Two important classes of mutants that lead to aberrant chromosomal pairing or abnormal maintenance of chromosomal pairing have been identified in soybean. Genes that affect synapsis when mutated can cause various combinations of male and female sterility (Slattery et al. 2011). A male-sterile, female-sterile soybean mutant is the subject of this study.

Male and female sterility that are caused by abnormal chromosome number can be due to asynapsis or desynapsis. In sterile plants, homologous chromosome pairing can be variable during pachytene. Often there are major differences in diakinesis in chromosome pairing between sterile and fertile plants. Male-sterile and female-sterile synaptic mutants will give rise to gametophytic cells that have abnormal chromosome numbers because of the loss or gain of chromosomes. A variation in pollen grain size from sterile plants is characteristic of these desynaptic mutants (Ilarslan et al. 1997).

Study of these desynaptic mutants can help to better understand chiasma and its maintenance. In the formation of chiasma, crossing-over is essential. The effects of early appearance of univalents prior to metaphase I can lead to small extra mini-spindles to tri-polar or quadri-polars, while unpaired chromosomes can cause multiple spindle defects. Unpaired chromosomes can also cause spindle abnormalities (Bione et al. 2002). Since most synaptic mutants also affect female fertility, it has been suggested that the set of genes that controls homologous chromosome pairing and recombination works in both ovules and anthers. Gamete imbalance is one of the major consequences of synaptic mutations (Bione et al. 2002). This is seen, especially when the chromosomes separate incompletely and can lead to various forms of aneuploidy. Aneuploids have been observed in the offspring of synaptic mutants (Bione et al. 2002).

Mer3

One tool researchers are using to better map genes is the use of transposons. These transposons are DNA fragments that can move throughout the genome. There are two classes of transposons, of which class 2 is of interest since this class consists of DNA transposons that are transposed directly from DNA to DNA, using a cut and paste system. Therefore, this class is of a low-copy number making it easier to track its movements around the genome. 'CACTA' type, that contains the conserved sequence in its outermost terminal repeat, is a Class 2 transposon (Fang-Ping 2006).

Raval et al. (2013) found that the male- and female-sterility was the result of an insertion of the transposon, *Tgm9,* into a fertility locus in soybean. Baumbach et al. (2016) cloned the gene that was disrupted by this transposon and found that it had a high corresponding match to the *MER3* gene in *Arabidopsis* encoding a helicase enzyme.

MER3 encodes a ZMM protein that is associated with crossing-over. Research has suggested that *MER3* could act in at least two stages during meiotic recombination. The first occurs with the initiation of strand invasion or in extending the length of early heteroduplex recombination intermediates. Secondly, it further promotes the formation of a recombination intermediate required for crossing over and/or it could function to promote the resolution of Holliday junctions to yield crossovers. Studies indicating that *MER3* can unwind dsDNA from blunt ends suggest that *MER3* is involved in unwinding the ends of meiotic double strand breaks prior to their resection, a helicase function (Nakagawa and Kolodner 2002).

A mutation in *MER3* in *Arabidopsis* has been shown to be specific to recombination events. The *MER3* function appears to be conserved across yeast and plants. (Mercier et al. 2005). In rice *MER3* mutants, leptotene and zygotene stages of meiosis were found to be similar to that in wild-type. In pachytene, the mutant aligned normally, but some regions of the homologous chromosomes began to separate. This separation became more evident as the condensation of chromosomes occurred. While some normal bivalents were seen, there was an increase in univalents in metaphase I. This trend continued until uneven chromosome numbers were observed in anaphase I and telophase I (Wang et al. 2009). These phenotypes have been observed in *Arabidopsis*, rice, and yeast (Mercier et al 2005, Nakagawa et al. 2002, Wang et al. 2009).

The research conducted in this study, was to determine the microscopic phenotypes induced in the male-sterile female-sterile soybean mutant and then to compare it to the known *MER3*, phenotypes (Mercier et al 2005, Nakagawa et al. 2002, Wang et al. 2009). To confirm this phenotype, would not only support the genetic and

molecular work done by Baumbach et al. (2016), but it would also validate the use of the transposon-induced sterility mutants, as an approach to map and identify other potentially useful fertility genes in soybean.

References

Bilyeu KD 2008 Forward genetics in soybean. Pages 135-139 *in* G Stacey ed; Genetics and Genomics of Soybean. New York.

Bione NC, MS Pagliarini, LA Almeida 2002 A new and distinctive male-sterile, female-fertile desynaptic mutant in soybean (*Glycine max*). Hereditas 136:97-103.

Buntman D, HT Horner 1983 Microsporogenesis of normal and male (ms_3) mutant soybean (*Glycine max*). Scan Electron Microsc II:913-922.

Carlson JB, NR Lersten 2004 Reproductive morphology Pages 59-95 *in* HR Boerma, JE Specht eds, Soybeans: Improvement, Production Madison, Wisconsin.

Cohn NS 1969 Elements of cytology. Harcourt, Brace & World Inc, New York

Cui Y, S Barampuram, MG Stacey, CN Hancock, S Findley, M Mathieu, Z Zhang, WA Parrott, G Stacey 2013 *Tnt1* retrotransposon mutagenesis: a tool for soybean functional genomics. Plant Physiol 161(1):36-47.

Fang-Ping T 2006 Progress in plant CACTA elements Acta Genetica Sinica 33:765-774.

Graybosch RA, RG Palmer 1988 Male sterility in soybean - an overview. Amer J Bot 75:144-156

Hancock CN, F Zhang, K Floyd, AO Richardson, P Lafayette , D Tucker, SR Wessler, WA Parrott 2011 The rice miniature inverted repeat transposable element *mPing* is an effective insertional mutagen in soybean. Plant Physiol157(2):552-562.

Ilarslan H, HH Skoripska, RG Palmer 1997 Cytology and genetics of a tissue culture-derived soybeam genic male-sterile, female-sterile. J Hered 88:129-138.

Johnson HW, RL Bernard 1962 Soybean Genetics and Breeding. Pages 149-218 *in 23 ed,* Advances in Agronomy, Press, City.

Lersten NR, JB Carlson 2004 Vegetative morphology Pages 15-57 *n* HR Boerma, JE Specht eds, Soybeans: Improvement, Production Madison, Wisconsin.

Mathieu M, EK Winters, F Kong, J Wan, S Wang, H Eckert, D Luth, M Paz, C Donovan, Z Zhang 2009 Establishment of a soybean (*Glycine max* Merr. L) transposon-based mutagenesis repository. Planta 229(2):279-289.

Mercier R, S Jolivet, D Veson, E Huppe, L Chelysheva, M Giovanni, F Nogue, MP Doutriaux, C Horlow, M Grelon, C Mezard 2005 Two meiotic crossover classes cohabit in *Arabidopsis:* one is dependent on *MER3,* whereas the other one is not. Curr Bio 12:692-701.

Mercier R, M Grelon 2008 Meiosis in plants: ten years of gene discovert. Cytogenet Genome Res 120:281-290.

Nakagawa T, RD Kolodner 2002 *Saccharomyces cerevisiae* Mer3 is a DNA helicase involved in meiotic crossing over. Mol and Cell Bio 22,10:3281-2191.

Orf J 2010 Introduction Pages 1-18 *in* K Bilyeu, MB Ratnaparkhe, C Kole, *Genetics, Genomics and Breeding of Soybean*. CRC Press. New York.

Palmer RG, J Gai, VA Dalvi, MJ Suso 2011 Male sterility and hybrid production technology. Pages 193-207 *in* A Pratap, K Jitendra eds, Biology and Breeding of Food Legumes, Press, City.

Ross K, P Fransz, G Jones 1996 A light microscopic atlas of meiosis in *Arabidopsis thaliana*. Chromosome Res 4:507-516.

Slattery RA, S Pritzl, K Reinwand, B Trautschold, RG Palmer, D, Sandhu 2011 Mapping eight male-sterile, female-sterile soybean mutants. Crop Sci 51:231-236

Shivanna K 2003 Pollen sterility Page 77. *in* eds, Pollen Biology and Biotechnology Science Publishers.New Hampshire.

Wang K, D Tang, M Wang, J Lu, H Yu, J Liu, B Qian, Z Gong, X Wang, J Chen, M Gu, Z Cheng 2009 MER3 is required for normal meiotic crossover formation, but not for presynaptic alignment in rice. J Cell Sci 122:2055-2063.

CHAPTER II

MICROSCOPIC CHARACTERIZATION OF A TRANSPOSON-INDUCED MALE-STERILE, FEMALE-STERILE MUTANT IN *GLYCINE MAX* L.

Katherine A. Thilges[1, 2, 3], Mark A. Chamberlin[2], Marc C. Albertsen[2], Harry T, Horner[1]

[1]Department of Genetics, Development and Cell Biology & Microscopy and NanoImaging Facility, Iowa State University, Ames, IA 50011

[2]DuPont Pioneer, 7300 NW 62nd Avenue Johnston, IA 50131-1004

[3]Primary researcher and author

Manuscript to be submitted to the: *International Journal of Plant Sciences*

Abstract

Premise of research. A male-sterile, female-sterile mutant was discovered in a *w4*-m mutable line of *Glycine max* L. The mechanism of its sterility was not well understood. Therefore, different cytological and microscope techniques were undertaken to better understand the process of mutant phenotype development. Molecular research indicated that *mer3* was responsible for the sterility.

Methodology. Macro images were collected of whole plants, flowers, anthers, pods and ovules. Anthers squashes and optical slices through whole anthers and ovules were made using bright-field and confocal scanning laser microscopy. Whole mature anthers and isolated pollen images were collected and studied with scanning electron microscopy.

Pivotal results. In observations of the mutant, male cell development was found to begin normally and then digress when reaching metaphase I, when abnormal segregation of chromosomes with reduced bivalent formation was observed. It was the distribution of univalents and bivalents that led to male sterility. On the female side of development, the

progression of development was arrested in the megagametophyte stage, likely due to abnormal meiosis, leading to ovule abortion and female sterility.

Conclusions. The *Glycine max* male-sterile, female-sterile mutant was shown to have the same phenotype of *mer3* sterility already shown in Arabidopsis, rice and yeast, and some animal systems.

Brief Title: Transposon-induced MSFS in *Glycine max*

Keywords: *Glycine max,* female sterility (FS), male sterility (MS), *mer3*, soybean, transposon

Acknowledgements: We would like to thank DuPont Pioneer for supporting this study and use of its facilities for growing the plants and carrying out some of the microscopy, and the Microscopy and NanoImaging Facility, Iowa State University, for use of its scanning electron microscope.

Introduction

The *w4*-mutable line (T322) of soybean was found, in an F_7 generation of a cross between two breeding lines of Asgrow Seed Co (Palmer et al. 1989). In these plants, flowers were either white, purple or variegated in color. Analysis revealed that the floral traits were conditioned by an unstable recessive allele, *w4*-m, caused by insertion of a CACTA-type active transposable element in the *DFR2* gene located in the *w4* locus. *DFR2* encodes dihydroflavonol-4-reducatse 2 involved in anthocyanin biosynthesis (Xu et al. 2010). Mutable plants of the *w4*-mutable line are homozygous for mutable alleles at the *W4* locus. Wild-type progeny (germinal revertants) are produced by mutable plants when the *w4*-m allele reverts to wild type (Palmer et al. 1989). The *w4*-mutable line is

wild-type for the anthocyanin. This unstable allele, *w4*-m, reverts at high frequency to a the wild-type *W4* allele (Palmer et al. 1989).

Xu et al. (2010) confirmed that when the transposon *Tgm9* was excised from *DFR2* intron II, an anthocyanin locus, the *DFR2* expressed and gave a purple flower phenotype. *Tgm9* is a low copy number element like most CACTA elements. These CACTA elements excise at a high frequency (Xu et al. 2010).

A male-sterile, female-sterile mutant from a germinal revertant of the *w4*-m line bearing purple flowers was found at Iowa State University (Raval et al. 2013). The locus was mapped to molecular linkage group J (Chromosome 16) through bulked segregant analysis. A 62-kb region was found and fine mapping indicated that the region contained only five genes. One of the genes in that region, identified as *Glyma.16G072300*, encodes a helicase (Raval et al. 2013). A rice homolog of this helicase gene has been shown to control crossing over and a sterility phenotype (Wang et al. 2009). *Glyma.16G072300* is then most likely the gene regulating the male and female sterilty phenotype in the mutant soybean (Raval et al. 2013) . DNA blot analysis was done for individuals that were segregating for *Tgm9.* A perfect association was shown between sterility in the plant and the presence of the transposon *Tgm9* in the sterility locus (Raval et al. 2013).

Molecular analysis of two independent revertant plants confirmed excision of *Tgm9* from *Glyma.16G072300* had resulted in the fertile branches, confirming that mutation in the helicase gene causes male and female sterility (Raval et al. 2013). Phylogenetic analysis placed the gene in a clade with the Arabidopsis and rice MER3 proteins and suggested that it is the only *MER3*-like gene in soybean (Baumbach et al

2016). This gene is expressed in flower buds, trifoliolate leaves and stems; however, there was no transcript detected in cotyledons or roots (Baumbach et al. 2016).

The purposes of this study were three-fold: to conduct a microscopic study to determine phenotypes of the male-sterile and female-sterile mutant caused by insertion of *Tgm9* in a *MER3*-like gene recently identified by Baumbach et al. (2016); to identify any abnormalities during meiosis that were *MER3*-like; and to follow the development of anthers and ovules to pinpoint when sterility occurs and what tissues may be affected in the mutant. These results were compared to the molecular study (Baumbach et al. 2016) to understand the fertility mechanisms in soybean.

Materials and Methods

Plant material: The *w4*-mutable line of soybean is an unstable mutation for purple flower color at the *W4* locus. It was found in the F_7 generation of a cross between breeding line X1878 (Amsoy 17 X AG52109) and X2717 (Corsoy X Essex) (Palmer et al. 1990). From this progeny of the *w4*-m line, a male-sterile, female-sterile (MSFS) mutant was identified, ASR-10-181 (A05-221, segreating background number) (Baumbach et al. 2016). This *w4*-m line (MT) and a comparable wild-type (WT) line (Minsoy) were used in this study.

Growth of Plants: All plants (seven rounds of plantings) were grown under identical greenhouse conditions at the DuPont Pioneer Soy Research Facility (Johnston, IA). The lighting schedule started at 16 h of light for 25 d and then descreased in the following sequence: 15.5 h for 3 d; 15 h for 3 d; 14.5 h for 3 d; 14 h for 25 d; 13.5 h for 3 d; 13 h for 3 d; 12.5 h for 3 d; and 12 h for approximately 25 d or until complete dry down and then harvest. Throughout the growing cycle the daytime temperature was set

for 81°F- 83°F and the nighttime temperature was 71°F - 73°F. Seeds were initially started in flats with a standard soil mix and then were transplanted into pots with a Fafard 3B growing mix (www.fafard.com)

Greenhouse whole plants and flowers: Whole WT and MT plants were imaged in the greenhouse with a Nikon DSLR model D70 camera starting at 4 w post germination and were imaged once a week for 5 w to monitor for any vegetative differences between the WT and MT plants. Whole flowers and buds were collected at different stages and imaged fresh with the Zeiss Axio Zoom V16 (www.zeiss.com) and LeicaM165FC (www.leica.com) stereo microscopes.

Sampling and DNA Extraction: Plants from all plantings were sampled at 2 w post germination on dry ice with two leaf punches for each plant. Samples were stored at -80°C until DNA extraction. DNA extraction was done using the DNeasy® Plant Mini Kit from Qiagen (www.qiagen.com).

DNA analysis to identify WT and MT plants: PCR was set up using the Rev1, TransR1, and TransR2 primers (Baumbach et al. 2016). The reaction was completed using a Bio-Rad C100 Thermal Cycler and the samples were run on a 1.5% agarose gel. The gels were imaged on an Imagen Gel Imager (www.bio-rad.com).

*Chromosome spreads***:** Young anthers of both WT and MT plants were fixed in 3:1 (EtOH [ethanol]: glacial acetic acid) solution. Samples were then processed according to Ross, Fransz & Jones (1996). The slides were stained with Vectashield with DAPI (www.vectorlabs.com) and then imaged with the Leica DMRXA fluorescence microscope (www.leica.com) with a Hammatsu Orca Flash 4.0LT camera (www.hammastu.com).

Acetocarmine staining: Anthers were collected at different developmental stages and fixed in 3:1 (EtOH [ethanol]:glacial acetic acid), and the fixative solution was changed until it was clear. The anthers were dissected to release the male cells into 2% acetocarmine and imaged on the Leica DMRXA with a Leica DFC450 camera (www.leica.com).

Fixation and clearing for anther and ovule anaylses using the CSLM: Whole flowers were collected at different developmental stages and fixed in 2% paraformaldedye: 4% glutaraldehyde (www.emsdiasum.com) in PBS buffer and vacuum infiltrated at 10 psi to increase the penatration of fixative. Samples were rinsed in PBS and dehydrated in a graded EtOH series. Upon reaching 100% EtOH the samples were cleared in benzyl alcohol:benyl benzoate (BABB) (http://www.seas.upenn.edu/~confocal/Clearing_agents.html#babb). Samples were mounted on slides in BABB and imaged using the Leica TCS SPE confocal scanning laser microscope (CSLM) (www.leica.com) with 405 nm, 488 nm, and 532 nm laser lines.

Scanning electron microscopy: Mature WT and MT anthers and gynoecia at pollen dehiscence were fixed in FAA (formalin, acetic acid, EtOH) (Ruzin 1999) and dehydrated in a graded EtOH series to pure EtOH. At this point the samples were critical point dried using liquid carbon dioxide (www.denton.com). Some of the anthers were teased open and mounted on aluminum stubs with double-sided sticky tabs, and surrounded with silver paint. The samples were then sputter-coated (www.denton.com) with palladium-gold (60:40) and observed using a JEOL 5800 scanning electron microscope (SEM) (www.jeolusa.com) at 10 kV.

Results

Various microscopic techniques and three different chemical fixation procedures were used to provide information on the developmental sequence of events that occur during WT and MT anther and ovule development. All three fixation procedures provided comparable anatomical results. Once the WT soybean developmental patterns for anthers and ovules were established, they were compared with the same developmental patterns in the MT soybean plants. A total of 430 plants were grown and genotyped (Table 1 and fig 1). Flowers were collected from MT and WT plants at various stages of development for different microscopic procedures (Table 2).

Whole plant images: were taken beginning four weeks post planting and then weekly, until 8 w post planting. No differences were discerned between the WT and MT vegetative plants (figs 2A and 2B). The only visible difference at the time of flowering was the lack of pod formation on the MT plants (figs 2C and 2D). Inspection of the early pods (gynoecia) on the WT and MT plants revealed that in the MT pods, the ovules were arrested at an early stage of development (figs 2E, 2F, 2J and 2K). Development of the flowers progressed normally for both the WT and MT plants (fig 2L) and visible shed pollen (or microspores) was observed from both the WT and MT anthers (figs 2G and 2H).

Acetocarmine staining: was carried out on the WT and MT male cells from the pre-meiotic sporogenesis mass stage to the mature pollen stage (figs 3A-3J). The MT male cells progressed normally (figs 3B, 3D and 3F) until the tetrad stage when a mixture of normal-appearing tetrads and abnormal-appearing triads and pentads were observed; some of the latter with mini-microspores were evident (fig 3H). Counts were done to

quantify the number of WT and MT male cells that appeared at the tetrad stage (Table 3). Upon release from their callose the MT microspores displayed various sizes with some were collapsed. By the comparable WT pollen stage this variable-size condition on the MT was clearly evident with many collapsed and/or misshapen male cells (fig 3J).

Chromosome spreads: of fixed anthers revealed no visual differences between WT and MT meiotic male cells (figs 4A-4D and 4H-4K) until metaphase I (figs 4E, 4F, 4L, 4M). At this stage, the MT chromosomes displayed significantly fewer bivalents (3-6), unpaired homologous chromosomes, than the normal complement of WT bivalents (figs 4E, 4F). This reduction in bivalents was noted across numerous samples. Ovule squashes to observe chromosomes were not attempted but their early abnormal development using CSLM (see later) strongly suggested that the same abnormal synapses occurred during female meiosis at metaphase I as occurred on the MT male side (fig 6).

CSLM of cleared WT and MT anthers and ovules: the aldehyde-fixed (and BABB cleared) anthers and ovules, by over-fixation of glutaraldehyde induced autofluorescence of all of the components of the internal cells and tissues of the anthers and ovules.

WT and MT anther development: WT pollen development began with the differentiation of pre-meiotic sporogenous mass within the anther (fig 5A); followed by continued differentiation of the meiocytes and the formation of a thick callose wall that surrounds each meiocyte (fig 5B). The meiocytes underwent two meiotic events to produce dyads and then tetrads of haploid microspores (fig 5C). This was followed by breakdown of the callose and release of the young microspores (fig 5D). As the microspores progressed into mid-microspore stage their small vacuoles began to

coalesce. The union of these small vacuoles as well as thickening of the surrounding exine wall signaled their progression to the late microspore stage when mitosis gives rise to vacuole pollen (fig 5E). The continuing development of the exine wall and the accumulation of starch grains completed the progression into mid-pollen and finally mature pollen where the generative nucleus then divided to form two sperm (fig 5F).

The MT male cells, like the WT male cells, developed normally (figs 5G, 5H) until meiosis when (cytologically) chromosome pairing abnormalities occurred that translated into malformed, different sized tetrads, as well as triads and pentads (fig 5J). These abnormalities accumulated in the microspore stage after release from the tetrads where the released microspores show abnormal sizes ranging from small to large when compared to uniform-sized WT microspores (fig 5K). Even though MT development continued, the microspores of different sizes, high vacuolation, and collapse did not become viable pollen (figs 5K, 5L).

WT and MT ovule development: WT ovule primordium was comprised of several hypodermal archesporial cells (fig 6A) where one cell enlarged (the archesporial cell) (fig 6B) that became the functional megasporocyte or megaspore mother cell. This cell underwent meiosis I to produce a dyad (fig 6C) then meiosis II to produce a linear tetrad of megaspores. The three cells closest to the micropylar end aborted leaving the functional megaspore (fig 6D) to undergo mitosis and form a two-nucleate megagametophyte with a large vacuole in the center of the cell. This caused the nuclei within the megagametophyte to move to the opposite ends of the cell. The binucleate megagametophyte then underwent two karyotic divisions and then cellularization to become the eight-cell megagametophyte (fig 6E). After fertilization the

megagametophyte became the embryo sac (fig 6F) and the ovule develops into the mature seed (Kennell & Horner 1985).

The MT female ovule development begins similar to that of the WT ovule (figs 6G-6I). Meiosis was not observed even though young megagametophytes were observed to form before they showed signs of collapse and eventual abortion (figs 6J, 6K). These ovules did not continue developing (fig 6L) and contributed to cessation of pod development as already shown in fig 1D.

Scanning electron microscopy: Anthers in both WT and MT showed no outward visible difference (fig 7A, 7D). WT pollen was observed with pollen tubes on the stigma (fig 7B). In the WT, the pollen grains were uniform in size and most were germinated either in the dehiscing anthers or on the stigma (fig 7B). The WT pollen exine was tricolporate with even distribution of the architecture of the wall (fig 7G).

The MT male cells (microspores or pollen) were, of varying sizes and state of collapse. No germination, whether in the anthers (fig 7F) or on the stigma of the gynoecia (fig 7E), indicating they were sterile. The MT male cell exine displayed patterns different from the WT pollen exine, large pollen showed abnormally formed exine and sometimes collapsed colpi and pores (fig 7H); medium-sized male cells were collapsed (fig 7I) and small male cells showed abnormal shapes and surface exine (fig 7J).

Discussion

In this study a full cytological investigation was done on the MSFS soybean mutant. This mutant originated from a *w4*-m line that contains the transposon *Tgm9.* In the *w4*-m line *Tgm9* was located in the *DFR2* gene that is responsible for anthocyanin pigment in flowers (Xu et al. 2010). With the insertion of *Tgm9* into this locus white

flowers are produced. Since *Tgm9* is a highly active element excision occurs frequently, restoring the purple color to flowers. When this restored purple flower line progresses to the next generation, the progeny with purple flowers are called germinal revertants. It is from these germinal revertants that the MSFS mutant was identified. The fertility gene was mapped to the *St8* position on a molecular linkage group J (Raval et al. 2013). This *St8* gene was then cloned using *Tgm9*. One possible candidate, *Glyma.16G072300,* encodes *MER3* which is a DNA helicase gene (Wang et al. 2009). Genetic and molecular work done by Baumbach et al. (2016) showed the encoded protein has a high similarity to the Arabidopsis Rock-N-Rollers (RCK) MER3 DNA helicase. *MER3* is found to be critical in meiotic crossing over, so that mutant *mer3* shows a reduction in bivalent formation during meiotic chromosome pairing in Arabidopsis (Mercier et al. 2005). The results of the present study confirm this phenotype as described in other *mer3* mutants (Terasawa et al. 2007).

There were no visible vegetative differences between the growth of the WT and MT plants (fig 2), indicating that the gene expression is gametophytic. The MT floral development was normal until pod formation, when the WT formed pods that enlarged with developing seed(s) while the MT line formed small sterile pods that never developed further.

The acetocarmine staining of MT male cells showed abnormalities by the tetrad stage with uneven distribution of the chromosomes leading to triads, tetrads and pentads, and other various combinations. The chromosome spreads during meiosis I helped to solidify the phenotype of *mer3* (Mercier et al. 2005), and that meiosis proceeds normally in the MT line until metaphase I, where there were a reduced number of bivalents. In WT

there are typically 20 bivalents, while in the MT the number of bivalents ranged from 3-6 in total. This significant reduction in bivalents and increased number of univalents created an unbalanced distribution of chromosomes and resulted in abnormal tetrad formation.

The CSLM clearing observations defined which developmental stages proved fatal due to uneven chromosome distribution. On the male side the MT progressed past the tetrad stage, but showed varying sizes of male cells. This eventually led to collapse of the male cells. On the female side, MT development occurred normally until the megagametophyte stage where collapse and abortion was evident. This is presumed to be a *mer3*-like effect leading to unbalanced chromosome numbers. This present study, therefore, confirms the presence of the *mer3* gene phenotype for the first time in soybean. It also confirms the use of the *w4*-m line as a useful system to study genes in soybean, by identifying new phenotypes as the transposon inserts into other gene loci.

In addition, this present study of a meiotic mutant causing MSFS in soy adds to the knowledge base of sterility mutants already existing in soy (Ilarslan et al. 1999). Lastly, it opens the possibility for continued work that could lead to differential expression of sterility on just the male side in development of a stable hybrid soybean.

References

Baumbach J, RN Pudake, C Johnson, K Kleinhans, A Ollhoff, RG Palmer, MK Bhattacharyya, D Sandhu 2016 Transposon tagging of a male-sterility, female-sterility gene, *St8*, revealed that the meiotic MER3 DNA helicase activity is essential for fertility in soybean. PLOS One 11(3): e0150482.

Bilyeu KD 2008 Forward genetics in soybean. Pages 135-139 *in* G Stacey ed; Genetics and Genomics of Soybean. New York.

Bioengineering Confocal and Multiphoton Imaging Core Facility 2000 *Clearing Agents*. Retrieved 2014, from University of Pennsylavania School of Engineering and Applied Science:http://www.seas.upenn.edu/~confocal/Clearing agents.html#babb

Ilarslan H, HT Horner, RG Palmer 1999 Genetics and cytology of a new male-sterile, female-sterile soybean mutant. Crop Sci 39:58-64

Kennell JC, HT Horner 1985 Megasporogenesis and megagmetogenesis in soybean, *Glycine max*. Amer. J. Bot 72:1553-1564.

Palmer RG, HB Benavente, RW Groose 1989 w4-mutable Line in soybean. Develop Gen 10:542-551.

Raval, J, J Baumbach, AR Ollhoff, RN Pudake, RG Palmer, MK Bhattacharyya, D Sandhu2013 A candidate male-fertility female-fertility gene tagged by the soybean endogenous transposon, Tgm9. Funct Integr Genomics 13:67-73.

Ross K, P Fransz, G Jones 1996 A light microscopic atlas of meiosis in *Arabidopsis thaliana*. Chromosome Res 4:507-516.

Terasawa M, H Ogawa, Y Tsukamoto, M Shinohara, K Shirahlge, N Kleckner, T Ogawa 2007 Meiotic recombination-related DNA synthesis and its implication for cross-over and non-cross-over recombinant formation PNAS 104:5965-5970.

Xu M, HK Brar, S Grosic, RG Palmer, MK Bhattacharyya 2010 Excision of an active CACTA-like transposable element from DFR2 causes variegated flowers in soybean (*Glycine max* [L.] Merr.). Genetics 184:53-63.

Wang K, D Tang, M Wang, J Lu, H Yu, J Liu, B Qian, Z Gong, X Wang, J Chen, M Gu, Z Cheng 2009 MER3 is required for normal meiotic crossover formation, but not for presynaptic alignment in rice. J Cell Sci 122:2055-2063.

Table 1. Genotyping Results from *w4*-m lines in soybean

Total	WT	WT%	Het	Het%	MT	MT%
A05-221-1	30	27.77%	46	42.59%	32	29.62%
A05-221-3	36	34.61%	48	46.15%	20	19.23%
A05-221-4	106	99.06%	1	0.93%	0	0.00%
A05-221-5	28	25.22%	51	45.94%	32	28.82%

Note. Each line represents a different line of the *w4*-m soybean line containing the *Tgm9* transposon.

Table 2. Number of WT and MT plants collected and observed for different microscopic techniques.

	Plants Collected	Plants Observed for Chromosome Spreads	Plants Observed for Acetocarmine Staining	Plants Observed for CSLM	Plants Observed for SEM
MT	84	15	12	32	6
WT	26	8	8	10	2

Note. Each plant was collected at multiple times during flowering to obtain developmental stages for observations.

Table 3. WT and MT male cells tetrad stage counts from acetocarmine staining

	Tetrads	Uneven Tetrads	Triads	Pentads
WT	50	0	0	0
MT	7	18	11	14

Note. These counts were done across multiple anthers from multiple plants.

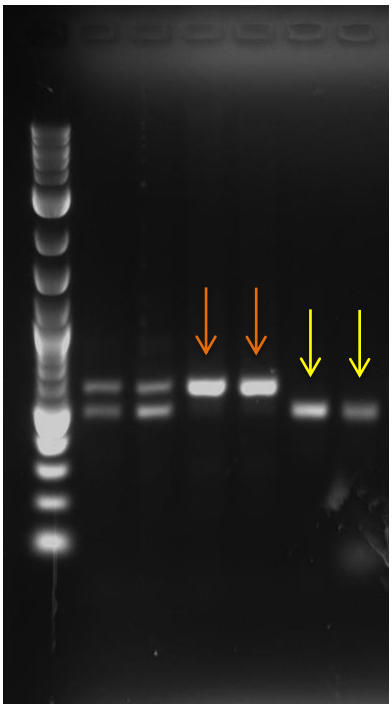

Fig 1. PCR results from genotyping of *w4*-m soybean lines. Lane 1 contains log20 DNA ladder. Lanes 2-3 show banding pattern of a heterozygous line. Lanes 4-5 show banding pattern of *Tgm9* male-sterile female-sterile mutant (MT) (orange arrows). Lanes 6-7 show wild-type (WT) banding pattern (yellow arrows).

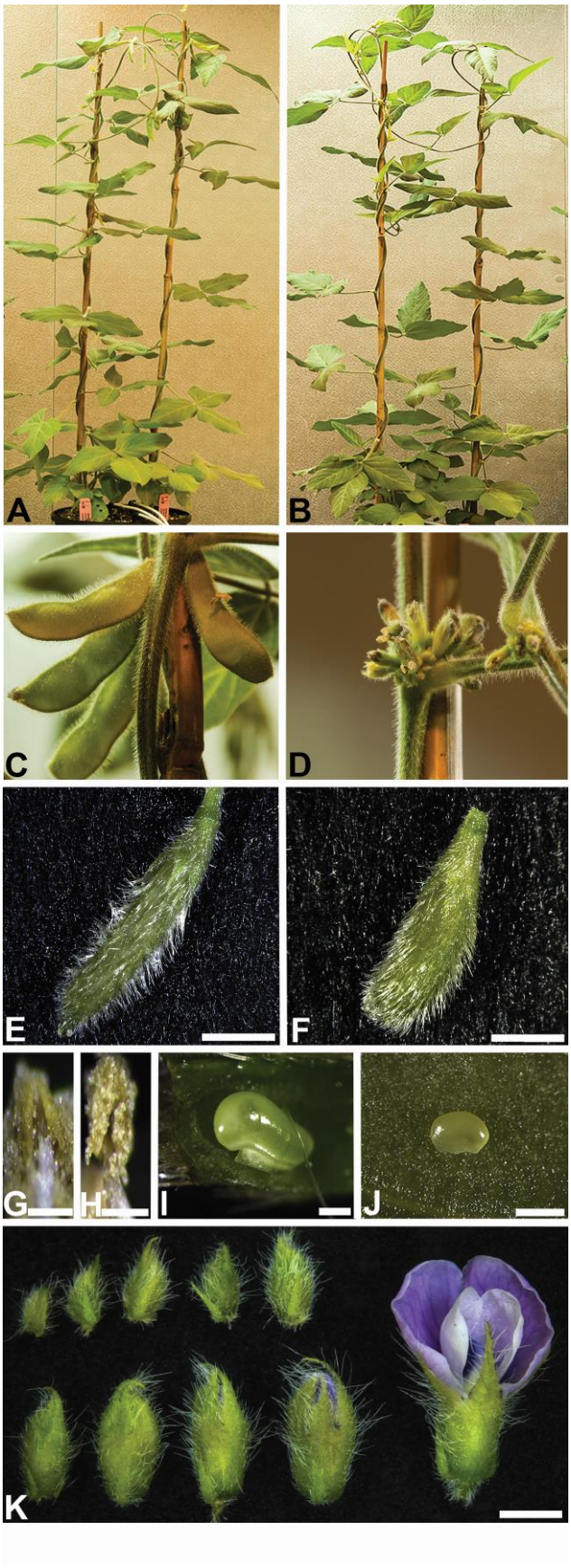
A
B
C
D
E
F
G
H
I
J
K

Fig 2. Whole plant and flower images of WT and MT soybean plants. *A,* Whole WT plant, 5 weeks after planting. *B,* Whole MT plant, 5 weeks after planting. *C,* Close-up of pods on a WT plant, 6 weeks after planting. *D,* Lack of pod formation on a MT plant, 6 weeks after planting. *E,* An early pod from a WT plant. *F,* An early pod on a MT plant. *G,* A WT anther, showing dehisced male cells. *H,* A MT anther, showing dehisced male cells. *I,* An ovule from a WT plant pod, in comparison to *J,* a MT ovule, where development is arrested at a young stage. *K,* Bud to flower progression of an MT plant; appears identical to a WT plant. Scale bars: *E*, *F* = 5 mm; *G*, *C* = 200 μm; *I*, *J* = 500 μm; *K* = 2 mm.

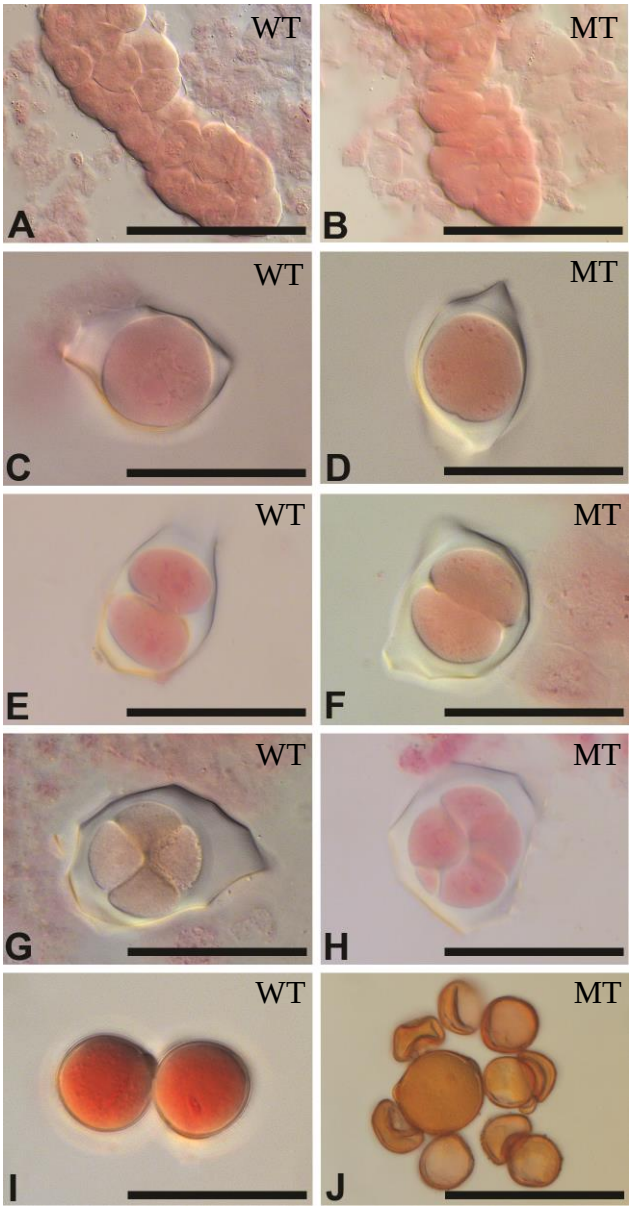

Fig 3. Acetocarmine staining of WT (*A*, *C*, *E*, *G*, *I*) and MT (*B*, *D*, *F*, *H*, *J*) male cell development, *A*, WT = sporogenous mass. *C*, WT = meiocytes. *E*, WT = dyad. *G*, WT = tetrad. *I*, WT = pollen. *B*, MT = sporogenous mass *D*, MT = meiocytes. *F*, MT = dyad *H*, MT = pentad. *J*, MT = variable-sized and collapsed pollen. Scale bars: *A*, *B* = 50 µm; *C-J* = 20 µm

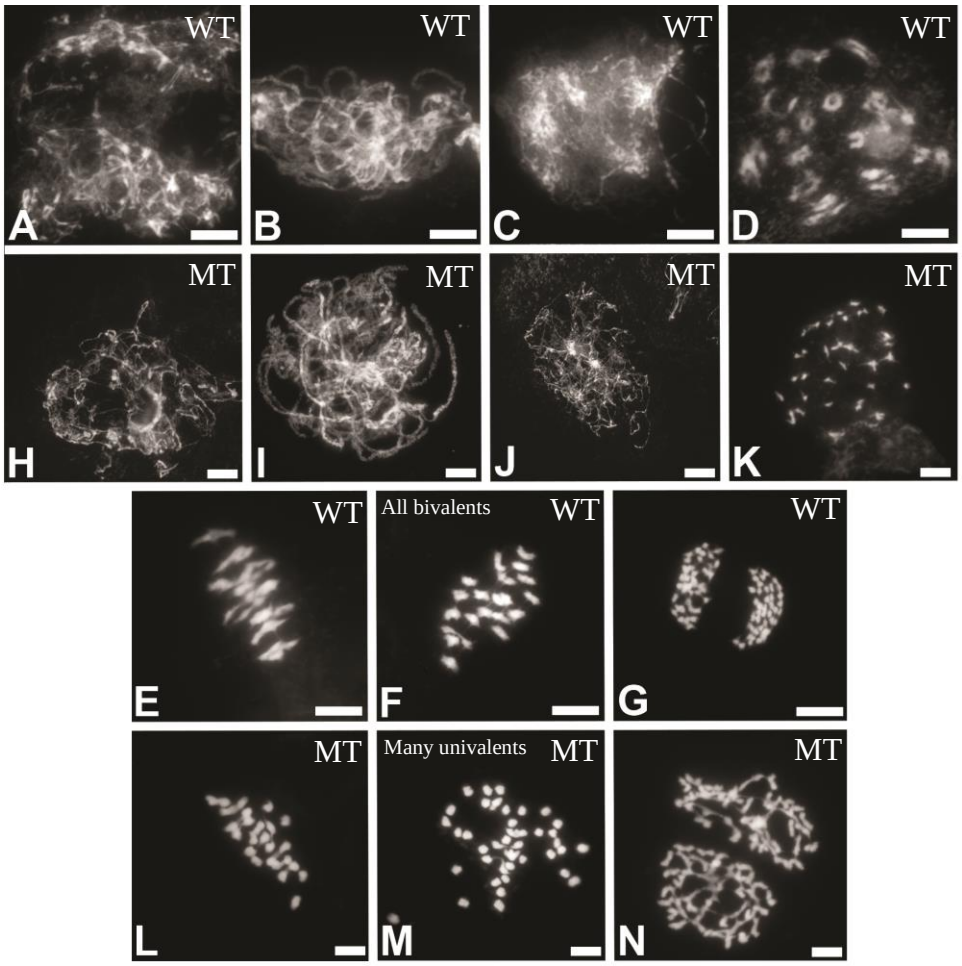

Fig 4. Chromosome spreads of WT and MT male cells at meiosis. *A* (WT), *H* (MT) = leptotene. *B* (WT), *I* (MT) = pachytene. *C* (WT), *J* (MT) = diplotene. *D* (WT), *K* (MT) = diakinesis. *E* (WT), *L* (MT), *F* (WT), *M* (MT) = metaphase I. *G* (WT), N (MT) = anaphase I. Scale bars: *A*–*N* = 20 μm

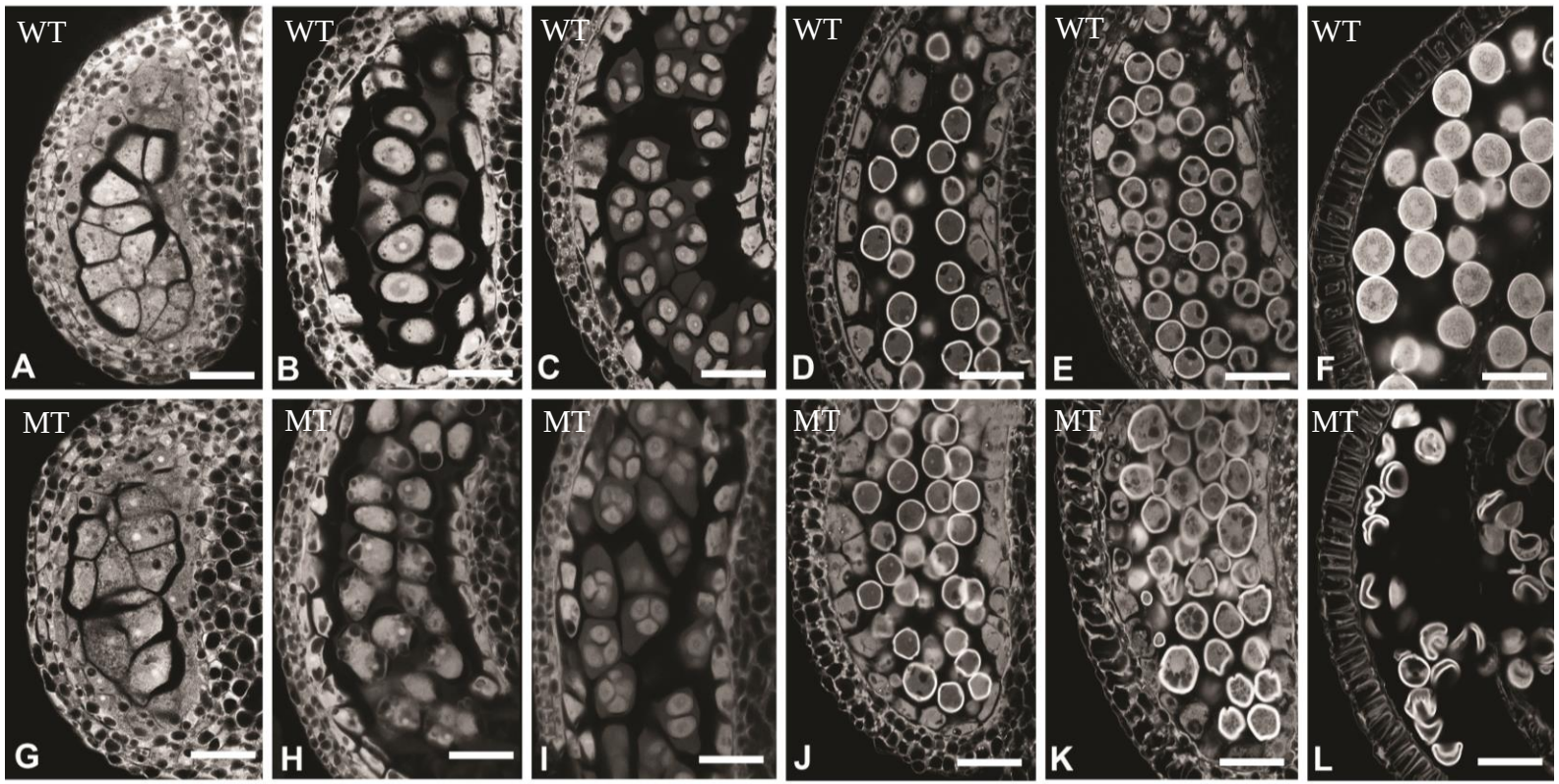

Figure 5. *A-L*. CSLM Clearings of soybean WT AND MT male anthers and female ovules. *A-F*. WT anther male cell development. *A*, WT sporogenous mass. *B*, WT meiocytes. *C*, WT tetrads. *D*, WT early microspores. *E*, WT mid- to late uninucleate microspores. *F*, WT binucleate pollen. *G-L* MT anther male cell development. *G*, MT sporgenous mass. *H*, MT meiocytes. *I*, MT tetrads. *J*, MT early microspores. *K*, MT mid-microspores. *L*, MT late-uninucleate aborted microspore-aborted pollen. Scale bars: 25 μm

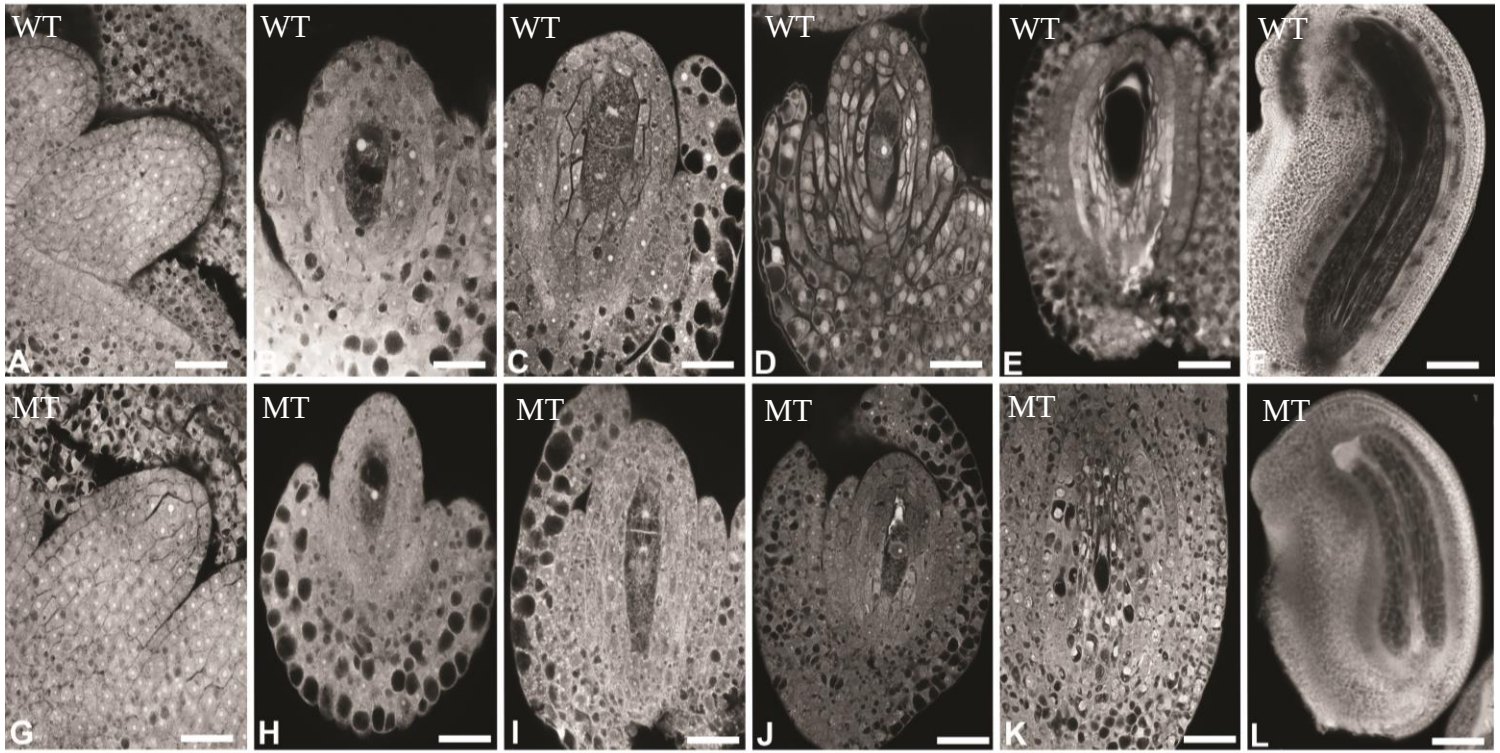

Figure 6. *A-L*. CSLM Clearings of soybean WT AND MT female ovule development. *A-F*. WT ovule development. *A.* WT primordial. *B*, WT megaspore mother cell/ *C*, WT dyad. *D*, WT functional megaspore. *E*, WT 8-celled megagametophyte. *F*, WT embryo sac. *G-L*. MT ovule development. *G*, MT ovule primordial. *H*, MT megaspore mother cell. *I*, MT dyad. *J*, MT functional megaspore. *K*, MT megagametophyte (partial collapse). *L*, MT megagametophyte (complete collapse). Scale bars: 25 µm

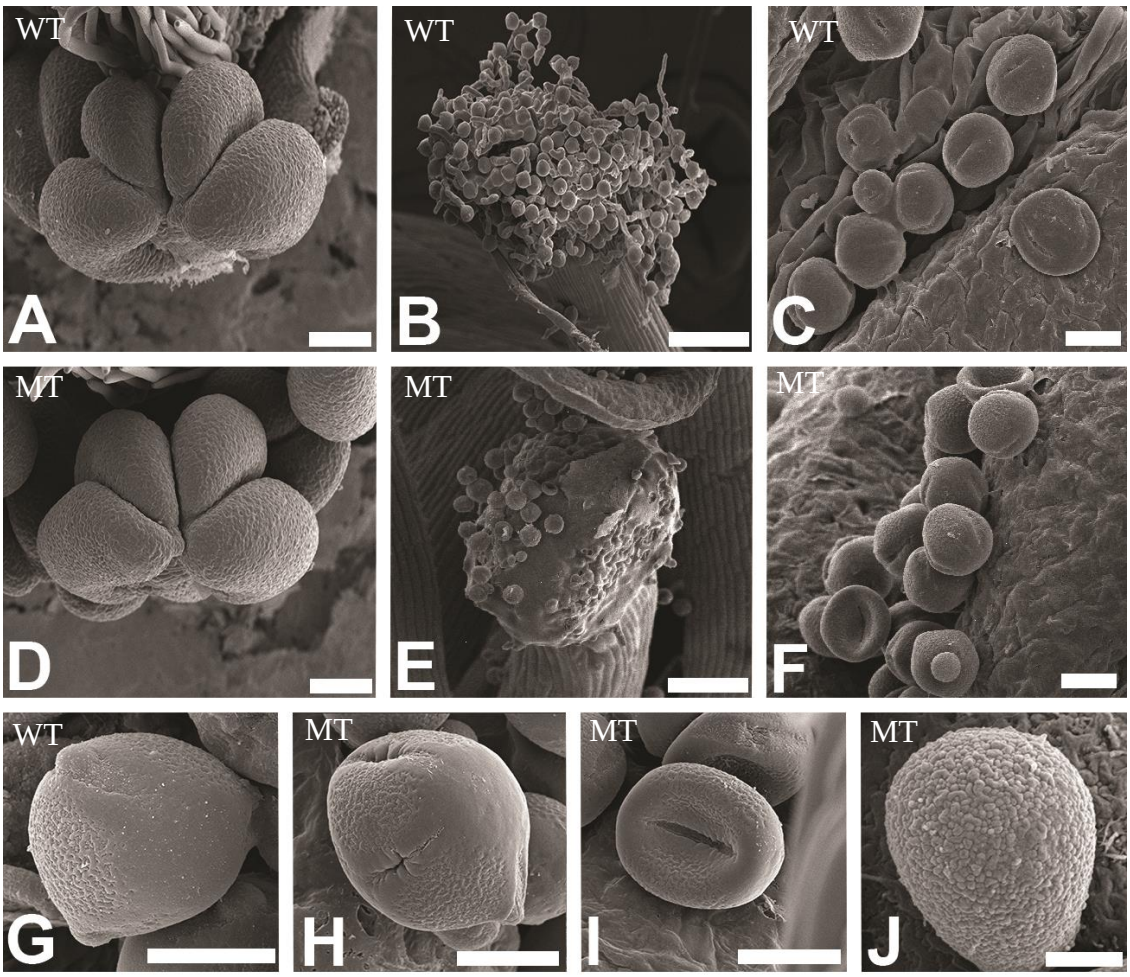

Fig 7. Scanning electron micrographs of WT (*A-C, G*) and MT (*D-F, H-J*) anthers, male cells and pollen. *A,* WT anther top view. *B,* WT germinating pollen on stigma. *C,* WT dehisced anther with normal-sized pollen. *D,* MT anther top view. *E,* MT male-cells of different sizes on stigma. *F,* MT dehisced anther with collapsed and different sized male cells. *G,* WT tricolporate pollen. *H,* MT large aborted male cell. *I,* MT medium, collapsed male-cell. *J,* MT small male cell. Scale bars: *A, B, D, E* = 100 µm; *C, F* = 20 µm; *G, H, I* = 10 µm; *J* = 2 µm.

CHAPTER III

CONCLUDING REMARKS

The MSFS soybean mutant was studied in this thesis to better understand the mechanism(s) of both male and female sterility and when abortion occurs during development. In Baumbach et al. (2016), the *Tgm9* transposon was found to have inserted into the locus of molecular linkage group J (Chromosome 16). Fine mapping enabled them to flank the locus to a 62-kb region that contains only five predicted genes (Raval et al. 2013). One of the genes in this region, *Glyma.16G072300*, codes for a helicase (Raval et al. 2013). *MER3* is a DNA helicase gene (Mercier et al. 2005). This transposon insertion into this gene adversely affects crossing over at meiosis disrupting bivalent formation. The microscopic work done in this thesis confirms this phenotype. Since the gene is gametophytic, there were no visible vegetative differences observed between the growth of the WT and MT plants as was shown in fig 2 (Chapter II). The floral development was also shown to progress normally toward pod formation as the WT produced pods that enlarged with enclosed seeds, while the MT line formed small sterile pods that never progressed any farther. The acetocarmine staining showed on the male side that abnormalities occurred by the tetrad stage with uneven distribution of the chromosomes leading to pentads and other various combinations (Table 3, Chapter II). The chromosome spreads helped to solidify the phenotype of *mer3* (Mercier et al. 2005) by identifying that male cell meiosis proceeded normally in the MT until metaphase I, where there was a reduced number of bivalents. In WT there are typically 20 bivalents, while in the MT the number of bivalents ranged from 3-6 in total (observed in numerous chromosomes spreads). This resulted in an unbalanced distribution of chromosomes and

supported the observations of abnormal ‘tetrads’ that were seen with the acetocarmine staining. The CSLM clearings of anthers and ovules at different developmental stages pinpointed the times following meiosis (due to uneven chromosome distribution) where cessation of development occurred (figs 5, 6). On the male side the MT line progressed past the tetrad stage, but showed varying sizes of male cells (microspores). This led to the collapse of the male cells and formation of non-viable pollen. No abnormalities were seen in the tapetum cell layer unlike what was suggested in other work on the MSFS lines (Palmer & Horner 2000). This confirms that the defect in *MER3* is responsible for the gametic phenotype seen in this MSFS mutant and not due to any possible defects in the tapetum which does have an important role in pollen development (Pacini 2010). On the female side, MT development occurred normally until the megaspore stage that produced abnormal, collapsed megagametophytes. Through this study, the confirmation of the *mer3*-like gene phenotype represents the first-time observation in soybean. It also confirms the potential use of the *w4*-m line as a system to study genes in soybean by identifying phenotypes as the transposon inserts into different loci. This study of the *Tgm9*-induced meiotic mutant adds to the knowledge of sterility lines already existing in soybean, and expands the significance of meiosis as a focal point for disruption of both male and female development. Thus, this study adds to continued research undertaken on the stages and genes involved in meiosis and its potential role in affecting normal development.

ENTIRE THESIS REFERENCES

Baumbach J, RN Pudake, C Johnson, K Kleinhans, A Ollhoff, RG Palmer, MK Bhattacharyya, D Sandhu 2016 Transposon tagging of a male-sterility, female-sterility gene, *St8*, revealed that the meiotic MER3 DNA helicase activity is essentialfor fertility in soybean. PLOS One 11(3): e0150482

Bilyeu KD 2008 Forward genetics in soybean. Pages 135-139 *in* G Stacey ed; Genetics and Genomics of Soybean. New York.

Bioengineering Confocal and Multiphoton Imaging Core Facility 2000 *Clearing Agents*. Retrieved 2014, from University of Pennsylavania School of Engineering and Applied Science:http://www.seas.upenn.edu/~confocal/Clearing_agents.html#babb

Bione NC, MS Pagliarini, LA Almeida 2002 A new and distinctive male-sterile, female-fertile desynaptic mutant in soybean (*Glycine max*). Hereditas 136:97-103.

Buntman D, HT Horner 1983 Microsporogenesis of normal and male (ms_3) mutant soybean (*Glycine max*). Scan Electron Microsc II:913-922.

Cui Y, S Barampuram, MG Stacey, CN Hancock, S Findley, M Mathieu, Z Zhang, WA Parrott, G Stacey 2013 *Tnt1* retrotransposon mutagenesis: a tool for soybean functional genomics. Plant Physiol 161(1):36-47.

Carlson JB, NR Lersten 2004 Reproductive morphology Pages 59-95 *in* HR Boerma, JE Specht eds, Soybeans: Improvement, Production Madison, Wisconsin.

Cohn NS 1969 Elements of cytology. Harcourt, Brace & World Inc, New York

Graybosch RA, RG Palmer 1988 Male sterility in soybean - an overview. Amer J Bot 75:144-156

Hancock CN, F Zhang, K Floyd, AO Richardson, P Lafayette , D Tucker, SR Wessler, WA Parrott 2011 The rice miniature inverted repeat transposable element *mPing* is an effective insertional mutagen in soybean. Plant Physiol157(2):552-562.

Ilarslan H, HH Skoripska, RG Palmer 1997 Cytology and genetics of a tissue culture-derived soybeam genic male-sterile, female-sterile. J Hered 88:129-138.

Ilarslan H, HT Horner, RG Palmer 1999 Genetics and cytology of a new male-sterile, female-sterile soybean mutant. Crop Sci 39:58-64

Johnson HW, RL Bernard 1962 Soybean Genetics and Breeding. Pages 149-218 *in 23 ed*, Advances in Agronomy, Press, City.

Kennell JC, HT Horner 1985 Megasporogenesis and megagametogenesis in soybean, *Glycine max*. Amer J Bot 72:1553-1564.

Lersten NR, JB Carlson 2004 Vegetative morphology Pages 15-57 *in* HR Boerma, JE Specht eds, Soybeans: Improvement, Production Madison, Wisconsin.

Mathieu M, EK Winters, F Kong, J Wan, S Wang, H Eckert, D Luth, M Paz, C Donovan, Z Zhang 2009 Establishment of a soybean (*Glycine max* Merr. L) transposon-based mutagenesis repository. Planta 229(2):279-289.

Mercier R, S Jolivet, D Veson, E Huppe, L Chelysheva, M Giovanni, F Nogue, MP Doutriaux, C Horlow, M Grelon, C Mezard 2005 Two meiotic crossover classes cohabit in *Arabidopsis:* one is dependent on *MER3*, whereas the other one is not. Curr Bio 12:692-701.

Mercier R, M Grelon 2008 Meiosis in plants: ten years of gene discovert. Cytogenet Genome Res 120:281-290.

Nakagawa T, RD Kolodner 2002 *Saccharomyces cerevisiae* Mer3 is a DNA helicase involved in meiotic crossing over. Mol and Cell Bio 22,10:3281-2191.

Orf J 2010 Introduction Pages 1-18 *in* K Bilyeu, MB Ratnaparkhe, C Kole, *Genetics, Genomics and Breeding of Soybean*. CRC Press. New York.

Pacini E 2010 Relationships between tapetum, loculus, and pollen during development. Int JPlant Sci 171:1-11.

Palmer RG, J Gai, VA Dalvi, MJ Suso 2011 Male sterility and hybrid production technology. Pages 193-207 *in* A Pratap, K Jitendra eds, Biology and Breeding of Food Legumes, Press, City.

Palmer RG, HT Horner 2000 Genetics and cytology of a genic male-sterile, female-sterile mutant from a transposon-containing soybean population. Amer Gen Assoc 91:378-383.

Palmer RG, HB Benavente, RW Groose 1989 w4-Mutable Line in Soybean. Develop Gen 10:542-551.

Raval, J, J Baumbach, AR Ollhoff, RN Pudake, RG Palmer, MK Bhattacharyya, D Sandhu2013 A candidate male-fertility female-fertility gene tagged by the soybean endogenous transposon, *Tgm9*. Funct Integr Genomics 13:67-73.

Ross K, P Fransz, G Jones 1996 A light microscopic atlas of meiosis in *Arabidopsis thaliana*. Chromosome Res 4:507-516.

Slattery RA, S Pritzl, K Reinwand, B Trautschold, RG Palmer, D, Sandhu 2011 Mapping eight male-sterile, female-sterile soybean mutants. Crop Sci 51:231-236

Shivanna K 2003 Pollen sterility Page 77. *in* eds, Pollen Biology and Biotechnology Science Publishers.New Hampshire.

Terasawa M, H Ogawa, Y Tsukamoto, M Shinohara, K Shirahlge, N Kleckner, T Ogawa 2007 Meiotic recombination-related DNA synthesis and its implication for cross-over and non-cross-over recombinant formation PNAS 104:5965-5970.

Wang K, D Tang, M Wang, J Lu, H Yu, J Liu, B Qian, Z Gong, X Wang, J Chen, M Gu, Z Cheng 2009 MER3 is required for normal meiotic crossover formation, but not for presynaptic alignment in rice. J Cell Sci 122:2055-2063.

Xu M, HK Brar, S Grosic, RG Palmer, MK Bhattacharyya 2010 Excision of an active CACTA-like transposable element from DFR2 causes variegated flowers in soybean (*Glycine max* [L.] Merr.). Genetics 184:53-63.

APPENDIX
RECIPIES AND PROCEDURES

Chemical Fixation of specimens for confocal scanning laser microscopy:

- 2% glutaraldehyde: 2% paraformaldehyde, in 100 mM phosphate buffer (pH 7.2).
- To make a 100 mL final volume of fixative:
- In 50 mL distilled water add 2 g paraformaldehyde;
- In fume hood, continuously stir and heat solution to 65°C on a hot plate/stirrer for a minimum of 30 min, the solution will become cloudy white;
- To make the paraformaldehyde go into solution, add 10µL of 1N NaOH. Continue to stir and heat for 5-10 min, if solution does not begin to clear add 5-10 µL more of 1N NaOH add 10 µL of 1N NaOH;
- When solution clears, allow to come to room temp on ice or running water before adding other components;
- Add 50 mL of 0.2M Phosphate buffer;
- Add 4 mL of 50% glutaraldehyde.

Phosphate Buffer Saline Recipe

5x PBS – 1 L, pH 7.2

Amount	Chemical	5X Molarity
40 g	NaCl	684 mM
1.0 g	KCl	13 mM
7.2 g	Na_2HPO_4	50 mM
1.2 g	KH_2PO_4	9 mM

- If solution is too acidic (1–6); use drops of stock 1N KOH
- If solution is too basic (8-10); use drops of 1N HCl
- This recipe is for 1 L. The total should be divided into two 1-L bottles containing 500 mL. The bottles then need to be autoclaved.

Chemical fixation for anther squashes

3:1 Solution

- 3 parts 100% Ethanol;
- 1 part Glacial Acetic Acid;
  - Change fixative until clear.

Stain for anther squashes

Aceto-Carmine

- 2% Carmine (Sigma C6152) in 50% glacial acetic acid;
- Bring to boil in fume hood for 5 min;
- Filter and store at RT.

Chromosome Spreads (Ross, Fransz, & Jones, 1996)

- Fixed samples in 3:1 (ethanol: acetic acid) then rinsed 2x with H_2O
- Dissect out anthers

- Place in 10 µL of 60% glacial acetic acid;
- Place slide on hot plate (45°C) and stir droplet for 2 min;
- At 1 min add 10 µL of 60% acetic acid
- After 2 min -
    - Make a ring with 3:1 around the droplet and then place some in the middle;
    - Then rinse off slide;
    - Let slide dry;
    - Then can stain with Vecta shield + DAPI.

Clearing fixed specimens for confocal scanning laser microscopy: (Bioengineering Confocal and Multiphoton Imaging Core Facility, 2000)

BABB (Benzyl alcohol–Benzyl benzoate; 1:2 ratios (Benzyl alcohol: Benzyl benzoate)

- After fixing in buffered 2% paraformaldehyde and 4% glutaraldehyde (see first fixation procedure)
    - 25% EtOH for 15 min
    - 50% EtOH for 15 min
    - 75% EtOH for 15 min
    - 100% EtOH for 15 min (2x)
    - Sink tissue into BABB solution at room temperature.

DNA Extraction (Plantings 1 – 3)

DNeasy® Plant Mini Kit

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C);
- If necessary, redissolve any precipitates in Buffer AP1 and Buffer AW1 concentrates;
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates;
- Preheat a water bath or heating block to 65°C.
- Disrupt samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the TissueRuptor®, the TissueLyser II, or a mortar and pestle;
- Add 400 µL Buffer AP1 and 4 µl RNase A. Vortex and incubate for 10 min at 65°C. Invert the tube 2–3x during incubation;

  Note: Do not mix Buffer AP1 and RNase A before use
- Add 130 µL Buffer P3. Mix and incubate for 5 min on ice;
- Recommended: Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm;
- Pipet the lysate into a QIAshredder spin column placed in a 2 mL collection tube, Centrifuge for 2 min at 20,000 x g;
- Transfer the flow-through into a new tube without disturbing the pellet if present, Add 1.5 volumes of Buffer AW1, and mix by pipetting;

- Transfer 650 µL of the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge for 1 min at ≥6000 x g (≥8000 rpm). Discard the flow-through. Repeat this step with the remaining sample;
- Place the spin column into a new 2 mL collection tube. Add 500 µL Buffer AW2, and centrifuge for 1 min at ≥6000 x g. Discard the flow through;
- Add another 500 µL Buffer AW2. Centrifuge for 2 min at 20,000 x g;
  Note: Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.
- Transfer the spin column to a new 1.5 mL or 2 mL microcentrifuge tube;
- Add 100 µL Buffer AE for elution. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g;

PCR

- 20 uL reaction
  - 0.5 µL Rev 1 Primer
  - 0.5 µL Rev 2 Primer
  - 0.5 µL Trans1 Primer
  - 12.5 µL PCR Master Mix
  - 4.0 µL DNA
  - 2.0 µL dH_2O

Primer Name	**Primer Description**	**Primer Sequence (5' to 3')**
Trans R1	Outer Transposon primer	CGTCGTGGGTGAAGAGTGGGTGAAGAGTG
Rev1	Revertants Primer 1	GGCTGAGAGAGGCTTCTTTATCTTG
Rev2	Revertants Primer 2	GAGCGGTCATATTCCATATAGAGAC

- Run on the Bio-Rad C100 Thermal Cycler
- 94.0°, 2 min;
- 94.0°, 30 sec;
- 60.0°, 30 sec;
- 50.0°, 30 sec;

Note: Back to step 2, 35x;

- 72. 0°, 5 min;
  4.0°, 10 min.

Gel

PCR was run on a 1.5% agarose gel at 80 volts for 70 min