Characterization of *fuzzy tassel* male sterility defects implicates novel miRNA-regulated pathways in maize stamen development

by

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The maize *fuzzy tassel* (*fzt*) mutant is caused by a mutation in *dicer-like1* and has broad developmental defects. *dicer-like1* encodes a key enzyme for microRNA (miRNA) biogenesis and many miRNAs are reduced in *fzt* plants. *fzt* plants have reduced stature compared to normal siblings and make shorter, narrower leaves. *fzt* also has striking inflorescence defects; the inflorescence meristem is fasciated, and other meristem types in the inflorescence are indeterminate. In addition, *fzt* is male and female sterile. To further investigate the *fzt* stamen defects, I compared development of *fzt* and normal siblings stamens in fixed, sectioned tissue. Early stamen development in *fzt* was indistinguishable from normal siblings. However, later in development, *fzt* stamens arrested after microspores became vacuolated but before dehiscence. At maturity, some *fzt* stamens contained pollen that appeared morphologically normal, but most of the pollen was dead based on Alexander staining. Normal pollen is tricellular at maturity; *fzt* pollen was a mixture of uni-, bi-, and tri- cellular pollen, indicating pollen development was arrested at multiple developmental stages. Pollen in normal siblings is loaded with starch before dehiscence. *fzt* pollen, however, failed to accumulate starch, suggesting pollen that developed to late stages arrested before maturity.

I hypothesize that misexpression of specific miRNA targets underlies the *fzt* stamen defects. The GAMYBs are regulated by miR159 and have key roles in stamen development in other species, and misexpression of the GAMYBs might contribute to male sterility in *fzt*. *ZmMyb74* and *ZmMyb138* encode GAMYBs that are regulated by miR159. I obtained a transposon insertion allele in *ZmMyb138* (*ZmMyb138-m1::Mu1*) and two insertion alleles in *ZmMyb74* (*ZmMyb74-m1::Mu1; ZmMyb74-m2::Mu1*). Single mutants in both genes are phenotypically normal, suggesting these genes might function redundantly. I am generating *ZmMyb138 ZmMyb74* double mutants for further analysis. Better characterization of the phenotype and molecular interactions of the GAMYBs has the potential for improving maize breeding systems in industry in a similar fashion to patented male sterile alleles that allow selectivity in hybrid corn breeding.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Introduction

Significance of Maize Reproductive Development Research

Research conducted on reproductive development in *Zea mays* (maize) allows a better understanding of grass-specific mechanisms and has a direct application for use in agriculture. Maize grain production was over 117 million tons and worth over $62 billion in 2013 (USDA). Maize is in the family Poaceae, with agriculturally important relatives including rice (*Oryza sativa*), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*).

My thesis focuses on characterizing the stamen defects in the maize male-sterile *fzt* mutant, with my work contributing to the long-term goal of understanding the genetic cause of *fzt* male-sterility. One hypothesis is that male-sterility in *fzt* is caused by misregulation of two gibberellic acid (GA) inducible MYB transcription factors (GAMYBs). The upstream and downstream targets of the GAMYBs have been studied in *Arabidopsis*, rice, and barley (Chhun, et al. 2007; Gocal, et al. 2001; Plackett, et al. 2011; Murray, et al. 2003; Gubler, et al. 1995), but no research has been performed on their homologs in maize. Better characterization of the phenotype and molecular interactions of the GAMYBs has the potential for improving maize breeding systems in industry in a similar fashion to patented male sterile alleles that allow selectivity in hybrid corn breeding (Albertsen, et al. 2011; Albertson, et al. 2010).

Plant Growth Requires Meristems

Meristems are regions of rapidly dividing undifferentiated cells where plant organs are initiated. The shoot apical meristem (SAM) is at the apex of the plant, which gives rise to the

vegetative tissue, the tassel, and to lateral meristems on the sides of the stalk that give rise to the ears. Reproductive development begins with transition of the vegetative meristem, which forms leaves and stem tissue, to an inflorescence meristem (IM). In maize, the IM gives rise to spikelet pair meristems (SPMs). In the tassel, the IM also gives rise to branch meristems (BM), which form long branches at the base of the tassel (McSteen, et al. 2000; Alonso-Peral, et al. 2010). SPMs give rise to two spikelet meristems (SMs), which produce two floral meristems (FM) that produce the floral organs, including stamens and carpels (McSteen, et al. 2000). Male and female reproductive organs are both initiated in the tassel and ear, and sex determination occurs through selective abortion of pistils (female) in the tassel and stamens (male) in ears (McSteen, et al. 2000; Barazesh and McSteen 2008).

Plant Reproduction Involves Two Life Stages: Alternation of Generations

Plants go through two independent life stages (alternation of generations), the diploid sporophyte and the haploid gametophyte. Unlike animals, which set aside a germ line early in development, plants produce organs and germ cells for sexual reproduction from vegetative tissue. The gametophyte is produced from the sporophyte; the male gametophyte is produced from meiosis in the stamen, the female gametophyte is produced from meiosis in the ovary. The male gametophyte (pollen) is released, lands on the style of the stigma, germinates and travels to the female gametophyte (embryo sac) in the ovary at the base of the stigma. Fusion of the male (sperm cells) and female (egg) gametes gives rise to the sporophyte, which will grow until mature and produce the next generation (the gametophyte). Male gametogenesis occurs in the stamen: a mature stamen is composed of the anther, a four lobed structure that houses pollen, which is connected to the floret and the rest of the plant by the filament.

Stamen Development in Maize

Stamen development is divided into 15 stages based on cellular and morphological events (Koltunow, et al. 1990; Sanders, et al. 1999). During stages 1 through 5, tissue layers within the locule are differentiated. Stamen development begins with emergence of stamen primordia from the floral meristem (Stage 1; Figure 1). Stage 2 begins with differentiation of archesporial cells, a cell type that will give rise to the gametophyte, in the four lobes of the anther. The L2 layer of cells surrounds the archesporial cells divides periclinally (parallel to the epidermal layer; Kelliher and Walbot 2011) in stage 3, then in stage 4 the L2 layer divides anticlinally (perpendicular to the epidermis; Kelliher and Walbot 2011; stage 4), by stage 5 the tissue layers from these divisions will give rise to the endothecium, the middle layer, and the tapetum (Sheridan, et al. 1999). The archesporial cells will give rise to the pollen mother cells (PMC; stage 5) that will later give rise to the pollen (Ma, et al. 2007; Freeling and Walbot 1996); at this stage all tissue layers of the anther locule are present.

Early stages of pollen development occur between stages 6 through 10 of stamen development. The PMC enters meiosis (stage 6) while simultaneously being coated in β-1,3-glucan (callose), a polysaccharide secreted from the tapetum, resulting in a coat around the PMC (Abramova, et al. 2003). PMC meiosis results in four microspore cells trapped within the callose coat, termed a tetrad (stage 7; Scott, et al. 2004; Hsu and Peterson 1981). The tapetum becomes binucleate during pollen meiosis while also secreting β-glucanase (callase), an enzyme that breaks down callose, into the locule (Scott, et al. 1991). Callase breaks down the PMC callose coat, freeing the microspores for further development and growth within the locule (stage 8). The exine, an extracellular matrix of sporopollenin, is deposited on the microspores from the tapetum

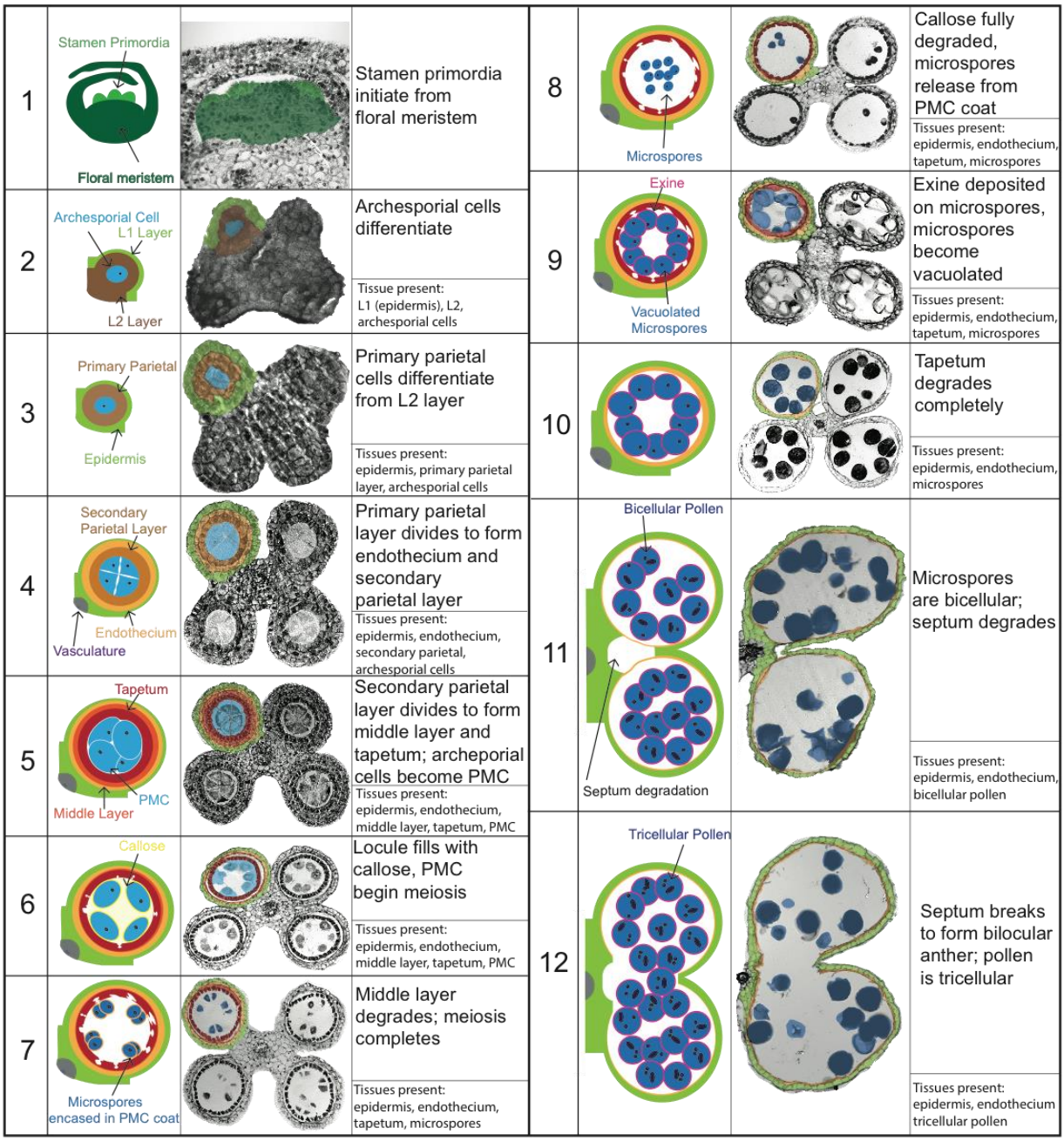

Figure 1. Summary of maize stamen development. Major cellular events of stamen anther development are shown; left column, cartoon of locule; middle column, anther cross section with colours overlaid to indicate cell types (same colours used in cartoons and cross sections); right column, description of cell types present and defining characteristics for each stage. Stages based on Sanders et al., 1999.

(Stage 9; Li, et al. 2012). The tapetum in the anther goes through programmed cell death (stage 10), followed by the microspores going through two rounds of mitosis.

Late stages of pollen development occur from stage 11 through 13. The first microspore mitosis results in a large vegetative cell and a small generative cell (pollen is bicellular; stage 11), additionally the septum between locules begins to degrade. The second round of microspore mitosis yields an additional haploid generative cell (both are now referred to as sperm cells; pollen is tricellular; stage 12). Bicellular and tricellular pollen accumulates starch granules while developing (Chang and Neuffer 1989; Bedinger 1992). The septum between locules is fully degraded during stage 12, after which the stamen is composed of two large locules (bilocular). Mature pollen is then released through the programed opening at the ends of the anther locules (stomium; stage 13). Stamen development finishes with senescence of the stamen (stage 14) and the stamen falling off of the plant (stage 15).

fuzzy tassel (fzt) Contains a Mutation in DCL1

Mutants that cannot make normal levels of microRNAs (miRNAs) have broad developmental defects. Null mutants in miRNA biogenesis enzymes are often lethal, but some hypomorphic alleles are viable. Expanded expression of organ specific genes and fused organs are seen in *Arabidopsis* miRNA biogenesis pathway mutants (Yang, et al. 2007). In *Arabidopsis*, *carpel factory1* (*caf1*) mutants contain a hypomorphic allele of DCL1, and are smaller than wildtype in stature and leaf size, and have dramatic reproductive defects, including indeterminate carpel production and extra stamens that have only two locules instead of four (Jacobsen, et al. 1999). CAF1 is essential for miRNA biogenesis; *caf1* mutants have reduced miRNA accumulation compared to wildtype (Park, et al. 2002).

The maize *fzt* mutant has a mutation in the gene encoding DCL1, leading to decreased DCL1 function (Thompson, et al. 2014). The decrease in DCL1 functionality leads to reduced processing of miRNAs (Thompson, et al. 2014). The *fzt* phenotype is shorter than normal siblings. The IM is fasciated in *fzt* plants, indicating a lack of homeostasis in rapidly dividing meristem cells (Thompson, et al. 2014). The SPMs initiate more than two SMs, which in turn initiate multiple FMs that have abnormal numbers of defective floral organs (Thompson, et al. 2014). These severe reproductive defects result in both male and female sterility (Thompson, et al. 2014).

MicroRNA Biogenesis

MiRNAs are a class of short, non-coding RNAs that regulate gene expression post-transcriptionally (Bartel 2004). Synthesis of miRNAs begins with transcription of the primary RNA (pri-miRNA), which is cleaved by DCL1, resulting in the pre-miRNA hairpin (Reinhart, et al. 2002; Nag and Jack 2010; Yang, et al. 2007). DCL1 cleaves the pre-miRNA a second time, resulting in a miRNA-miRNA* (miRNA duplex) complex (Bartel 2004). The miRNA duplex is exported into the cytoplasm, the complex dissociates and the miRNA is incorporated into the RNA-Induced Silencing Complex (RISC), a large macromolecular complex containing the endonuclease ARGONAUT (David 2013; Bartel 2004). The RISC complex then targets specific mRNAs for cleavage, the miRNA provides the sequence specificity while AGO cleaves the transcript, resulting in the degradation of targeted mRNAs (Ren and Yu 2012; Rogers and Chen 2012).

MiRNAs in Stamen Development

MiRNAs (miRNAs) are essential for stamen development. miR159, miR319, and miR167 regulate stamen or pollen development (Palatnik, et al. 2007). Overexpression of

miR159 resulted in stunted stamens that failed to dehisce in *Arabidopsis* (Achard, et al. 2004; Palatnik, et al. 2007). Reduced expression of miR319 results in decreased stamen length (Nag, et al. 2009). miR167 targets two auxin response factors (arf), *arf6 arf8* double mutants fail to release pollen (Nagpal, et al. 2005).

MiR159 Regulates Stamen Development Through Gibberellic Acid-Induced MYBs

MiR159 targets MYB transcription factors that regulate reproductive development in plants (Achard, et al. 2004). MYBs are a large group of transcription factors that function in plant development, metabolism, hormone response, and reproduction. One clade within the MYBs are the gibberellic acid (GA-) inducible MYBs (Dubos, et al. 2010; Du, et al. 2012). Gibberellic acid is a plant hormone that regulates cell elongation, seed dormancy, and flowering (Chhun, et al. 2007; Plackett, et al. 2011; Schwechheimer and Willige 2009).

Mutations in *Arabidopsis* and rice GAMYB genes result in male sterility (Woodger, et al. 2003; Plackett, et al. 2011). Two redundant GAMYBs in *Arabidopsis*, *AtMyb33* and *AtMyb65*, are essential for tapetum regulation and pollen viability (Miller and Gubler 2005). The *AtMyb33 AtMyb65* double mutant had enlarged tapetum that crushed the developing pollen (Miller and Gubler 2005). In rice stamens, *OsGAMyb* had middle layer and tapetal degradation defects, and stamens were shorter (Tsuji, et al. 2006; Kaneko, et al. 2004; Liu, et al. 2010; Aya, et al. 2009). GAMYBs are essential for male-fertility by maintaining the tapetum.

Overexpression of GAMYB can also lead to stamen defects. Overexpression of the barley GAMYB, *HvGAMyb*, resulted in small stamens (Murray, et al. 2003). *HvGAMyb* developed normally until stamen stage 9, when the tapetum was being degraded and microspores were vacuolated, but stamens never dehisce because the septum between locules did not degrade (Murray, et al. 2003). The pollen in *HvGAMyb* stamens was viable and generated seeds, but at a

very low frequencies (Murray, et al. 2003). Unlike GAMYB underexpression, GAMYB overexpression results in male-sterility by preventing septum degradation.

Objectives

My two objectives were to characterize the *fzt* male-sterile phenotype, and begin to investigate candidate miRNA-targeted genes that contribute to the male-sterile phenotype. I conducted a detailed analysis of stamen development in *fzt* mutants and I found that *fzt* is required at multiple stages of stamen development, and is essential for the final stages of pollen maturation and release. One common phenotype in *fzt* stamens were locules that arrested at stage 9, this similarity with the HvGAMYB overexpression line implicated two related maize GAMYBs, *ZmMyb74* and *ZmMyb138*, as candidates for further investigation. I have developed tools for analyzing *ZmMyb74* and *ZmMyb138* expression *in situ*, and isolated maize lines with mutant alleles in both genes. The ultimate goal of the mutant lines will be to demonstrate that *ZmMyb74* and *ZmMyb138* are causing part of the *fzt* stamen phenotype by crossing them into *fzt* to rescue part of the male-sterile phenotype.

CHAPTER 2: Methods

Plant Tissue and Growth Conditions

Plants for analyses were grown in the Howell Biological Science Complex Biological Greenhouses under long day growth conditions (16 hours light) between March 2014 and March 2015. *fzt* homozygous mutant plants were always grown alongside normal sibling plants and co-grown plants were used as controls for all experiments and analyses. *fzt* plants were grown in the A619 (BT1398) and Mo17 (BT688) inbred background after several generations of introgression.

Developmental Series

Florets from tassels before emergence from the stalk were fixed in FAA (Ruzin 1999) modified: 50 100% ethanol: 35 water: 10 37% formaldehyde: 5 glacial acetic acid: 0.5 Triton: 1 DMSO. Florets from tassels fully emerged from the stalk were fixed in Carnoys fixative (Ruzin 1999). Stamens were dehydrated through a graded ethanol series, and embedded in paraffin (Electron Microscopy Science). Embedded tissue was sectioned 8μm thick, on a Reichert-Jung Biocut 2030 Paraffin Microtome. Tissue sections were dewaxed with xylene and stained using Toluidine Blue (0.05%w/v in water), dried, and mounted with Permount.

Pollen Viability

Pollen Viability was assessed using a modified Alexander stain as previously described (Peterson, et al. 2010) on anthers fixed with Carnoys fixative. At least two biological replicates were performed for each timepoint.

Pollen Germination Assay

Pollen germination media was made according to the Maize Handbook (Freeling and Walbot 1996). Pollen was collected between 8am to 9am from greenhouse grown plants in January and February 2015. Two to three biological replicates were performed for each time point, all stamens from a whole spikelet were used. Stamens were cut on a glass slide to liberate pollen. After incubation, a pollen germination media fixative (Pfahler 1981) was added to the plates to kill and preserve the pollen. Plates were stored at 4°C until all samples were collected and analyzed together, counts were taken of all pollen in $5cm^2$ section of the plates.

Nuclei Visualization

All stamens from florets from at least two biological replicates were used. Pollen was manually collected from stamens fixed in Carnoys fixative on a glass cover slide, all plant debris was discarded. Pollen was incubated in the dark with 0.5ug/mL DAPI (D1306; Life Technologies) in 1xPBS (Sigma; BP2944-100 tablets) at 4°C overnight (~16 hours). Florescence nuclei were observed at 509nm.

Starch Staining

All stamens from whole spikelets were used from two replicates per a timpoint. Stamens fixed in Carnoys fixative were incubated with I/KI 0.33%/0.66% (Gram's Iodine Solution; Sigma-Aldrich), and incubated at room temperature overnight before analysis (Ruzin 1999).

RNA Extraction

Total RNA was extracted from pools of 8 to 24 stamens using Tri-Reagent (Sigma-Aldrich). Total RNA was DNase treated (Quiagen) and cleaned over a column (RNEasy Minelute; Quiagen). cDNA was synthesized with random oligo(dT) primers using SuperScript III First-Strand Synthesis System (Invitrogen) on 1μg of clean-RNA. cDNA was synthesized

from 1µg of RNA using oligo(dT) primers. cDNA corresponding to ZmMyb74 and ZmMyb138 was amplified using gene specific primers. cDNA was used with CJMu2F/R primers for *ZmMyb138*, and Zm028054.2F/R for *ZmMyb74* (Table 1).

RNA *in situ* Hybridization Probe Design and Synthesis

RNA was extracted using a trizol-based method, total RNA was treated with DNase Treatment & Removal (Life Technologies) and cleaned up over a RNA Mini Cleanup Kit (Qiagen). cDNA was synthesized using Oligo(dT) primers from a Superscript III First Strand RT-PCR Synthesis kit (Life Technologies).

I created probes for RNA *in situ* hybridization by amplifying regions of the 5' and 3' untranslated regions (UTRs) or the protein coding sequence of *ZmMyb74* and *ZmMyb138* using A619 inbred cDNA. Amplicons were 125 to 500 basepairs in length. Primers for *ZmMyb138* were Zm139688.1F/R and Zm139688.2 F/R (both target the 3' transcript, between 125 to 200bp long probes), 5P139688.2 F/R (targeting the coding sequence; 500bp long probe) (Table 1). Primers for *ZmMyb74* were ZmMyb028054.1 F/R and ZmMyb028054.2 F/R, both target the 3' end of the transcript, probes were between 125 to 200bp long (Table 1). Amplicons were cloned into the pGEM-T Easy vector (Promega) and correct clones were verified by sequencing. I generated template for *in vitro* transcription by linearizing the plasmid using either Nco1 or Nde1 (New England Biolabs), depending on the insertion orientation. *In vitro* transcription was performed using digoxigenin-dUTP (DIG labeled uracil; Roche) and T7 or Sp6 polymerase (Promega), depending on the insertion orientation.

Table 1. Primers used and their conditions.

Primers for in situ probes	Conditions	Product size (bp)
Zm139688.1F/R	95°C2'/(95°C45"/53°C45"/72°C20")x35/72°C5'/4°C	127
Zm028054.1F/R	95°C2'/(95°C45"/53°C45"/72°C20")x35/72°C5'/4°C	107
Zm028054.2F/R	95°C2'/(95°C45"/53°C45"/72°C20")x35/72°C5'/4°C	140
MYB138CDS1F/R	95°C2'/(95°C1'/55°C45"/72°C1')x35/72°C5'/4°C	501
Primers for UniformMu lines		
Cj-Mu2F/R	95°C2'/(95°C45"/58°C45"/72°C35")x35/72°C5'/4°C	202
Cj-Mu1R/TUSC	95°C2'/(95°C45"/58°C45"/72°C35")x35/72°C5'/4°C	298
Mu054CR/TUSC (mu1045931)	95°C2'/(95°C45"/58°C45"/72°C35")x35/72°C5'/4°C	1077
Mu054CR/TUSC (mu1016361)	95°C2'/(95°C45"/58°C45"/72°C35")x35/72°C5'/4°C	276
MuMYB74.2F/MuMYB74.1R	95°C2'/(95°C45"/65°C45"/72°C35")x35/72°C5'/4°C	586

RNA *in situ* Hybridization

Developing stamens were fixed with FAA and embedded in paraffin, protocol from Jackson et al. 1994, modified by Beth Thompson. Tissue was sectioned 8μm to 10μm thick, using a Reichert-Jung Biocut 2030 microtome. Hybridization was performed as described in Pollack et al. 1992. After hybridization, sections were washed, incubated with anti-DIG antibodies (Roche), and incubated with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; Roche).

Imaging

Images for the developmental series and florescence were taken using a LSM Zeiss 700. Starch and pollen viability images were taken on Zeiss Axio Observer Z1.

Phylogenetic Analysis

Predicted CDS of MYB transcription factors was obtained from online public databases (MaizeGDB, Grameme, and TAIR), and were aligned with MegAlign using the Clustal V alignment (Gap penalty: 10, Gap Length Penalty:10). The phylogenetic tree was generated from the aligned sequences as a cladogram, using straight branches, and is weighted.

CHAPTER 3: *fzt* is Required for Multiple Stages of Stamen Development

I characterized the stamen phenotypes found in *fzt*. *fzt* plants have severe stamen development defects. Defects arising in the diploid sporophytic tissue likely underlie the stamen defects in *fzt* plants. Homozygous *fzt* progeny are obtained in a 3:1 phenotypic ratio when heterozygous parents are crossed (Thompson, et al. 2014). This ratio indicates that the problem in male gametogenesis is not in haploid gametophytic tissue, nor is the *fzt* allele pollen-lethal, so the focus of my investigation was on development of the sporophytic stamen. I hypothesize that the issue in *fzt* stamen development is in the diploid sporophytic tissue. This work implicates miRNAs in multiple stages of stamen development and demonstrates that the complex regulatory pathway involving miRNAs in male development has yet to be fully understood.

fzt is Male Sterile and Makes Abnormal Stamens

Grass florets are borne in spikelets, which contain one or more florets. Maize spikelets initiate two florets, which are the product of the upper and lower floral meristem. Both tassel and ear floral meristems initiate both stamen and carpel primordia, and sex determination occurs via stamen arrest in the ear and carpel abortion in the tassel (Barazesh and McSteen 2008). At maturity, maize tassel florets are composed of two bract-like organs (lemma and palea), two lodicules (analogous to petals) and three stamens (Fig. 2C). In normal spikelets, the upper floret and lower floret each produce three stamens, resulting in six stamens per spikelet. Stamens in the UFM develop about one day ahead of LFM (Hsu, et al. 1988), although there are no morphological differences between stamen development in the upper and lower floret at maturity

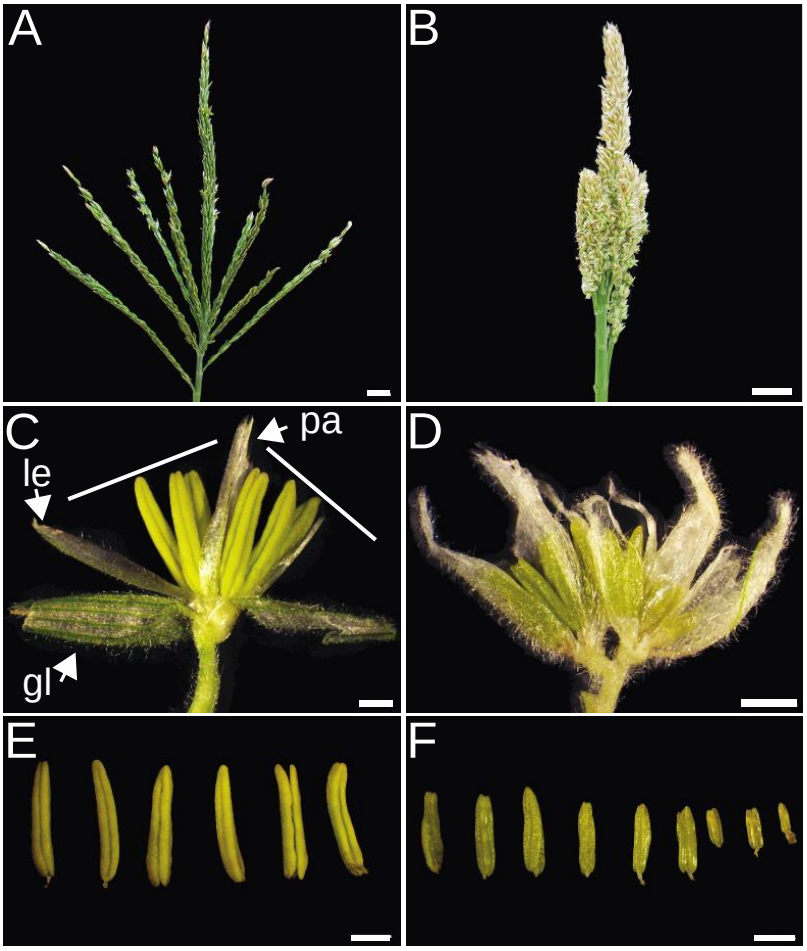

Figure 2. Male reproductive defects in *fzt*. A) Normal sibling. B) *fzt* tassels are shorter than normal siblings and have increased branching. C) Normal sibling florets are composed of the upper and lower floret (lines). Each floret is composed of the palea (pa), lodicules (not visible), and stamens. The glumes are from the spikelet and cover the floral organs. D) *fzt* differentiates floret-like structures that lack glumes and contain many 'florets'. E) Stamens from florets in C. Mature normal sibling stamens are uniform in size and shape from the upper and lower floret. Mature stamens are 6mm long, yellow in color, and have four plump locule lobes. F) Stamens from florets in D. *fzt* initiates abnormal numbers of stamens. Stamens from *fzt* arrest at several sizes, no stamens reach full size. Locule lobes in *fzt* are not uniformly plump, often are twisted or shriveled. A and B scale bars are 5cm. C, D, E, and F scale bars are 2mm.

(Chang and Neuffer 1989). In normal plants, the three stamens from a single floret initiate and develop synchronously. Normal anthers are a uniform yellow color and are composed of four locules, which support pollen development and contain pollen at maturity (Fig. 2E). In contrast, *fzt* florets contained too many or too few stamens with abnormal morphology (Fig. 2D, F). Whereas normal anthers were between 5mm-6.5mm and uniform in shape, *fzt* anthers varied in size from less than 1mm to 4mm (Table 2).

fzt is Required for Late Stages of Stamen Development

Male sterility can result from multiple defects, including defects in microsporogenesis itself, as well as defects in somatic cell developmental programs in the developing stamen (Zhou, et al. 2011, Skibbe and Schnable 2005, Horner and Palmer 1995). Stamen development follows a stereotypical program of cell proliferation, differentiation, and degradation and has been well-characterized in maize (Kelliher and Walbot 2011, Chang and Neuffer 1989). The development and degradation of the tapetum, which provides nutrients to the developing pollen, plays a particularly important role in supporting development of viable pollen, and mutants that affect tapetum function are often male sterile (Kawanabe, et al. 2006, Vizcay-Barrena and Wilson 2006, Shi, Zhao and Yao 2009). To gain a better understanding of the cause of male sterility in *fzt*, I examined normal and *fzt* anthers in transverse sections during development. Anther length and developmental stage are usually tightly correlated in maize and anther length is often used as a proxy for developmental stage. *fzt* mutants, however, are much smaller than normal and I did not observe this close correlation between anther length and developmental stage (for example, some *fzt* stamens contain tricellular pollen, but never reach the size of normal anthers with tricellular pollen). Therefore, I used defining hallmarks of stamen development (Sanders, et al. 1999) to determine developmental stage of *fzt* anthers (Fig. 1). *fzt* anthers often included both

Table 2. Anther length of mature normal and *fzt* anthers.

Anther size (mm)	<1	1-2	2-3	3-4	4-5	5-5.5	5.5-6.5
Normal (n=28)	0	0	0	0	0	36%	64%
fzt (n=150)	11%	49%	25%	15%	0	0	0

normal and defective locules in the same anther. Therefore, I scored the number of locules with developmental defects, rather than the number of defective anthers. In approximately 70% of *fzt* anther locules, development was normal until late stages of anther/pollen development (stage 9; Fig. 3)**,** at which point microspores have become vacuolated (Table 3). I never observed *fzt* locules that progressed past stage 9, the stage immediately preceding complete degradation of the tapetum (stage 10), breakdown of the septum (stage 11 and 12), and dehiscence (stage 13; Fig. 4). In arrested locules, the tapetum either fails to degrade at all, or fails to degrade completely since I always observed at least some tapetal remnants in arrested locules. In the remaining ~30% of locules (not arrested at stage 9), I observed phenotypes consistent with slightly earlier defects in development, including collapsed, shrunken locules (Fig. 5B, C), and locules with enlarged and vacuolated cell layers (Fig. 5D). Collapsed locules often had one to three somatic cell layers and contained crushed pollen (based on auto-fluorescence of the native exine; Fig. 6). In addition, I often observed locules in developing anthers with three cell layers, a degrading inner cell layer and degenerating microspores (Fig. 5E). These phenotypes are consistent with defects in the anther wall, including aberrant (premature or late) degradation of cell layers, vacuolated cells or cells that fail to differentiate normally. I never observed phenotypes that indicated defects in pre-meiotic anthers (Fig. 3).

***fzt* anthers produce non-viable that arrests at multiple developmental time points**

My developmental analysis indicated that approximately 70% of *fzt* locules developed normally until the final stages of anther development, however pollen did not appear to fully mature and dehisce. To ask if this immature pollen was viable, I stained *fzt* anthers with a modified Alexander stain to assess pollen viability (Peterson, Slovin and Chen 2010). Pollen that contains a cytoplasm (viable) stains light fuchsia, whereas pollen that lacks a cytoplasm

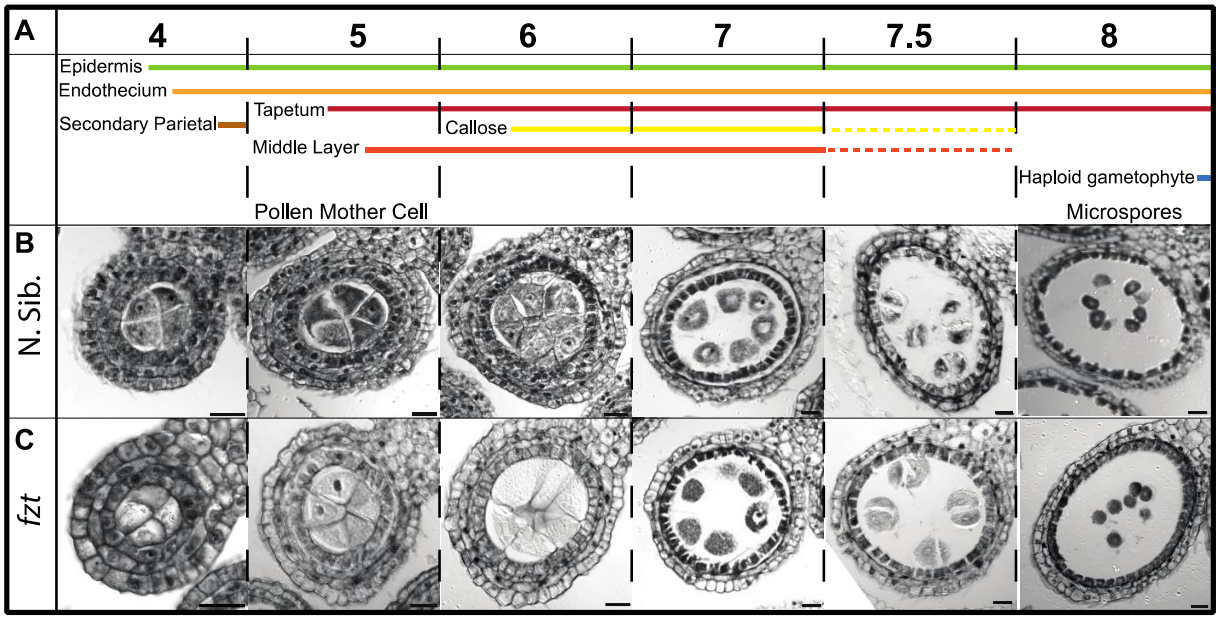

Figure 3: Early anther development in *fzt* is normal. (A) Stage of anther development is indicated, as well as the cell types and physiological events that occur at each stage. (B) Normal anther development. (C) In ~70% of *fzt* locules, development is indistinguishable from normal through stage 8. Scale bars = 20μm.

Table 3. Frequency of locule phenotypes in mature *fzt* anthers.

	Morphologically normal stage 9 locules	Collapsed locules	Locules with vacuolated inner layers(s)
fzt (n=100)	71%	21%	8%

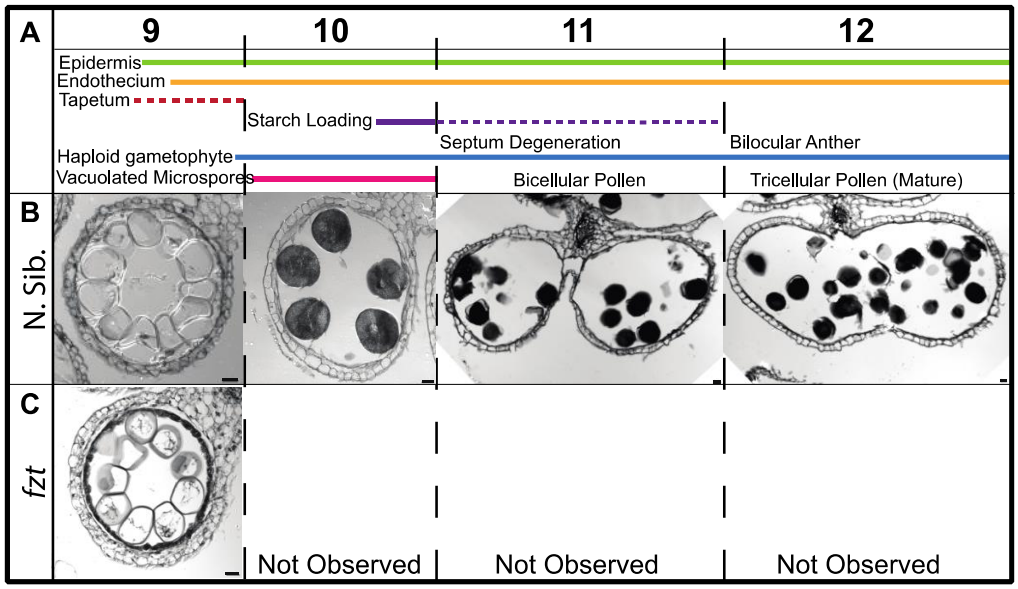

Figure 4. *fzt* anthers arrest at late stages of stamen maturation and do not dehisce.
(A) Stage of anther development is indicated, as well as the cell types and physiological events that occur at each stage. (B) Normal anthers fully mature and produce mature pollen; the septum degrades immediately prior to dehiscence. (C) *fzt* anthers often progress to stage 9, however never progress past stage 9 and do not dehisce. Scale bars = 20μm.

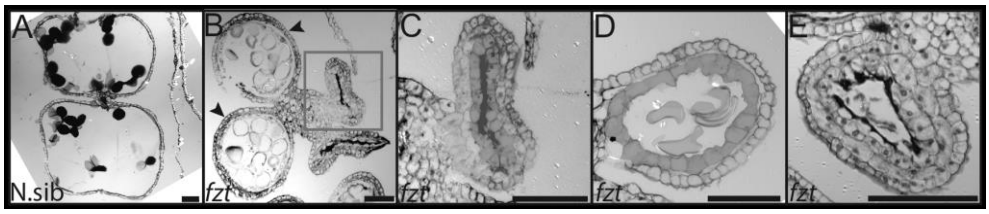

Figure 5. *fzt* anthers have abnormal locules at maturity.
(A) Normal anther immediately before dehiscence contains mature pollen and is bilocular. (B-D) Examples of abnormal locules in *fzt* anthers from mature plants. (B) *fzt* anther contains two "normal" locules arrested at stage 9 (arrowheads), and two collapsed locules. (C) Close-up of collapsed locule in (B); collapsed locule contains three tissue layers, indicating defects in cell layer degradation. (D) Locule from *fzt* anther has three cell layers; cells are vacuolated. (E) Locule from developing *fzt* anther contains three cell layers, a degrading inner cell layer and degenerating microspores. Colours indicate cell types: green = epidermis; yellow and orange = cell layers of unknown identity (endothecium, middle layer or tapetum). A single *fzt* anther has morphologically normal stage 9 locules (arrowheads) and collapsed locules. Scale bars = 100μm.

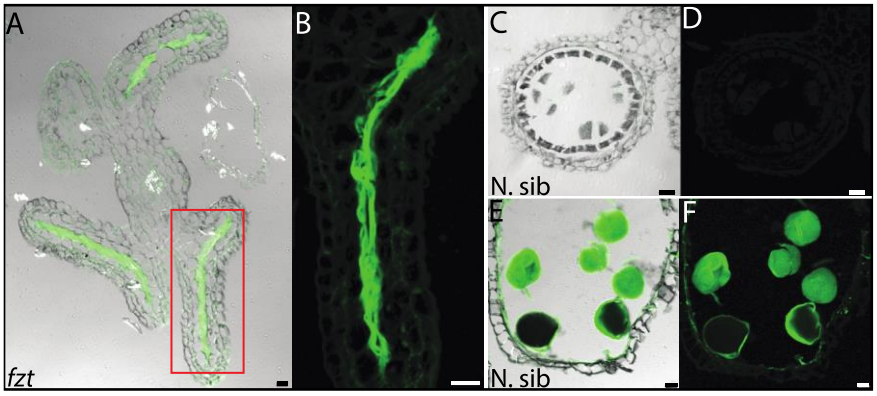

Figure 6: Collapsed locules in *fzt* anthers contain crushed pollen. (A) *fzt* stamen with three collapsed locules. Exine in crushed pollen auto-fluoresces. B) Close-up of collapsed locule in (A, red box). (C-D) Normal stage 7 locule prior to exine deposition. (C) Normal stage 7 locule, bright field with fluorescence overlaid. Pollen does not auto-fluorescence because it lacks a pollen coat with exine. (D) Normal stage 7 locule in (C), showing only fluorescence. (E) Normal stage 12 locule, bright field with fluorescence overlaid. Mature pollen auto-fluoresces because pollen coats contains exine. (F) Normal stage 12 locule shown in (E), showing only fluorescence. All images were taken using the same confocal conditions normalized to exine fluorescence. Scale bars = 20μm.

(nonviable) stains blue. I assayed viability of pollen from *fzt* anthers at multiple time points relative to dehiscence in normal siblings, including immediately before dehiscence (dehiscence minus 0.5 weeks; D-0.5), during dehiscence (D 0.0) and several time points after dehiscence (D+0.5, D+1.0, D+2.0). Normal anthers immediately before (D-0.5) and during dehiscence (D 0.0) contained pollen that stained light fuchsia and was uniformly plump, indicating that pollen was viable (Fig. 7A; Table 4). In contrast, many *fzt* anthers produced shrivelled pollen that stained dark blue, indicating the pollen was not viable (Fig. 7B, C). *fzt* anthers differed in the amount of viable pollen they contained and I classified *fzt* anthers based on the ratio of viable/nonviable pollen. Class I anthers contained >90% viable pollen, class II anthers contained a mixture of viable and nonviable pollen and class III anthers contained >90% nonviable pollen (Fig. 7B, C). At early time points (D -0.5, D+0.0), ~25% of *fzt* anthers were class I (mostly viable pollen), ~25% class II (mixture of viable/nonviable pollen) and ~50% class III (mostly nonviable pollen; Fig. 7B, C; Table 4). At later time points (D+0.5, D+1.0, and D+2.0), <5% of *fzt* anthers were class I, ~47% were class II and ~47% were class III. Class I anthers were usually the largest anthers in the florets, however even *fzt* class I anthers contained more nonviable pollen than normal anthers. Class III anthers were typically the smallest anthers in florets and were thin, with shrivelled or flat locule lobes.

Although Alexander staining indicated that *fzt* anthers contain some viable pollen, *fzt* homozygotes are male sterile. Therefore, I performed *in vitro* germination assays to determine if pollen from *fzt* anthers could germinate. Nearly all pollen (~92%) collected from normal anthers at dehiscence (D 0.0) germinated (Table 5); pollen grains from normal anthers were uniformly large, pale yellow spheres. Since *fzt* anthers never dehisce, I manually collected pollen by breaking open anthers to release the pollen. I assayed pollen germination at four time points to

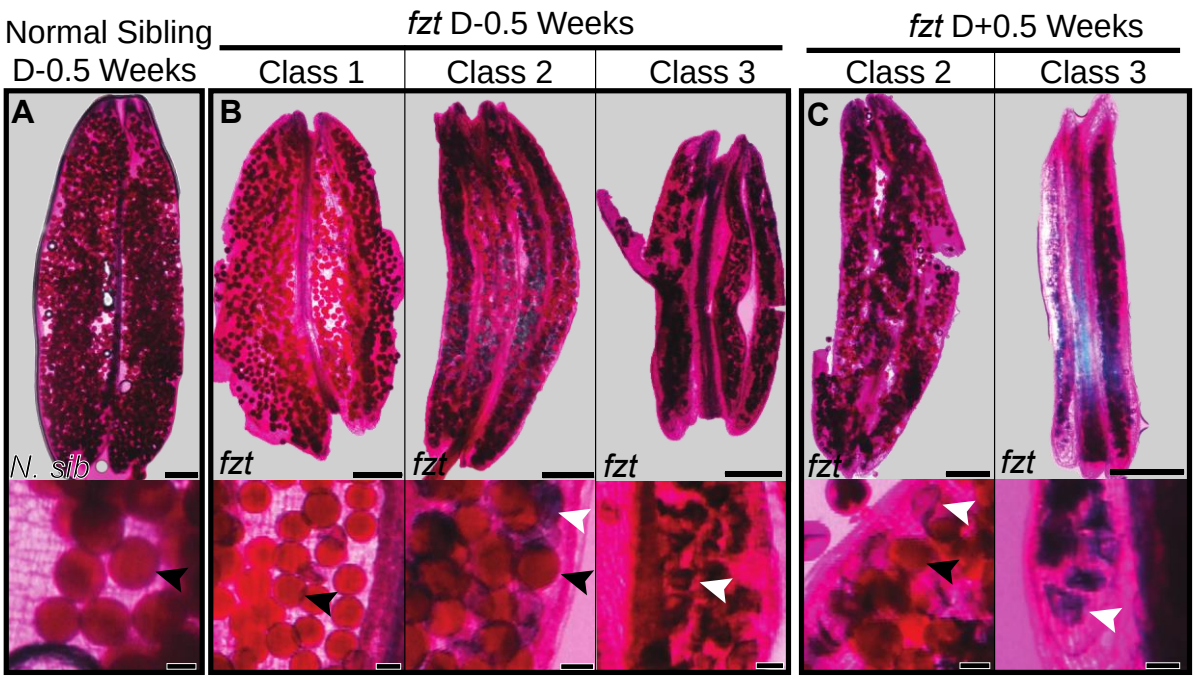

Figure 7. Pollen in *fzt* anthers has reduced viability.
Modified Alexander stain to assess pollen viability; viable pollen stains pink (black arrowheads), nonviable pollen stains blue (white arrowheads). Top box depicts whole anther, bottom box depicts close-up of pollen grains. (A) Almost all pollen from normal anthers is viable; pollen are round and plump in shape and stain pink. (B) *fzt* anthers (D-0.5) are heterogeneous. Class 1 anthers contain almost entirely viable pollen, similar to normal anthers. Class 2 anthers contain a mixture of viable and non-viable pollen grains. Nonviable pollen stains blue and is shrivelled. Class 3 anthers contain almost entirely nonviable pollen. C) *fzt* anthers (D+0.5) are all Class 2 or Class 3. Scale bars: top row = 500 μm; bottom row = 50 μm.

Table 4. Pollen viability based on Alexander staining.

Genotype (time point)	Class 1	Class 2	Class 3
Normal Sibling D-0.5 (n=7)	100%	0.0%	0.0%
Normal sibling D+0.0 (n=11)	100%	0.0%	0.0%
fzt D-0.5 (n=20)	10.0%	40.0%	50.0%
fzt D+0.0 (n=32)	31.2%	21.9%	46.9%
fzt D+0.5 (n=25)	0.0%	52.0%	48.0%
fzt D+1.0 (n=46)	4.3%	37.0%	58.7%
fzt D+2.0 (n=19)	5.2%	52.6%	42.1%

Table 5. Germination frequencies of pollen from normal and *fzt* anthers.

Genotype (time point)	% pollen germinated (n)
Normal (D+0.0)	92.7% (466)
fzt (D+0.0)	2.9% (136)
fzt (D+1.0)	0.9% (656)
fzt (D+1.5)	0.2% (720)
fzt (D+2.5)	0% (73)

ensure any apparent germination defect reflected a real defect and not simply a developmental delay in *fzt*. At all time points, <3% of pollen from *fzt* anthers germinated (Table 5). Pollen from *fzt* anthers was heterogenous in appearance and included small, shrivelled, clear pollen grains, as well as larger, opaque, pale yellow pollen grains (Figure 8). Thus, even though *fzt* stamens make some viable pollen, very little pollen can germinate.

Based on my developmental analysis, *fzt* anthers fail to develop past stage 9, at which point unicellular microspores are vacuolated. To ask if pollen maturation also prematurely arrest in *fzt* anthers, I stained pollen with DAPI to visualize the number of nuclei. Normal mature pollen have undergone two rounds of mitosis, yielding tricellular pollen with two compact germ nuclei and one large vegetative nucleus. Nearly all pollen from normal anthers at dehiscence (D 0.0) were tricellular (94%; Table 6). In contrast, pollen from *fzt* anthers of the same age contained a large proportion of unicellular and bicellular pollen, indicating that pollen from *fzt* anthers fails to develop to maturity (Table 6). I examined pollen from *fzt* anthers at multiple time points to account for any variability in developmental timing. At the first two developmental time points (D -0.5, D+0.0), approximately 75% of pollen from *fzt* anthers was unicellular, and the remaining pollen was bicellular (24%), suggesting that pollen development in *fzt* anthers is delayed or arrested. At the final time point assayed (D+1.0), a third of the pollen from *fzt* anthers was tricellular, while the remaining pollen was unicellular or bicellular (Table 6). I could not examine later time points because pollen would not separate from the anther.

During the final stages of anther maturation, pollen accumulates starch, which functions both as an energy source and as a terminal regulator of pollen maturation (Datta et al., 2002). Pollen that fails to accumulate normal starch levels often arrests prematurely (Datta et al., 2002).

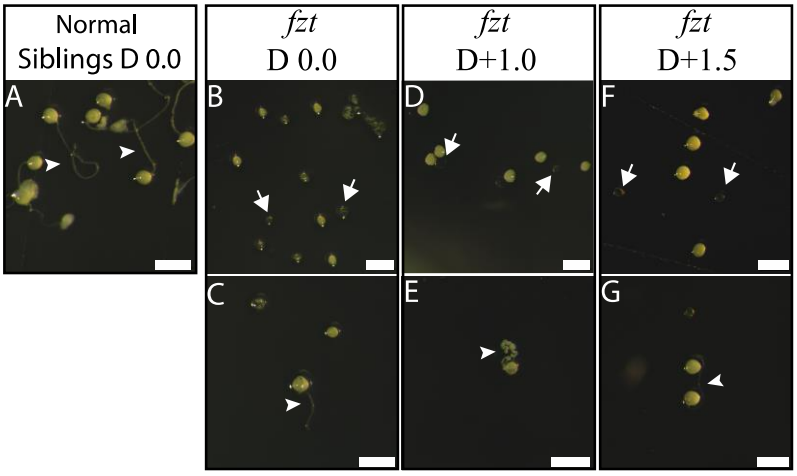

Figure 8. *fzt* produces pollen grains that germinate. (A) Normal sibling pollen germinates and extends a pollen tube (arrowhead). Pollen in normal siblings is uniformly in shape and size, and is pale yellow. (B-G) *fzt* at several time points has large round pollen grains as well as smaller, shriveled, and transparent grains (arrows). (C, E, G) *fzt* has pollen grains that germinate and extend a pollen tube (arrowhead). 200μm scale bars.

Table 6. *fzt* pollen arrests at the unicellular and bicellular stage.

Genotype (time point)	One nuclei	Two nuclei	Three nuclei
Normal (D 0.0) (n=51)	0.0%	5.8%	94.1%
fzt (D-0.5) (n=154)	75.3%	24.0%	0.6%
fzt (D 0.0) (n=66)	68.1%	30.3%	1.5%
fzt (D+1.0) (n=107)	34.6%	36.4%	28.9%

The failure of most of the pollen from *fzt* anthers to mature into tricellular viable pollen could be due to insufficient starch accumulation (Datta, Chourey, et al. 2001). To test this hypothesis, I assayed starch accumulation in *fzt* and normal sibling anthers by staining pollen with iodine (Datta et al., 2002). Pollen grains fully loaded with starch stain dark brown or black, while pollen without starch do not stain and appear clear or light brown. Pollen from normal and *fzt* mutant anthers had dramatic differences in starch accumulation (Fig. 9; Table 7). Half a week before dehiscence (D -0.5), most anthers from normal plants contained pollen grains that stained black (81%), indicating these pollen grains were fully loaded with starch (Fig. 9A). The remaining anthers contained a mixture of pollen that stained brown and black, consistent with partial starch loading. Similar to previous assays, I assayed starch accumulation in *fzt* anthers at multiple time points to account for any developmental delay in *fzt*. At all developmental time points, pollen in *fzt* anthers had dramatically reduced starch accumulation compared to normal anthers. Approximately 50% of *fzt* anthers produced pollen with no detectable starch, while pollen from the other 50% of *fzt* anthers had minimal starch accumulation (Fig. 9B-D). Thus, *fzt* anthers fail to accumulate starch and arrest prior to maturation.

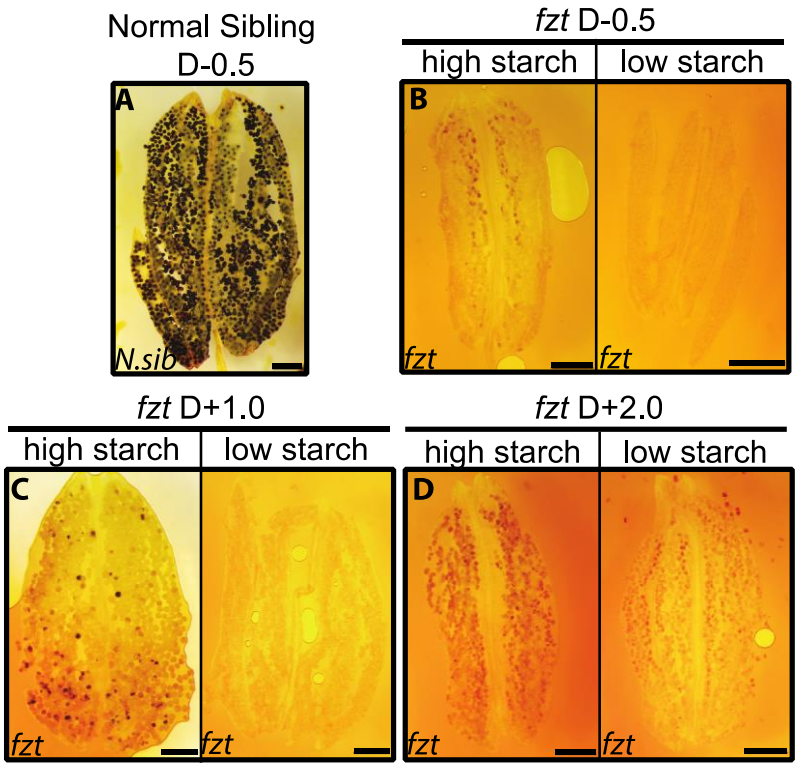

Figure 9. Pollen in *fzt* anthers does not accumulate starch. (A) Pollen from normal anthers (D-0.5) stain black with iodine indicating high starch accumulation. (B) Pollen from *fzt* anthers (D-0.5) do not stain or stain light brown indicating minimal starch accumulation. (C, D) Pollen from *fzt* anthers one week (C) and two weeks (D) after normal siblings dehisced still had minimal starch accumulation. Scale bars = 500µm.

Table 7. Pollen in *fzt* anthers do not accumulate starch.

Genotype (time point)	All pollen stains black	Mixture of pollen that stains black and brown	Faint staining	No staining
Normal Sibling (D-0.5) (n=11)	81.8%	18.1%	0	0
Normal Sibling (D+0.0) (n=7)	71.4%	28.6	0	0
fzt (D-0.5) (n=9)	0	0	55.5%	44.4%
fzt (D+0.0) (n=18)	0	0	5.5%	94.4%
fzt (D+1.0) (n=6)	0	0	66.6%	33.3%
fzt (D+2.0) (n=26)	0	0	42.2%	57.7%

CHAPTER 4: Development of Genetic Tools to Investigate the Cause of Male Sterility in *fzt*

The *fzt* stamen phenotype is similar to GAMYB overexpression lines in barley. MYBs are regulated by miR159, and several MYBs are overexpressed in *fzt*. The MYB genes predicted to be targeted by miR159 (Thompson, et al. 2014) are closely related to the GAMYB clade of MYB transcription factors (Du, et al. 2012; Du, et al. 2009; Dubos, et al. 2010; Fig. 10). I am particularly interested in *ZmMyb74* and *ZmMyb138*. *ZmMyb138* is up-regulated 3.4 fold in *fzt* (Thompson, et al. 2014), and expressed in developing tassels and in mature anthers (Sekhon, et al. 2011). *ZmMyb74* is likely redundant with *ZmMyb138*. *ZmMyb74* and *ZmMyb138* have publicly available UniformMu transposons inserted in the gene or upstream of the 5'UTR, allowing isolation of mutant alleles and characterization of the phenotype resulting from these mutant alleles.

Location of Transposon Insertions in *ZmMyb74* and *ZmMyb138*

I obtained one UniformMu insertion line in *ZmMyb138* and two insertion lines in *ZmMyb74* (Fig. 11; Table 8). To begin to characterize the UniformMu insertion lines I determined where the transposon insertions are within *ZmMyb138* or *ZmMyb74*. I confirmed the transposon in *ZmMyb138* using CjMu2F and TUSC, a gene-specific primer and a transposon-specific primer, respectively. The transposon in *ZmMyb138-mum1* is on Chromosome 3 at 187,005,320, which is an intron in the 5'UTR. I confirmed *ZmMyb74-m1::Mu1* (*ZmMyb74-mum1*) has the transposon insertion on Chromosome 8 at 17,175,120, within the 5'UTR of *ZmMyb74* with MuMYB74.1R and TUSC. Using Mu054AR and TUSC primers I confirmed

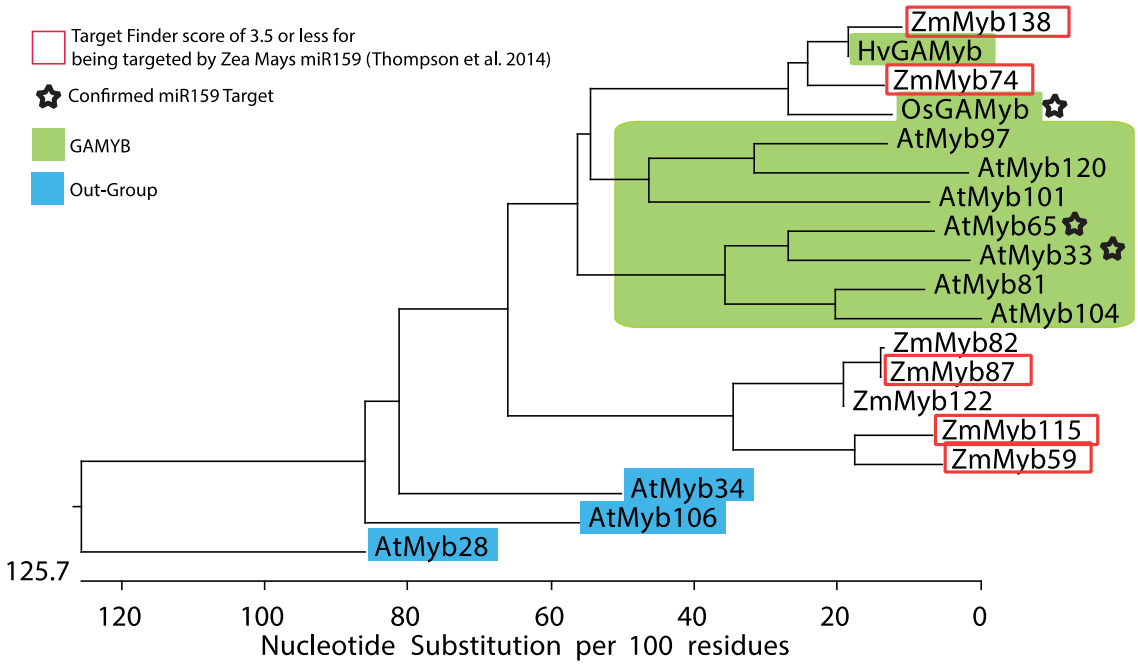

Figure 10. Phylogenetic tree of GAMYBs.
Relationship between maize miR159 targets and Mybs from other plants. miR159 targets (red box) closely associate with previously studied GAMybs (green) and confirmed targets of miR159 in other species (star). *AtMyb28* and *AtMyb34* are an out-group. Grouping of *Arabidopsis* Mybs is supported by Du et al. 2012 and Dubose et al, 2010.

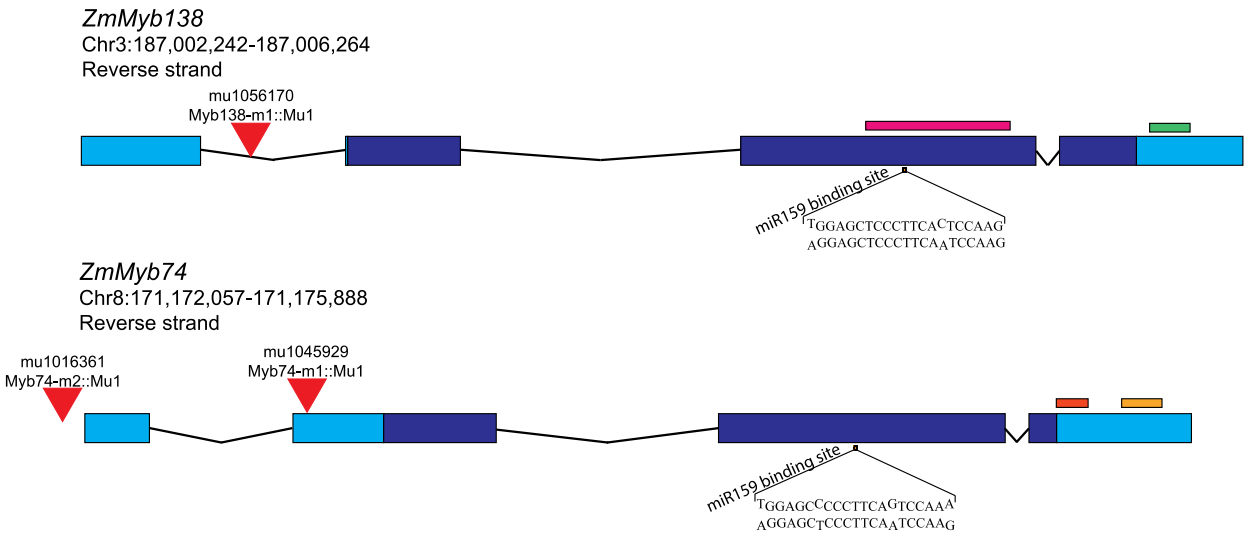

Figure 11. Gene Model of *ZmMyb138* (top) and *ZmMyb74* (Bottom).
Genes are aligned 5' to 3'. Lines are introns. Light blue are untranslated regions, dark blue are protein coding regions. Typical for plants, the miR159 binding site is in the coding sequence for both genes. Sequence in the binding site: top is gene target, lower sequence is miR159.
Colored lines are where RNA *in situ* hybridization probes target.
Triangles indicates insertion of UniformMu transposons, the specific insertion is named on top of the triangle.

Table 8. Name of *ZmMyb74* and *ZmMyb138* insertions and alleles

Loci	Gene	UniformMu line	Other name	Insertion name
GRMZM2G139688	*ZmMyb138*	*ZmMyb138-m1::Mu1*	*ZmMyb138-mum1*	Mu1056170
GRMZM2G028054	*ZmMyb74*	*ZmMyb74-m1::Mu1*	*ZmMyb74-mum1*	Mu1045929
		ZmMyb74-m2::Mu1	*ZmMyb74-mum2*	Mu1016361

ZmMyb74-m2::Mu1 (*ZmMyb74-mum2*) has a transposon insertion on Chromosome 8 at 17,175,920, roughly 20 bases upstream from the *ZmMyb74* 5'UTR.

ZmMyb138-mum1 is a Null Allele

Progeny of heterozygous *ZmMyb138mum1/+* segregate *ZmMyb138* and *ZmMyb138-mum1* alleles in a stereotypical 1:2:1 ratio (Table 9). *ZmMyb138-mum1* homozygous plants did not have any noticeable differences from normal sibling in overall plant stature or in reproductive development.

Since I did not see an altered phenotype in *ZmMyb138-mum1* homozygotes I wanted to determine if *ZmMyb138* RNA was expressed. To test for *ZmMyb138* expression, I extracted RNA from developing *ZmMyb138-mum1* homozygous and *ZmMyb138-mum1* heterozygous tassels, and synthesize oligo(dT)-primed cDNA. Using primers specific to *ZmMyb138*, I was able to amplify cDNA corresponding to *ZmMyb138* from *ZmMyb138-mum1* heterozygous plants, but I was not able to generate a PCR product from *ZmMyb138-mum1* homozygous, suggesting that the *ZmMyb138-mum1* allele is a RNA null allele (Fig. 12). As a control, I amplified *ZmMyb74* cDNA from both *ZmMyb138-mum1* homozygous and heterozygous plants to verify I generated high quality cDNA. This experiment was repeated using RNA from older tassels and yielded the same result. This experiment demonstrated that the *ZmMyb138-mum1* allele is likely a null allele.

Tools for expression analysis

Previous work showed that*ZmMyb74* and *ZmMyb138* are expressed in the tassel (Sekhon, et al. 2011), but the specific stage and cell type these genes are expressed in maize stamen

Table 9. *ZmMyb138-m1::Mu1* is not haploid lethal. N=36

	ZmMyb138/ZmMyb138	*ZmMyb138*/*ZmMYB138-Mu1*	*ZmMyb138-Mu1*/*ZmMyb138-Mu1*
Number of Progeny (%)	7 (20%)	20 (55%)	9 (25%)

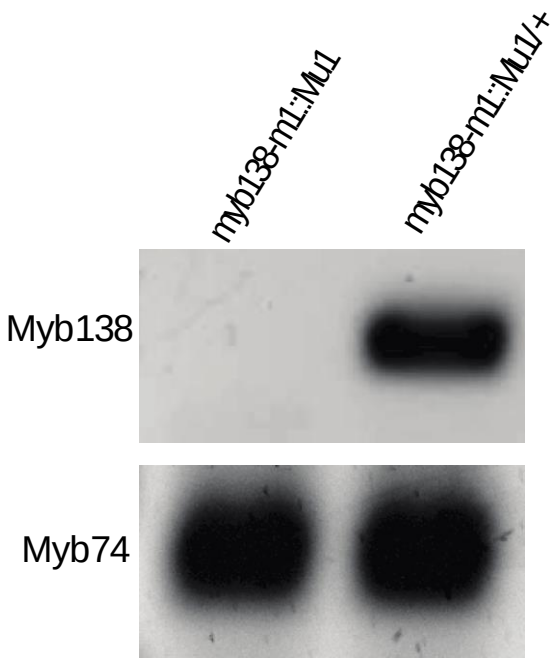

Figure 12. *ZmMyb138-m1::Mu1* is a null allele. RT-PCR using cDNA synthesized from RNA from young tassels homozygous and heterozygous for the transposon insertion in *ZmMyb138* (*Myb138-m1::Mu1; ZmMyb138-mum1*). Primers specific for *ZmMyb138* did not amplify a product using cDNA from homozygous *ZmMyb138-mum1* plants, but primers specific for *ZmMyb74* did generate a product. *ZmMyb138* and *ZmMyb74* products were amplified using cDNA from plants homozygous for *ZmMyb138-mum1*. No *ZmMyb74* or *ZmMyb138* products were amplified using the negative RT control (data not shown).

development are unknown. To determine when and where these two GAMYBs are expressed I designed and synthesized RNA *in situ* hybridization probes specific for each gene.

Probes specific for *ZmMyb138* target the 5' and 3' UTRs (two probes, ~150bp each) and the protein coding sequence (one probe, ~500bp). The DNA sequence for the probes are stored on pGEM T-Easy plasmids, and kept in the -80°C in Science & Technology 575. I have performed RNA *in situ* hybridization using combinations of these probes, but high background and unspecific signals prevent high confidence in the results (see appendix A).

I also designed two probes that target the *ZmMyb74* 3'UTR (~150bp each). The DNA sequence for the probes are stored on pGEM T-Easy plasmids in the -80°C freezer in Science & Technology 575. Expression of *ZmMyb74* in meiotic stamens is localized to the tapetum, but high background and characteristics of the tissue (the tapetum stains with most probes) prevents high confidence in RNA *in situ* hybridization results (see appendix A).

CHAPTER 5: Discussion

I conducted a detailed characterization of stamen and pollen development in *fzt* plants to gain a better understanding of the role of microRNAs in maize stamen development. MiRNAs are likely to regulate anther development at multiple points in development to support normal pollen development.

fzt is Required at Multiple Stages of Stamen Development

fzt has defects in several stages of male development, demonstrating *fzt* is required at multiple stages of stamen development. *fzt* male-sterility is caused by defects in the somatic tissue that surrounds the developing pollen since pollen from heterozygous siblings transmits the *fzt* allele in normal Mendelian ratios. I observed several defects in the somatic tissue of *fzt*, including premature tapetal degradation, enlargement of one of the inner tissue layers, failure to accumulate starch, and failure to dehisce. Together these results demonstrate multiple steps that miRNA-regulated pathways function at multiple parts in stamen development. Numerous mutants that cause male sterility in maize have been documented (Beadle 1932; Skibbe and Schnable 2005; Timofejeva, et al. 2013; Wang, et al. 2010; Chaubal, et al. 2000)), however the *fzt* phenotype does not resemble any reported mutants, suggesting that future studies on *fzt* will provide valuable insight on uncharacterized pathways in maize.

One *fzt* stamen morphological defect was premature tapetum degeneration. The tapetum serves the essential role of providing nutrients to the developing pollen (Li, et al. 2012; Scott, et al. 1991). Degeneration of the tapetum in maize occurs through programmed cell death (PCD)

(Skibbe, et al. 2008), and premature degeneration causes sterility (Wilson and Zhang 2009). PCD of the tapetum releases stored materials that become the pollen coat (Li, et al. 2012). In *Arabidopsis*, two glutaredoxin genes, *roxy1* and *roxy2*, have roles in tapetal differentiation (Xing and Zachgo 2008). Gleutrodoxins defend against abiotic stress and some glutrodoxins are induced by the plant hormone auxin (Sharma, et al. 2013), which has been linked to miRNA signaling (Eckardt 2005). Some *fzt* stamens exhibited multiple defects in a single stamen. Similarly, the double mutant *roxy1 roxy2* differentially affected the abaxial and adaxial locules of the stamen. *roxy1 roxy2* adaxial lobes had defects in the cell formation of the tissue layers, while the abaxial lobes have defects in the PMC and the tapetum (Xing and Zachgo 2008). These two glutrodoxin genes are good leads to follow up with in maize because the *roxy1 roxy2* mutant has similarities to *fzt*, like different locule phenotypes in the same stamen, and tapetal degradation issues, and *roxy1* and *roxy2* are induced by auxin, connecting them to the miRNA regulatory pathway.

Starch accumulation was severely reduced in *fzt* compared to normal siblings, likely contributing to male-sterility. Pollen starch is synthesized from glucose and fructose in locular fluid and serves as an energy source for maturing pollen (Castro and Clement 2007) and as a terminal regulator of pollen development (Datta, et al. 2002). While several genes in the anther starch synthesis pathway have been studied, none have been connected to miRNA regulation. *starch-synthase* expression occurs in pollen grains in late stages of pollen development (Olmedilla, et al. 1991). Starch loading is normal in *fzt* normal siblings, despite half of the haploid pollen harboring the *fzt* allele, suggesting the starch accumulation defect is due to a defect in somatic tissue sugar partitioning. A male-sterile sorghum line, IS1112C, makes normal pollen, but fails to accumulate starch (Datta, et al. 2001). IS1112C is caused by somatic tissue

defects, but the gene has not been identified (Datta, et al. 2001). The *carbon starved anther* (*csa*) is a rice anther sugar-partitioning mutant that is male-sterile; *csa* encodes a MYB transcription factor (Zhang, et al. 2010). Some MYBs are regulated by miRNAs, but *csa* has not been linked to miRNA regulation. An informative next step would be to observe a difference between sugar partitioning into the locular fluid of normal siblings and *fzt*. If the problem is sugar partitioning, then I would expect *fzt* to have reduced sugar content, resulting in pollen that is unable to synthesize and accumulate starch. While starch synthesis in pollen has yet to be connected to miRNA pathways, future work in *fzt* stamen development would benefit from investigating the expression of sugar transporters and genes involved in sugar partitioning, such as the *csa* ortholog, in *fzt* stamens to see if there is a difference from normal sibling expression.

All *fzt* stamens that develop to late stages failed to dehisce. Mutants that only affect dehiscence have been reported in *Arabidopsis*. GTR1 encodes a hormone transporter in *Arabidopsis*, loss-of-function *gtr1* mutants have shorter stamens that wildtype and never dehisce (Saito, et al. 2015). GTR1 transports jasmonic acid and gibberellic acid, both hormones are connected to miRNA regulated pathways (Tabata, et al. 2010; Achard, et al. 2004). In *delayed-dehiscence1* (*dde1*) and *delayed-dehiscence2 (dde2)*, pollen developed normally, but the delay in dehiscence resulted in degraded pollen when the stomium did break (Sanders, et al. 1999). *dde1* and *dde2* are both encode jasmonic acid biosynthesis enzymes (Sanders, et al. 2000). *ARF6* and *ARF8* promote jasmonic acid biosynthesis (Tabata, et al. 2010), and application of jasmonic acid to *dde1* anthers restored dehiscence (Sanders, et al. 2000). One possibility is that the failure of *fzt* stamens to dehisce is due to reduced hormone levels or signaling. One simple experiment to test the hypothesis that failure to dehisce is due to reduced hormone levels is to apply exogenous gibberellic acid or jasmonic acid to stamens to see if part of the non-dehiscence phenotype is

rescued. If exogenous application of these hormones restores dehiscence then future work would heavily focus on genes involved in biosynthesis and transport of jasmonic acid and gibberellic acid.

Possible MiRNAs to Underlie the *fzt* Stamen Defects

Work in multiple plant species demonstrates miRNAs are required for normal stamen development. miRNA networks involving miR159-MYB33/65, miR167-ARF6/8, and miR319-TCP4 in *Arabidopsis* regulate floral development (Rubio-Somoza and Weigel 2013). MiR159, miR167, and miR319 all have reduced accumulation in *fzt*, and some of their targets are overexpressed in *fzt* (Thompson, et al. 2014). MiR159 overexpression results in stunted stamens that failed to dehisce in *Arabidopsis* (Achard, et al. 2004; Palatnik, et al. 2007). ARFs regulate stamen development in *Arabidopsis* (*arf6 arf8*), mutants have underdeveloped stamens (Rubio-Somoza and Weigel 2013) that fail to dehisce (Nag and Jack, 2010). Mutants that have loss of function in miR319 in *Arabidopsis* develop stunted and misshapen stamens that lack the typical four lobed structure (Nag and Jack, 2010). Overexpression of miR319, which targets TCPs, results in stunted stamens that do not dehisce (Palatnik, et al. 2007). All of these phenotypes resemble *fzt* stamen phenotypes, and documented misregulation of miRNAs and genes in *fzt* suggest these miRNA pathways are excellent leads for further investigation.

miR159 Targets GAMYBs Essential for Male Fertility

A likely cause of part of the *fzt* phenotype is overexpression of the miR159 regulated GAMYBs. GAMYBs have been studied in barley, rice and *Arabidopsis*.

The *Arabidopsis* GAMYB clade is composed of *AtMyb33*, *AtMyb65*, *AtMyb81*, *AtMyb97*, *AtMyb101*, *AtMyb104*, and *AtMyb120*. *AtMyb104* and *AtMyb81* have not been studied. *AtMyb97*, *AtMyb101* and *AtMyb120* are expressed in mature pollen and essential for pollen tube elongation

(Liang, et al. 2013). *AtMyb33* and *AtMyb65* are confirmed targets of miR159 (Allen, et al. 2007), and they both are most closely related to the GAMYB in barley and rice (Gocal, et al. 2001). The *AtMyb33 AtMyb65* double mutant makes smaller stamens than wildtype, and is male sterile (Miller and Gubler 2005). The *AtMyb33 AtMyb65* double mutant has two locule phenotypes that can occur in the same stamen; locules that develop normally, or locules that have tapetal hypertrophy before meiosis and have aborted pollen (Miller and Gubler 2005). Maize has a large GAMYB clade that groups closely with the *Arabidopsis* GAMYB clade, suggesting these genes may have conserved functions.

Rice has a single GAMYB gene, *OsGAMyb*. *OsGAMyb* is required to maintain the tapetum, underexpression of *OsGAMyb* causes male sterility (Kaneko, et al. 2004). Interestingly, the GAMYB mutant in rice had varying floral phenotypes in each plant, with the most severe forming abnormal stamen structures (Kaneko, et al. 2004). Similarly, *fzt* had varying stamen phenotypes per each plant. The *OsGAMyb* mutant phenotype is similar to *AtMyb33 AtMyb65*, suggesting that the grass GAMYB has a similar roles some of the *Arabidopsis* GAMYBs

fzt has reduced miRNA accumulation, resulting in overexpression of miRNA-targeted genes. In barley, overexpression of GAMYB (*HvGAMybOx*) has a similar phenotype to *fzt*: lack of dehiscence and a decrease in anther size (Murray, et al. 2003). In *HvGAMybOx* plants, the pollen grains accumulate starch similar to normal siblings, but manual opening of the anthers only resulted in successful pollination at less than 1% (Murray, et al. 2003). A major difference between *fzt* and *HvGAMybOx* was that *HvGAMybOx* pollen loaded with starch (Murray, et al. 2003). Despite the difference in starch loading, the similar phenotype between *fzt* and the *HvGAMybOx* line suggest this is a good area for further investigation. *ZmMyb74* and *ZmMyb138*

are two GAMYBs that have similar sequences to *HvGAMyb*. RNA sequencing on *fzt* demonstrated *ZmMyb138* is overexpressed (Thompson, et al. 2014).

Goal of the *ZmMyb138 ZmMyb74* Double Mutant

A major goal of the Thompson lab is to link specific *fzt* phenotypes to misregulation of specific miRNA target genes. *fzt* has decreased accumulation of at least some miRNAs, leading to overexpression of some miRNA targets. Misregulation of *ZmMyb74* and *ZmMyb138* are excellent candidates to underlie at least a portion of the male sterility in *fzt* plants. Definitively establishing this link requires functional analysis with mutants in these genes. A *ZmMyb138* null allele does not have any obvious phenotype, suggesting that one or more other genes function redundantly with *ZmMyb138*. I have isolated the *ZmMyb74 ZmMyb138* double mutant and these plants will be ready for analysis this summer.

In addition to analyzing single and double mutants in *ZmMyb74* and *ZmMyb138*, I will also cross the single and double mutant to *fzt*. If overexpression of these genes contributes to the *fzt* phenotype, I hypothesize that reduced expression of *ZmMyb74*/*ZmMyb138* will suppress a subset of *fzt* phenotypes. I have crossed single mutants in *ZmMyb74* and *ZmMyb138* to *fzt* and are in the process of identifying double mutants. I have also initiated crosses to generate a *ZmMyb74*; *ZmMyb138*; *fzt* triple mutant. Due to the long generation time of maize, analysis of the triple mutant is outside the scope of this thesis.

Conclusions and Future Work

My research demonstrates that miRNAs are needed for male development and I have developed tools to investigate miR159-regulated GAMYBs contributing to the *fzt* phenotype. This work has provided novel insights into the role of miRNAs in stamens, suggesting that miRNAs regulate pathways involved in maintenance of the tapetum, starch accumulation, and

degradation of the septum. A likely cause of part of the *fzt* male phenotype is *ZmMyb74* and *ZmMyb138*. I hypothesize that the double mutant will have defects in stamen and pollen development. A definitive link between misregulation of the GAMYBs and *fzt* male sterility defects can be established by crossing the *ZmMyb74-mum1 ZmMyb138-mum1* double mutant into *fzt*; these crosses are in progress. Reducing *ZmMyb74* and *ZmMyb138* expression in *fzt* will relieve any phenotypes caused from overexpression of these two MYBs, and rescuing part of the male-sterile phenotype. If *ZmMyb74-mum1* and *ZmMyb138-mum1* in *fzt* does suppress part of the male-sterile phenotype, then it will demonstrate that these genes were responsible for part of the phenotype.

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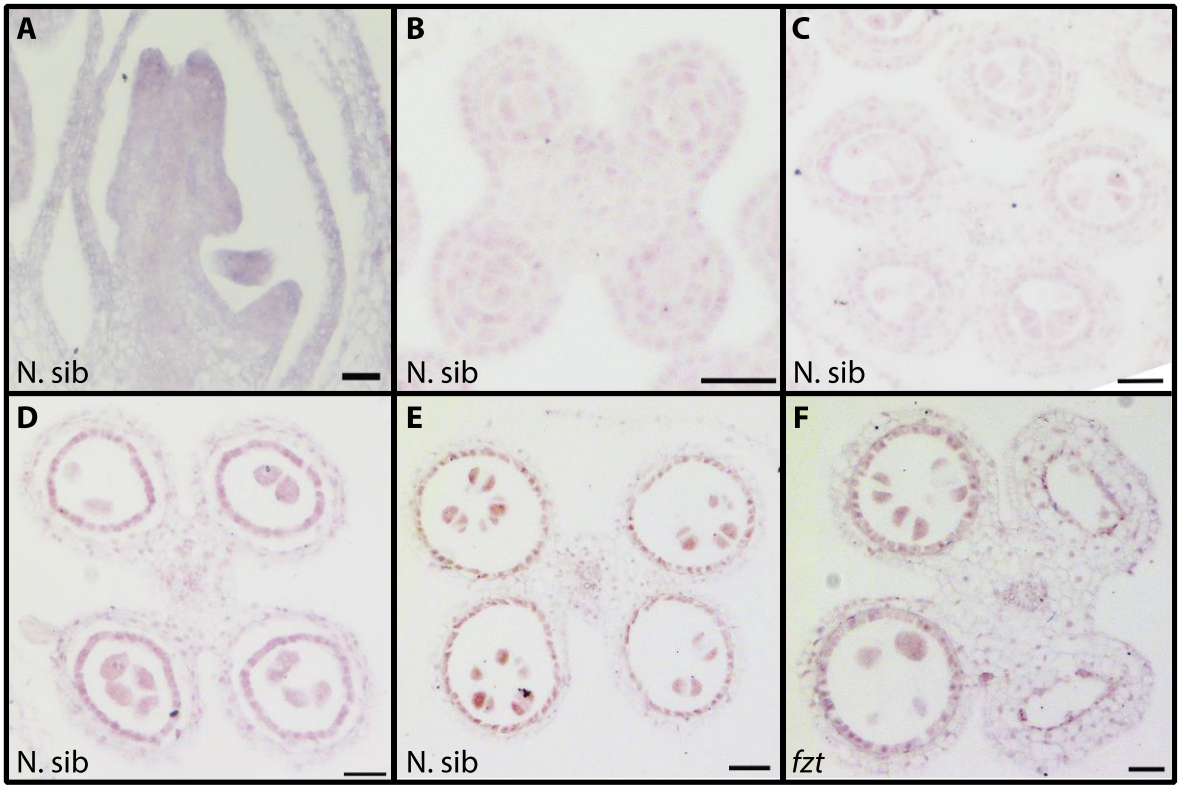
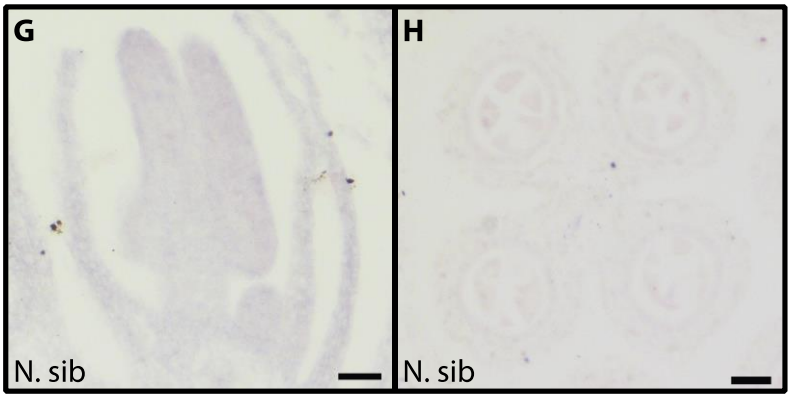
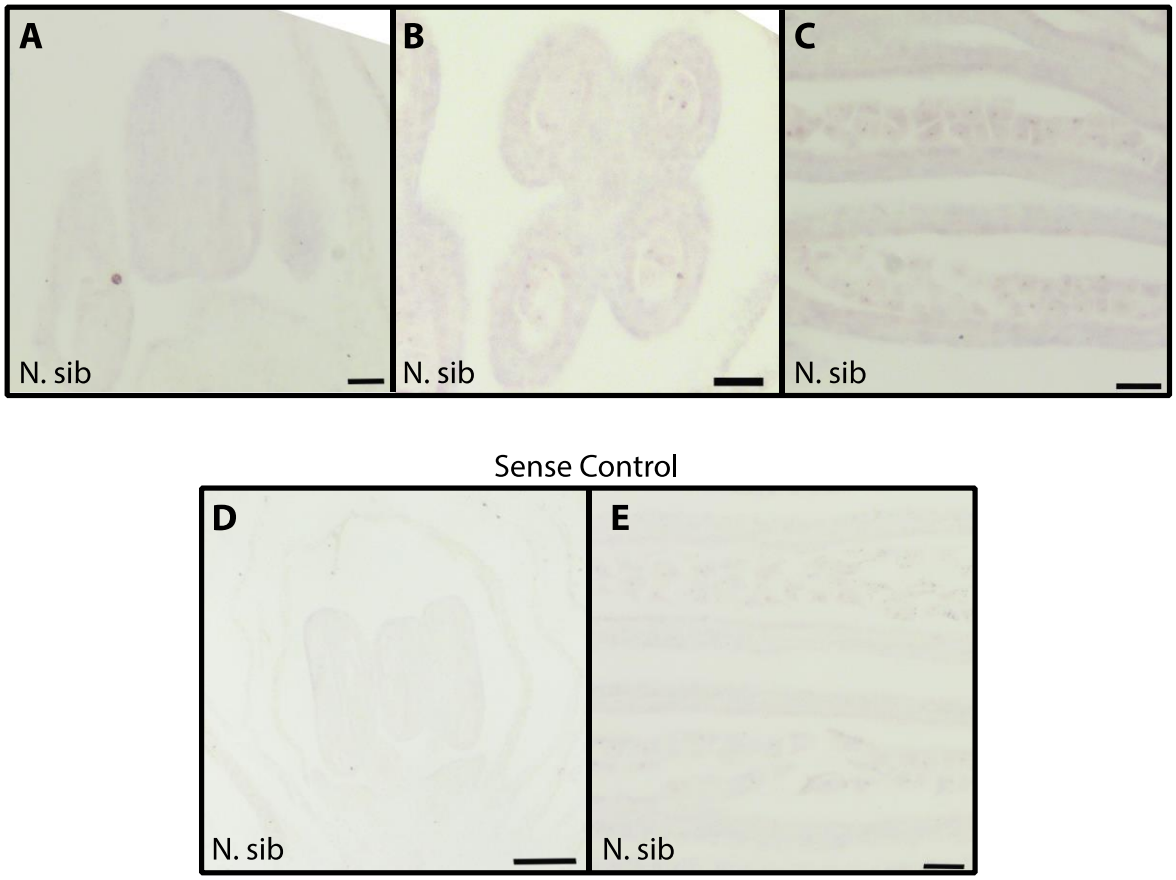
APPENDIX A: RNA *in situ* hybridization



Sense control



RNA *in situ* hybridization of *ZmMYB74*. Expression of *ZmMYB74*. Antisense *ZmMyb74* probe A-F. A) *ZmMyb74* is expressed in recently initiated stamens. B) *ZmMyb74* is expressed in the secondary parietal layer in stage 4 stamens. C) *ZmMyb74* is expressed in the tapetum and the PMC in stage 5 stamens. D) Strong *ZmMyb74* expression is seen in the tapetum and meiocytes. E) Expression of *ZmMyb74* is still seen in the tapetum and dyads of stage 7 stamens. F) *ZmMyb74* expression is seen in the tapetum and dyads of *fzt* normal locules, as well as in the degenerating tapetum. G and H) Sense controls for tissues. Scale bars are 50μm.



RNA *in situ* hybridization of *ZmMyb138*. Expression of *ZmMyb138* in stamens. Antisense *ZmMyb138* probe A-C. A) *ZmMyb138* is expressed in recently initiated stamens. B) Transverse section of stamen. *ZmMyb138* is expressed in all tissue layers of the stamen as well as the PMC. C) Longitudinal section of stamens. *ZmMyb138* is expressed in all tissue layers. D and E) Sense control for tissue layers. Scale bars are 50μm.

APPENDIX B: Gene sequences

ZmMyb74

tatatatatatagttcaggagctttatcagcttgttattactctggaacaaggttttaaataaccggttataaaggtcgatatatcagccttttttag
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upstream of *ZmMyb74*
intron
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Mu1016361
Mu054C.R
Zm028054.1F/R PROBE
Zm028054.2F/R PROBE
Mu054A.R
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ZmMyb138

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introns
EXONS
Mu1056170
CjMu2R
CjMu1/2F
CjMu1R
MYB138CDS1F/R Probe
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