ABSTRACT

MADS-box transcription factors are important regulators of flower development in all flowering plants. In the grasses, flowers (called florets) are contained in spikelets. Maize spikelets contain two florets (the upper and lower florets) that are morphologically identical, although development of the lower floret is delayed compared to the upper floret. Floral meristems are groups of undifferentiated cells that give rise to floral organs. *bearded-ear (bde)* encodes a MADS-box transcription factor required for multiple aspects of floral development. *bde* mutants affect the upper and lower florets differently, suggesting the gene regulatory network in the upper and lower floral meristems are different. In addition, two other MADS-box transcription factors (*zmm8* and *zmm14*), are expressed only in the upper floral meristem (UFM), but not in the lower floral meristem (LFM). Together, these data suggest that the gene regulatory networks in the UFM and LFM are distinct and some genes, including MADS-box genes are differentially expressed. The long-term goal of this research is to globally identify genes specifically expressed in the UFM and LFM. Floral meristems cannot be manually dissected, so we are using laser capture microdissection (LCM) to specifically isolate UFM and LFM. LCM allows specific cells to be isolated from fixed, sectioned tissue using a laser. This tissue can then be used for downstream applications, including RNA isolation. The goal of this project is to isolate UFM and LFM using LCM and test for the expression of maize MADS-box transcription factors using RT-PCR and qPCR.

I have optimized fixation and RNA isolation protocols for LCM, and we have isolated RNA from sectioned tissue. In addition, we have successfully isolated UFM and LFM from sectioned tissue using LCM, and extracted RNA for amplification. We have tested for the expression of several control genes using quantitative RT-PCR (qRT-PCR). *zmm8* and *zmm14*, which were initially thought to be expressed exclusively in the UFM, were observed in the LFM albeit at much lower levels. Other spikelet meristem genes like *ids1* and *bd1* were also expressed in the UFM and LFM. However, *ral* and *ra2*, which are expressed in the SPM and in the anlagen of the SM were not observed in the mixed meristems (MM) containing a mixture floral meristems and spikelet meristems or the UFM and LFM. *Pepcase1* and *zmTIP2-3*, which are expressed in the leaves and roots respectively were not observed in any floral meristems. qRT-PCR results showed that expression of *zmm8* and *zmm14* in the LFM is much lower than in the UFM.
zmMADS3 was observed in both UFM and LFM but there was no significant difference in levels of expression in the two floral meristems. Together, these data suggests differential gene expression in the UFM and LFM may be studied by looking at the expression level of genes in the two floral meristems and not simply by looking at the absence or presence of a gene.
USING LASER CAPTURE MICRODISSECTION (LCM) TO EXAMINE MADS-BOX GENE EXPRESSION IN THE UPPER AND LOWER FLORAL MERISTEMS OF MAIZE.

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Master of Science in biology

by
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USING LASER CAPTURE MICRODISSECTION (LCM) TO EXAMINE MADS-BOX GENE EXPRESSION IN THE UPPER AND LOWER FLORAL MERISTEMS OF MAIZE.

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DEDICATION

I dedicate this thesis to my husband Kafui, our daughter Eyiram and our son Mawuli. I love you guys so much.
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CHAPTER 1
INTRODUCTION

Maize (corn) is an important crop in the modern world. It belongs to the grass family Poaceae (alongside wheat, rice, millet and sorghum). It is one of the most economically important and extensively grown grain crops in the world. Its uses range from fuel production to food for both man and livestock. Additionally, the widespread availability of molecular resources and modern genetic tools makes maize indispensable model plant for studies within the grasses. In this project, I used maize as a model to understand the molecular regulation of floral development in the grasses. Specifically, I investigated the expression of a key class of floral regulators, MADS-box transcription factors, in the upper and lower floral meristem of maize using Laser Capture Microdissection (LCM).

The ABC model of floral development.

Flower formation in eudicots is a complex process, which is regulated by several genetic factors. Flowers develop from a group of undifferentiated stem cells called meristems which give rise to floral organs. There are floral meristem (FM) identity genes (like AP1) which specify floral meristems identity and there are also floral organ identity genes which are responsible for specifying floral organ identities (Irish, 1998).

A typical eudicot flower is composed of four different types of organs arranged in four whorls. The first whorl is composed of a leaf-like structure called the sepal, the second whorl is composed of petals, the third whorl is composed of stamens (the male reproductive organ), and the fourth whorl is composed of carpels (the female reproductive organ) from which the ovules and seeds develop (Fig. 1) (Theissen, 2001).
Early research into two eudicot species, *Arabidopsis thaliana* and *Antirrhinum majus* resulted in the development of the ‘ABC’ model to explain the genetic establishment of floral organ identity (Bowman *et al*., 1991; reviewed by Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). The research led to the identification of mutants which had homoetic transformation of floral organs.

Three main classes of mutations were identified and designated the letters A, B and C. Class A mutants (*i.e.*, *APETALAI* (*AP1*) and *AP2*) affect whorls 1 and 2 resulting in carpels in place of sepals and stamens instead of petals in whorl 2 (Bowman *et al*., 1991; Goto *et al*., 2001). Class B mutants (*i.e.*, *PISTILLATA* (*PI*) and *AP3*) affect whorls 2 and 3 resulting in the homeotic conversion of petals to sepals in whorl 2 and stamens to carpels in whorl 3 (Coen and Meyerowitz, 1991, Theissen, 2001). The Class C mutant, *AGAMOUS* (*AG*,) affect whorls 3 and 4 in which petals are developed in the third whorl instead of stamens and the carpels are replaced by sepals in the fourth whorl. In addition to controlling stamen and carpel organ identities, *AG* is also required for meristem determinacy (Weigel and Meyerowitz, 1994; Bowman *et al*., 1991). Meristem determinacy occurs when a group of undifferentiated cells produce a definite number of organs and therefore fail to initiate extra meristems or organs.

The ABC model suggests that all three classes of genes work in a combinatorial fashion to specify floral organ identity. Class A genes specify sepal identity, class A and B genes work together to specify petal identity, class B and C genes specify stamen identity and finally, class C genes specify carpel identity (Whipple and Schmidt, 2006). In recent times, two other classes of genes have been added to the ‘ABC’ model. These are class E and class D genes. Class D genes are required for ovule identity and class E genes work together with the ABCD genes to specify all the floral organs (Ditta *et al*., 2004; Dreni *et al*., 2007). The A, B, C, D and E classes of genes
are members of the MADS-box genes except for the class A gene, *AP2* (Reichmann and Meyerowitz, 1997; reviewed by Litt and Kramer, 2010; Ng and Yanofsky, 2001).

![Floral ABC model](image)

**Figure 1**: Floral ABC model: Class A and E genes specify sepal identity; class A, B and E genes specify petal identity; class B, C and E genes specify stamen identity; class C and E genes specify carpel identity; class D and E genes specify ovule identity.

**MADS-box Transcription Factors**

MADS-box genes are transcription factors, which control several developmental processes including inflorescence and flower development in plants are important regulators of flower development in flowering plants (reviewed by Shore and Sharrocks, 1995). The MADS-box domain is identified by a conserved 56 amino acid region found within the DNA-binding motif of a wide variety of eukaryotes. The term MADS refers to the founding members: MCM1 (yeast), AGAMOUS (Arabidopsis), DEFICIENS (Antirrhinum) and SRF (human) (Schwarz-Sommer *et al.*, 1990). There are two main classes of MADS-box genes, type I and type II genes. The type I genes include MCM1 from yeast and SRF from humans. Type II genes include MEFI and plant MIKC MADS domain genes. In addition to the MADS-box, the MIKC MADS-box genes contain four functional domains (Fig. 2). The structure of the domain consists of the MADS-box, Intervening, Keratin-like and C-terminal domains (Theissen *et al.*, 1996; Becker
and Theissen, 2003). The MADS-box domain is highly conserved and its function is to bind DNA. The I and K domains are protein-protein interaction domains and required for dimerization. The C-domain is more variable and is involved in the formation of high-order protein complexes and transcriptional activation of some MADS domain proteins (reviewed by Kaufmann et al., 2005; Immink et al., 2010). The binding site for MADS-box transcription factors is an A-T rich region in the DNA called the CArG box (Shore and Sharrocks, 1995; Reichmann and Meyerowitz, 1997). Most plant MADS-box genes are known to recognize and bind specifically to the main consensus sequence, CC(AT)$_n$GG (de Folter and Angenet, 2006; Reichman and Meyerowitz, 1997; Shore and Sharrocks, 1995). All MADS-domain proteins have the ability to form homo- or heterodimers that bind to DNA. The MIKC MADS-box proteins can form dimers and high order complexes (reviewed by Kaufmann et al., 2005, Egea-Cortines et al., 1999).

| DNA binding |
|---|---|---|---|
| **MADS** | **I** | **K** | **C** |

|  | Dimerization | Multimerization and transcriptional activation of some MADS-domain proteins |

**MADS-box Intervening Keratin-like C-terminus domain**

Figure 2: MIKC MADS-box gene domains: The MADS-domain binds DNA; the I and K-domains are required for dimerization; the C-domain is required for the formation of higher order protein complexes and the transcriptional activation of some MADS domain proteins.
Maize Inflorescence development

Maize is a monoecious plant that develops two distinct mature inflorescences, the tassel and the ear. The tassel bears the male (staminate) flowers and is located at the apex of the plant whereas the ear bears the female (pistillate) flowers and is found in the axils of vegetative leaves (Bonnet, 1953). Despite the morphological differences between the two mature inflorescences, development of the tassel and ear is similar. Inflorescence development is dependent on the activity of meristems, groups of undifferentiated cells that initiate other meristems or plant organs. Determinate meristems give rise to a defined number of floral organs whereas indeterminate meristems continuously produce meristems or organs (Bortiri and Hake, 2007).

The shoot apical meristem (SAM) at the apex of the plant converts into an inflorescence meristem (IM) that will form the tassel. The IM produces spikelet pair meristems (SPM), which in turn produce the SM (Fig.3). In the tassel, the IM initiates indeterminate branch meristems (BM) before switching to produce the SPM. Each SM first produces a pair of bract like organs known as the glumes and then initiates an upper and lower floral meristem (FM). The floral meristems make the floral organs. During floral development, the lower floral meristem (LFM) is initiated first, followed by the upper floral meristem (UFM). However, development of the LFM is delayed relative to the UFM. FM in both the tassel and the ear initiate both stamens and ears, and sex determination occurs by carpel abortion in the tassel and stamen arrest in the ear (Cheng et al., 1983; reviewed in Dellaporta and Calderon-Urrea, 1994). In addition, the lower floret aborts in ears resulting in a spikelet with a single floret (reviewed in Thompson et al., 2009).
Figure 3. Maize inflorescence development: A. Schematic of branching in the tassel and ear. B. SEM of immature tassel and ear showing meristems involved in maize inflorescence development. IM-Inflorescence meristem; BM-branch meristem; SPM-spikelet pair meristem; SM-spikelet meristems; FM-floral meristems; UFM-upper floral meristem; LFM-lower floral meristems.

MADS-box genes and grass floral development

Flowering plants are grouped into two subclasses, the eudicots and monocots. The eudicots and the monocots diverged about 200 million years ago (Wolfe et al., 1989). The grasses, which are monocots, have a derived floral structure called the floret. Core eudicot flowers are made up of the sepals, petals, stamen and carpel. However, grass flowers contain lemma, palea and lodicules in addition to stamens and carpels (Fig. 4). Lodicules are analogous to petals (Ambrose et al., 2000). Class B genes are highly conserved between the monocots and the dicots (Ambrose et al., 2000). The function of class C genes has been partly conserved but
subfunctionalized due to a duplication event in grasses (Mena et al., 1995; Yamaguchi et al., 2006). Despite the difference in floral morphology between the eudicots and the grasses, studies centered on floral development mutants suggests the conservation of some regulatory network that controls floral development between the two (Nagasawa et al., 2003; Ambrose et al., 2000; Li et al., 2011).

Although there is inadequate information about the role Class A genes play in grass floral development, research suggests they are expressed in the meristems of grasses other than floral meristems (Whipple and Schmidt, 2006). In core eudicots, class B genes specify stamen and petal identity. In class B mutants, there is homoetic transformation of stamens into carpel and petals into sepals (Bowman et al., 1991).

In addition to controlling stamen and carpel organ identities, Class C genes play a role in floral meristem determinacy. In Arabidopsis, both class C functions are controlled by a single gene AGAMOUS (AG) (Yanofsky et al., 1990). In maize, AG has two co-orthologs, zag1 and zmm2. zag1 mutants have indeterminate floral meristems indicating that zag1 is required for FM determinacy and that C-class function is at least partially conserved in maize. zag1 mutants do not have defects in floral organ identity, although it has been proposed that Class C function has been subfunctionalized in the grasses and zmm2 regulates floral organ identity (no zmm2 mutants exist). Indeed in rice Class C function has been subfunctionalized with OsMADS3 (Zmm2 ortholog) functioning in floral organ identity and OsMAD58 (zag1 ortholog) in floral meristem determinacy (Yamaguchi et al., 2006).
The AGL6 group of MADS-box genes also has key functions in floral development in monocots. AGL6-like genes play major roles in meristem determinacy and floral organ development in the grasses (Ohmori et al., 2009; Thompson et al., 2009).

Figure 4. Grass floral morphology: A. Cartoon of a grass floret showing the various floral organs. B. male spikelet showing the upper floret (UF) and lower floret (LF). Arrow heads indicate stamens and * indicate palea. Carpels are aborted in the tassel.

*bearded-ear (bde)*

*bearded-ear (bde)* is a MADS-box gene, which belongs to the AGL6 family of MADS-box genes (Becker and Theissen, 2003; Thompson et al., 2009). BDE is required for multiple aspects of floral development in maize. In *bde* mutants, the LFM in the tassel initiates extra FM which in turn make extra floral organs and occasionally silks. Additionally, *bde* mutants make extra silks (which are often sterile) in the ear (Thompson et al., 2009). Despite the morphological similarities between the UFM and LFM, *bde* (which is expressed in both florets) affects the two florets differentially. In the UFM, *bde* mutants initiate extra floral organ primordia; thus *bde* is required for FM determinacy and floral organ development in the UFM. In the LFM, *bde* mutants initiate additional meristems. FMs never initiate meristems indicating that in the LFM, BDE is required for FM fate (Thompson et al., 2009).
MIKC MADS-box proteins form dimers and high order complexes to regulate transcription of target genes (reviewed by Kaufmann et al., 2005; Honma and Goto, 2001). Dimerization is required for DNA-binding (Reichmann et al., 1996). According to the quartet model, MADS-box dimers come together to form a tetramer to regulate gene expression (Theissen and Saedler, 2001). This tetramer structure is achieved by the binding of two MADS-box dimers to different CArG boxes on the same molecule of DNA, which are brought close to each other by a mechanism of DNA bending (Honma and Goto, 2001; Theissen and Saedler, 2001). Thus, the different phenotypes observed in the UFM and the LFM are likely the result of the disruption of distinct BDE-containing complexes with distinct target genes in the UFM and LFM (Thompson et al., 2009). The long-term goal of this research is to globally identify genes differentially expressed in the UFM and LFM.

Laser Capture Microdissection (LCM)

To help isolate UFM and LFM, we are using laser capture microdissection (LCM). Floral meristems cannot be manually dissected, so we are using laser capture microdissection (LCM) to specifically isolate these cells. LCM is a recently developed technique, which allows for the isolation of specific organs, cells or tissue for molecular analysis (Emmert-Buck et al., 1996). LCM has been used successfully to isolate a variety of plant cell types including maize (Nakazono et al., 2003; Ohtsu et al., 2007; reviewed by Day et al., 2005, Nelson et al., 2006; Schnable et al., 2004). Protocols exist for fixing tissue and isolating maize tissue using LCM (Kerk et al., 2003, Yu et al., 2007, Inanda and Wildermuth, 2004). Briefly, tissue is fixed, embedded in wax, sectioned and mounted on membrane slides (that allow for easier tissue capture after laser microdissection). During LCM, an instrument that uses UV laser energy is used to cut cells from the tissue mounted on the membrane slides and then catapult the dissected
cells into an adhesive cap (Fig.5). RNA from laser captured cells can be isolated using a variety of commercially available kits. For gene expression studies, several techniques such as microarrays, RNA in situ hybridization and northern blot analysis have been developed. Although these techniques provide information about the expression profile of the gene, the ability to dissect cell specific regions and analyze several genes at the same time has been a major drawback of some of these techniques. Additionally, some of these techniques are limited by their ability to quantify RNA expressed in specific domains, the amount (concentration) of starting RNA to be used and also the number of samples tested per given time. Small amounts of RNA can however be amplified to increase RNA quantity. Reverse transcription-PCR (RT-PCR) can be performed and subsequently used for molecular analysis (Fig.6). LCM has extensively been used in plants for gene expression studies (Nakanzo et al., 2003; Asano et al., 2002; Kerk et al., 2003).

Figure 5. Example of LCM dissection process A. B73 ear showing cells to be dissected (circled in yellow) B. Selected cells cut using laser energy. C. Selected cells catapulted in collecting microfuge tube cap. D. Laser-captured samples collected in microfuge tube cap.
The purpose of this thesis was to study the expression of a key class of floral regulators, MADS-box transcription factors, in the upper and lower floral meristem of maize using Laser Capture Microdissection (LCM). The importance of this experiment is based on knowledge that bde differentially affects the upper and lower florets of maize (Thompson et al., 2009).
OBJECTIVES

The objectives of this project were to:

1. Establish the feasibility of using Laser Capture Microdissection (LCM) to dissect maize upper and lower floral meristems.

2. Determine the expression of MADS-box genes in the upper and lower floral meristems of maize.
CHAPTER 2
MATERIALS AND METHODS

Seedling preparation for greenhouse planting

B73 seeds were bleached sterilized prior to planting in the greenhouse. Seeds were shook in distilled water for 2 hours. Viable seeds (seeds which did not float) were soaked in 10% bleach for 15 minutes followed by a wash in distilled water for 15 minutes (done five times) on a shaker. The seeds were air dried on a clean paper towel for about 12 minutes (making sure to turn them every four minutes) until totally dry. The seeds were then coated lightly with Captan® (an anti-fungal powder) in the hood and laid on clean wet (not over saturated) paper towels with the embryo-side facing upward in a glass dish. The glass dish was covered with saran wrap, secured tightly with a rubber band and placed in an incubator at 29°C for 2 days. After two days, some of the seeds had sprouted, and those which had not, had their pericarps removed using a pair of forceps. The seeds were put back into the incubator for 3 more days after which they were removed and planted in small pots with their shoots exposed above the soil and the roots completely submerged in the soil. A small amount of Osmocote plant food was added to the soil and watered. The pots with the seeds were transferred to a growth chamber (26°C) for about a week and half (and were watered daily). When the seedlings had developed they were transferred to the greenhouse where they were transplanted into larger pots under the supervision of the greenhouse manager until harvesting.

Harvesting tissue.

Approximately 1-2cm immature ears were harvested at 7-8 weeks from B73 plants in the greenhouse. This was done by making a horizontal cut through the second/third node above the roots of the maize plant using a sharp razor blade. The plants were submerged in water with the
bottom of the plant (freshly cut nodes) inside the water and top part (with the tassels) up. Leaves were removed from the plant and more cuts made from the bottom of the plant upward. Plants were brought into the lab shortly before fixation. Only immature ears (about 1-2cm) at the uppermost two nodes were harvested for fixation (gloves were worn at all times).

Tissue fixation.

I tested two types of fixatives, acetone fixative and Farmer’s Fixative (ethanol and acetic acid in a 3:1 ratio). However, upon mounting on slides after sectioning, the acetone fixed samples became brittle and shattered thereby disrupting the integrity of sectioned inflorescence. Therefore I concluded that the ethanol: acetic acid fixative was more suitable for this experiment. Immediately after harvesting tissue samples, they were quickly placed into a vial containing 15mls of fresh ice-cold mixture of ethanol and acetic acid in a ratio of 3:1. Tissue samples (8-10 samples per vial) placed in the vial were kept on ice at all times. Total preparation time of seedlings took about 8-12 minutes. A small container containing ice and the vial (opened) were subjected to vacuum infiltration of 400mm Hg for 15 minutes and then slowly brought back to room pressure. After vacuum infiltration, old fixative was removed and fresh fixative was added; samples were incubated in fixation solution on a rotator at 4°C for 1 hour. The vacuum infiltration process and fixative change were repeated two more times. After the final vacuum infiltration, fixation change, samples were incubated overnight at 4°C on a rotator.

Dehydration

After fixing tissues, the samples are dehydrated down to 100% ethanol. This was done by pouring away half the volume of fixative in the vial and replacing it with 100% ethanol at room temperature (ice-cold 100% ethanol was used for subsequent changes). The vials with the samples are then incubated at 4°C for 2 hours on a rotator. These steps were repeated twice. All
of the remaining solution was poured off and replaced with 15mls of ice-cold 95% ethanol and incubated at 4°C for 2 hours on a rotator. All of the 95% ethanol was replaced with ice-cold 100% ethanol and incubated at 4°C for 2 hours on a rotator. Another 100% ethanol change was performed and left on a rotator overnight in a cold room.

Xylene infiltration

The vials were removed from the cold room and allowed to warm up to room temperature on a rotator. All of the ethanol was poured off and replaced with 100% ethanol at room temperature (all subsequent incubations were performed in a hood at room temperature). Samples were incubated for 1 hour on a rotator. ¼ the volume of ethanol was removed, replaced with 100% xylene (this was performed in the hood) and incubated at room temperature for 1 hour on a rotator. I continued to replace ¼ the volume of ethanol/xylene mixture 4 more times. The remaining solution was poured off and replaced with 100% xylene at room temperature and incubated on a rotator for 1 hour. This process was repeated one more time. The solution in the vial was replaced with fresh 100% xylene, filling it less than half way. Paraplast chips (McCormick Scientific paraplast plus Tissue embedding medium) were added to xylene in the vial up to the level of the liquid and left to dissolve overnight on a rotator at room temperature.

Paraplast infiltration and embedding

The vial was placed in a 60°C oven to melt chips which had not dissolved. When all the chips had melted, the vial was gently inverted back and forth to evenly mix the xylene and the paraplast. After about an hour and a half, more paraplast chips were added to the vial for melting and left in the oven for an additional hour and a half to melt. Half of the mixture was then poured off and replaced with 100% molten paraffin, mixed by inversion and left overnight in the 60°C oven. The next day, all of the mixture was replaced with pure molten paraplast. A total of four
pure paraplast changes were made, each lasting 4 hours. The following day, one more pure paraplast change is made. After the final paraplast infiltration, metal weight boats were placed on a slide warmer at 60°C. Molten paraplast containing samples was poured into the weigh boat less than half way. Half of the paraplast in the vial was discarded and the rest of the solution containing the tissue, quickly poured into the weigh boat with the molten paraplast. The tissues were oriented in the weigh boat using a pair of RNAse treated tweezers. The slide warmer was turned off, and the weigh boats were allowed to cool down to room temperature. Once thoroughly cooled, embedded samples were stored in a plastic bag at 4°C.

Sectioning

Blocks containing inflorescences were cut from paraffin blocks using a razor blade. Paraplast blocks containing individual inflorescences were melted onto already prepared wax blocks and allowed to cool down to room temperature. The blocks were kept in a plastic bag at 4°C until they were ready to be sectioned. For sectioning, tissues in paraplast blocks were trimmed down into a narrow trapezoid shape with parallel horizontal cuts at top and bottom and slanted cuts on the sides. The blocks were sectioned with on a Reichert-Jung (Leica) 2030 rotary microtome. Sections were cut 8-10µm thick; 8um sections catapulted more successfully in LCM. Ribbon sections were placed on a Zeiss Membrane Slide 1.0 polyethylene naphthalate -PEN (D). Slides are baked in an oven at 180°C for 2 hours and then UV treated for 30 minutes to make them hydrophilic and also kill RNAses. The ribbons were floated on 70% ethanol (made with RNAse free water) on a slide warmer for 5 minutes until the sections stretched. To remove the ethanol, a pipette was used to suck up the water on the slides (while being careful not to puncture the membrane). Residual ethanol was removed by tipping the end of the slide onto a kim wipe.
The slides were immediately placed back onto the slide warmer and allowed to dry for 30-40 minutes. Slides were used for LCM immediately after drying.

Laser Capture Microdissection (LCM)

LCM was performed at Brody School of medicine using a Zeiss PALM Micro Beam System. Prior to dissection, sections were deparaffinized in two changes of 100% xylene, each lasting for 3 minutes. The slides were dried in the hood and placed on the microscope stage to select tissue regions to be collected. UFM and LFM were collected at a similar developmental stage in which lemma primordia were present, but no stamen primordia had been initiated. The cells were marked out using a drawing tool on the computer and then catapulted in a Zeiss Adhesive Cap 500 clear (D) using the RoboLPC feature on the computer. A minimum of 300,000 µm² of tissue was collected for each floral meristem replicate. UFM1 was dissected from two different inflorescences from batch 1 and 2, making a total of 363855 µm². UFM2 was dissected from one inflorescence from batch 2 (different inflorescence used in UFM1), making a total of 469844 µm². UFM3 was dissected from two different inflorescences from batch 2 (same inflorescence used in UFM2) and 3, making a total of 400000 µm². UFM4 was dissected from one inflorescence from batch 4, making a total of 409588 µm². LFM1 was dissected from one inflorescence from batch 5, making a total of 383234 µm². LFM2 was dissected from one inflorescence from batch 4 (different inflorescence used in UFM4), making a total of 392885 µm². LFM3 was dissected from two inflorescences from batch 4, making a total of 393507 µm². LFM4 was dissected from two different inflorescences from batch 4 (same inflorescence used in LFM3) and 5, making a total of 391040 µm². Remaining tissue on the slide was scrapped off the slide as control for RNA integrity and RT-PCR (LS).
RNA extraction and isolation

RNA was extracted and isolated using the Arcturus Pico Pure RNA extraction kit. Immediately after collecting tissue, the caps were removed from the microscope stage and 25 µl of extraction buffer was added to the samples. For tissue scrapped off the slide, 50 µl of extraction buffer was added. Tubes were inverted and incubated on a slide warmer at 42°C for 30 minutes. Samples were then centrifuged at 800 x g for two minutes to collect cell extract into the microcentrifuge tube. After centrifugation the tubes were kept on dry ice and stored at -80°C. To isolate RNA, the RNA purification column was preconditioned by pipetting 250µl conditioning buffer onto the purification column filter membrane and incubated for 5 minutes. The purification column in a collection tube was centrifuged at 16,000 x g for 1 minute. For laser captured samples, 25µl of 70% ethanol was pipetted into the cell extract in the microcentrifuge tube and mixed well by pipetting up and down (samples were not centrifuged). 50 µl of 70% ethanol was pipetted into cell extract from tissue scrapped off the slide. The cell extract and ethanol mixture was pipetted onto the preconditioned purification column. To bind RNA, the purification column was centrifuged at 100 x g for 2 minutes, immediately followed by a centrifugation at 16,000 x g for 30 seconds to remove flow through. 100µl of wash buffer 1 was pipetted into the purification column and centrifuged at 8,000 x g for 1 minute. To DNase treat samples, the Qiagen RNAse-Free DNase set was used. 5µl of DNase I stock solution was added 35µl of Buffer RDD and mixed by inverting. 40µl of the DNase incubation mix directly into the purification column membrane and was incubated at room temperature for 15 minutes. 40µl of the Pico Pure RNA Kit wash buffer 1 was pipetted into the purification column membrane and centrifuged at 8,000 x g for 15 seconds. 100µl of Wash buffer was pipetted into the purification column and centrifuged for 1 minute at 8,000 x g. Another 100µl of wash buffer 2 was pipetted
into the column and centrifuge for 2 minutes at 16,000 x g. An additional spin at 16,000 x g for 1 minute was performed to remove residual wash buffer. The purification column was transferred to a new 0.5 ml microcentrifuge tube provided in the kit. 15 µl of elution buffer was pipetted directly onto the membrane of the purification column and allowed to incubate at room temperature for 1 minute. The column was centrifuged for 1 minute at 1,000 x g to distribute elution buffer in the column and then for 1 minute at 16,000 x g to elute RNA. 1 µl of RNase inhibitor was added to the sample.

To check the concentration of RNA isolated from tissue scrapped off the slide, the Qubit® 2.0 Fluorometer and Qubit™ RNA Assay kit were used. The Qubit working solution was made by diluting the Qubit RNA reagent 1:200 in the Qubit RNA buffer in a clean plastic tube. Two standards were used for this experiment. 190µl of Qubit working solution was carefully pipetted into tubes for the two standards and then 10µl of each Qubit standard was added to the appropriate tube. The tubes were mixed by vortexing 2-3 seconds, being careful not to create bubbles. 199µl of Qubit working solution was carefully added to assay tubes and 1µl of sample was pipetted into the appropriate tubes. Sample tubes were mixed by vortexing 2-3 seconds, being careful not to create bubbles. They were allowed to incubate at room temperature for 2 minutes after which the concentrations of the assay tubes were measured using the Qubit® 2.0 Fluorometer. To assess the quality of RNA isolated from tissue scraped off LCM slides, samples were sent to the Genomics Core Facility in the biology department of East Carolina University to be ran on the Agilent 2100 Bioanalyzer. Floral meristems from tissue samples with RNA integrity number of 4 and above were used for subsequent experiments.
To amplify RNA isolated from laser captured upper and lower floral meristems, the Epicenter TargetAmp 2-Round aRNA Amplification Kit 2.0 was used. Isolated floral meristem RNA was concentrated down to a volume of ~1-1.5µl using a Thermo Scientific Savant DNA 120 SpeedVac concentrator at low heat. The first step was to reverse transcribe the poly (A) RNA component of the total RNA sample isolated from the upper and lower floral meristems using a synthetic oligo (dT) primer containing a phage T7 RNA polymerase promoter sequence at its 5’ end (T7-Oligo (dT) primer). The starting concentration of total RNA was 10-500pg. To anneal the TargetAmp T7-Oligo (dT) primer B to the RNA sample a mixture of 0.5µl of RNase-free water, 1.5 µl of total RNA sample and 1µl of the TargetAmp T7-Oligo (dT) primer B was incubated at 65ºC for 5 minutes in a thermocycler. The reaction mix was chilled on ice for 1 minute and briefly centrifuged in a microcentrifuge. The Round-One, 1st – Strand cDNA Synthesis Master Mix was prepared by mixing on ice, 1.5µl of TargetAmp Transcription PreMix-SS, 0.25µl of DTT and 0.25µl of SuperScript III Reverse Transcriptase (200U/µl) from Life Technologies. The reaction mix was gently mixed and 2µl of it added to each reaction. The reaction was mixed gently and incubated at 50ºC for 30 minutes in a thermocycler. The 2nd – Strand CDNA synthesis master mix was made by combining on ice 4.5µl TargetAmp DNA Polymerase Pre-Mix-SS1 and 0.5µl of TargetAmp DNA Polymerase-SS1. 5µl of the mixture was added to each reaction, gently mixed and incubated at 65ºC for 10 minutes in a thermocycler. The reaction was briefly centrifuged after the incubation period and then incubated at 80ºC for 3 minutes. The reaction was centrifuged again after incubation and chilled on ice for 2 minutes. 1µl of TargetAmp cDNA Finishing Solution-SS was added to each reaction gently mixed and then incubated at 37ºC for 10 minutes. The reaction was transferred to 80ºC for 3 minutes,
centrifuged briefly and chilled on ice for 2 minutes. To prepare the Round-One *In Vitro* Transcription Mater Mix, all reagents were thawed at room temperature and the TargetAmp T7 RNA Polymerase warmed to room temperature. 4µl of TargetAmp T7 Transcription buffer, 27µl of TargetAmp *In Vitro* Transcription Premix A, 4µl of DTT, 4µl of TargetAmp T7 RNA Polymerase and 1µl of RiboGuard RNase Inhibitor were combined at room temperature and gently mixed. 40µl of the mixture was added to each tube, gently mixed and incubated at 42°C for 4 hours in a thermocycler. 2µl of RNase-Free DNase I was added to each tube, gently mixed and incubated at 37°C for 15 minutes.

To purify RNA, the Qiagen RNeasy MinElute Cleanup Kit (Qiagen cat. no. 74204) was used. 350µl of RLT/β-ME Solution was prepared for each sample by combining 1ml of buffer RLT (from the MinElute kit) with 10µl of β-ME (β-mercaptoethanol). 650µl of RPE solution was prepared for each sample by diluting 1 volume of buffer RPE with 4 volumes of 100% ethanol. To each sample, 47.5µl of RNase-Free water, 0.5 µl of Poly (I), and 350µl of RLT/β-ME solution and 250 µl of 100% ethanol was added. The mixture was applied to an RNeasy MinElute spin column in a 2ml collection, centrifuged at 10,000 x g for 15 seconds and the flow-through discarded. 650µl of RPE solution was applied to the column, centrifuged at 10,000 x g for 15 seconds and the flow-through discarded. 650µl of 80% ethanol was added to the column, centrifuged at 10,000 x g and the flow-through discarded. The RNeasy MinElute spin column was transferred into a new collection tube and centrifuged at full speed for 5 minutes. The spin column was transferred to a 1.5 ml collection tube. Amplified RNA (aRNA) was eluted by applying 14µl of RNase-Free water directly onto the center of the silica gel membrane, incubated at room temperature for 5 minutes and centrifuged at full speed for 1 minute.
For Round-Two, 1\textsuperscript{st} Strand cDNA synthesis 2\mu l of TargetAmp Random primers was added to each sample. The mixture was transferred into a 0.2-0.6ml sterile tube and the volume of each aRNA sample adjusted to 3\mu l using a speed vacuum centrifugation with low heat. The reactions were incubated at 65\(^\circ\)C for 5 minutes in a water bath. Next, the samples were chilled on ice and centrifuged briefly in a microcentrifuge. To prepare the Round-Two, 1\textsuperscript{st} Strand cDNA synthesis master mix, 1.5\mu l of TargetAmp Reverse transcription PreMix-ss, 0.25\mu l of DTT.0.25\mu l of SuperScript II Reverse Transcriptase from Life technologies (200U/\mu l) was combined on ice. 2\mu l of the mixture was added to each sample, gently mixed and incubated at 23\(^\circ\)C for 10 minutes and then transferred to 37\(^\circ\)C for 1 hour in a thermocycler. 0.5\mu l of TargetAmp RNase H-SS was added to each sample, gently mixed and incubated at 37\(^\circ\)C for 20 minutes. The reactions were transferred to 95\(^\circ\)C for 2 minutes in a thermocycler, chilled on ice for 2 minutes and then briefly centrifuged in a microcentrifuge. To each reaction, 1\mu l of TargetAmp T7-Oligo(dT) Primer C was added, the reactions were gently mixed, incubated at 70\(^\circ\)C for 5 minutes and then at 42\(^\circ\)C for 10 minutes in a thermocycler. The Round-Two 2\textsuperscript{nd} Strand cDNA Synthesis master mix was prepared by combining 13\mu l of TargetAmp DNA Polymerase PreMix-SS 2 and 0.5\mu l of TargetAmp DNA polymerase-SS 2. The reaction was gently mixed and 13.5\mu l of it was added to each tube. The tubes were mixed gently and incubated at 37\(^\circ\)C for 10 minutes in a thermocycler after which they were centrifuged briefly. The reactions were transferred to 80\(^\circ\)C for 3 minutes, centrifuged briefly and chilled on ice for 2 minutes.

For final in vitro transcription of amplified RNA, reagents for this reaction were thawed to room temperature. The Round-Two \textit{In Vitro} Transcription (IVT) master mix was prepared by combining at room temperature 13.6 \mu l of RNase-Free water, 4\mu l of TargetAmp T7 transcription
buffer, 3.6µl of ATP, 3.6µl CTP, 3.6µl GTP, 3.6µl UTP, 4µl of DTT, 4µl TargetAmp T7 RNA Polymerase and 1µl of RiboGuard RNase Inhibitor. 40µl of the round-two IVT was added to each reaction, gently mixed and incubated at 42ºC for 9 hours in thermocycler. 2 µl of RNase-Free DNase I to each reaction, gently mixed and incubated at 37ºC for 15 minutes.

To purify aRNA, the Qiagen RNeasy MinElute Cleanup Kit (Qiagen cat. no. 74204) was used. A mixture of 38µl RNase-Free water, 350µl RLT/β-ME Solution and 250µl of 100% ethanol was added to each sample. The samples were applied to the RNeasy spin column in a 1.5ml collection tube, centrifuged at 10,000 x g for 15 seconds and the flow through was discarded. 700µl of RPE solution was applied to the column, centrifuged at 10,000 x g for 15 seconds and the flow through discarded. Another 700µl of RPE solution was applied unto the column, centrifuged for 10,000 x g for 2 minutes and the flow through discarded. The 1.5ml collection tube was reattached and the samples were centrifuged at full speed for 1 minute. The RNeasy spin column was transferred to a new 1.5ml collection tube. 25µl of RNase-Free water was pipetted directly into the column and then incubated at room temperature for 5 minutes. Next, the samples were centrifuge for 1 minute at 10,000 x g to collect aRNA. Amplified RNA was immediately placed on ice and its concentration measured using a Thermo Scientific NanoDrop LITE Spectrophotometer. To assess the size of aRNA produced, an aliquot of the aRNA was diluted to approximately 100ng/µl. Using the Agilent RNA 6000 Nano Labchip, 1µl of diluted aRNA was loaded per well. Only aRNA samples with size above 200bp or a smear between 200-500bp on the bioanalyzer were used for subsequent experiments.

Genomic DNA removal form UFM and LFM aRNA

The Thermo Scientific RapidOut DNA Removal Kit was used to remove genomic DNA contamination from amplified RNA samples. 4-5µl of aRNA was mixed with 10µl of 10X
DNase buffer with MgCl₂, 5µl of DNase I, RNase-free (5 U) and 84-85µl of nuclease-free water to make a final volume of 100µl. The mixture was vortexed gently and incubated at 37°C for 30 minutes. To remove DNase, 10µl of completely resuspended DNase Removal Reagent (DRR) was added to each reaction and incubated at room temperature for 2 minutes, gently mixing the reaction 2-3 times to resuspend the DRR. The reaction was centrifuged at 1000 x g for 1 minute to pellet DRR. The supernatant, containing DNA-free and DNase-free RNA was carefully transferred into a new tube without transferring the DRR.

To cleanup DNase treated aRNA, the Qiagen RNeasy MinElute Cleanup Kit (Qiagen cat. no. 74204) was used. A mixture of 38µl RNase-Free water, 350µl RLT/β-ME Solution and 250µl of 100% ethanol was added to each sample. The samples were applied to the RNeasy spin column in a 1.5ml collection tube, centrifuged at 10,000 x g for 15 seconds and the flow through was discarded. 700µl of RPE solution was applied to the column, centrifuged at 10,000 x g for 15 seconds and the flow through discarded. Another 700µl of RPE solution was applied unto the column, centrifuged for 10,000 x g for 2 minutes and the flow through discarded. The 1.5ml collection tube was reattached and the samples were centrifuged at full speed for 1 minute. The RNeasy spin column was transferred to a new 1.5ml collection tube. 25µl of RNase-Free water was pipetted directly into the column and then incubated at room temperature for 5 minutes. Next, the samples were centrifuge for 1 minute at 10,000 x g to collect aRNA. Amplified RNA was immediately placed on ice and its concentration measured using a nanodrop.

cDNA synthesis

The Invitrogen Superscript III First-Strand Synthesis System was used to perform RT-PCR on DNase treated aRNA. Depending on concentration, 3-5µl of DNase treated aRNA was combined with 1µl of 50ng/µl random hexamer primer, 1µl of 10mM dNTP mix and 3-5µl of
DEPC-treated water to make a final volume of 10µl. The reaction mix was incubated at 65°C for 5 minutes, then placed on ice for 1 minute. The cDNA Synthesis Mix was prepared by combining 2µl of 10X RT buffer, 4µl of 25mM MgCl₂, 2µl of 0.1 M DTT, 1µl RNaseOUT™ (40U/µl) and 1µl of Superscript™ III RT (200U/µl). 1µl of DEPC-treated water was substituted for Superscript™ III RT with the no RT reactions. 10µl of the mixture was added to each reaction, mixed gently and centrifuge briefly. The reaction was incubated at 25°C for 10 minutes, followed by a 50°C incubation for 50 minutes. The reactions were terminated by 85°C for 5 minutes and chilled on ice. The reactions were collected by brief centrifugation. 1µl of RNase H was added to each tube and incubated at 37°C for 20 minutes. 20µl of nuclease free water was added to each sample.

PCR

Reagents from the Promega GoTaq® Flexi DNA Polymerase kit were used for PCR. A reaction mix containing 10.9µl of BBL™ water, 5µl of 5X Green GoTaq® Flexi Buffer, 2.5µl of 25mM MgCl₂ solution, 2.5µl of 2mM dNTPs, +/- 1µl of DMSO, 1µl of 10mM forward primer, 1µl of 10mM reverse primer and 0.1µl of GoTaq® DNA Polymerase (5U/µl) was added to 1µl of cDNA to make a final volume of 25µl. The PCR program was set to run at 95°C for 2 minutes (initial denaturation), 95°C for 30 seconds (denaturation), 55°C/58°C for 30 seconds (depending on annealing temperature of primer), 72°C for 45 seconds (extension) and 72°C for 5 minutes (final extension) in an Eppendorf vapo.protect Mastercycler pro thermocycler. Two replicates of both UFM and LFM were used for PCR. Both RT and no RT controls for each sample were used. Other controls used for PCR include SM cDNA, LS cDNA, B73 gDNA and no template control (ntc). Amplicons were run on a 1% gel made by dissolving 1.5g of Seakem®
LE Agarose in 150mls of 1X Sodium Borate (SB) buffer. 1.5µl of Ethidium Bromide was added the mixture and the gel was ran at 100v for 1-1.5 hours.

Primer Design

The Invitrogen OligoPerfect™ Designer (http://tools.lifetechnologies.com/content.cfm?pageid=9716) was used to design primers for PCR and qPCR. Primers were designed to amplify the 3’ end of target regions with size, ~150-230 bp and an annealing temperature of 60ºC. cDNA sequences for various targets were obtained from Maize GDB (http://www.maizegdb.org), Maize sequence (http://maizesequence.org/index.html), and NCBI (http://www.ncbi.nlm.nih.gov). The accession number and primer sequence for each target gene is shown in Table 4 in the appendix. MADS-box primers designed for pcr were tested using B73 genomic DNA (see figure 23 in appendix).

Real time PCR

SsoFast™ EvaGreen® Supermix from Bio-Rad was used for real time PCR. Reagents including primers, cDNA from UFM/LFM and EvaGreen were thawed before use. A total reaction volume of 15µl was made by mixing 7.5µl of EvaGreen, 4.5µl highly purified BBL water, 1µl of forward and reverse primers (10µm) and 1µl of cDNA. For standard curves cDNA dilution were made 1:10, 1:100, 1:1000 and 1:10000. 96 well plates from Bio-Rad were used and sealed with a microseal ‘B’ film. The plates were loaded into the Bio-Rad CFX96 thermocycler. The plate program was set to run at 95ºC for 30 seconds, 95ºC for 5 seconds and 59ºC for 5 seconds (primer annealing and extension) 40 times. The melt curve was set at 65-95ºC for 0.5 second interval. The Bio-Rad CFX manager software was used to calculate the standard curves and primer efficiencies. Wells that were used for standard curve analysis were assigned ‘standard’ and those fit to the standard curve were designated ‘unknown’. R² values below 0.980
or primer efficiency values below 85% or above 120% were not used in data analysis. The CFX manager software was used to calculate the fold change in gene expression between the three biological replicates of the UFM and LFM by calculating the \( \Delta Ct \) and normalizing it relative to GAPDH (reference gene).
CHAPTER 3

RESULTS

Objective 1: Establish the feasibility of using Laser Capture Microdissection (LCM) to dissect maize upper and lower floral meristems

Fixation and embedding

To establish a fixation protocol, I used a combination of protocols from the Schnable lab (http://schnablelab.plantgenomics.iastate.edu/) and Nathalie Bolduc (by personal communication) for tissue fixation. I tested two types of fixatives, acetone fixative and Farmer’s Fixative (ethanol and acetic acid in a 3:1 ratio). Other labs have used either ethanol: acetic acid fixative or acetone fixative for LCM (Ohtsu et al., 2007; Kerk et al., 2003), but for this project I used both fixatives to determine which one was more suitable for the experiment. I initially fixed two batches with acetone and three batches with ethanol: acetic acid. Compared to the ethanol: acetic acid fixative, the acetone fixed samples became brittle and shattered upon mounting on slides after sectioning. Therefore I concluded that the ethanol: acetic acid fixative was more suitable for this experiment. For all experiments, I embedded tissue in wax, sectioned (at 8-10µm thickness) and mounted on special UV treated membrane slides from Zeiss for LCM.

RNA isolation

RNA is a very unstable molecule, which is easily subject to degradation. To assess the RNA integrity after fixation/embedding but prior to LCM, I sectioned samples and scrapped off whole sections mounted on normal slides and then RNA was extracted from them. I determined the amount of RNA extracted using the Qubit® Fluorometer from Invitrogen®. RNA quantity ranged from 0.8 ng/µl to 11 ng/µl based on size and number of sections used (Table 1). To check the quality of RNA extracted, samples were run on a gel and the degradation of RNA was
measured using the Agilent 2100 Bioanalyzer (Fig. 7). For a good RNA, a dark upper and lower band corresponding to 28s and 18s ribosomal bands is expected. Samples with RNA integrity number (RIN) of ~4 to 8 (Table 1) were used for LCM.

Table 1. Concentration of RNA from some B73 tissue samples (fixed with ethanol: acetic acid) using the Qubit® Fluorometer with RINs.

<table>
<thead>
<tr>
<th>Tissue samples (Batches)</th>
<th>Concentration ng/µl</th>
<th>RNA integrity Number (RIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>11.1</td>
<td>7.8</td>
</tr>
<tr>
<td>Batch 2</td>
<td>9.86</td>
<td>6.2</td>
</tr>
<tr>
<td>Batch 3</td>
<td>8.43</td>
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<td>Batch 4</td>
<td>6.75</td>
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</tr>
<tr>
<td>Batch 5</td>
<td>0.83</td>
<td>5.1</td>
</tr>
</tbody>
</table>
Figure 7. Assessing RNA integrity using the Agilent bioanalyzer: A. Gel representation of total RNA from 5 individual samples. Arrows indicate 18s and 28s bands as predicted. The presence of two distinct ribosomal bands indicates good quality RNA. B. Electropherogram corresponding to B3 from A. RNA integrity number (RIN) of 7.2 is an indication of good quality RNA.
Isolation of UFM and LFM using LCM, and RNA amplification.

I successfully isolated four replicates of upper and lower floral meristems using LCM (Fig.8). I have also isolated RNA from LCM-dissected cells and scraped off tissue from the slide. To generate sufficient quantities of RNA for RT-PCR, I amplified isolated RNA using the TargetAmp™ 2-Round aRNA Amplification Kit 2.0 from Epicenter. The integrity of RNA isolated from tissue scraped from LCM slide was assessed by running the RNA on an Agilent Bioanalyzer (Fig.9). RINs from tissues scraped of LCM slides ranged from 4.3 to 7.1 (Table 2). I quantified amplified RNA (aRNA) using the nanodrop and initial concentrations of amplified UFM and LFM were diluted down to 1µg/µl. After DNase treatment, I found aRNA concentrations ranging from 7.0 to 30.9 ng/µl (See table 4 in appendix). The integrity of aRNA was also assessed using the bioanalyzer. The average size of the aRNA was ~220-350 bp, indicative of good amplification (Fig.10 and 11).

Table 2. RINs of tissue scrapped of LCM slides

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS(UM1)</td>
<td>6.1</td>
</tr>
<tr>
<td>LS(UM2)</td>
<td>5.5</td>
</tr>
<tr>
<td>LS(UM3)</td>
<td>7.1</td>
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<td>5</td>
</tr>
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<tr>
<td>LS(LM3)</td>
<td>5.2</td>
</tr>
<tr>
<td>LS(LM4)</td>
<td>4.3</td>
</tr>
</tbody>
</table>
Figure 8. Dissection of upper and lower floral meristems using LCM: A. selected LFM to be dissected. Lemma has initiated (arrow). B. Selected UFM to be dissected. Lemma has initiated (arrow). C. Dissected LFM using LCM. D. Dissected UFM using LCM.
Figure 9. Bioanalyzer results from tissue scraped of LCM slides: Gel representation of total RNA from tissue scraped off LCM slides of UFM and LFMs using the Total RNA Nano chip. The presence of two distinct ribosomal bands indicates good quality RNA.
Figure 10. Assessing integrity of upper floral meristem amplified RNA using the Agilent bioanalyzer: A-D. Electropherograms of four replicates of UFM amplified RNA (aRNA). Size distribution of 300-500bp is an indication of good amplified RNA. UFM-upper floral meristem.
Validation of dissected of UFM and LFM

To validate the dissection of upper and lower floral meristems, I tested the expression of several marker genes by RT-PCR. I synthesized cDNA for PCR, and designed primers to test for the expression of selected marker genes including *knotted (kn1)*, *indeterminate spikelet1 (ids1)*, *zmm8*, *zmm14*, *bearded-ear (bde)*, *branched silkless1 (bd1)*, *ramosa1 (ra1)*, *ramosa2 (ra2)*, *Phosphoenol pyruvate carboxylase1 (pep1-1)* and *zmtip2-3 (zmtip-1)*. *kn1* is expressed in all meristem types in the inflorescence (Jackson et al., 1994) and serves as a meristem marker; *ids1* is expressed in the spikelet meristem (Chuck et al., 1998); *zmm8* and *zmm14* are expressed
exclusively in the upper floral meristem but not the in the lower floral meristem (Cacharron et al., 1999); bde is expressed in both the upper and lower floral meristems (Thompson et al., 2009); bd1 is expressed in the SM (Chuck et al., 2002); ra1 and ra2 are expressed in the spikelet pair meristem and in the anlagen of the spikelet meristem (Botiri et al, 2006); pep1 is expressed in the mesophyll cells of maize leaves (Martineau and Taylor, 1985) and zmTIP2-3 is expressed in the roots of maize plants (Lopez et al., 2004). None of the primers used spanned introns, and thus the same size amplicon was expected using both genomic DNA and cDNA as a template. Genomic DNA was used as a control template with all primers to assess primer specificity and as a positive control for the PCR.

kn1, which is expressed in all meristem types in the inflorescence (Jackson et al., 1994), was expressed in amplified RNA isolated from UFM and LFM. Indeed, I found that kn1 is amplified from all UFM and LFM samples, as well RNA from mixed meristems (MM; note that MM contains FM and contaminating SM) and RNA isolated from tissue scraped off the slide (LS). Genomic DNA was used a positive control for PCR (note the primers do not span an intron so the kn1 amplicon from cDNA and genomic DNA is the same expected size). kn1 was not amplified from no RT controls, indicating there was no contaminating genomic DNA in the RNA samples (Fig. 12).
Figure 12. *kn1* expression pattern: A. RNA in situ hybridization showing *kn1* expression in all meristems in the inflorescence. Image taken from Jackson et al., *Dev (1994)*; B. *kn1* expected pcr results; C. *kn1* actual pcr results. *kn1* is observed in all UFM and LFM samples; it is also observed in the LS and MM. UF-Upper Floral meristem; LF-Lower Floral meristem; MM-Mixed meristem containing spikelet meristem (SM) and floral meristem; LS-tissue scrapped off LCM slide; gD- genomic DNA; ntc- no template control; + - rt control; - - no rt control.

*nde*, which is expressed in both the UFM and LFM (Thompson et al., 2009), was expressed in amplified RNA isolated from UFM and LFM. I found that *nde* is amplified from all UFM and LFM samples, as well RNA from MM and LS (Fig. 13).

Figure 13. *nde* expression pattern: A. RNA in situ hybridization showing *nde* expression in the upper and lower floral meristems (arrows indicating floral meristems). Image taken from Thompson et al., *The Plant Cell (2009)*; B. *nde* expected pcr results; C. *nde* actual pcr results.
*bde* is expressed in both upper and lower floral meristems as expected, it is also expressed in the LS and MM.

Interestingly, *ids1*, which is expressed in the SM (Chuck et al., 1998), was expressed in amplified RNA isolated from UFM and LFM. I found that *ids1* is amplified from all UFM and LFM samples, as well RNA from MM and LS (Fig. 14).

Figure 14. *ids1* expression pattern: A. RNA in situ hybridization showing *ids1* expression in the spikelet meristem. Image taken from Chuck *et al.*, *Genes Dev* (1998); B. *ids1* expected pcr results; C. *ids1* actual pcr results. *ids1* is observed in both UFM and LFM samples as well as the MM and LS. UF/lfm-Upper Floral meristem; LF/lfm-Lower Floral meristem; SM-spikelet meristem, og-outer glume; ig-inner glume; ol-outer lemma; il-inner lemma.

Similarly, *zmm8* and *zmm14*, which are expressed exclusively in UFM (Cacharron et al., 1999), were expressed in amplified RNA isolated from both UFM and LFM. Similar to *ids1* expression, I found that *zmm8* and *zmm14* were amplified from all UFM and LFM samples, as well RNA from MM and LS (Fig. 15 and 16).
Figure 15. *zmm8* expression pattern: A. RNA in situ hybridization showing *zmm8* expression in the upper floral meristem. Arrows show *zmm8* is expressed in only the upper floret. It is also seen in the floral organs of the upper floret and nowhere in the lower floral meristem. Image taken from Cacharron *et al.*, *Dev* (1999); B. *zmm8* expected pcr results; C. *zmm8* actual pcr results. *zmm8* is observed in both UFM and LFM samples as well as the MM and LS. *gl*-glume; *ufp*-upper floret primordia; *lfp*-lower floret primordial; *c*-carpel; *st*-stamen; *p*-palea.

Figure 16. *zmm14* expression pattern: A. RNA in situ hybridization showing *zmm14* expression in the upper floral meristem. Arrows show *zmm14* is expressed in only the upper floret. It is also seen in the floral organs of the upper floret and nowhere in the lower floral meristem. However, unlike *zmm8*, expression is observed more in the carpel than the other floral organs. Image taken from Cacharron *et al.*, *Dev* (1999); B. *zmm8* expected pcr results; C. *zmm8* actual pcr results. *zmm8* is observed in both UFM and LFM samples as well as the MM and LS.
The observed expression patterns of *ids1*, *zmm8* and *zmm14* was unexpected. To ensure unwanted products were not being amplified during the amplification process, I designed primers for additional markers which were not originally part of my list of control markers. These included *ra1*, *ra2*, *pepcase1* and *zmtip2-3*.

*ra1*, which is expressed in the spikelet pair meristem (SPM) (Vollbrecht *et al.*, 2005), was not expressed in amplified RNA isolated from UFM, LFM and MM. I found that *ra1* is amplified from only LS samples (Fig. 17).

Figure 17. *ra1* expression pattern: A. RNA in situ hybridization showing *ra1* expression in the spikelet pair meristems (arrow heads) .Image taken from Vollbrecht *et al.*, Nature (2005) ; B. *ra1* expected pcr results; C. *ra1* actual pcr results. As expected, *ra1* is observed in only the LS.

Similar to *ra1* expression, *ra2*, which is expressed in the spikelet pair meristem (SPM) and in the anlagen of the SM (Bortiri *et al.*, 2006), was not expressed in amplified RNA isolated from UFM, LFM and MM. I found that *ra1* is amplified from only LS samples (Fig. 18).
Figure 18. *ra2* expression pattern: A. RNA in situ hybridization showing *ra2* expression in the spikelet pair meristems. It is also observed in the anlagen of the spikelet meristem (arrow). Image taken from Bortiri et al., The Plant cell (2006); B. *ra2* expected pcr results; C. *ra2* actual pcr results. As expected, *ra2* is observed in only the LS.

*pepcase1* which is expressed in the mesophyll cells of maize leaves (Martineau and Taylor, 1985), was not expressed in amplified RNA isolated from UFM, LFM, MM or LS. I found that *pep1* is expressed only in genomic DNA. Similarly, *zmtip2-3* which is expressed in the roots of maize plants (Lopez et al., 2004), was observed only in the genomic DNA (Fig. 19).

Figure 19. *pepcase1 and zmtip2-3* expression pattern: A. *pep-1* expected pcr results (upper panel); *zmtip-1* expected results (lower panel); B. *pep-1* (upper panel) and *zmtip-1* (lower panel)
actual pcr results. *pep*-1 is observed in gD. No expression is observed in the LS. *zmtip*-1 expected expression is observed.

The observed expression patterns of *ra1, ra2, pep1* and *zmtip2-3* convinced me that indeed we dissected UFM and LFM and that we were just not amplifying unwanted products. Moreover, the reported expression patterns of the selected markers (*ids1, zmm8* and *zmm14*), are based on RNA in situ hybridization assays, which are biased and tend to measure the relative abundance of transcripts. RNA in situs also have a minimum threshold, however, below which you are unable to detect any expression. PCR, on the other hand is a more sensitive assay which can detect very small amounts of transcripts. Secondly, the UFM and LFM are morphologically very distinct and photographic evidence from laser captured samples shows that only upper and lower floral meristems in which the lemma had been initiated were dissected (Fig. 8). Based on these reasons, I was very confident about my FM samples. These results therefore suggested differential gene expression in the UFM and LFM may not necessarily be the case of the presence or absence of a gene but rather, the difference in level of expression of these genes in the two floral meristems which could not be detected by in situ hybridization experiments, so I decided to look more closely at *zmm8* and *zmm14* using qPCR.

Objective 2: Study the expression of MADS-box genes in the upper and lower floral meristems of maize.

**Primer design for RT-PCR and qPCR**

The maize genome contains 43 annotated MIKC MADS-box genes (Zhao et al., 2010; Zhao et al., 2011). I designed primers for 32 of these MADS-box genes for qRT-PCR (see Table 3 in appendix). Primers were designed to amplify the 3’ end of target regions with size, ~150-
230 bp long. Out of the 32 designed primers, 28 of them worked with PCR when tested with genomic DNA. I tested the primers with genomic DNA using qPCR, and 15 out the 32 primers worked. A total of 9 out of the 15 worked with qPCR when I used cDNA from FMs (see Table 5 and 6 in appendix for qRT-PCR reaction conditions).

Studying MADS-box gene expression in the upper and lower floral meristems of maize

Previous worked indicated that at least two MADS-box genes, *zmm8* and *zmm14* were exclusively expressed in the UFM (Cacharron et al., 1999). Therefore, I originally proposed to examine expression of the remaining MADS-box genes by RT-PCR and visualizing the results of a gel. However, when I tested the expression of *zmm8* and *zmm14* in the UFM and LFM-dissected samples, I detected expression of both genes in both the UFM and LFM (Fig. 15 and 16). This suggests that genes are unlikely to be exclusively expressed in the UFM or LFM, but rather that the differential expression is likely due to a difference in expression levels in the UFM and LFM. Therefore, I developed a qRT-PCR protocol to study MADS-box gene expression in the upper and lower floral meristems by looking at the level of expression of these genes in the two floral meristems. The expression of nine MADS-box genes was studied using this approach. These included three genes with known expression patterns (*zmm8, zmm14* and *zmMADS3*) (Fig. 20, 21 and 23) and six genes with no reported expression pattern (*CA483635, zagl1, zmm20, zmm24, zmm27 and zmm29*) (Fig. 22). Three biological replicates of upper and lower floral meristems were used for the qPCR assay. The Bio-rad CFX manager software was used to calculate the standard curves and primer efficiencies. The CFX manager software was used to calculate the fold change in gene expression between the three biological replicates of the UFM and LFM by calculating the ∆Ct and normalizing it relative to GAPDH. To calculate the fold change, the relative normalized expression levels of all three biological replicates were averaged.
As expected, \textit{zmm8} and \textit{zmm14} was expressed at a much higher level in the UFM than the LFM. \textit{zmm8} and \textit{zmm14} expression was higher in the UFM of all three biological replicates tested than the LFM, although there was variation among biological replicates (Fig. 20 and 21). On average, \textit{zmm8} was enriched 44-fold and \textit{zmm14} was enriched 20-fold in the UFM compared to the LFM. These differences were significant using a one-tailed student t-test and \( p < 0.05 \). The \( p \)-values were high for the two genes because the sample size used was 3, which is small for this kind of statistical analysis.

I also tested the relative expression of seven additional MIKC MADS-box genes in the UFM and LFM-dissected samples. For these initial experiments, I used two biological replicates and assumed a primer efficiency of 100\% to calculate the fold change between UFM and LFM samples. In these experiments, I was focusing on genes with a large differential expression between the UFM and LFM (such as \textit{zmm8} and \textit{zmm14}), and these large differences should be detectable even there is variation in primer efficiencies between primer sets. None of the genes tested showed a large expression difference in the UFM and LFM. Interestingly there was not much change in the levels of expression of the other MADS-box genes (Fig. 22). One gene, \textit{zmMADS3}, appeared slightly increased in the UFM than the LFM (Fig. 22). I therefore decided to look more critically at the expression levels of this gene in the UFM and LFM by calculating the standard curve and primer efficiencies. \textit{ZmMADS3} expression was higher in the UFM of all three biological replicates tested than the LFM, although there was variation among biological replicates (Fig. 23). On average, \textit{zmMADS3} was enriched 3-fold in the UFM compared to the LFM. These differences were not significant using a two-tailed student t-test and \( p > 0.05 \). Again, due to the small sample size of the two FM, not much can be inferred from the \( p \)-value.
Figure 20. *zmm8* qPCR data: A. GAPDH standard curve showing primer efficiency of 98.2%. B. *zmm8* standard curve showing primer efficiency of 102.1%. C. *zmm8* expression in three biological replicates of the UFM and LFM. Expression is low but variable in all three biological replicates of LFM as compared to the UFM. D. Normalized relative expression of *zmm8* in the UFM and LFM. There is high expression of *zmm8* in the UFM (44-fold) as compared to the LFM. * indicates statistical significance difference with p < 0.05.
Figure 21. *zmm14* qpcr data: A. GAPDH standard curve showing primer efficiency of 98.2%. B. *zmm14* standard curve showing primer efficiency of 97.4%. C. *zmm14* expression in three biological replicates of the UFM and LFM. Expression is low but variable in all three biological replicates of LFM as compared to the UFM. D. Normalized relative expression of *zmm14* in the UFM and the LFM. There is high expression of *zmm14* in the UFM (20-fold) as compared to the LFM. * indicates statistical significance difference with p < 0.05.
Figure 22. qPCR results showing the expression of CA483635, zagl1, zmm20, zmm24, zmm2 , zmm29 and zmMADS3 in the upper and lower floral meristems of maize: Calculated fold change between UFM and LFM samples using two biological replicates and an assumed a primer efficiency of 100%. None of the genes tested showed a large expression difference (as observed in zmm8 and zmm14) in the UFM and LFM. zmMADS3 however, appears slightly increased in the UFM than the LFM.
Figure 23. zmMADS3 qPCR data: A. GAPDH standard curve showing primer efficiency of 94.2%. B. zmMADS3 standard curve showing primer efficiency of 110.6%. C. zmMADS3 expression in three biological replicates of the UFM and LFM. Expression is variable in all three biological replicates of LFM and UFM. D. Normalized relative expression of zmMADS3 in the UFM and the LFM. There is a slight increase in zmMADS expression in the UFM (3-fold) as compared to the LFM. ** indicates not statistically significant difference with p > 0.05.
CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

The long-term goal of this research is to study the differential expression of MADS-box genes in the upper and lower floral meristems of maize. UFM and LFM cannot be manually dissected and therefore I used laser capture microdissection (LCM) to specifically isolate UFM and LFM cells. MADS-box genes are transcription factors, which control several developmental processes including inflorescence and flower development in plants and are important regulators of flower development in flowering plants (reviewed by Shore and Sharrocks, 1995). In maize, flowers develop from a group of undifferentiated cells called floral meristems, which give rise to floral organs.

Several lines of evidence suggest that the gene regulatory networks in the UFM and LFM are distinct. First, bde mutants affect the upper and lower florets differently, suggesting the gene regulatory network in the upper and lower floral meristems are different. Second, zmm8 and zmm14, are expressed only in the upper floral meristem (UFM), but not in the lower floral meristem (LFM). Third, we also know that many stamen genes are differentially expressed and two MADS-box genes are differentially expressed. bearded-ear (bde) is a MADS-box gene, which is known to affect the upper and lower floral meristems differently. In the UFM, bde mutants initiate extra floral organ primordia; thus bde is required for FM determinacy and floral organ development in the UFM (Thompson et al., 2009). In the LFM, bde mutants initiate additional meristems. FMs never initiate meristems indicating that in the LFM, BDE is required for FM fate (Thompson et al., 2009). The different bde phenotypes observed in the UFM and the LFM are likely the result of the disruption of distinct BDE-containing complexes with distinct target genes in the UFM and LFM (Thompson et al., 2009). The long-term goal of this research
is to identify UFM and LFM specific MADS-box complexes and their target genes to understand the development of the UFM and LFM in maize.

To study the differential expression of MADS-box genes in the two floral meristems, I used LCM to isolate UFM and LFM. LCM is a technique, which allows for the isolation of specific organs, cells or tissue for molecular analysis (Emmert-Buck et al., 1996). This technique has extensively been used in plants for gene expression studies (Nakanzo et al., 2003; Asano et al., 2002; Kerk et al., 2003).

I isolated four biological replicates of pools of UFM and LFM using LCM. I dissected FMs during a narrow developmental window in which lemma primordia had been initiated, but stamen primordia had not yet been initiated (Fig. 8). To validate my dissections, I used several markers with reported RNA in situ hybridization expression patterns. My results showed that several markers previously reported to only be expressed in the SM (by in situ) are also expressed in FMs and genes previously reported to be expressed exclusively in the UFM, expressed in both the UFM and LFM. This led to the conclusion that from a technical standpoint, to study differential gene expression in the two floral meristems, we have to look at level of expression of a particular gene (not just the absence or presence of a gene).

**SM, UFM and LFM genes are expressed in both the UFM and LFM**

My results indicate that several genes previously reported to be expressed in the SM (e.g. *ids1* and *bd1*) are also present in the UFM and LFM. Furthermore, *zmm8* and *zmm14* previously reported to only be expressed in the UFM are also present in the LFM. There are two possibilities for my results: 1) contamination of FM samples with SM and UFM samples with LFM and 2) these genes are expressed in the UFM and LFM. I favor the second possibility for
several reasons. First, most of the reported expression pattern of these genes is based on RNA in situ hybridization data. This technique is however biased as it measures the relative abundance of mRNA and has a minimum threshold, below which you are unable to detect any expression. PCR is a more sensitive assay which detects very small amounts of transcripts. The detection of zmm8 and zmm14 in the LFM indicates there relatively small amounts of these genes in the LFM which are below the detection limits of in situ hybridization assays. The same can be said of bd1 and ids1. Second, one could argue that the reason for detecting these genes in the upper and lower floral meristems is due to SM contamination in laser captured samples, but the UFM and LFM are morphologically very distinct and photographic evidence from laser captured samples shows that only upper and lower floral meristems in which the lemma had been initiated were dissected (Fig. 8) Third, I tested the expression patterns of additional control markers (ra1, ra2, pepcase1 and zmtip2-3) to ensure unwanted products were not amplified during the amplification process. The accumulation of ra1 and ra2 (genes expressed in the spikelet pair meristem) in tissue scraped off the slide (LS) not the spikelet meristem and similar expression of pepcase1 (expressed in the leaves) and zmTIP2-3 (expressed in the roots) made me more confident about my dissections, as it indicated the amplification process did not result in the amplification of unwanted products.

Possible role of zmm8 and zmm14 in the LFM

My results indicate zmm8 and zmm14 are expressed in both the UFM and LFM. Previous work by Cacharron et al. (1999), hypothesized that zmm8 and zmm14 could be acting as “upper floret determinacy genes” rather than “upper floret identity genes” If this were the case we would expect a gene in the lower floret to have a similar function. So far, no gene has been identified in the lower floret to have this function. Despite the fact that they are expressed at very low levels
in the LFM, could zmM8 and zmM14 be the same genes that control this function in the lower floret? Another important observation made by Cacharron et al. (1999), was that zmM8 expression was visible in all floral organs of the developing upper floret but nowhere in the lower floret. On the other hand, zmM14 had very strong expression in the carpels of the upper floret but weak expression in other floral organs. Their data showed that even though zmM8 and zmM14 transcripts have a similar spatial expression pattern, the expression level of zmM14 is significantly lower than that of zmM8. Our results confirmed this as zmM8 was highly expressed in the UFM (44-fold) as compared to zmM14 (20-fold).

zmMADS3 expression in the UFM and LFM

zmMADS3 is an AP1-like gene that is co-expressed with zmMADS1 in all ear spikelet organ primordia at intermediate stages, to help in flower development, egg cell formation and early embryogenesis (based on RNA in situ hybridization data and northern blot analyses using transgenic plants) (Heuer et al, 2001). zmMADS3 expression is detectable in the upper and the lower floret only at intermediate stages of development (Heuer et al, 2001). At earlier stages of spikelet primordia development, zmMADS3 is not observed by RNA in situ analysis in the ear, but is seen at the latter stages when the spikelet is fully developed and floral meristem are initiated (Heuer et al, 2001). There is no data however, on expression levels in the two floral meristems. In the UFM and LFM, zmMADS3 is visible in all floral organs of the two florets until at latter stages of development when the silks develop that it disappears (Heuer et al, 2001). Additional function of zmMADS3 observed in transgenic maize plants ectopically expressing the gene suggests it plays a role in meristem identity. zmMADS3 and its orthologue in rice, osMADS15 belong to the squamosa group of MADS-box genes which is closely related to the Arabidopsis AP1 gene (A-class gene). zmMADS3 has about 95% similarity with zap1 (A-class
gene) in maize (Heuer et al, 2001). The rice ortholog of the gene is known to affect plant architecture and flowering time just like in maize (Lu et al., 2012). A-class genes are known to be expressed in the meristems of grasses and as such would be expected to be expressed in both UFM and LFM. The qPCR results confirmed this as zmMADS3 was observed in both UFM and LFM.

Future Directions

I have successfully isolated upper and lower floral meristems from maize using LCM, isolated and amplified RNA for downstream amplifications. I have verified my dissections by looking at the expression of several control genes in the UFM and LFM and have good quality RNA for large-scale expression analyses. I identified MADS-box genes (zmM8 and zmM14) expressed at different levels in the UFM and the LFM. This suggests regulation of gene expression in the two floral meristems may be dependent on levels of expression of interacting genes. Out of a total of 32 designed MADS-box primers, only 9 could be tested using qRT-PCR. In order to study the differential expression of MADS-box genes in the UFM and LFM, future work to be done include using RNA seq to globally identify remaining MADS-box genes that are differentially expressed (in terms of levels expression and presence or absence) in the UFM and LFM.

RNA-seq is a technique, which uses sequencing technology to measure RNA expression levels in tissues. This technique will be used to globally identify genes that are differentially expressed in the UFM and LFM. MADS-box TFs form complexes to regulate the transcription of downstream target genes. A long-term goal of this research is to determining what MADS-box containing complexes are present in the UFM and LFM and their target genes. To determine protein/protein interactions we could perform yeast-2-hybrid (Y2H), co immunoprecipitation
(Co-IP) and Bimolecular florescence complementation (BiFC) experiments and integrate this with expression data. The quartet model proposes that MADS-box genes form dimers, which bind separate CArG boxes, and that these dimers form a tetramer (Theissen, 2001). The use of yeast two-hybrid and BiFC assays will allow us to determine the interaction between MADS-box dimers in maize.

The RNA seq data could also help distinguish between proposed models concerning the origin of UFM and LFM. The lateral branching model suggests the upper and lower floral meristems have a similar origin and that they are both lateral products of the SM (Chuck et al., 1998). In contrast, the terminal upper floret model suggests that the upper and lower floral meristems have different origins and that the spikelet meristem first initiates the LFM and then the SM transitions into the UFM where it is later degenerated (Irish, 1997). I argue that the results presented in this thesis support the lateral branching model. First of all, we observed that the UFM and LFM express similar genes, although at very different levels. If indeed the upper and lower floral meristems are of the same origin and are the lateral products of the SM, it could explain why they are identical morphologically and as such we would expect to see similar genes expressed in the two floral meristems, which is what we observed. From the RNA –seq data sets I expect to see a few MADS-box genes that are exclusively expressed in UFM or LFM and a number of genes with similar expression as zmm8 and zmm14 in the UFM and LFM.

In a summary, I have successfully established the feasibility of using LCM to examine gene expression in the UFM and LFM of maize. I found that in order to study differential gene expression, we have to look at the level of expression of a particular MADS-box gene and not just the absence or presence of a gene. The RNA samples generated in this thesis research can be used for RNA-seq experiments to globally identify differential expression genes in the UFM and
LFM, including MADS-box genes. Knowledge from this work can be used to study the effect of gene expression on agriculturally important traits in maize and also help identify new genes important for improving crop yield.
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APPENDIX

Figure 24. Testing designed primers with B73 genomic DNA.

Gel showing designed primers tested with B73 genomic DNA. The numbered lanes represent the following primers:

1=ra1-1  12=KN7  23=KN25  34=KN34
2=pep1-1  13=KN23  24=KN24  35=KN35
3=zmtip-1  14=KN16  25=KN27  36=KN36
4=bd1-2  15=KN10  26=KN26  37=KN37
5=bd1  16=KN10  27=KN27  38=KN38
6=ra2  17=KN11  28=KN28  39=KN39
7=KN8  18=KN12  29=KN29
8=KN8b  19=KN13  30=KN30
9=KN5  20=KN14  31=KN31
10=KN6  21=KN15  32=KN32
11=KN9  22=KN19  33=KN33
Table 3. Designed primers for PCR and qPCR.

a) Sequences of control primers for PCR with accession numbers and sizes.

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<th>Gene</th>
<th>Accession #</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>cDNA Size (bp)</th>
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<td>bd1-2</td>
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<td>171</td>
</tr>
<tr>
<td>bde</td>
<td>GRMZM2G160565</td>
<td>KN8b</td>
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<tr>
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</tr>
<tr>
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<td>Kn1utr</td>
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<td>156</td>
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<tr>
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<tr>
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<td>Zmm8utr</td>
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</tr>
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</tr>
<tr>
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<td>AF326503</td>
<td>Zmtip-1</td>
<td>F: AGCTGTTTCATCGCTCCTAC R: GTGGGAAATGCAACAGGA</td>
<td>165</td>
</tr>
</tbody>
</table>

b) Primers sequences of MADS-box genes for qPCR with accession number and sizes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>cDNA Size (bp)</th>
<th>Type of MADS-box gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY109828</td>
<td>GRMZM2G079727</td>
<td>KN2</td>
<td>F: GCCTGGAACTCAAACTGGGT R: TCCATCTCAATCCATGCAGT</td>
<td>259</td>
<td>B-sister</td>
</tr>
<tr>
<td>CA483635</td>
<td>GRMZM2G097059</td>
<td>KN1a</td>
<td>F: GATCGCAGTGCTGACAGAT R: AAGCAACTAGCCCCGTGCGATG</td>
<td>162</td>
<td>SEP</td>
</tr>
<tr>
<td>sil1</td>
<td>GRMZM2G139073</td>
<td>KN5</td>
<td>F: CGAGATCAGCGGAGATGCAGA R: CTGCTGGTACCAGGTTCAAGA</td>
<td>202</td>
<td>AP3</td>
</tr>
<tr>
<td>zag1</td>
<td>GRMZM2G052890</td>
<td>KN6</td>
<td>F: GCTGAGGCGACAGATCTGTTA R: TCGACCTGACACACGGCACA</td>
<td>165</td>
<td>AG</td>
</tr>
<tr>
<td>zag2</td>
<td>GRMZM2G160687</td>
<td>KN7</td>
<td>F: CGTGGGAAAACCTGCTAAGTACAGA R: TCGCTGGTAGGCTAGGAGTCA</td>
<td>178</td>
<td>AG</td>
</tr>
<tr>
<td>zag3</td>
<td>GRMZM2G160565</td>
<td>KN8b</td>
<td>F: TTATCTATGCGCCGTTGCT R: GGGCCAGTAGAAAGGGACA</td>
<td>150</td>
<td>AGL6</td>
</tr>
<tr>
<td>Symbol</td>
<td>Accession</td>
<td>Ref</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Length</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>-----</td>
<td>----------------</td>
<td>----------------</td>
<td>--------</td>
</tr>
<tr>
<td>zag5</td>
<td>GRMZM2G003514</td>
<td>KN9</td>
<td>F: ACGCAACTTATGATGAGCAGC</td>
<td>R: AGGCCGGATGCACCTTTGATAGG</td>
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</tr>
<tr>
<td>zagl1</td>
<td>GRMZM2G026223</td>
<td>KN11</td>
<td>F: GAAGGATGTCGGGGATGGACG</td>
<td>R: CAGTTTCGCCGACCACAGAT</td>
<td>166</td>
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<tr>
<td>zap1</td>
<td>GMZMR2G148693</td>
<td>KN10</td>
<td>F: CTTGGGAGAGACGTGGAGCAAGG</td>
<td>R: AGTGCGCTGCTGCTGGG</td>
<td>214</td>
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<tr>
<td>zmm1</td>
<td>GRMZM2G010669</td>
<td>KN13</td>
<td>F: AGGTACAAGAAGGACACGC</td>
<td>R: AGATGCACTTTTCAAGGCGG</td>
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<tr>
<td>zmm2</td>
<td>GRMZM2G359952</td>
<td>KN12</td>
<td>F: TGAAGCTGCTATAGCTGAGT</td>
<td>R: GAGGCATGCTCCTGCTGCA</td>
<td>206</td>
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<tr>
<td>zmm3</td>
<td>GRMZM2G113202</td>
<td>KN19</td>
<td>F: TGTCCTGGGCAAATCAGTG</td>
<td>R: TGCTGAACCTTTCAACTTG</td>
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<tr>
<td>zmm4</td>
<td>GRMZM2G032339</td>
<td>KN14</td>
<td>F: GACCAAAAATCAACGGAGACC</td>
<td>R: CGGTGAGGGCAACCCACAG</td>
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<tr>
<td>zmm5</td>
<td>GRMZM2G171365</td>
<td>KN15</td>
<td>F: CGAGTAAAGCTGATGCCGAGT</td>
<td>R: TTCACGCAAATCTTGTTGC</td>
<td>226</td>
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<td>zmm6</td>
<td>GRMZM2G159397</td>
<td>KN16</td>
<td>F: GGAGGAGACGCAACCAGGTTAT</td>
<td>R: ATCTCATGGGCAACCCAGG</td>
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<td>zmm7</td>
<td>GRMZM2G097059</td>
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<td>F: ACAGGAAACCTTGAGGATCG</td>
<td>R: ACCCAATCTCCACACAGCA</td>
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<tr>
<td>zmm8</td>
<td>GRMZM2G102161</td>
<td>zmm8UTR</td>
<td>F: CCCCCAATAATGTCGATGC</td>
<td>R: TCGTGAACACACGACACAGCA</td>
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<tr>
<td>zmm14</td>
<td>GRMZM2G099522</td>
<td>zmm14UTR</td>
<td>F: AGCTGAAACAGGAGACCTG</td>
<td>R: TCGCACAACACACACACCA</td>
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<tr>
<td>zmm15</td>
<td>GRMZM2G553379</td>
<td>KN24</td>
<td>F: GGGCTGTGGCCGCAAGTA</td>
<td>R: TGCTGCAAGCAACACACAA</td>
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<tr>
<td>zmm16</td>
<td>GRMZM2G110153</td>
<td>KN23</td>
<td>F: ACTCCTGGAACACCAAGAC</td>
<td>R: GGTCAGGATGGTAACCCAGCA</td>
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<tr>
<td>zmm17</td>
<td>GRMZM2G130382</td>
<td>KN25</td>
<td>F: ATCGAGAAACTCGACAGACCG</td>
<td>R: CCTGCAAGTGGCCTGTTGGTA</td>
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<tr>
<td>zmm19</td>
<td>GRMZM2G370777</td>
<td>KN26</td>
<td>F: ACATACCCAGTGGTGGCAAG</td>
<td>R: CACCCAGATCAACACACAGCA</td>
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<tr>
<td>zmm20</td>
<td>GRMZM2G471089</td>
<td>KN27</td>
<td>F: ACGCAAGACGCACGTAATC</td>
<td>R: CTGGCAACGCGGCTTGGAAAA</td>
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<tr>
<td>zmm21</td>
<td>GRMZM5G814279</td>
<td>KN28</td>
<td>F: ATTCAGGAGCTCGCTCGAC</td>
<td>R: GACATGCACAAAGGACACAC</td>
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<tr>
<td>zmm22</td>
<td>GRMZM2G171622</td>
<td>KN29</td>
<td>F: GGTTCTGGGAGTCCGACAGT</td>
<td>R: CCAGGCGCGCTTGGTTTGTGA</td>
<td>206</td>
</tr>
<tr>
<td>zmm23</td>
<td>GRMZM2G471089</td>
<td>KN30a</td>
<td>F: GCAAGCAGCGAGTAAATCAT</td>
<td>R: CTGGCAACGCGTGGGAAAA</td>
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<tr>
<td>zmm24</td>
<td>GRMZM2G087095</td>
<td>KN31</td>
<td>F: CCTCTCTCTCCCCGACTTAGC</td>
<td>R: GGGACCTGGAAAGGGTACGA</td>
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<tr>
<td>zmm25</td>
<td>GRMZM2G018589</td>
<td>KN34</td>
<td>F: TGGAACAGCTCCAGTGACAG</td>
<td>R: GATTCCTCCCAATGCCTCTT</td>
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<tr>
<td>zmm26</td>
<td>GRMZM2G046885</td>
<td>KN32</td>
<td>F: CAGATACCCCAAAGCGCCAG</td>
<td>R: ACCCACATCCACACCCAGAA</td>
<td>218</td>
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<tr>
<td>zmm27</td>
<td>GRMZM2G129034</td>
<td>KN33</td>
<td>F: GAGGAGACCGCAACCAGG</td>
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<td>159</td>
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</table>
### Table 4. Concentrations of amplified RNA using the nanodrop

<table>
<thead>
<tr>
<th>RNA Sample</th>
<th>Before DNase treatment(ng/µl)</th>
<th>A260/280</th>
<th>After DNase treatment (ng/µl)</th>
<th>A260/280</th>
<th>Amount used for DNAse treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFM1</td>
<td>2050</td>
<td>2.39</td>
<td>103</td>
<td>2.12</td>
<td>4µg</td>
</tr>
<tr>
<td>UFM2</td>
<td>2101.9</td>
<td>2.41</td>
<td>70.4</td>
<td>2.27</td>
<td>4µg</td>
</tr>
<tr>
<td>UFM3</td>
<td>2303</td>
<td>1.34</td>
<td>30.9</td>
<td>2.20</td>
<td>1µg</td>
</tr>
<tr>
<td>UFM4</td>
<td>2197.8</td>
<td>1.18</td>
<td>20.6</td>
<td>2.16</td>
<td>1µg</td>
</tr>
<tr>
<td>LFM1</td>
<td>2076.9</td>
<td>2.40</td>
<td>64.3</td>
<td>2.21</td>
<td>4µg</td>
</tr>
<tr>
<td>LFM2</td>
<td>2131</td>
<td>2.39</td>
<td>18.6</td>
<td>2.08</td>
<td>1µg</td>
</tr>
<tr>
<td>LFM3</td>
<td>1541</td>
<td>2.37</td>
<td>73.3</td>
<td>2.21</td>
<td>4µg</td>
</tr>
<tr>
<td>LFM4</td>
<td>2117.3</td>
<td>2.39</td>
<td>7.9</td>
<td>2.09</td>
<td>1µg</td>
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</tbody>
</table>
Table 5. Reaction conditions for genes used for PCR: Gene highlighted in red did not work with PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression pattern (Insitu)</th>
<th>Rxn conditions</th>
<th>Annealing temp</th>
<th>cycle #</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>zmm17</td>
<td></td>
<td>35</td>
<td>homeotic gene function between maize and Arabidopsis. Development 131, 6083–6091</td>
<td></td>
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<tr>
<td>--------</td>
<td>----</td>
<td>----</td>
<td>--------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>zmTIP2-3</td>
<td></td>
<td>(+) DMSO</td>
<td>55</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>
Table 6. Reaction conditions for genes used for qPCR

<table>
<thead>
<tr>
<th>Good qPCR genes with good melt curves using genomic DNA</th>
<th>Rxn conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN1-CA483635-KN5-silky1-KN6-zag1-KN7-zag2-KN8-zag3</td>
<td>Enzyme activation-98°C for 2 min</td>
</tr>
<tr>
<td>Zmm8utr-Zmm14utr-KN10-zap1-KN11-zagl1-KN13-zmm1</td>
<td>Denaturation-98°C for 5 secs</td>
</tr>
<tr>
<td>KN33-zmm27-KN35-zmm29-KN37-ZmMADS2-KN39-ZmMADS3</td>
<td>Go to step 2 – 40x</td>
</tr>
<tr>
<td></td>
<td>Melt curve- 65-95°C in 0.5°C increment for 5 sec/step</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes with good melt curves but require DMSO using genomic DNA</th>
<th>Rxn conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN15-zmm5-KN19-zmm3</td>
<td>Enzyme activation-98°C for 2 min</td>
</tr>
<tr>
<td></td>
<td>Denaturation-98°C for 5 secs</td>
</tr>
<tr>
<td></td>
<td>Annealing/extension-62°C for 5 secs</td>
</tr>
<tr>
<td></td>
<td>Go to step 2 – 40x</td>
</tr>
<tr>
<td></td>
<td>Melt curve- 65-95°C in 0.5°C increment for 5 sec/step</td>
</tr>
</tbody>
</table>
| Genes with double melts curve peaks using genomic DNA | Gene activation-98°C for 2 min  
Denaturation-98°C for 5 secs  
Annealing /extension-59°C for 5 secs  
Go to step 2 – 40x  
Melt curve- 65-95°C in 0.5°C increment for 5 sec/step |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KN12-zmm2</td>
<td></td>
</tr>
</tbody>
</table>

| Genes that did not work with genomic DNA when performing qPCR | Gene activation-98°C for 2 min  
Denaturation-98°C for 5 secs  
Annealing /extension-59°C for 5 secs  
Go to step 2 – 40x  
Melt curve- 65-95°C in 0.5°C increment for 5 sec/step |
|---|---|
| KN2-AY109828  
KN9-zag5  
KN14-zmm4  
KN16-zmm6  
KN17-zmm7  
KN25-zmm17  
KN26-zmm19  
KN28-zmm21  
KN32-zmm26  
KN34-zmm25  
KN36-zmm31  
KN38-zmm28 | |

<table>
<thead>
<tr>
<th>Genes that produced good melt curves with UFM and LFM cDNA</th>
<th></th>
</tr>
</thead>
</table>
Table 7. Annotated cDNA sequences for genes used in research. Exons are in red and introns in black. Forward primers are highlighted in red and reverse primers in blue.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Region</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN1-CA483635</td>
<td>Zmm8utr</td>
<td>Enzyme activation-95°C for 30 secs</td>
</tr>
<tr>
<td>Zmm14utr</td>
<td>Denaturation-95°C for 5 secs</td>
<td></td>
</tr>
<tr>
<td>KN11-zagl1</td>
<td>Annealing /extension-59°C for 5 secs</td>
<td></td>
</tr>
<tr>
<td>KN27-zmm20</td>
<td>Go to step 2 – 40x</td>
<td></td>
</tr>
<tr>
<td>KN31-zmm24</td>
<td>Melt curve - 65-95°C in 0.5°C increment for 5 sec/step</td>
<td></td>
</tr>
<tr>
<td>KN33-zmm27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KN39-ZmMADS3</td>
<td></td>
<td></td>
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<tr>
<td>KN35-zmm29</td>
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<td></td>
</tr>
</tbody>
</table>

AY109828  [GRMZM2G079727]

CTCTCCCCCTGCGCGGTCCACGCAAGCAACGGGAGTACGGCGGTGTAGCTTCTTACATCCGTACACGGACTTTAATCCTTTCTTTTGCTTAAAGAAGCTGCAAGGCCTGCAGATCACTTCCAGTCTTCAAAAGTACAGTTCGCGAAAGTTGAAACGAGCGAGCGACGAACCGAACCAACCAACCGGCGGTGGCCACGTAGCCCCCCCTGACACAAAG

Table 7. Annotated cDNA sequences for genes used in research. Exons are in red and introns in black. Forward primers are highlighted in red and reverse primers in blue.
ZmTIP2-3 (AF326503)

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