ANALYSIS OF THE MIR319 TARGET ZMTCPTF24 IN THE MAIZE INFLORESCENCE

by

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A Senior Honors Project Presented to the

Honors College

East Carolina University

In Partial Fulfillment of the

Requirements for

Graduation with Honors

by

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December 2014

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Honors College Biology Thesis

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December 10, 2014

I hereby declare that I am the sole author of this work. It is the result of my own original work and research and has not previously been submitted elsewhere for another degree.

Signature:_____ Date:_____

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Abstract:

Maize inflorescences are essential for reproduction and also produce seeds that are consumed as food. To understand the genetics pathways that control normal inflorescence development, we study mutants with abnormal inflorescence development. A major focus of the Thompson laboratory is the maize *fuzzy tassel* (*fzt*) mutant, which has severe inflorescence defects. *fzt* contains a mutation in *dicer-like 1 (dcl1),* which encodes a key enzyme required for microRNA (miRNA) biogenesis. miRNAs are 20-22-nucleotide long RNAs that repress gene expression by directed RNA cleavage or translational inhibition . In *fzt* mutants, some miRNAs are dramatically decreased, while others are moderately decreased or unchanged. MiR319 is reduced approximately 8-fold in *fzt* mutants, and is predicted to target mRNAs that encode TCP transcription factors. We hypothesize that reduced miR319 levels may lead to increased or ectopic expression of *TCP* target genes and be responsible for a subset of the *fzt* defects. My project focuses on one miR319 target, *Zmtcptf24.* I used RNA in situ hybridization to examine expression of *Zmtcptf24* in normal tassel primordia. Preliminary experiments indicate that *Zmtcptf24* is expressed in the carpal, stamens, and lodicules, suggesting that *Zmtcptf24* may play a role in maize floral development.

Acknowledgements

I would like to thank Katherine Novitzky for teaching me various laboratory techniques and helping me work through the research necessary for the project. I would also like to extend thanks to my mentor Beth Thompson for taking me under her wing and allowing me to conduct research in her laboratory. Without the guidance from both, I would not be the researcher I am today.

Table of Contents

List of Tables

List of Figures

Introduction

Maize is a major food crop, which humans are highly dependent on. In maize there are two flowering structures called inflorescences, the ear and tassel. The ear makes female flowers and produces the kernels that are harvested to provide nutrition. The tassel makes male flowers and produces pollen that fertilizes female flowers. The Thompson laboratory studies the genetic control of maize inflorescence development.

Plant growth and development depends on the activity of meristems (Steeves & Sussex, 1989). Meristems are groups of indeterminate stem cells. The architecture of the inflorescence is determined by the meristem activity (McSteen & Hake et al., 2001; Bartlett & Thompson, 2014). The inflorescence meristem (IM) is at the apex (tip) of the inflorescence and initiates long g branch or short branch meristems. Long branches are produced only in the tassel. The short branch meristems are called spikelet pair meristems (SPM). Each SPM gives rise to two spikelet meristems (SM), and each SM initiates two terminal floral meristems (FM) (Figure 1). The IM and branch meristems are indeterminate meristems because the stem cells are not consumed in the production of meristem or organ primordia**.** The SPM, SM, and FM are determinant meristems because stem cells are consumed in the production of primordia (Steeves & Sussex, 1989). The Thompson lab is interested in the genetic control of meristem fate and determinacy in the inflorescence.

fuzzy tassel Mutant (fzt)

One mutant we study in the Thompson lab is the *fuzzy tassel (fzt)* mutant, which has severe inflorescence defects. *fzt* plants have reduced plant stature and also have shorter, narrower leaves than normal siblings. *fzt* inflorescences have increased indeterminacy in multiple

meristems (SPM, SM, FM) and abnormal floral organs. Scanning electron micrographs (SEM) of the tassel and ear show a lack of stem cell homeostasis in the IM creating abnormal growth (Thompson et al., 2014).

DICER-LIKE1 *(*DCL1*)* is a key enzyme required for miRNA biogenesis (Kurihara & Watanabe, 2004). *fzt* has a mutation in *dcl1,* predicted to cause a missense mutation in the RNAse III domain of DCL1 (Thompson et al., 2014). MiRNAs are 20-22 nucleotide long RNAs that play a role in plant growth, stress response, and development (Bartel, 2004; Kim, 2005; Bushati & Kohen, 2007; Sunkar et al., 2007). miRNAs are transcribed as long primary miRNAs (pri-miRNA) that can form a stem-loop or hairpin structure (Kurihara & Watanabe, 2004). The pri-miRNA transcript is cleaved by DCL1 to release the hairpin section, the pre-miRNA (Kurihara et al., 2006). The pre-miRNA hairpin is cleaved again by DCL1 resulting in the miRNA/miRNA* duplex. (Bartel et al., 2004, Krol et al., 2010) The duplex is exported out of the nucleus and one strand of the small RNA duplex is incorporated into the RNA Induced Silencing Complex (RISC) (Meister & Tuschl, 2004; Chen, 2009). The miRNA guides RISC to the mRNA with a complimentary sequence. ARGONAUTE (AGO), one of the proteins associated with RISC, is the catalytic component of RISC and cleaves the mRNA to the bound miRNA (Vaucheret et al., 2004; Miyoshi, 2005; Kurihara & Watanabe, 2004).

To understand how miRNA processing was affected in *fzt*, small RNA sequencing was performed in *fzt* and examined against normal tassels (Thompson et al., 2014). The data supported the hypothesis that not all miRNAs are affected equally. In plants, miRNAs target mRNAs for cleavage and degradation so miRNAs may be responsible for some mutant phenotypes or start a cascade of change (Bartel, 2004). The data also indicated that some miRNAs are dramatically reduced compared to others (Thompson et al., 2014). The miRNA in

question for my project is miR319, which is decreased about 8-fold in *fzt* mutant tassel primordia compared to normal controls (Thompson et al., 2014). In other plant species, miR319 targets a plant-specific group of transcription factors called TCPs (Schommer et al., 2008; Palatnik et al., 2007; Nag et al., 2009; Martín-Trillo & Cubas, 2010).

TCPs are a group of plant-specific transcription factors contain a similar basic-helix-loop-helix domain (Martín-Trillo & Cubas, 2010). TCPs are divided into two subgroups, class I and class II, based on their protein sequence (Danisman et al, 2012). Within class II TCPs, a subset contain a miR319 binding site and are targeted by miR319 (Nag et al., 2009; Ori et al., 2007). In Arabidopsis, out of 24 TCPs, five are targeted by miR319 including *TCP3, TCP4, TCP5, TCP10, and TCP24* (Nag et al., 2009). This miR319 binding site is located near the 3' end of the coding region (Schommer et al., 2012).

My gene of interest in maize is *Zmtcptf24*, which is closely related to *TCP4* in Arabidopsis, based on previous phylogenetic analyses (K.Novitzky, unpublished). Proper regulation of *TCP4* by miR319 is required for petal growth and development in Arabidopsis (Nag et al., 2009). Decreased miR319 activity results in reproductive and vegetative defects of Arabidopsis (Ori et al., 2007; Schommer et al., 2008). Direct overexpression of *TCP4* by absent miR319, and the lack of a regulator of the five *TCPs*, results in a complete loss of petal growth (Nag et al., 2009)

Objective

The objective of my project is to analyze the role *Zmtcptf24* plays in maize. Through in vitro and in situ experiments the research questions to answer are what is the normal role of *Zmtcptf24* in maize development and does over expression contribute to the *fzt* phenotype. RNA

in situ hybridization will give some insight into where *Zmtcptf24* is expressed in the mRNA, and give an idea of normal gene function. I hypothesize that since *TCP4* plays a key role in of floral development in Arabidopsis, , *Zmtcptf24* may also play a role in maize floral development.

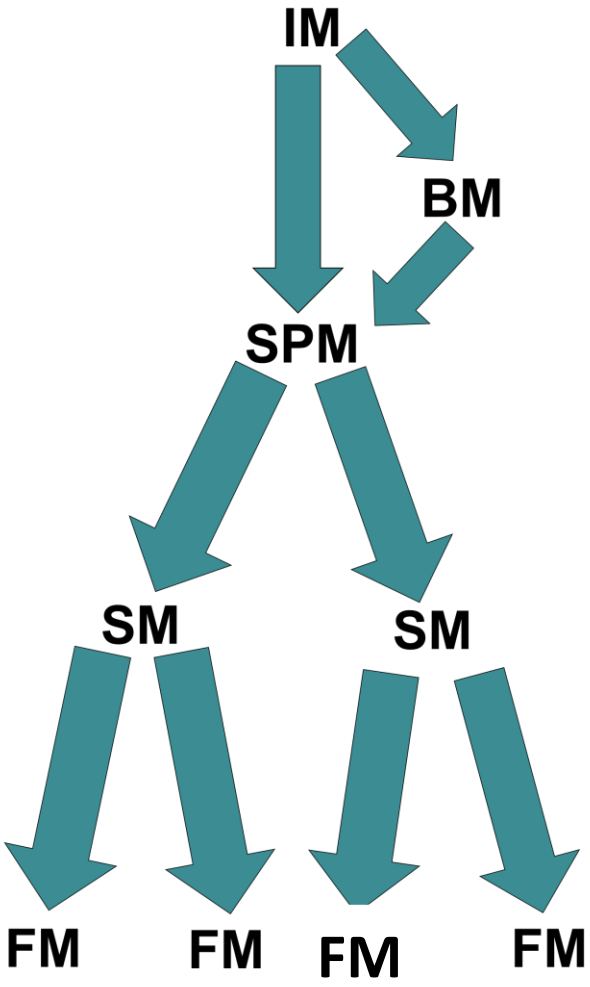

Figure 1: Schematic of inflorescence development

Tassels and ears arise from a collection of indeterminate stem cells at the apex of the inflorescence called the inflorescence meristem (IM). The IM initiates the spikelet pair meristems (SPM); each SPM gives rise to two spikelet meristems (SM); each SM gives rise to two floral meristems (FM). In the tassel, the IM also initiates long branch meristems (BM).

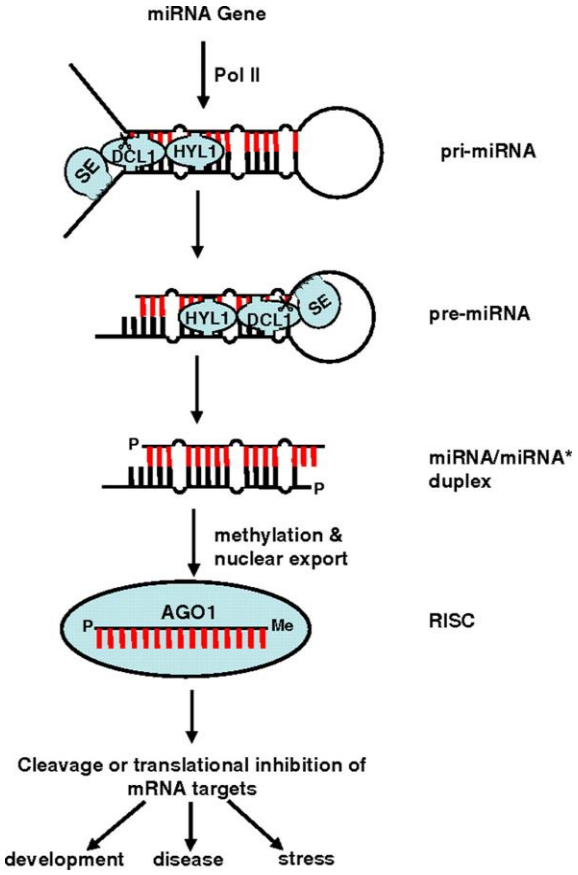

Figure Credit Jian-Kang Zhu (2008)

Figure 2: **The function of DCL1 in miRNA biogenesis**

DCL1 is responsible for two cleavage points in miRNA biogenesis. The first cleavage releases the hairpin from the pri-miRNA to release the pre-miRNA and the second cleavage releases the small RNA duplex, consisting of the miRNA and its complement.

Materials and Methods:

Probe Design and Synthesis

Probes for RNA in situ hybridization were designed to target the 5' and 3' UTR of *Zmtcptf24*. UTRs were targeted because these are sequences unique to *Zmtcptf24* and not shared with closely related *tcp* genes. PCR primers were designed to amplify ~150-200 base pairs (bp) of *Zmtcptf24* cDNA (see Table 1 for primers used) using the Oligoperfect primer design program (www.lifetechnologies.com/oligoperfect). To ensure *Zmtcptf24* amplicons were unique to *Zmtcptf24*, these sequences were BLASTED against known maize genomic sequences.

To obtain sufficient amounts of the gene-specific DNA for cloning, PCR was used to amplify the targeted regions of *Zmtcptf24*. DNA from the A619 inbred was used as a template for PCR because in situs were carried out on A619 tissue. The targeted regions did not span any introns, so the genomic and cDNA sequences should be identical in these regions. To minimize PCR errors, Phusion high-fidelity DNA polymerase (Thermofisher) was used to amplify the expected fragments (see protocol in Appendix 1A). 5 μl of PCR product was run on a 1% agarose gel to visualize the size of the PCR product. Reaction products were purified over a BIONEER purification column in a PCR purification clean up kit to remove unwanted components. These unwanted components included genomic DNA, primers and dNTPs. The reaction products with multiple amplification regions were purified using BIONEER gel purification clean up kit, which required the 150-200bp fragments to be excised from the agarose gel and purified.

Prior to cloning into the pGEM-T easy vector, the sequences had to be a-tailed. The A tail ensured the sequence would insert and bind to the 3' thymine overhang of the pGEM-T easy

vector (see Appendix 1 for exact protocol). PCR products were cloned into the pGEM-T easy vector system (Promega) according to manufacturer's protocols (see Appendix 1C for exact protocol). 2µl of ligation reactions were then transformed into 25µl competent cells, plated on LB/CARB plates coated with 7µl (200mg/ml) IPTG and 40µl (20 µg/µl) X-Gal, and incubated overnight at 37° C.

Putative positive colonies were selected based on blue/white colony screening (See Figure 3). Plasmids that have insert present interrupt the formation of β-galactosidase by blocking the lacZ operon. The blue colonies did not have an interruption because of the lack of insert and were able to produce the β-galactosidase. The white colonies were considered successful because they disrupted the gene due to the placement of an insert, however the correct insert had to be verified through later steps. To verify clones were correct, 4-6 white colonies were cultured overnight in selective media and plasmid DNA isolated using BIONEER Plasmid clean up kit. Plasmids were sequenced with T7 and SP6 sequencing primers to confirm sequence and insert orientation. Although the insert is expected to enter in random orientation, all plasmids tested (6/6) were in the same orientation and the SP6 RNA polymerase was used to generate anti-sense probes. Cultures of selected plasmids for probe synthesis were regrown and purified using BIONEER Plasmid Cleanup kit. The plasmid cultures were saved as stocks in 60% glycerol for a final concentration of 30% and stored at -80° C for long-term storage.

In Vitro Transcription Reaction

In order to detect *Zmtcptf24* mRNAs during tassel development, I used RNA in situ hybridization. Probes for in situ hybridization are antisense transcripts that contain digoxigenin (DIG)-labeled uridines. The DIG can be detected by an anti-DIG antibody, which is labeled with

alkaline phosphatase (AP). In the presence of its substrate AP forms a blue precipitate and thus provides a method to visualize bound probe. To generate a template for in vitro transcription, linear DNA fragments were amplified off of the select plasmids using M13 forward and reverse primers. All of the plasmids I generated required the use of SP6 RNA polymerase to generate the RNA antisense probe. To maximize probe synthesis, in vitro transcription reactions were incubated for 2.5 hours (See Appendix 1E for full protocol). To compare whether the DNAse treatment was effective, 3µl was removed from the in vitro reaction before stopping the reaction by adding DNAse. After the 15 minute DNAse reaction, 2µl were removed. The 3µl before and 2 µl after samples were loaded onto a 1% Agarose gel and were run to check for degradation of DNA contamination by comparing the two samples. The first sample should show a smear after the gel is imaged, indicating the presence of RNA. Probe was precipitated with 1µl 10mg/mL of yeast tRNA, 75µl 100% EtOH, and 2.5µl 3M NaOAc overnight, spun down the following day, washed in 70% EtOH, and resuspended in 40µL of 50% formamide.

To determine efficiency of probe synthesis and estimate probe concentration, a dot blot was performed. 20-fold serial dilutions were made for both control (DIG-labeled control RNA, Roche, 100µm/ml initial concentration) and synthesized probe. 1µl of each dilution was spotted and allowed to air dry before being cross-linked to a nylon membrane using UV light. The cross-linked RNA probes on the nylon membrane were subjected to a series of washes to prepare for hybridization following Dot Blot protocol detailed in Appendix 1F, and developed using the same detection techniques as those used for in situ hybridization. Synthesized probe intensities are compared to Roche manufacturers control to determine starting concentrations for in situ hybridization. As expected, T7 -transcribed probes had more robust synthesis (approximately 20 ng/µl) than SP6 probes (approximately 100 pg/µl) (see figure 4).

In Situ Hybridization

In situ hybridization is a laboratory technique to visualize which cells in a tissue an RNA is expressed. I briefly describe the RNA in situ hybridization method below; a complete protocol can be found in Appendix 1E. First, an RNA probe complementary to the mRNA of interest (antisense), is synthesized with DIG-labeled UTPs and the labeled probe is hybridized to fixed tissue. Sense probes are not expected to hybridize to any cellular RNAs and serve as a negative control. The antisense RNA probe will hybridize to the RNA transcript with a complementary sequence. To visualize where the probe is bound within the tissue, the tissue is incubated with an anti-DIG antibody that is coupled to AP. AP will form a blue precipitate when incubated with its substrate. The blue staining identifies cells within the tissue where the synthesized RNA that corresponds to *Zmtcptf24* is present.

4µl of probe at 2X concentration was used for each slide pair. Hybridization and posthybridization are the two steps the slide pairs go through and are detailed in Appendix 1G. The slide pairs were taken through a variety of steps within the initial hybridization and the posthybridization. The hybridization step includes fixing the slides with formaldehyde to improve tissue fixation on the slides, and a proteinase K treatment to allow the probe to penetrate the tissue, thus obtatining better signal. During the posthybridization steps the slides are treated with the detection buffer and substrate and allowed to incubate before the reaction is stopped. The slides are allowed to develop overnight before expression is visible. A transcript with a low concentration can require more detection buffer and a longer period of incubation to detect clear signal. After the slides are mounted with permout overnight, they can be imaged under the microscope and evaluated for expression.

Primer	Sequence	Date	Region
TCP24-F1	ATCCAACCAGCATGACCATT	5/20/2014	3'
TCP24-R1	AATGGGGACAACAGAACTGC	5/20/2014	
TCP24-F2	CCCATTCCAGGAGAGAAGAG	5/20/2014	3'
TCP24-R2	TCTTGTGTCGTTCTCGCAGT	5/20/2014	
TCP24-F3	CCCGGTACCAGCTTCTCC	6/19/2014	Coding
TCP24-R3	ATCTGTGCTCCACTGCTGCT	6/19/2014	
TCP24-F4	GAGACGGAAACCCCTTCC	6/19/2014	5'
TCP24-R4	ACCTTGTCGGGGACCATAAT	6/19/2014	
TCP24-F5	TGGCTTCCTTTGCGTTAAAT	6/19/2014	5'
TCP24-R5	CCTGATGGGTGCGATTAAGT	6/19/2014	

Table 1: Primers ordered and tested. The purple highlighted primer sets used to generate probes for in situ hybridization.

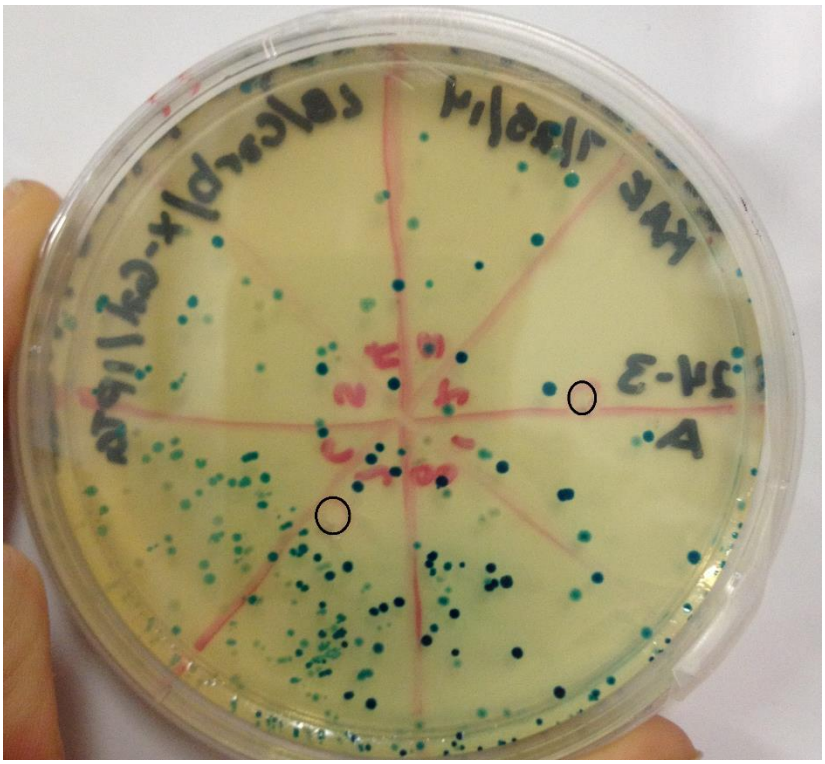

Figure 3: Example of a Blue/White Screening

Insert+pGEMT Easy ligations were transformed into JM 109 High efficiency competent cells. The white colonies (black circles) were considered potential positives and cultured. The blue colonies contained plasmids that lacked insert.

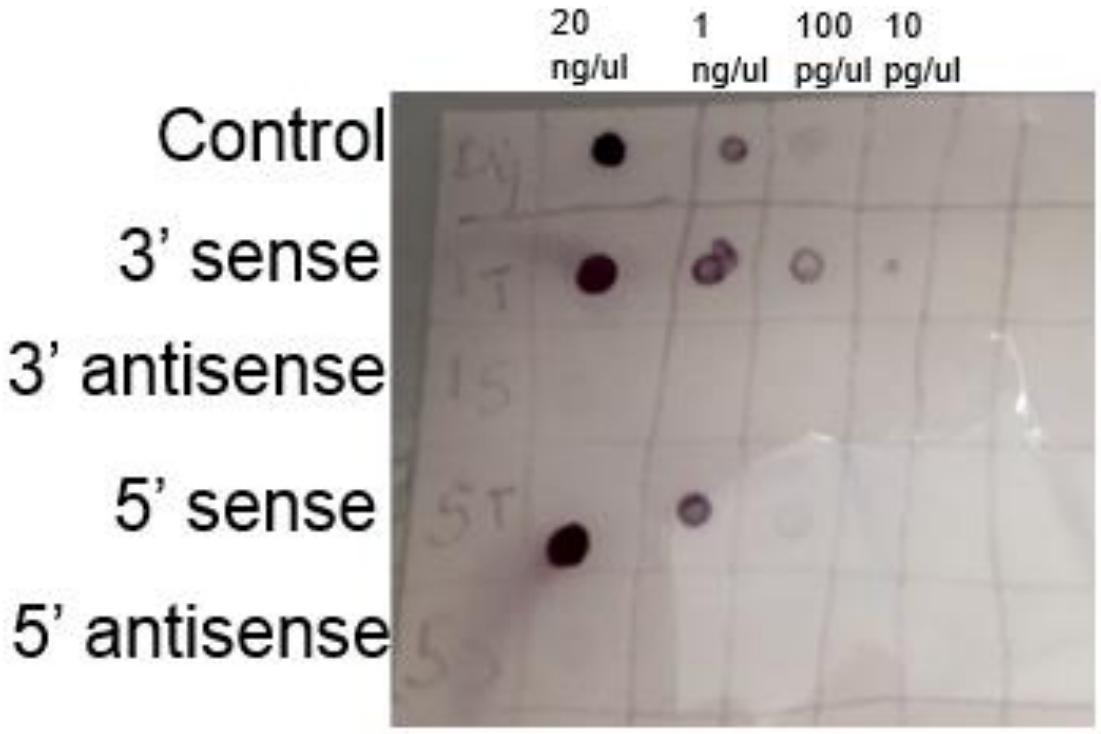

Figure 4: Dot Blot to determine probe concentration

The purpose was to quantify the amount of probe present for use in in situ hybridization. Although the antisense probes were weak they were recorded at about 100 pg/µl and used for in situ hybridizations.

Results

As referenced in the introduction, *Zmtcptf24* is closely related to *TCP4* in Arabidopsis. In plants with reduced miR319 levels, *TCP4* is overexpressed, resulting in loss of petal growth (Nag et al., 2009). Due to their phylogenetic relatedness, I hypothesize that *Zmtcptf24* in maize functions similarly in floral development. We are investigating *Zmtcptf24* and if/how it contributes to the developmental defects found in *fzt*.

fzt has decreased levels of several miRNAs that are thought to have key roles in development, including miR319. We hypothesize that misregulation of mRNAs targeted by the miR319 and other miRNAs are responsible for the inflorescence defects in *fzt.* Based upon RNA-seq data in *fzt*, miR319 target mRNAs are not upregulated. RNA-seq functions by taking a snapshot of the transcriptome at a certain point in time (Wang et al., 2009). Predicted miR319 targets may not have been upregulated because the time the data was accrued was not a developmental period miR319 regulated *tcps* were functioning at a high level. In situ hybridization allows specific cells to be analyzed for expression at multiple developmental stages. This allows us to examine if miR319 regulation only occurs in a few cells in a critical time period, which would not be revealed in RNA-seq data, which uses whole tissues.

Based on previous RNA-seq analysis for GRMZM2G015037, *Zmtcptf24* is expressed strongly in the immature tassel and immature cob. Mild expression was also found at the base of the stage two leaf and immature leaf (Sekhon et al., 2011). To determine where *Zmtcptf24* is expressed during tassel development, I performed in RNA situ hybridization. I designed probes that targeted the 5' and 3' UTRs of *Zmtcptf24* because they were unique to the *Zmtcptf24*

mRNA. A dot blot was performed to determine the starting concentration of the antisense probe, which will bind to the *Zmtcptf24* mRNA, and it was 100 pg/µl.

I have performed in RNA in situ hybridization on tassel primordia that contains floral meristems and developing floral organs. Within the developing floret, expression is seen in developing floral primordia, including the carpel and stamens. Expression of *Zmtcptf24* appears in developing lemma and palea. Figure 5B shows a magnified floret from figure 5A and clearly identifies structures within the upper and lower florets enclosed by the glume. *Zmtcptf24* is not expressed in spikelet glumes (Figure 5B). The expression in figure 5C also shows two florets, an offset upper floret and a lower floret, that reinforce the observations seen in the other sections; there is clear expression in the two carpels and stamens. Staining is observed in the vasculature at the base spikelet pairs (Figure 5D). These results are consistent with the hypothesis that *Zmtcptf24* has a potential role in maize floral development. Thus far, I have only performed in situs on one developmental stage of developing tassels. Further investigation in different aged tissue will need to be conducted to reinforce the function of *Zmtcptf24* in maize and its function in floral development.

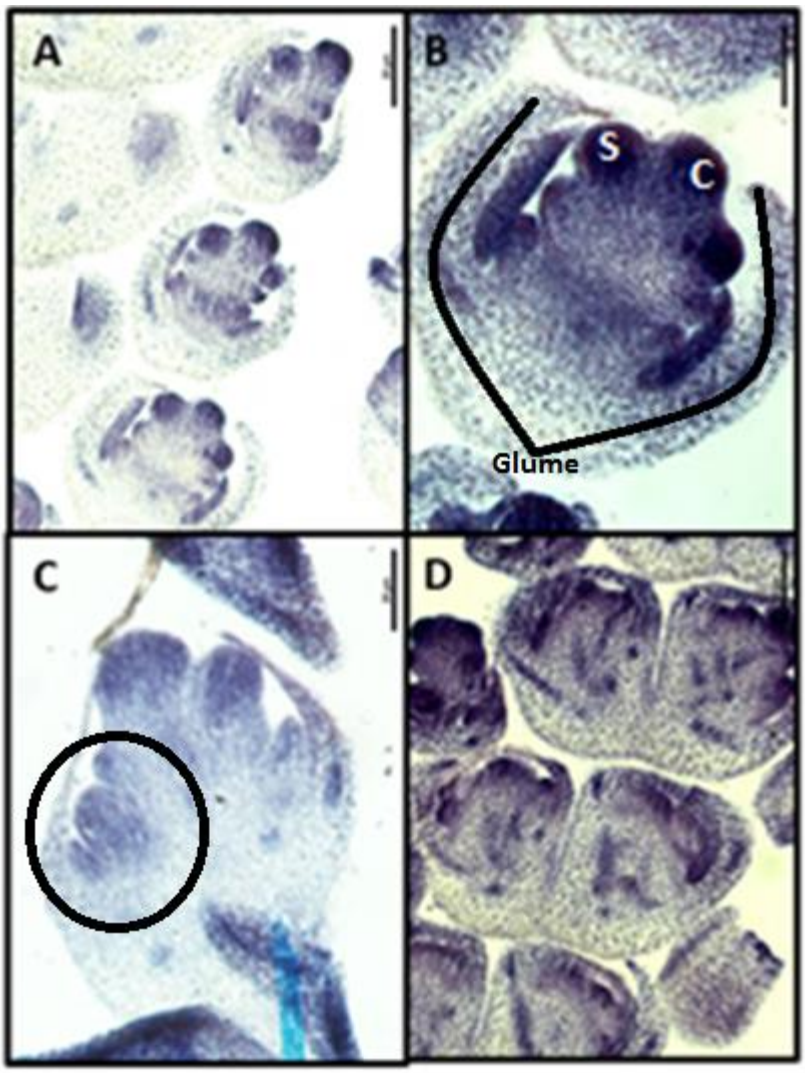

Figure 5: **Results of in situ hybridization on *Zmtcptf24***

(A) A section of three spikelets on a normal tassel. (B) A magnified view of the bottom spikelet on image A. The carpal is identified by a ‘C’ and the stamens are identified by an ‘S’. The glume, which did not exhibit any staining, is outlined in black. (C) A section of another spikelet that distinguishes an upper and lower floret, which is outlined with a black circle. (D) A section of a spikelet pair exhibiting vascular expression.

Discussion

TCPs are plant-specific transcription factors that contain a basic-helix-loop-helix domain. TCPs are named for their founding members: TEOSITE BRANCHED 1 (TB1) from maize, CYCLOIDEA (CYC) from snapdragons, and PROLIFERATING CELL FACTORS 1 and 2 (PCF1 & PCF2) from rice (Cubas et al, 1999). There are two classes of TCPs, class I and class II, that are differentiated by their amino acid sequence (Danisman et al., 2012). Class I TCPs include TCP transcription factors with a PCF1 domain. The class II TCPs include TCP transcription factors with a CIN domain, along with TB1/CYC (Martín-Trillo & Cubas, 2010). Both classes of TCPs regulate various aspects of plant development, including cell division, differentiation, and floral development (Schommer et al., 2012; Nag et al., 2009)

In Arabidopsis and other known species, class II TCPs are targeted by miR319 and have key roles in leaf and flower development (Nag et al., 2009; Ori et al., 2007). The focus of this study, *Zmtcptf24*, is closely related to Arabdiopsis *TCP4*, suggesting that *Zmtcptf24* might share some roles with *TCP4* (Nag et al., 2009). *TCP4* functions by regulating the inflorescences and patterning stamens and carpels. *TCP4* mutants that are resistant to miR319, exhibit severe developmental defects, and the inability to regulate *TCP4* results in the failure of stamen and petal development (Nag et al., 2009; Palatnik et al., 2003).

The maize genome is predicted to contain 7 class II *tcp* genes, 5 of which are predicted targets of miR319. *Zmtcptf24* is closely related to *Zmtcptf33*, suggesting that the genes might function redundantly (K. Novitzky, unpublished). Other redundancies have been identified through analysis of *TCP* genes in Arabidopsis. In the *jaw*-D mutant of Arabidopsis, miR319 is overexpressed and expression multiple *TCP* genes are reduced. resulting in a crinkled leaf phenotype (Rodriguez et al., 2013). In contrast, single mutants in *TCP* genes have very mild or

no phenotypes and the leaf shape is normal (Schommer et al., 2008). These analyses support the conclusion that *TCP* genes function redundantly (Schommer et al., 2008; Martín-Trillo & Cubas, 2010).

To investigate possible function of *Zmtcptf24* in maize, I performed RNA in situ hybridization on developing maize tassels. In these preliminary experiments, I observed *Zmtcptf24* expression in multiple organ primordia; carpels, stamens, palea, and lemma of both upper and lower floral meristems. Possible expression was seen in the lodicules, which are analogous to petals (Ambrose et al, 2000). The expression of *Zmtcp24* in the developing primordia is not a confirmation of gene function. In combination with the known function of *TCP4* in Arabidopsis, however, this analysis supports the hypothesis that *Zmtcptf24* plays a role in floral development.

To further identify and verify the location in the tassel where *Zmtcptf24* is expressed, in situ hybridization needs to be performed on tassels in various stages of development. *Zmtcptf24* may only be identified during a short time period in development and a sequence of different aged tassels would help identify what stage of development it is most expressed in, if not all. It is also important to take sections of the ears to see if expression is found in both inflorescences. To be accurate, multiple ages of the ears need to be examined as well, to see if *Zmtcptf24* expression is patterned similar to the tassels, and what role that could play in organ primordia development.

With the first round of in situs complete, early results showed possible expression in the vasculature at the base of the florets. To verify this putative vascular expression, transverse sections would help confirm this expression. Transverse sections would also be helpful to confirm the putative expression in developing carpels, stamens, palea, lemma, and lodicules (See Figure 6A).

The shoot apical meristem (SAM) is required for all aerial development and growth. Like all meristems, the SAM maintains a population of stem cells and also produces organ primordia (leaves). Because TCPs, have well-established roles in leaf development, *Zmtcptf24* might also be expressed in SAMs or leaf primordia. To analyze the role *Zmtcptf24* plays in the SAM, in situ hybridization can be performed on transverse and longitudinal sections of shoot apeices. Since most leaf patterning is completed in young leaves, TCP expression will most likely be identified in developing leaves

fzt mutants contain a mutation in DCL1, which encodes a key enzyme required for miRNA biogenesis. Some miRNAs are dramatically reduced in *fzt*, including miR319, which is reduced approximately 8-fold in tassel primordia (Thompson et al., 2014). Due to the known roles of TCPs targeted by miR319 in Arabidopsis, we hypothesize that some of the *fzt* phenotypes are due to increased expression of miR319 target mRNAs, including *Zmtcptf24.* RNA-seq data indicates that *Zmtcptf24* or other TCPs are not overexpressed in *fzt* mutants, at least at the level of whole tassel primordia. Further analysis through in situ hybridization is necessary to give us a spatial view of *Zmtcptf24* expression. *Zmtcptf24* may only be expressed in a few cells within a tissue or at a specific developmental time period. Although the RNA-seq data did not differentially express the presence of TCPs in whole tissue, early results show that *Zmtcptf24* is expressed in the developing tassel.

To support hypotheses that *Zmtcptf24* has a role in developmental defects in the *fzt* mutant, in situ hybridization would need to be performed on the *fzt* mutant. Knowing what expression is seen in the normal maize tassels and developing primordia, and comparing it to the expression in *fzt* tassels and developing primordia, will give insight into the genes functions. To do this however, in situ hybridization would need to be performed on the same aged *fzt* tissue

and tissue sections to be successful. If the function of *Zmtcptf24* is repressed in normal tassels due to regulation by miR319, I would hypothesize that altered expression of *Zmtcptf24* would be identified in *fzt*. Since DCL1 is not functioning in *fzt* miR319 is unable to regulate the targeted TCPs, including *Zmtcptf24*. With early in situs complete on developing normal tassels, and expression identified in developing floral primordia, I hypothesize that floral expression in more regions would be identified in *fzt*, due to the suppressed function of miR319 by DCL1.

The overall goal of this project is to determine the expression *Zmtcptf24* in maize inflorescence development in both normal and *fzt* mutant inflorescences. These experiments will potentially give insights into the role of *Zmtcpt24* in normal development and also test the hypothesis that overexpression of *Zmtcptf24* contributes to *fzt* phenotypes.. The purpose of learning more about the genes required for normal inflorescnce development is because they can provide insight into future improvement of the crop. *TCPs* that function in organ primordia may give insight into how modification and maintainence can improve robust floral development. As with any crop, maize crops have limitations that can affect a high yield. Seeds (kernels) are the product of a fertilized ovule, and determining seed number relies on learning about the activity of meristems and the number of flowers, thus determining plant structure. Overall, an ear that produces a higher amount of kernels would be admirable, and modification of these *TCP* genes in future crops could ensure that end result.

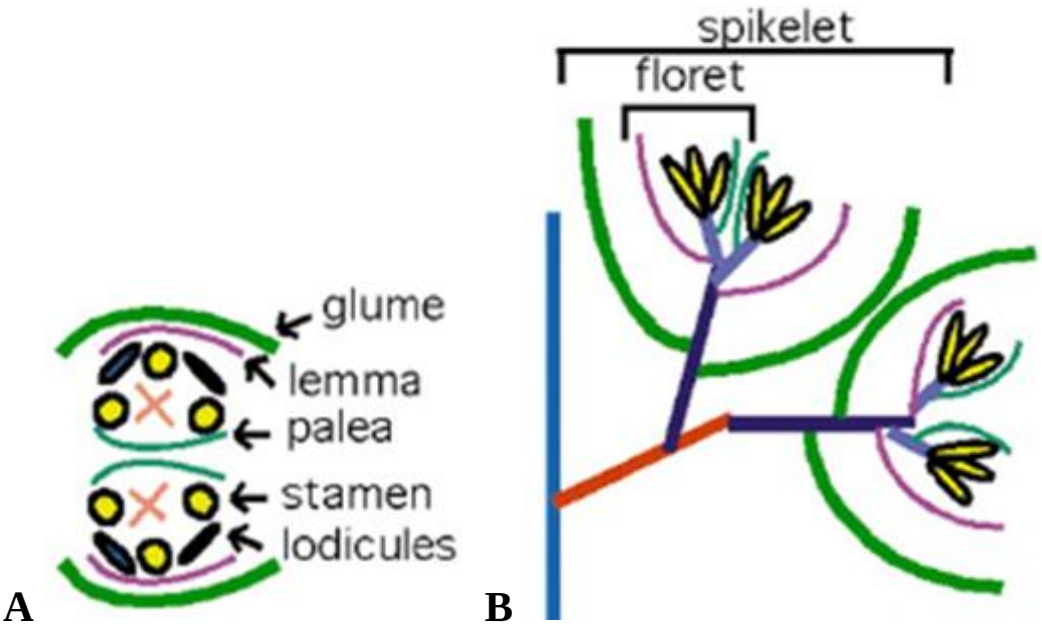

Figure 6: Transverse section and structure of an upper and lower floret

In figure 6A a hypothetical transverse section of the florets would show the above image, all developing structures in the floret would be visible. The sections used for in situs are taken longitudinal and use a whole spikelet, see figure 6B. Since the actual spikelet is three-dimensional, structures like the stamen jut out, making it difficult to get an accurate section showing all developing structures.

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Appendix:

A. Phusion PCR

- Individually add 2µl 10mM Forward Primer and 2µl 10mM Reverse Primer to the microcentrifuge tubes (Primer sequences found in Table 1)
- Design a master mix of n+1, where n is the number of reactions, of template, dNTP's, 5X HF Buffer, and water
- Each 50µl reaction will contain 1µl A619 template, 5µl 2mm dNTPs, 2µl 10µM forward primer, 2µl 10µM reverse primer, 10µl 5X HF Buffer, 29.5µl nuclease free water, and 0.5µl Phusion polymerase
- Start the cycler, so it can get up to the proper temperature, prior to adding Phusion to the master mix (0.5µl per reaction)
- Add Phusion to the master mix and join by flicking
- Aliquot 50µL master mix to individual microcentrifuge tubes
- Place in cycler under the following cycling conditions:

    1. Initial Denature= 98˚C – 2:00 minutes
    2. Denature= 98˚C – 10 seconds
    3. Annealing = 62˚C – 15 seconds
    4. Extension = 72˚C – 15 seconds
    5. Repeat steps 2-4 35 times.
    6. Final Extension = 72˚C – 5:00 minutes
    7. Hold = 4˚C

B. A tailing PCR Product

- Make a 10µl mixture of 2µl DNA Fragment, 2µl 2mm dATP's, 2µl 5X HF Buffer, 0.6µl $MgCl_2$, 2.4µl nuclease free water, and lastly 1µl Taq Polymerase
- Incubate in cycler for 30 minutes at 70˚C
- Remove and store on ice at 4˚C

C. Ligation and Transformation to clone insert into pGEM-T Easy (Promega)

- Centrifuge vector and control insert
- Vortex 2X rapid ligation buffer
- Add 5µl of 2X rapid ligation buffer, 1µl pGEM easy, 3µl PCR product, and 1µl of T4 DNA ligase into a microcentrifuge tube
- Set up a positive control with 5µl of 2X rapid ligation buffer, 1µl pGEM easy, 2µl control insert, 1µl T4 DNA ligase and 1µl of water to bring up to a 10µl reaction
- Mix reactions by pipetting
- Incubate at 4˚C overnight
- Prep LB/CARB plates by spreading 7µl (200mg/ml) IPTG and 40µl (20 µg/µl) X-Gal per plate.
- Centrifuge ligates and add 2µl to new labeled tubes on ice
- Remove JM109 high efficiency cells from -80 ˚C freezer and place on ice
- After cells have thawed, mix by flicking and remove 25µl to be added to ligation
- Incubate 20 minutes on ice
- Heat shock for 45 second in 42˚C water bath
- Return to ice for 2 minutes

- Add 950µl of SOC medium to reaction
- Incubate at 37˚C with shaking for 90 minutes
- Plate 100µl onto the prepared LB/CARB/XGAL/IPTG plates
- Incubate overnight at 37˚C

D. Miniprep protocol

- Pour cultured cells into labelled microcentrifuge tubes and centrifuge for 2 minutes at 8,000 rpm
- Remove excess media
- Resuspend cells in 250µl of Buffer 1
- Add 250µl of Buffer 2 and invert tubes 3-4 times to mix thoroughly
- Add 350µl of Buffer 3 and quickly mix by inverting 3-4 times
- Centrifuge tubes for 10 minutes at 4˚C and 13,000 rpm
- Pipette out the cleared lysate and transfer to a labelled DNA binding column
- Centrifuge at 13,000 rpm for one minute
- Pour off flow-through and reassemble
- Add 700µl of Buffer 4 to the DNA binding column
- Centrifuge at 13,000 rpm for one minute
- Pour off flow-through and reassemble
- Dry by centrifuging at 13,000 rpm for an additional minute
- Transfer the binding column to a new microcentrifuge tube and add 75µl of water and wait at least one minute for elution
- Centrifuge at 13,000 rpm for one minute
- Spec the DNA on the Nanodrop Lite to determine final concentration

E. In Vitro Transcription

- Mix a reaction using 4µl transcription buffer (with DTT and magnesium ions), 2µl DIG labeling mix, 1µl RNAsin, 1µl SP6/T7 polymerase, 1000ng of DNA template, and H_2O to 20µl
- Incubate the reaction at 37˚C for 2-3 hours in the thermocycler
- Remove 3µl of reaction to run on a gel later
- To stop the reaction add 75µl DEPC H2O, 1µl tRNA, and 1µl DNAse
- Incubate at 37˚C for 10 minutes
- Remove 2µl of the reaction to run against the 3µl initially removed to ensure the DNA template is removed
- Precipitate probe with 100µl 3M NaOAc and 200µl 100% EtOH
- Incubate for 1 hour at -20˚C
- Spin probe at 4˚C at maximum speed for 10 minutes
- Look for a pellet
- Wash pellet with 70% 100µl EtOH and spin again for 10 minutes
- Resuspend in 50µl of 50% foramide
- Speed Vac to remove excess ethanol
- Freeze at -20˚C

F. Dot Blot

- Start by preparing the five solutions as directed below:
    - Prepare a solution (1) of 100mM Tris (pH=7.5) +150mM NaCl by mixing 100ml of 1M Tris pH 7.5, 30ml of 5M NaCl, and DEPC water up to 1L

    - Prepare a block solution (2) with 20ml 100mM Tris pH 7.5 microwaved until warm. Add 0.04g of blocking Reagent and vortex until it goes into solution and store on ice
    - Prepare a Block + αDig Solution (3) with 10ml blocking solution and 2µl of αDig Store at 4˚C
    - Prepare a G3 solution (4) with 100mM Tris pH 9.5 (25ml), 100mM NaCl (5 ml), 50mM MgCl2 (12.5ml), and add DEPC water up to 250ml
    - Prepare a G3+NBT/BCIP solution (5) with 10 ml of G3 solution, and 235µL NBT/BCIP. Due to light sensitivity, wrap in foil and store at 4˚C
- Make a series of five tubes per primer being tested, along with a DIG control
- Perform a 1:20 serial dilution through each of the tubes
- Remove 1µl from each tube an sequentially dot on a nylon membrane
- Crosslink the RNA with UV light twice or incubate at 80˚C for 30 minutes
- Dampen the membrane with Solution 1 and add 10ml of Solution 2. Incubate with shaking at room temperature for five minutes
- Pour off the solution and add 10ml of Solution 3 and incubate with shaking at room temperature for another five minutes
- Pour off solution and wash twice with Solution 1 for a total of ten minutes
- Incubate at room temperature and shaking for five minutes with Solution 4
- Add Solution 5 and incubate at room temperature in the dark for 30 minutes - 2 hours
- Check dot for probe precipitation and to estimate the concentrations

G. In Situ Hybridization

Hybridization

- Dewax slides in histoclear for 10 minutes each. While they are dewaxing prepare the following:
    - Start by preparing a Pronase solution with 47ml of DEPC water, 2.5ml 1M Tris pH 7.5, 0.5ml 0.5M EDTA, and 157µl of 40mg/ml Pronase Stock
    - Prepare a 2% Glycine Solution with 0.1g Glycine, 5ml 10X PBS, and 45ml DEPC water
    - Prepare a Formaldehyde solution with 5ml 37% formaldehyde, 45ml PBS
- Place slides in a graded ethanol series for 2 minutes per step
    - 100% EtOH
    - 100% EtOH
    - 95% EtOH
    - 90%EtOH (5ml 8.5% NaCl, 45 ml 100% EtOH)
    - 80% EtOH (5ml 8.5% NaCl, 5ml DEPC water, 40ml EtOH)
    - 70% EtOH (5ml 8.5% NaCl, 10ml DEPC water, 35ml EtOH)
    - 50% EtOH (5ml 8.5% NaCl, 20ml DEPC water, 20ml EtOH)
    - 30% EtOH (5ml 8.5% NaCl, 30ml DEPC water, 15ml EtOH)
    - 0.85% NaCl (5ml 8.5% NaCl, 45ml DEPC water)
    - 1X PBS (5ml 10X PBS, 45ml DEPC water)
- Wash in the Pronase solution for 15 minutes and prepare the acetic anhydride treatment (592ml DEPC water, 8ml triethanolamine then pH to 8.0 with about 2ml 6M HCl)
- 2 minutes in 2% Glycine (50ml 1X PBS and 0.1g of Glycine)

- Under the hood, wash for 10 minutes in the 3.7% formaldehyde solution
- Wash for 2 minutes in 1X PBS
- Suspend slide in acetic anhydride treatment over a stir bar. Slowly drip 3ml of Acetic anhydride over the slides and incubate for 10 minutes
- Grab a bucket of ice and warm the heat block to 80˚C during the Acetic Anhydride step
- Wash for 2 minutes in 1X PBS
- Repeat the two minute incubation of the graded ethanol series in reverse (0.85% NaCl to 100% EtOH)
- Dry with Kimwipes
- Wet paper towels with 50% foramide and prepare in a box
- Defrost Hybe solutions
- Make Hybe solution with 151µl in situ salts, 500µl deionized foramide, 250µl dextran sulfate after heating to 80˚C, 25µl denhardts solution, 12.5µl tRNA, and 87.5µl nuclease free water
- Prepare a probe solution of 4µl tiled probe 1, 4µl tiled probe 2, and 78µl 50% foramide
- Mix 160µl of hybe solution and 40µl of the probe solution for a total of 200µl. Apply to one slide in a T shape and sandwich them together.
- Suspend sandwiches in the slide box with dampened towels and incubate overnight at 55˚C
- Prepare a NTE solution with 50ml 4M NaCl, 4ml 1M tris, 800µl 0.5M EDTA, and water up to 400ml. Store overnight at 37˚C.
- Prepare 0.2X SSC with 3ml 20X SSC, 297ml milliQ water, and store overnight at 55˚C

Posthybridization

- Dip slides in warmed 0.2X SSC solution to separate
- Wash for 30 minutes at 55˚C in the hybe oven with gentle agitation
- Repeat once more and prepare the following solutions:
    - Blocking Reagent with 0.2g Roche blocking reagent, and 40ml TBS. Heat to 60˚C and stir for about an hour
    - RNase (20µg/ml) with 50ml NTE, 100µl RNase, and hold at 4˚C
    - Buffer A with 9.375ml 4M NaCl, 0.75ml Triton, 25ml Tris pH 7.5, 2.5g BSA, and 215ml water. Stir for about five minutes.
    - Detection Buffer with 25ml Tris pH 9.6, 6.25ml 4M NaCl, and water to 250ml
    - Prepare 50ml of 1X PBS and hold at 4˚C
    - Anti-DIG antibody with 1000µl Buffer A and 2µl anti-DIG fragments. Hold at 4˚C.
    - Prepare the detection buffer with substrate with 1000µl detection buffer and 20µl NBT/BCIT stock. Hold at 4˚C
- Wash slides for 5 minutes in NTE at 37˚C with agitation
- Repeat
- Rinse 30 minutes with RNase at 37˚C with gentle agitation
- Rinse 5 minutes in NTE at 37˚C with gentle agitation
- Repeat
- Wash 1 hour in 0.2X SSC at 55˚C with gentle agitation
- Incubate at 4˚C in 1X PBS

- Place slides in a Copeland jar with blocking reagent and rock at room temperature for 45 minutes
- Replace blocking reagent with Buffer A and wash at room temperature for 45 minutes with rocking in Tupperware
- Make a regular sandwich with anti-DIG fragments
- Incubate in slide box over wet paper towels at room temperature for one hour
- Drain slides with kimwipes and place in Tupperware
- Wash with Buffer A for 20 minute at room temperature
- Repeat three times
- Wash for 10 minutes in detection buffer with rocking at room temperature
- Make sandwiches with detection buffer and substrate (about 100-150µl)
- Incubate overnight in the dark in the slide box at room temperature

Stopping Reaction

- Dip the slides in water and carefully separate
- Perform a graded ethanol series for 20 seconds each from 0.85% NaCl to histoclear
- Allow slides to dry on kimwipes
- Mount slides with long coverslips and approximately 100µl permount
- Keep in slide box and allow to dry overnight.