MERISTEM SIZE AND DETERMINACY IN MAIZE

A Dissertation Presented

by

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ABBREVIATIONS

AM, axillary meristem; BM, branch meristem; CLE, CLV3/ESR; CLV, CLAVATA; CZ, central zone; FM, floral (flower) meristem; IM, inflorescence meristem; LRR, leucine-rich repeat; OC, organizing center; PZ, peripheral zone; P1, P2, plastochron 1, 2; SAM, shoot apical meristem; SM, spikelet meristem; SPM, spikelet pair meristem; RAM, root apical meristem; WOX, WUSCHEL-related homeobox.

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CHAPTER 1: GRASS MERISTEMS I

Abstract

The vegetative and reproductive shoot architectures displayed by members of the grass family are critical to reproductive success, and thus agronomic yield. Variation in shoot architecture is explained by the maintenance, activity, and determinacy of meristems, pools of pluripotent stem cells responsible for post-embryonic plant growth. This review summarizes recent progress in understanding the major properties of grass shoot meristems, focusing on vegetative phase meristems and the floral transition, primarily in rice and maize. Major areas of interest include: the control of meristem homeostasis by the CLAVATA-WUSCHEL pathway and by hormones such as cytokinin; the initiation of axillary meristems and the control of axillary meristem dormancy; and, the environmental and endogenous cues that regulate flowering time. In an accompanying paper in this issue, Tanaka et al. (2013) review subsequent stages of shoot development, including current knowledge of reproductive meristem determinacy and the fate transitions associated with these meristems.

1.1 Introduction

All post-embryonic plant tissues are derived from meristems, structures that harbor pluripotent stem cells. Shoot structures are created by the shoot apical meristem (SAM), while the root apical meristem (RAM) gives rise to root structures. Shoot development occurs in repeating modules called phytomers, consisting of a leaf, an axillary meristem (AM), and an internode. Plant architecture is largely dictated by the activity and determinacy of the SAM and AMs. For example, the shape of the plant can be determined by spatial and temporal patterns of leaf initiation from the SAM, and by elaboration of secondary shoots from AMs. This review will focus on the genetic networks controlling meristem maintenance and organization during the vegetative phase. It will also cover another major process in meristem biology: the transition of the SAM from vegetative to reproductive fate. The determinacy and fate transitions of reproductive meristems are reviewed in an accompanying paper by Tanaka et al. (2013).

Stem cells in the SAM are continuously self-maintained, and supply cells that will differentiate into lateral organs. The stem cells are located in the upper region of the central zone (CZ) of the meristem, in which cells divide slowly. The progeny produced from the division of the stem cells are used to replenish the stem cells themselves and are also displaced into the peripheral zone (PZ), where they start to divide more rapidly and lateral organs are initiated. Stem cell maintenance is achieved by the balance between self-replacement and organ initiation. The elucidation of the molecular mechanisms of meristem function, including stem cell maintenance, is currently a major area of interest in plant development.

1.2 WUS-CLV negative feedback loop in *Arabidopsis*

In *Arabidopsis*, the CLAVATA (CLV)-WUSCHEL (WUS) negative feedback loop is a major genetic mechanism to maintain stem cell populations in the meristem (Brand et al. 2000; Schoof et al. 2000; for review, see Ha et al. 2010; Aichinger et al. 2012). Mutations in the *CLV*

genes, such as *CLV1, 2* and *3*, cause enlargement of the meristem by an over-accumulation of stem cells, whereas mutation in the *WUS* gene results in premature termination of the meristem. Thus, CLVs and WUS are negative and positive regulators for stem cell maintenance, respectively (Fig. 1A). *CLV3* encodes a small protein containing the conserved CLE domain while *CLV1* and *2* encode an LRR-receptor kinase and an LRR –receptor like protein, respectively (Clark et al. 1997; Fletcher et al. 1999). CLV3 is processed into a small peptide, followed by chemical modifications such as proline hydroxylation and glycosylation (Kondo et al. 2006; Ogawa et al. 2008;Ohyama et al. 2009). It acts as a mobile signal, which is perceived by numerous receptors including CLV1, CLV2, which forms a complex with the pseudokinase CORYNE (CRN)/SOL2, and RPK2/TOAD2 (Clark et al. 1997; Miwa et al. 2008, Müller et al. 2008; Kinoshita et al. 2010; Betsuyaku et al. 2011). *WUS* encodes a homeodomain-containing transcription factor (Mayer et al. 1998). *WUS* promotes stem cell identity and the expression of CLV3, while the CLV pathway negatively regulates it by restricting the expression of *WUS* (Schoof et al. 2000). Thus, stem cell maintenance is regulated by the WUS-CLV negative feedback loop, which is associated with communication between different domains of the meristem: the stem cell region where CLV3 is expressed, and the organizing center (OC) where *WUS* is expressed (Mayer et al. 1998; Fletcher et al. 1999). Signaling between these different domains is likely achieved by intercellular movement of the CLV3 peptide and of the WUS transcription factor (Yadav et al. 2011).

1.3 CLV-related pathway in grasses

Stem cell maintenance is also studied in grasses, where the framework of meristem maintenance is principally conserved, with some interesting differences (for review, see Bommert et al. 2005b; Hirano 2008). Mutations in the maize genes *THICK TASSEL DWARF* (*TD1*) and *FASCIATED EAR2* (*FEA2*) predominantly affect the maintenance of the inflorescence meristems (IM) (Taguchi-Shiobara et al. 2001; Bommert et al. 2005a). In the tassel, the IM enlarges,

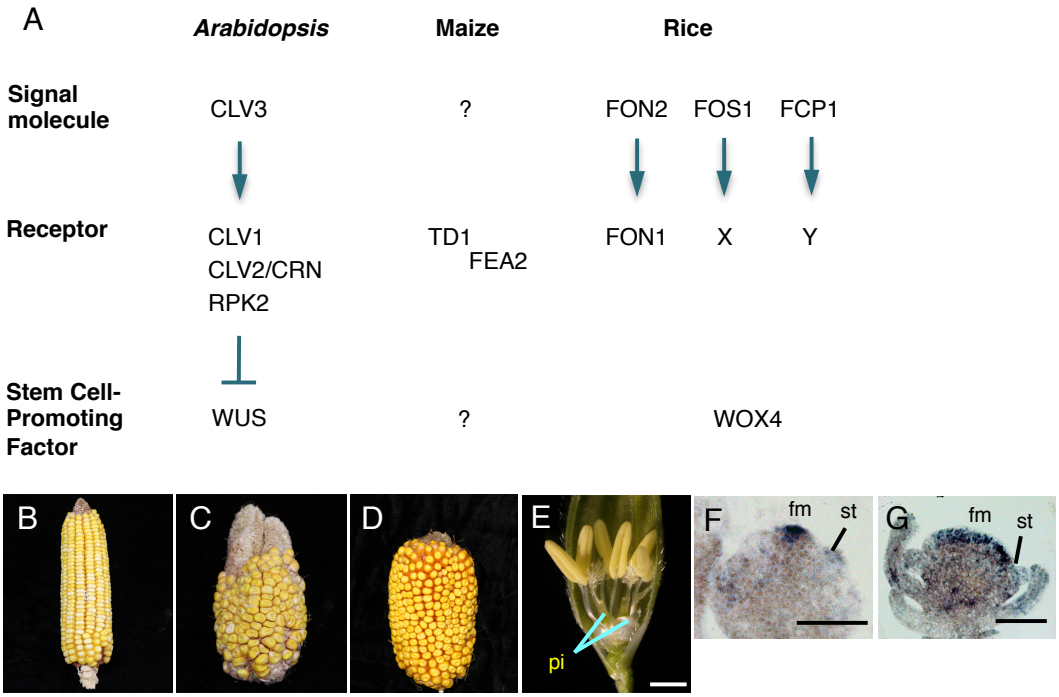

Figure 1: Genes involved in stem cell maintenance.

(A) Models of stem cell maintenance in *Arabidopsis*, maize, and rice. Receptor X and Y are different from FON1, but it is unknown whether X and Y are equivalent. (B-D) Ears of maize in wild type (B), and fasciated ears of *td1* (C), and *fea2* mutants (D). (E) Flower of rice *fon1* mutant, showing an increase in the number of floral organs (F, G) Expression of rice *FON2* in wild type (F) and *fon1* mutant (G), showing expansion of the *FON2* domain (Images kindly provided by T. Suzaki).

fm, flower meristem; pi, pistil; st, stamen. Bars= 1 mm in (E) and 100μm in (F, G).

resulting in an increased spikelet density and a thicker central spike. In the ear, the inflorescence is fasciated and seed row number is increased (Fig. 1B-1D). Floral meristems (FM) are also enlarged, resulting in an increase in the number of floral organs, such as stamens. *TD1* and *FEA2* encode a CLV1-like LRR-receptor kinase and a CLV2-like LRR receptor- like protein, respectively. *td1 fea2* double mutants show an enhanced phenotype relative to each single mutant, suggesting that these two genes function in different genetic pathways (Bommert et al. 2005a). This maize double mutant phenotype initially implied a difference in genetic mechanism between maize and *Arabidopsis*, since it was once thought that *CLV1* and *CLV2* act in the same genetic pathway (Kayes and Clark 1998). However, recently, it has been revealed that CLV2 acts in a signaling pathway independent of CLV1, by forming a receptor complex with CRN, also known as SUPPRESSOR OF LLP2 (SOL2) (Miwa et al. 2008; Müller et al. 2008). Another interesting aspect of the work in maize is the finding that *FEA2* maps to a QTL for kernel row number, suggesting that natural variation in these genes contributed to crop improvement (Taguchi-Shiobara et al. 2001). This QTL association has been confirmed by the characterization of weak *fea2* alleles that enhance kernel number without causing fasciation (Bommert et al, Nature Genetics, in press).

In rice, *floral organ number* (*fon*) mutants have also contributed to understanding stem cell maintenance (Nagasawa et al. 1996). The FM enlarges in both *fon1* and *fon2* mutants, resulting in an increase in the number of floral organs, such as stamens and carpels (Fig. 1E) (Nagasawa et al. 1996; Suzaki et al. 2004; Suzaki et al. 2006). Molecular cloning has revealed that *FON1* encodes a gene orthologous to *CLV1* and maize *TD1*, whereas *FON2* encodes a CLE protein related to *Arabidopsis* CLV3. The independently isolated *fon4* mutant is allelic to *fon2* (Chu et al. 2006). *FON1*, like maize *TD1*, is expressed throughout the meristem, whereas *FON2* is expressed in the apical region of the meristem. (Suzaki et al. 2004; Chu et al. 2006; Suzaki et al. 2006). The expression domain of *FON2* is highly expanded in the enlarged floral meristem of *fon1* mutant (Fig. 1F, 1G) (Suzaki et al. 2006), reminiscent of *CLV3* behavior in *Arabidopsis*

(Fletcher et al. 1999; Brand et al. 2000).

The phenotype of *fon1 fon2* double mutants resembles that of each single mutant, indicating that *FON1* and *FON2* function in the same genetic pathway (Suzaki et al. 2006). Overexpression of *FON2* causes severe reduction in the number of floral organs, probably because of a decrease in size of the floral meristem. This overexpression effect is not observed in the *fon1* mutant background, suggesting that FON2 acts through the putative receptor encoded by *FON1* (Suzaki et al. 2006). Thus, the genetic relationship and molecular function of *FON1* and *FON2* in rice are very similar to those of *CLV1* and *CLV3*.

These studies in both maize and rice suggest that the CLV pathway that negatively regulates stem cell maintenance is conserved in grasses. Despite a common mechanism, there are differences in the mutant phenotypes between maize and rice: the inflorescence meristem is severely affected in both maize *td1* and *fea2* mutants, whereas such defects are not evident in rice *fon1* and *fon2* mutants. These differences may be due to genetic redundancy, or to a high sensitivity of the maize IM to these mutations owing to selection for increase in the number of rows of seeds on the ear during maize domestication (Taguchi-Shiobara et al. 2001; Brown et al. 2011).

In rice, the *FON2 SPARE1* (*FOS1*) gene was identified as a modifier of the *fon2* mutation; the floral phenotype of *fon2* is suppressed when *FOS1* from *indica* is present (Suzaki et al. 2009). *FOS1* encodes a CLE protein like FON2, but the protein encoded by the *japonica* allele is likely to have no or weak function due to a defect in a putative processing site of the signal peptide. Thus, FON2 and FOS1 are likely to act redundantly in the maintenance of the FM in *indica*, and an enlargement of the FM in the original *fon2* mutant (*japonica* background) results from mutations occurring in both the *FON2* and *FOS1* genes. The mutant allele of *FOS1* is distributed in all *japonica* strains examined, whereas all *indica* strains and wild rice species examined have wild-type *FOS1* (Suzaki et al. 2009). Therefore, the FM is robustly maintained by parallel redundant signaling pathways in rice (the genus *Oryza*) in general, whereas a mutation might

have occurred in *FOS1* during domestication of *japonica* rice.

Unlike the *clv* mutants in *Arabidopsis*, no obvious abnormalities have been described in the vegetative meristems in the grass mutants described above. In addition to *FOS1*, *FON2-LIKE CLE PROTEIN1* (*FCP1*), a gene that encodes a protein containing the CLE domain with high similarity to that of *FON2,* is likely to be involved in the maintenance of the rice vegetative SAM (Suzaki et al. 2008). Constitutive expression of either *FCP1* or *FOS1* causes termination of the vegetative SAM in shoots regenerated from calli (Suzaki et al. 2008; Suzaki et al. 2009). By contrast, *FON2* overexpression causes no abnormality in the vegetative SAM, although the FM is severely affected, as described above (Suzaki et al. 2006). Therefore, *FCP1* and *FOS1* negatively regulate the maintenance of the vegetative SAM, whereas *FON2* function is restricted to reproductive meristems (IM and FM). In addition, FCP1 and FOS1 likely act through a receptor other than FON1, because constitutive expression of either *FCP1* or *FOS1* shows a similar effect on shoot regeneration in the *fon1* mutant to that observed in wild type (Suzaki et al. 2008; Suzaki et al. 2009). These observations demonstrate that stem cell maintenance is likely to be regulated by at least three related negative pathways in rice, and each pathway seems to contribute differently to this regulation depending on the type of meristem.

1.4 Genes that promote stem cell identity

In contrast to negative pathways in meristem maintenance, current understanding of factors that promote stem cell identity is still lacking. It is probable that *WUS* orthologs, or *WUSCHEL RELATED HOMEOBOX* (*WOX*) genes, may also have such function in grasses. Although a few studies concerning the expression patterns of *WOX* genes have been published, no genetic or functional analysis has been reported in grasses. However, the presence of two *WUS* paralogs, *ZmWUS1* and *ZmWUS2*, with different expression patterns, suggests some degree of sub-functionalization has occurred (Nardmann and Werr, 2006). Functional identification of stem cell-promoting factors, such as *WUS*, would be helpful to elucidate the genetic mechanism that

regulates stem cell maintenance in grasses. A recent study reports that *WOX4*, a distinct member of the rice *WOX* gene family, acts as a positive factor in shoot meristem maintenance and is negatively regulated by *FCP1* in rice (Ohmori et al., in press).

1.5 Cytokinin action in the meristem

One of the first indications of the role of cytokinin in meristem maintenance came from the maize mutant *aberrant phyllotaxy1* (*abph1*), which has a defect in phyllotaxy, the geometric pattern of leaf initiation, and an enlarged meristem (Jackson and Hake 1999). The *ABPH1* gene encodes a type-A response regulator functioning in cytokinin signaling (Giulini et al. 2004). Cytokinin signal transduction is regulated by a two-component feedback system where cytokinin-inducible B-Type Response Regulators (RRs) activate the expression of a set of cytokinin-responsive genes, including A-type RRs, which inhibit cytokinin signaling (For review, see Argueso et al. 2010). In *Arabidopsis*, WUS promotes cytokinin signaling by repressing the A-type genes *ARABIDOPSIS RESPONSE REGULATOR7* (*ARR7*) and *ARR15*, whereas cytokinin positively regulates the expression of *WUS* (Leibfried et al. 2005; Gordon et al. 2009).

In rice, the *lonely guy* (*log*) mutant produces small panicles with a reduced number of branches and spikelets (Kurakawa et al. 2007). Analysis of *LOG* function provided an important breakthrough, since it was revealed that *LOG* encodes an enzyme that catalyzes the final step of cytokinin biosynthesis, which had not been found by biochemical studies. *LOG* is expressed in the tip of the reproductive meristem, and the expression of cytokinin-inducible genes is dramatically reduced in the meristem of severe *log-1* mutants. Maintenance of the meristem is compromised, especially in the reproductive phase; expression of the meristem marker *Oryza sativa HOMEOBOX GENE1* (*OSH1)* is highly reduced in the FM, and the shape of the FM is altered. A severe reduction in the number of floral organs, especially in the inner whorls, is observed in *log-1*. In a weak allele, *log-3*, the ovule does not develop, due to a failure to maintain the FM after carpel initiation (Yamaki et al. 2011). The floral phenotype of *log-1* resembles that

of the *FON2*-overexpressing plant, whereas a *fon1* mutation suppresses the *log-3* phenotype (Suzaki et al. 2006; Kurakawa et al. 2007; Yamaki et al. 2011). These observations suggest involvement of cytokinin action in FON signaling. The importance of *LOG* function in meristem organization has been recently reported in *Arabidopsis*. The biologically active form of cytokinin, which is probably catalyzed by *LOG4* expression in the SAM epidermis, acts as a positional cue for patterning the *WUS* expression domain (Chickarmane et al. 2012).

1.6 KNOX genes promote meristem identity

Another important layer of regulation in the SAM is imposed by the homeobox-containing transcription factor KNOTTED1 (KN1) and related KNOTTED1-like homeodomain (KNOX) proteins. Originally identified as a dominant gain-of-function mutation in maize with knotted protrusions on vegetative leaves, *KN1* is required for maintenance of the SAM, as loss-of-function alleles cause meristem termination in a background dependent manner (Kerstetter et al. 1997; Vollbrecht et al. 2000). *KNOX* genes positively regulate meristem identity in both monocots and dicots as the *Arabidopsis* ortholog of *KN1*, *SHOOTMERISTEMLESS,* and the rice ortholog, *OSH1*, display conserved meristem termination phenotypes (Long et al. 1996; Tsuda et al. 2011).

There has been considerable interest in the mechanism by which *KNOX* genes promote meristematic activity. In several different model species, KNOX proteins have been shown to directly bind and either activate or repress giberellic acid (GA) biosynthesis genes, modifying levels of active GA in meristems and boundary regions (Sakamoto et al. 2001; Chen et al. 2004; Bolduc et al. 2009). KNOX proteins also regulate cytokinin biosynthesis in *Arabidopsis* by activating isopentenyl transferase genes (Jasinski et al. 2005; Yanai et al. 2005). In addition, inducible overexpression of the KNOX gene *OSH15* upregulates expression of several cytokinin biosynthesis genes in rice (Sakamoto et al. 2006). Tsuda et al. (2011) also showed that *OSH1* and *OSH15* activate their own expression, and are positively regulated by cytokinin. Taken together,

the data suggest that *KNOX* genes and cytokinin mutually reinforce SAM identity.

A genome-wide binding profile for KN1 was recently identified by Chromatin Immunoprecipitation-sequencing (ChIP-seq), and targeted genes were compared to a list of genes differentially expressed in the *kn1* loss-of-function mutant (Bolduc et al. 2012). This analysis revealed that KN1 targets genes involved in four major hormone pathways (auxin, cytokinin, GA, and brassinosteroids), orchestrating a careful balance that promotes meristem maintenance. Direct targets also included many other transcription factors, placing KN1 at the summit of a regulatory cascade controlling shoot meristem function (Bolduc et al., 2012).

1.7 Additional pathways required for meristem maintenance

The *FLATTENED SHOOT MERISTEM* (*FSM*) gene is another factor required for meristem maintenance in rice, as mutants have a flatter and smaller SAM than wild type plants (Abe et al. 2008). *FSM* encodes a Chromatin Assembly Factor-1 (CAF1) subunit, and is the ortholog of the *Arabidopsis* gene *FASCIATA1 (FAS1)*. Interestingly, *FAS1* displays an enlarged meristem, suggesting that this layer of meristem maintenance may function quite differently in the monocot and dicot lineages (Abe et al. 2008).

Several classes of small RNAs and associated biosynthetic machinery have been implicated in meristem maintenance in rice. Mutants in trans-acting small interfering RNA (ta-siRNA) biogenesis components, such as *SHOOTLESS4 (SHL4)*/ *ARGONAUTE7 (AGO7)* and *SHOOT ORGANIZATION1 (SHO1)/ DICER-LIKE4 (DCL4)*, fail to maintain a SAM through embryogenesis (Nagasaki et al. 2007). The meristem defect in these plants is partially explained by a strong down-regulation of class III HD-ZIP genes, caused by an accumulation of miR166 (Nagasaki et al. 2007). In addition, mutants in *WAVY LEAF1 (WAF1)*, which encodes the ortholog of the *Arabidopsis* RNA methyltransferase *HUA ENHANCER1* (*HUA1*), have reduced levels of ta-siRNAs and microRNAs, due to decreased RNA stability (Abe et al. 2010). *waf1* mutants enhance the meristem maintenance defects of hypomorphic *sho* mutants, further

demonstrating the importance of the ta-siRNA population for meristem function.

1.8 Phyllotaxy and Plastochron regulation

Most members of the grass family display an alternate phyllotaxy, or pattern of leaf initiation, with one organ initiated at the flank of the meristem at a time, resulting in one leaf per node (Jackson and Hake, 1999). The pattern of leaf arrangement is important for plant traits such as stalk strength and optimal light capture. Maize *aberrant phyllotaxy1* (*abph1)* was the first mutant cloned that has a specifically altered phyllotaxy program (Giulini et al. 2004), although other *aberrant phyllotaxy* mutants await molecular identification (DJ, unpublished). *ABPH1* is expressed in the SAM during embryonic development, and in the incipient leaf primordium (P0) post-germination (Guilini et al. 2004). Mechanistic studies showed that *ABPH1* expression is dependent on polar auxin transport, and that *ABPH1* activates expression of the auxin transporter PIN1, suggesting that a complex interplay between auxin and cytokinin signaling regulates phyllotaxy and leaf initiation (Lee et al. 2009).

In rice, *decussate* (*dec*) mutants also display a transformation to opposite phyllotaxy, as well as a larger SAM characterized by an increased rate of cell division (Itoh et al. 2012). Conversely, the mutants have a smaller RAM, and the mutants die before reaching the reproductive phase. It is well established that cytokinin exerts an opposite effect on cell division in the SAM compared to the RAM (Werner et al. 2003). Consistent with this, *dec* mutants have decreased expression of several type A-ARRs, as well as decreased sensitivity to applied cytokinin (Itoh *et al.* 2012). *DEC* encodes a glutamine-rich protein with domains of unknown function shared with fungi and animals (Itoh et al. 2012), thus it will be interesting to further explore connections with cytokinin and auxin signaling.

Another property of organ initiation from the meristem is plastochron, the elapsed time between the initiation of two leaves. Three rice mutants, *plastochron1, 2,* and *3*, display greatly reduced plastochron length, with a large increase in the number of leaves originating from the

SAM (Miyoshi et al. 2004; Kawakatsu et al. 2006; Kawakatsu et al. 2009). The plastochron phenotype is associated with larger meristems, with much higher rates of cell division than wild-type plants (Miyoshi et al. 2004). Similarly, the *shoot organization* (*sho1, 2, 3*) mutants generate an abnormally high number of leaves in a random phyllotaxy (Itoh et al. 2000). Loss-of-function *aberrant panicle organization1* (*apo1*) mutants also have decreased plastochron length, while dominant gain-of-function alleles display a corresponding increase in plastochron time (Ikeda-Kawakatsu et al. 2009).

An outstanding question is to what degree changes in meristem size and structure are correlated with, or causative of, changes in phyllotaxy and plastochron. Larger meristems are present in the *abph1* and *dec* mutants described above, but a larger vegetative meristem does not always produce a change in phyllotaxy in other mutants. Changes in plastochron length are also usually accompanied by changes in meristem size (Wang et al. 2008; Kawakatsu et al. 2009). An analysis using various mutants with defects in the rate of leaf initiation found a correlation between meristem shape parameters (ie. height/width ratios) and phyllotaxy and plastochron parameters; however, no such relationship existed with meristem size *per se* (Itoh et al. 2000).

1.9 Axillary meristem dormancy and tillering

Tillering, the production of secondary shoots by axillary meristems (AMs), is a widespread property of grasses. Maize domestication selected very strongly for untillered maize plants, with dormant AMs, with the exception of one to two ear shoots per plant. This architecture is extremely important for achieving high planting density while maintaining ease of harvest. In contrast, an appropriate degree of tillering is essential to high yield production in rice. Secondary shoot production is determined first by the initiation of AMs, and then by factors controlling dormancy of the axillary shoots.

Several key regulators of AM initiation have been identified based on loss-of-function mutant phenotypes. The maize mutant *barren stalk1* (*ba1*) encodes a bHLH transcription factor

that is required to establish axillary meristems in vegetative and reproductive stages (Gallavotti et al. 2004). The orthologous rice gene *LAX PANICLE1* (*LAX1*), is required only to initiate AMs in the inflorescence (Komatsu et al. 2003). Both of these grass-specific transcription factors are expressed in boundary domains associated with all AMs, but not in the meristems, and act non-cell autonomously (Komatsu et al. 2003; Gallavotti et al. 2004). The LAX1 protein moves directionally into the developing AM in a stage-specific manner, and this trafficking is required for its function (Fig. 2A) (Oikawa and Kyozuka, 2009). The role of *LAX1* in vegetative AMs is more clearly revealed when *lax1* is combined with the *monoculm1* (*moc1*) or *lax panicle2* (*lax2*) mutants (Li et al. 2003; Tabuchi et al. 2011). Vegetative AMs are completely abolished in the *lax1 moc1* double mutant, and tillers are very strongly reduced in the *lax1 lax2* double mutant (Tabuchi et al. 2011). *moc1* encodes the rice ortholog of the GRAS family transcription factor *LATERAL SUPPRESSOR* (*LAS*) of *Arabidopsis* (Li et al. 2003), and recent work has implicated a role for proteasome-mediated degradation of MOC1 in regulating AM dormancy (Lin et al. 2012; Xu et al. 2012). *LAX2* encodes a plant-specific nuclear protein that physically interacts with LAX1 to cooperatively regulate AM formation (Tabuchi et al. 2011).

Maize underwent strong selection for AM dormancy during domestication from its highly branched ancestor, teosinte. Five classical QTL differentiate the architecture of modern maize from its wild progenitor (Doebley, 2004). One of these QTL maps to *teosinte branched1* (*tb1*), a mutant with a teosinte-like morphology due to elaboration of axillary shoots (Doebley et al. 1997). *TB1* is a founding member of the TCP (*TB1- CYCLOIDEA (CYC)- PROLIFERATING CELL FACTOR (PCF)*) family of transcription factors, which modulate rates of cell division (Martin-Trillo and Cubas, 2010). *TB1* orthologs in rice and *Arabidopsis* play conserved roles in regulating plant branching architecture (Takeda et al. 2003; Aguilar-Martinez et al. 2007). Population genetic studies have identified a selective sweep signature upstream of the *TB1* gene in maize, and a region approximately 70-kb upstream was shown to act as a transcriptional enhancer (Clark et al. 2006). Recent work has narrowed down the enhancer activity to a

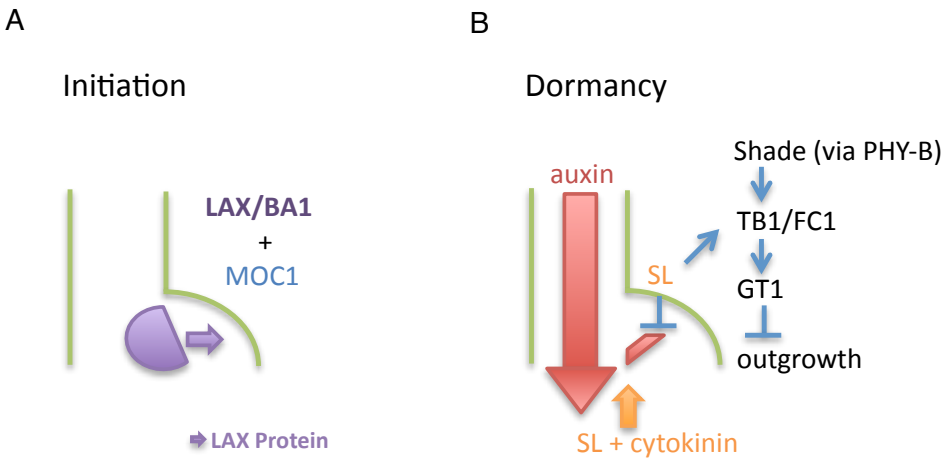

Figure 2: Tillering is controlled by a two-step process of axillary meristem (AM) initiation and dormancy.

(A) Initiation of AMs is controlled by the co-operative action of *LAX PANICLE1* (*LAX1*)/*BARREN STALK1*(*BA1*) and *MONOCULM1* (*MOC1*). Initiation depends on the stage-specific trafficking of the LAX1 protein into the meristem. (B) AM dormancy is controlled by the antagonistic interactions of three phytohormones and two genes that are responsive to shade signals (*TEOSINTE BRANCHED1* and *GRASSY TILLERS1*). Auxin (red arrow), which is transported basipetally through the polar auxin transport stream, inhibits the outgrowth of axillary buds. Cytokinin is transported in the opposite direction, and directly promotes growth. Strigolactones (SLs) are hypothesized to limit outgrowth by mitigating polar auxin transport out of AMs, and may also act by activating *FINE CULM1 (FC1)*.

HOPSCOTCH retrotransposon insertion in this upstream region. The *HOPSCOTCH* insertion pre-dates the domestication of maize by approximately 10,000 years, indicating that selection during domestication acted on standing variation in the teosinte gene pool (Studer et al. 2011).

Another likely target of selection for reduced tillering is the *GRASSY TILLERS1* (*GT1*) gene, encoding a HD-ZIP I protein (Whipple et al. 2011). *GT1* appears to be under the transcriptional control of TB1, as its expression is greatly reduced in the *tb1* mutant. Furthermore, in teosinte and *Sorghum bicolor*, *TB1* and *GT1* appear to inhibit axillary bud outgrowth in response to shade signals perceived by phytochrome B (Fig. 2B). The shade avoidance pathway represses axillary bud outgrowth in many grasses, but axillary buds are constitutively dormant in domesticated maize (Whipple et al. 2011).

AM dormancy is also influenced by the antagonistic action of several classes of plant hormones (Fig. 2B). The phenomenon of apical dominance plays an important role in regulating axillary shoots. Auxin, synthesized at the growing tip of the plant, is transported basipetally through the polar auxin transport (PAT) stream, and indirectly suppresses bud outgrowth (McSteen and Leyser 2005). In contrast, cytokinin is transported acropetally through the xylem system, into the AMs, where it promotes growth. The mechanisms by which these two hormones influence AM determinacy are well established, and are reviewed by McSteen and Leyser (2005).

A third hormone plays a central role in regulating AM dormancy. The existence of this signal was postulated based on a collection of *Arabidopsis*, rice, and pea mutants with increased branching, which encoded biosynthetic machinery for an unknown carotenoid-based hormone (for review see, Ongaro and Leyser, 2008). Reciprocal grafting experiments provided evidence that this hormone moved acropetally from the roots into the shoot. Levels of root-synthesized terpenoid hormones called strigolactones (SLs) were reduced in these biosynthetic mutants, and exogenous application of SLs rescued the shoot branching phenotypes (Gomez-Roldan et al. 2008; Umehara et al. 2008). Thus, SLs are a novel and specific inhibitor of axillary meristem outgrowth.

An important unanswered question in the field has been the nature of the SL receptor. Several candidate genes were identified in rice based on insensitivity to exogenous SLs, including *DWARF14* (*D14*) (Arite et al. 2009). Homology modeling showed that this alpha-beta (α-β) fold hydrolase could potentially interact with a natural SL ligand (Gaiji et al. 2012). Recently, the pea ortholog of *D14*, *DAD2*, was shown to encode a catalytically active candidate SL receptor, which physically associates with *PhMAX2A,* a key signal transduction component (Hamiaux et al. 2012). Therefore, it is likely that D14 and related proteins represent authentic SL receptors in grasses and dicot species.

One putative downstream effector of SL signaling in rice is *FINE CULM1* (*FC1*), as mutants in this *TB1* ortholog are insensitive to exogenously applied SL (Minakuchi et al. 2010). Interestingly, treatment with cytokinin reduces expression of *FC1*, suggesting that this gene may be important in integrating multiple hormonal signaling pathways in axillary buds (Minakuchi et al. 2010). Further work is needed to elucidate the downstream consequences of SL signaling in the AM. For example, the relationship between SLs and auxin is still not fully understood. It has been suggested that SLs prevent axillary shoot branching by limiting auxin polar transport, such that auxin export cannot be established from axillary buds, a process that is essential for outgrowth (Fig. 2B)(Crawford et al. 2010).

1.10 The floral transition

Grasses have evolved a spectrum of different pathways that coordinate the floral transition in response to environmental and endogenous cues. Some features of grass flowering pathways are conserved between all flowering plants, while others represent innovations specific to various grass lineages. For example, different species of grasses have different sensitivities and thresholds for daylength-dependent flowering. Rice is considered a photoperiod-sensitive species, with a facultative short-day requirement. On the other hand, floral induction in maize reflects its domestication from a tropical grass, but subsequent breeding and improvement over a wide range

of temperate environments. Most temperate maize inbred lines are essentially day-neutral, whereas tropical lines respond to short-day inductive cues (Colasanti and Coneva, 2009). Other temperate grasses, such as wheat and barley, have a long-day requirement with a vernalization switch (for review, see Cockram et al. 2007); this section will focus on flowering pathways in maize and rice.

Much of what we know about the floral transition comes from studies in *Arabidopsis*. The *CONSTANS* (*CO*) gene integrates the main outputs of the circadian clock, and serves to synchronize flowering time with long day photoperiods (For review, see Turck et al. 2008). Under long day conditions, CO, a zinc finger transcription factor, is stable and activates the expression of *FLOWERING LOCUS T* (*FT)* in leaves. Subsequently, the FT protein product is translocated through the phloem to the SAM, where it interacts with the bZIP transcription factor FLOWERING LOCUS D (FD) and targets floral regulators. FT is regarded to fulfill the criteria for the universal leaf-derived flowering signal, "florigen" (Corbesier et al. 2007; Turck et al. 2008).

This extensively characterized photoperiod-responsive flowering module is conserved in grasses, however there are obvious differences in the daylength perception (Fig. 3). A major rice QTL for photoperiod responsiveness, *HEADING DATE1* (*HD1*), was cloned and revealed to encode an ortholog of *CO* (Yano et al. 2000). HD1 is an activator of the rice *FT* ortholog *HEADING DATE 3a* (*HD3a*) under short day conditions, but is a repressor of *HD3a* expression under long day conditions (Tamaki et al. 2007). Another factor, *EARLY HEADING DATE1* (*EHD1*), which encodes a B-type cytokinin response regulator, also activates the expression of *HD3a* under short day conditions, independently of *HD1*. The exquisitely sensitive daylength response of rice flowering is conferred by *EHD1* regulation via the opposing action of blue-light-mediated floral promotion and phytochrome-mediated floral repression pathways (Itoh et al. 2010). *GRAIN NUMBER, PLANT HEIGHT AND HEADING DATE7* (*GHD7*), a CCT-domain protein, which is induced through phytochrome signaling, represses the expression of *HD3a*

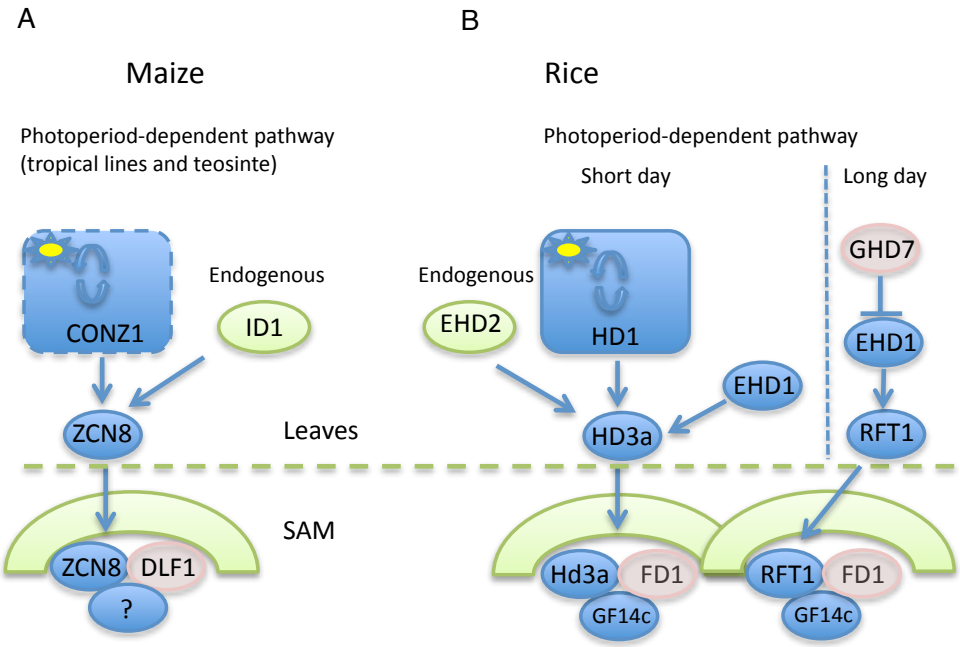

Figure 3: **Regulation of the floral transition in maize and rice.**

(A) Flowering in maize is controlled by an endogenous pathway regulated by *INDETERMINATE1* (*ID1*), and a short-day inducible pathway specific to tropical inbred lines. The output of the circadian clock is integrated by *CONZ1* (Miller et al. 2008). *ZCN8* is a putative maize FT (florigen) ortholog, which is induced in the leaves and predicted to translocate to the SAM where it interacts with DLF1, a FD homolog (Muszynski et al. 2006). (B) The floral transition in rice is sensitive to changes in photoperiod. The output of the circadian clock is integrated by *HD1*. Short-day conditions induce flowering via *EHD1* and *EHD2*-dependent up-regulation of *HD3a* (florigen). A parallel pathway involving *GHD7* represses the expression of EHD1 under long-days to prevent flowering, but the transition may proceed under long days via a second florigen protein, RFT1. Activation of floral regulators is achieved by the Florigen Activation Complex (FAC) comprised of HD3a, FD1, and GF14c.

CONZ1, CONSTANS OF ZEA MAYS1; ZCN8, ZEA CENTRORADIALIS8 ;FT, FLOWERING LOCUS T; DLF1, DELAYED FLOWERING1; FD, FLOWERING LOCUS D; EHD1, EARLY HEADING DATE 1; EHD2, EARLY HEADING DATE2; HD3a, HEADING DATE 3a; GHD7, GRAIN NUMBER PLANT HEIGHT HEADING DATE7; RFT1, RICE FLOWERING LOCUS T1.

under long days by suppressing blue-light induction of *EHD1* in the morning. This results in a highly sensitized system where an increase in daylength of only 30 minutes is sufficient to delay floral induction (Itoh et al. 2010). A paralog of *HD3a*, *RICE FLOWERING LOCUS T1* (*RFT1*), is induced under long day conditions by *EHD1* and *OsMADS50*, and also acts as a transmissible florigen signal (Komiya et al. 2009). Thus, in contrast to *Arabidopsis*, two separate photoperiod-sensing pathways converge on two FT/ florigen genes in rice (Fig. 3).

Recent work has identified an intracellular receptor for the rice FT protein HD3a (Taoka et al. 2011). HD3a and OsFD1 do not directly interact at the apex, but rather are bridged together by the 14-3-3 protein GF14c, to form the Florigen Activation Complex (FAC). Once assembled in the nucleus, the FAC is responsible for activating the expression of *OsMADS15*, the rice ortholog of *APETALA1* (*AP1*), a key floral regulator (Taoka et al. 2011).

Different cultivars of rice display natural variation in flowering time under SD conditions (Tsuji et al. 2011). These differences are explained by variation in sequence and expression levels of members of the photoperiodic flowering pathway, namely *HD3a*, *HD1*, and *EHD1* (Takahashi et al., 2009). Natural variation in the floral repressor *GHD7* is correlated with different geographical areas of cultivation, and hypomorphic alleles have allowed the expansion of rice cultivation into more temperate northern latitudes (Xue et al. 2008).

An endogenous pathway regulating the floral transition operates in parallel with the photoperiod pathway in grasses, and takes on an increased importance in day-neutral temperate maize. A central player in this pathway, *INDETERMINATE1* (*ID1*), was identified as a mutant that failed to transition to the reproductive phase (Colasanti et al. 1998) (Fig. 3). This zinc finger transcription factor is localized to developing leaves, and acts non cell-autonomously to induce flowering at the apex. ID1 may also serve to connect the endogenous and photoperiod-dependent pathways, as expression of the *FT* homolog *ZEA CENTRORADIALIS 8* (*ZCN8*) is greatly reduced in the *id1* mutant (Lazakis et al. 2011). *ZCN8* displays circadian fluctuations in photoperiod-sensitive tropical lines, and is upregulated in the leaves of teosinte under inductive short-day

conditions (Meng et al. 2011; Lazakis et al. 2011). Similarly, the rice ortholog of *ID1*, *OsID1/EHD2*, is required to activate expression of *EHD1* (Park et al. 2008; Matsubara et al. 2008), indicating a conserved connection between the endogenous and photoperiod-dependent pathways in grasses.

1.11 Inflorescence Meristem Identity

Following the vegetative to reproductive transition, the IM functions much like the vegetative SAM, initiating lateral leaf (bract) primordia in a regular phyllotaxy, which are accompanied by axillary meristems. Grasses have a program of bract suppression to limit leaf outgrowth, and thus the dominant features of the inflorescence are all derived from the axillary meristems (e.g. spikelet and floral meristems) (Whipple et al. 2010). Not much is known about genes that regulate the identity and determinacy of the IM. A recent study revealed that *PANICLE PHYTOMER2* (*PAP2*) and three other AP1-like MADS-box genes are required to specify the identity of the rice inflorescence meristem downstream of the florigen signal (Kobayashi et al., 2012). Properties such as the determinacy, or persistence, of the IM have the ability to greatly influence panicle size and ear length, and thus grain yield.

1.12 Perspective

Great progress has been made towards understanding the genetic factors that control meristem regulation in maize and rice; however, several fundamental questions remain unanswered. An important area of focus is identification of genes required for positive regulation of stem cell identity in grasses. Little is known about positive regulators of stem cell maintenance, such as *WUS*, in grasses, although understanding of negative regulators has accumulated, as described above. Where are the genes responsible for the positive regulation expressed in the meristem? Does the grass meristem have a domain corresponding to the organizing center, where

WUS is expressed? How do the positive and negative regulators interact with each other to regulate stem cell homeostasis? These questions are especially interesting in light of the fact that the structure of the meristem in grasses differs from that of eudicots, since grass meristems generally lack a clonal L2 cell layer. Another salient question is which CLE peptides encoded in the maize genome function to negatively regulate meristem size. It is likely that many outstanding questions in grass meristem biology will be answered in the next few years through a combination of forward and reverse genetics, QTL mapping, and functional genomics experiments, such as mRNA-seq, proteomics and ChIP-seq.

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CHAPTER 2: GRASS MERISTEMS II

2.1 Introduction

The plant body plan is governed by the activities of the shoot apical meristem (SAM) and the root apical meristem (RAM), which are formed during embryogenesis. All shoot parts of the plant, such as leaves, stems and flowers, develop from the SAM, whereas the root system is formed from the RAM. Postembryonic development depends on the function of the meristems; therefore, regulation of meristem maintenance and fate is very important for plant growth.

In the vegetative phase, the SAM sequentially initiates leaf primordia. The axillary meristems (AM) are formed in the leaf axil, and then develop the secondary shoots, or tillers. After transition from the vegetative to the reproductive phase, the SAM changes its fate, and converts into the IM. In some species, the IM initiates the FM directly, in the axil of a bract. In *Arabidopsis*, transition of the meristem appears to be simple, as if the FM was directly formed from the IM because of suppression of bract growth. Unlike *Arabidopsis*, there are several intermediate types of meristem between the IM and the FM in the grasses, as described in the next section. Therefore, the transitions of meristem fate are complex, and involve a number of genes regulating this process. Grass inflorescences, such as the rice panicle and maize tassel, consist of a main axis, long branches, and spikelets. These unique inflorescence units develop from specialized meristems: the BM and the SM.

In the accompanying paper, Pautler et al. (2013) describe the genetic and hormonal regulation of the meristem, as well as the transition from vegetative to inflorescence fate. In this review, we focus on the inflorescence, first considering the regulation of the initiation and determinacy of the BM. In the next section, we describe the genes involved in changes in meristem fate: transition from the BM to the SM and from the SM to the FM, respectively, and in determinacy of the SM. Then, we focus on the genes involved in both the regulation of determinacy of the FM and specification/development of floral organs. Finally, we briefly mention the communication between the meristem and lateral organ primordia. Although there

are several excellent reviews on grass meristems (Bortiri and Hake 2007; Thompson and Hake 2009; Yoshida and Nagato 2011), the accumulation of the papers describing meristem function and flower development has been very rapid. We have tried to summarize the related studies, including new findings.

2.2 Flower development and meristem transitions in grasses

Grass inflorescences are complex, and formed from several types of meristems. After transition from the vegetative phase to the reproductive phase, the SAM converts into the IM, which initiates the BMs and forms the main axis of the inflorescence (Fig. 4A). The BMs initiate the SMs, followed by the initiation of the floret (flower) meristems (FMs) from the SM. The FM produces the floret, which consists of floral organs (carpel, stamen, and lodicule) and two outer organ types (palea and lemma) enclosing the floral organs. The spikelet is composed of one to several florets and two glumes that enclose them. Thus, the SM initiates the glume primordia in addition to the FM. Although this is a general scheme of the inflorescence and flower development in grasses, various modifications result in diverse structures of the inflorescence depending on species.

In maize, for example, several major differences occur. First, there are two types of inflorescence: the male inflorescence, or tassel, which forms long branches; and the female inflorescence, or ear, which does not (Fig. 4A). Second, the IM initiates a novel kind of meristem, the SPMs, that are responsible for making paired spikelets typical of the Andropogoneae. SPMs are made in a spiral phyllotaxy in the tassel after the formation of several branch meristems with an indeterminate branch meristem fate. The SPMs subsequently divide into two SMs (Fig. 4A, 4C). Next, the SM initiates two FMs (Fig. 4A, 4E). The two florets normally develop in the tassel, whereas the lower FM aborts in the ear, such that only one floret is formed in the female spikelet (Fig. 4B, 4E, 4F) (for review see, Bommert et al. 2005; McSteen et al. 2000; Thompson and Hake

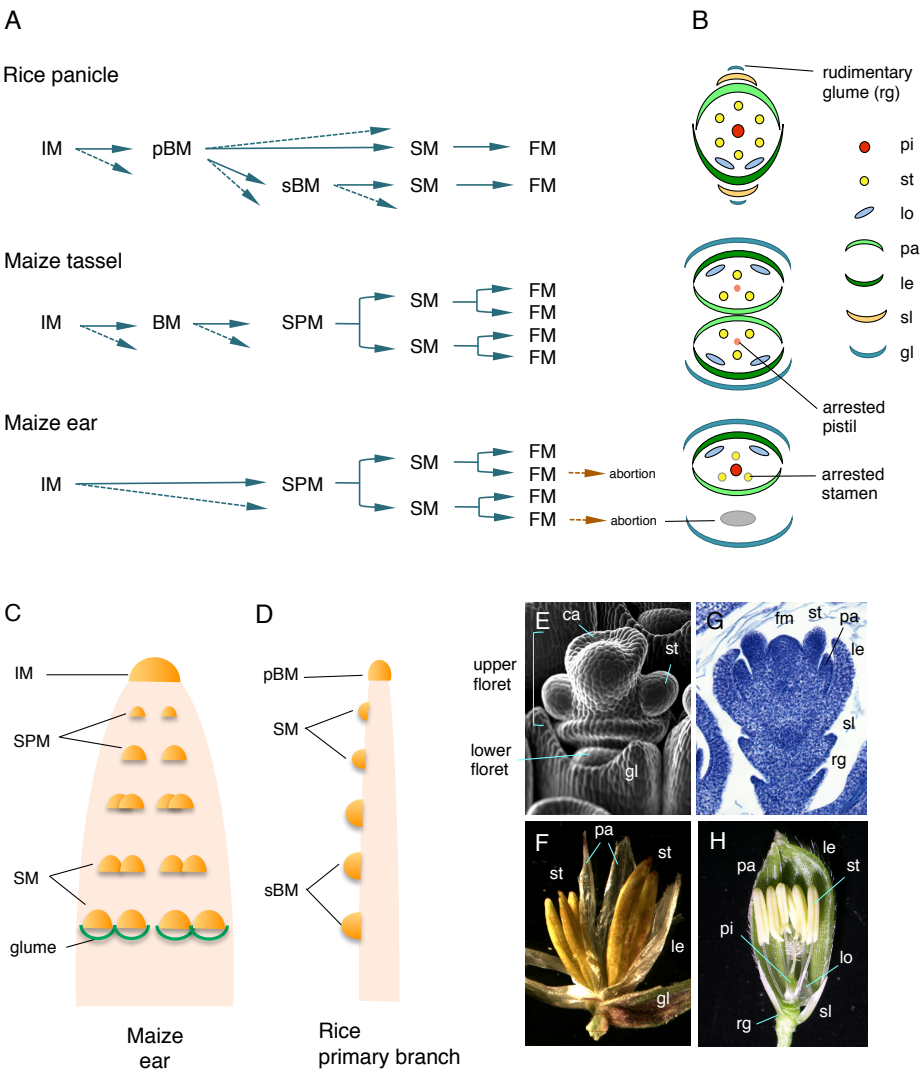

Figure 4: Transition of the reproductive meristems, and the flower and inflorescence in rice and maize.

(A) Transition of the meristems. Dashed arrow indicates multiple formation of the meristem. (B) Schematic representation of the spikelet of rice (upper) and maize (male, middle; female, lower). (C) Schematic representation of the ear in maize. (D) Schematic representation of the primary branch of rice panicle. (E) Spikelet of maize ear at early developmental stage. (F) Mature maize male flower. (G) Cross section of rice flower at the initiation stage of the stamen. (H) Mature flower of rice.

BM, branch meristem; ca, carpel; FM, flower meristem; gl, glume; IM, inflorescence meristem; le, lemma; lo, lodicule; pa, palea; pi, pistil; rg, rudimentary glume; sl, sterile lemma; SM, spikelet meristem; SPM, spikelet pair meristem; st, stamen.

2009).

In rice, the SMs are initiated from the primary or secondary BMs, which are formed from the IM or primary BMs, respectively (Fig. 4A, 4D). The SM initiates one fertile floret, two sterile lemmas, and two glumes (Fig. 4B, 4G, 4H) (for review see, Bommert et al. 2005; Thompson and Hake 2009). The glumes are highly reduced, and are called rudimentary glumes. The sterile lemma, a tiny flap-like organ, is thought to be a reduced lemma of two sterile lateral florets, and develops as a lateral organ of the SM (Yoshida et al. 2009). Because the SM initiates a single FM, the transition from the SM to FM is less clear in rice, compared with maize.

2.3 The *ramosa* pathway

Branch formation in the inflorescence is a key determinant of plant architecture. The branching pattern of the rice panicle is created by the formation of primary branch meristems (pBMs) by the inflorescence meristem, and secondary branch meristems from the pBMs (Fig. 4A). In maize, several long branches are formed in the tassel, but these are completely absent in the female inflorescence, which allows efficient seed packing (Fig. 4A, 4C) (Sigmon and Vollbrecht 2010). BMs and SPMs are formed around the same time, and are virtually indistinguishable at initiation. Each SPM forms two SMs, and each SM forms two FMs upon which a determinate fate is imposed.

Three classical mutants of maize, *ramosa* (*ra*)*1, ra2,* and *ra3* are characterized by increased long branches in the tassel and ear; thus, these genes function to enforce the determinacy of SPMs to limit the production of long branches (Fig. 5A-D). *ra1* and *ra2* encode putative transcription factors of the C2H2 Zinc Finger and Lateral Organ Boundary (LOB) domain-containing families, respectively (Bortiri et al. 2006; Vollbrecht et al. 2005). Interestingly, *ra3* encodes a Trehalose-6-Phosphate Phosphatase (TPP), an enzyme that catalyzes the production of trehalose sugar (Satoh-Nagasawa et al. 2006). Genetic analysis has placed all three genes into a pathway, with *ra2* and *ra3* acting in parallel upstream of *ra1* (Satoh-Nagasawa et al.

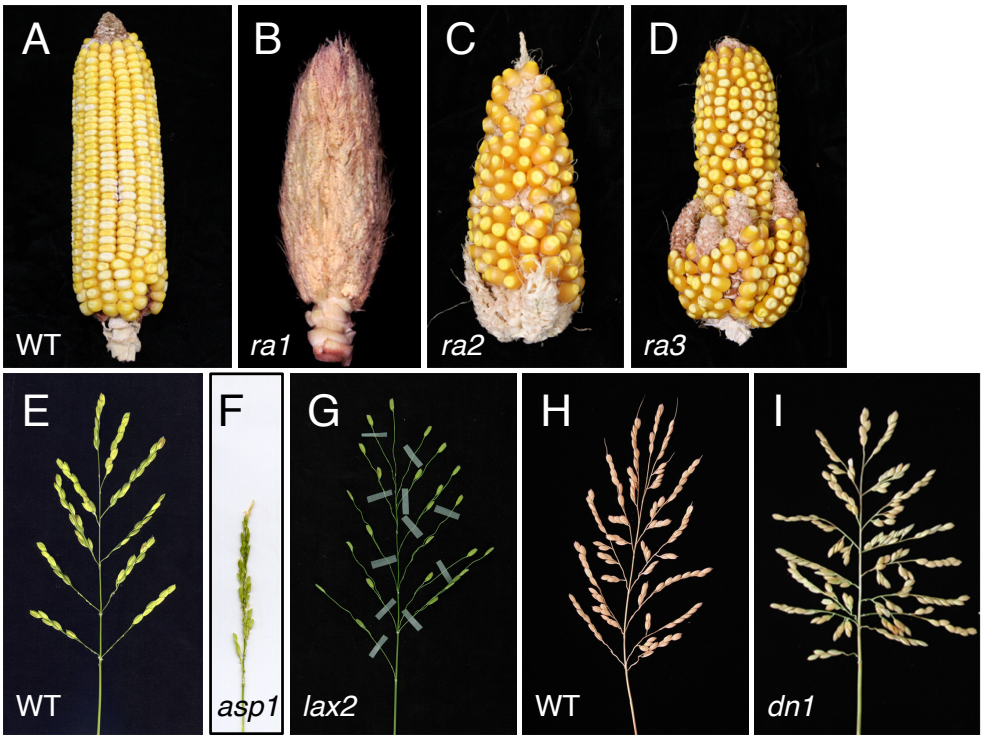

Figure 5: Branching mutants of maize and rice. (A-D) Maize ears. (A) Wild type, (B) *ramosa1* (*ra1*), (C) *ra2*, and (D) *ra3*. (E-I) Rice panicles. (E) Wild type, (F) *aberrant spikelet and panicle1* (*asp1*), (G) *lax panicle2* (*lax2*), (H) wild type (Koshihikari), and (I) *Dense panicle1* (*Dn1*) on Koshihikari background. Images are kindly provided by Drs. Akiko Yoshida (E), Yutaka Sato (G), and Fumio Taguchi-Shiobara (H, I).

2006). The three genes are expressed in overlapping, but distinct domains, either within or directly subtending the SPMs and SMs that they regulate, pointing to cell non-autonomous signals emanating from these domains.

Two possible mechanisms for a mobile signal regulating SPM determinacy include a RA3-dependent sugar signal, or a RA1-dependent protein or small molecule signal. RA3 catalyzes the final step in the production of trehalose: removal of a phosphate group from the intermediate metabolite trehalose-6-phosphate (T6P) (Paul et al. 2008). Both trehalose and T6P have been proposed to act as sugar signals, due to their low abundances relative to primary metabolites. These molecules have been shown to regulate enzymes involved in central carbon metabolism, and may serve to couple sugar availability and plant growth (Paul et al. 2008). *ra3* is a member of a large TPP gene family, whose members possess unique and diverse expression patterns in *Arabidopsis*, indicating some degree of sub-functionalization has occurred (Vandesteene et al. 2012). It is not feasible to measure changes in T6P and trehalose levels within these specific domains *in situ*. Nevertheless, key questions, such as whether the enzymatic activity of RA3 is required for function, can be addressed in maize. An alternative hypothesis positions RA3 as a transcriptional regulator, much like some glycolytic enzymes, such as *Arabidopsis HEXOKINASE1* (Cho et al. 2006; Satoh-Nagasawa et al. 2006). Transcriptome profiling by Digital Gene Expression (DGE) signatures revealed a list of differentially expressed genes that could be responsible for mediating the *ra3* phenotype (Eveland et al. 2010). Candidate genes included those involved in primary carbon metabolism as well as those involved in hormone response pathways, such as Ethylene Response Factor (ERF) family members. Overall, this study suggests an interesting link between sugar sensing, hormone signaling, and growth and development (Eveland and Jackson 2012; Eveland et al. 2010). A hint at the mechanism of action of RA1 comes from the identification of *ramosa enhancer locus2* (*rel2*) that encodes a TOPLESS (TPL) -like co-repressor (Gallavotti et al. 2010). RA1 and REL2 physically interact through the C-terminal EAR domains of RA1 (Gallavotti et al. 2010). Therefore, it is likely that

this complex plays a role in repressing transcription of target genes. A rice mutant named *aberrant spikelet and panicle1* (*asp1*), which displays a range of vegetative and reproductive defects (Fig. 5E, 5F), encodes the rice ortholog of *REL2* (Yoshida et al. 2012). Several of the mutant phenotypes, as well as molecular knowledge of TPL function in *Arabidopsis*, strongly implicate defects in auxin signaling in the genesis of the *asp1* phenotype (Yoshida et al. 2012). Several hormones have long been known to modify the number of long branches in the maize tassel upon exogenous application, including auxin and gibberellic acid (GA) (McSteen 2006; Nickerson 1959). Therefore, hormone biosynthesis and signaling components are potential downstream effectors of the *ramosa* pathway. Moving forward, it will be important to determine direct transcriptional targets of RA1 through methods such as Chromatin Immunoprecipitation-sequencing (ChIP-seq).

2.4 Genes regulating determinacy, maintenance and initiation of the BM

While the *ramosa* pathway defines the principal mechanism of branch meristem regulation in maize, several additional genetic factors can modulate the number of long branches produced in grass inflorescences. Constitutive overexpression of several TERMINAL FLOWER-related (TFL) genes in maize increases indeterminacy of axillary meristems in the inflorescence, consistent with TFL function in *Arabidopsis* (Bradley et al. 1997; Danilevskaya et al. 2010). Branch number is decreased in the mutant or transgenic knockdowns of the rice *LEAFY* homolog *RFL/ABBERANT PANICLE ORGANIZATION2* (*APO2*) (Ikeda-Kawakatsu et al. 2012; Rao et al. 2008) as well as double mutants of the duplicated maize orthologs *zfl1* and *zfl2* (Bomblies et al. 2003). These phenotypes imply close integration of flowering time regulation and inflorescence architecture, for example there may be a competency period for production of branches, such that the number produced depends on the rate of progression through the transition.

Inflorescence architecture is also fundamentally dependent on the initiation and maintenance of the axillary meristems of the inflorescence. There are a number of mutants in

maize and rice that display a defect in forming BMs and SMs, leading to a reduced number of long branches, or even a completely barren inflorescence. *barren inflorescence2* (*bif2*), which encodes a maize ortholog of the *Arabidopsis* serine-threonine kinase *PINOID*, fails to initiate all axillary meristems of the inflorescence (McSteen et al. 2007). Mutants of the orthologous bHLH transcription factors BARREN STALK1 (BA1) and LAX PANICLE1 (LAX1), of maize and rice, respectively, fail to initiate axillary meristems in both the vegetative and reproductive phases (Gallavotti et al. 2004; Komatsu et al. 2003a). Branching is also reduced in *monoculum1* (*moc1*)/*small panicle* (*spa*) and *lax2* mutants of rice (Fig. 5E, 5G), in addition to reduced tillering (Komatsu et al. 2003a; Li et al. 2003; Tabuchi et al. 2011). *MOC1/SPA* and *LAX2* encode a GRAS family transcription factor and a novel nuclear protein, respectively. Combinations of *lax2* mutants with *lax1* or *moc1* mutants show enhanced sparse panicle phenotypes, suggesting synergistic genetic interaction between these genes (Tabuchi et al. 2011).

Quantitative Trait Loci (QTL) and genome-wide association studies examining inflorescence architecture traits in maize have hinted at the contribution of other loci, such as *liguleless1,* which do not have strong loss-of-function branching phenotypes (Brown et al. 2011). It is important to pursue these types of powerful approaches in parallel with forward genetic studies, as genetic redundancy can limit the utility of traditional screens.

2.5 Inflorescence architecture and yield

Meristematic activity in the inflorescence has a profound influence on grain yield. Characteristics such as the size and determinacy of the IM, BMs, and SMs may drastically affect the number of spikelets, and eventually mature grains, per plant. Grain number per plant is a continuous trait that can be modified by a large number of genes controlling a range of developmental and physiological responses. Positional cloning has been successfully employed to identify several genes underlying yield QTL in rice, and a few of these cases are highlighted below.

A major yield QTL on chromosome 1, *GRAIN NUMBER 1a* (*GN1a*) was fine-mapped to a single open reading frame encoding a cytokinin oxidase gene, OsCKX2 (Ashikari et al. 2005). High yielding rice cultivars had less CKX2 activity, and therefore higher cytokinin levels, particularly in the IM of the panicle. This increased meristematic activity results in a higher number of long branches and spikelets, leading to higher grain yield per panicle (Ashikari et al. 2005). A dominant-negative truncation of a phosphatidylethanolamine-binding protein (PEBP) underlies a yield QTL at the *DENSE AND ERECT PANICLE* (*DEP1*)/*DENCE PANICLE1* (*DN1*) locus (Huang et al. 2009; Taguchi-Shiobara et al. 2011). This mutation is responsible for creating high yielding inflorescence architecture in many *japonica* rice varieties (Fig. 5H, 5I).

Another grain yield QTL called *WEALTHY FARMER'S PANICLE* (*WFP*)/*IDEAL PLANT ARCHITECTURE* (*IPA1*) corresponds to *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14* (*OsSPL14*) (Jiao et al. 2010; Miura et al. 2010). High branching accessions overexpress *OsSPL14* in the panicle in a domain associated with BMs (Miura et al. 2010). A single point mutation that relieves *OsSPL14* from its miR156-mediated repression is sufficient to decrease plant tiller number, while increasing panicle branching and grain number, thus creating the "ideal plant" for agriculture (Jiao et al. 2010).

Many forward genetic screens uncover strong loss-of-function mutants with very severe phenotypes. While these "monstrous" mutants are useful for uncovering the normal function of genes, they rarely provide useful substrate for breeding efforts, because they frequently display negative pleiotropic traits. For example, the increase in size of the IM in maize fasciated ear mutants is accompanied by a decrease in the length of the ear, as well as disorganized seed rows, which limit the number of seeds per ear. Reverse genetic resources, such as TILLING, can facilitate the discovery of hypomorphic alleles, which may have a beneficial effect on crop yield (Weil, 2009). Bommert *et al*. (2012, under submission) isolated a weak allele of the fasciated ear mutant *fea2* and showed that this allele increases kernel row number and kernels per ear, without causing a fasciated IM or shorter ear. Furthermore, this study implicated natural variation in *fea2*

expression in modifying meristem size in diverse inbred lines of maize (Bommert et al, 2012 under submission).

Dominant mutant alleles of the *aberrant panicle organization1* (*apo1*) gene of rice produce extra primary and secondary branches in the inflorescence, whereas loss-of-function alleles display the opposite phenotype (Ikeda et al. 2007; Ikeda-Kawakatsu et al. 2009). The contrasting effect on branching is explained by significantly different rates of cell division in each respective mutant (Ikeda-Kawakatsu et al. 2009). The beneficial panicle architecture of the gain-of-function mutant is associated with other negative traits, such as fewer panicles per plant. However, positional cloning of a QTL for culm strength, *STRONG CULM2* (*SCM2*), identified a dominant allele of *apo1* that conferred the improved panicle, without decreasing panicle number (Ookawa et al. 2010). The plant architecture QTLs described above reinforce the value of exploiting natural variation for yield improvement.

2.6 Tunicate

The classical pod corn mutant of maize, *Tunicate1* (*Tu1*), has pleiotropic inflorescence phenotypes, but is most obviously characterized by elongated glumes that completely enclose the seeds (Han et al. 2012). This dominant mutant is caused by a chromosomal rearrangement, resulting in the MADS box gene *ZMM19* gaining a novel expression pattern from a neighboring gene (Han et al. 2012; Wingen et al. 2012). Ectopic expression of ZMM19 in a *ramosa*-like domain confers indeterminacy to SMs and results in the production of long branches. Therefore, when misexpressed in the *Tu1* mutant, ZMM19 plays a role in promoting growth and indeterminacy, in opposition to the *ramosa* genes (Han et al. 2012). The fact that a gene not normally expressed in the inflorescence can dictate such dramatic changes in inflorescence architecture is indicative of the modularity underlying developmental programs.

2.7 Transition from the BM to the SM

The *branched silkless1* (*bd1*) and *FRIZZY PANICLE* (*FZP*) genes regulate the transition from the BM to the SM, in maize and rice, respectively (Chuck et al. 2002; Komatsu et al. 2003b). In both *bd1* and *fzp* mutants, indeterminate branches are formed instead of spikelets (Fig. 6). Thus, it is likely that these two genes control the determinacy of the BM and establish the identity of the SM. *bd1* and *FZP* encode orthologous transcription factors in the AP2/ERF family. These two genes are expressed at the junction of the SM and the initiation site of the glume, but are not expressed in the meristem *per se*. This finding raises the possibility that the expression domain of these two genes might be important for the establishment of the SM identity.

In *Arabidopsis*, the *PUCHI* gene is the ortholog of *bd1* and *FZP*. A partial conversion from the FM to the IM is observed in the *puchi* mutant, in addition to other additional phenotypes (Karim et al. 2009). Although the phenotypes of *puchi* in *Arabidopsis* are different from those of *bd1* in maize and *fzp* in rice, it seems likely that a fundamental function of these genes in controlling meristem transitions and/ or determinacy is conserved in both grasses and *Arabidopsis*.

2.8 Identity of the SM and its determinacy

In maize, the transition from the SM to the FM is regulated by *indeterminate spikelet1* (*ids1*), and its close paralog, *sister of indeterminate spikelet1* (*sid1)* (Fig. 6) (Chuck et al. 2008; Chuck et al. 1998). In the *ids1* mutant, a few extra florets are formed within a spikelet, both in the tassel and the ear, suggesting that the regulation of SM determinacy is compromised (Chuck et al. 1998). In *ids1 sid1* double mutants, glumes (bracts) are continuously formed, implying that the transition of the SM to the FM is strongly inhibited (Chuck et al. 2008). Therefore, *ids1* and *sid1* redundantly act to control the transition from the SM to the FM, and the determinacy of the SM. Long branch number is also reduced in *ids1 sid1* double mutant, suggesting that both genes regulate the determinacy of the BM in the inflorescence (Chuck et al. 2008). The *ids1* and *sid1*

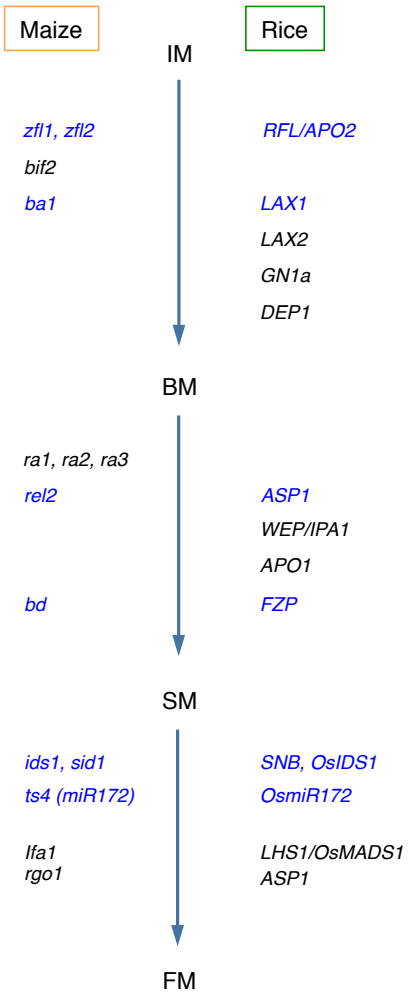

Figure 6: Genes responsible for transition of meristem fate in maize and rice. Orthologs are indicated in blue. BM, branch meristem; FM, flower meristem; IM, inflorescence meristem; SM, spikelet meristem.

genes also encode AP2/ERF transcription factors, but they belong to a class different from that of *bd1* (Chuck et al. 2008; Chuck et al. 1998).

In rice, *SUPERNUMERARY BRACT* (*SNB*) and *OsIDS1* have functions similar to those of *ids1* and *sid1* in maize (Fig. 6) (Lee and An 2012; Lee et al. 2007). The *snb* mutant displays repetitious production of rudimentary glumes (bracts) or extra lemma/palea, and, in rare cases, forms two florets in a spikelet (Lee et al. 2007). These phenotypes are enhanced in *snb osids1* double mutant, suggesting that the transition from the SM to the FM is extremely delayed, and that SM determinacy is compromised (Lee and An 2012). *SNB* and *OsIDS1* encode homologs of *sid1* and *ids1* (Lee and An 2012; Lee et al. 2007), thus, the genetic mechanism controlling the transition from the SM to the FM appears to be conserved between maize and rice.

The classical maize mutants, *tasselseed4* (*ts4*) and *Tasselseed6* (*Ts6*), have been characterized as defective in sex determination (Chuck et al. 2007). These two mutants exhibit similar phenotypes: a failure in carpel abortion in the male flower and an increase in meristem branching. Gene isolation revealed that *ts4* encodes a member of the *miR172* microRNA family that restricts the function of AP2 domain transcription factors. *ids1* is broadly expressed in the *ts4* mutant, compared with wild type, and the branching and sex determination phenotypes of *ts4* are almost completely suppressed by *ids1* mutation. Therefore, *ids1* is likely to be a key target of miR172. The dominant *Ts6* mutant, on the other hand, has a mutation in the binding site of miR172 in the *ids1* gene. This mutation probably results in ectopic expression of *ids1* in *Ts6* mutant, because of the release from the restriction of miR172 (Chuck et al. 2007). In rice, overexpression of the *OsmiR172* genes largely phenocopies the *snb osids1* double mutant (Lee and An 2012; Zhu et al. 2009). These observations indicate that the function of *IDS1*-like genes and their proper regulation by *miR172* play important roles in specifying the fate of the SM in both maize and rice (Fig. 6).

In rice, *LEAFY HULL STERILE1* (*LHS1*)/*OsMADS1* is involved in SM identity, because loss-of-function mutants show reiterative formation of lemma/palea or extra floret formation in

spikelets, in addition to defects in the identity of the lemma/palea (Jeon et al. 2000). *lhs1* phenotypes, including loss-of-determinacy of the SM, are enhanced by mutation in the *MOSAIC FLORAL ORGAN1* (*MFO1*)/*OsMADS6* gene (Li et al. 2010; Ohmori et al. 2009) (see below).

Whereas many of the mutations already described are in genes encoding specific transcription factors, more general transcriptional machinery is also important in control of meristem fate. Mutation in *ABERRANT SPIKELET AND PANICLE1* (*ASP1*) causes pleiotropic phenotypes in spikelet development and inflorescence architecture (Yoshida et al. 2012). These phenotypes are closely associated with various defects in meristem function, such as precocious transition of the BM to SM, partial failure in maintenance of the BM and SM, and disturbed initiation of the axillary meristem (AM). In a rare case, a reversion from the SM to the BM is observed. *ASP1* encodes a transcriptional corepressor, similar to *Arabidopsis* TPL. Therefore, it is likely that derepression of multiple genes in the *asp1* mutant causes the various defects in meristem fates.

In maize, the *INDETERMINATE FLORAL APEX1* (*IFA1*) and *REVERSED GERM ORIENTATION1* (*RGO1*) genes are responsible for the determinacy of the SM (Kaplinsky and Freeling 2003; Laudencia-Chingcuanco and Hake 2002). In *ifa1* and *rgo1* mutants, the SPM and the SM initiate extra SMs and FMs, respectively. Double mutants of *rgo1* and *ids1* show a more and Freeling 2003). Reversion of the SM to the SPM or the BM is also observed in the double mutant of *ifa1* and *ids1*(Laudencia-Chingcuanco and Hake 2002). Thus, *IDS1*, *IFA1*, and *RGO1* redundantly regulate the fate of the reproductive meristems in maize, however the latter two genes have not yet been cloned.

2.9 Floral meristem determinacy

The vegetative SAM initiates leaf primordia, whereas the FM initiates floral organs. Apart from the type of lateral organs that these meristems produce, the essential difference

between the SAM and the FM is determinacy. The SAM and IM are indeterminate, because the meristems continue to replace stem cells and to repeatedly initiate lateral organs and the axillary meristems. By contrast, the FM is determinate, because the stem cells are consumed by the formation of final floral organs, such as the carpel and the ovule.

2.10 Function of C-Class MADS-box genes

In *Arabidopsis*, *AGAMOUS* (*AG*), which encodes a C-class MADS-box transcription factor, is a key gene responsible for meristem determinacy (for review, see Barton 2010; Sun and Ito 2010). The *ag* mutant produces indeterminate flowers in which a set of floral organs (sepal-petal-petal) are repeatedly formed (Bowman et al. 1989; Yanofsky et al. 1990). *WUS* expression persists in the FM of the *ag* mutant at a late stage of flower development, whereas it disappears after formation of the carpel in wild type (Lenhard et al. 2001; Lohmann et al. 2001). Therefore, in the FM, *AG* regulates meristem determinacy by repressing *WUS*. *KNUCKLES* (*KNU*), which encodes a transcriptional repressor, has an important role to mediate the repression of *WUS* by *AG* (Sun et al. 2009). A recent study suggests that possibility that *AG* in turn directly represses *WUS* (Liu et al. 2011).

C-class MADS-box genes have increased in number during evolution of the grasses. Maize has at least three *AG* orthologs, and, among them, *zag1* has shown to be partially responsible for FM determinacy, as multiple carpels are formed in the *zag1* ear (Mena et al. 1996; Zanis 2007). Rice has two *AG* orthologs, *OsMADS3* and *OsMADS58*, and their functions are diversified (Yamaguchi et al. 2006). Whereas FM determinacy is partially compromised in an *osmads3* mutant, a floral phenotype similar to that of *zag1*, a severe loss of determinacy, was observed in knockdown lines of *OsMADS58*. In this line, a set of floral organs (lodicules, stamens, and partial carpels) is repeatedly formed in the flower, and an FM like structure remains even in the mature flower (Yamaguchi et al. 2006). Although in a different genetic background, the *osmads58* mutation has little effect on floral phenotypes, but dramatically enhances the indeterminate

phenotype of the *osmads3* single mutant, suggesting the importance of *OsMADS58* in FM determinacy in rice (Dreni et al. 2011). Taken together, the evidence suggests C-class MADS-box genes play crucial roles in regulating the determinacy of the FM in both maize and rice.

OsMADS13, a MADS-box gene in the D-class lineage, is required for specification of the ovule (Dreni et al. 2007; Lopez-Dee et al. 1999; Yamaki et al. 2011). In the *osmads13* mutant, determinacy is partially lost, because *OSH1* expression is prolonged and multiple pistils are formed. Mutation of *osmads13* enhances the indeterminate phenotype observed in the *osmads3* or *osmads3 osmads58* double mutant (Dreni et al. 2011).

2.11 Additional genes responsible for FM determinacy

MADS-box genes in the *AGL6* subfamily, including rice *MOSAIC FLORAL ORGAN1* (*MFO1*)/*OsMADS6* and maize *bearded-ear* (*bde*)/*zag3*, are also responsible for FM determinacy (Li et al. 2010; Ohmori et al. 2009; Thompson et al. 2009). Mutation in *MFO1* causes production of extra carpels and spikelets in the center of the flower in rice (Li et al. 2010; Ohmori et al. 2009). *OsMADS17*, a close paralog of *MFO1*, seems to have a weaker function similar to that of *MFO1*, because RNA-silencing of this gene enhances abnormal flower phenotypes in *mfo1* mutant but does not cause any obvious phenotype in wild type (Ohmori et al. 2009). In the maize *bde* mutant, the upper FM forms extra floral organs, whereas the lower FM initiates additional FMs (Thompson et al. 2009). An evo-devo study has indicated that *AGL6*-like genes are expressed similarly in the FM in all grass species, whereas they are expressed differently in the floral organ primordia depending on the species (Reinheimer and Kellogg 2009). Thus, it is possible that the regulation of meristem determinacy is the ancestral function of *AGL6*-like genes in grasses.

Mutation of C-class MADS-box genes enhances the floral phenotype of *mfo1* and *bde* in both rice and maize (Li et al. 2011; Thompson et al. 2009). In maize, it has been demonstrated that BDE protein physically interacts with ZAG1 protein (Thompson et al. 2009). In rice, by

contrast, it has been reported that C-class genes are regulated by *MFO1* (Li et al. 2010). However, this is inconsistent with the results of another group who found expression levels of two C-class genes are unchanged in the *mfo1* mutant, as compared to wild type (Ohmori et al. 2009). More detailed analysis is required in rice to resolve this discrepancy. Combination of mutations in *MFO1* and *OsMADS13* enhances defects in FM determinacy in each single mutant (Li et al. 2011). As described above, *LHS1* is also involved in FM determinacy, and *lhs1* mutations enhance the *mfo1* phenotype (Jeon et al. 2000; Li et al. 2010; Ohmori et al. 2009), suggesting that multiple MADS-box genes redundantly regulate determinacy of the FM in rice.

In rice, carpel identity is specified by a *YABBY* gene, *DROOPING LEAF* (*DL*), whereas this identity is regulated by *AG* orthologs in eudicots (Yamaguchi et al. 2004). Because spatial expression patterns of *DL* orthologs in maize and wheat resemble that of *DL* in rice, the function of *DL* orthologs is likely to be conserved in grasses (Ishikawa et al. 2009). In loss-of-function *dl* mutants, the carpels are homeotically transformed into variable numbers of stamens, and *OSH1* continues to be expressed after production of central stamens (Yamaguchi et al. 2004). Therefore, *DL* is also partially involved in the regulation of FM determinacy. Furthermore, the indeterminate nature of *dl* is also promoted when combined with *mfo1* (Li et al. 2011).

ABERRANT PANICLE ORGANIZATION1 (*APO1*), which encodes a F-BOX protein similar to *Arabidopsis UFO,* has pleiotropic functions in both inflorescence and flower development (Ikeda et al. 2007; Ikeda et al. 2005). One function of *APO1* is the regulation of determinacy, as carpels are reiteratively formed in the *apo1* mutant. *APO1* promotes the expression of *OsMADS3*, and this may partially explain the loss-of-determinacy of the FM in *apo1* mutants. Defects in floral determinacy observed in each single mutant are strongly enhanced in *dl apo1* double mutants, in addition to homeotic change of stamens into lodicules (Ikeda et al. 2007).

2.12 Signal from the meristem to lateral organs

Gene activity in the meristem is not restricted to the meristem itself, but also affects lateral organ development. Pioneering surgical experiments indicated that the leaf primordium develops into a radially symmetric abaxialized leaf, when an incision is made between the meristem center and the incipient leaf (P0) (Sussex 1951, 1954). This result suggests that a putative signal arising in the meristem specifies adaxial identity in the leaf primordia. Recent laser ablation studies have demonstrated the importance of the L1 layer of the meristem for the movement of the putative signaling molecule (Reinhardt et al. 2003; Reinhardt et al. 2005). This signaling seems to be transient, because the leaf develops normally without abaxialization when the incision is made between the meristem and an older leaf primordium (P2).

Molecular markers of adaxial and abaxial identity are expressed at early stages of stamen development in rice, with patterns similar to those observed in leaf development (Toriba et al. 2010). Subsequently, however, the expression patterns change, suggesting that rearrangement of adaxial-abaxial domains occurs. The former patterning likely depends on a signal from the meristem, whereas the latter patterning may result after release from the control of the meristem (Toriba et al. 2011). Therefore, the rearrangement of adaxial-abaxial polarity in the stamen might represent the transition from meristem-dependent development to organ-autonomous development.

Although the molecular nature of this signal is still unknown, *MIR390* may be a candidate for this signal in *Arabidopsis* (Chitwood et al. 2009). Identification of the signal emanating from the meristem is critical for elucidation of the mechanism underlying communication between the meristem and lateral organs.

2.13 Signal from lateral organs to the meristem

Conversely, meristem activity is likely to be also affected by a signal from the lateral

organs. Recent studies suggest that a class of *YABBY* (*YAB*) genes are involved in this process (Goldshmidt et al. 2008; Tanaka et al. 2012a).

In *Arabidopsis*, *FILAMENTOUS FLOWER* (*FIL*) and its related *YABBY* genes (*YAB2, YAB3, YAB5*) regulate leaf development, including establishment of adaxial–abaxial polarity and lamina expansion (Sarojam et al. 2010; Stahle et al. 2009). In addition, mutations in these genes result in defects associated with meristem function. For example, the expression domain of *CLAVATA3* and *WUSCHEL* is markedly expanded in the SAM of *fil* or *fil yab3* mutants, whereas the primary SAM fails to be maintained in triple and quadruple mutants of these *YABBY* genes.

In rice, mutation in the *TONGARI-BOUSHI1* (*TOB1*) gene results in pleiotropic defects in spikelet development, such as reduction in palea and lemma growth, formation of a seamless lemma/palea-like organ, and production of two florets within a spikelet (Tanaka et al. 2012a; Tanaka et al. 2012b). In severe cases, the SM is arrested after formation of the sterile lemma. Formation of the seamless organs and the two-florets containing spikelet is likely to be associated with a disorganized meristem. *TOB1* corresponds to *OsYABBY5*, which belongs to the same subclass as FIL and YAB3.

Both rice *TOB1* and *Arabidopsis YABBY* genes are expressed in lateral organs, but not expressed in the meristem (Goldshmidt et al. 2008; Tanaka et al. 2012a). The meristem defects observed in the above *YABBY* mutants are therefore likely to be caused by non-cell autonomous action of the *YABBY* genes from the lateral organs. In *Arabidopsis*, mobility of YABBY protein or mRNA is not detected (Goldshmidt et al. 2008). Therefore, the FIL-clade *YABBY* genes are involved in the communication between lateral organs and the meristem, possibly by producing a signaling molecule that travels from the lateral organ to the meristem.

2.14 Perspective

In the past decade, much progress has been made towards understanding the molecular mechanism underlying the regulation of the fate, determinacy and maintenance of grass

meristems. Many genes responsible for these activities have been isolated and their functions have been revealed together with genetic relationships between the genes. As discussed in the accompanying paper (Pautler et al. 2013), identification of positive regulators of stem cell maintenance, such as WUS, is also critical for understanding the determinacy of the FM. In *Arabidopsis*, determinacy is achieved by the repression of the positive regulator *WUS* by *AG*, after specification of the carpel. Although Class C genes such as rice *OsMADS58* and maize *zag1* are partially involved in this process, these genes do not specify the carpel in grasses, and meristems still persist after carpel specification by *DL*. Therefore, a complex mechanism might be required for repressing the putative WUS-like positive factor.

Furthermore, the genetic mechanism that regulates communication between the meristem and lateral organs is an intriguing question in meristem biology. What genes are involved in the production and transduction of the signal connecting meristem maintenance and lateral organ development?

Little is known about these important questions, even in *Arabidopsis*. We are expecting that increasing molecular genetic studies of maize and rice will contribute to understanding this issue.

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CHAPTER 3: FEA4 ENCODES A BZIP TRANSCRIPTION FACTOR REQUIRED TO CONTROL MERISTEM SIZE IN MAIZE

3.1 Introduction

Plant development is a plastic process that depends on the activity of pluripotent stem cells resident within the shoot apical meristem (SAM). Differences in the initiation, determinacy, and size of different classes of meristems are responsible for variation in vegetative and reproductive architecture within the plant kingdom. Plant architecture, especially inflorescence architecture, is critical for reproductive success and is therefore a primary determinant of crop yield.

The stems cells contained with the SAM have two roles: first, to divide to give rise to daughter cells that supply founder cells for organ initiation; and second, self-replacement. An imbalance in positive or negative signals for stem cell maintenance can result in enlarged or consumed meristem phenotypes; therefore, the stem cell population in the SAM must be precisely regulated. The principal mechanism of stem cell counting in angiosperms is the CLAVATA (CLV)-WUSCHEL (WUS) negative feedback pathway, which was first characterized in *Arabidopsis thaliana* (Brand et al. 2000; Schoof et al. 2000*)*. WUS is a homeodomain protein expressed in the organizing center beneath the stem cell niche, which acts non-cell autonomously to promote stem cell fate in the central zone, likely by repressing expression of genes involved in organ differentiation (Mayer et al. 1998; Schoof et al. 2000; Yadav et al. 2013). These stem cells express a small secreted peptide, CLV3, which is perceived by a battery of receptors, principally CLV1, CLV2, and RPK2/TOADSTOOL, resulting in the repression of WUS transcription (Brand et al. 2000; Clark et al. 1997; Kinoshita et al. 2010; Schoof et al. 2000).

The framework of this pathway is conserved in the grasses, including the major crop species rice and maize. Two maize fasciated ear mutants, *thick tassel dwarf1* and *fasciated ear2*, have lesions in the maize orthologs of CLV1 and CLV2, and the rice mutant *fon1* harbors a mutation in the CLV1 ortholog (Bommert et al. 2005; Suzaki et al. 2004; Taguchi-Shiobara et al.

2001). The CLAVATA pathway in rice represents an interesting variation on the theme, with three distinct clavata3-like peptides acting to restrict stem cell populations in vegetative, inflorescence, and floral meristems (Suzaki et al. 2009). Aside from the identification of two clv-like receptors, a small number of additional meristem size regulators have been identified in maize. One of these factors, *compact plant2* (*ct2*), is probably directly involved in the clavata pathway, as it codes for the alpha subunit of a heterotrimeric GTPase, which physically interacts with FEA2 (Bommert et al., submitted). Furthermore, double mutants between *ct2* and *fea2* are not significantly stronger than either single mutant with respect to tassel spikelet density (Bommert et al., submitted). The association of G-protein signaling with a LRR- Receptor-like protein provides a plausible mechanism of signaling for FEA2, which lacks an active kinase domain (Bommert et al., submitted). Another mutant, *aberrant phyllotaxy1* (*abph1*), which encodes an A-type cytokinin response regulator, displays increased SAM size as well as a switch from alternate to decussate phyllotaxy (Giulini et al. 2004). Molecular cloning of this mutant was the first direct evidence that the cytokinin response regulates meristem size in plants (Giulini et al. 2004). Subsequent studies have clarified a role for Arabidopsis Response Regulators (ARRs) in integrating cytokinin and auxin signals with the CLV-WUS stem cell-counting loop (Zhao et al. 2010).

At the opposite end of the phenotypic spectrum are mutants that have a reduced meristem size or fail to maintain a productive SAM throughout development. KNOTTED1 is a homeodomain protein that is required to maintain meristematic fate, as loss-of-function alleles exhibit smaller meristems, and meristem termination in some genetic backgrounds (Kerstetter et al. 1997; Vollbrecht et al. 2000). A recent genome-wide chromatin IP and mRNA-seq analysis suggested that KNOTTED1 promotes meristematic activity by regulating a cascade of transcription factors and hormone biosynthesis/signaling components (Bolduc et al. 2012).

Despite these findings, less is known about the factors controlling meristem size in maize compared to *Arabidopsis* and rice. For example, it is not clear which CLE peptides restrict the

size of the stem cell niche, or which WUSCHEL-related Homeobox (WOX) genes promote stem cell fate (Pautler et al. 2013). The functional sub-domains of the meristem, such as the central zone, organizing center, and peripheral zone, are not well defined in maize. Furthermore, the dynamics of cell division and differentiation between the central zone and the incipient lateral organ are relatively poorly understood. The maize community has a rich tradition of collecting developmental mutants, some of which display larger meristems and fasciated ears and tassels. Identification of new meristem regulators through positional cloning of these mutants can potentially address these unanswered questions.

Here, we describe the phenotypic and molecular characterization of *fasciated ear 4* (*fea4*), a maize mutant with enlarged meristems. *fea4* encodes a bZIP transcription factor, orthologous to the Arabidopsis gene *PERIANTHIA*, a mutant affected in floral organ patterning, but not meristem size *per se* (Chuang et al. 1999; Running and Meyerowitz 1996). Expression analysis by *in situ* hybridization and fluorescent protein fusions suggests that *fea4* is dynamically and specifically expressed in different meristem types. Genetic analysis of double mutants demonstrates that *fea4* acts in parallel to the canonical CLV-WUS pathway. Expression profiling by RNA-seq suggests that *fea4* may buffer meristem homeostasis by acting as a transcriptional activator of other important developmental transcription factors.

3.2 Results

3.2.1 Phenotypic Characterization of the *fea4* mutant

fea4 was originally isolated as a fasciated ear mutant in a screen of EMS-mutagenized A619 inbred maize. Further phenotypic characterization was carried out in the A619 inbred background and the B73 inbred background, two lines in which the phenotype is particularly expressive. *fea4* plants are characterized by larger than normal SAM diameter (Fig. 7A,B). This increase in vegetative SAM size may explain the decreased plant stature observed in the A619 inbred background (Fig. 7C), as larger SAM size is correlated with decreased plant height in several mutants (Bommert et al. 2005). However, the semi-dwarf phenotype is less pronounced

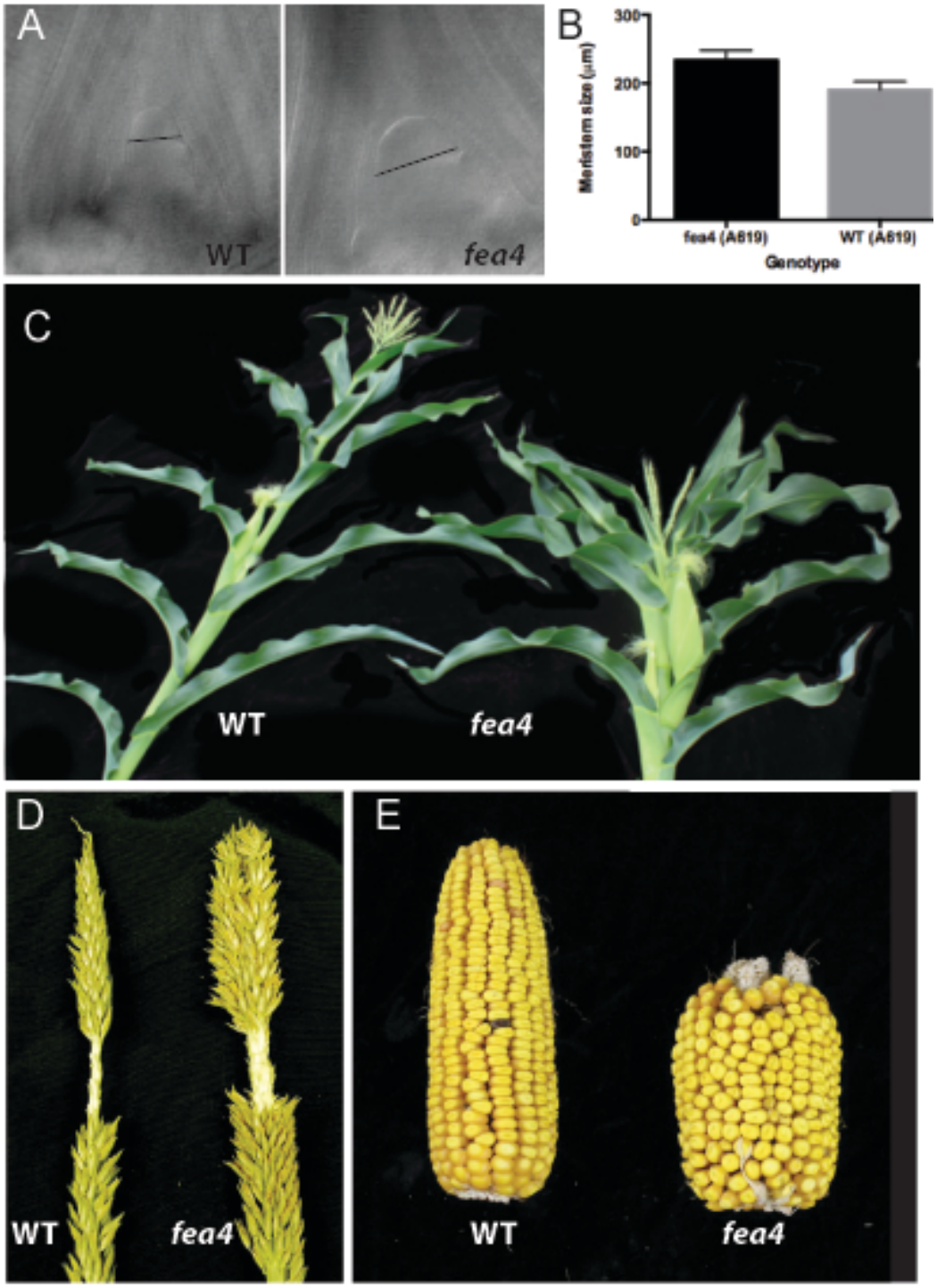

Figure 7: Phenotypes of *fea4* mutants. *fea4* mutants have a larger vegetative SAM than wild type siblings 14 days after germination (p-value<0.05) (A, B). *fea4* is a semi-dwarfed mutant in the A619 inbred background (C). Following transition to flowering, *fea4* tassels have a higher spikelet density and thicker rachis diameter than wild type tassels (D). *fea4* ears are massively fasciated and shorter than wild type ears, and have disorganized seed rows (E).

in B73 and several other common inbred lines, even when larger SAM size is manifest (Table 1). The most dramatic phenotypes in the *fea4* mutant are a greatly thickened tassel and massively fasciated ears. Mutant tassels have a greater main rachis diameter, and these tassels have a much higher spikelet density than wild-type siblings (Fig.7D; Table 1). *fea4* ears are massively fasciated and shorter than wild-type ears, and have disorganized seed rows (Fig. 7E). In general, the vegetative and reproductive phenotypes are fully recessive, and are not detectable in heterozygous plants (Table 1).

To better understand the development of the enlarged inflorescence structures, we subjected developing *fea4* ears and tassels to Scanning Electron Microscopy (SEM). This analysis revealed that the fasciated inflorescences were caused by enlarged tassel and ear inflorescence meristems (IMs)(Fig. 8). The large, flattened IMs in the *fea4* mutant appear to function similarly to wild-type IMs, as they initiate rows of axillary meristems, and also express the meristem marker *knotted1* in a pattern resembling the wild-type (Fig. 8C, 8F). The fasciated inflorescence meristem becomes progressively more severe as ear development progresses, culminating in an elongated and folded structure that bears little resemblance to the wild type ear tip (Fig. 8G). The axillary meristems of the inflorescence, including spikelet pair (SPM), spikelet (SM), and floral meristems (FM), were not obviously enlarged in the mutant; furthermore, there is no increase in floral organ number in the *fea4* mutant, unlike in other fasciated ear mutants, such as *fea2* and *td1* (Figure 9)(Bommert et al. 2005; Taguchi-Shiobara et al. 2001). However, approximately 25% of *fea4* (A619) florets contained a reduced number of stamens, whereas variation in stamen number was not observed in wild-type florets (Fig. 9; Table 1). No further abnormalities were observed in floral organ number or patterning, suggesting that FMs were only subtly affected in the balance of organ specification or differentiation.

Table 1: *fea4* mutant phenotype quantification in three different inbred backgrounds. Plant phenotypes were quantified at Uplands Farm in Summer 2012. Plants were grown in trays in the greenhouse for SAM measurements. Measurements represent mean ± standard deviation.

Trait	**n**	**WT**	***fea4/+***	***fea4***
B73				
SAM width 14 DAG (μm)	20		179.83 ± 8.38	226.16±13.07b
Plant height	49		249.69 ± 11.1	213.13±23.94b
Spikelet density/cm	22	21±1.97		29.8±1.98a
Kernel row number	32	16.29 ± 1.9	17.14 ± 1.51	28 ± 2.31ab
Tassel branch number	51	7.67±1.19	7.86 ± 1.66	6.89±2.21
Stamen Number per floret	50	3.0 ± 0		3.0 ± 0
A619				
SAM width 14 DAG (μm)	20		190.67±11.97	233.97±14.2b
Plant height (cm)	54	204.28±12.19	190.75±15.1	144.61±13.88ab
Spikelet density/cm	24	18.8±1.39		33.5±8.8a
Kernel row number	28	14.33 ± 0.82	15.80 ± 1.48	19.67 ± 2.39ab
Tassel branch number	33	9.5±1.84	9.4±3.43	4.07±1.75ab
Stamen Number per floret	50	3.0 ± 0		2.78±0.42a
Mo17				
Plant height	29	207.09±8.43	198.75±10.99	190.33±6.86a
Spikelet density/cm	28	16.33±2.38		20.1±4.56a
Tassel branch number	28	6.67±2.14	7.67±2.45	9.0±1.41

a- significantly different than wild-type value, Student's t-test, p-value <0.05
b- significantly different than fea4/+ value, Student's t-test, p-value<0.05

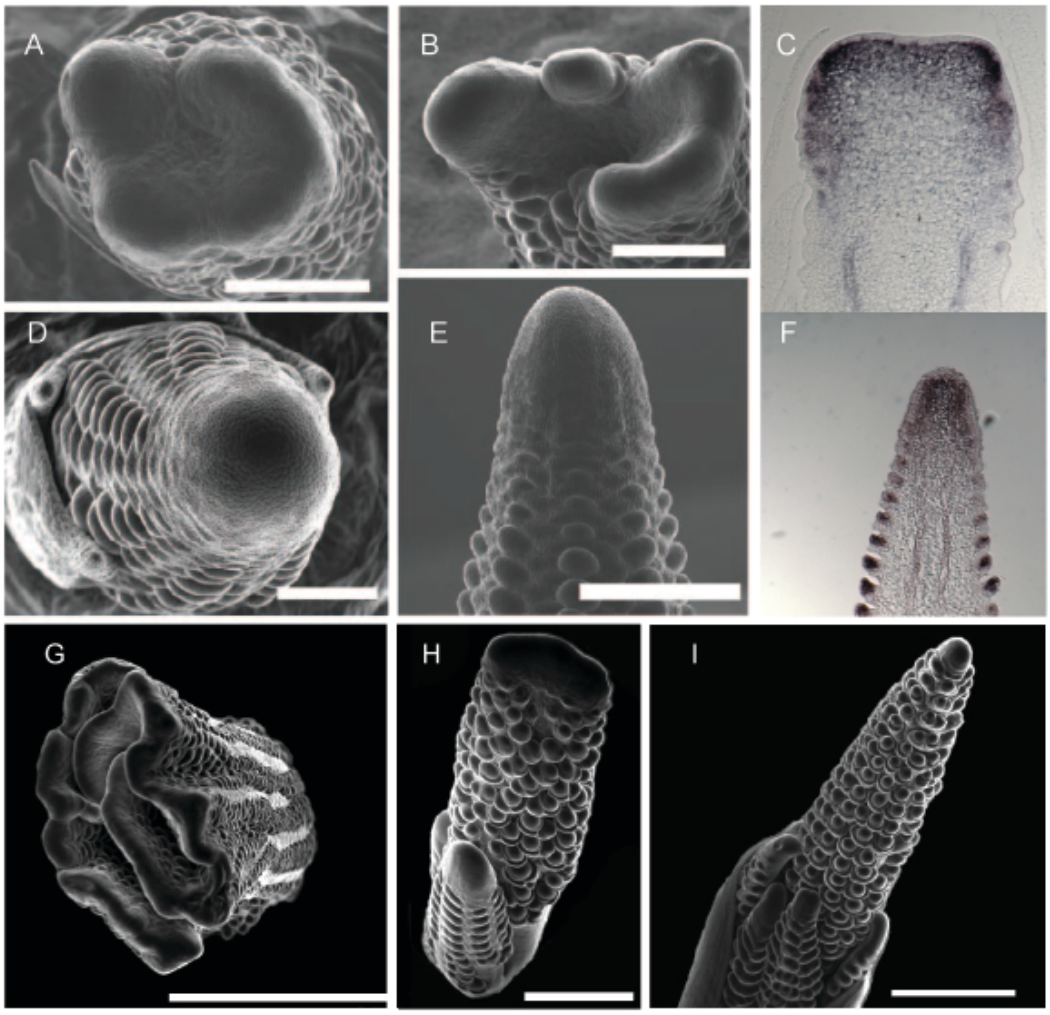

Figure 8: Microscopic phenotypes of *fea4* mutants. *fea4* inflorescence meristems are enlarged and flattened (A, B) whereas wild type ears have tapered conical shaped inflorescence meristems (D, E). *in situ* hybridization with the *knotted1* meristem marker shows an expanded domain of expression throughout the enlarged inflorescence meristem in *fea4* ears (C) compared to wild type ears (F). *fea4* meristem fasciation becomes progressively more severe as ear development progresses (G). Tassel inflorescence meristems are similarly affected, with mutants (H) displaying fasciation relative to wild type (I). Scale bars= 500 um in A,B,E; 250 um in D; 2mm in G; 1mm in H and I.

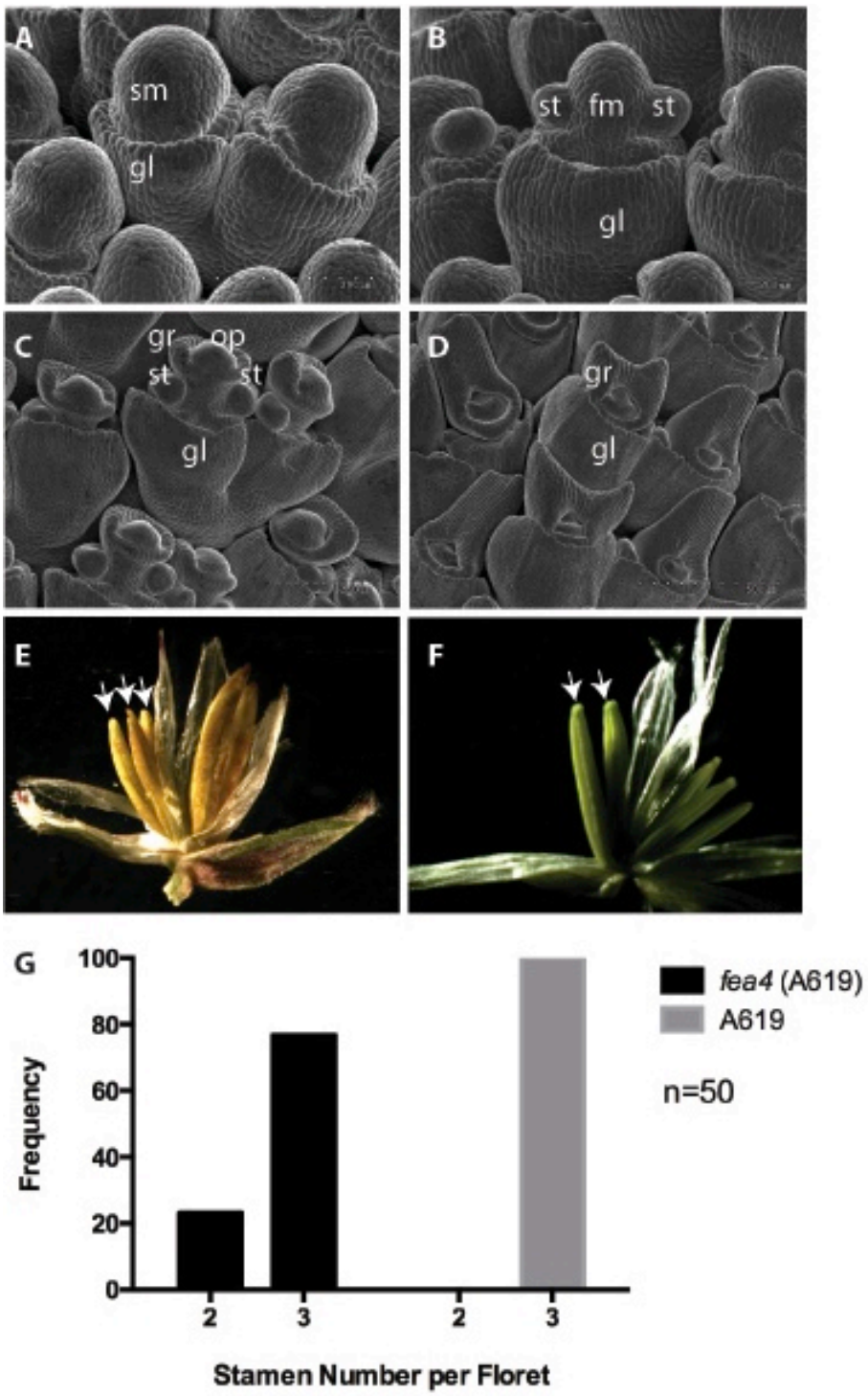

Figure 9: *fea4* floral phenotypes. There is a normal progression of floral development in *fea4* mutants (A-D; wild type not shown). Wild type A619 male florets each contain 3 stamens (E), whereas *fea4* (A619-5) florets frequently contain only 2 stamens (F,G). sm= spikelet meristem; gl= glume; fm= floral meristem; st= stamen primordium; op= ovule primordium; gr= gynoecial ridge.

3.2.2 Molecular Cloning of *fea4*

We mapped *fea4* to the long arm of chromosome 6 by bulked segregant analysis, and carried out fine mapping by genotyping 528 mutants from an F2 mapping population (Fig. 10A). Two CAPS markers delineated a 2.7Mb region containing approximately 30 gene models. We sequenced a basic-region leucine zipper (bZIP) transcription factor, and found that the reference mutant (*fea4-ref*) harbored an EMS-induced C-T transition, which caused a premature stop codon. A second independently derived allele (*fea4-rel*09-5171*) produced another early stop codon, confirming the identity of the gene underlying the mutant phenotype (Fig. 10B). Both of the stop codons fall after the bZIP domain, but before two glutamine-rich regions, which are associated with transcriptional activation and mediate post-translational regulation of bZIP proteins (Li et al. 2009). Subsequently, we isolated five additional alleles from various sources, including EMS and transposon-mutagenized seed stocks, which demonstrated non-complementation with the reference mutant allele (Fig. 10B, Table 2).

Functional characterization of related genes in other model plants can illuminate the function of maize genes. A maximum likelihood phylogenetic tree was constructed from a CLUSTAL alignment of the top 100 BLAST hits from NCBI (Fig. 10C). This analysis revealed that *fea4* encodes the ortholog of the *Arabidopsis* gene *PERIANTHIA* (*PAN*). FEA4 and PAN share approximately 59% amino acid identity, but have very divergent N-terminal sequences, a common feature of TGA-class bZIP proteins (Figure 11)(Jakoby et al. 2002). *pan* is an *Arabidopsis* mutant characterized by an increase in floral organ number, without a corresponding increase in FM size (Running and Meyerowitz 1996). These mutants also have more flowers per inflorescence due to a mild increase in IM size, but this increase is not as severe as the massive fasciation observed in *fea4* (Figure 12) (Maier et al. 2011). Therefore, there is evidence for some conservation of function between *fea4* and *pan* in controlling meristem size.

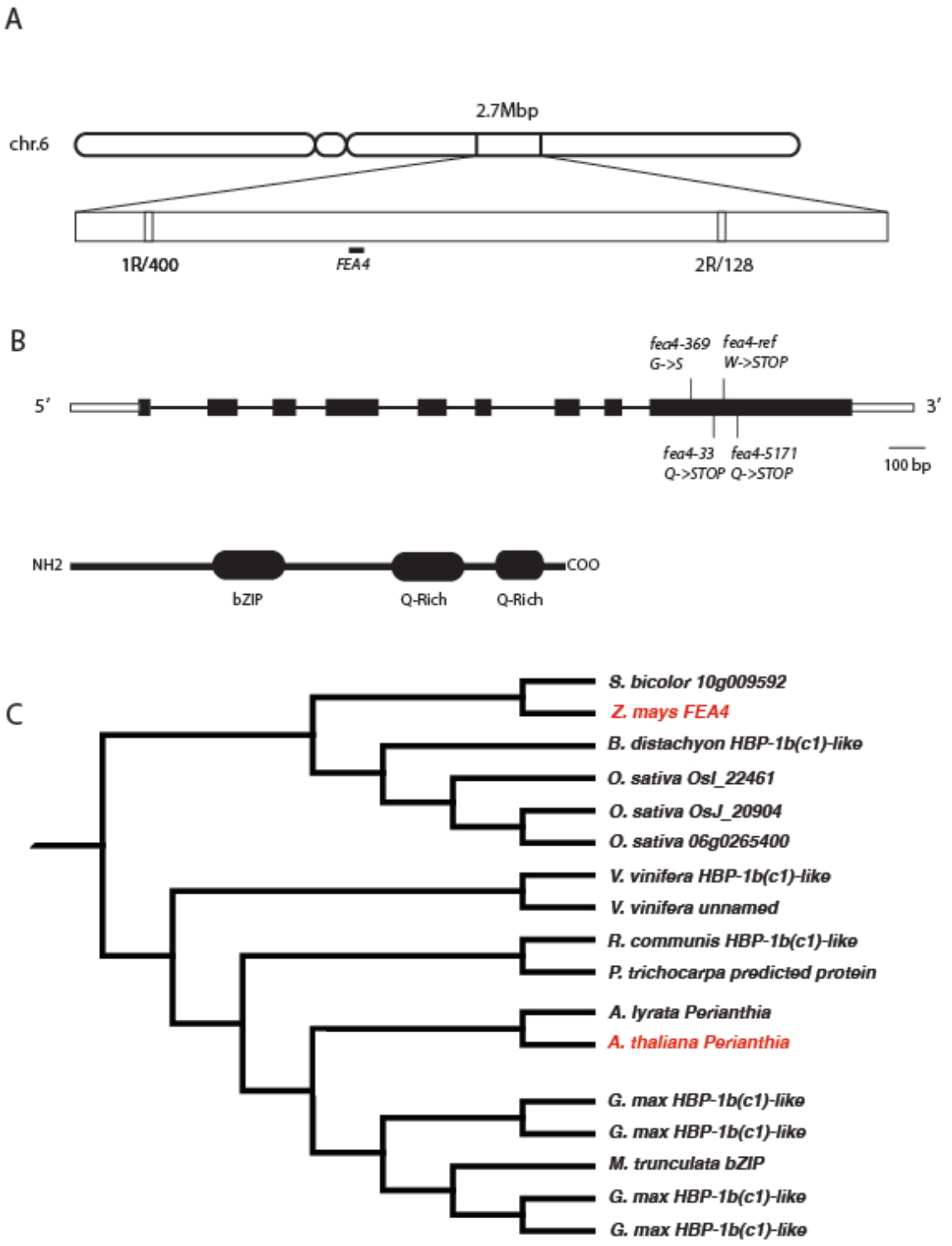

Figure 10: Mapping and molecular cloning of *fea4*. Two CAPS markers delineated a 2.7 Mbp mapping interval on the long arm of chromosome 6, containing approximately 30 genes (A). A gene encoding a bZIP transcription factor in this interval (GRMZM2G133331) contained multiple independent lesions (B). *fea4* encodes a TGA-class bZIP transcription factor orthologous to the *Arabidopsis* gene *PERIANTHIA* (C).

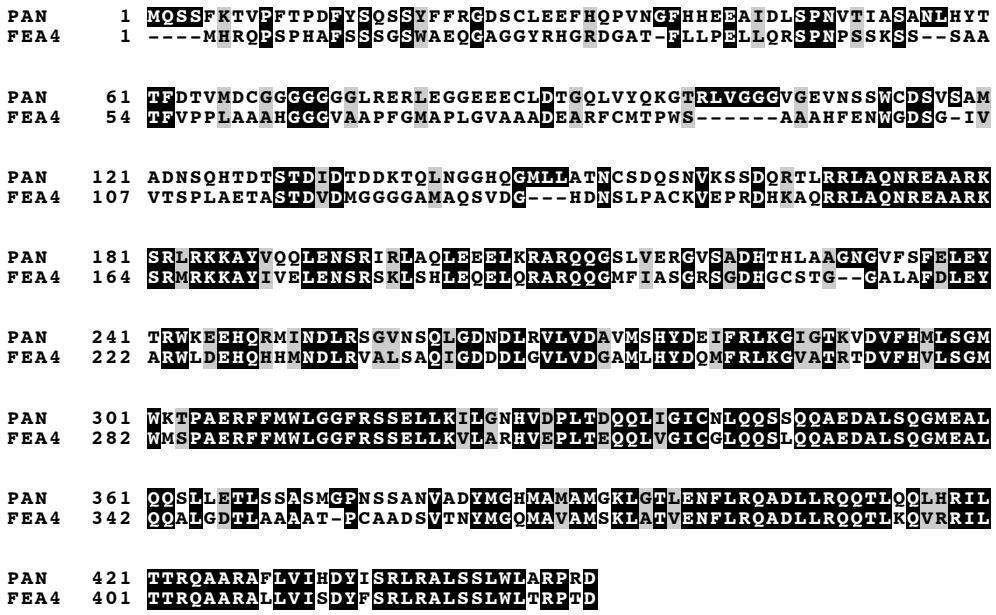

Figure 11: A CLUSTAL alignment of FEA4 and PERIANTHIA visualized with the Boxshade program. Black boxes represent identical residues, grey boxes conservative substitutions, and white boxes non-conservative substitutions. FEA4 and PAN share approximately 59% identity

Table 2: Summary of *fea4* mutant alleles. In total, seven independent *fea4* mutant alleles were obtained from various sources, including Ethyl Methane Sulfonate (EMS)-induced mutagenesis, and the Trait Utility Scoring in Corn (TUSC) population.

Name	Source	DNA	Protein	Complementation	Allelism
fea4-ref	EMS	C-T	W➔STOP	N/A	N/A
*fea4-rel*09-5171*	EMS	G-A	Q➔STOP	All fasciated, n=20	All fasciated, n=40
*fea4-rel*07-167*	EMS	G-A	R➔R	All fasciated	All fasciated n=20
fea4-369	EMS	G-A	G➔S	All fasciated	
fea4-33	EMS	C-T	Q➔STOP		
fea4-TUSC1	TUSC	Mu	?	Fasciated	
fea4-TUSC2	TUSC	Mu	?	Fasciated	

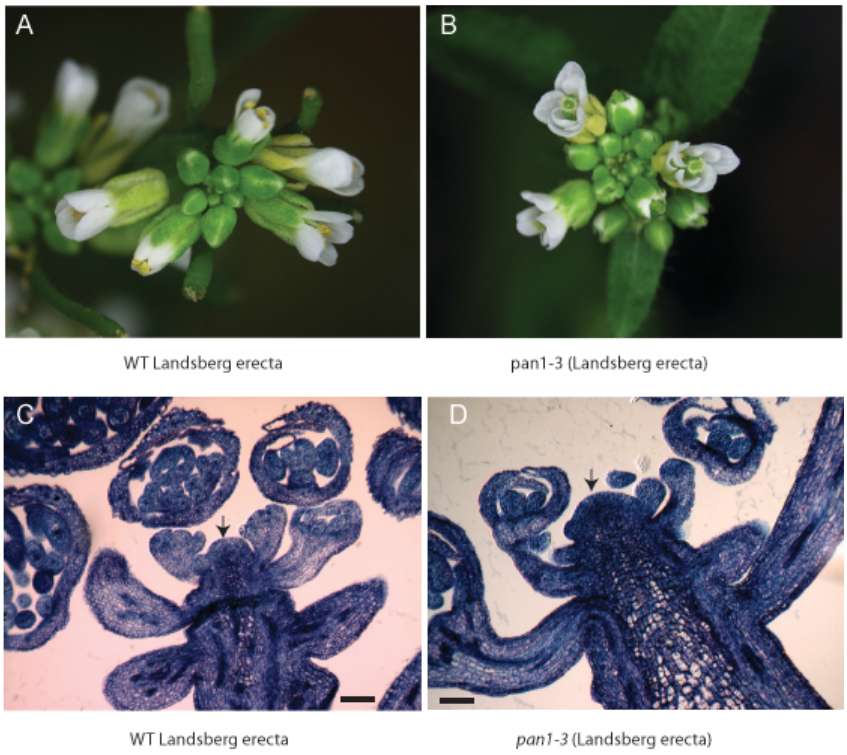

Figure 12: Reproductive phenotypes of the *perianthia (pan)* mutant of *Arabidopsis*. Mutant flowers (B) have extra petals and sepals in the outer whorls compared to wild type flowers (A)(Running et al. 1996). Mutant inflorescences also contain an increased number of flowers compared to the wild type, likely due to larger inflorescence meristems (C,D). Histological sections were stained with Toluidine Blue O. Scale bars = 100 μm in C and D.

3.2.3 Expression Analysis of *fea4*

We carried out RNA *in situ* hybridization with a *fea4* antisense probe to determine the expression pattern of *fea4* throughout various stages of development. During the vegetative phase, *fea4* is expressed specifically in the peripheral zone of the SAM and in the vasculature of immature leaves (Fig. 13A). *fea4* is conspicuously excluded from the stem cell niche at the tip of the SAM, excluded from the incipient leaf primordium (P0), and strongly enriched in a domain beneath the P0 (Fig. 13B,C). This peripheral zone expression pattern is present in various embryonic stages examined (data not shown), and persists until the SAM undergoes the floral transition (Fig 13D). Following transition to reproductive fate, *fea4* is expressed throughout the entire IM of the tassel and ear, and also throughout the SPMs, SMs, and FMs (Fig. 13E,F,G).

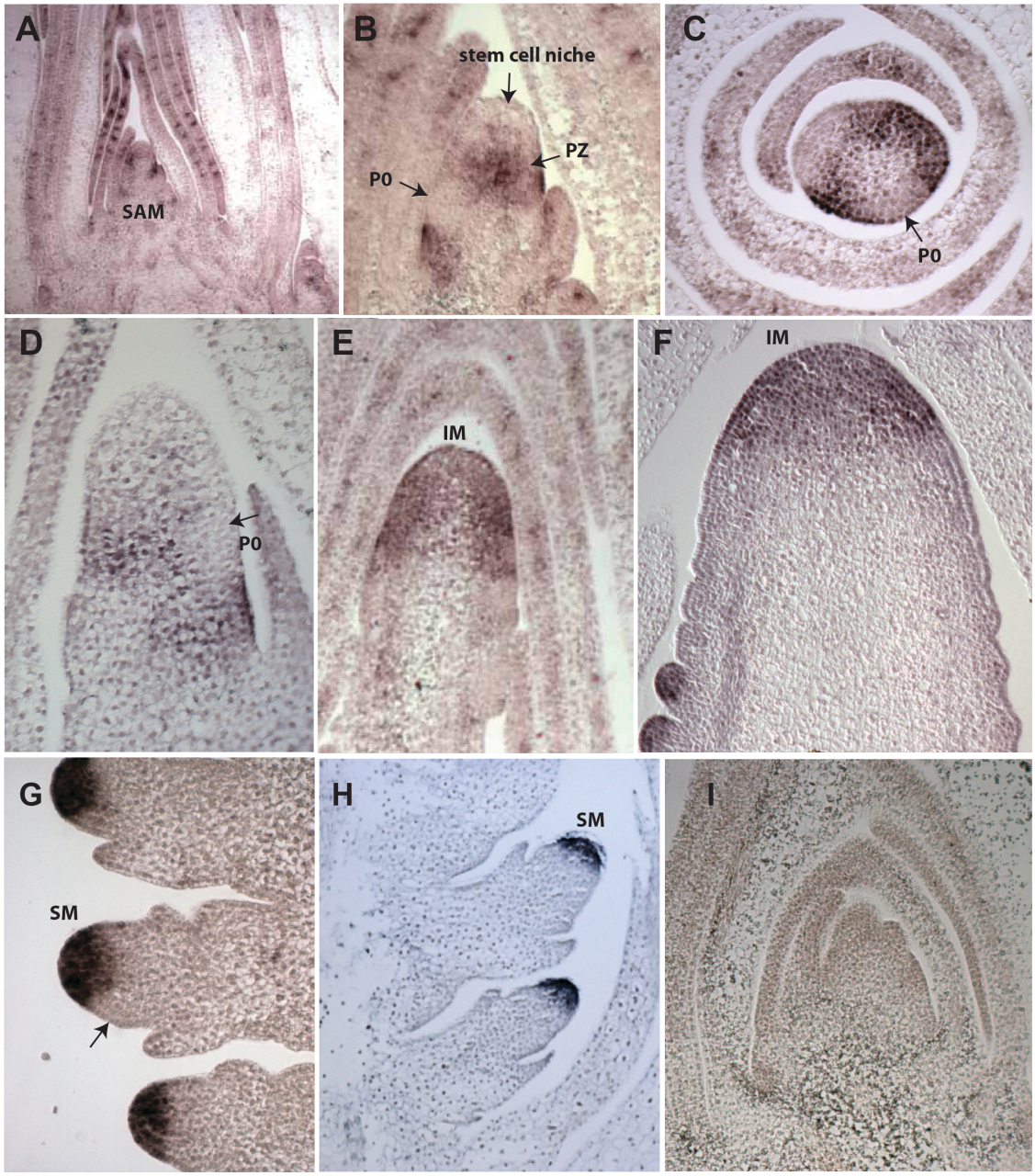

Figure 13: Expression analysis of *fea4*. *in situ* hybridization with a *fea4* antisense probe showing expression in the SAM and vasculature of young leaves surrounding the meristem (A). *fea4* is expressed predominantly in the peripheral zone of the SAM, excluded from the stem cell niche and the incipient leaf P0 (arrows, B). Transverse SAM section, showing exclusion from the PO(C). The peripheral zone specific expression pattern persists through the transition stage (D), but *fea4* is expressed throughout the entire inflorescence meristem of the tassel (E) and ear (F) following the floral transition. It is subsequently expressed throughout the entire spikelet pair (F), spikelet and floral meristems (G), but is again excluded from the site of lateral organ initiation (arrow, G). Sense *fea4* probe produces no signal after overnight hybridization (H).

Similar to the pattern observed in the SAM, *fea4* is down regulated at the site of incipient lateral organ formation (Fig. 13G, Arrow). In control experiments, a short antisense probe created from the 5' portion of the *fea4* cDNA gave rise to a signal identical to the full-length probe (Fig. 13H), and a sense orientation probe produced no detectable signal (Fig. 13I).

To examine the subcellular and tissue-scale localization of the FEA4 protein product, we constructed a translational fusion of the FEA4 coding sequence and yellow fluorescent protein (YFP), under the control of the native promoter (Fig. 14A). This construct was transformed into the HiII inbred maize background, and backcrossed twice to the *fea4-ref* mutant to assess complementation. The presence of this transgene was sufficient to rescue the tassel fasciation phenotype in two independent events, indicating that the fusion protein is functional *in planta* (Figure 14B, Table 3). Wild-type phenotype plants were genotyped for the *fea4-ref* mutation to verify the complementation result (n=8). YFP-FEA4 expression recapitulated the pattern of expression observed by *in situ* hybridization. Strong nuclear expression was observed in all stages of meristem examined, from embryo to inflorescence, and was also present in young leaves surrounding the SAM (Fig. 14C-F). The protein was absent from the vegetative SAM stem cell niche and generally absent from sites of lateral organ initiation, in accordance with the mRNA expression pattern (Fig. 14C, 14F).

In *Arabidopsis*, *in situ* hybridization with a *pan* antisense probe revealed an expression pattern strongly enriched in the peripheral zone of the IM (Maier et al. 2011). However, immunolocalization of PAN protein using a peptide antibody showed accumulation throughout all cell layers of the IM (Chuang et al. 1999); this discrepancy suggests that PAN could move from cell to cell, similar to other plant transcription factors (Gallagher et al. 2004; Lucas et al. 1995; Maier et al. 2011). We did not observe any evidence of FEA4 protein movement, as the expression pattern of the YFP-FEA4 translational fusion line closely matched the *in situ* mRNA pattern. Furthermore, *in situ* hybridization of YFP-FEA4 transgenic plants using a YFP antisense

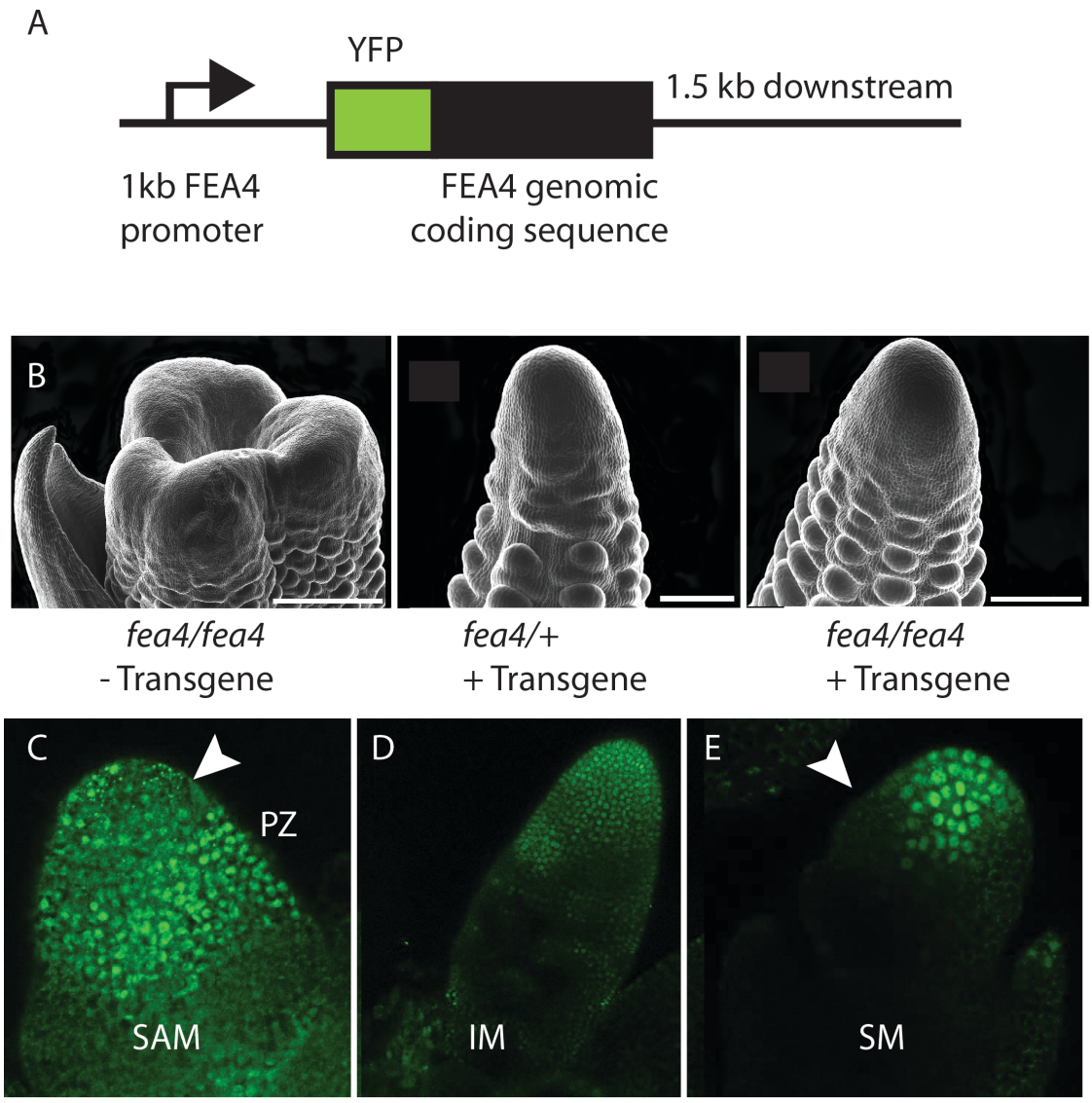

Figure 14: Protein accumulation of YFP-FEA4 translational fusion recapitulates the mRNA expression pattern. YFP was fused in frame to the N-terminus of FEA4 and expressed under the regulation of 1kb of native upstream sequence and 1.5kb of downstream sequence (A). Expression of the transgene was sufficient to rescue the mutant phenotype in the tassel (B). Strong nuclear fluorescence was observed in the peripheral zone of the SAM (C), and fluorescence was absent from the stem cell niche (arrow, C). Fluorescent signal also accumulated in an ear inflorescence meristem (D). FEA4 was also expressed in the spikelet meristems of the ear (E) and was excluded from the site of lateral organ initiation (arrow, E).

Table 3: Expression of YFP-FEA4 is able to rescue the *fea4* mutant phenotype. Tassel phenotypes were scored in families segregating 1:1 for the *fea4-ref* mutation and 1:1 for the YFP-FEA4 transgene. Two independent events were used to rule out segregation distortion caused by linkage of the transgene and the *fea4* locus. Chi-square tests were performed with the null hypothesis that the four categories would occur at a 1:1:1:1 ratio, and that fasciated and normal phenotypes should occur at 50% frequency in both the Basta-sensitive and Basta-resistant categories. A p-value less than 0.05 was chosen for significance cutoff. Yates' correction for small sample sizes was applied to the Chi-square statistic for comparison.

Event #13	**n= 23**	
	Basta-sensitive	**Basta-resistant**
Fasciated	6	13
Normal	4	0
χ^2	15.435	
p-value	0.0015	
χ^2_{Yates}	3.818	
p-value	0.05	

Event #7	**n=15**	
	Basta-sensitive	**Basta-resistant**
Fasciated	4	9
Normal	2	0
χ^2	11.993	
p-value	0.0076	
χ^2_{Yates}	3.818	
p-value	0.3	

probe demonstrated that the mRNA and protein expression patterns were in concordance (Fig 15). The YFP transcripts were localized to the peripheral zone of the SAM in one-week old seedlings, and were clearly absent from the stem cell niche and P0 (Fig. 15). Localized expression was also observed in the vasculature of young leaves, reminiscent of the endogenous gene expression pattern.

We also generated transgenic plants harboring C-terminally tagged FEA4-YFP or FEA4-mRFP constructs. These constructs have not yet been tested for mutant complementation, but show an expression pattern matching the mRNA expression pattern and transgenic constructs described above (Figure 16). The mRFP-tagged translational fusion line will enable co-localization studies, co-immunoprecipitation experiments, and fluorescence activated cell sorting (FACS).

3.3.3 Double mutant genetic analysis

We took a genetic approach to understand how *fea4* interacts with other factors that regulate meristem size. We created F2 populations segregating *fea4* and *fasciated ear2 (fea2)*, the maize ortholog of CLAVATA2. Double mutants displayed a wide range of synergistic phenotypes in vegetative and reproductive structures, including extreme dwarfism and split shoots (Fig. 17). In order to acquire a quantitative readout of the genetic interaction, we genotyped plants from a segregating family, and measured the size of the SAM at 14 days after planting. SAM size was increased by approximately 33% in the *fea4* and *fea2* single mutants relative to wild type (p-value<0.05, Student's t-test), and by 120% in the double mutants (p-value<0.05, Fig 17A,B). The mean meristem size of 347.25 μm in double mutants far exceeded the additive expectation of 275 μm. From the synergistic genetic interaction, we conclude that *fea4* acts in parallel to *fea2*, and by extension we hypothesize that *fea4* is not involved in transducing *clavata*-like signals (Laufs et al. 1998; Prigge and Wagner 2001)

Similarly, combinations of *fea4* with *td1* and *ct2* mutants also displayed enhanced meristem size, height defects, tassel spikelet density, and ear fasciation (data not shown).

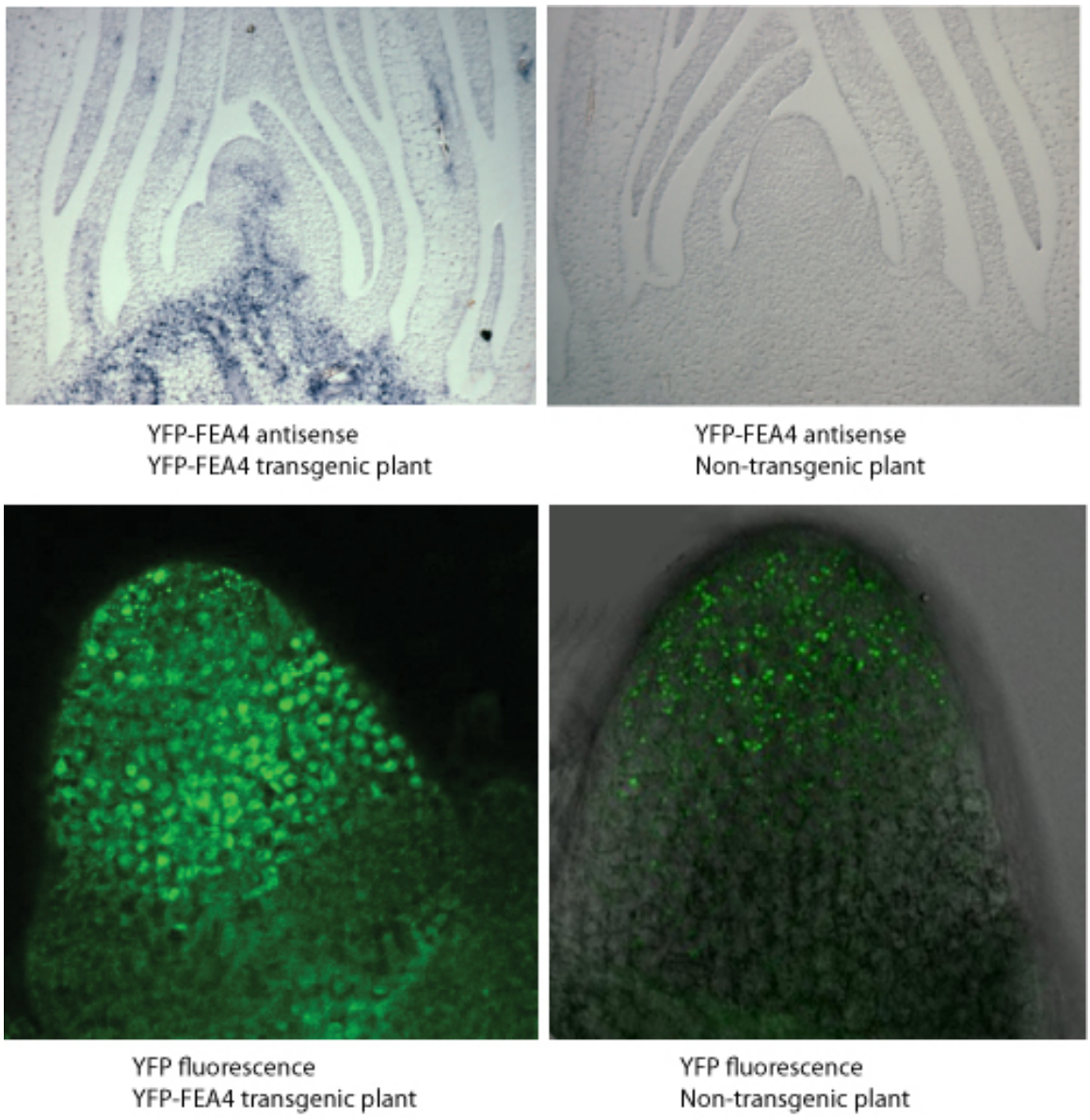

Figure 15: YFP anti-sense *in situ* hybridization demonstrates concordance between mRNA and fusion protein accumulation. Apices from transgenic YFP-FEA4 plants and non-transgenic sibling plants were hybridized with a YFP anti-sense probe, which showed peripheral zone specific expression in the transgenic plants, and no signal in the control. YFP-FEA4 protein was visualized using a Zeiss 710 confocal microscope.

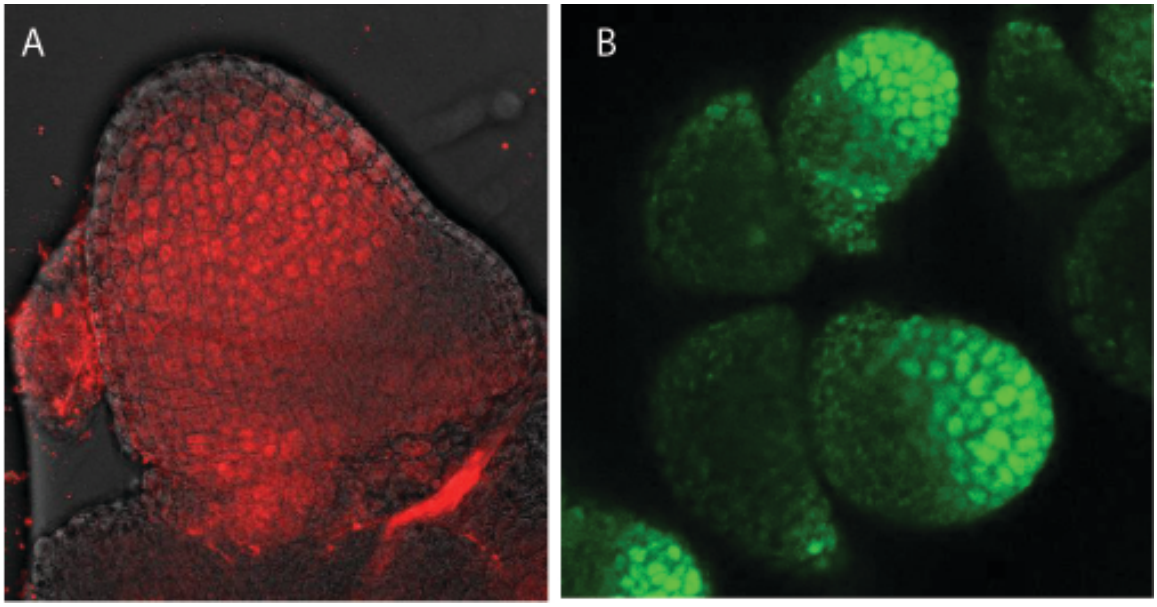

Figure 16: Protein accumulation of FEA4-mRFP (A) and FEA4-YFP (B) translational fusion closely matches N-terminal YFP-FEA4 constructs and endogenous gene expression pattern. YFP or mRFP were fused in frame to the C-terminus of FEA4 and expressed under 1kb of native upstream sequences and 1.5kb of downstream regulatory sequences. A vegetative SAM, 14 days after planting, is shown in (A), and a spikelet/floral meristem is shown in (B).

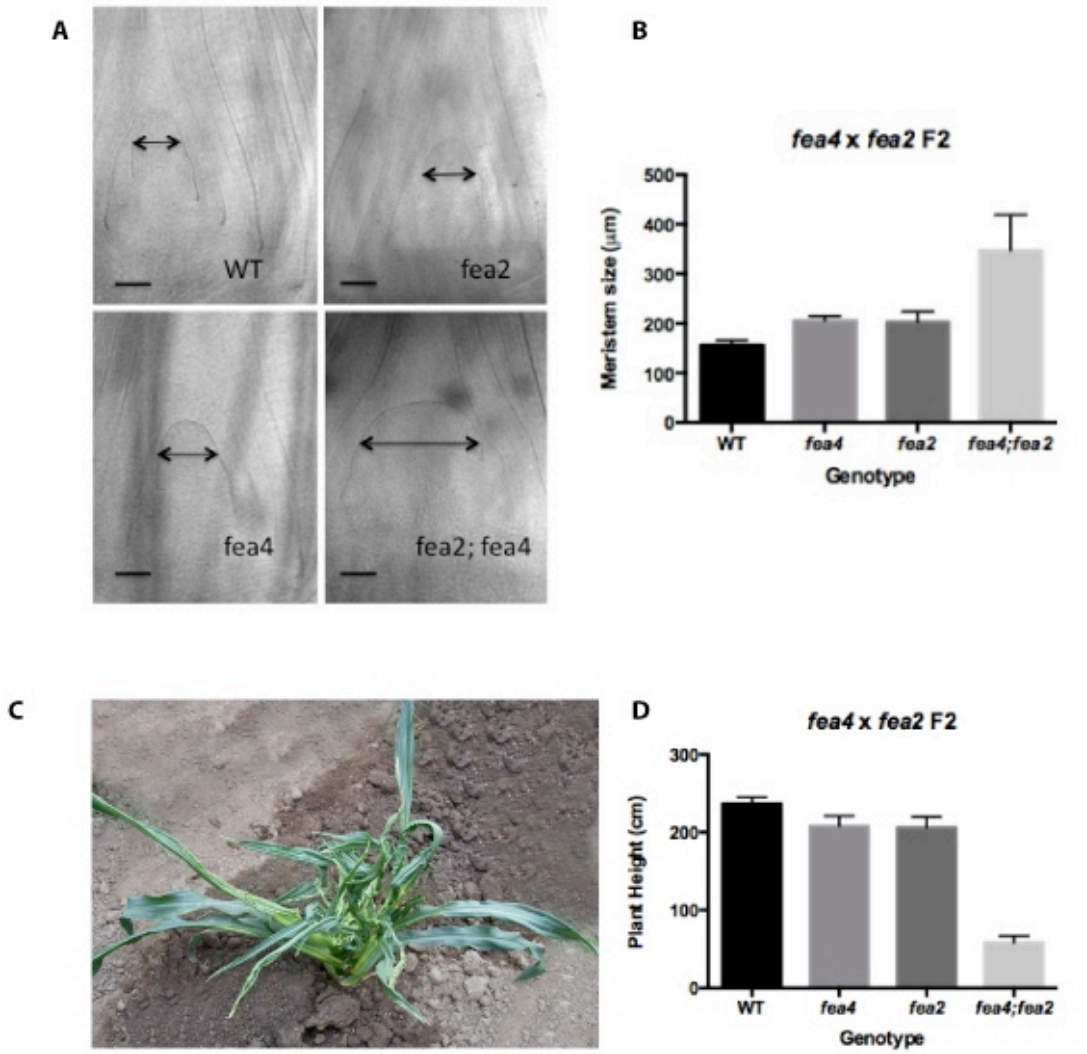

Figure 17: SAM size in F2 families segregating *fea4* and *fea2* mutants. Meristems were dissected and cleared with methyl salicylate 14 days after germination (A). *fea4* and *fea2* meristems were significantly larger than wildtype meristems (p-value<0.05). *fea2;fea4* double mutants were significantly larger than both single mutants (p-value<0.05), and also much higher than the predicted additive point (B). n= 4-16 of each genotype. Mature double mutants (C) showed extreme dwarfism (D) and split shoots.

In contrast, epistasis between factors directly involved in the *clavata* signaling pathway is readily observed, for example, between *fea2* and *ct2* (Bommert et al., submitted).

3.3.4 Transcriptome profiling of developing *fea4* inflorescences

We performed high-throughput mRNA-sequencing on developing ear primordia in order to obtain a global picture of transcriptional changes in the *fea4* mutant relative to wild-type (Fig. 18A,B; Table 4). Two biological replicates of *fea4* and wild-type libraries were very closely correlated with each other, with Spearman rank correlation values greater than 0.98 within genotypes (Fig 18C,D). Differential gene expression analysis revealed that differentially expressed genes were split almost evenly between the up-regulated and down-regulated classes (Fig 18E). Down-regulated genes were significantly enriched for biological processes including "regulation of transcription" and "gene regulation" (Table 5), and included several previously characterized developmental regulators. These include transcription factors belonging to the Wuschel-related Homeobox (WOX), Auxin Response Factor (ARF), and Homeodomain-Leucine Zipper Class III (HD-ZIPIII) families.

As a parallel approach to the differential gene expression analysis, we also tracked the expression of known meristem markers, primarily to establish the effects of *fea4* mutation on the *clv-wus* pathway (Table 6). While only *WOX3A* was differentially expressed beyond the significance threshold, trends in expression levels may be informative. *fea4* transcripts are not abolished in the *fea4-ref* mutant; this serves as a control for changes in gene expression resulting from architectural changes in mutant ears. *fea4* expression was approximately two-fold upregulated in the mutant, consistent with an expansion in inflorescence meristem volume (Table 6). In contrast, meristem markers that show decreased expression, despite an increase in meristem volume, such as *ct2*, may be downstream effectors of *fea4* (Table 6). Overall, it was not possible to capture one clear trend for all members of the *clv-wus* pathway, suggesting that *clv* and *wus* orthologs may not be primary drivers of the *fea4* phenotype.

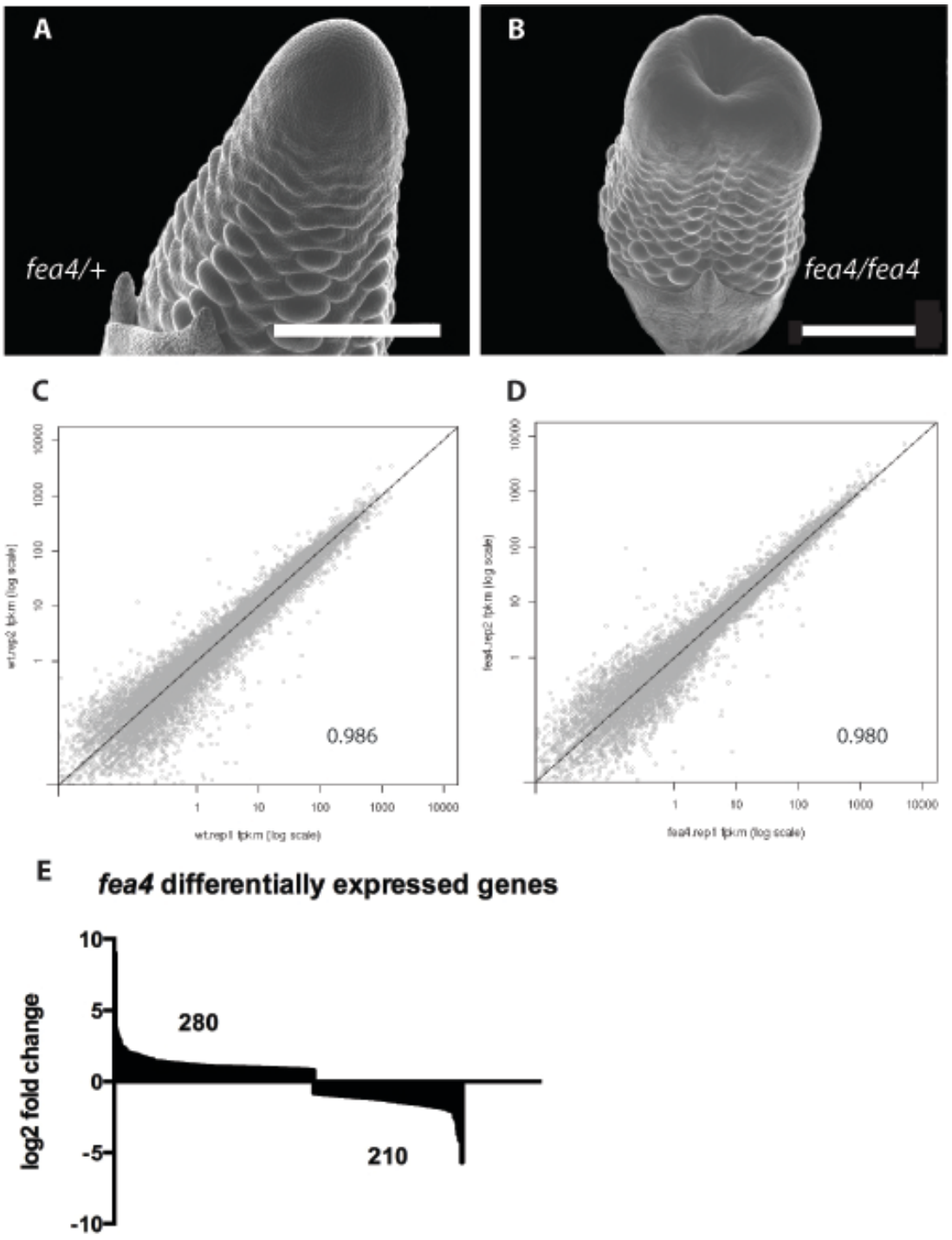

Figure 18: Summary of RNA-seq profiling of *fea4* inflorescences. Duplicate pools of 1 mm ears were harvested from *fea4* homozygous mutants and *fea4/+* heterozygous siblings (A,B). RNA-seq biological replicates showed very close correlation, with Spearman rank coefficients >0.98 (C,D). Cuffdiff 2.0 identified 490 genes that were differentially expressed between genotypes, including 280 upregulated and 210 downregulated genes (E). Scale bars= 500μm.

Table 4: Summary of *fea4* RNA-seq read mapping.

Group	Total paired end reads	Filtered out	Total used Reads	Total mapped Reads	%	Properly Paired
WT-1	39,487,678	466,735	78,041,886	63,720,499	81.6	51,146,876
WT-2	46,070,091	32,320	92,075,542	71,440,057	77.58	59,854,378
fea4-1	49,746,832	648,167	98,197,330	76,107,257	77.50	61,854,220
fea4-2	52,853,684	214,476	105,278,416	69,289,656	65.81	59,567,958

Table 5: Significantly enriched biological processes among genes down regulated in *fea4* mutant ears relative to wild type. Categories involved in gene regulation and transcription are highlighted in bold.

GO #	GO category annotation	p-value
GO:0032774	RNA biosynthetic process	4.30E-05
GO:0006350	**Transcription**	**4.86E-05**
GO:0006807	Nitrogen compound metabolic process	6.57E-05
GO:0006355	**Regulation of transcription, DNA-dependent**	**6.70E-05**
GO:0010468	**Regulation of gene expression**	**1.46E-04**
GO:0019219	Nucleic acid metabolic process	1.93E-04
GO:0005992	Trehalose biosynthetic process	5.81E-04
GO:0019222	Regulation of metabolic process	7.88E-04

Table 6: Expression analysis of 19 meristem marker genes via RNA-seq of *fea4* and wild type inflorescences. Expression values in the far right columns are stated in fragments per kilobase length of exon per million reads (FPKM). The statistical output of Cuffdiff 2.0 is shown with a p-value corrected for multiple tests. Out of the marker gene set, only WOX3A was significantly differentially expressed.

Gene	Gene id	ln(fold-change)	p-value	WT FPKM	*fea4* FPKM
fea4	GRMZM2G133331	0.927031	0.271026	9.92016	18.8618
ct2	GRMZM2G064732	-0.335299	0.682967	25.6752	20.3507
kn1	GRMZM2G017087	0.482607	0.605748	270.959	378.601
fea2	GRMZM2G104925	-0.185804	0.868632	11.4876	10.0994
fea3	GRMZM2G166524	0.0103214	0.995409	9.41784	9.48546
td1	GRMZM2G300133	-0.599264	0.404721	8.41309	5.5534
wus1	GRMZM2G047448	-0.158718	0.956981	1.98071	1.77436
wus2	GRMZM2G028622	-0.0854497	1	0.890206	0.839011
wus-like	GRMZM2G069028	0.772906	1	0.630794	1.07784
wus-like	GRMZM2G108933	-0.944964	1	0.416259	0.216222
wus-like	GRMZM2G069274	0.172583	0.903295	8.52377	9.60692
wox3A	**GRMZM2G122537**	**-1.23229**	**0.00429884**	**21.3722**	**9.09688**
wus-like	GRMZM2G409881	-0.351461	0.766103	8.76754	6.87191
wus-like	GRMZM2G133972	-1.16845	1	0.291799	0.129821
wus11	GRMZM2G170958	-1.79769e+308	1	0.0133571	0
clv1_like	GRMZM2G123178	0.00787122	0.996622	2.91072	2.92665
clv1_like	GRMZM2G066248	0.303465	0.831821	2.91072	3.59214
zm CLV3	GRMZM2G315601	0	1	0	0
zm FCP1	GRMZM2G165836	-0.451825	1	1.24435	0.909769

3.3 Discussion

3.3.1 *fea4* acts in parallel to the clv-wus pathway to regulate meristem size

In this study, we isolated a new fasciated ear mutant, contributing to the growing repertoire of factors controlling meristem size in maize, the world's most widely cultivated crop species. Most of the previously identified factors are directly involved in perceiving or transducing signals in the *clavata* pathway, such as *fea2*, *td1*, and *ct2* (Bommert et al. 2005; Taguchi-Shiobara et al. 2001). Based on a strong synergistic genetic interaction between *fea4* and *fea2*, we propose that *fea4* acts in parallel to the *clavata* pathway to regulate meristem size homeostasis (Laufs et al. 1998; Prigge et al. 2001). Interestingly, *fea4* is the most severe fasciated ear mutant isolated to date, pointing to a critical requirement for this parallel function. It is not surprising that there are partially redundant pathways regulating meristem size in plants, as meristem maintenance is so fundamental for plant development. In the absence of epistasis between *fea4* and any known components of the *clv* signaling pathway, we conclude that *fea4* acts somewhat separately, that is, not directly upstream or downstream of *clv* signaling.

3.3.2 *fea4* encodes the ortholog of the Arabidopsis gene *perianthia*

Phylogenetic analysis revealed that *fea4* encodes the ortholog of the previously characterized *Arabidopsis* gene *PERIANTHIA (pan)*. *pan* mutants are most obviously characterized by increased floral organ number in the outer whorls of petals and sepals (Running and Meyerowitz 1996). Careful analysis of floral meristems by Running and Meyerowitz (1996) revealed no change in meristem size, cell size, or cell number. The authors hypothesized that *pan* mutants were compromised in their ability to specify the identity of floral organs in response to positional cues (Chuang et al. 1999; Running and Meyerowitz 1996).

Changes in the *pan* phenotype in different environmental conditions provide further insight regarding PAN function. *pan* floral meristems adopt an extremely indeterminate fate under short day conditions, producing many ectopic organs, due to sustained expression of WUS

(Das et al. 2009; Maier et al. 2009). PAN is an activator of the floral regulator AGAMOUS, which is required to turn off WUS expression in order to terminate the floral meristem (Lohmann et al. 2001; Maier et al. 2009). It is not clear whether altered WUS expression is responsible for the subtle floral organ number phenotype under long day conditions. During the course of another study, it was noted that *pan* mutants had larger IMs than wild type plants (Maier et al. 2011). These larger IMs maintain their organization and produce floral meristems in a regular phyllotaxy. The magnitude of change in IM size is much less in *pan* mutants than in *fea4* mutants (compare Figure 8 to Figure 12).

fea4 loss-of-function differentially affects different classes of meristems; for example, the IM is severely affected, while the axillary meristems of the inflorescence are not. Indeterminate meristems may be more sensitive to loss of *fea4* than shortly lived, determinate meristems, such as SMs. However, the indeterminate vegetative SAM is relatively mildly affected compared to the IM of the tassel and ear. One possibility is that the maize inflorescence meristem is sensitized to genetic perturbation due to selection for increased meristem size during domestication and subsequent improvement of maize (Bommert et al. 2013; Brown et al. 2011; Taguchi-Shiobara et al. 2001). It remains a point of contention whether the same selective pressure was applied indirectly to the tassel (Brown et al. 2011).

The absence of a phenotype in certain classes of meristems in *Arabidopsis* and maize may reflect the redundant activities of other bZIP proteins or other unrelated proteins. One cannot assume that maize has more genetic redundancy than *Arabidopsis* due to a complex pattern of gene gain and loss in different lineages (Bennetzen 2007). Instead, it is more accurate to consider that each species has a unique set of redundancies, arising either from individual gene function, or emergent network-level properties.

There are both striking similarities and differences between the *fea4* and *pan* expression patterns. Both genes demonstrate a PZ-specific expression pattern: *fea4* in the vegetative SAM, and *pan* in the IM. In addition, *fea4* is expressed throughout the entirety of reproductive

meristems, whereas *pan* is expressed in this manner in the vegetative phase. Therefore, the expression patterns for the two genes are conserved, but shifted in development. Changes in the expression pattern of conserved proteins often underlie morphological differences between lineages (Carroll 2008). In addition to regulatory changes, it is interesting to consider whether differences in phenotype between maize and *Arabidopsis* can be explained by protein coding changes. FEA4 and PAN are vastly different in the N-terminal region of the proteins, which could translate into different DNA-binding activities or protein-protein interactions.

3.3.3 *fea4* and *perianthia* as buffers in meristem function

Several lines of evidence support a role for *PAN* as an important buffer in meristem homeostasis (Maier et al. 2011). First, overexpression of *PAN* with the 35S promoter complements the *pan* mutant, but does not confer any other phenotypes (Chuang et al. 1999). Second, *pan* mutants show incredible sensitivities to environmental conditions, particularly photoperiod regimes (Das et al. 2009; Maier et al. 2011; Maier et al. 2009). It is worth noting that TGA-clade bZIP transcription factors are mostly involved in pathogen response and other environmental surveillance capacities (Zander et al. 2010). Third, *PAN* is expressed in the peripheral zone of the meristem, spatially separated from the core components of the CLV-WUS pathway; in addition, subtle gradients in this expression pattern suggest that relative amounts of transcript or protein may be important (Maier et al. 2011). Finally, microarray profiling revealed that *PAN* regulates cytokinin and auxin pathways (Maier et al. 2011). The balance of these two hormones throughout the meristem is a key modulator of meristematic versus differentiated fate (Su et al. 2011).

Several pieces of more circumstantial evidence reinforce the idea of *fea4* buffering meristem function. For example, *fea4* mutant vegetative phenotypes are stronger in winter Mexico fields under short day conditions. Furthermore, preliminary evidence suggests that loss of *fea4* sensitizes maize plants to the dosage of other meristem regulators, but this effect may also

be environmentally dependent.

3.3.4 Significance of the peripheral zone

The peripheral zone (PZ) is defined as the population of cells that has been displaced to the flanks of the meristem by cell division of stem cell daughter cells (Reinhardt et al. 2003). It can be further subdivided into the inner and outer PZ, based on the ability of cells to de-differentiate into a pluripotent state following removal of the central zone (Reinhardt et al. 2003). Studies involving inducible WUS expression suggest that the stem cell promoting function of WUS can be attributed to its role in repressing the expression of organ differentiation genes (Yadav et al. 2013). Therefore, the cells in the PZ may be more simply defined as the population of cells that are relieved of this repression and are on their way towards a differentiated fate. Meristem size phenotypes of both the *pan* and *fea4* mutants suggest that disruptions in the PZ can have dramatic developmental consequences. In *Arabidopsis*, mutations in the *LOST MERISTEMS* genes, which encode GRAS domain transcription factors, prevent differentiation of cells in the PZ and cause an over-accumulation of meristematic cells (Schulze et al. 2010). Beyond this, the contribution of the PZ to meristem size control is not well established.

3.3.5 Potential targets and effectors of the *fea4* phenotype

One potential caveat in interpreting the results of the RNA-seq experiment is the use of heterozygous wild type siblings instead of true homozygous wild type controls. This decision was predicated on the belief that the benefit of having completely isogenic controls outweighs the risk of gene dosage effects. Many regulatory genes have dosage effects and molecular phenotypes owing to their participation in macromolecular complexes (Birchler et al. 2007). While heterozygous plants may have molecular phenotypes relative to wild type, many changes in gene expression can be expected between heterozygous and homozygous mutants.

A general issue with highly variable RNA-seq data is that conservative multiple test

corrections generate an extremely high false negative rate (Trapnell et al. 2012). One approach to mitigate this would be to reduce the number of tests, for example by only testing genes above an expression level cutoff.

An additional concern is that architectural changes in mutant inflorescences may mask significant changes in gene expression. For example, *fea4* expression is elevated two-fold in the *fea4-ref* mutant, as the transcript is not subject nonsense-mediated decay (NMD). The elevation in *fea4* expression level is roughly equivalent with the increase in IM volume in the mutant. Given the architectural change, a meristematic marker expressed at the same level in mutant and wild type inflorescences may be effectively down regulated.

The RNA-seq analysis revealed that several interesting developmental regulators were differentially expressed in *fea4* mutants, including several homeodomain-containing proteins, and proteins related to auxin response. Perhaps the most intriguing candidate is a gene annotated as WOX3A, which represents one of two duplicate *narrow sheath* genes in maize. These homeobox genes are required for lateral organ founder cell recruitment from the SAM (Nardmann et al. 2004). The *narrow sheath* genes are expressed at the margins of all lateral organs, including floral organs (Nardmann et al. 2004). Both *fea4* and *ns1/2* are expressed strongly in the marginal leaf domain beneath the P0. It is therefore tempting to speculate that defects in founder cell specification could result in both meristem size defects and floral organ number defects.

RNA-seq analysis of *fea4* and wild type siblings at different developmental stages may validate differentially expressed genes, and may also reveal interesting stage-specific differences. YFP-FEA4 transgenic lines will be used for ChIP-seq experiments, which will ascertain bound and modulated targets of FEA4.

3.3.6 FEA4 and PAN as transcriptional co-activators

Many developmentally relevant transcription factors were down regulated in *fea4* inflorescences relative to wild type, suggesting that FEA4 could be an important activator of

these genes. In support of this, PAN was shown in a yeast trans-activation assay to activate transcription of a reporter gene, but only in the presence of co-activators, such as GARP-domain proteins (Maier et al. 2009). Furthermore, PAN has also been shown to physically interact with the BTB-POZ domain transcriptional activators BLADE ON PETIOLE1 (BOP1) and BOP2 (Hepworth et al. 2005). ChIP experiments demonstrated that BOP1/BOP2 co-localize with TGA-class bZIP binding sites in the genome (Xu et al. 2010). FEA4 has two glutamine-rich domains that are traditionally associated with transcriptional activators (Xiao and Jeang 1998). These same domains have been shown to be indispensable for the post-translational redox regulation of PAN by the glutaredoxin proteins ROXY1 and ROXY2 (Li et al. 2009).

3.3.7 Meristem size, kernel row number, and crop yield

This work provides another target for manipulation of inflorescence meristem size and kernel row number, as has been recently demonstrated for *fea2*. Hypomorphic *fea2* alleles do not produce obviously fasciated ears, but significantly increase kernel row number around the diameter of the ear, and kernels per ear (Bommert et al. 2013). Achieving gains in kernel row number without other negative pleiotropic effects is a great challenge, but represents a potentially fruitful path to increasing crop yield. The dramatic *fea4* mutant phenotype suggests that it plays a very important role in regulating meristem size, but at the same time it has not been implicated in kernel row number or ear diameter association studies (Brown et al. 2011; Upadyayula et al. 2006). It would be useful to further study if natural variation in *fea4* expression or activity could contribute to variation in meristem size, in order to deepen understanding of meristem biology and to facilitate crop improvement.

3.4 Materials and Methods

3.4.1 Plant stocks and growth conditions

fea4-ref was isolated from an M2 screen of EMS-mutagenized A619 inbred maize and is deposited in the Maize Genetics Co-Op Stock Center as *fea179*. The mutation was introgressed 4-5 times into various inbred lines for phenotypic analysis in segregating families. *fea4-5171* and *fea4*-167 were found in a screen for enhancers of *ramosa2* in the A619 inbred background. Trait Utility Scoring in Corn (TUSC) alleles were identified from a PCR-based screen of Mutator element mutagenized populations, using *fea4* and Mu-specific primer sets. Plants were grown in field locations in: Cold Spring Harbor, NY; Berkeley, California; Valle de Banderas, Mexico; or under standard greenhouse conditions.

3.4.2 SEM analysis

Fresh tissues were dissected, mounted on stubs with silver conductive paint, and kept on ice before imaging on a Hitachi S-3500N variable pressure Scanning Electron Microscope.

3.4.3 Mapping and molecular cloning

fea4-ref (A619) was crossed to the B73 and W23 inbred lines and the F1 plants were self-pollinated to produce F2 mapping populations. F2 individuals with the mutant phenotype were selected and subjected to bulked segregant analysis and further genotyping with CAPS markers. CAPS markers based on single nucleotide polymorphisms in the introns of GRMZM2G166366 and GRMZM42889 were used to screen for recombinants and establish an interval of 2.7Mbp. The *fea4* coding sequence was amplified in 1 kb fragments from genomic DNA extracted from a pool of F2 mutants, and sequenced by Sanger sequencing (Cold Spring Harbor Genome Center).

3.4.4. Phylogenetic Analysis

A phylogenetic tree was constructed with the PHYML program (http://www.atgc-montpellier.fr/phyml/) (Guindon et al. 2005) using a CLUSTAL alignment of the top 100 BLAST hits with FEA4 protein sequence as input. Branch support was determined by the aLRT SH-like fast likelihood-based method.

3.4.5 Histological Staining

Paraformaldehyde-fixed and paraffin-embedded tissues were sectioned at ten-micron thickness using a Leica microtome. Following adherence to Probe on Plus slides, the sections were stained with Toluidine Blue O (TBO) for 15 minutes, briefly de-stained in dH_20, de-waxed with Histoclear, and mounted with Cytoseal mounting media.

3.4.6 *in situ* hybridization

The coding sequence of *fea4* was amplified from cDNA using primers MP815 and MP816 (see Table 8) and TOPO cloned into pCR2.1. Digoxigenin-labelled riboprobes in the sense and anti-sense orientation were synthesized by *in vitro* transcription from the T7 promoter. Hybridization was carried out according to Jackson et al. (1991), with the addition of 8% polyvinyl alcohol (PVA) to the detection buffer to minimize diffusion of reaction products. Slides were exposed for approximately 12-15 hours before mounting and imaging. The short *fea4* probe specific to the 5' region of the gene was synthesized as above from a cloned fragment amplified with primers MP833 and MP834. *knotted1 in situ* hybridizations were performed using a mix of three probes according to Jackson et al (1994).

3.4.7 Fluorescent protein fusions and confocal microscopy

A translational fusion of the coding sequence of *fea4* and the Yellow Fluorescent Protein

(YFP) driven by the native promoter was created with the Multisite Gateway Pro kit (Invitrogen). Approximately 1 kb of upstream promoter sequence, the entire coding region plus introns, and 1.5kb of downstream genomic sequence were included. Primers used are listed in Table 7. The fragments were cloned into a gateway compatible version of PTF101 (pAM1006-RL) and this binary vector was transformed into the maize HiII line at the Iowa State Plant Transformation Facility (Ames, IA). Plant apices and inflorescences were dissected, mounted on glass slides, and imaged on a Zeiss 710 Confocal microscope.

3.4.8 Double mutant analysis

fea4-ref (B73-4) was crossed to *fea2-o* (B73-6), and F1 plants were self-pollinated to create F2 mapping populations segregating both single mutants, as well as double mutants at a frequency of 1/16. Seedlings were genotyped for *fea2* using gene specific (fea2-D, fea2-ASA) and Mu-element specific (Mu58) primers (see Table 7). *fea4* genotype was determined using primers MP900 and MP901, followed by Cac8I digestion. Shoot apices were coarsely dissected from seedlings after 14 days of growth. Meristems, with surrounding leaves still attached, were fixed in FAA, dehydrated in an ethanol series, and cleared for several days with methyl salicylate (Jackson and Hake 1999). Apices were mounted on glass slides and imaged on a light microscope with attached camera. Meristem width was measured just above the bulge of the P1 leaf primordium, using a global scale in ImageJ. For mature plant analysis, F2 families were grown in the field in Newark, Delaware; or Valle de Banderas, Mexico.

3.4.9 RNA-seq Library Preparation

Duplicate pools of ten 1mm ears were harvested from homozygous *fea4* mutants and heterozygous wild-type sibling plants. Freshly dissected ear tissue was fixed in ice-cold acetone, followed by vacuum infiltration for 20 minutes, and three acetone changes of one hour each. Total RNA was extracted from pools of ear tissue using the PicoPure RNA Isolation kit,

according to manufacturer instructions. Messenger RNA was enriched by two successive purifications with oligo-dT coupled dynabeads (Invitrogen). Approximately 50ng of mRNA was used as input for the ScriptSeq v2 RNAseq system (Epicentre). This kit allowed the addition of barcoded adapters (Index #4,5,6,7) to enable multiplexed sequencing in a single lane of an Illumina HiSeq2000 machine at the Cold Spring Harbor Laboratory Genome Center. Prior to sequencing, the average size distribution of the libraries was verified on a high sensitivity bioanalyzer chip. The libraries were diluted to 10 nM and this concentration was verified with the KAPA qPCR library quantification kit.

3.4.10 RNA-seq Data Analysis

Following sequencing, paired end reads of 101 base pairs were separated according to barcode. Reads were mapped to the maize B73 v2 reference genome using TOPHAT (Trapnell et al. 2009), which allows mapping of spliced (ie. exonic) reads.

Differentially expressed genes were determined by implementing Cufflinks 2.0 (Trapnell et al. 2012). Significantly differentially expressed genes were called with an adjusted p-value cutoff of 0.05 after multiple testing corrections.

3.4.11 ChIP-seq

Developing tassel and ear primordia, approximately 2-5mm in size, were harvested from FEA4-YFP plants grown under green house conditions. These experiments used families containing a transgene integration event that complemented the mutant (A399S1-7), and expression of the transgene was verified by wide-field epi-fluorescence microscopy. Following dissection, inflorescences were immediately cross-linked in buffer containing 1% formaldehyde for 15 minutes under vacuum. Glycine was added to a concentration of 0.1 M and infiltrated for 5 minutes. Following three washes with distilled water, the cross-linked tissues were dried with paper towels and flash frozen in liquid nitrogen.

Table 7: Primers used in this study.

Primer Name	Primer Sequence	Purpose
MP 510	ACTGGATTCCTTGGGAAGGT	CAPS marker for mapping
MP 511	TGGAGTGCACAATCCACAAT	CAPS marker for mapping
MP 516	GCATCCACTTTAGCTTCTGGA	CAPS marker for mapping
MP 517	CGACAACTGGTTCTGTTACCAA	CAPS marker for mapping
MP 900	TGATCCTGTGCAATGTAAAGC	*fea4-ref* genotyping (Cac8I)
MP 901	CAGCTGCTGCTCCGTCAG	*fea4-ref* genotyping (Cac8I)
FEA2-D	AACCTGCAGTCCCTGCCTCCA	*fea2-o* genotyping
FEA2-ASA	AATAGGTCAGGTTCCCTATC	*fea2-o* genotyping
Mu58	CCAWSGCCTCYATTTCGT	*fea2-o* genotyping
MP 815	ATGCATCGTCAGCCATCTC	*fea4* full length probe
MP 816	TCAATCCGTCGGCCGCGTC	*fea4* full length probe
MP 833	TCCCATTCGAAAACAAAAGC	*fea4* short probe
MP 834	GAAGCTCCTGCTCAAGATGG	*fea4* short probe
MP 220	GGGGACAAGTTTGTACAAAAAAGCAGG-CTTCATAAATTTGATTTAGGGGGTGTT	YFP-FEA4 construction
MP 201	GGGGACAACTTTTGTATACAAAGTTGT-CATCGGGCACGGATCAGAGCG	YFP-FEA4 construction
MP 202	GGGGACAACTTTTCTATACAAAGTTGTC-ATGCATCGTCAGCCATCTC	YFP-FEA4 construction
MP 203	GGGGACAACTTTATTATACAAAGTTGT-GCACCGAAATCGCTCTACTC	YFP-FEA4 construction
MP 204	GGGGACAACTTTGTATAATAAAGTTGTC-TCTGTATCCGTTGTGAGATGG	YFP-FEA4 construction
MP 205	GGGGACCACTTTGTACAAGAAAGCTGGGT A- GCGAAAGCAAACATTAAATCA	YFP-FEA4 construction

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CHAPTER 4: IDENTIFICATION OF DIRECT TARGETS OF RAMOSA1

Author Statement: This chapter represents a collaborative effort involving the work of several different people. Michael Pautler designed and constructed the tagged RA1 transgenic lines, and tested independent events for transgene expression by confocal and western blot. He harvested materials for all ChIP experiments and participated in the ChIP and library construction for the tassel tissue. Kengo Morohashi (Ohio State) performed ChIP experiments and library construction. Andrea Eveland performed all of the bioinformatics analysis for the ChIP-seq and *ra1* RNA-seq. Christophe Liseron-Monfils and Andrea Eveland performed the cis-regulatory motif analysis together. For LG1 immunolocalization, Michael Pautler fixed, embedded, and sectioned tissue; immunolocalization was performed by Michael Lewis (University of California Berkeley).

4.1 Introduction

4.1.1 *ramosa1* shapes inflorescence architecture in the grasses

Grass inflorescences display a great diversity of form, and this architectural diversity may contribute to reproductive success and crop yield. All grasses share a common floral unit called the spikelet and inflorescence architecture is largely dictated by the presence or absence of long branches, which bear the spikelets. *ramosa1* (*ra1*) is a C2H2 zinc finger transcription factor responsible for imposing short branch (spikelet pair) identity in the inflorescence of maize and related grasses. Spikelet pair determinacy in the maize ear is essential for the creation of straight, organized rows of seeds (Sigmon and Vollbrecht 2010). The *ra1* locus was a target of selection during domestication. Similar to other domestication factors, selection acted upon standing variation in the teosinte gene pool, and not *de novo* mutations (Sigmon and Vollbrecht 2010; Studer et al. 2011).

4.1.2 The role of SUPERMAN in floral development in *Arabidopsis thaliana*

Phylogenetic analysis revealed that SUPERMAN is the most closely related *Arabidopsis* protein to RA1 (Vollbrecht et al. 2005). Even if RA1 and SUP are not orthologous in the strict sense, it is still worthwhile to review what is known about SUP phenotype, interaction partners, and DNA-binding activity. This is because shared molecular features, such as protein-protein interaction domains or DNA binding domains, can be repeatedly co-opted throughout evolution (Plavskin and Timmermans 2013; True and Carroll 2002). Therefore, related proteins can share mechanisms of action in regulating developmentally analogous processes.

sup was first described as a floral mutant with increased stamen numbers, due to an inability to maintain a precise boundary between the stamen and carpel whorls (Bowman et al. 1992). In an interesting parallel to *ra1* mutants, sup mutants are also characterized by indeterminate floral meristems (Bowman et al. 1992). SUP encodes a single C2H2 zinc finger protein, which may function by negatively regulating expression of B-class homeotic genes (Bowman et al. 1992; Sakai et al. 1995). Alternatively, *sup* loss-of-function may cause increased cell proliferation in the third whorl, at the expense of cell division in the fourth whorl, leading to mixexpression of B-class genes (Sakai et al. 2000).

4.1.3 DNA-binding activities of ramosa1, superman, and EPF-class zinc finger proteins

C2H2 zinc finger domains create platforms for specific contacts between amino acids and DNA base pairs (Razin et al. 2012). The cysteine and histidine residues coordinate a zinc ion, which enforces a defined structural arrangement of two beta sheets and one short alpha helix. Many years of structure-function studies have contributed to the creation of “rules” for DNA-binding specificity, based on the identity of amino acids at certain positions on the alpha helix. Amino acids at helix positions -1,3, and 6 initiate specific contacts with bases in the major groove of the DNA, with each zinc finger specifically interacting with three DNA bases (Razin et al.

2012). Individual proteins often contain multiple C2H2 zinc fingers, which together contribute to the specificity of the transcription factor- DNA target interaction; this property has been exploited for use in biotechnology applications, such as custom-designed zinc-finger nucleases. For example, a six zinc-finger protein can make 18 specific base contacts, which is predicted to create a binding site that occurs only once in every 68 billion base pairs (4^{18}), enough to provide specificity in a maize or human-sized genome.

The founding members of the EPF zinc finger family were identified in *Petunia* as floral organ-specific transcription factors (Takatsuji and Matsumoto 1996; Takatsuji et al. 1992). These *Petunia* proteins possess two zinc fingers with a QALGGH helix-forming sequence, separated by short linkers. Each zinc finger specifically interacts with the short nucleotide sequence AGT and further specificity is imparted by the spacing of these nucleotide motifs (Takatsuji and Matsumoto 1996). Some EPF proteins have only a single zinc finger, as is the case for RA1 and SUP (Sakai et al. 1995; Vollbrecht et al. 2005). An important question is whether sequence-specific DNA-binding activity can be derived from a single zinc finger motif. The first report of specific DNA-binding for a single C2H2 zinc finger was the *Drosophila melanogaster* protein GAGA (Pedone et al. 1996). The single zinc finger and a series of basic amino acids mediate GAGA's DNA-binding activity (Pedone et al. 1996). Similarly, the minimal DNA-binding domain of SUP appears to consist of the zinc finger as well as two flanking basic regions (Dathan et al. 2002). The zinc finger specifically interacts with the nucleotide sequence AGT, as mutating this sequence abrogates DNA-binding in a gel shift assay (Dathan et al. 2002). The flanking basic regions are likely responsible for stabilizing the protein-DNA interaction, but are not likely to impart specificity. A three base-pair motif is not sufficient to provide specificity in a large eukaryotic genome; therefore, binding partners of SUP and RA1 are likely to play a key role in determining targets.

The Drosophila protein GAGA was proposed to cooperatively bind to multiple sites in promoters in order to displace nucleosomes (Katsani et al. 1999; Omichinski et al. 1997). A

chromatin-modifying role for EPF-class zinc fingers would be especially intriguing given the very short temporal requirement for *SUP* activity, but long lasting developmental effects of *sup* loss-of-function (Sakai et al. 2000).

4.1.4 Order of action in the *ramosa* pathway

The existence of three mutants with similar phenotypes immediately suggests a possible common pathway in controlling inflorescence branching. Double mutants of a weak *ra1* allele and strong *ra2* allele display a strong *ra1* phenotype. Furthermore, expression levels of *ra1* are reduced in the *ra2* mutant, and *in situ* hybridization shows that *ra1* expression is also spatially restricted to a smaller than usual domain in the *ra2* background (Vollbrecht et al. 2005). A similar relationship exists between *ra1* and *ra3*: a loss of function *ra3* allele enhances a weak *ra1* allele, and levels of *ra1* transcript are reduced in *ra3* mutants (Satoh-Nagasawa et al. 2006). One interpretation of these data is that *ra2* and *ra3* act in parallel upstream of *ra1* to activate its expression. This is consistent with an almost complete loss of *ra1* expression in a *ra2;ra3* double mutant (Satoh-Nagasawa et al. 2006). It appears that the ramosa pathway converges on *ra1*; therefore, identifying direct targets of *ra1* is of paramount importance in understanding the regulation of meristem determinacy.

4.1.5 Evidence for a non-cell autonomous mechanism of action for *ramosa1*

From the loss-of-function mutant phenotype, we know that *ra1* is required to impose determinacy upon the axillary meristems of the maize inflorescence. However, *ra1* is expressed in a discrete crescent-shaped domain subtending these axillary meristems and not within the meristem proper (Vollbrecht et al. 2005). This observation suggests that RA1 may act non-cell autonomously by controlling a mobile signal. Classically, formal evidence of non-cell autonomy in plants is provided by clonal mosaic analysis. For example, X-ray irradiation can induce aneuploidy in cells that will divide and give rise to clonal sectors of the plant. Visible markers on

the same chromosome arm as the gene of interest can demarcate sectors of wild type and mutant genotype, and the extent and boundaries of these sectors can be compared to the phenotype of the sectors (Becraft et al. 1990). Vollbrecht et al. (2005) took advantage of mutable *Spm* element-derived *ra1* alleles, which, after somatic excision, gave rise to mosaic plants with sectors of phenotypically mutant and wild type tissues. The authors found that pollen from phenotypically normal tassel sectors could often transmit the mutant allele, indicating that a sector could have a wild type phenotype even if its L2 clonal layers had a mutant genotype. This result suggests that RA1 activity in the L1 layer is sufficient to impose determinacy on spikelet pair meristems, providing direct evidence for a non-cell autonomous mode of action. Possible mechanisms for non-cell autonomy include cell-to-cell movement of the protein in question, movement of a downstream target protein, or regulation of another mobile signal, such as a hormone, sugar, or small RNA.

4.1.6 The role of *ramosa1* in activating or repressing target gene expression

The primary role of a transcription factor is to provide sequence specificity to complexes that promote or repress the transcription of target genes. This can involve direct interaction with RNA Polymerase II (Pol II)-containing complexes, or interaction with chromatin modifying complexes that compact or loosen local chromatin structure (Spitz and Furlong 2012). In addition to the C2H2 zinc finger domain and associated basic regions, RA1 possesses two Ethylene-responsive element-binding Amphiphilic Repression (EAR) motifs (Vollbrecht et al. 2005). These classical repression domains are required for physical interaction with the TOPLESS family of transcriptional co-repressors (Szemenyei et al. 2008). This group of co-repressors probably functions by recruiting Histone Deacetylases (HDACs), which remove acetyl marks from Histone tails, thus rendering associated DNA transcriptionally inactive (Long et al. 2006). *ramosa enhancer locus2* (*rel2*) was isolated in a screen for enhancers of a weak *ra1* allele, and was found to encode a co-repressor orthologous to the Arabidopsis protein TOPLESS

(Gallavotti et al. 2010). RA1 and REL2 physically interact via the two EAR domains and deletion of these motifs abolishes the interaction (Gallavotti et al. 2010). Therefore, genetic and physical interactions suggest that RA1 may act to repress transcription of target genes.

Is the presence of canonical repression domains sufficient to sentence RA1 to a life of transcriptional repression? Domain swapping experiments have shown that EAR domains can transform strong transcriptional activators into dominant transcriptional repressors (Hiratsu et al. 2003). However, there are counter examples of transcriptional regulators that can either activate or repress transcription of targets, depending on developmental context or binding partners. The homeodomain protein WUSCHEL is one such example, as it contains an acidic activation domain and an EAR-like motif (Kieffer et al. 2006). WUS activates expression of the floral patterning gene AGAMOUS in a process required for floral meristem termination (Lohmann et al. 2001). However, WUS has also been shown to bind to and repress transcription of ARABIDOPSIS RESPONSE REGULATOR7 (ARR7) to mediate cytokinin homeostasis in the SAM (Leibfried et al. 2005). Inducible expression of WUS coupled with chromatin IP has also uncovered up- and down-regulated target genes (Busch et al. 2010; Yadav et al. 2013). Thus, there is a precedent for a single factor mediating context-dependent transcriptional activation and repression in plants.

RNA-seq analysis of 1 mm *ra1* mutant ears relative to wild type revealed several hundred differentially expressed genes, including many that were upregulated (Eveland et al., unpublished). The upregulated genes may represent indirect targets; for example, RA1 may normally repress a repressor of these genes. Indirect effects are difficult to eliminate, as for practical reasons this developmental stage is the earliest stage at which ears can sampled, and inducible gene expression systems are not yet widely available in maize. As an alternative, RNA-seq based differential gene expression can be coupled with ChIP-seq to derive a list of "bound and modulated targets" that are both differentially expressed in the mutant and bound by the transcription factor (see Bolduc et al. 2012).

4.2 Materials and Methods

4.2.1 Generation of YFP-RA1 and HA-FLAG-RA1 constructs

Tagged RA1 constructs were created with the Multisite Gateway® Three-Fragment Vector Construction kit (DeBlasio et al. 2010). 2.9kb of promoter sequence was amplified using primers RA1-attB4 and RA1-attB1r and cloned into entry vector p4-p1r (see Table 8). A 3xHA-FLAG tag was amplified from a plasmid template using primers HA-FLAG-attB1 and HA-FLAG-attB2, and cloned into entry vector p221 (p1-p2). A version of the Yellow Fluorescent Protein (YFP) with poly-alanine linkers (TT-YFP) had previously been cloned into entry vector p221 (p1-p2). A 2.5 kb fragment representing the RA1 coding sequence and 3'UTR was amplified using primers RA1-attB2r and RA1-attB3, and cloned into entry vector p221 (p2r-p3). The three fragments were combined by LR recombination, according to manufacturer instructions, into a 3-way gateway compatible version of the PTF101 binary vector. This binary vector was transformed into *Agrobacterium tumefasciens* by electroporation. The resulting clones were checked for undesirable recombination before being sent to the Plant Transformation Facility at Iowa State University (Ames, IA).

4.2.2 Confocal Microscopy

Tassel or ear primordia were dissected immediately before imaging and mounted on glass slides with cover slip. YFP-RA1 samples were imaged on a Zeiss 510 confocal microscope, with an excitation of 488 nm and 514 nm, and acquisition tuned for a maximum emission at 527 nm.

4.2.3 Western Blots

Frozen samples were ground into a fine powder and resuspended in an SDS-containing sample buffer (Lammeli). 30uL of sample buffer was loaded into a 10% SDS-polyacrylamide gel and run for 1.5 hours. HA-FLAG-RA1 fusion protein was detected by using a monoclonal anti-HA antibody (Sigma H3663) at a dilution of 1:1000, and HRP-conjugated anti-mouse antibody

Table 8: Primers used in this study.

Primer Name	Primer Sequence	Purpose
RA1-attB4	GGGGACAACTTTGTATAGAAAAGTTGCG-ACAGTAACACGGGTGCCAATC	RA1 promoter
RA1-attB1r	GGGGACTGCTTTTTTGTACAAACTTGG-CATAGCTGCTAGCTAGTCGAG	RA1 promoter
HA-FLAG-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGG-GATTATAAAGATGATGAT	HA-FLAG tag
HA-FLAG-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC-GGCGTAGTCCGGAACGTC	HA-FLAG tag
RA1-attB2r	GGGGACAGCTTTCTTGTACAAAGTGGCG-ACGAAGCACGCCGCCTACTCC	RA1 cds+utr
RA1-attB3	GGGGACAACTTTGTATAATAAAGTTGCGC-TCCACGCTATTCATGACG	RA1 cds+utr
RA8	TGCTCTATCTTGCCTCTTCATGC	*ra1-R* CAPS
RA11	TGCACTGCACGTACCCATTGTAG	*ra1-R* CAPS

at 1:5000 (Pierce #31457).

4.2.4 Chromatin Immunoprecipitation and Library Construction

Basta-resistant transgenic plants were selected in T1 or T2 families that were either heterozygous or segregating 1:1 for the *ra1-R* mutation. One to five mm tassel primordia were harvested approximately four weeks after planting, and similarly sized immature ears were harvested after six weeks. Approximately 200-300 mg of tissue was used per biological replicate. ChIP was performed according to methods described in Morohashi et al. (2012) and Bolduc et al. (2012). Chromatin extracts from YFP-RA1 and HA-FLAG-RA1 plants were immunoprecipitated with anti-GFP (ab290, Abcam) or anti-HA (H3663, Sigma). Protein A agarose-salmon sperm DNA beads (Millipore, catalog # 16-157) were used to capture antibody-chromatin complexes.

Following ChIP, DNA was end-repaired and A-tailed, and multiplex Illumina adapters were ligated. Libraries were amplified by Phusion Hot Start II DNA polymerase (Thermo Scientific) using MltplxPCR1.0 and PCR2.0 (index1-7) primers. Amplified products were subjected to gel-size fractionation by electrophoresis to obtain libraries with size distributions between 200bp and 500bp.

4.2.5 Illumina Sequencing

Libraries were sequenced on Illumina GA or HiSeq2000 machines with single end reads of 50 base pairs at The Ohio State University Nucleic Acid Shared Resource (OSUCCC, Columbus, OH). Read counts and mapping statistics are summarized in Table 9.

4.2.6 Bioinformatic Analysis

Following sequencing, filtered reads were mapped to the maize reference genome using the memory-efficient short read alignment program Bowtie (Langmead et al. 2009). Duplicate tags likely to arise from amplification artifacts were collapsed. For peak calling, MACS v1.0.4

(Zhang et al. 2008) utilitizes an algorithm that computationally shifts read tags from the sequenced ends towards a predicted summit. MACS uses an underlying Poisson distribution to control for local biases in the genome, in order to confidently call peaks of transcription factor occupancy (Zhang et al. 2008). For derivation of "high confidence peaks" found in multiple libraries, we required that two significant peak summits were found within a 300bp window.

4.2.7 Immunolocalization of LG1 protein

B73 and *ra1-R* ears were dissected at 1-5mm, fixed in 4% paraformaldehyde, embedded in paraplast, and sliced into 10 micron thick sections with a Leica microtome. Sections were adhered to Probe-on Plus charged slides (Fisher Scientific) without heating, in order to avoid protein denaturation. Immunolocalization of LG1 was carried out according to Jackson et al. (2002), using purified peptide antisera raised against full-length LG1 at a dilution of 1:500 (Cocalico Biologicals, Inc., Reamstown, PA).

4.3 Results

We created two translational fusion constructs in order to drive the expression of tagged RA1 proteins in the endogenous mRNA expression domain. Previously generated pRA1-RA1::CFP and pRA1-RA1::3xYFP failed to complement the *ra1* mutant. This could be due to: 1) insufficient protein expression levels; 2) inappropriate spatial expression pattern; 3) impaired cell-to-cell movement; or 4) steric hindrance of protein-protein interactions at the C-terminus. As an alternative strategy, we fused the YFP and HA-FLAG tags in frame with the RA1 coding sequence at the N-terminus (Fig. 19A). Constructs were transformed into the HiII genetic background at the Iowa State University Plant Transformation Facility (Ames, IA). T0 generation transformed plants were crossed to the *ra1-R* mutant allele, which had been introgressed into the B73 background seven times. Subsequently T1 plants were backcrossed to the mutant to create a T2 generation segregating 1:1 for the transgene and 1:1 for the *ra1-R* mutant allele.

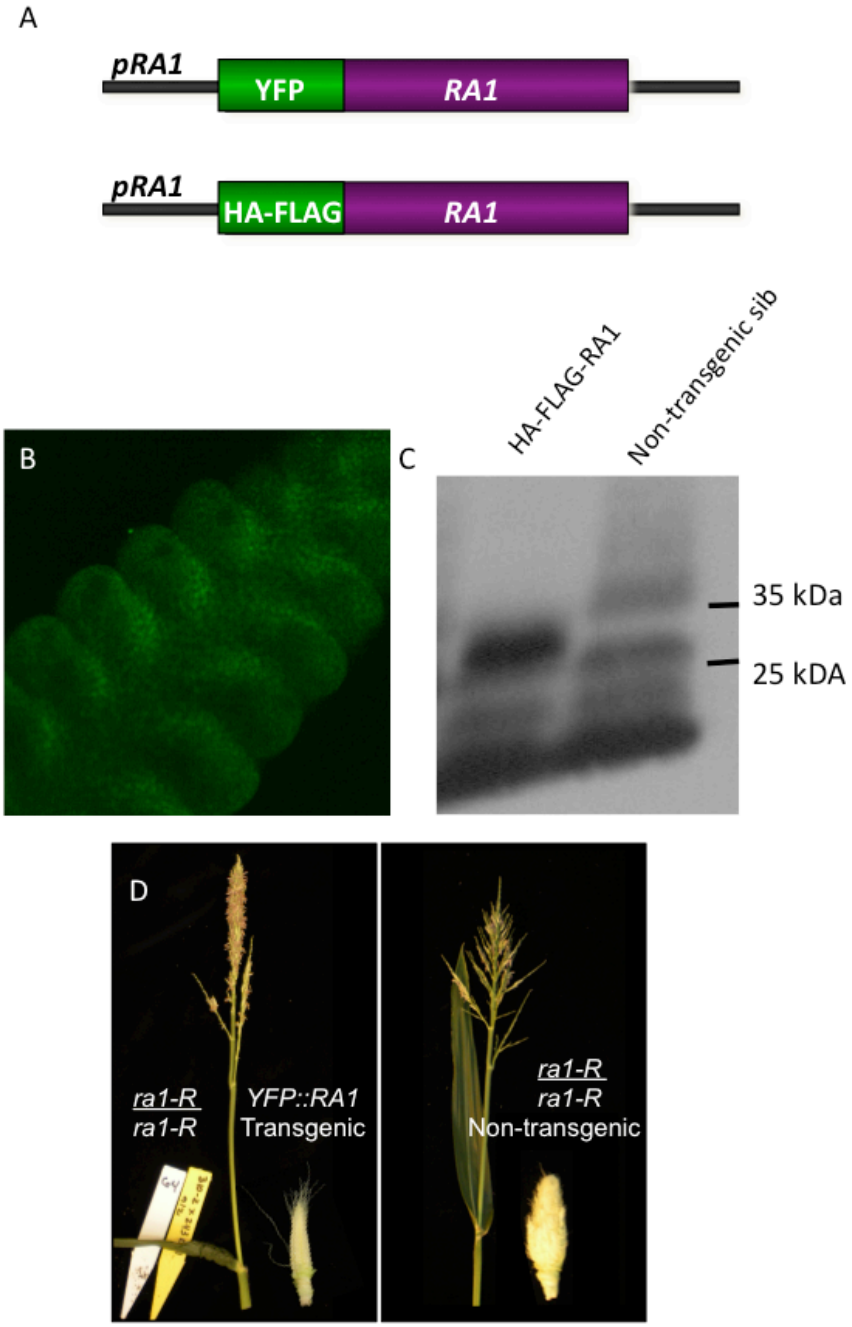


Figure 19: Constructs used for ChIP experiments. YFP and HA-FLAG tags were fused in frame with the RA1 coding sequence, under control of the native promoter (A). Constructs were transformed into the HiII genetic background at the Iowa State University Plant Transformation Facility. YFP-RA1 was expressed in an adaxial domain subtending the spikelet pair and spikelet meristems of developing inflorescences, and was localized to the nucleus (B). HA-FLAG-RA1 expression was confirmed by detection of a ~30kDa fusion protein on a western blot of immature ear extracts (C). Expression of the YFP::RA1 transgene complements the *ra1-R* mutant (D).

As expected**,** YFP-RA1 was expressed in an adaxial domain subtending the spikelet pair and spikelet meristems of developing inflorescences, and was localized to the nucleus (Fig. 19B). HA-FLAG-RA1 expression was confirmed by detection of a ~30kDa fusion protein on a western blot of immature ear extracts (Fig. 19C). Analysis of plant phenotypes in T2 families segregating the transgene and the *ra1* mutant revealed that the transgenic constructs are capable of complementing the mutant (Fig. 19D). This result was confirmed by the wild type phenotypes of hundreds of transgenic plants in T2 families segregating 1:1 for *ra1-R*.

We performed ChIP-seq using pools of YFP-RA1 and HA-FLAG-RA1 tissue in order to establish a genome-wide binding profile for RA1. The two different epitope tags served as pseudo-biological replicates and helped rule out artifacts arising from either the HA or YFP antibody. Both tassel and ear primordia were assayed in parallel in order to identify overlapping targets, and also to detect differences associated with indeterminate branch meristems.

Across all experiments, more than 10,000 RA1 binding peaks were identified and called significantly enriched ($p<1x10^{-05}$) relative to input (Table 9). However, in an effort to minimize false positives, we adopted a combinatorial approach, and selected only peaks present in both HA- and YFP-ChIP libraries in one tissue, or two or more libraries overall. This allowed us to analyze the distribution of 2,105 high-confidence binding sites throughout the maize genome. These binding peaks were present in various genomic contexts, including proximal promoter regions, 5'UTRs, exons and introns of gene bodies, and intergenic regions (Figure 20). We searched for maize filtered gene set models within 10kb of each peak in order to derive a list of candidate target genes. By this criterion, we catalogued 1094 putative RA1 target genes in tassel and ear tissue, with an overlap of 305 genes between tissues.

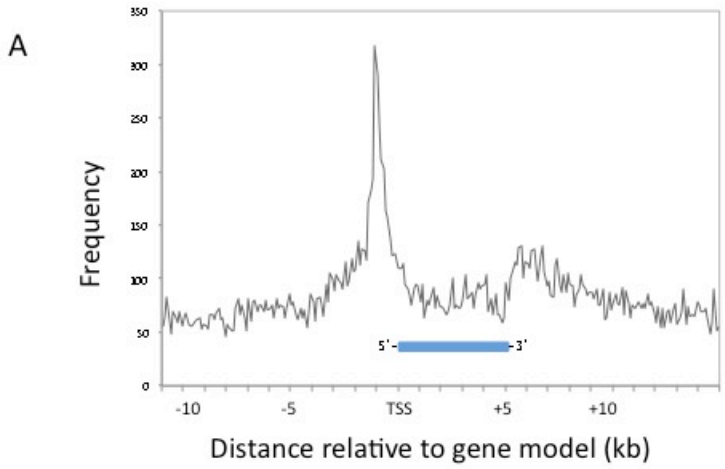
In addition to the issue of false positive binding peaks, there is also not always a straightforward relationship between transcription factor binding and changes in gene expression (Farnham 2009). For this reason, we further restricted our analysis to genes that were both bound by RA1 and significantly differentially expressed in *ra1* mutant transcriptome profiling

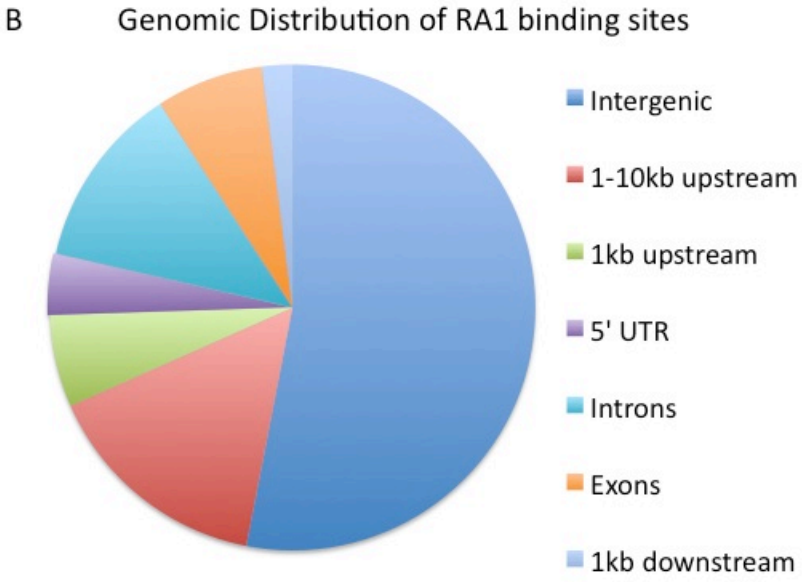
Table 9: ChIP-seq library sequencing and alignment summary statistics.

EAR		Reads sequenced	Reads aligned	Suppressed alignments[a]	Reads post filter	Redundant rate[b]	Called peaks[c]
HA	ChIP	18,486,261	3,645,239 (19.72%)	9,083,295 (49.14%)	2,954,164	0.19	6648
	Input	44,368,810	12,884,081 (29.04%)	3,0109,827 (67.86%)	12,822,480	0	
YFP	ChIP	27,095,082	6,429,599 (23.73%)	15,706,318 (57.97%)	5,313,815	0.17	12856
	Input	47,206,737	13,853,924 (29.35%)	32,062,291 (67.92%)	13,792,123	0	

TASSEL		Reads sequenced	Reads aligned	Suppressed alignments[a]	Reads post filter	Redundant rate[b]	Called peaks[c]
HA	ChIP	42,088,728	5,054,621 (12.01%)	11,147,684 (26.49%)	433,236	0.91	4829
	Input	9,993,333	3,135,271 (31.37%)	5,948,047 (59.52%)	2,215,753	0.29	
YFP	ChIP	55,285,732	2,817,477 (5.1%)	7,414,078 (13.41%)	440,594	0.84	2913
	Input	19,428,094	5,775,749 (29.73%)	10,908,448 (56.15%)	4,628,587	0.2	

[a] Suppressed alignments due to multiple mapping reads; only unique alignments were kept.
[b] Rate of tag redundancy in ChIP-seq libraries; only unique tags were used for peak-calling with MACS v1.0.4.
[c] Significance threshold for calling enriched peaks was $p < 1.0e-05$.





confidence peaks were strongly enriched just before the transcriptional start sites (TSS) of genes (A). Overall, the predominant binding locations were located in intergenic regions and from 1 to 10kb upstream of gene models (B). The category "1 kb upstream" is particularly enriched (6%), as it represents approximately 1.3% of the genome space (assuming ~40,000 genes in a 3Gbp genome).

(Eveland et al., submitted). Bolduc et al. (2012) similarly identified "bound and modulated targets" of KN1 in the first published ChIP-seq study in maize. In our study, out of 1094 potential targets, 240 genes were differentially expressed in *ra1* mutants, indicating they are likely directly modulated targets. We found that 70% (167 out of 240) of modulated targets were expressed at significantly lower level in *ra1* mutant ears, implying that the normal function of RA1 is to activate expression of these genes (Figure 21A). The remaining 30% of targets were up regulated in *ra1* mutants, suggesting that RA1 represses expression of these genes, perhaps in concert with the REL2 co-repressor.

Several functional Gene Ontology (GO) categories were significantly enriched among RA1 targets (Figure 21B). The most over-represented category was "nucleic acid-related", dominated by targets that are repressed by RA1. Conversely, the "transcription factor" category mostly contained targets that would be activated by RA1 in wild type inflorescences.

RA1 targets included several classical maize mutant genes of note, including meristem regulators such as *compact plant2,* and the sex-determination factor *tasselseed2*. RA1 also bound broadly to a regulatory intron of the master meristem regulator *kn1*, but levels of *kn1* were not significantly different in the *ra1* mutant.

Another theme that emerged was that RA1 regulated components of hormone biosynthesis and signal transduction pathways. In particular, it appears RA1 may modulate and provide fine-tuning for the giberellic acid (GA) pathway. RA1 binds to and activates *ga3-oxidase* and *ga2-oxidase*, which encode a rate-limiting GA biosynthesis enzyme and a GA catabolism enzyme, respectively. RA1 also binds to and represses expression of *spindly*, which is a negative regulator of GA signaling (Jacobsen et al. 1997).

One target of particular interest is the *liguleless1* (*lg1*) locus, where RA1 binds to the first intron of a gene encoding a SPB transcription factor (Fig. 22A) (Moreno et al. 1997). *lg1* is required for the specification of the leaf-sheath boundary during vegetative development and has been shown to control rates and planes of cell divisions in the pre-ligular band (Sylvester et al.

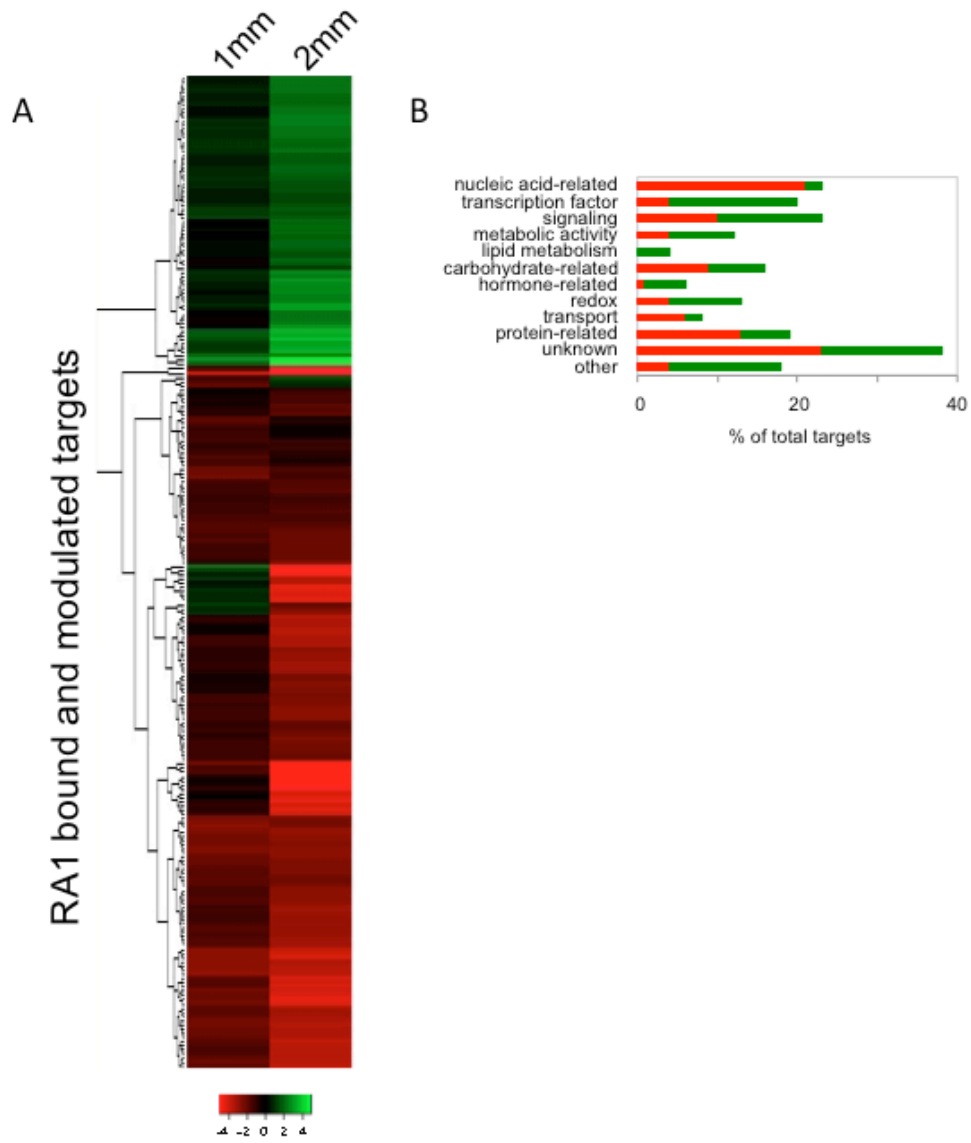


Figure 21: Bound and modulated targets of RA1. 240 out of 1094 target genes were significantly differentially expressed in *ra1* mutant ears relative to wild type (A; Eveland et al., unpublished). The heat map demonstrates that 70% of these genes were down regulated in *ra1* mutants, whereas 30% were up regulated. Nearly all of the genes were more strongly differentially expressed in 2mm ears than 1mm ears. Several functional categories were significantly enriched among RA1 targets (B). The most over-represented category was "Nucleic Acid-related", and this was dominated by targets that are repressed by RA1.

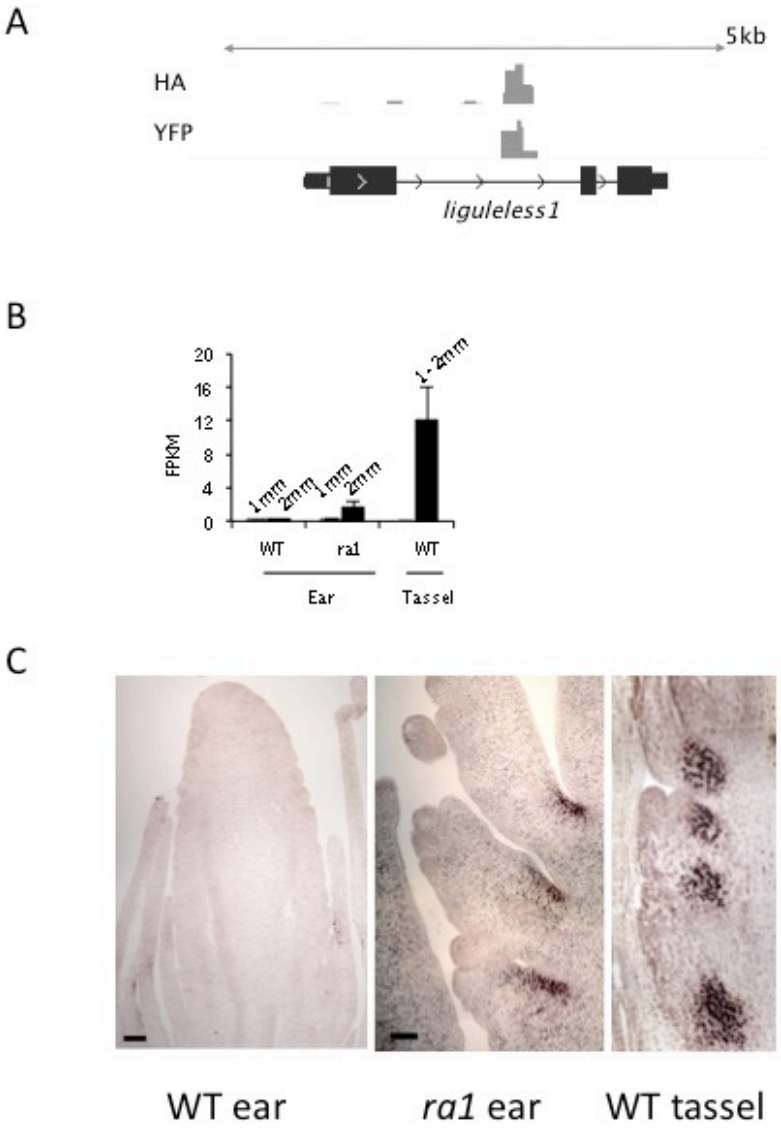


Figure 22: *liguleless1 (lg1)* is a putative effector of RA1. RA1 was strongly bound to the first intron of *lg1,* which encodes a SBP-box transcription factor, in multiple libraries (A). *lg1* is upregulated in *ra1* mutant ears relative to wildtype, correlating with the presence of long branches in the mutant (B). *lg1* is also expressed at a high level in young tassels, which feature indeterminate branch meristems. Immunolocalization of LG1 protein using a peptide antibody confirms an association with LG1 expression and indeterminate branches (C). The protein is not present in young ear primordia, which lack long branches, but can be detected in the husk leaves surrounding the ear. LG1 protein accumulates at the base of indeterminate branches in *ra1* mutant ears. Similarly, tassels also express LG1 at the base of long branches.

1990). No tassel branch number phenotype has been described for the loss of function mutant; however, *lg1* tassels express upright tassel branch angles, a defect associated with the development and expansion of the pulvinus (Bai et al. 2012). A recent association mapping study of maize inflorescence traits found a very strong association between alleles of *lg1* and tassel branch number (Brown et al. 2011). This association was supported by a very high model re-inclusion probability and a large effect size of approximately one branch per tassel (Brown et al. 2011). Transcriptome profiling of *ra1* mutant inflorescences revealed a strong up regulation of *lg1* relative to wild type (Fig. 22B) (Eveland et al., submitted).

We performed immunolocalization of LG1 protein using a peptide antibody to further refine the link between *lg1* and inflorescence branching. LG1 protein was not detected in young ear primordia, which lack long braches (Fig. 22C). Expression of the protein in the husk leaves surrounding the ear served as a positive control. LG1 protein accumulates at the base of indeterminate branches in *ra1* mutant ears, closely matching the expression pattern of LG1 at the base of tassel branches.

Next, we sought to uncover regulatory motifs underlying peaks of RA1 occupancy in proximal promoter regions by implementing the recently published Promzea pipeline (Liseron-Monfils et al. 2013)(www.promzea.org). Four significant motifs were discovered by this method (Figure 23A). The most significantly enriched motif was the GAGA motif, which was most frequently found in the geographic center of the peak (Figure 23B). We also detected the ID1-like binding site characterized by Kozaki et al. (2004), which was frequently displaced to the flanks of the peak (Figure 23B), as well as CAG-box and TGTG motifs.

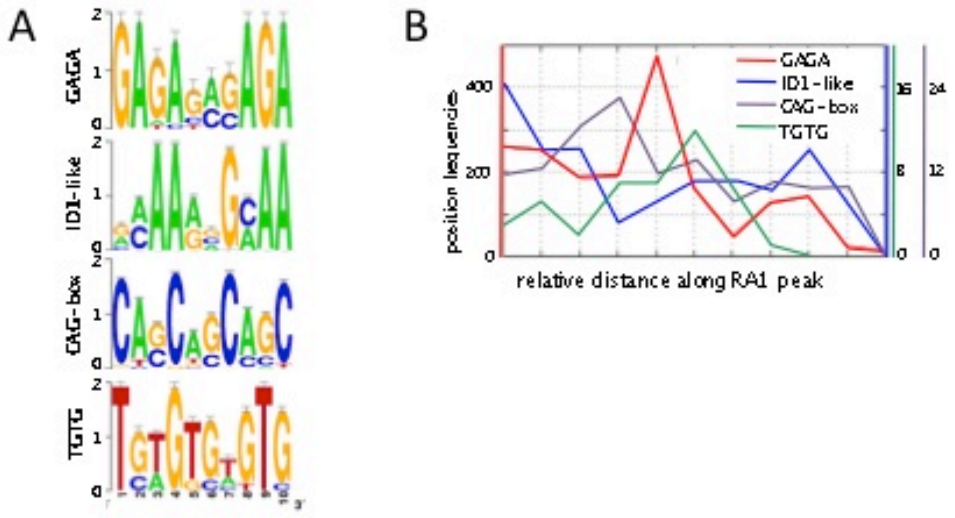


Figure 23: Significantly enriched DNA motifs underlying RA1 binding peaks. Promzea (Liseron-Monfils et al. 2013) was implemented to detect significantly enriched regulatory motifs associated with RA1 occupancy. Position-weight matrices for four regulatory motifs (A). The relative distance along the RA1 peak is shown for each of the four co-regulatory motifs (B). The GAGA motif was the most centrally located.

4.4 Discussion

4.4.1 Distribution of RA1 binding sites in the genome

In this study, we catalogued the genome-wide binding profile for a maize transcription factor that regulates inflorescence branching, a potentially important yield trait. We found that RA1 binds to thousands of sites in a variety of genomic contexts, consistent with recently published ChIP-seq studies in different model organisms (MacQuarrie et al. 2011). The majority of high confidence RA1 peaks were found in intergenic regions, as opposed to proximal promoters. It is difficult to determine which genes may be targeted by long-range interactions due to these binding events (Smallwood and Ren 2013). Another surprising result was that 7% of predicted peaks fell in protein coding exons, much higher than expected based on published studies. However, a recent ChIP-seq study found convincing evidence of exonic enhancers (Birnbaum et al. 2012). The authors suggested that exons serve both as protein coding sequences and enhancers depending on cell type (Birnbaum et al. 2012). Alternatively, some of the exonic RA1 binding peaks may fall in introns, due to the uncertainty of shifting tags towards a predicted summit (Zhang et al. 2008).

4.4.2 Binding sites of RA1 in male and female inflorescences

Given the nearly identical developmental progression of tassel and ear structures, it is somewhat surprising that there was not higher overlap in targets between tassel and ear tissues. The lack of overlap likely represents technical and experimental variation rather than dramatic differences in RA1 function in tassels versus ears. This hypothesis is supported by similar differences between the pseudo-biological replicates using different epitope tags. Developmental differences between the ear and the tassel are limited to: 1) the presence of long branches in the tassel; 2) the sex determination pathways that are imposed later in inflorescence development; and 3) axis thickening in the ear (Bommert et al. 2005). Increased branching in the tassel relative to the ear is more likely to result from delayed expression of *ra1* than from major differences in

RA1 DNA-binding activity, as timing of *ra1* expression drives variation in branching architecture in different grass species (Vollbrecht et al. 2005).

4.4.3 RA1 as an activator and repressor of gene expression

This study erred on the side of caution in focusing on differentially expressed genes associated with peaks found in libraries derived from multiple tissues or epitope-tags. Several studies have found that as few as 1-10% of genes bound by a transcription factor are differentially regulated in the mutant (Farnham 2009). Targets that are bound but not differentially expressed may be subject to redundant transcriptional regulation or may differ under various environmental conditions (Bolduc et al. 2012; Farnham 2009). A transcription factor may always bind to the same site in the genome, but only have functional consequences in the presence of a co-factor in a certain cell type or condition. In our study, 22% of high confidence RA1 target genes were mis-regulated in the *ra1* mutant. This compares well to the KN1 ChIP-seq study where approximately 17.5 % of strongly bound genes were modulated in mutant tissues (Bolduc et al. 2012).

RNA-seq profiling revealed that genes could be either up and down regulated in the *ra1* mutant (Eveland et al, unpublished). However, in the absence of ChIP-seq data, it is not clear whether these genes represent indirect or direct targets of RA1. Considering both the RNA-seq and ChIP-seq experiments, the data argue for a model where RA1 both activates and represses transcription of target genes. A genome-wide occupancy map for the co-repressor REL2 would shed light on the question of whether the consequence of RA1 binding varies depending on co-factors. Profiling histone acetylation in *ra1* and wild type tissues may also clarify how RA1 represses or activates targets.

4.4.4 Modulation of hormone biosynthesis and response pathways

RA1 appears to bind to and modulate components of the GA biosynthesis and signaling pathways, possibly to impose fine-tuning of GA homeostasis. Hormones have long been

suspected to play a role regulating branching in the maize inflorescence. Exogenous application of giberellic acid (GA) decreases long tassel branch number, and applied GA is capable of suppressing the *ra1* mutant phenotype (Nickerson 1959). In addition, exogenously applied auxin has also been reported to reduce tassel branching (McSteen 2009; Nickerson 1959), but this is most likely due to a direct effect on axillary meristem initiation, rather than an effect on meristem determinacy *per se*. Local manipulation of GA levels represents a plausible explanation for the non-cell autonomous activity of RA1.

4.4.5 lg1 is a putative effector of the ra1 phenotype

If *lg1* is a *bona fide* effector of *ra1*, one might predict that the *lg1* mutant would suppress the *ra1* mutant. Preliminary data suggests that this is not the case, as branch number is not significantly different in *ra1* mutants and *lg1;ra1* mutants (Fang Bai, pers. comm). However, this can easily be explained by redundancy, which is plausible given the lack of *lg1* single mutant branching phenotype. Many examples reinforce the fact that genes without a mutant phenotype can nonetheless drive a phenotype when misexpressed. For example, single mutants in the HD-ZIP III class transcription factor *PHABULOSA (PHAB)* are aphenotypic, but overexpression of *PHAB* produces dramatic leaf polarity defects (Prigge et al. 2005). Ultimate proof that LG1 dictates inflorescence branching can be gained by ectopically driving LG1 expression in the ear through the use of a two-component trans-activation system (Moore et al. 1998). Our lab has a pRA3::Lhg4 driver line available for this purpose, and a pOp::LG1 responder line is under construction (Jackson and Sylvester, unpublished).

Identification of LG1 as a putative effector of RA1 is not entirely satisfying, as it does not address the non-cell autonomy of RA1 function. Similar to *ra1*, *lg1* is also expressed in a domain subtending the branch meristem, indicating LG1 may also control a non-cell autonomous signal. LG1 appeared to act cell autonomously in mosaic analyses, but this could vary by developmental context and co-factors (Becraft et al. 1990). Performing ChIP-seq with the peptide

antibody used in this study may identify key targets of LG1. It would be extremely informative to perform ChIP in multiple tissue types to dissect similarities and differences related to LG1 regulation of meristem determinacy and ligule specification. There are potentially interesting parallels between the regulation of meristem determinacy, rates of cell division in the ligule, and rates of cell division in the pulvinus. Interestingly, *liguleless2* (*lg2*), another factor involved in ligule specification, has a reduced tassel branching phenotype (Walsh and Freeling 1999), and genetic analysis of double mutants suggests that *lg1* and *lg2* function in a common pathway (Harper and Freeling 1996).

4.4.6 cis-acting motifs underlying RA1 binding peaks

The four motifs identified in this analysis may represent RA1 binding sites or sites of co-regulation involving RA1 and binding partners or antagonists. We restricted our analysis to high confidence peaks falling in proximal promoter regions. This very conservative approach is likely to minimize false positives. Identification of the GAGA motif serves as a validation, as it was previously associated with single zinc finger proteins (Pedone et al. 1996). Due to the central location of GAGA motifs relative to RA1 binding peaks, it is temping to speculate that they could represent the site of RA1 occupancy. However, it is not very practical to look for the co-occurrence of the AGT-core of the predicted RA1 binding site, as this tri-nucleotide motif occurs once every 64bp by chance. Future work could include gel shift experiments to confirm binding of RA1 to GAGA-containing oligonucleotides and simultaneously test the contribution of GAGA and AGT-containing motifs. Alternatively, the GAGA motif could represent the binding site of a binding partner of RA1. Studies in *Drosophila* have emphasized the importance of *cis*-regulatory modules that integrate inputs from multiple transcription factors to influence gene expression (Sandmann et al. 2006).

4.4.7 Future Directions

Future work will focus on qPCR validation of a subset of peaks, followed by ordering insertional mutants in confirmed target genes from publically available resources. Quantifying branching phenotypes in double mutant plants will be informative, as effectors of RA1 should suppress the *ra1* phenotype in the simplest case. The *cis*-acting motif analysis will be expanded to include all genomic contexts, not just proximal promoter regions. This will allow dissection of the triggers and consequences of RA1 binding throughout the genome. Newly discovered binding motifs can be validated by gel shift experiments.

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary

This body of research reflects an integrative approach to developmental genetics and genomics, with an underlying theme of moving from mutants to genes to networks.

In Chapter 3, we presented a detailed phenotypic and molecular characterization of *fea4*, a novel fasciated ear mutant of maize. *fea4* encodes a bZIP transcription factor with a dynamic expression pattern that suggests a role regulating differentiation in the PZ. Genetic analysis places *fea4* acting in parallel with the principal stem cell counting machinery in the meristem. We performed RNA-seq on immature *fea4* ear primordia, and found that FEA4 may activate the expression of other developmental regulators. ChIP-seq experiments using YFP-FEA4 are underway, and should elucidate the network of genes under the control of FEA4.

The direct transcriptional targets of the zinc finger protein RA1 were investigated in Chapter 4. Although ChIP-seq identified thousands of binding sites across the genome, we focused on peaks located near genes that were differentially expressed in the *ra1* mutant. By integrating the *ra1* transcriptome and RA1 cistrome, we accumulated evidence that RA1 is involved in both direct repression and activation of gene expression. We observed some evidence that RA1 regulates genes involved in giberellic acid biosynthesis and signaling, suggesting that RA1 may control a mobile hormone signal. We also identified the SBP-box transcription factor *liguleless1* as a potential effector protein. Finally, we characterized *cis*-regulatory motifs in the promoters of high confidence targets.

This chapter will provide an overview of some preliminary studies as well as future work, and attempt to contextualize results presented in previous chapters.

5.2 Connections between meristem size and meristem determinacy

As the title of this dissertation is “Meristem size and determinacy in maize,” it is worthwhile to probe for links between the processes controlling meristem size and those

controlling meristem determinacy. The inspiration for this line of investigation comes from the identification of two alleles of *fea4* in a screen for enhancers of the determinacy mutant *ra2*. This screen took advantage of the weak phenotype of the *ra2-R* mutant upon introgression into the A619 inbred line. Two putative enhancers, *rel*07-167* and *rel*09-5171* were identified on the basis of enhanced tassel branching and branched ear tips (Figure 24). After outcrossing to A619, a single mutant fasciated ear phenotype was evident in the F2 (Figure 24). Both putative enhancers showed non-complementation and allelism with the *fea4-ref* mutant. Subsequent sequencing revealed that these two mutants harbored lesions in the *fea4* gene.

We confirmed the genetic interaction between *fea4* and *ra2* by crossing *fea4-ref* (B73-4) to *ra2-R* (B73), which produced similar double mutant phenotypes. We carried out SEM analysis of *fea4;ra2* mutants to investigate the origin of the split ear tips, which is an emergent property not seen in either single mutant. Analysis of 1-3 mm ear primordia showed a progressive splitting of the inflorescence meristem, as opposed to an outgrowth of spikelet pair or branch meristems (Figure 25). Later in development, these split inflorescence meristems give rise to long branch-like structures, which have masculinized features (Figure 25). The degree of fasciation is greatly reduced in *fea4;ra2* double mutants; therefore, *ra2* partially suppresses the *fea4* phenotype. Furthermore, the split inflorescence phenotype may suggest a role in meristem cohesion for *ra2*. This is perhaps related to the early pulse of expression in the anlagen of the suppressed bracts, marking the positions of the incipient SPMs (Bortiri et al. 2006). Upon close inspection, tassel phenotypes appear additive. The *fea4;ra2* double mutant tassels have a thickened main rachis, give rise to many long branches, and also have many spikelet pairs and multimers borne on elongated pedicels.

Given the interesting genetic interaction between *fea4* and *ra2*, we subsequently analyzed *fea4;ra1* double mutants. Loss of function in *ra1* suppressed the fasciated ear phenotype of *fea4* in mature ears; however, more careful analysis revealed that *fea4* and *ra1* mutants were additive in early stages of development (Figure 26). *ra1* mutants also appear to suppress the ear fasciation

Figure 24: Genetic interaction between *fea4* and *ra2*. A putative enhancer of *ra2-R* (*rel*07-167*) has a single mutant fasciated ear phenotype after outcrossing. This mutant is allelic to *fea4-ref*. Photographs courtesy of Becky Weeks.

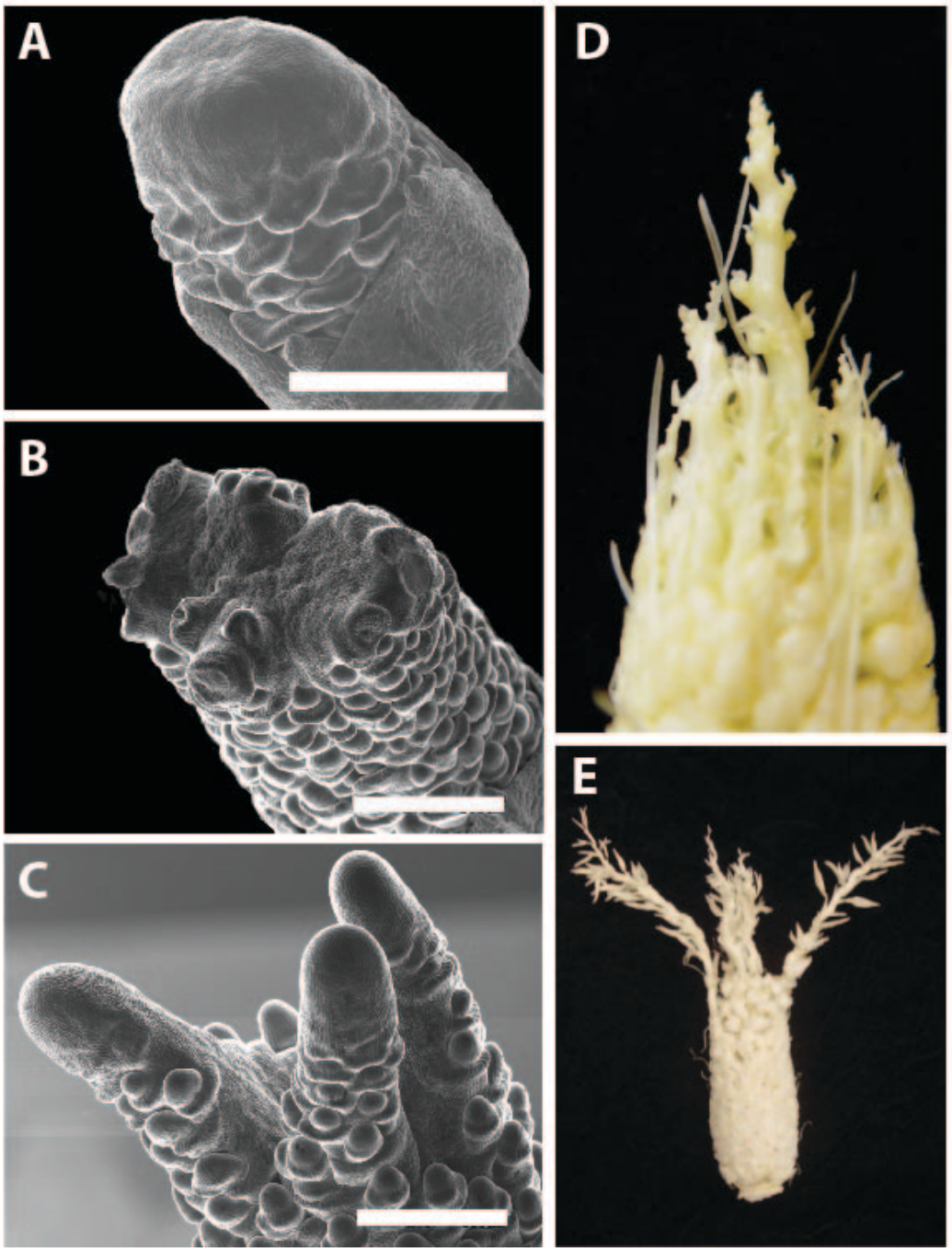

Figure 25: Developmental time course of inflorescence meristem splitting in *fea4;ra2* (A619) double mutants (A-E). The double mutant inflorescence meristem begins to split around the 2mm stage (B), leading to the creation of branch-like structures (C). At maturity, these branches are masculinized (D-E). Scale bars= 500um in A and B, 1 mm in C.

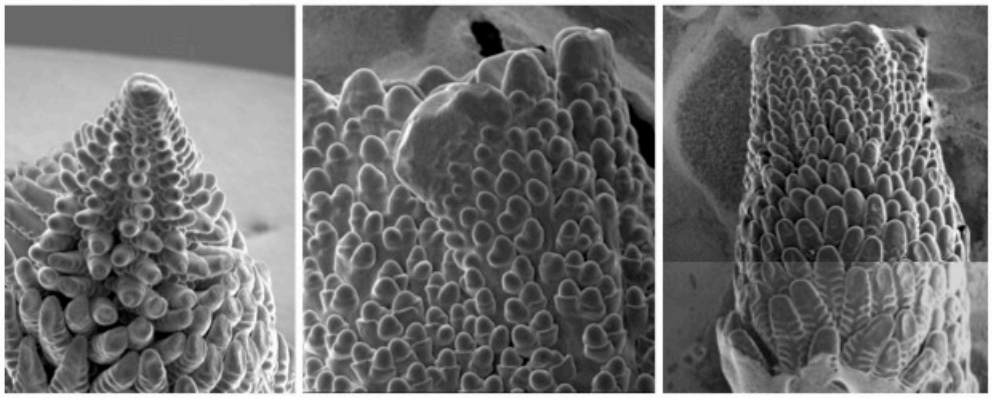

Figure 26: Genetic interaction between *fea4* and *ra1*. Although at maturity *ra1* appears to suppress fasciated ear mutants, SEM of early developmental stages reveals an additive genetic interaction.

phenotype of *fea2* and *td1* mutants at maturity (data not shown). One possible explanation is that the increased branching in the *ra1* mutant consumes energy and/or cells, leading to partial consumption of the meristem over time.

In light of the genetic interactions between *fea4* and the *ramosa* mutants, one might ask to what degree meristem size and meristem determinacy are coupled. Both determinate and indeterminate meristems must control the size of the meristem and balance stem cell self-renewal with the production of daughter cells. A proper balance between stem cell promoting and stem cell restricting factors is a basic pre-requisite of any functional meristem. Does adopting a determinate fate necessarily involve putting the brakes on stem cell promoting factors, such as WUS?

Floral meristem determinacy in *Arabidopsis* involves actively shutting down expression

of *WUS* to cause termination of the stem cell population (Lohmann et al. 2001). Floral meristem determinacy is a relatively simple problem to solve, because it involves consumption of the meristematic cells into floral organs. Determinacy of SPMs is somewhat more complicated, as it involves transitioning from one meristem type to another. This situation is specific to the Andropogoneae as other members of the grass family bear spikelets on indeterminate long branches. In maize, it is not clear what role the central meristem size regulators play, due to a lack of informative markers.

One common thread is that indeterminacy represents the default meristem state, and determinacy factors are layered on to enforce specific fates or transitions (Wakana et al. 2013). For example, the *ra* genes create determinate spikelet pairs as opposed to indeterminate branch meristems, and *branched silkless1 (bd1)* enforces SM determinacy (Chuck et al. 1998). In the absence of determinacy factors, as in the *bd1-Tu1* double mutant, indeterminate meristems grow unchecked, limited only by physiological constraints.

5.3 Natural variation and *fea4*

An emerging theme in plant developmental genetics is exploring natural variation in developmental processes (Alonso-Blanco et al. 2009). This can take the form of mapping quantitative trait loci (QTL) in phenotypically diverse lines, or introgressing mutants into diverse genetic backgrounds. Maize is an excellent system for dissecting natural variation due to the tremendous level of genomic diversity between inbred lines. Common inbred lines may have a nucleotide diversity rate greater than 1% (Tenaillon et al. 2001), and large amounts of presence-absence variation have been catalogued (Springer et al. 2009). In the course of this study, *fea4* was introgressed 4-5 times into several common inbred lines, including B73, A619, W22, and Mo17. Mutants displayed a range of severity with respect to inflorescence and vegetative phenotypes (Figure 27; Table 1 in Chapter 3).

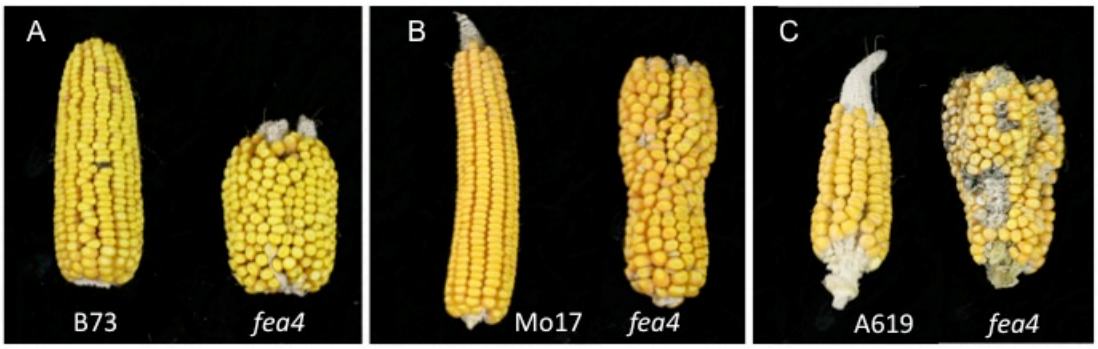

Figure 27: Natural variation in the *fea4* ear fasciation phenotype. Phenotypes of *fea4-ref/fea4-ref* ears next to *fea4-ref*/+ siblings upon 4-5 introgressions into various inbred backgrounds.

In addition, we performed a screen for naturally occurring modifiers by crossing *fea4-ref* (B73) to 22 diverse inbred lines, representing 22 out of 25 founding lines of the maize Nested Association Mapping (NAM) population. The concept of using the severity of a mutant phenotype as a visual reporter of gene function has been termed Mutant-Assisted Gene Identification and Characterization (MAGIC) (Chaikam et al. 2011).

100 F2 plants were screened for each *fea4* (B73) x NAM founder cross. The *fea4* phenotype was penetrant in all F2 families, as expected in the absence of a strong dominant suppressor. While the degree of ear fasciation varied between families, no strong ear enhancers were identified. For comparison, a similar screen for *fea2* modifiers produced one strong enhancer of ear fasciation (Bommert and Jackson, unpublished). Several families harbored weaker *fea4* inflorescence phenotypes, which could be explained either by a segregating suppressor, or by a dominant modifier. Genotyping F2 plants with weak phenotypes for *fea4-ref* can distinguish these possibilities.

fea4 mutants have essentially no vegetative abnormalities upon introgression into B73. In contrast, plants with strong vegetative defects such as dwarfism, crooked stems, and decussate

fea4 (NC350-enhanced)

Figure 28: Enhanced *fea4* vegetative phenotype segregating in F2 population derived from *fea4* (B73) and NC350. Enhanced plants display dwarfism, split stems, and aberrant phyllotaxy.

phyllotaxy segregate in an F2 population derived from crossing *fea4* (B73) and NC350 (Figure 28). In the winter 2013 field season, 13 out of 250 plants showed such an enhanced vegetative phenotype; this ratio closely approximates the expected segregation ratio of 1/16 for a single recessive enhancer locus. *fea4-ref* will be introgressed 4-5 times into the NC350 background for more careful phenotypic analysis, and to exclude the possibility that the enhanced phenotype is caused by interaction between the B73 and NC350 genomes.

Two pools were collected for bulked segregant analysis (BSA), including 13 strongly enhanced *fea4* mutants and 25 representative unenhanced *fea4* mutants. These pools were genotyped quantitatively with a set of 1016 markers on the Sequenom MassArray system (Liu et al. 2010), but no clear linkage was observed. Possible reasons for this include small sample size, insufficient phenotypic resolution, and lack of informative polymorphic markers to distinguish the B73 and NC350 genomes. Rough mapping will be repeated with larger pools of individuals restricted to the phenotypic extremes to maximize phenotypic resolution. Various bulk mapping techniques may be employed, including Sequenom MassArray, RNA-seq, or genotyping by sequencing (GBS) (Elshire et al. 2011; Liu et al. 2010; Liu et al. 2012). The enhancer can be fine mapped by generating CAPS or SSR markers from available genome sequence, or by crossing *fea4-ref* (B73) to the B73 x NC350 NAM Recombinant Inbred Lines (RILs)(Yu et al. 2008). Ultimately, positional cloning of the modifier based on the B73 reference genome may prove difficult due to presence-absence variation. This would traditionally be surmounted by construction of a NC350 Bacterial Artificial Chromosome (BAC) library, but this could perhaps be avoided by performing RNA-seq with *de novo* transcript assembly (Grabherr et al. 2011). To date, no modifiers have been successfully cloned by the MAGIC approach, although several strong QTL were detected for naturally occurring modifiers of the maize hypersensitivity response in one study (Chaikam et al. 2011).

The strong suppression of the *fea4* phenotype in the Mo17 inbred background provides another interesting natural variation case study (Figure 27). *fea4* (B73-4) plants have been

crossed to *fea4* (Mo17-5) plants to determine whether this suppression is dominant or recessive. Once this is established, the suppression can be mapped in F2 or F1BC1 mapping populations. Sequenom BSA may work well in this case, as the set of 1016 SNP markers were derived from B73-Mo17 polymorphisms (Liu et al. 2010). Fine mapping can leverage the Intermated B73 x Mo17 (IBM) RILs. One important question is whether *fea4* mutants have a weaker phenotype in Mo17 relative to B73 because Mo17 plants have smaller meristems than B73 plants. Previous work has demonstrated that natural variation in *fea2* expression probably explains much of the difference in meristem size and kernel row number between B73 and Mo17 (Bommert et al. 2013). It would be valuable to generalize the relationship between the severities of fasciated mutants in different inbred backgrounds with the normal meristem size in those inbred lines.

In general, the penetrance and expressivity of phenotypes is of great interest in many areas of biology, including agriculture and human medicine. Deleterious mutations or beneficial traits may be expressive in one genetic background, but completely suppressed in another. For example, the loss-of-function *kn1-e1* allele causes meristem termination in the W23 background, but only a small reduction in meristem size in B73 (Vollbrecht et al. 2000). One key question surrounds the genetic architecture of this phenomenon: can it be discretized into Mendelian loci or QTL, or does it reflect emergent properties of different genetic networks? Attempting to map naturally occurring enhancers and suppressors by the methods outlined above, and closely following segregation ratios, is likely to help answer this question.

5.4 Integrating *fea4* with the current state of meristem knowledge

Many key questions remain unanswered in the realm of meristem biology. The problem is exacerbated in maize due to a lack of knowledge about the basic components of meristem homeostasis. Not only do we lack function information about CLE peptides and *wus* homologs, but also the lack of visual markers for these genes limits characterization of other mutants. Forward genetic screens have identified nearly 50 fasciated ear mutants of maize, which await

molecular isolation (B. Je and D. Jackson, unpublished). We expect to clone the genes underlying these mutations in the coming years. However, genetic redundancy may prevent us from identifying all of the relevant players by forward genetics alone. Functional experiments, such as *in vivo* CLE peptide response assays, can help fill in the gaps (Kinoshita et al. 2007). This will help us achieve a deeper understanding of the full complement of CLE peptides and receptors active in different meristem types. Transgenic approaches, such as overexpression of candidate genes, may also be necessary. Two-component transactivation systems can be employed to circumvent the problem of manipulating genes that may be required for in *vitro* regeneration of transgenic plants.

An integrative view of meristem homeostasis takes into account many different layers of regulation. The *clv-wus* pathway is involved in an explicit stem cell counting function, which directly influences meristem size. KNOX genes, such as KN1 and STM, conspire with cytokinin to promote an undifferentiated meristematic environment in a positive feedback pathway (Sakamoto et al. 2006). Likewise, cytokinin and WUS also engage in a positive feedback pathway (Chickarmane et al. 2012; Gordon et al. 2009). Apically derived cytokinin signals activate expression of WUS, helping to position the WUS expression domain in the organizing center (Chickarmane et al. 2012). WUS directly represses type-A Response Regulators, which potentiates the cytokinin response (Leibfried et al. 2005). Zhao et al. (2010) demonstrated that type-A response regulators integrate cytokinin and auxin signals with the CLV-WUS pathway, at least in part through the activation of CLV3. The outputs of the pathways described above give rise to patterns of cell fate and cell division throughout the meristem. A small population of slowly dividing pluripotent stem cells, one to three per cell layer by some estimates, resides in the central zone (CZ) (Stewart and Derman, 1970). Rates of cell division are higher in the PZ, and these cells start to express markers of organ differentiation on a journey towards differentiated fate (Yadav et al. 2013).

Where does *fea4* fit into this integrated picture? FEA4 may act as a counterweight to the

meristem promoting factors WUS or KN1 by accelerating the differentiation of cells in PZ (Figure 29). It may accomplish this by activating genes associated with differentiation, possibly sharing targets with WUS, such as KANADI, ASYMMETRIC LEAVES2, and YABBY3 (Yadav et al. 2013). It will also be interesting to determine if FEA4 targets either WUS or KN1 in an antagonistic manner. KN1 appears to target FEA4 by binding in the promoter, approximately 100bp from the transcriptional start site (Bolduc et al. 2012)(N. Bolduc personal communication). We can directly test hypotheses regarding FEA4 targets with ChIP-seq using YFP-FEA4 transgenic lines. Comparing FEA4 binding profiles in the SAM versus inflorescence tissues may elucidate different roles for FEA4 in the CZ versus PZ.

fea4 may also affect rates of cell division in the PZ. This can be tested with *in situ* hybridization using markers of cell division, such as HISTONE4 and CYCLIN D. Alternatively, *fea4* may affect recruitment of founder cells into lateral organs, given the overlap with the *narrow sheath1/2* expression pattern (Nardmann et al. 2004). This could be responsible for the subtle changes in floral organ number and patterning in the *pan* mutant. Finally, *fea4* activity may alter the balance of cytokinin and auxin throughout the meristem. Profiling of *pan* inflorescences in Arabidopsis revealed that PAN regulates cytokinin response regulators and auxin biosynthesis genes in a direction that favours differentiation over meristem proliferation (Maier et al. 2011). No cytokinin response genes were differentially expressed in *fea4* RNA-seq profiling, but future profiling experiments may solidify this link.

Several lines of evidence suggest that *fea4* plays an important role in buffering meristem development. It will be exciting to elucidate a mechanism of action by continuing to integrate genetic and genomic analyses, as *fea4* represents an attractive target for manipulations that may ultimately improve crop yields and help feed a growing population.

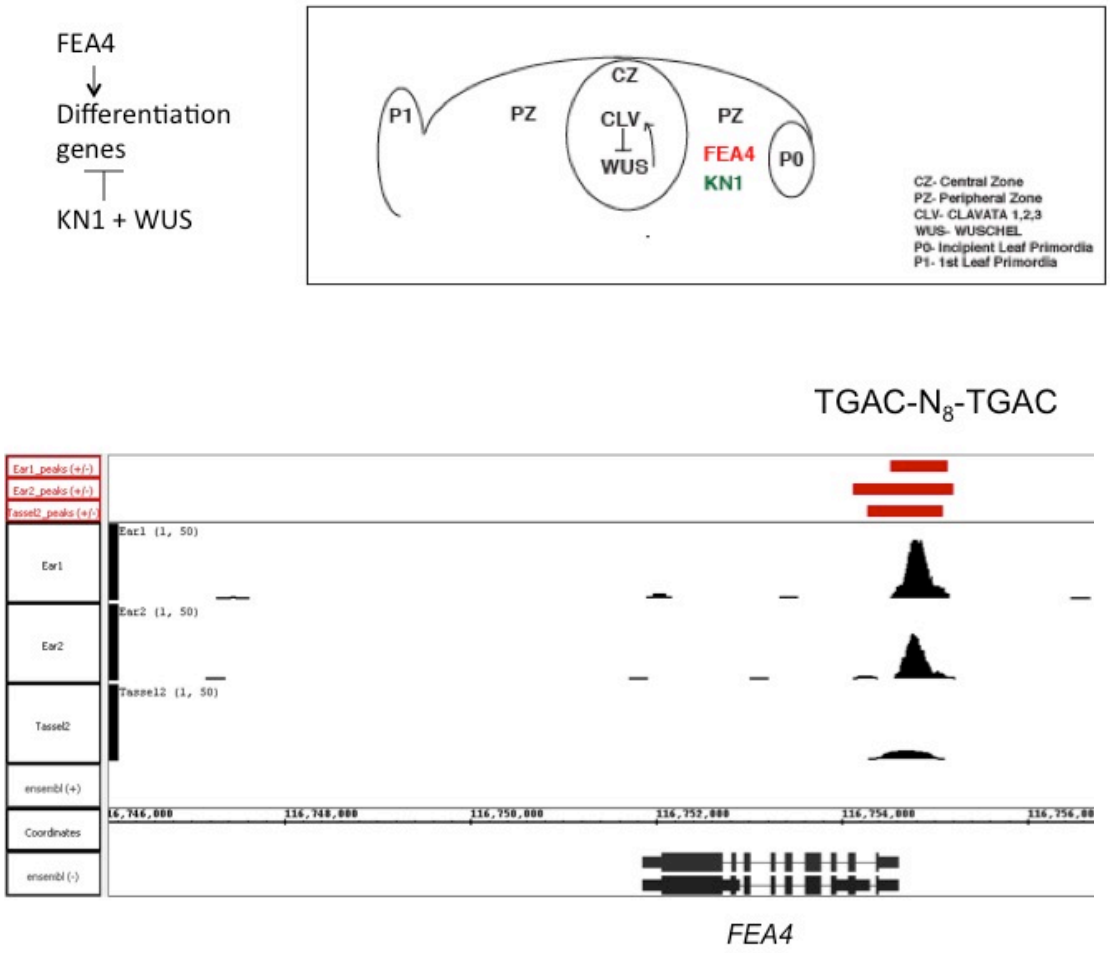

Figure 29: Model for FEA4 activity. FEA4 may act predominantly in the peripheral zone or throughout the meristem in opposition to the meristem-promoting factors WUS and KN1 (top). FEA4 may directly activate genes involved in organ differentiation, which are normally repressed by WUS (Yadav et al. 2013). This will be tested directly with ChIP-seq using YFP-FEA4 transgenic lines. FEA4 may act as an antagonist to KN1. The two genes have opposite loss-of-function phenotypes and KN1 binds to the FEA4 promoter just upstream of the transcriptional start site (bottom).

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