

Control of maize development by microRNA and auxin regulated pathways

by

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Plant architecture and inflorescence architecture, in particular, are major determinates of yield. Plant architecture is dependent upon the activity of meristems. Meristems are vital to plant development because they not only maintain groups of undifferentiated cells, but they also produce cells that differentiate to give rise to new organs. This process is called organogenesis. The pattern and timing of organogenesis is a major contributor to plant architecture. The plant hormone auxin plays a major role in determining organogenesis. Auxin regulates position and number of primordia that form on the flanks of a meristem. Certain transcriptions factors have been found to effect leaf morphology such as TCP transcriptions factors. To better understand the genetic pathways that regulate inflorescence architecture I characterized single, double, and triple mutants from *Zea mays*.



Control of maize development by microRNA and auxin regulated pathways

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## **CHAPTER 1: Introduction**

### **Significance of developmental research on flowering grass species, specifically, Maize**

Grass species are essential domesticated crops worldwide. Corn, rice, barley, and wheat, part of the Poaceae family of flowering grasses, produce seeds consumed as food for humans and livestock. As the human population expands so does the need for improved crops (Yang C. et al 2016). Studying the development of seed-bearing grasses may lead to the improvement of crop plants to meet worldwide food demand. Plant and inflorescence architecture are major determinates of yield. Understanding the genes and pathways involved in the inflorescence architecture of flowering grass species can help improve growth, and potentially yield.

*Zea mays* (maize) is an excellent model to understand molecular mechanisms that regulate development involved in the grasses (Colosanti J, 2009). The information we uncover in maize may be translated into other systems to improve agricultural production.

### **Maize inflorescence development**

Grasses from the Poaceae family develop inflorescences that produce flowers. Maize is a monoecious plant, meaning it produces unisexual (male and female) flowers on separate inflorescences (Bartlett *et al.*, 2015; Li and Liu, 2017; Mao Y et al, 2017). The tassel produces male flowers and is located in the apex of the plant. The tassel consists of a main spike that is surrounded by long branches several branches at the base. Each branch contains spikelets and flowers (Figure 1). The ear produces female flowers and is located in the axils of the leaves (Figure 1). The ear contains many rows of kernels and two lateral branches (Bortini E, 2007).

Flowers (called florets in grasses) in both the tassel and ear are contained in spikelets (Bartlett *et al.*, 2015; Bortini E, 2007). Both the tassel and ear produce paired spikelets. In the tassel, each spikelet contains an upper and lower floret with three stamens, a lemma, palea, and lodicules (Figure 1) (Smoczynska and Szweykowska-Kulinska, 2016; Bartlett *et al.*, 2015; Bonnett O.T. 1954). In the ear, the lower floret aborts, resulting in a spikelet with a single floret. (Smoczynska and Szweykowska-Kulinska, 2016; Bartlett *et al.*, 2015; Bonnett O.T. 1954).

#### *Inflorescence development is dependent upon meristem activity*

The formation of the tassel and ear are dependent upon the activity of meristems (Vollbrecht, E 2005; Bortiri, E 2007; Sun, W. *et al*, 2017). Meristems are groups of undifferentiated cells in the plant that initiate the formation of new plant organs (Fletcher, 2018; Zhang, Z 2017). Meristems can be determinate or indeterminate (Zhang, Z 2017; Vollbrecht, E 2005; Bortiri, E 2007). Determinate meristems are consumed after making a specific number of organs or meristems, whereas indeterminate meristems make an indefinite number of organs or meristems (Zhang, Z 2017; Bortiri, E *et al* 2007). The regulation between determinate and indeterminate meristems is necessary for normal patterning of maize organs. Indeterminacy in meristems leads to abnormal development including characteristics such as increased proliferation and increased branching (Bommert, P *et al* 2017; S. J Park, Y 2014, Thompson, B 2014).

The shoot apical meristem (SAM) produces all above ground organs such as the leaves, stem, and axillary meristems. The SAM is an indeterminant meristem because it produces an unlimited number of leaves during its vegetative stage (Fletcher, J. 2018; Zhang, Z 2017; Bortini E. *et al* 2007). Later during development, the shoot apical meristem and axillary meristems transition from a vegetative state into a reproductive state and the SAM transitions to the inflorescence meristem (IM). The IM is also indeterminate but produces determinate meristems.

The tassel is the product of the apical IM and the ear is the product of an axillary meristem (S.J Park, Y 2014; Thompson BE. 2014). The IM contains stem cells that initiate additional meristems that ultimately give rise to the reproductive structures needed for reproductive development. In both the tassel and the ear, the IM initiates spikelet pair meristems (SPM) (Figure 2). In the tassel the IM also gives rise to branch meristems (BM) which forms the branches at the base of the tassel; in the ear the IM does not produce BMs. SPMs give rise to two spikelet meristems (SM) leading to the formation of two floral meristems (FM) (Figure 2) (Thompson BE et al. 2014). These two floral meristems give rise to the upper floret and lower floret. Each floret contains a lemma, palea, and lodicule that consists of the reproductive organs, stamens and carpels (Figure 2) (McSteen, et al. 2000).

### **Mutant analysis gives insight into normal gene functions during inflorescence development**

MicroRNAs (miRNAs) and their target mRNAs are key regulators of inflorescence development (Zhao, Yunde, 2010). Many key genes that function in inflorescence development are a part of miRNA dependent pathways. The isolation of mutants to study the function of the genes and miRNA targets involved in these developmental pathways is a common technique used to study inflorescence development in plants. Seeing how a gene specific mutant affects development allows us to tell what the function of a that gene is in normal development. miRNAs regulate many developmental processes and target mRNAs that control developmental processes such as the auxin signaling pathway, as well as plant specific transcription factors such as TCPs.

The auxin biosynthesis, transport, and signaling pathways have been extensively studied and many of the key players identified. The auxin signaling pathway is important in inflorescence development because it plays a prominent role in the initiation of primordia. The plant hormone

auxin modulates lateral plant growth and development within those developmental processes (Zhao, Yunde 2010). In the absence of auxin, ARFs are complexed with AUX-IAAs proteins and inhibit transcription of target genes. In the presence of auxin, auxin binds to TIR1 and in the SCF-TIR1 complex to form a coreceptor. Aux/IAA repressors then enter the pathway, bind to the SCF-TIR1 complex, and are polyubiquitinated which leads to the degradation of Aux/IAA. This leaves the ARF activators unbound and leads to the subsequent activation of auxin-response genes leading to transcription. The most commonly researched auxin signaling pathway is the TIR1 pathway which controls transcriptional responses to auxin. During the auxin signaling pathway, AUXIN RESPONSE FACTORS (ARFs) bind to auxin-response elements that are in the promoters of auxin-response genes. Aux/IAA transcripts are rapidly and strongly induced by auxin treatment in peas, soybeans, and other plants. In order to have auxin-response gene activation there needs to be a sufficient amount of auxin.

Auxin regulates cell division, cell expansion, cell differentiation, lateral root formation, flowering, and trophic responses (Mashiguchi, Kiyoshi et al. 2011). Mutants in genes that control auxin signaling, as well as other plant hormone growth regulators have been characterized

Maize mutants exist in genes required for both auxin biosynthesis as well as transport and signaling, which we can use to understand the roles of auxin in maize inflorescence development. For example, *sparse inflorescence 1 (spi1)* is an auxin biosynthesis mutant that has defects during vegetative and reproductive development such as fewer branches and spikelets on tassels, smaller ears, and low yield suggesting a defect in branch meristem (BM), SPM, SM, FM, and floral organ initiation. *Vanishing tassel 2 (vt2)* is a co-ortholog of TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1) which functions in the auxin biosynthesis pathway to convert tryptophan to indole-3-pyruvic acid and has a similar phenotype to *spi1* with severe barren

inflorescences without branches or spikelets (Phillips, K.A. 2011). Other examples, such as the characterization of the *barren inflorescence1 (ba1)* mutant led to the discovery of genes required for lateral meristem initiation in inflorescence development (Barazesh S. 2008, Gallavotti A, 2010). These auxin mutants show that auxin biosynthesis is required for lateral meristem initiation in maize inflorescences.

#### *fuzzy tassel mutation affects miRNA biogenesis*

The maize *fuzzy tassel (fzt)* mutant has a lack of meristem determinacy and severe inflorescence and vegetative defects. (Thompson BE et al, 2014) (Figure 5). Positional cloning showed the *fzt* phenotype is caused by a mutation in the *dicer-like1 (dcl1)* gene. *dcl1* encodes an enzyme required for microRNA (miRNA) biogenesis (Figure 6). miRNAs are small, non-coding RNAs that function in RNA silencing and posttranscriptional regulation of gene expression. Many miRNAs have already been characterized and have known roles that contribute to maize inflorescence development (Djami-Tchatchou, Arnaud T et al., 2017). A common mutant used to study the effects of miRNA targets on maize inflorescence development is *tasselseed4 (Ts4)* which targets *indeterminate spikelet1 (ids1)* and *Tasselseed6 (Ts6)* (G. Chuck, 2007). Maize *tasselseed4* encodes a miRNA that targets a gene required for spikelet meristem determinacy (G. Chuck, 2007). *Ts4* mutants have inflorescence defects such as irregular branching within the inflorescence (G. Chuck, 2007). Some of the roles of these miRNAs include sex determination, stamen development, and meristem maintenance and initiation leading to establishment of the reproductive phase and organ patterning (Banks, J. 2008). Two miRNAs that are reduced in *fzt* are miR167 and miR319 (Thompson BE et al, 2014) (Figure 7).

## miR319 and miR167 regulate development in other plants

When we look at miR167, miR167a-d-5p specifically, it is dramatically reduced in *fzt* mutants. miR167a-d-5p specifically, is a miRNA that targets mRNAs that encode for auxin response factors (ARFs). Sequencing was done to analyze small RNA populations from 14-day old seedlings and tassel primordia of *fzt* mutants and normal siblings. This data showed many differentially expressed targets were increased in *fzt* mutants. Although many miRNAs were reduced, they were not all reduced at the same levels, some were more dramatically reduced such as miR167a-d-5p which was reduced 20-30 fold in *fzt* mutants. From analyzing the RNA sequencing data, it was found that miR167 targets two and these are statistically increased in *fzt* mutant tassels (Thompson, B. 2014). Since miR167 targets ARFs and they are statistically increased in *fzt* mutants one possibility is that the increased branching and primordia seen in *fzt* is an effect of the increased auxin signaling.

Seven miR319-regulated TCPs are expressed in tassel primordia; however, these mRNAs are not significantly upregulated in *fzt* tassel primordia. To determine the function of miR319-regulated TCPs during development, we obtained transposon insertions in six of seven miR319-targeted TCP genes from the uniform mu project (Chapter 2) (Andorf CM *et al*, 2015).

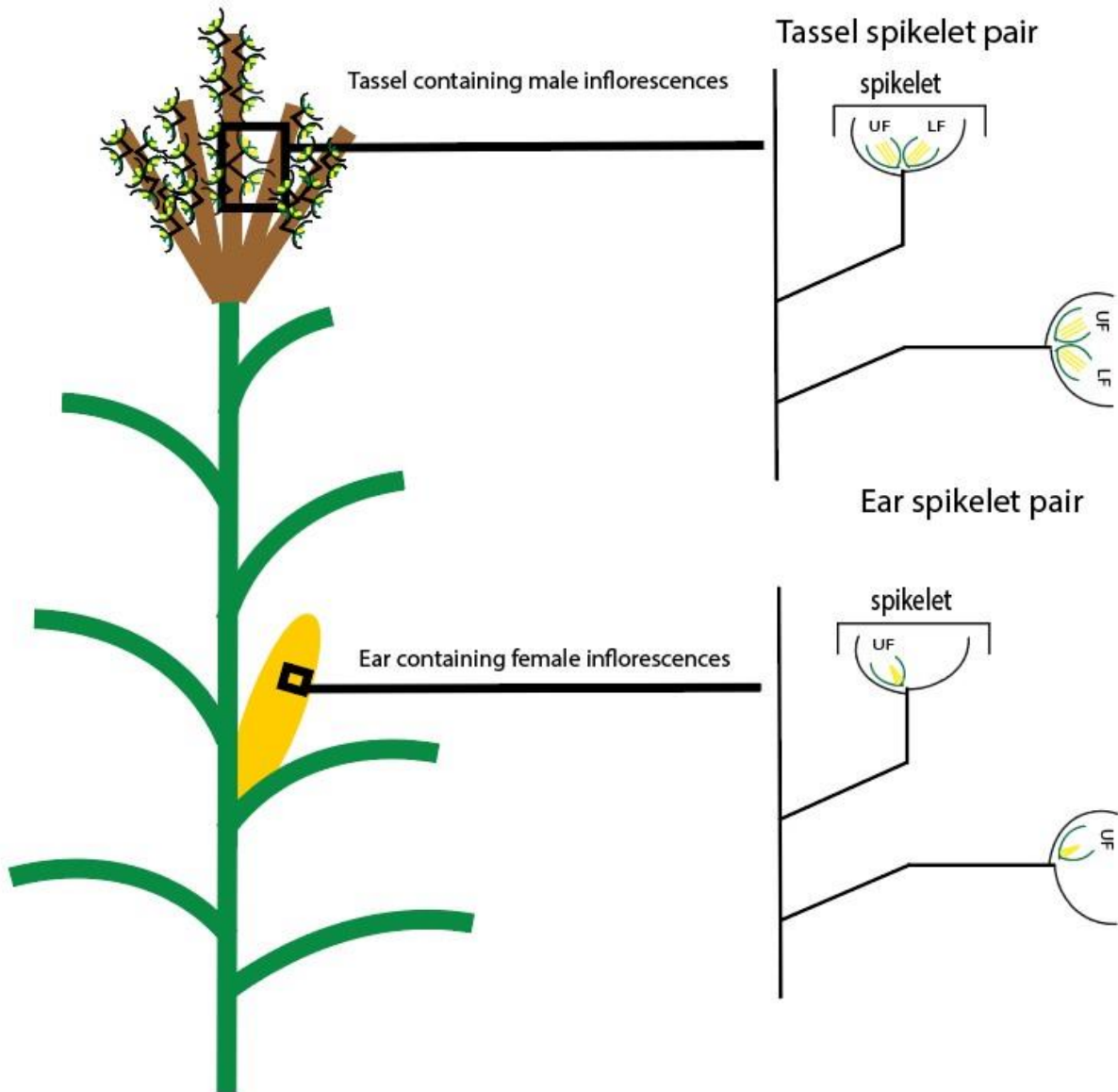
In multiple plant species, TCP genes have key roles in vegetative and reproductive development. In general, plants with reduced TCP levels have increased proliferation resulting in notched leaf margins as well as wavy leaves (Palatnik *et al.*, 2003). In general, overexpression of miR319 or knock-downs of multiple *tcps* results in abnormal curvature and excess leaf growth. (Efroni *et al.*, 2008; Nag *et al.*, 2009; Palatnik *et al.*, 2003). For example, the Arabidopsis, *jaw-D* mutant overexpresses miR319, resulting in reduced *tcp* levels, and has jagged and wavy leaves. *Antirrhinum* (snapdragon) *cin* mutant contains a loss-of-function mutation in a miR319-regulated

TCP transcription factor and have a similar phenotype as well as larger leaves with an undulating edge due to excessive growth in marginal regions (Crawford BC, 2004).

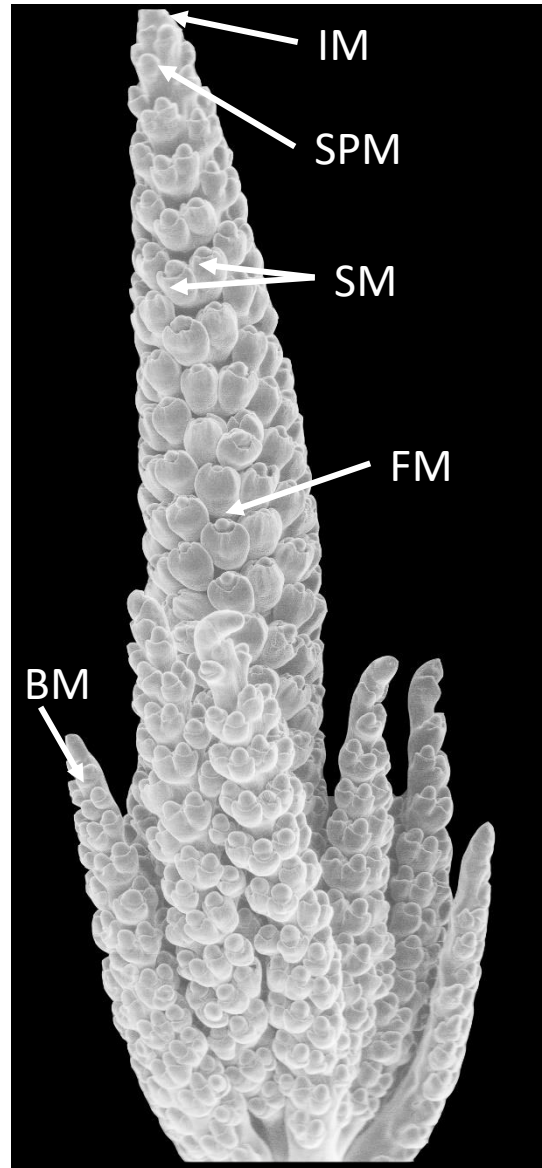
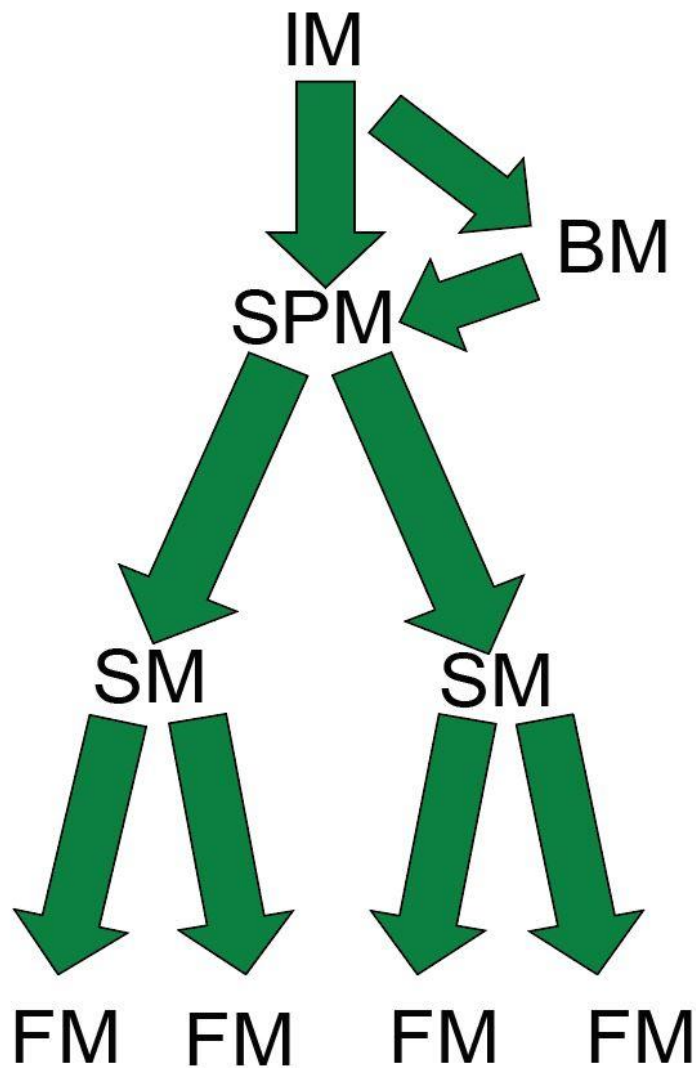
In contrast, plants with increased *tcp* levels have smaller leaf margins, narrower and shorter petals, as well as impaired stamen and anther development (Crawford et al., 2004; Nag et al., 2009). For example, in *Solanum lycopersicum* (tomato), the classical, partially dominant, *Lanceolate (La)* mutant harbors a mutation in the miR319-binding site, resulting in overexpression of *tcp*. In *La* mutants, the normally large compound leaves are converted into small simple leaves (Ori et al., 2007). The regulation network of miRNAs using miR319 regulated TCPs is very complex but necessary for proper vegetative and reproductive development.

I investigated the function of miR319-targeted TCPs to test my hypothesis that TCP transcription factors are necessary to maintain the balance of differentiation and proliferation in maize development.





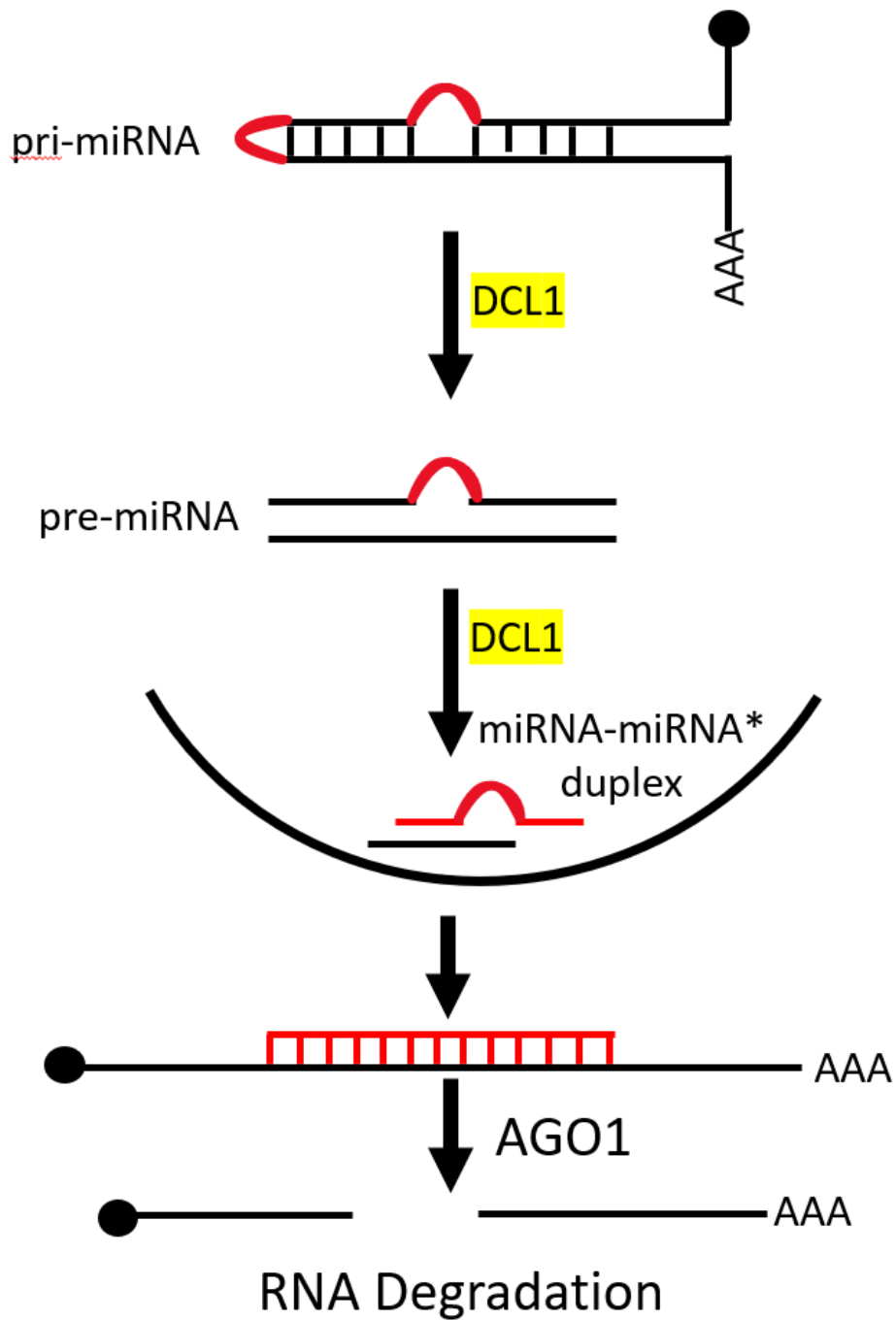
**Figure 1:** The tassel is located at the apex of the maize plant. The ear is located in the axil of the leaf. The tassel and ear produce paired spikelets. In the tassel, each spikelet produces an upper and lower floret. In the ear, the lower floret aborts, resulting in a spikelet with a single.



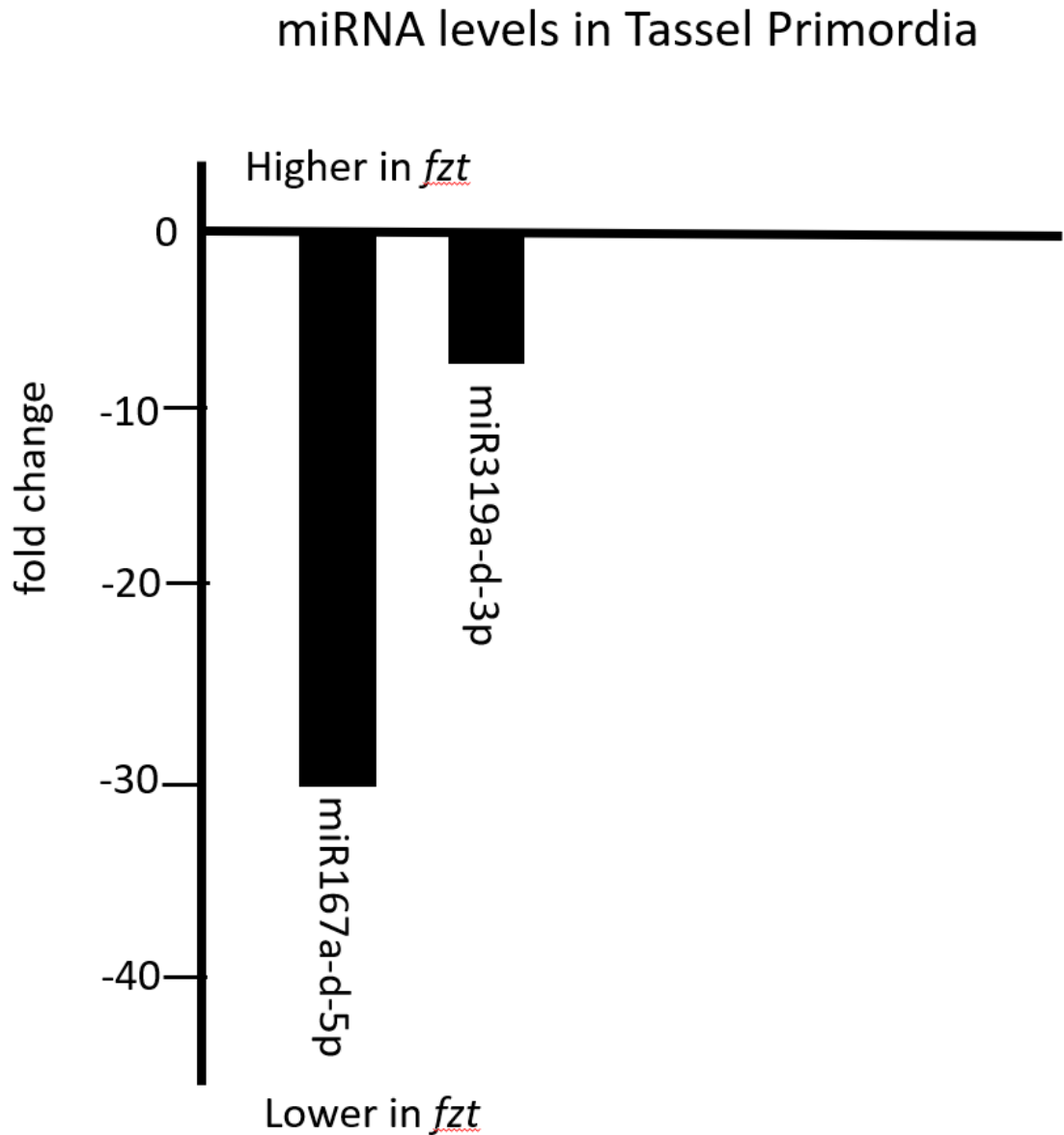
**Figure 2:** The inflorescence meristem (IM) contains stem cells that initiate additional meristems. In both the tassel and the ear, the IM initiates spikelet pair meristems (SPM). In the tassel the IM also gives rise to branch meristems (BM) which form all the branches on the tassel; the ears IM does not produce any BMs. SPMs give rise to two spikelet meristems (SM) leading to the formation of two floral meristems (FM).



**Figure 3: *fzt* has severe vegetative and reproductive defects.** (Left) Scanning electron micrograph of *fzt* tassel primordia showing indeterminate meristems and increased branching (Right) *fzt* plant at maturity (8 weeks). *fzt* plants are shorter than normal and have shorter, narrower leaves.



**Figure 4: *dcl1* is required for miRNA biogenesis.** *fzt* has a mutation in an enzyme named *dicer-like1* (*dcl1*) which is required for miRNA biogenesis. The miRNA biogenesis pathway above highlights where *dcl1* acts in this process.



**Figure 5: miRNAs are significantly reduced in *fzt*.** Both miR319, which targets mRNAs that encode TCP transcription factors, and miR167, which targets mRNAs that encode for auxin response factors, are reduced in *fzt* mutants. Data from Thompson et al, 2014.

## CHAPTER 2: Characterization of miR319 TCPs in Development

### Introduction:

TCPs are plant-specific transcription factors and have known roles in leaf and floral development in maize and other plant species (Parapunova V, 2014). TCP targets maintain a balance between proliferation and differentiation of miR319 to enable proper leaf development (Koyama, T. 2017; Schommer *et al*, 2012) (Fig 6). TCP transcription factors are named after the proteins where they were first identified: *Teosinte Branched 1 (tb1)* from maize, *Cyclodia (cyc)* from *Antirrhinum*, and *Proliferating Cell Factors 1 and 2 (pcf1/2)* from rice (Martin-Trillo, 2010) The TCP domain is a 59-amino acid basic helix-loop-helix motif that binds directly binds DNA and regulates transcription of genes that control the cell cycle regulators and interacts with cell cycle proteins (Martin-Trillo and Cubas, 2010). There are two classes of TCPs distinguished by a 4 amino acid change within the TCP domain, Class I and Class II (Cubas et al. 1999, Martin-Trillo and Cubas, 2010).

A subset of class II TCPs are regulated by miR319 in maize and other plants. Maize contains seven TCP genes with a putative miR319 binding site (Martin-Trillo and Cubas, 2010; Novitzky, K. 2016) (Figure 7).

In this study, I sought to characterize the phenotype of maize TCP transcription factors. A previous graduate student obtained lines with transposon insertions in six of the seven predicted miR319-targeted TCPs. (Novitzky, K., 2016). To this end, I examine single and double mutants to determine if any of these mutants affected maize development. In addition, I analyzed RNA from each of the mutant alleles to determine if any of these mutants are likely RNA null alleles.

## Methods

### *Plant Growth and Maintenance*

Seeds were germinated by soaking for 2 hours in milli-Q water on the shaker and then placed in the incubator on wet paper towels covered with Captan fungicide. They were incubated at 28 ° Celsius for ~4 days until their shoots emerged at which point, they were transplanted in flats in soil from the greenhouse. Plants were grown in a growth chamber (Percival Intellus Control System) at 26 ° Celsius, 80% humidity, and 12-hour light and watered once daily.

Plants grown for phenotypic analysis were grown in the greenhouse for eight weeks to maturity or in the field during Summer of 2017 at Central Crops Research Station (Clayton, NC). During this time, once the plants had grown to maturity around eight weeks, I measured plant height, total leaf number, leaf length, and leaf width to look for subtle phenotypic differences between tcp single mutants and normal siblings. I then performed a two-tailed t-test for each variable to see if any of the differences were statistically significant.

### *Genotyping*

A PCR-based assay following Taq Touchdown cycling conditions was used to screen for homozygous TCP mutants by using a gene-specific primer and TUSC (mu-specific primer) (Table 1). To detect the wild type gene, without the mu transposon insertion, forward and reverse gene-specific primers that flank the insertion site were used. A product from a PCR reaction with the forward and reverse primers indicates the presence of at least one wild type allele. A product from a PCR reaction with TUSC (mu-specific primer) indicates that individual

is either heterozygous or homozygous (Fig 8). No PCR product with the forward and reverse gene specific primers indicates the individual is homozygous for the mu insertion.

The first approach to characterizing *tcp* single mutant phenotype was to isolate single mutants for each of the six TCPs (*tcp33*, *tcp24*, *tcp38*, *tcp44*, *tcp43*, *tcp5*) from known segregating families. Heterozygotes or normal siblings were also isolated to be used as controls during the Fall of 2016.

#### *RNA Extraction*

Shoot apices from plants of the appropriate genotype were dissected and immediately flash frozen in liquid nitrogen and stored at -80°C. RNA was extracted using the miRNeasy kit by Qiagen following manufacturer's instructions. The optional DNase treatment step was completed as well. RNA was extracted from three individuals of each genotype.

#### *RNA Analysis*

RNA quality was verified by running 3µl a 1% agarose gel visualizing two discrete bands corresponding to ribosomal RNA. Reverse transcription was done using the Invitrogen Superscript III 1st Strand RT-PCR kit according to manufacturer's instructions using 1µg of RNA. A reaction lacking reverse transcriptase was included to control for genomic DNA contamination. After the cDNA was synthesized, cDNA corresponding to 1µg TCP RNA was used as a template for PCR using primers listed in Table 2. Primer specificity you can determine by aligning the cDNA sequences of the closest related TCPs (*tcp44* and *tcp5*, *tcp33* and *tcp24*) to one another using the DNASTAR's SeqMan and EditSeq from the Genomics Suite Version 12.0 to make sure the primer sequences had multiple mismatches between TCPs. I made cDNA from the *tcp33;tcp24;tcp38* individuals, the *tcp44;tcp43;tcp5* individuals, W22 normal siblings as controls.



## Results and Discussion

I first characterized the six miR319-regulated TCPs in maize by phenotype. Discovering which mutants are likely loss-of-function alleles would all me to focus my efforts when isolating mutants. I hypothesized that the single and double mutants would not have any phenotypic differences from the normal plants because TCPs are closely related and redundant but, the triple mutants would have subtle phenotypic characteristics directly related to the mutated *tcp* gene (Figure 9). I concluded that *tcp44* was a loss-of-function allele although I did not see any phenotypic abnormalities with the *tcp44* mutants. *tcp33* and *tcp24* did not show to be loss-of-function from the RTPCR so they were sent for sequencing. *tcp5*, *tcp38*, and *tcp43* were not loss-of-function but could have reduced levels of RNA but would need to be quantified with qPCR since RT-PCR is only qualitative.

### *Phenotypic Characterization of tcp mutants*

Looking at the phenotype of the individuals that are homozygous for the *tcp* genes that are loss-of-function is important because they may have abnormal characteristics compared to normal siblings. I screened through 16 families segregating these *tcp* genes to isolate single, double, and triple mutants (Table 3); *tcp* mutants should segregating 1:4 for each allele. To determine if *tcp* genes in maize have functions like *tcp* genes in other species, I examined the phenotype of *tcp33*, *tcp24*, *tcp38*, *tcp44*, *tcp43*, *tcp5*, *tcp43;tcp44*, *tcp43;5*, *tcp33;24*, *tcp33;tcp38*, *tcp38;tcp24*, *tcp33;tcp24;tcp38*, and *tcp44;tcp43;tcp5* field grown plants (Table 3).

During the summer 2017 field season, I planted 162 seeds from families segregating *tcp33*, *tcp24* and *tcp38*, and *tcp44*, *tcp43* and *tcp5*. I measured several traits from single, double, and triple mutants to detect any subtle defects in vegetative development. Specifically, I measured leaf width and length because TCPs are known to affect leaf development, as well as

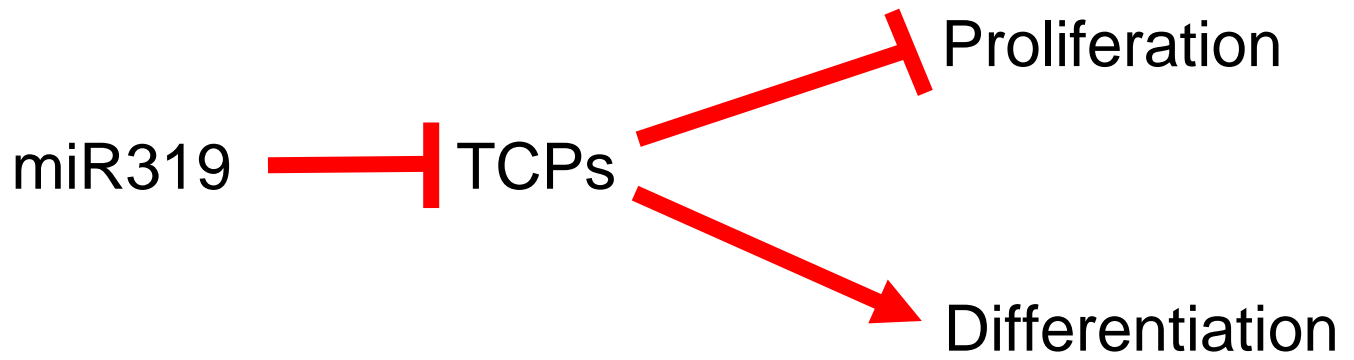
plant height and leaf number. From my research on TCP transcription factors I was unable to statistically conclude that leaf development in maize was affected by TCPs (Figure 10A). Looking at *tcp33* leaf length and width it looks like there may be a difference between the normal siblings and *tcp* mutants but according to the data (Figure 10A) I could not make any conclusions regarding this. I isolated one triple mutant (*tcp33;tcp24;tcp38*) in the greenhouse that had abnormal phyllotaxy, curling back of oracles, and severe ruffling of the leaves (Figure 11). These phenotypic characteristics were consistent with what has been seen in other *tcp* mutants in other plant including tomato and Arabidopsis but I was not able to confirm this phenotype with field grown plants. I isolated three triple mutants during my field season since the families we had available were only segregating one triple mutant to every 64 plants. I saw no difference between the double or triple mutants and the normal siblings according to the data collected (Figure 10A&B). I contributed to this research by isolating the first *tcp33;tcp24;tcp38* triple mutant in the greenhouse as well as one in the field that we were able to self- pollinate to generate seed stocks for future field seasons. Those seeds can be planted to look at triple mutants since 100% of those plants are *tcp33;tcp24;tcp38* individuals. Without the ability to look at more than three triple mutants, I cannot confidently conclude that TCPs effect maize development. It is possible that the severe phenotype seen in the greenhouse could have low penetrance and only show in some plants and not all. Looking at ~twelve more triple mutants would determine whether it was a real triple mutant phenotype. Now that we have a family that is homozygous for *tcp33*, *tcp24*, and *tcp38* as well as two families that are homozygous for *tcp5* and segregating *tcp43* and *tcp44* it will be possible to look at a greater number of triple mutants at one time.

To investigate the molecular nature of the allele miR319-regulated TCPs in maize development, I synthesized cDNA from plants homozygous for mu transposon insertions in six

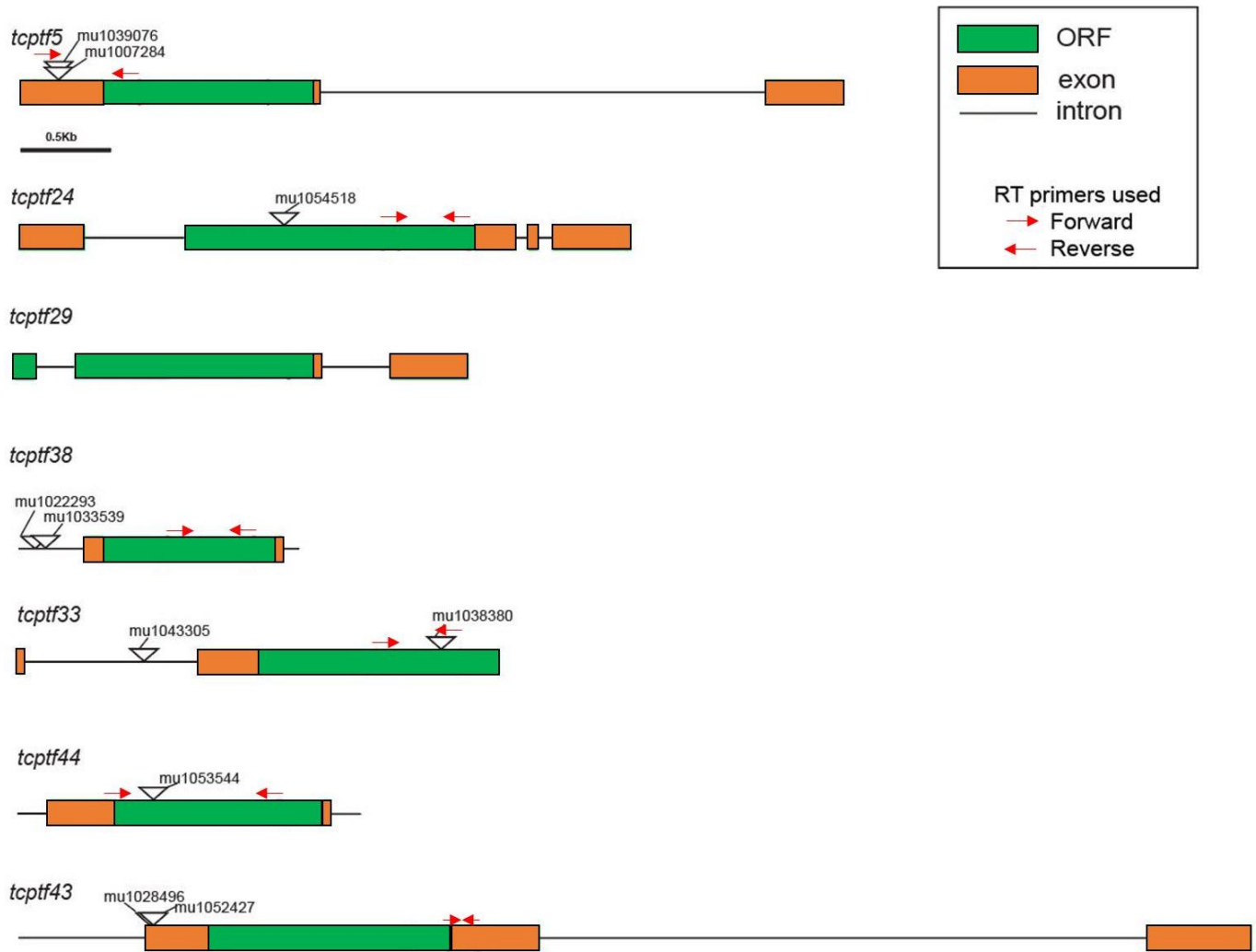
of the seven predicted miR319-targeted *tcp* genes. Of the seven miR319 regulated TCPs in maize, we have alleles in/near six of them. *tcptf24*, *tcptf33*, and *tcptf44* have insertions in exons corresponding to the open reading frame and *tcptf5*, *tcptf43*, and *tcptf33* have an insertion in the predicted 5' UTR and *tcptf38* has an insertion in the upstream promoter region (Figure 7). I hypothesized that *tcp44*, *tcp33*, and *tcp24* are strong loss-of-function alleles and likely RNA null alleles because of the location of the mu insertions in the open reading frame. The insertion sites of *tcp5* and *tcp43* are in the 5' UTR. Finally, *tcp38* has a transposon insertion in the upstream promoter region, which could potentially decrease transcription. The transposon insertions in the 5' UTR and upstream promoter region could be null alleles, could have reduced levels of RNA, or they may not have any effect.

The high similarity between the *tcp* genes made it difficult to design gene-specific primers that would specifically amplify the cDNA corresponding to a single *tcp*. I was able to design gene-specific primers for *tcp33*, *tcp38*, *tcp44*, *tcp43*, and *tcp5*, but not *tcp24*. Therefore, I synthesized cDNA using mutants homozygous for three *tcp* mutant alleles, (*tcp33; tcp24; tcp38*) and (*tcp44;tcp43;tcp5*). Using cDNA from the *tcp44;tcp43;tcp5* triple mutant and *tcp33; tcp24; tcp38* triple mutant PCR reactions were set up to verify cDNA synthesis; wildtype controls were also used. From performing RT-PCR I was not able to amplify cDNA corresponding to *tcp44* but I was able to amplify other *tcp*s, indicating that the *tcp44* allele is an RNA null (Figure 12B). I could not conclude that *tcp33* and *tcp24* are loss-of-function alleles because from my RT-PCR I was able to amplify cDNA corresponding to *tcp33* and *tcp24*. I concluded that *tcp43*, *tcp5*, and *tcp38* were not loss-of-function alleles based on the RT-PCR because I was able to amplify cDNA corresponding to those alleles as well. Since RT-PCR is not quantitative, these alleles could still have reduced levels. When I did my RT-PCR for *tcptf33* and *tcptf24*, bands we

present in the *tcp33;tcp24;tcp38* triple mutant individuals which shows that RNA was present showing that they are not null alleles (Figure 12B). This is hard to believe though because of the location of their mu insertion, so I submitted these samples for sequencing. A few possibilities are that *tcp33* could be making mRNA but it's spliced out or there could be multiple *tcp33* transcripts that the RT-PCR is picking up. The band in the *tcp24* RT-PCR could be a product from *tcp33* because they are closely related but this was not confirmed due to sequencing issues. The other TCP genes: *tcptf5*, *tcptf43*, and *tcptf38* all produced a PCR product with the RT-PCR for their correlated triple mutant individual (Figure 12B). This means they are not null alleles, although they could still form a non-functional protein. Since they are not null alleles and RT-PCR only shows presence or absence of RNA for that gene qRT-PCR is necessary to complete in the future to determine if these TCPs have decreased levels of RNA expression.



**Figure 6: TCPs regulate the balance between proliferation and differentiation in leaf development.** Analysis of TCP function in other species led to a model in which TCP promotes cell differentiation and represses proliferation.

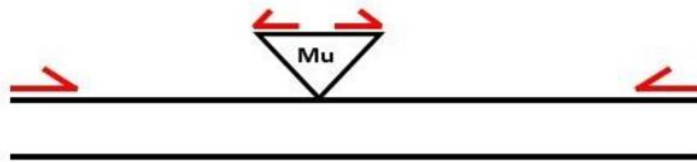


**Figure 7: Gene models for the miR319-regulated *tcp* genes in maize.** Mu transposon insertions are indicated by upside down triangles with the uniform mu identification number above them. Red arrows indicate primers used for RT-PCR. ORF=Open Reading Frame.

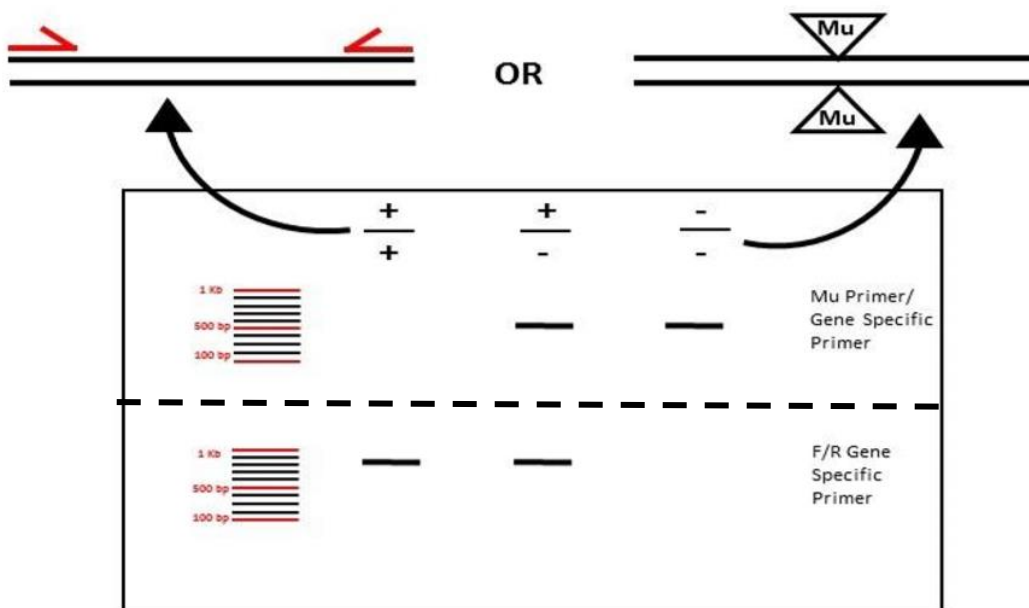
Sequence	Primers	TCP	Mu ID	Gene specific Length (bp)	TUSC/GSP length (bp)	Positive Control
TCGCTCGACTCGGACTCCATC	tcp33mu2F4	<i>tcptf33</i>	1038380	758 bp	~190 bp	W22 3370 #1,#9
CACCATCACGAGCATTTCAG	tcp33R3					
<b>CTCTCCCAGACCCCATGTT</b>	<b>08229F2</b>					
GAGGGGCATCAAAGAATCAA	tcp24muF1	<i>tcptf24</i> (also picks up <i>tcp33</i> )	1054518	~1.1 kb	~300 bp	W22 3448 #1
CAGTTCTACGACGTGCAGGA	tcp24CR1R					
<b>ACACCGCCATCCAGTTCTAC</b>	<b>tcp24muR3</b>					
TTCCAAAAGAACCAATCGG	02232F1	<i>tcptf38</i>	1022293	362 bp	~100 bp	W22 3368 #9
<b>TGAGCTCCGAATCTTCGTCT</b>	<b>02232R1</b>					
CATTCCTCCTCACGGCTATC	tcp44muF1	<i>tcptf44</i>	1053544	~1 kb	~700 bp	W22 3378 #9
<b>TACAGCCTCGGTTTCTCCAT</b>	<b>tcp44muR1</b>					
CGAGTGTCCAGTCCAGTGTG	tcp5CR2F	<i>tcptf5</i>	1007284	~600 bp	~150 bp	W22 3378 #9
AAGGTGTACACGACCAAGGG	tcp5CR2R					
<b>GCGTGAGGAGAGACGGTGATACA</b>	<b>00503F1</b>					
<b>AGTAGCCAGTGGGGTCTCT</b>	<b>tcp43muF1</b>	<i>tcptf43</i>	1052427	258 bp	594 bp	W22 3379 #10
CACTAGGACGGGCGTAATTC	tcp43muR1					

**Table 1:** Genotyping primer sequences and positive controls used. Primers sequences in bold were used in combination with the TUSC primer to detect individuals with the transposon insertion.

**1. Screen individuals for Mu transposon insertion:**



**2. Determine Genotype:**



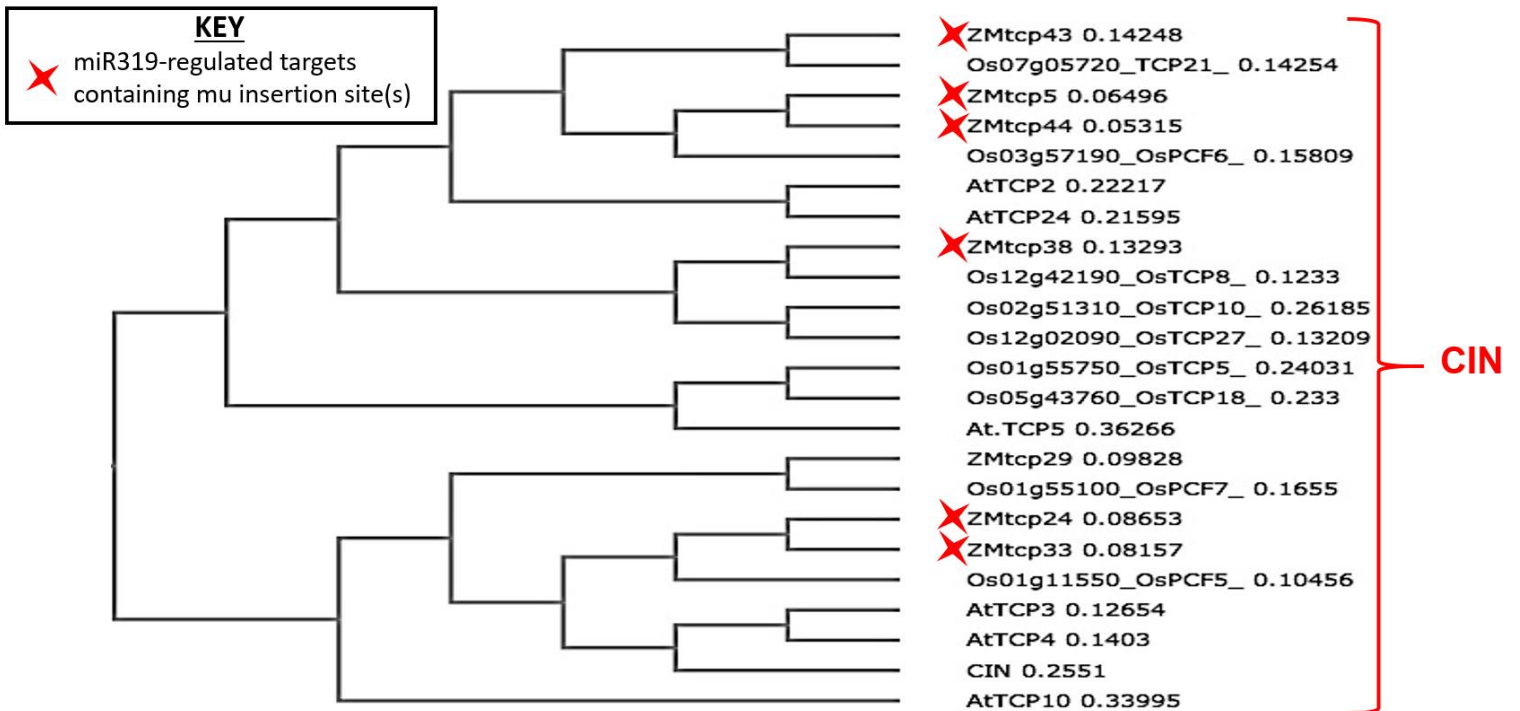
**Figure 8: Strategy to genotype families segregating mutator insertions.** Two PCR reactions are completed to determine whether the individual is homozygous or heterozygous for the mu insertion or homozygous for the wildtype allele. One reaction is using two gene-specific primers that flank the mu insertion and the other reaction uses a gene-specific primer and a mu specific primer.



Sequence	Primers	Gene	Mu ID	Gene specific Length (bp)
TCGCTCGACTCGGACTCCATC	mu2F4	<i>tcptf33</i>	1038380	493 bp
TAAGTCCAGTAACCAGCCCT	CR3R			
TACCAAAGCTACACGCCTGA	CR2F	<i>tcptf24</i>	1054518	565 bp
GTCTGTTTCCTCGGCTTCTC	CR2R			
CTCGACTGCTCCTTCGTCTT	CR1F	<i>tcptf38</i>	1022293	520 bp
GTTCTTTCTTGAAGCGGTG	CR1R			
CATTCCTCCTCACGGCTATC	muF1	<i>tcptf44</i>	1053544	995 bp
TACAGCCTCGGTTTCTCCAT	muR1			
CGAGTGTCAGTCCAGTGTG	CR2F	<i>tcptf5</i>	1007284	584 bp
AAGGTGTACACGACCAAGGG	CR2R			
GAAACTGGAAAGGCGTCAAA	CR2F	<i>tcptf43</i>	1052427	174 bp
CTCGTCGTCCTTACTCTGGC	CR2R			

**Table 2:** Primer sequences used for RT-PCR.

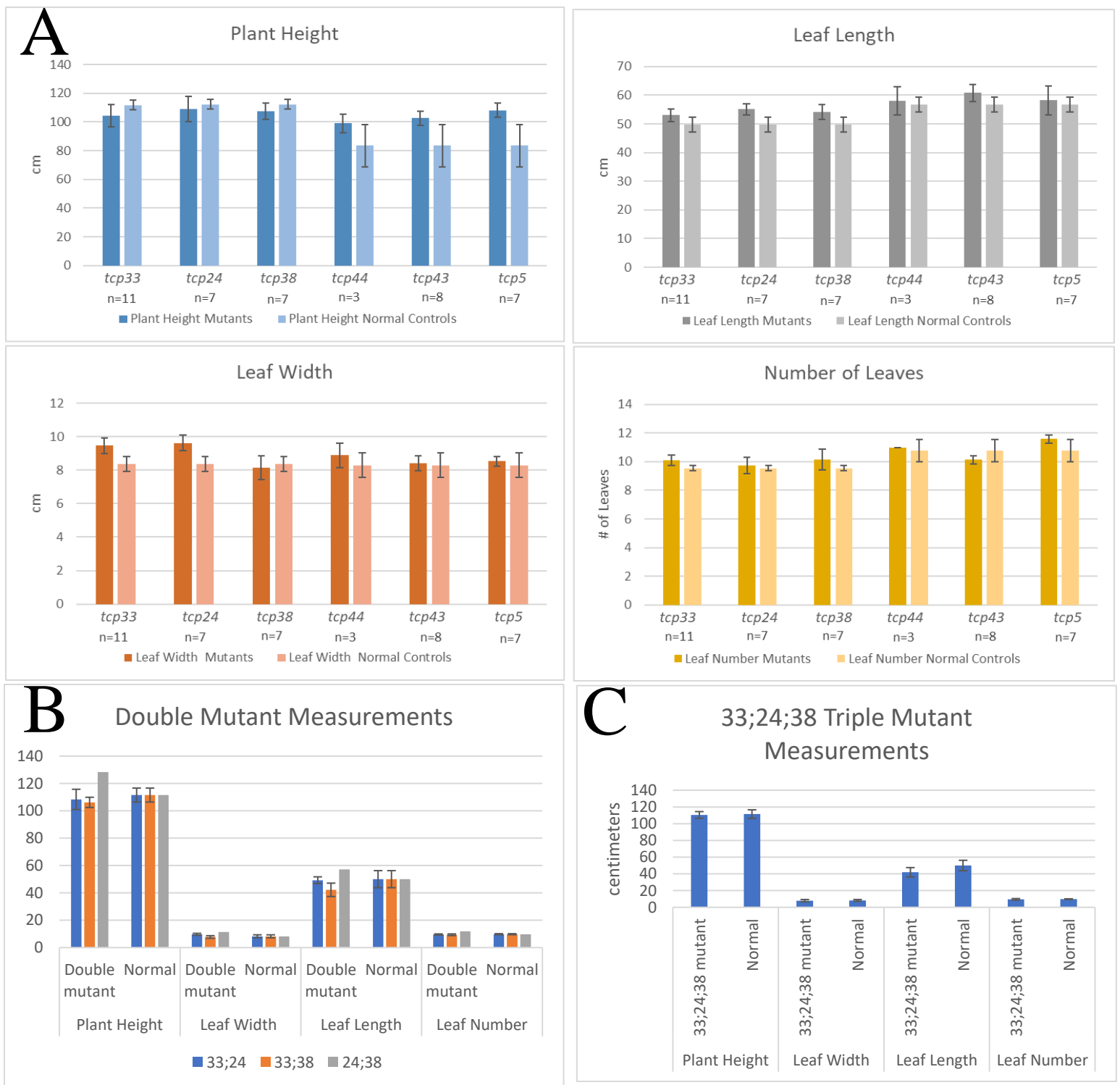
## Class II TCPs



**Figure 9: MiR319-targeted TCPs are closely related and likely redundant.** Phylogenetic tree showing miR319-regulated TCPs and their close relationship to other known miR319-regulated TCPs. Maize miR319-regulated TCPs with known mu insertions are starred and are very closely related. Phylogeny constructed by former graduate student Katherine Novitzky.

TCP Mutants Observed					
Single mutants		Double mutants		Triple mutants	
<i>tcp44</i>	n=3	<i>tcp44;tcp43</i>	n=5	<i>tcp44;tcp43;tcp5</i>	n=1
<i>tcp43</i>	n=8	<i>tcp44;tcp5</i>	n=0	<i>tcp33;tcp24;tcp38</i>	n=2
<i>tcp5</i>	n=7	<i>tcp43;tcp5</i>	n=3		
<i>tcp33</i>	n=11	<i>Tcp33;tcp24</i>	n=5		
<i>tcp24</i>	n=7	<i>tcp33;tcp38</i>	n=5		
<i>tcp38</i>	n=7	<i>tcp24;tcp38</i>	n=1		

**Table 3:** Single, double, and triple mutants were grown for each of the six miR319 regulated TCPs in maize to observe phenotypic characteristics.



**Figure 10: *tcp* mutants do not exhibit obvious developmental phenotypes.** Measurements were taken from all individuals that were planted during the 2016 field season. Single mutant (A), double mutant (B), and triple mutant (C) measurements for each *tcp* (*tcp33*, *tcp38*, *tcp24*, *tcp43*, *tcp44* and *tcp5*) were taken and a two-tailed t-test was done to look for any statistically significant difference between the single mutants and normal controls. Error bars represent



Abnormal  
phyllotaxy



Curling back  
of auricles

Severe  
ruffling of  
leaves

**Figure 11:** *tcp33;tcp24;tcp38* triple mutant that was isolated in the greenhouse has **multiple developmental defects**. The phenotype had defects that correlated with the other defects seen in *tcp* mutants in tomato and Arabidopsis such as abnormal phyllotaxy, curling back of auricles, and severe ruffling of leaves.

A

Gene & Primers Used	Expected Results	Observed Results																											
<i>tcptf44</i> (muF1/muR1)	<table border="1"> <thead> <tr> <th>1</th> <th>2</th> <th>3</th> <th>4</th> <th>5</th> <th>6</th> <th>7</th> <th>8</th> <th>9</th> </tr> </thead> <tbody> <tr> <td>44;43;5</td> <td>44;5</td> <td>44;5</td> <td>33;24;38</td> <td>33;24;38</td> <td>33;24;38</td> <td>+/+</td> <td>+/+</td> <td>+/+</td> </tr> <tr> <td></td> <td></td> <td></td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> </tr> </tbody> </table>	1	2	3	4	5	6	7	8	9	44;43;5	44;5	44;5	33;24;38	33;24;38	33;24;38	+/+	+/+	+/+				—	—	—	—	—	—	
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<i>tcptf5</i> (CR2F/CR2R)	<table border="1"> <thead> <tr> <th>1</th> <th>2</th> <th>3</th> <th>4</th> <th>5</th> <th>6</th> <th>7</th> <th>8</th> <th>9</th> </tr> </thead> <tbody> <tr> <td>44;43;5</td> <td>44;5</td> <td>44;5</td> <td>33;24;38</td> <td>33;24;38</td> <td>33;24;38</td> <td>+/+</td> <td>+/+</td> <td>+/+</td> </tr> <tr> <td></td> <td></td> <td></td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> </tr> </tbody> </table>	1	2	3	4	5	6	7	8	9	44;43;5	44;5	44;5	33;24;38	33;24;38	33;24;38	+/+	+/+	+/+				—	—	—	—	—	—	
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<i>tcptf43</i> (CR2F/CR2R)	<table border="1"> <thead> <tr> <th>1</th> <th>2</th> <th>3</th> <th>4</th> <th>5</th> <th>6</th> <th>7</th> <th>8</th> <th>9</th> </tr> </thead> <tbody> <tr> <td>44;43;5</td> <td>44;5</td> <td>44;5</td> <td>33;24;38</td> <td>33;24;38</td> <td>33;24;38</td> <td>+/+</td> <td>+/+</td> <td>+/+</td> </tr> <tr> <td></td> <td></td> <td></td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> <td>—</td> </tr> </tbody> </table>	1	2	3	4	5	6	7	8	9	44;43;5	44;5	44;5	33;24;38	33;24;38	33;24;38	+/+	+/+	+/+				—	—	—	—	—	—	
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<i>tcptf33</i> (mu2F4/CR3R)	<table border="1"> <thead> <tr> <th>1</th> <th>2</th> <th>3</th> <th>4</th> <th>5</th> <th>6</th> <th>7</th> <th>8</th> <th>9</th> </tr> </thead> <tbody> <tr> <td>44;43;5</td> <td>44;5</td> <td>44;5</td> <td>33;24;38</td> <td>33;24;38</td> <td>33;24;38</td> <td>+/+</td> <td>+/+</td> <td>+/+</td> </tr> <tr> <td>—</td> <td>—</td> <td>—</td> <td></td> <td></td> <td></td> <td>—</td> <td>—</td> <td>—</td> </tr> </tbody> </table>	1	2	3	4	5	6	7	8	9	44;43;5	44;5	44;5	33;24;38	33;24;38	33;24;38	+/+	+/+	+/+	—	—	—				—	—	—	
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<i>tcptf24</i> (CR2F/CR2R)	<table border="1"> <thead> <tr> <th>1</th> <th>2</th> <th>3</th> <th>4</th> <th>5</th> <th>6</th> <th>7</th> <th>8</th> <th>9</th> </tr> </thead> <tbody> <tr> <td>44;43;5</td> <td>44;5</td> <td>44;5</td> <td>33;24;38</td> <td>33;24;38</td> <td>33;24;38</td> <td>+/+</td> <td>+/+</td> <td>+/+</td> </tr> <tr> <td>—</td> <td>—</td> <td>—</td> <td></td> <td></td> <td></td> <td>—</td> <td>—</td> <td>—</td> </tr> </tbody> </table>	1	2	3	4	5	6	7	8	9	44;43;5	44;5	44;5	33;24;38	33;24;38	33;24;38	+/+	+/+	+/+	—	—	—				—	—	—	
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<i>tcptf38</i> (CR1F/CR1R)	<table border="1"> <thead> <tr> <th>1</th> <th>2</th> <th>3</th> <th>4</th> <th>5</th> <th>6</th> <th>7</th> <th>8</th> <th>9</th> </tr> </thead> <tbody> <tr> <td>44;43;5</td> <td>44;5</td> <td>44;5</td> <td>33;24;38</td> <td>33;24;38</td> <td>33;24;38</td> <td>+/+</td> <td>+/+</td> <td>+/+</td> </tr> <tr> <td>—</td> <td>—</td> <td>—</td> <td></td> <td></td> <td></td> <td>—</td> <td>—</td> <td>—</td> </tr> </tbody> </table>	1	2	3	4	5	6	7	8	9	44;43;5	44;5	44;5	33;24;38	33;24;38	33;24;38	+/+	+/+	+/+	—	—	—				—	—	—	
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**Figure 12:** RT PCR results from that *tcp24*, *tcp33*, and *tcp44* were null alleles of TCPs. *tcp44* is a loss-of-function allele. The RTPCR product of the *tcp33;tcp24;tcp38* triple mutant

## CHAPTER 3: The Effects of Auxin on the *fzt* Phenotype

Introduction:

### *Plant Structure and Development*

Plant development is characterized by continuous initiation of tissues and organs (Bohn-Courseau 2010). The initiation of tissues and organs is controlled by meristems. Meristems hold the undifferentiated stem cell populations that are involved in organogenesis. The SAM, specifically, holds the self-replenishing undifferentiated stem cells that control the initiation of all above ground organs and tissues of the plant (Thompson A.M, 2008; Leiboff S. et al, 2016). Studies and experiments about meristem signaling in the SAM and how it gave rise to lateral primordia began seventy years ago. From these experiments, they concluded that existing leaf primordia determines future sites of organ formation in adjacent regions on the SAM. This discovery lead to further research about mechanisms that control phyllotaxy, i.e. the way leaves or primordia are arranged on a plant (Golz, J. F., 2006)

Auxin is a plant growth hormone that plays many different roles during development including gametogenesis, embryogenesis, seedling growth, vascular patterning, and flower development (Zhao, Yunde 2010). Auxin regulates cell division, cell expansion, cell differentiation, lateral root formation, flowering, and trophic responses (Mashiguchi, Kiyoshi et al. 2011). Plant hormones are important because they influence physiological processes in plants. These “chemical messengers” can affect the plant’s ability to respond to its environment by acting like plant growth regulators since they are responsible for stimulating or inhibiting plant growth.

### *Auxin biosynthesis and transport*

The main naturally occurring auxin is indole-3-acetic acid (IAA). IAA is synthesized when tryptophan (Trp) is first converted to indole-3-pyruvate (IPA) by the TAA family of amino transferases (Zhao, Y., 2010). IAA is then produced from IPA by the YUCCA Flavin monooxygenase, which is a key auxin biosynthesis enzyme. This pathway is the main auxin biosynthesis pathway that operates in many developmental processes in plants (Figure 13) (Zhao, Y. 2010).

Auxin maxima and minima are what triggers organ formation and is important for determining the cells structure and function (Truskina, J. 2018). There is also evidence that an important role for auxin minima in stem cell fate maintenance and organ patterning (Wang Y, Jiao Y. 2018). Auxin maxima are formed in part though active polar auxin transport. Polar auxin transport leads to auxin accumulation which initiates subsequent primordia to develop (Wang Y, Jiao Y. 2018). Polar auxin transport is an active process that transport auxin into different regions of cells and tissues. Organ primordia are formed by patterns of auxin accumulation and depletion in different sites of the shoot apical meristem (Davies, 2005). At the beginning of organ initiation, primordia are called sinks. Sinks are areas where auxin is reduced. As the organ primordia matures it switches from an auxin sink to a source (Golz, J. F., 2006). Changes in auxin levels occur rapidly and are transported via transport proteins called PIN proteins. One of the most well-known auxin transporters is the PINFORMED1 (PIN1) protein of *Arabidopsis thaliana*. PIN1 is an auxin efflux transporter, meaning it actively transports auxin out of the cells and then determines the direction of the auxin based upon its' polar localization. The changes in polarity are thought to be the reason for the creation of the maxima of auxin that accumulates after the initiation of new primordia (Gallavotti, A *et al* 2008).



One way we have identified genes involved in auxin synthesis, transport, and signaling is through mutant analysis. By studying these mutants, we can characterize the developmental defects caused by auxin deficiency. Many auxin mutants were found and used to help elucidate the role of auxin in lateral primordia initiation. Auxin-deficient mutants have a phenotype that lacks lateral primordia; because of their lack of auxin they are unable to initiate lateral primordia. Commonly studied auxin mutants are *pinformed1* (*pin1*) mutants from Arabidopsis. The study of these mutants has told us what happens when auxin is not transported properly in and out of the cells. Available cloned mutants available in maize include: *spi1*, *vt2*, *Bif1*, *Bif4*, *bif2*, and *ba1*. Out of those, I looked at *sparse inflorescence 1* (*spi1*) and *vanishing tassel 2* (*vt2*). *spi1* is an auxin biosynthesis mutant that has defects during vegetative and reproductive development such as fewer branches and spikelets on tassels, smaller ears, and low yield suggesting a defect in branch meristem (BM) and SPM initiation. *vt2* is a co-ortholog of TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1) which functions in the auxin biosynthesis pathway to convert tryptophan to indole-3-pyruvic acid. *vt2* has a similar phenotype to *spi1* with severe barren inflorescences without branches or spikelets (Phillips, K.A. 2011). Both of those auxin mutants have been used to show that auxin is required for proper plant development. Mutants that disrupt auxin synthesis or transport have reduced numbers of primordia or completely lack primordia. Other known auxin maize mutants include *bif2*, *Bif1* and *Bif4* (Galli, M. *et al* 2015; McSteen, P. *et al* 2007). *bif2* is a co-ortholog of PINOID from Arabidopsis, which regulates auxin transport. It is also required for the initiation of axillary and lateral primordia (McSteen, P. *et al* 2007). *Bif1* and *Bif4* encode Aux/IAA proteins that are essential to the auxin signaling pathway because they regulate early steps of inflorescence development (Galli, M. *et al*, 2015).

In this study, I sought to analyze the interaction between *fzt* and auxin. Since miR167 targets ARFs and they are statistically increased in *fzt* mutants one possibility is that the increased branching and primordia seen in *fzt* is an effect of the increased auxin signaling. The first approach I took to analyze the interaction between *fzt* and auxin mutants was to use the *spi1* and *vt2* auxin mutants to make *spi1;fzt* and *vt2;fzt* mutants. This approach would enable me to look at where *fzt* functions in the auxin pathway. The second approach I took was to inhibit auxin transport using N-1-Naphthylphthalamic Acid (NPA) to observe the effects that it had on the *fzt* phenotype.

## Methods

### *Isolation of spi1;fzt and vt2;fzt double mutants*

Since miR167 targets ARFs and they are statistically increased in *fzt* mutants one possibility is that the increased branching and primordia seen in *fzt* is an effect of the increased auxin signaling. The first approach to look at this was to isolate double mutants with *fzt* and auxin mutants, *spi1* and *vt2*, respectively. Seeds segregating for *fzt;spi1* double mutants and *fzt;vt2* double mutants were planted. In collaboration with another graduate student, *fzt* homozygotes were identified by their phenotype. All plants were planted 36 to a flat in soil and grown in the growth chamber (Percival Intellus Control System) at 26 ° Celsius, 80% humidity, and 12-hour light and watered once daily while being genotyped. To see if the *fzt* homozygotes were also homozygous for *spi1* or *vt2* I genotyped them with gene specific primers. *fzt* plants were able to be identified by their phenotype. To isolate double mutants, the *fzt* plants were genotyped for either *spi1* or *vt2*. Isolated double mutants were grown to maturity (8 weeks) and then phenotypically characterized. They were moved to the greenhouse to grow to maturity. In the greenhouse they were transplanted into large pots (5.68 L Classic 600 (C600) pots, 50%

Fafard 3B soil and 50% Turface) for growth in the greenhouse (16 hours light). Greenhouse-grown plants (two plants/pot) were watered using drippers (2.0 GPH Woodpecker pressure compensating junior drippers (model number 01WPCJL8) from Netafim) eight times a day for three minutes and were supplemented with Peter's Excel 15-5-15 Cal-Mag Special at 400PM nitrogen. Pictures and measurements including leaf number, height, internode lengths, and tassel height were taken for each double mutant identified (Table 4).

#### *NPA Treatment*

There were four different conditions: *fzt* with NPA, *fzt* without NPA, normal siblings with NPA, and normal siblings without NPA. Three separate batches of plants were used to gather all the information needed to answer my hypothesis (Table 5). *fzt* homozygotes were planted aside normal siblings and were watered daily with an 80uM concentration of NPA in 150mL of water at 2 ½ weeks old at which point, tassel primordia had not started to initiate spikelet pair meristems (SPM) or spikelet meristems (SM) according to previous literature (Wu, X., and P. McSteen 2007). Plants were watered before the SPM and SM started to initiate in order to see how NPA would affect the increase in branching seen in *fzt*. The concentration used was determined because it was the minimum concentration tested by Wu X. and McSteen P. that was previously shown to cause a strong inhibition of lateral primordia (Wu, X., and P. McSteen 2007). Plants were watered daily for 14 consecutive days. After the 14 days of NPA treatment, tassels were dissected and visualized on the dissecting scope or using a scanning electron microscope under low vacuum conditions on an FEI Quanta 200 Mark 1 scanning electron microscope at an accelerating voltage of 10 to 15 k.

### *Plant Growth Conditions*

Seeds were taken from a family segregating for *fzt* in A619 inbred background and soaked for 2 hours in water and then bleach sterilized with 2% bleach for 15 minutes followed by five fresh water washes. They were then placed on wet paper towels, coated with Captan fungicide, and incubated at 28°C until a shoot emerged. Seedlings were transferred to soil in individual pots and grown in a Percival Intellus growth system at 26 ° C, 12-hour light dark cycle and watered daily. *fzt* seedlings were identified based on phenotype and transplanted to large pots (5.68 L Classic 600 (C600) pots, 50% Fafard 3B soil and 50% Turface) for growth in the greenhouse (16 hours light) along with an equal number of normal sibling seedlings. Greenhouse-grown plants (two plants/pot) were watered using drippers (2.0 GPH Woodpecker pressure compensating junior drippers (model number 01WPCJL8) from Netafim) eight times a day for three minutes and were supplemented with Peter's Excel 15-5-15 Cal-Mag Special at 400PM nitrogen.

### **Results and Discussion**

My objective was to test the hypothesis that auxin biosynthesis, and transport is necessary for normal development of inflorescences in maize. From this hypothesis, I wanted to see whether the increase in branching and primordia seen in *fzt* mutants require auxin biosynthesis or transport. I first used a genetic approach. Several mutants that perturb auxin biosynthesis (*vt2*, *spi1*) and signaling (*bif2*, *Bif1*, *Bif4*) exist in maize (Galli, M. 2015; McSteen, P. *et al* 2007; Gallavotti, A 2008; Phillips, K. A. 2011) No mutants that affect auxin transport have been reported. I first tried to isolate *spi1;fzt* and *vt2;fzt* double mutants. As mentioned earlier, *spi1* and *vt2* are already characterized maize auxin mutants. Analyzing the interaction between these auxin mutants and *fzt* will determine how the *fzt* mutants interacts with the auxin biosynthesis. I

planted 100 seeds segregating for *vt2* and 100 seeds segregating for *spi1* in the growth chamber. *fzt* seedlings were identified based on phenotype and transplanted to larger pots to grow to maturity. According to Mendelian genetics 1/16<sup>th</sup> out of the 100 planted for each gene should be *vt2;fzt* or *spi1;fzt* double mutants. From the plants I screened through I identified one *spi1;fzt* double mutant and one *vt2;fzt* double mutant. Another graduate student in the lab isolated the *vt2;fzt* double mutant before I took over this project. The phenotype of the *vt2;fzt* double mutant lacked lateral primordia and had characteristics of the *vt2* single mutant (Figure 14A). From this result, it looked as if *vt2* is likely epistatic to *fzt* but in order to confidently conclude this I would need to look at more than one plant. The phenotype of the *spi1;fzt* also had characteristics that corresponded to the characteristics seen in *fzt* homozygotes. The tassel of the *spi1;fzt* double mutant had branches but the *spi1* homozygous phenotype is not barren like *vt2* and does have some branches (Figure 14B). I could not conclude without doubt that the phenotype was more similar to *fzt* than *spi1* without having more double mutants to observe. Since I did not see the same affect with the *spi1;vt2* double mutant this result suggests that the auxin biosynthesis pathway may not be as simple as expected. Measurements including plant height, leaf length and width, internode length, and tassel length were taken from the *spi1;fzt* and *vt2;fzt* double mutants but no specific differences between the measurements of the two double mutants stood out (Table 4).

Since I could only isolate one double mutant per genotype I could not come to a confident conclusion about how auxin and *fzt* were interacting. I used an alternative approach to ask how the inhibition of auxin transport affects the *fzt* phenotype. NPA is a common polar auxin transport inhibitor (Wu, X., and P. McSteen 2007). I adapted this approach as well to look at the effects of auxin transport inhibition in *fzt* mutants compared to normal siblings. Auxin transport is very

important because it enables initiation of lateral primordia. In the experiments that Wu X. and McSteen P. performed, they were able to conclude that auxin transport is required for the initiation of inflorescences during reproductive development (Wu X., McSteen P. 2007). Their research, done on wildtype maize, demonstrated that auxin is required for the formation of lateral primordia on inflorescences. I used conditions previously shown to severely inhibit lateral primordia in maize inflorescences.

After two weeks of NPA treatment, I visualized the inflorescences using scanning electron microscopy. In both wildtype and *fzt* mutant plants the roots had grown upwards out of the soil and were visible (Figure 15). The agravitropism seen in the roots was a good indication that the plant was taking up the NPA because this characteristic has been seen in other auxin experiments using NPA as the auxin transport inhibitor (Wu X. and P. McSteen. 2007). As expected, A619 plants treated with NPA had decreased lateral primordia (Figure 16) whereas untreated A619 plants had normal inflorescence morphology and patterning. *fzt* mutants showed decreased primordia and branching in the *fzt* homozygotes watered with NPA (Figure 17).

From these experiments I was able to conclude that the inhibition of auxin transport does reduce branching in *fzt* mutants. Also, the auxin pathway may not be a simple linear pathway since *vt2* and *spi1* have different genetic interactions with *fzt*.

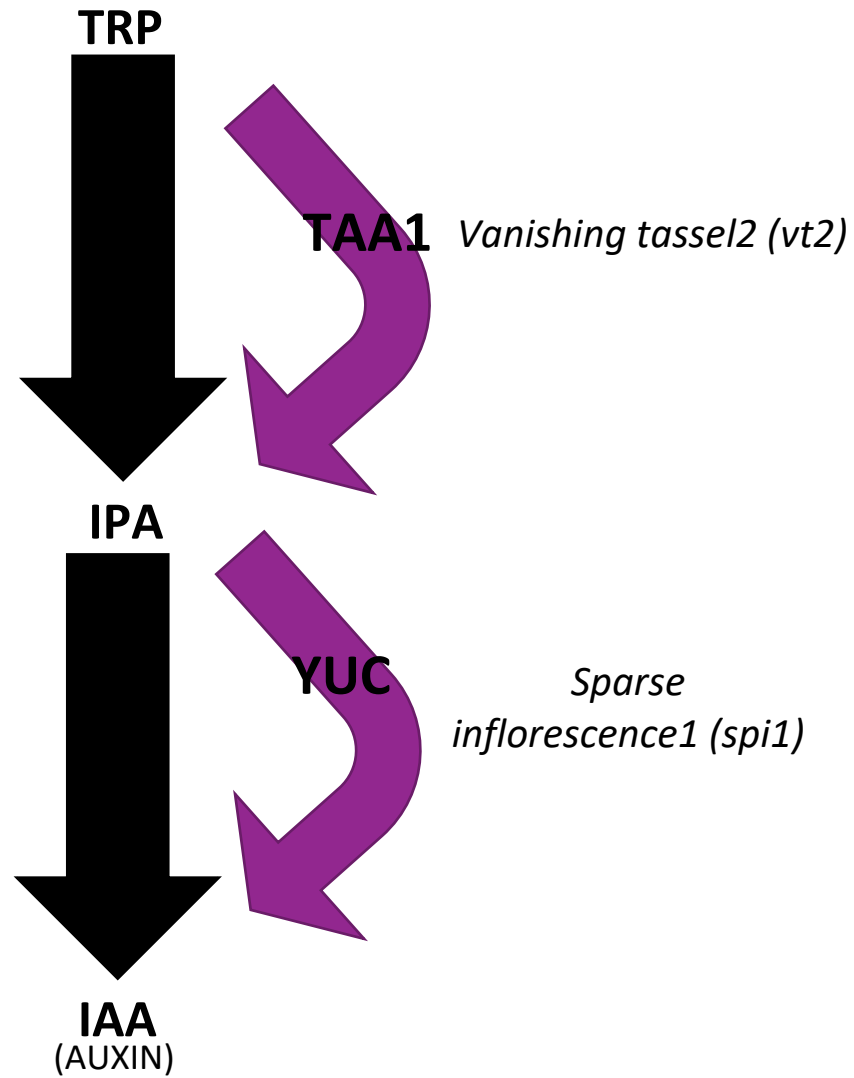
	<b>Leaf length</b>	<b>Leaf width</b>	<b>Height</b>	<b>Internode lengths</b> <i>(1=first internode above root)</i>	<b>Tassel height</b>
<i>fzt;fzt</i>	30.5 cm	5.5 cm	65 cm	1 (2.50 cm) 2 (2.50 cm) 3 (2.50 cm) 4 (2.0 cm) 5 (3.0 cm) 6 (3.0 cm) 7 (3.2cm) 8 (2.0 cm) 9 (9.5 cm)	_____
<i>spi1;fzt</i>	_____	_____	49.53 cm	1 (1.27 cm) 2 (3.81 cm) 3 (8.25 cm) 4 (8.25 cm) 5 (8.25 cm) 6 (8.89 cm) 7 (12.06 cm)	5.39 cm
<i>vt2;fzt</i>	42.5 cm	4.75 cm	62 cm	1 (4.0 cm) 2 (5.0 cm) 3 (11.50 cm) 4 (6.50 cm) 5 (8.25 cm) 6 (8.89 cm) 7 (12.06 cm)	_____

**Table 4:** Measurements of *vt2;fzt* mutant and *spi1;fzt* mutant.

	<b># of WT with water</b>	<b># of <i>fzt</i> with water</b>	<b># of WT with NPA</b>	<b># of <i>fzt</i> with NPA</b>
<b>Batch 1</b>	2	2	2	6
<b>Batch 2</b>	4	4	2	3
<b>Batch 3</b>	2	3	3	3

**Table 5:** Number of batches indicative of how many individuals I treated and what treatment they received.





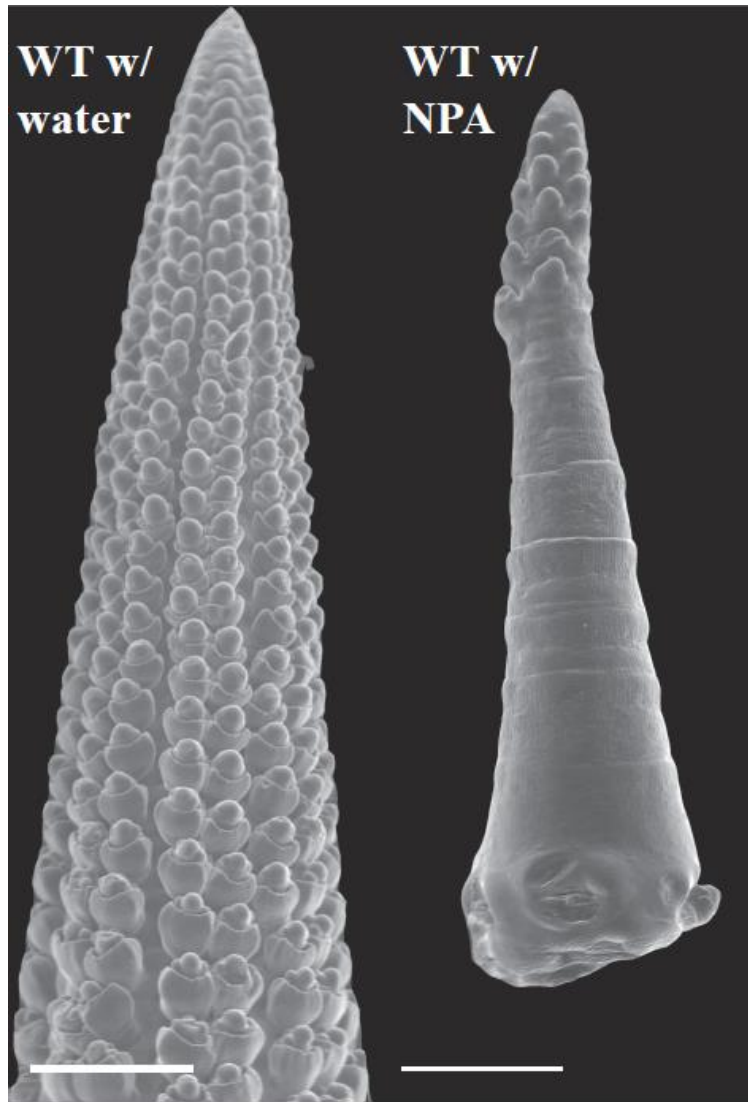
**Figure 13:** *spi1* encodes an enzyme in the tryptophan pathway for TRP dependent auxin biosynthesis. *vt2* is a coortholog of TAA1 that converts TRP to IPA. *vt2* is a coortholog of TAA1 which converts Trp to IPA. *spi1* encodes an enzyme in the tryptophan pathway for TRP dependent auxin biosynthesis.



**Figure 14:** Phenotypes of (A) *vt2;fzt* and (B) *spi1;fzt* auxin mutants that I isolated.

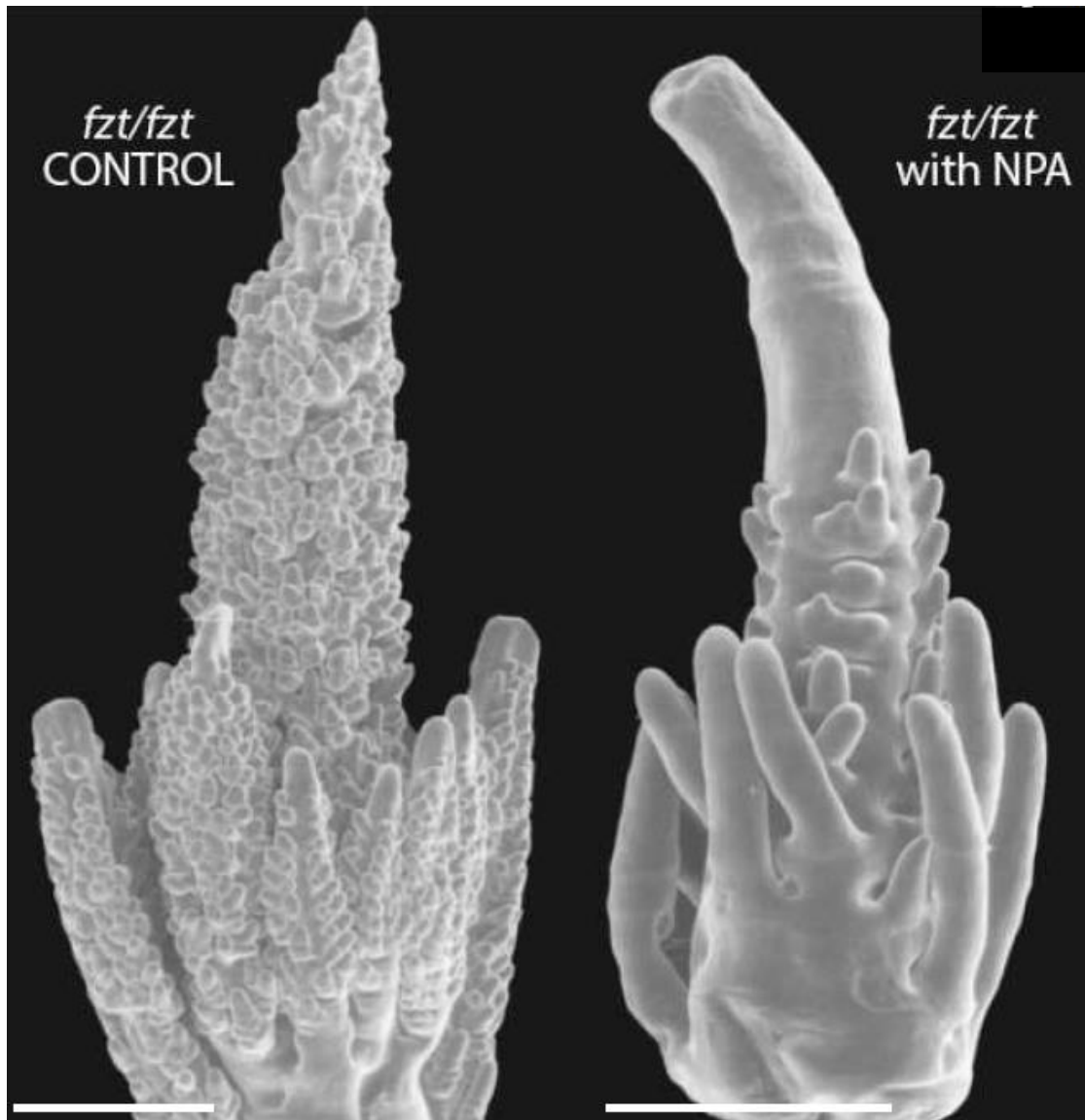


**Figure 15:** The immediate effect of watering the NPA auxin transport inhibitor. A (WT watered without NPA), B-C (*fzt;fzt* watered without NPA), D-I (*fzt* watered with NPA)



**Figure 16:** A-B (WT treated with only DMSO), C-D (WT treated with NPA)





**Figure 17:** A-B (*fzt;fzt* treated only with DMSO), C-D (*fzt;fzt* treated with NPA)

## CHAPTER 4: Summary and Future Directions

### *Objective 1: Summary and Future Directions*

One part of my Masters research consisted of an RNA analysis and phenotypic characterization of miR319-regulated TCPs in maize inflorescence development. The goal of this research was to gather information on how TCPs effect leaf development of maize and function in normal development. I first determined which miR319-regulated TCPs were null alleles and from that I was able to conclude that *tcp44* is a loss-of-function allele. I also isolated *tcp* triple mutants including *tcp44;tcp43;tcp5* as well as *tcp33;tcp24;tcp38* which had not been done before in the Thompson lab. This enabled me to look for subtle phenotypic characteristics due to the reduction of *tcp* expression. From all of the *tcp* mutants I isolated, the only abnormal phenotype seen was from one *tcp33;tcp24;tcp38* triple mutant grown in the greenhouse. The phenotypic characteristics were consistent with other already characterized *tcp* mutants although this phenotype did not reappear in the two other triple mutants isolated in the field.

Now that I have discovered that *tcp44* is a loss-of-function allele it can be studied further by looking at more triple mutants to see if there are subtle phenotypic characteristics due to the reduction in *tcp44* expression. As far as *tcp33* and *tcp24*, sequencing data needs to be obtained to determine if the RT-PCR product for *tcp24* is actually *tcp33* product. The sequencing data also needs to be analyzed to determine why the *tcp33* RT-PCR product is larger than expected. The location of the mu transposon insertion for both of those make them most likely loss-of-function alleles but that cannot be verified until the sequencing data has been analyzed for those. Lastly for the complete characterization qRT-PCR will need to be performed to obtain quantitative data on the RNA expression of *tcp33*, *tcp24*, *tcp38*, *tcp43*, and *tcp5*. Although they did not show that

they were loss-of-function alleles from the RT-PCR is it possible that they still have reduced levels of RNA.

*Objective 2: Summary and Future Directions*

The second part of my research focused on miR167, a mRNA target that encodes for auxin response factors. I was able to determine the effects of inhibited auxin transport on the *fzt* phenotype. By inhibiting auxin transport in *fzt* mutants I observed a reduction in branching and lateral primordia. From isolating *vt2;fzt* and *spi1;fzt* double mutants I was able to conclude that *vt2* is epistatic to *fzt* but, the *spi1;fzt* double mutant did not have a phenotype that reassembled a *spi1* mutant. I was also able to demonstrate that the auxin signaling pathway is not as simple as we thought. Future directions would be to continue to examine the interactions of *fzt* with the auxin pathway.

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