A MAIZE THIAMINE AUXOTROPH IS DEFECTIVE IN MERISTEM MAINTENANCE AND LEAF BLADE DEVELOPMENT

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A MAIZE THIAMINE AUXOTROPH IS DEFECTIVE IN MERISTEM MAINTENANCE AND LEAF BLADE DEVELOPMENT

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Plants undergo organogenesis throughout their life cycle via the perpetuation of stem cell pools called shoot meristems (SMs). SM maintenance requires the coordinated equilibrium between stem cell division and differentiation, and is feedback-regulated by integrated networks of gene expression, hormonal signaling, and metabolite sensing. Here I show that maize mutant bladekiller1 (blk1) is defective in meristem maintenance and leaf-blade development. The blk1 mutants exhibit a progressive reduction in SM size, resulting in premature shoot termination. Molecular markers for stem cell maintenance (including *knotted1*, *Zmwuschel1-1*, and *abphyll1*) and organ initiation (including *Zm*PIN1a~YFP and *narrow sheath1*) reveal that these meristematic functions are progressively compromised in mutant plants, especially in inflorescence and floral SMs. Positional cloning of the *blk1* mutation identified a predicted missense mutation in a highly-conserved amino acid encoded by the maize *thiamine biosynthesis2* (*thi2*) gene located on chromosome 3L. Consistent with chromosome dosage studies suggesting that *blk1* is a null allele of *thi2*, biochemical analyses confirm that the non-mutant THI2 enzyme co-purifies with a thiazole substrate whereas the mutant enzyme does not. A nearly identical paralog of *thi2*, named *thiamine biosynthesis1* (*thi1*) is located on chromosome 8. Although both paralogs are expressed ubiquitously, transcript accumulation of *thi2* is significantly more abundant than *thi1* in vegetative and inflorescence SMs. Heterologous expression studies reveal that THI2 is targeted to chloroplasts, providing additional

evidence for the synthesis of thiamine in plastids. All blk1 mutant phenotypes are rescued by exogenous thiamine supplementation, suggesting that blk1 is thiamine auxotroph. These data reveal that the inhibition of thiamine accumulation blocks the proliferative growth of stem cell populations in the maize shoot, and provide additional evidence for the integral role of carbohydrate metabolism and signaling during meristem development.

BIOGRAPHICAL SKETCH

John Woodward was born in St. Louis, Missouri to Patricia and Gary Woodward. He has two sisters, Genna Byerly and Kate Woodward, and one brother, Clayton Woodward. John graduated from McCluer High School, Florissant, MO in 2000. In May of 2004, John earned a Bachelor of Science degree in Biology from Truman State University, Kirksville, MO. In August of 2004, John entered the Ph.D. program in the Department of Plant Biology at the University of Georgia and joined Mike Scanlon's lab. He subsequently transferred with Mike to Cornell University in December of 2006. He will graduate with a Ph.D. degree in Plant Biology from Cornell University in May of 2010.

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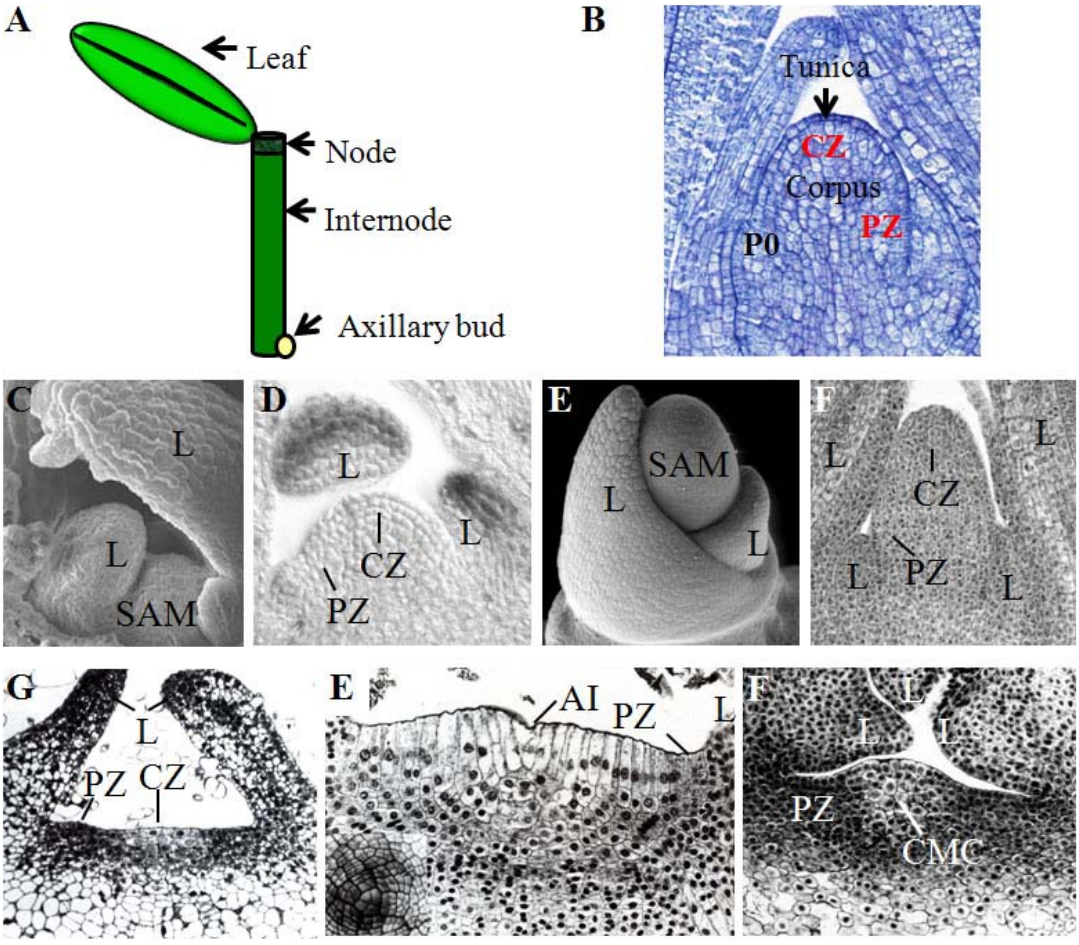
CHAPTER 1

Introduction

Introduction

The plant shoot comprises repeated base units called phytomers, wherein each segment contains a leaf, node, stem, and lateral bud (Figure 1.1A, Galinat, 1959). Phytomers are initiated from a group of self-renewing pluripotent cells known as the shoot apical meristem (SAM) (Figure 1.1B, reviewed by Steeves and Sussex, 1989). A survey of plant taxa reveals widespread variation in SAM size and shape, ranging from the small conical SAM found in *Arabidopsis* and *Gingko*, to the elongated SAM of maize and other grasses, and the flattened, nearly concave SAM seen in sunflower (Figure 1.1C-I). Despite these disparities in meristem morphology, multicellular plant shoot meristems share an ancestrally conserved histological zonation that is intrinsically linked to SAM function (Figure 1.1). The angiosperm SAM comprises a central zone (CZ), which is composed of slowly dividing stem cells that function in maintaining the meristem, and a surrounding peripheral zone (PZ), which contains smaller and more rapidly dividing cells that are recruited for lateral organ formation. The basis of the angiosperm SAM morphology is manifested in the evolutionary history of land plants. *Chara* is a group of aquatic multicellular green algae that is sister to land plants (Kenrick and Crane, 1997; Karol et al., 2001). During *Chara* development, a single apical meristematic cell divides periclinally to produce a basal derivative that will undergo additional divisions to form leaf-like lateral branches and stem-like filaments (Pickett-Heaps, 1967). Similarly, seedless plants such as ferns and *Equisetum* feature a prominent and enlarged apical-initial cell at the meristem summit that ultimately gives rise to all the cells in the shoot (Figure 1.1H, Gifford, 1983). Gymnosperms such as ginkgo and pine form a cluster of large, highly vacuolated cells near the SAM sub-surface called central mother cells, which provide cells to the surrounding peripheral zone that are then used for shoot development (Figure 1.1I, Johnson, 1951).

Figure 1.1: While SAM architecture varies, zonation is a shared feature of plant meristems
A. Phytomers, which comprise a leaf, node, internode, and axillary bud, are continuously generated from SAM to form the plant body. In maize (*Zea mays*) the axillary bud is found at the base of the internode that subtends the leaf.
B. The SAM is composed of two functional zones, the central zone (CZ) and the peripheral zone (PZ). Leaf founder cells (P0) are recruited from the PZ. Stratification of the SAM into the tunica and corpus is common feature of angiosperms. The maize SAM (shown) has a single layered tunica, which covers the multi-layered corpus.
C-D. The SAM of the eudicot *Arabidopsis* is much smaller the tall domed SAM of maize (**E-F.**)
C. SEM of the *Arabidopsis* SAM with two leaf primordia (L)
D. The *Arabidopsis* SAM has a two-layered tunica as seen in a longitudinal section
E. SEM of the maize SAM with two leaf primordia
F. Longitudinal section of the maize SAM
G. Some SAMs are flattened such as that of the sunflower (*Helianthus annuus*)
H. The fern (*Osmunda cinnamomea*) SAM has a single apical initial (AI). Inset: a top view of the fern SAM showing the AI and its recent derivatives.
I. Gymnosperms, such as *Ginko biloba* (shown), have a core of central mother cells(CMC), which provide cells to the PZ


A
Leaf
Node
Internode
Axillary bud
B
Tunica
CZ
Corpus
PZ
P0
C
L
L
SAM
D
L
L
CZ
PZ
E
SAM
L
L
F
L
L
CZ
L
PZ
L
G
L
PZ
CZ
E
AI
PZ
L
F
L
L
L
PZ
CMC

The SAM of angiosperms and some gymnosperms is further patterned by distinct layers into a tunica-corpus hierarchy (Figure 1.1B, 1.1D; Schmidt, 1924). The tunica forms one or more peripheral layers with cells that primarily divide anticlinally to create a sheet of cells of identical ancestry. The inner corpus, on the other hand, contains cells which divide in all planes to generate the SAM body. Generally, the outermost layer of the tunica, or L1, provides cells for the epidermis of plant organs, while internal layers and the corpus contribute to inner tissue.

In order to maintain SAM function, the cells that are recruited from the PZ for organogenesis must be replaced by cells propagated from the CZ. Failure to perpetuate the stem cell pool results in SAM consumption and the termination of plant development. Conversely, overproduction of stem cells leads to meristem bifurcation, altered phyllotaxy, and increased organ number. Thus, an equilibrium between stem cell division and differentiation is coordinated by integrated networks of transcription regulation, metabolite sensing, and hormone signaling (Figure 1.2; reviewed by Barton, 2009). The following dissertation introduction describes analyses performed on the angiosperm model organisms *Arabidopsis thaliana* and *Zea mays* to provide a review of shoot meristem structure and function and a summary of thiamine biosynthesis and function during plant development.

WUS/CLV Feedback network

The homeobox gene *wuschel* (*wus*) is the earliest described regulator of stem cell activity in *Arabidopsis*. Expression of *wus* first occurs in the center of the 16-cell stage embryo, and is then gradually restricted during development to a presumptive SAM organizing center (OC) that lies just beneath the central zone (Lenhard and Laux 2003; Mayer et al. 1998). Loss of WUS function in the OC results in a failure to propagate stem cells in the CZ above (Laux et al.,1996), suggesting that *wus* functions

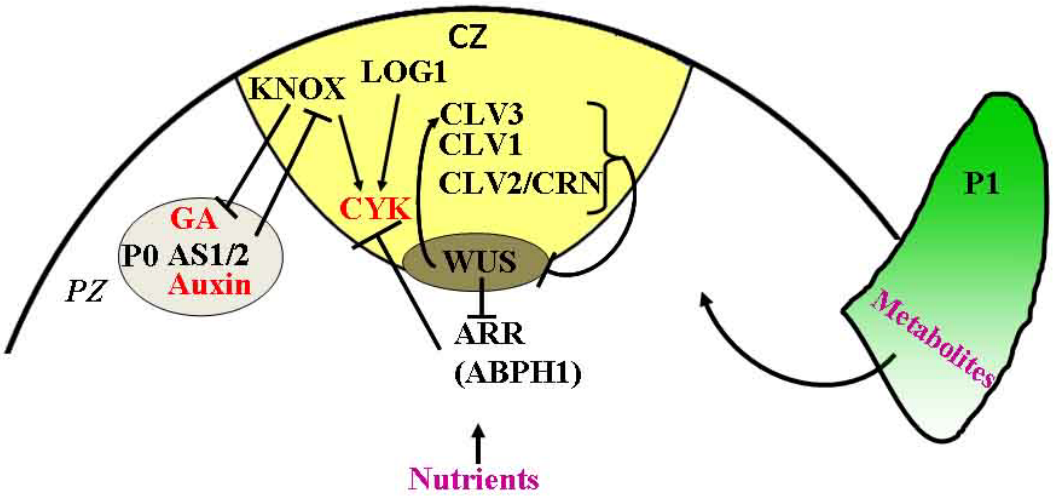


Figure 1.2: The two essential functions of the SAM, self-renewal and lateral organ formation, are tightly regulated by integrated networks of transcriptional regulation, hormone signaling, and resource utilization. Terms: gibberellin (GA), cytokinin (CYK), LONELY GUY1, CLAVATA1-3 (CLV1-3), CORYNE (CRN), WUSCHEL (WUS), KNOTTED1-LIKE HOMEOBOX (KNOX) factors, ASSYMETRIC LEAVES1 and ASSYMETRIC LEAVES2 (AS1/2), ARABIDOPSIS RESPONSE REGULATOR (ARR)

non-cell autonomously to maintain the organization of the SAM (Lenhard and Laux 2003; Mayer et al. 1998).

WUS activity induces the expression of *clavata3* (*clv*3), a negative regulator of stem cell proliferation (Figure 1.2; Fletcher et al., 1999; Brand et al., 2000; Schoof et al., 2000). The CLV3 protein is cleaved into a secreted 13 amino acid peptide that functions in a signaling cascade to restrict *wus* expression (Ogawa et al., 2008; Ohyama et al., 2009; Brand et al., 2000). Thus, *wus* and *clv*3 work in a negative feedback loop that precisely controls stem cell activity. The LLR receptor kinase CLAVATA1 (CLV1) is expressed within the SAM (Clark et al., 1997) and has been shown to directly bind the CLV3 peptide ligand (Ogawa et al., 2008; Ohyama et al., 2009). CLAVATA2 (CLV2) is similar in structure to CLV1, but lacks a cytoplasmic kinase domain (Jeong et al., 1999). CLV2 putatively interacts with CORYNE (CRN), a transmembrane domain containing protein with an intercellular kinase (Müller et al., 2008). It has been proposed that CLV2 and CRN form a receptor complex that acts in parallel with CLV1 homodimers in transmitting the CLV3 signal (Müller et al., 2008). Using real-time imaging techniques, Reddy and Meyerowitz (2005) found that the CLV3 signaling system is necessary for specification of the central zone boundaries. Accordingly, mutations to any of the *clv* loci or to *crn* result in over-proliferation of stem cells within both the SAM and floral meristems, and lead to extra floral organs and club-shaped siliques (Clark et al. 1993, 1995; Kayes and Clark, 1998; Müller et al., 2008).

Cytokinins and meristem maintenance

Shoot meristem maintenance also requires the precise regulation of a high cytokinin to low gibberellin and auxin hormone regime. This ratio is partially maintained by class I *knotted1-like homeobox* (*knox*) genes. The founding member of

the *knox* gene family, the maize *knotted1* (*kn1*) gene and its homologue in *Arabidopsis*, *shootmeristemless* (*stm*), are expressed throughout the meristem but are downregulated at organ initiation sites (Jackson et al. 1994; Long et al. 1996). Loss-of-function stm mutants have a drastically reduced meristem that is completely consumed following the initiation of cotyledons (Barton and Poethig, 1993). Likewise, kn1 mutants have a limited shoot phenotype in certain genetic backgrounds, which results in early stage seedling abortion (Vollbrecht et al., 2000). KN1 functions to maintain the SAM by preventing gibberellin accumulation through direct activation of the gibberellin catabolism gene *ga-2-oxidase1* (Buldoc and Hake, 2009). STM is similarly correlated with gibberellin repression as STM overexpression results in lower mRNA accumulation of the gibberellin biosynthetic gene *ga20ox1* (Hay et al., 2002). STM upregulation also induces *isopentyltransferase7*, a cytokinin biosynthesis gene, and cytokinin supplementation partially rescues stm mutant phenotypes. Furthermore, cytokinin levels increase in response to misexpression of the maize KN1 in tobacco leaves (Ori et al., 1999). These data, therefore, suggest that KNOX proteins simultaneously prevent stem cell differentiation through the repression of gibberellin activity and promote cell division via stimulation of cytokinin biosynthesis.

The phosphoribohydrolase LONELYGUY1 (LOG1) further controls cytokinin activity at the SAM apex by converting inactive cytokinin nucleotides to their active free base forms (Kurakawa et al., 2007). The log1 mutants have a smaller SAM and generate fewer floral organs than wild type siblings, emphasizing the necessity for localized cytokinin regulation during meristem maintenance (Kurakawa et al., 2007).

In plants, cytokinin is perceived at the plasma membrane by histidine kinase receptors (HKs), which then act to phosphorylate histidine phosphotransfer proteins (HP) that in turn activate cytokinin response regulators in the nucleus (Figure 1.3; reviewed by Müller and Sheen, 2007). Type-B response regulators (type-B rr) induce

transcriptional responses, including the upregulation of type-A response regulators (type-A rr) that act to dampen the cytokinin signal. Studies of the maize gene *aberrant phyllotaxy1* (*abph1*), which encodes a type-A RR, reveal that this feedback regulation is necessary for meristem size maintenance (Giulini et al. 2004). Mutations to *abph1* confer an increase in SAM size and altered phyllotaxy (Jackson and Hake, 1999), which is accompanied by increased levels of cytokinin intermediates and decreased levels of auxin (Lee and Johnston et al., 2009). Interestingly, WUS fine-tunes cytokinin responses in *Arabidopsis* meristems by direct transcriptional repression of *type-A Arabidopsis rr*s (*arr5*, *arr6*, *arr7*, and *arr15*) (Leibfried et al. 2005).

Organogenesis and leaf development

Plant phyllotaxy, or the spatial arrangement of leaves about the shoot axis, is determined by regulatory control of organ initiation events at the SAM PZ. The time elapsed between successive initiation events is called the plastochron, wherein the leaf anlage is designated as plastochron 0 (P0) (Figure 1.1). Prior to the histological indication of organogenesis, major shifts in transcriptional regulation and hormone signaling cue the developmental switch from indeterminate meristematic cells to determinate leaf founder cells. Using laser-microdissection coupled with microarray analysis, Brooks and Strable et al. (2009) found over 900 differentially regulated genes between the maize SAM proper and initiating leaves (P0/P1). Genes specifically upregulated during organogenesis included those involved in division/growth, cell wall biosynthesis, chromatin remodeling, RNA binding, and translation.

The earliest molecular marker of leaf initiation is the generation of an auxin maximum directed by the upregulation and polar localization of the auxin efflux carrier PINFORMED1 (PIN1) in the PZ (Reinhardt et al. 2000, 2003; Heisler et al.,

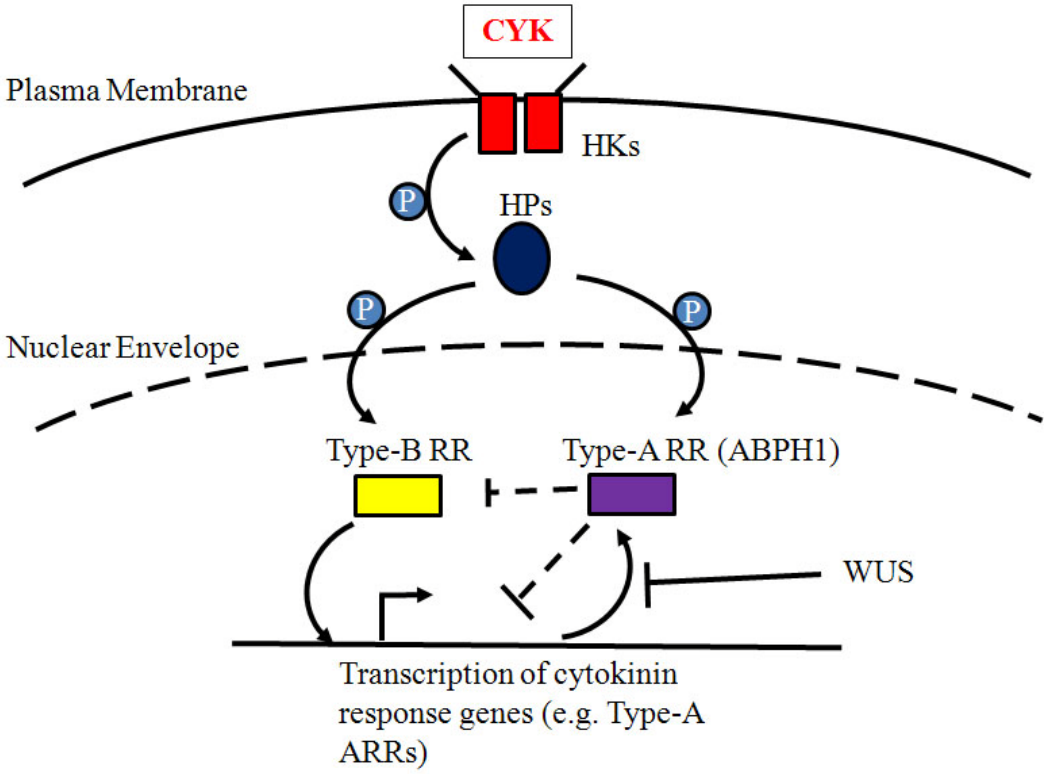


Figure 1.3: Cytokinin signaling in plants. Cytokinin reception by histidine kinase (HK) receptors generates a phosphate-transfer relay from histidine phosphotransfer proteins (HPs) to response regulators (Type-B and Type-A RR) in the nucleus. WUSCHEL (WUS) negatively regulates *type-A rr* suppressors of cytokinin signaling.

2005; Bayer et al., 2009). Auxin is both necessary and sufficient for organogenesis. Disruption of auxin transport with N-1-naphthylphthalamic acid (NPA) blocks new leaf initiation (Reinhardt et al. 2000; Scanlon, 2003), while local application of auxin to the SAM PZ induces new leaf formation (Reinhardt et al. 2000). Auxin functions alongside a complex composed of the MYB-domain containing protein ASSYMETRIC LEAVES1 (AS1) and the lateral organ boundary-domain protein ASSYMETRIC LEAVES2 (AS2) to repress *knox* gene expression (Figure 1.2; Guo et al., 2008; Hay et al., 2006). Although ectopic *knox* expression is observed in as1 mutant leaf primordia, *knox* downregulation proceeds normally at the P0 of mutant shoot apices (Byrne et al., 2002). These data indicate that initial *knox* downregulation in the incipient leaf is auxin dependent (Hay et al., 2006), whereas AS1 is required to maintain *knox* downregulation later in primordial development (Byrne et al., 2002; Timmermans et al., 1999; Tsiantis et al., 1999). Interestingly, STM functions to repress AS1 activity in SAM, thus creating a mutual repressing system that helps maintain the SAM/P0 boundary (Byrne et al., 2002).

Following leaf initiation, asymmetric cell division, expansion and differentiation occur along three developmental axes to give rise to the proximodistal (base-tip), mediolateral (midrib-margin), and adaxial/abaxial (Top/bottom) planes of the mature leaf (Figure 1.4A; reviewed by Hudson, 2000; Byrne, 2001). Adaxial/abaxial patterning in *Arabidopsis* is largely established by miRNA-mediated juxtaposition of the adaxial-promoting HD-ZIPIII transcription factors, REVOLUTA (REV), PHABULOSA (PHAB), and PHAVOLUTA (PHAV), and the abaxial-promoting *kanadi* (*kan*1-4) genes (Figure 1.4B). Intriguingly, phab; rev; phav triple mutants (Emery et al., 2003) and *kanadi1* overexpression lines (Eshed et al., 2001) have abaxialized leaves and fail to maintain the SAM. Conversely, loss of KANADI function (Eshed et al. 2001, 2004) or ectopic expression of HD-ZIPIIIs results in

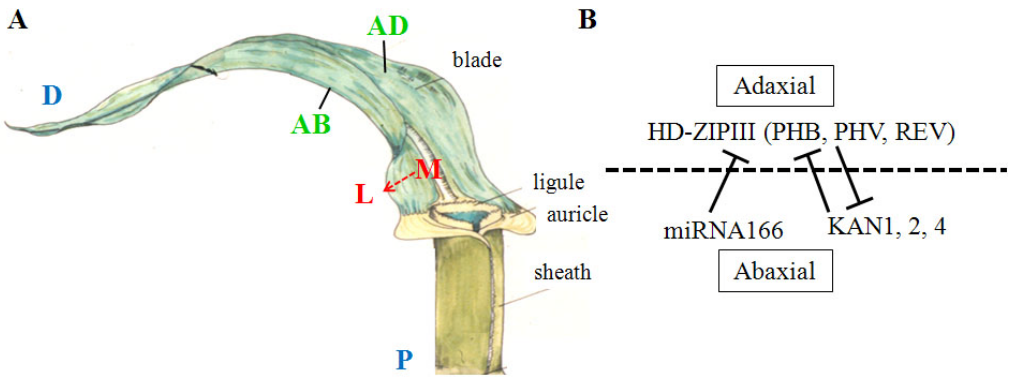


Figure 1.4: Leaf primordia develop along three axes to form leaves with distinct morphological domains.
A. The maize leaf is subdivided into the proximal (P) sheath and distal (D) blade by a fringe of epidermal tissue called the ligule and two wedge-like auricles. The mediolateral (M/L) axis extends from the midrib to margin. The adaxial (AD)/abaxial (AB) axis defines the top and bottom of the leaf respectively. Drawing by Mark Mooney.
B. Adaxial/abaxial leaf identity is determined by antagonistic interactions among the abaxial-promoting miRNA166 and KANADIs (KAN1,2, and 4) and the adaxial-promoting HD-ZIPIIIs PHABULOSA (PHB), PHAVOLUTA (PHV), REVOLUTA (REV).

adaxialized leaves and ectopic meristem formation (McConnell et al. 2001; Emery et al. 2003). These results demonstrate the interdependence of leaf adaxial identity and SAM function (Emery et al., 2003). Mediolateral patterning is partially dependent on the activity of WUSCHEL-LIKE HOMEOBOX (WOX) transcription factors. In maize, the duplicate *wox* genes *narrow sheath1 (ns1)* and *narrow sheath2 (ns2)* function non-cell autonomously from two foci within the meristem PZ to recruit cells that give rise to the marginal domains of the mature leaf (Scanlon, 1996; Nardmann and Ji, 2004). The *Petunia wox* gene *maewest* and the *Arabidopsis wox1* and *prs* paralogues confer similar functions in lateral outgrowth of the leaf (Vandenbussche et al. 2009)**.** The maize leaf is subdivided along the proximodistal axis by the proximal sheath and distal blade; these domains are separated by a conspicuous set of cells that make up the ligular region (Figure 1.4A). Disruption of the proximodistal axis occurs in the maize liguleless1(lg1) and liguleless2 (lg2) mutant backgrounds (reviewed in Freeling, 1992; Hake and Ori, 2002). In the absence of LG1 or LG2 function, the ligular boundary is not formed and a distal migration of the sheath tissue occurs (Emerson, 1912; Brink, 1933). The *knox* genes play particularly important roles in proximodistal patterning as gain-of-function Knox mutants in maize exhibit ectopic knots, and sheath and ligule characteristics within the leaf blade (Sinha and Hake, 1990 ; Becraft and Freeling, 1994 ; Schneeberger et al., 1995 ; Fowler et al., 1996 ; Foster et al., 1999). While these mutants have disproportionate blade to sheath ratios, no mutants have yet been described that exhibit a specific domain loss along the proximodistal axis

Metabolites and the SAM

SAM maintenance depends on the import of nutrients and metabolites from external sources (Figure 1.2). This point was clearly demonstrated in early work with

plant tissue culture, which revealed that meristematic activity requires supplemental minerals, vitamins, and sucrose (reviewed by White, 1951). Sucrose is particularly important for cell-cycle progression. Application of sucrose to *Arabidopsis* cells in suspension induces the expression of D-cyclins, cell-cycle regulators that function during the G1-S phase transition (Riou-Khamlichi et al., 2000). Interestingly, Pien et al. (2001) found that three genes encoding sucrose synthase, ADP-glucose pyrophosphorylase, and a SNF1-like kinase have specific expression patterns in the tomato SAM, suggesting that precise regulation of carbohydrate metabolism is correlated with meristem function. Studies of the *Arabidopsis* WOX transcription factor STIMPY also highlight the importance of sugar metabolism in meristem maintenance (Wu et al., 2005). External application of sucrose fully rescues the seedling lethal, stimpy mutant phenotype. Similarly, analyses of maize *ramosa3*, encoding a trehalose-6-phosphate phosphatase, revealed that determinacy within axillary meristems is dependent upon the production of a putatively mobile trehalose signal (Satoh-Nagasawa et al., 2006). Although these studies have provided some insight on the metabolic requirements of shoot meristems, additional research is needed to understand the mechanisms by which imported resources are incorporated into known pathways for meristem stability and function.

Thiamine and Plant Development

The phosphorylated derivative of the B-vitamin thiamine, thiamine-pyrophosphate (TPP), is a vital cofactor for enzymes that catalyze various carbohydrate metabolic pathways including the Calvin cycle, glycolysis, and non-oxidative pentose phosphate pathways (Friedrich, 1987). In plants, thiamine is implicated in both developmental and stress-related responses. Thiamine concentrations increase following salt stress, osmotic damage, and infection (Ahn et

al, 2005; Rapala-Kozik et al., 2008; Tunc-Ozdemir et al., 2009). Moreover, supplementing plants with exogenous thiamin increases tolerance to both salt stress and paraquat-induced oxidative damage (Sayed and Gadallah 2002; Tunc-Ozdemir et al., 2009). Auxotrophs demonstrate that thiamine is required for plant viability. Generally, thiamine auxotrophic mutants have metabolic defects, and seedlings die after the production of several albino leaves (Langridge, 1955; Feenstra, 1964; Redei, 1965; Li and Redei, 1969; Koornneef and Hanhart, 1981). Meristems have specific requirements for thiamine import as demonstrated by work with tissue culture. Wightman and Brown (1953) found that root tips required thiamine supplementation for meristematic growth in culture.

Thiamine is formed from the condensation of a substituted pyrimidine (2-methyl-4-amino-5-hydroxymethylpyrimidine phosphate; HMP-P) and a substituted thiazole (4-methyl-5-β-hydroxyethylthiazole phosphate; HET-P) using the enzyme 2-methyl-4-amino-5-hydroxymethylpyrimidine phosphate kinase/ thiamine-monophosphate pyrophosphorylase (HMPPK/TMPPase, or *At*TH1 in *Arabidopsis* and *Zm*THI3 in *Zea mays*) (Figure 1.5; reviewed by Roje, 2007 and Jurgenson et al., 2009). The enzyme THIC is implicated in the synthesis of the pyrimidine moiety from the precursor 5-aminoimidazole ribotide, AIR (Kong et al., 2008). HET-P is generated from glycine, cysteine, and nicotinamide adenine dinucleotide (NAD^+) using a thiazole synthase (White and Spenser, 1979a, 1979b, 1982; Praekelt et al., 1994).

Thiazole Synthase

A number of thiazole biosynthetic genes have been described, including the *Arabidopsis-Atthi1* (Papini-Terzi et al., 2003), *Saccharomyces cerevisiae-Scthi4* (Machado, 1997), and maize-*Zmthi1* and *Zmthi2* (Belanger et al. 1995). Crystallization

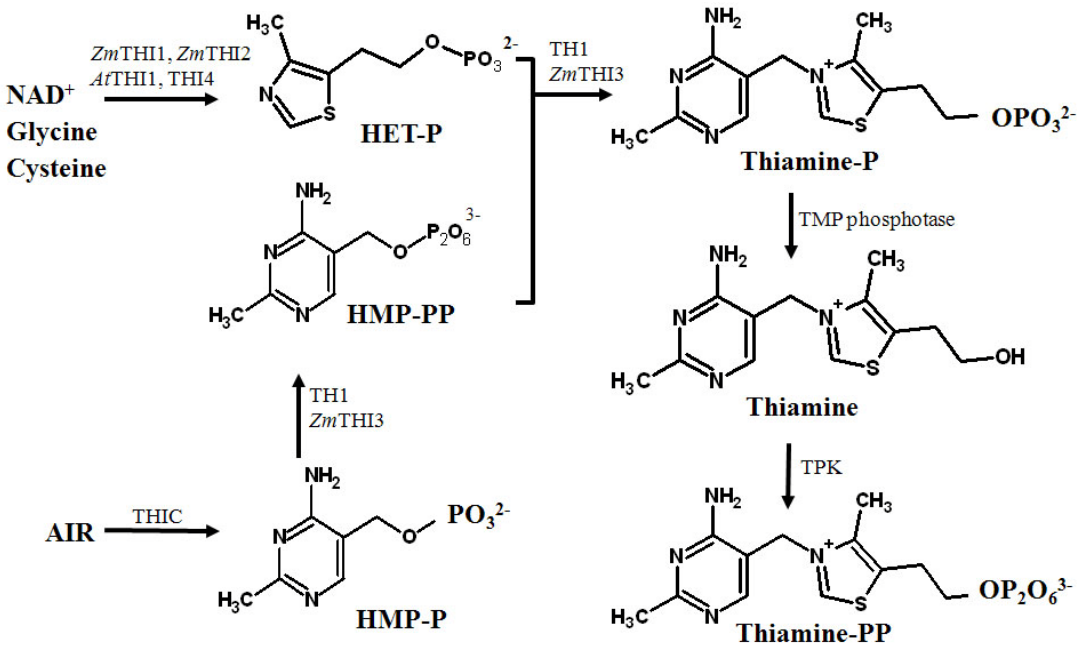


Figure 1.5: The proposed scheme for thiamine biosynthesis in *Arabidopsis* (*At*) and *Zea mays* (*Zm*) is shown. Biosynthesis of thiamine requires the condensation of thiazole (HET-P) and pyrimidine precursors (HMP-P).

of the *Sc*THI4 and *At*THI1 proteins reveal that they function as homooctomers, with each subunit containing a non-canonical NAD-binding domain that may have subfunctionalized from a FAD-binding site (Godoi et al., 2006; Jurgenson et al., 2006). The *At*THI1 and *Sc*THI4 proteins copurify with intermediates in the thiazole biosynthetic pathway, verifying their function in thiazole biosynthesis (Godoi et al., 2006; Jurgenson et al., 2006). Intriguingly, the *Arabidopsis thi1* gene was first recognized for its ability to partially repair DNA base-pair excision pathways in bacterial mutants (Machado et al., 1996). It was later shown that both the yeast *Scthi4* and *Atthi1* can rescue mitochondrial DNA instability conditioned in the *Sc*thi4 mutant background (Machado et al., 1996, 1997). While these results suggest a dual role for *thi1* homologues in thiamine biosynthesis and mitochondrial DNA stability, only the thiamine synthesis function has been demonstrated in plants.

The N-termini of many plant THI1-like proteins contain a plastid transit peptide that is followed by a mitochondria targeting pre-sequence, suggesting that these proteins are dual-targeted. Concordantly, chloroplast and mitochondrial specific isoforms of the *Arabidopsis* homologue, *At*THI1, are formed from two in-frame translation start codons, and truncated versions of these proteins localize to both mitochondria and chloroplasts in tobacco protoplasts (Chabregas et al., 2001, 2003).

Expression of the *thi* genes is developmentally regulated. The maize *thi1* and *thi2* transcripts accumulate highly in maize embryos, tassel primorida, and mature green leaves, but accumulate to lower levels in roots and endosperm (Belanger et al., 1995). Similarly, the *Arabidopsis thi1* accumulates in leaves, but is downregulated in roots (Papini-terzi et al., 2003). The *Citrus sinensis thi1* homologue is implicated in fruit ripening, as its expression increases during fruit maturation (Jacob-Wilk et al., 1997). Interestingly, brassinosteroid treatment of *Arabidopsis* plants decreases *thi1*

transcript and protein accumulation, suggesting that *thi1* is partially regulated by hormone signaling (Deng et al., 2007).

In *Arabidopsis, At*thi1 mutants produce green cotyledons; however, all true leaves are albino and plants die at the seedling stage. Similarly, thi mutants of *Nicotiana sylvestris* produce chlorotic leaves with severe disruption of plastid differentiation (McHale et al. 1990). Curiously, transgenic rice plants with RNA-interference mediated transcript reduction of the *thi1* homologue, *disease-responsive-gene8* (OsDR8) are viable, but have increased susceptibility to pathogens (Wang and Ding et al. 2006). All of the defects mentioned above are rescued with thiamine supplementation, suggesting that thiamine deficiency is the cause of the thiazole synthase mutant phenotypes in plants.

Purpose of study

In general, the mechanisms underlying meristem maintenance are conserved among well-studied monocot and eudicot model organisms. As mentioned above, *kn1* and *stm* confer similar functions in promoting an undifferentiated stem cell population. Furthermore, the putative maize orthologues of CLV1 and CLV2, *thick tassel dwarf1* (*td1*) and *fasciated ear2* (*fea2*) respectively, have conserved roles in restricting the size of the inflorescence and floral meristems (Bommert et al., 2005; Taguchi-Shiobara et al., 2001). Despite this general trend, a number of genes implicated in meristem function in *Arabidopsis* have non-homologous or redundant functions in maize. For example, *td1* is expressed within leaf primordia and on the flanks of the vegetative SAM, whereas the *Arabidopsis* ortholog *clv1* is expressed in the SAM CZ (Bommert et al. 2005). Accordingly, td1 mutants do not exhibit SAM over-proliferation phenotypes (Bommert et al. 2005). Moreover, recessive mutations to the maize *arr7* ortholog *abph1* confer SAM enlargement and altered phyllotaxy (Jackson and Hake,

1999), while sextuple arr mutants in *Arabidopsis* have no obvious mutant phenotypes (To et al., 2004).

Therefore, it is evident that the factors dictating meristem fate in plants cannot be uncovered using a single model system such as *Arabidopsis*. The primary goal of this research dissertation was to further investigate mechanisms of meristem maintenance in maize using the mutant bladekiller1 (blk1). Shoot meristems of blk1 mutant plants exhibit a progressive deficiency in stem cell maintenance and undergo premature abortion. Intriguingly, blk1 leaves are bladeless in upper adult nodes. Such a disruption in proximodistal leaf patterning has not yet been described in maize. Positional cloning revealed that the *blk1* reference mutation is in the *thiamine biosynthesis2* (*thi2*) gene, revealing a precise developmental role for thiamine during meristem maintenance in maize.

Chapter 2 is formatted for submission to *The Plant Cell* and contains a short introduction on meristem biology, data generated during the characterization of blk1, discussion of relevant findings, and a description of the materials and methods used for generating the data. Chapter 3 highlights the significance of this research and suggests future research directions. All of the work was completed by John Woodward under the advisement of Dr. Michael Scanlon with the following exceptions. The HPLC analysis of thiamine content in non-mutant and blk1 mutant seedlings was completed by Dr. Maria Rapala-Kozik (Jagiellonian University). The expression of *thi2* and *thi2-blk1* in *E. coli* and subsequent THI2 and THI2-blk1 protein purification was completed by Debmita Paul and Dr. Steve Ealick (Cornell University). The HPLC analysis of THI2 and THI2-blk1 bound substrate was completed by Dr. Dinuka Abeydeera and Dr. Tadhg Begley (Texas A&M University). Dr. Paula McSteen and Kim Phillips (Pennsylvania State University) provided the *thi2-tsl31* and *thi2-tsl32* mutant alleles.

REFERENCES

Ahn, I., Kim, S., and Lee, Y. (2005). Vitamin B1 functions as an activator of plant disease resistance. Plant Physiology **138,** 1505-1515.

Barton, M. (2009). Twenty years on: The inner workings of the shoot apical meristem, a developmental dynamo. Developmental Biology **341,** 95-113.

Barton, M., and Poethig, R. (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild type and in the shoot meristemless mutant. Development **119,** 823-831.

Bayer, E., Smith, R., Mandel, T., Nakayama, N., Sauer, M., Prusinkiewicz, P., and Kuhlemeier, C. (2009). Integration of transport-based models for phyllotaxis and midvein formation. Genes & Development **23,** 373-384.

Becraft, P., and Freeling, M. (1994). Genetic analysis of Rough sheath1 developmental mutants of maize. Genetics **136,** 295-311.

Belanger, F., Leustek, T., Chu, B., and Kriz, A. (1995). Evidence for the thiamine biosynthetic pathway in higher-plant plastids and its developmental regulation. Plant Molecular Biology **29,** 809-821.

Bolduc, N., and Hake, S. (2009). The maize transcription factor KNOTTED1 directly regulates the gibberellin catabolism gene ga2ox1. The Plant Cell **21,** 1647-1658.

Bommert, P., Lunde, C., Nardmann, J., Vollbrecht, E., Running, M., Jackson, D., Hake, S., and Werr, W. (2005). thick tassel dwarf1 encodes a putative maize ortholog of the *Arabidopsis* CLAVATA1 leucine-rich repeat receptor-like kinase. Development **132,** 1235-1245.

Brand, U., Grunewald, M., Hobe, M., and Simon, R. (2002). Regulation of CLV3 expression by two homeobox genes in *Arabidopsis*. Plant physiology **129,** 565-575.

Brand, U., Fletcher, J., Hobe, M., Meyerowitz, E., and Simon, R. (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by CLV3 activity. Science **289,** 617-619.

Brink, R. (1933). Heritable characters in maize: XLVI--Ligu1eless-2. Journal of Heredity **24,** 325-326.

Brooks III, J., Zhang, X., Ohtsu, K., Zhou, R., Sarkar, A., Hargreaves, S., Elshire, R., Eudy, D., Pawlowska, T., and Ware, D. (2009). Microdissection of shoot meristem functional domains. PLoS Genetics **5,** 1-14.

Byrne, M., Simorowski, J., and Martienssen, R. (2002). ASYMMETRIC LEAVES1 reveals knox gene redundancy in *Arabidopsis*. Development **129,** 1957-1965.

Byrne, M., Timmermans, M., Kidner, C., and Martienssen, R. (2001). Development of leaf shape. Current Opinion in Plant Biology **4,** 38-43.

Chabregas, S., Luche, D., Van Sluys, M., Menck, C., and Silva-Filho, M. (2003). Differential usage of two in-frame translational start codons regulates subcellular localization of *Arabidopsis thaliana* THI1. Journal of cell science **116,** 285-291.

Chabregas, S., Luche, D., Farias, L., Ribeiro, A., Van Sluys, M., Menck, C., and Silva-Filho, M. (2001). Dual targeting properties of the N-terminal signal sequence of *Arabidopsis thaliana* THI1 protein to mitochondria and chloroplasts. Plant molecular biology **46,** 639-650.

Clark, S., Running, M., and Meyerowitz, E. (1993). CLAVATA1, a regulator of meristem and flower development in *Arabidopsis*. Development **119,** 397-418.

Clark, S., Running, M., and Meyerowitz, E. (1995). CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as CLAVATA1. Development **121,** 2057-2067.

Clark, S., Williams, R., and Meyerowitz, E. (1997). The CLAVATA1gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell **89,** 575-585.

Deng, Z., Zhang, X., Tang, W., Oses-Prieto, J., Suzuki, N., Gendron, J., Chen, H., Guan, S., Chalkley, R., and Peterman, T. (2007). A proteomics study of brassinosteroid response in *Arabidopsis*. Molecular & Cellular Proteomics **6,** 2058-2071.

Emerson, R. (1912). The inheritance of the ligule and auricles of corn leaves. Ann. Rep. Nebr. Agr. Exp. Sta. **25,** 81-88.

Emery, J., Floyd, S., Alvarez, J., Eshed, Y., Hawker, N., Izhaki, A., Baum, S., and Bowman, J. (2003). Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and KANADI genes. Current Biology **13,** 1768-1774.

Eshed, Y., Baum, S., Perea, J., and Bowman, J. (2001). Establishment of polarity in lateral organs of plants. Current Biology **11,** 1251-1260.

Eshed, Y., Izhaki, A., Baum, S., Floyd, S., and Bowman, J. (2004). Asymmetric leaf development and blade expansion in *Arabidopsis* are mediated by KANADI and YABBY activities. Development **131,** 2997-3006.

Feenstra, W. (1964). Isolation of nutritional mutants in *Arabidopsis thaliana*. Genetica **35,** 259-269.

Fletcher, J., Brand, U., Running, M., Simon, R., and Meyerowitz, E. (1999). Signaling of cell fate decisions by CLAVATA3 in *Arabidopsis* shoot meristems. Science **283,** 1911-1914.

Foster, T., Yamaguchi, J., Wong, B., Veit, B., and Hake, S. (1999). Gnarley1 is a dominant mutation in the knox4 homeobox gene affecting cell shape and identity. The Plant Cell **11,** 1239-1252.

Fowler, J., Muehlbauer, G., and Freeling, M. (1996). Mosaic analysis of the Liguleless3 mutant phenotype in maize by coordinate suppression of Mutator-insertion alleles. Genetics **143,** 489-503.

Freeling, M. (1992). A conceptual framework for maize leaf development1. Developmental Biology **153,** 44-58.

Friedrich, W. (1988). Vitamins. Walter de Gruyter. Berlin. 445.

Galinat, W. (1959). The phytomer in relation to the floral homologies in the American Maydea. Bot Mus Leafl Harv Univ **19,** 1-32.

Gifford Jr, E. (1983). Concept of apical cells in bryophytes and pteridophytes. Annual Review of Plant Physiology **34,** 419-440.

Giulini, A., Wang, J., and Jackson, D. (2004). Control of phyllotaxy by the cytokinin-inducible response regulator homologue ABPHYL1. Nature **430,** 1031-1034.

Godoi, P., Galhardo, R., Luche, D., Van Sluys, M., Menck, C., and Oliva, G. (2006). Structure of the thiazole biosynthetic enzyme THI1 from *Arabidopsis thaliana*. Journal of Biological Chemistry **281,** 30957-30966.

Guo, M., Thomas, J., Collins, G., and Timmermans, M. (2008). Direct repression of KNOX loci by the ASYMMETRIC LEAVES1 complex of *Arabidopsis*. The Plant Cell Online **20,** 48-58.

Hake, S., and Ori, N. (2002). Plant morphogenesis and KNOX genes. Nature Genetics **31,** 121-122.

Hay, A., Barkoulas, M., and Tsiantis, M. (2006). ASYMMETRIC LEAVES1 and auxin activities converge to repress BREVIPEDICELLUS expression and promote leaf development in *Arabidopsis*. Development **133,** 3955-3961.

Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S., and Tsiantis, M. (2002). The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. Current Biology **12,** 1557-1565.

Heisler, M., Ohno, C., Das, P., Sieber, P., Reddy, G., Long, J., and Meyerowitz, E. (2005). Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. Current Biology **15,** 1899-1911.

Hudson, A. (2000). Development of symmetry in plants. Annual Review of Plant Biology **51,** 349-370.

Jackson, D., and Hake, S. (1999). Control of phyllotaxy in maize by the abphyl1 gene. Development **126,** 315-323.

Jackson, D., Veit, B., and Hake, S. (1994). Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development **120,** 405-413.

Jacob-Wilk, D., Goldschmidt, E., Riov, J., Sadka, A., and Holland, D. (1997). Induction of a Citrus gene highly homologous to plant and yeast thi genes involved in thiamine biosynthesis during natural and ethylene-induced fruit maturation. Plant molecular biology **35,** 661-666.

Jeong, S., Trotochaud, A., and Clark, S. (1999). The *Arabidopsis* CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. The Plant Cell Online **11,** 1925-1934.

Johnson, M. (1951). The shoot apex in gymnosperms. Phytomorphology **1,** 188-204.

Jurgenson, C., Begley, T., and Ealick, S. (2009). The Structural and Biochemical Foundations of Thiamin Biosynthesis. Annual review of biochemistry **78,** 569-603.

Jurgenson, C., Chatterjee, A., Begley, T., and Ealick, S. (2006). Structural Insights into the Function of the Thiamin Biosynthetic Enzyme Thi4 from Saccharomyces cerevisiae. Biochemistry **45,** 11061-11070.

Karol, K., McCourt, R., Cimino, M., and Delwiche, C. (2001). The closest living relatives of land plants. Science **294,** 2351-2353.

Kayes, J., and Clark, S. (1998). CLAVATA2, a regulator of meristem and organ development in *Arabidopsis*. Development **125,** 3843-3851.

Kenrick, P., and Crane, P. (1997). The origin and early diversification of land plants: a cladistic study. Smithsonian Institute Press Washington DC.: A cladistic study. Smithsonian Institute Press 441 pp **560,** 989-990.

Kong, D., Zhu, Y., Wu, H., Cheng, X., Liang, H., and Ling, H. (2008). AtTHIC, a gene involved in thiamine biosynthesis in *Arabidopsis thaliana*. Cell Research **18,** 566-576.

Koornneef, M., and Hanhart, C. (1981). A new thiamine locus in *Arabidopsis*. *Arabidopsis* Inf Serv **18,** 52-58.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., and Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445,** 652-655.

Langridge, J. (1955). Biochemical Mutations in the Crucifer *Arabidopsis thaliana* (L.) Heynh. Nature **176,** 260-261.

Laux, T., Mayer, K., Berger, J., and Jurgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in *Arabidopsis*. Development **122,** 87-96.

Lee, B., Johnston, R., Yang, Y., Gallavotti, A., Kojima, M., Travencolo, B., Costa, L., Sakakibara, H., and Jackson, D. (2009). Studies of aberrant phyllotaxy1 mutants

of maize indicate complex interactions between auxin and cytokinin signaling in the shoot apical meristem. Plant physiology **150,** 205-216.

Leibfried, A., To, J., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J., and Lohmann, J. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438,** 1172-1175.

Lenhard, M., and Laux, T. (2003). Stem cell homeostasis in the *Arabidopsis* shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. Development **130,** 3163-3173.

Li, S., and Redei, G. (1969). Thiamine mutants of the crucifer, *Arabidopsis*. Biochemical Genetics **3,** 163-170.

Long, J., Moan, E., Medford, J., and Barton, M. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. Nature **379,** 66-69.

Machado, C., Costa de Oliveira, R., Boiteux, S., Praekelt, U., Meacock, P., and Menck, C. (1996). Thi1, a thiamine biosynthetic gene in *Arabidopsis thaliana*, complements bacterial defects in DNA repair. Plant Molecular Biology **31,** 585-593.

Machado, C., Praekelt, U., de Oliveira, R., Barbosa, A., Byrne, K., Meacock, P., and Menck, C. (1997). Dual role for the yeast THI4 gene in thiamine biosynthesis and DNA damage tolerance1. Journal of Molecular Biology **273,** 114-121.

Mayer, K., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., and Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell **95,** 805-815.

McConnell, J., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M. (2001). Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature **411,** 709-713.

McHale, N., Kawata, E., and Cheung, A. (1990). Plastid disruption in a thiamine-requiring mutant of *Nicotiana sylvestris* blocks accumulation of specific nuclear and plastid mRNAs. Molecular and General Genetics MGG **221,** 203-209.

Muller, B., and Sheen, J. (2007). Advances in cytokinin signaling. Science Signaling **318,** 68-69.

Muller, R., Bleckmann, A., and Simon, R. (2008). The receptor kinase CORYNE of *Arabidopsis* transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. The Plant Cell **20,** 934-946.

Nardmann, J., Ji, J., Werr, W., and Scanlon, M. (2004). The maize duplicate genes narrow sheath1 and narrow sheath2 encode a conserved homeobox gene function in a lateral domain of shoot apical meristems. Development **131,** 2827-2839.

Ogawa, M., Shinohara, H., Sakagami, Y., and Matsubayashi, Y. (2008). *Arabidopsis* CLV3 peptide directly binds CLV1 ectodomain. Science **319,** 294.

Ohyama, K., Shinohara, H., Ogawa-Ohnishi, M., and Matsubayashi, Y. (2009). A glycopeptide regulating stem cell fate in *Arabidopsis thaliana*. Nature Chemical Biology **5,** 578-580.

Ori, N., Juarez, M., Jackson, D., Yamaguchi, J., Banowetz, G., and Hake, S. (1999). Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene knotted1 under the control of a senescence-activated promoter. The Plant Cell Online **11,** 1073-1080.

Papini-Terzi, F., Galhardo, R., Farias, L., Menck, C., and Van Sluys, M. (2003). Point mutation is responsible for *Arabidopsis* tz-201 mutant phenotype affecting thiamin biosynthesis. Plant and Cell Physiology **44,** 856-860.

Pickett-Heaps, J. (1967). Ultrastructure and differentiation in Chara sp. I. Vegetative cells. Australian Journal of Biological Sciences **20,** 539-551.

Pien, S., Wyrzykowska, J., and Fleming, A. (2001). Novel marker genes for early leaf development indicate spatial regulation of carbohydrate metabolism within the apical meristem. The Plant Journal **25,** 663-674.

Praekelt, U., Byrne, K., and Meacock, P. (2004). Regulation of THI4 (MOL1), a thiamine-biosynthetic gene of Saccharomyces cerevisiae. Yeast **10,** 481-490.

Rapala-Kozik, M., Kowalska, E., and Ostrowska, K. (2008). Modulation of thiamine metabolism in Zea mays seedlings under conditions of abiotic stress. Journal of Experimental Botany **59,** 4133-4143.

Reddy, G., and Meyerowitz, E. (2005). Stem-cell homeostasis and growth dynamics can be uncoupled in the *Arabidopsis* shoot apex. Science **310,** 663-667.

Redei, G. (1965). Genetic blocks in the thiamine synthesis of the angiosperm *Arabidopsis*. American Journal of Botany **52,** 834-841.

Reinhardt, D., Mandel, T., and Kuhlemeier, C. (2000). Auxin regulates the initiation and radial position of plant lateral organs. The Plant Cell Online **12,** 507-518.

Reinhardt, D., Pesce, E., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., and Traas, J. (2003). Regulation of phyllotaxis by polar auxin transport. Nature **426,** 255-260.

Riou-Khamlichi, C., Menges, M., Healy, J., and Murray, J. (2000). Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. Molecular and Cellular Biology **20,** 4513-4521.

Roje, S. (2007). Vitamin B biosynthesis in plants. Phytochemistry **68,** 1904-1921.

Satoh-Nagasawa, N., Nagasawa, N., Malcomber, S., Sakai, H., and Jackson, D. (2006). A trehalose metabolic enzyme controls inflorescence architecture in maize. Nature **441,** 227-230.

Sayed, S., and Gadallah, M. (2002). Effects of shoot and root application of thiamin on salt-stressed sunflower plants. Plant Growth Regulation **36,** 71-80.

Scanlon, M. (2003). The polar auxin transport inhibitor N-1-naphthylphthalamic acid disrupts leaf initiation, KNOX protein regulation, and formation of leaf margins in maize. Plant physiology **133,** 597-605.

Scanlon, M., Schneeberger, R., and Freeling, M. (1996). The maize mutant narrow sheath fails to establish leaf margin identity in a meristematic domain. Development **122,** 1683-1691.

Schmidt, A. (1924). Histologische studien an phanerogamen vegetationspunkten. Bot. Arch **8,** 345-404.

Schneeberger, R., Becraft, P., Hake, S., and Freeling, M. (1995). Ectopic expression of the knox homeobox gene rough sheath1 alters cell fate in the maize leaf. Genes & Development **9,** 2292-2304.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K., Jürgens, G., and Laux, T. (2000). The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell **100,** 635-644.

Sinha, N., and Hake, S. (1990). Mutant characters of knotted maize leaves are determined in the innermost tissue layers. Developmental Biology **141,** 203-210.

Steeves, T., and Sussex, I. (1989). Patterns in plant development. Cambridge Univ Pr. 388 pgs.

Taguchi-Shiobara, F., Yuan, Z., Hake, S., and Jackson, D. (2001). The fasciated ear2 gene encodes a leucine-rich repeat receptor-like protein that regulates shoot meristem proliferation in maize. Genes & Development **15,** 2755-2766.

Timmermans, M., Hudson, A., Becraft, P., and Nelson, T. (1999). ROUGH SHEATH2: a Myb protein that represses knox homeobox genes in maize lateral organ primordia. Science **284,** 151-153.

To, J., Haberer, G., Ferreira, F., Deruere, J., Mason, M., Schaller, G., Alonso, J., Ecker, J., and Kieber, J. (2004). Type-A *Arabidopsis* response regulators are partially redundant negative regulators of cytokinin signaling. The Plant Cell Online **16,** 658-671.

Tsiantis, M., Schneeberger, R., Golz, J., Freeling, M., and Langdale, J. (1999). The maize rough sheath2 gene and leaf development programs in monocot and dicot plants. Science **284,** 154-156.

Tunc-Ozdemir, M., Miller, G., Song, L., Kim, J., Sodek, A., Koussevitzky, S., Misra, A., Mittler, R., and Shintani, D. (2009). Thiamin confers enhanced tolerance to oxidative stress in *Arabidopsis*. Plant Physiology **151,** 421-432.

Vandenbussche, M., Horstman, A., Zethof, J., Koes, R., Rijpkema, A., and Gerats, T. (2009). Differential recruitment of WOX transcription factors for lateral development and organ fusion in *Petunia* and *Arabidopsis*. The Plant Cell Online **21,** 2269-2283.

Vollbrecht, E., Reiser, L., and Hake, S. (2000). Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, knotted1. Development **127,** 3161-3172.

Wang, G., Ding, X., Yuan, M., Qiu, D., Li, X., Xu, C., and Wang, S. (2006). Dual function of rice OsDR8 gene in disease resistance and thiamine accumulation. Plant molecular biology **60,** 437-449.

White, P. (1951). Nutritional requirements of isolated plant tissues and organs. Annual Review of Plant Physiology **2,** 231-244.

White, R., and Spenser, I. (1979a). Thiamin biosynthesis in Saccharomyces cerevisiae. Origin of carbon-2 of the thiazole moiety. Biochemical Journal **179,** 315-325.

White, R., and Spenser, I. (1979b). Biosynthesis of vitamin B1 in yeast. Origin of the thiazole unit. Journal of the American Chemical Society **101,** 5102-5104.

White, R., and Spenser, I. (1982). Thiamin biosynthesis in yeast. Origin of the five-carbon unit of the thiazole moiety. Journal of the American Chemical Society **104,** 4934-4943.

Wightman, F., and Brown, R. (1953). The effects of thiamin and nicotinic acid on meristematic activity in pea roots. Journal of Experimental Botany **4,** 184-196.

Wu, X., Dabi, T., and Weigel, D. (2005). Requirement of homeobox gene STIMPY/WOX9 for *Arabidopsis* meristem growth and maintenance. Current Biology **15,** 436-440.

Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A., and Ori, N. (2005). *Arabidopsis* KNOXI proteins activate cytokinin biosynthesis. Current Biology **15,** 1566-1571.

CHAPTER 2

A maize thiamine auxotroph is defective in meristem maintenance and leaf-blade development

Introduction

Plants undergo organogenesis throughout their life cycle via continual recruitment from a population of stem cells called the shoot apical meristem (SAM). In order to maintain the SAM, cells lost to organ formation at the SAM peripheral zone (PZ) must be replaced through cell divisions within the SAM central zone (CZ). Failure to maintain SAM size can result in meristem consumption, organ fusion and shoot termination. Conversely, overproliferation of stem cells can lead to altered phyllotaxy and supernumery lateral organs. Thus, an equilibrium between stem cell division and differentiation is maintained via integrated networks of gene expression, hormonal signaling, and metabolite sensing (reviewed by Fleming, 2006; Barton, 2009).

Continued stem cell activity depends on a high cytokinin to auxin and gibberellin hormone regime. Class-I *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) transcription factors help maintain this ratio by simultaneously preventing cell differentiation through the repression of gibberellin signaling (Sakamoto et al., 2001; Bolduc and Hake, 2009) and promoting cell division via stimulation of cytokinin biosynthesis (Jasinski et al., 2005; Yanai et al. 2005). The founding member of the *knox* gene family, the maize *knotted1* (*kn1*), is a useful marker of indeterminacy and is expressed throughout the meristem but downregulated at organ initiation sites (Jackson et al., 1994; Long et al., 1996). Loss of KN1 function leads to meristem consumption, underlining its importance in meristem maintenance (Vollbrecht et al., 2000). The phosphoribohydrolase LONELY GUY1 (LOG1) is necessary for localized activation of cytokinin analogs at the SAM apex (Kurakawa et al., 2007). In rice, mutations to *Oslog1* lead to a reduction in SAM size and fewer floral organs (Kurakawa et al., 2007).

WUSCHEL (WUS), a transcription factor required for meristem maintenance in *Arabidopsis*, refines cytokinin signaling by acting non-cell autonomously from a presumptive stem-cell organizing center to repress negative regulators of cytokinin signaling called *type-A Arabidopsis response regulators* (*arr5*, *arr6*, *arr7*, and *arr15*) (Leibfried et al., 2005). Accordingly, overexpression of *type-A arr*s results in a SAM consumption phenotype that mimics loss-of-function WUS mutants. The maize gene *aberrant phyllotaxy1* (*abph1*) encodes a cytokinin-inducible type-A ARR homolog that represses meristem size via the regulation of cytokinin and auxin signaling (Giulini et al., 2004; Lee and Johnston et al., 2009). Maize abph1 mutants have enlarged meristems and often generate a decussate leaf phyllotaxy (two leaves per node initiated perpendicular to leaves at adjoining nodes) rather than the distichous (one leaf per node in two ranks) pattern of non-mutant maize (Jackson and Hake, 1999).

A shift in hormone signaling accompanies the onset of organ initiation at the SAM flanks. Concordantly, localized transport of auxin, as indicated by the auxin efflux protein PIN-FORMED1 (PIN1) and the auxin reporter DR5, is the earliest known molecular marker for leaf initiation and vascular development (Reinhardt et al. 2000, 2003; Heisler et al., 2005; Bayer et al., 2009). The accumulation of auxin at the SAM periphery is both necessary and sufficient for leaf initiation. Exogenous auxin application to the meristem periphery can induce new leaf formation (Reinhardt et al. 2000), while blocking auxin transport with N-1-naphthylphthalamic acid (NPA) halts their initiation (Reinhardt et al. 2000; Scanlon, 2003).

Precise auxin regulation is also necessary for the initiation of axillary meristems from inflorescences. Mutations to the *Arabidopsis pin1* or the PIN1 regulator *pinoid* (*pid*) lead to a pin-like inflorescence that lacks flowers (Okada et al., 1991; Bennett et al., 1995). Likewise, treatment of the maize inflorescence with NPA

(Wu and McSteen, 2007) and mutations to the YUCCA-like auxin biosynthetic gene *sparse inflorescence1* (Gallavotti et al., 2008) or the *pid* homolog *barren inflorescence2* (McSteen et al., 2007) reduce axillary meristem initiation. Accumulation of the maize PIN1 ortholog, *Zm*PIN1a, and DR5 expression precedes meristem initiation events within the maize inflorescence, further supporting a role for auxin accumulation in this process (Wu and McSteen, 2007).

Less research has focused on the interplay of metabolites and meristem function. As an active center of cell division, regulation of carbohydrate metabolism is vital to SAM function. This interdependence is highlighted by analyses of the *Arabidopsis* gene *stimpy* (Wu et al., 2005), which encodes a WOX transcription factor that is necessary for maintaining cell divisions within the SAM. Sucrose application fully rescues the seedling-lethal phenotype of stimpy mutants, illustrating the importance of carbohydrate regulation in SAM function. A role for sugar signaling is further demonstrated by analyses of the meristem determinacy mutant ramosa3 (ra3), which causes abnormal branching of the female inflorescence (ear) (Satoh-Nagasawa et al., 2006). The *ra3* gene encodes a trehalose-6-phosphate phosphatase, which is expressed in a small domain below axillary meristems and is purported to regulate branching via production of a mobile trehalose signal (Satoh-Nagasawa et al., 2006).

The B vitamin thiamine is a necessary supplement for shoot meristems grown in culture (Addicott, 1939; Brown and Wightman, 1952; Wightman and Brown, 1953), suggesting its important role in meristem maintenance. Phosphorylated thiamine derivatives are essential cofactors for several enzymes involved in carbohydrate metabolism, including transketolase, alpha-ketoglutarate dehydrogenase, and pyruvate dehydrogenase. Thiamine auxotrophs in *Arabidopsis* illustrate that this cofactor is required for plant viability (Langridge, 1955; Feenstra, 1964; Redei, 1965; Li and Redei, 1969; Koornneef and Hanhart, 1981). Without thiamine supplementation, the

Arabidopsis th1, py, and *At*thi1 mutants die after the production of several albino leaves. The th2 and th3 auxotrophs are viable, but have chlorotic and necrotic regions in leaves. Several of these loci are known to encode enzymes in the thiamine biosynthetic pathway. *At*THI1, in particular, generates the thiazole precursor to thiamine, 4-methyl-5-B-hydroxyethylthiazole (HET) (Papini-Terzi et al., 2003).

Here, we describe the bladekiller1 (blk1) mutant of maize, a thiamine auxotroph with defects in meristem maintenance and a novel leaf-blade reduction phenotype. Positional cloning has revealed that mutations to *thiamine biosynthesis2* (*thi*2), a maize ortholog of *Atthi1* (Belanger et al. 1995), confer the blk1 mutant phenotypes. Biochemical analyses suggest a conserved role for THI2 in thiamine biosynthesis and that the reference mutant allele, *blk1-R*, is a null. Importantly, all blk1 mutant phenotypes are rescued by thiamine supplementation. These data provide a new perspective on the metabolic requirements of the shoot apical meristem.

Results

bladekiller1 mutants have defects in vegetative SAM maintenance

Plants homozygous for the *blk1-R* mutation were discovered as aborted shoot phenotypes segregating in the M2 generation of EMS-treated individuals. The *blk1-R* mutant allele was subsequently introgressed into the B73 inbred background for over nine generations. Longitudinal sections through seedlings at 7 and 21 days after-germination (DAG) reveal that while mutant and non-mutant SAMs are of similar height early in vegetative development, blk1-R SAMs fail to increase in height at the rate of their non-mutant siblings (Bassiri et al., 1992) and are on average 53 μm shorter at 21 DAG (Figure 2.1A-C). Despite this effect on SAM height, SAM width is unaffected in the blk1-R mutant background (Figure 2.1A).

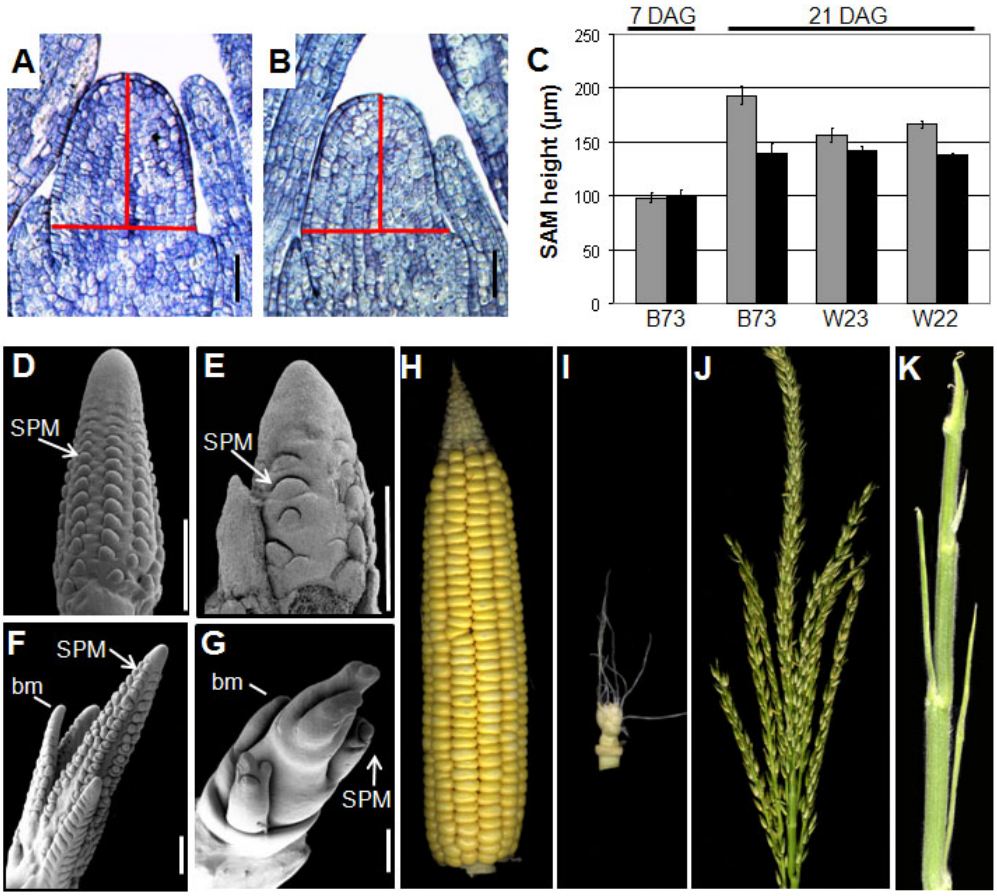


Figure 2.1: bladekiller1 (blk1) mutants exhibit progressive defects in shoot meristem maintenance

A-B. 21 days after germination (DAG) SAMs in B73 background; Red lines indicate reference points for measurement for height and width; scale bar = 50µm

A. Non-mutant SAM

B. blk1 SAM is reduced in height

C. blk1 meristems (black) are shorter than non-mutant SAMs (grey) in the B73, W22, and W23 inbred backgrounds at 21 DAG. Note that SAM height is essentially unaffected at 7 DAG. errors bars = ±standard error, SE (n=15)

D-G. Scanning electron microscopy (SEM) of immature maize inflorescences, spikelet pair meristems (SPM) and tassel branch meristems (BM); scale bar = 500µm

D. Non-mutant ear primordium

E. blk1 ear primordium is truncated and has fewer SPMs

F. Non-mutant tassel primordium

G. blk1 tassel primordium has aberrant branches and fewer SPMs

H-K. Mature maize inflorescences

H. Non-mutant ear

I. blk1 ear is greatly reduced in size and has few florets

J. Non-mutant tassel

K. blk1 tassel is truncated, barren, and has reduced branching

To further investigate the meristem size phenotype, the *blk1-R* mutant allele was introgressed for five generations into W22 and W23- inbred maize lines with shorter meristems than inbred B73 (Vollbrecht et al., 2000). In agreement with previously published data, non-mutant SAMs in W22 and W23 were smaller than B73 shoot meristems (Figure 2.1C). Introgression of the *blk1-R* mutation into W22 and W23 reduced the mean SAM height by 29 μm and 15 μm respectively (Figure 2.1C). Interestingly, the average height of blk1-R mutant SAMs after three weeks of growth (~140 μm) was not significantly altered by genetic background, suggesting BLK1 function is epistatic to factors leading to the enlarged meristem size observed in the B73 inbred background.

bladekiller1 mutants have highly reduced inflorescence meristems

Maize is a monoecious species with spatially separated male and female inflorescences, called the tassel and ear respectively (Figure 2A, Kiesselbach, 1949). The tassel develops at the apex of the plant following transition of the SAM into an inflorescence meristem. Maize ears develop from lateral meristems located in the axes of leaves. Each inflorescence comprises several types of axillary meristems (Figure 2.1D, 2.1F; reviewed by Bortiri and Hake, 2007): branch meristems produce the long branches located at the base of the maize tassel (Figure 2.1F, 2.1J) and are absent from the ear (Figure 2.1D, 2.1H); spikelet-pair meristems each generate two spikelet meristems; the spikelet meristems then yield short floral-bearing spikelets and give rise to floral meristems, which produce the floral organs. Mature blk1-R mutant tassels lack spikelets but have several shortened and barren branches (Figure 2.1K). Similarly, the mature mutant ear is severely truncated and either aborts without forming any spikelets (data not shown) or contains very few florets (Figure 2.1I). Scanning electron microscopy (SEM) of developing inflorescences was utilized to

determine that these mutant phenotypes result from a failure to initiate axillary meristems. Mutant tassel primordia generate branch-like structures, but the presumptive branches and central spike have very few SPMs and those that do form are located near the tips (Figure 2.1G). SPMs are formed at the base of the blk1-R ear primordium, which is similar to the development of non-mutant inflorescences (Figure 2.1E). However, the apex of the mutant inflorescence does not elongate, causing a reduction in SPM number. These observations highlight the progressive nature of the blk1 mutant phenotype, in that the most severe phenotypes occur in the latest emerging structures of the shoot.

bladekiller1 mutants have defects in leaf blade development

Maize leaves develop from approximately 250 initial cells recruited from the flank of the SAM (Poethig, 1984). Asymmetric cell division, expansion and differentiation within the leaf primordium give rise to a leaf with distinct morphological domains (reviewed by Hudson, 2000; Byrne et al., 2001). Mature leaves are subdivided along the longitudinal axis into a proximal sheath that encircles the stem and a distal blade; these two domains are separated by a hinge-like auricle and a fringe of epidermally-derived, adaxial tissue called the ligule (Figure 2.2A, 2.2C). The blk1 mutants exhibit a novel leaf-reduction phenotype wherein the basal leaves (L1-L7) are morphologically normal and each consecutive leaf thereafter (L8-L18) exhibits an increasing degree of blade truncation, which is accompanied by shredding and necrosis of the blade margins and tip (Figures 2.2B, 2.2D). Although the extent of leaf reduction is variable from plant to plant and from leaf to leaf, the last two to four mutant leaves immediately below the tassel frequently contain no leaf blade and are composed of only ligule and/or sheath tissue (Figures 2.2B, 2.2D).

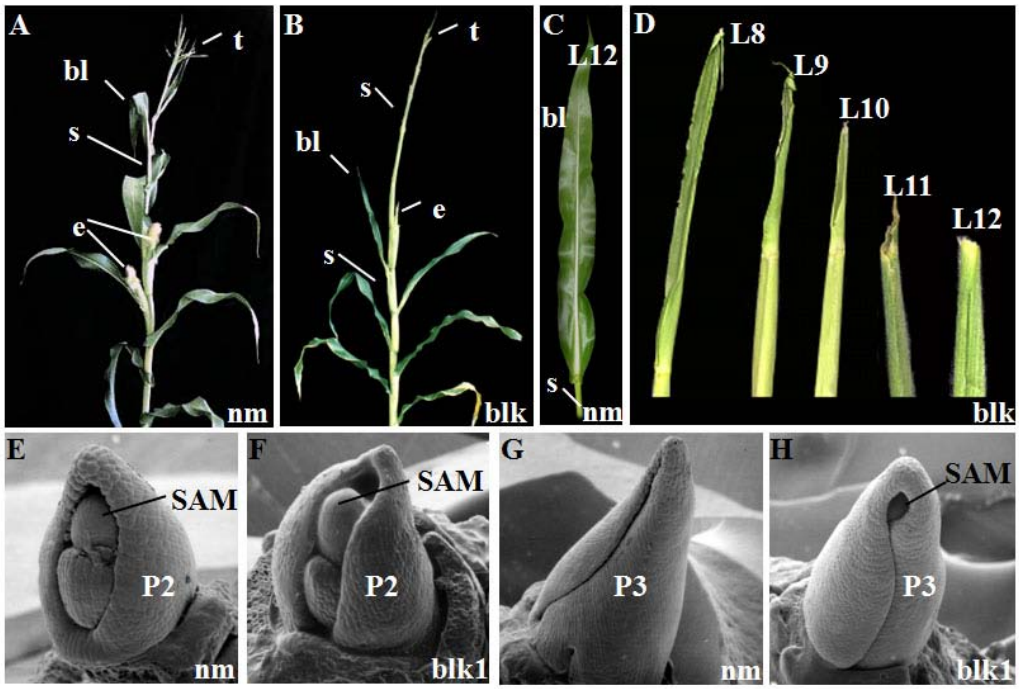


Figure 2.2: blk1 mutants exhibit a progressive loss of leaf blade

A. Mature non-mutant (nm) B73 plant. leaf blade (bl), leaf sheath (s), ears (e), and tassel (t)

B. Mature blk1 mutant plants have truncated leaf blade development in apical nodes

C. Mature maize leaf (L12 = 12th leaf from base)

D. Successive blk1 leaves exhibit increasing degree of blade truncation; L12 is bladeless.

E-H. SEM of dissected SAM apices from blk1 mutants and non-mutants; plastochrons 2 and 3 (P2, P3)

E. Non-mutant P2 has an apical hood covering the SAM

F. blk1 P2 leaf is reduced

G. Non-mutant P3 leaf envelops the underlying apex

H. blk1 P3 leaf is reduced and exposes the SAM

SEM analysis of developing leaf primordia reveals that the blk1-R blade truncation phenotype is evident as early as the second plastochron (P2). Non-mutant P2 leaves form an apical hood that covers the SAM (Figure 2.2E), however the blk1-R mutant P2 leaf fails to cover the SAM apex (Figure 2.2F). Whereas the margins of the P3 non-mutant leaf primordium completely surround the underlying shoot apex (Figure 2.2G), the mutant P3 primordium is truncated at the distal tip, exposing the SAM (Figure 2.2H). No evidence of tissue necrosis is observed in the blk1-R mutant primordial leaf, suggesting that the blade truncation phenotype results from a defect in either founder cell recruitment or cell division and not from an abnormal, early onset of senescence.

blk1 functions outside of known pathways for meristem size regulation

We further explored *blk1* gene function in meristem maintenance by creating double mutants with blk1-R and abph1, tassel dwarf1 (td1), and fasciated ear2 (fea2). The *td1* and *fea2* genes encode leucine-rich receptor-like proteins that function in overlapping pathways to repress stem-cell proliferation in the inflorescences (Bommert et al., 2005; Taguchi-Shiobara et al., 2001). Both td1 and fea2 single mutants have fasciated ear phenotypes, increased spikelet density, and extra floral organs (data not shown; Bommert et al., 2005; Taguchi-Shiobara et al., 2001). The phenotypes of blk1-R;td1 and blk1-R;fea2 double mutants are morphologically equivalent to those of blk1-R single mutants (data not shown), and exhibit leaf-blade reduction and shoot termination phenotypes. Moreover, the ears and tassels of blk1-R;td1 and blk1-R;fea2 double mutants abort early and do not produce SPM, suggesting that *blk1* gene function is epistatic to both *td1* and *fea2* during development.

The maize gene *aberrant phyllotaxy1* (*abph1*) encodes a cytokinin response regulator that functions to repress SAM size and mediate organ initiation (Jackson and Hake, 1999; Giulini et al. 2004; Lee and Johnston et al., 2009). Accordingly, abph1 single mutants display a significant increase in SAM width when compared to non-mutant siblings at 21 DAG (Figure 2.3A, Jackson and Hake, 1999), although SAM height is not significantly altered (Figure 2.3A). Like blk1-R single mutants, the SAMs of blk1-R;abph1 double mutants are significantly shorter than the SAMs of non-mutant siblings. While SAM width is slightly increased in double mutants when compared to the SAM width of non-mutants and blk1 single mutants, this change is not significant ($P > 0.5$, student's two-tailed t-test).

The SAM phenotypes of abph1 mutants correlate with alterations in organ initiation (Figure 2.3, Jackson and Hake, 1999). A small proportion (4/12) of abph1 mutants display decussate phyllotaxy, in which two leaves per node are present on opposite sides of the stem and successive pairs are aligned at right angles (Figure 2.3B). The majority of abph1 single mutants, however, retain distichous phyllotaxy. The first vegetative leaf of most abph1 mutant plants contains two midribs that extend into separate halves of a blade partially split along the presumptive leaf median (Figure 2.3C). This double-midrib phenotype is also frequently observed in the maize phyllotaxy mutant terminal ear1, and may result from the aberrant fusion of two leaves that were initiated adjacent to each other at the meristem (Veit et al., 1998; Jackson and Hake, 1999). The first leaf of blk1-R;abph1 double mutants similarly has two midribs (Figure 2.3D-E). Despite this similarity to abph1 single mutants, the majority of vegetative nodes in mature blk1-R;abph1 double mutant plants exhibit blk1-R phenotypes (data not shown). For example, leaves 8-13 of double mutants have reduced blades, and the leaves just below the apex (L14-16) are often devoid of blade

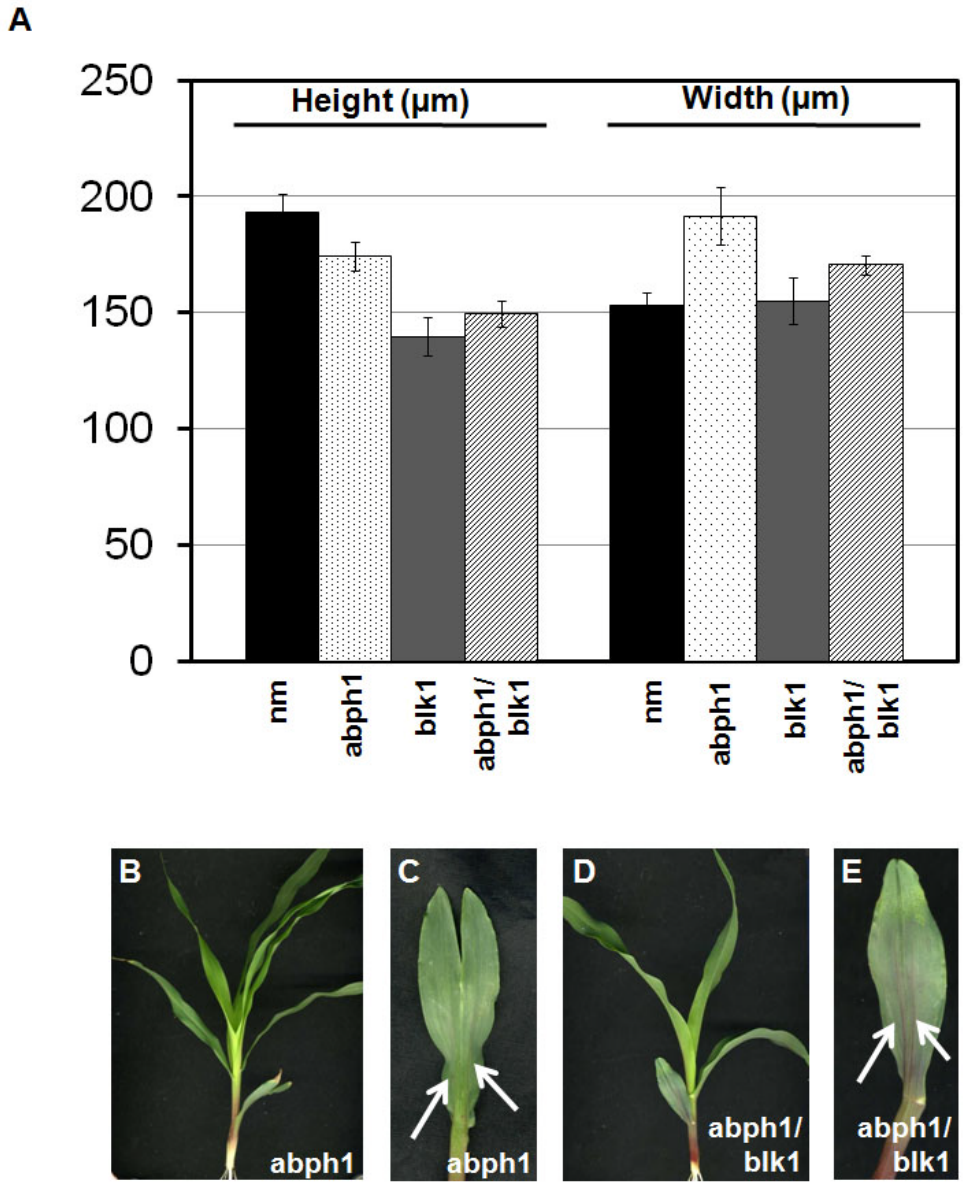


Figure 2.3: *blk1* and *abph1* function in independent pathways to control meristem size
A. Analysis of SAM height and width in non-mutants, abph1 single mutants, blk1 single mutants, and abph1;blk1 double mutants reveals that abph1 and blk1 have additive effects on meristem size
B. abph1 single mutant with decussate phyllotaxy.
C. First vegetative leaf of abph1 single mutant with two mid-ribs (arrows)
D. abph1;blk1 double mutants have a distichous phyllotaxy
E. Like abph1 single mutants, the first vegetative leaf of abph1;blk1 doubles has two mid-ribs (arrows)

tissue. Furthermore, none of the blk1-R; abph1 double mutants displayed decussate phyllotaxy (0/15).

Onset of blk1 developmental phenotypes correlates with the phase transition from juvenile to adult vegetative growth (phytomers 6-8) (reviewed by Poethig, 1990). To determine whether *blk1* gene function is linked to vegetative phase change, double mutants were constructed with blk1-R and the heterochronic mutant Teopod1 (Tp1). Gain-of-function *Tp1* mutations increase the number of phytomers expressing juvenile characteristics, and increase vegetative outgrowth from axillary meristems at basal nodes (tillering) (data not shown; Poethig, 1988). Furthermore, Tp1 mutant inflorescences are highly reduced, and develop vegetative leaves at the base of spikelet branches (data not shown; Poethig, 1988). The blk1-R;Tp1 double mutants exhibit additive phenotypes. Similar to Tp1 single mutants, double mutants have increased tillering in the basal nodes. However, the blk1 leaf-blade reduction phenotype occurs in blk1-R;Tp1 double mutants at equivalent nodes as observed in blk1 single mutants (L8-L16), suggesting that BLK1 function is not linked to phase change.

Molecular markers reveal bladekiller1 mutants are deficient in meristem maintenance.

Morphological analyses indicate that blk1-R mutants exhibit a progressive decrease in SAM size (Figure 2.1), which suggests a defect in stem cell maintenance. To explore this possibility, quantitative RT-PCR (qRT-PCR) analyses were used to compare the transcript accumulation of the meristem maintenance markers *kn1*, *abph1*, *Zmlog1* (a homolog of *Oslog1*), and *Zmwus1* (a *wus* homolog, Nardmann and Werr, 2006) in amplified RNA from laser-microdissected blk1-R mutant or non-mutant SAMs (Figure 2.4A). Mutant SAMs have a significant decrease in the

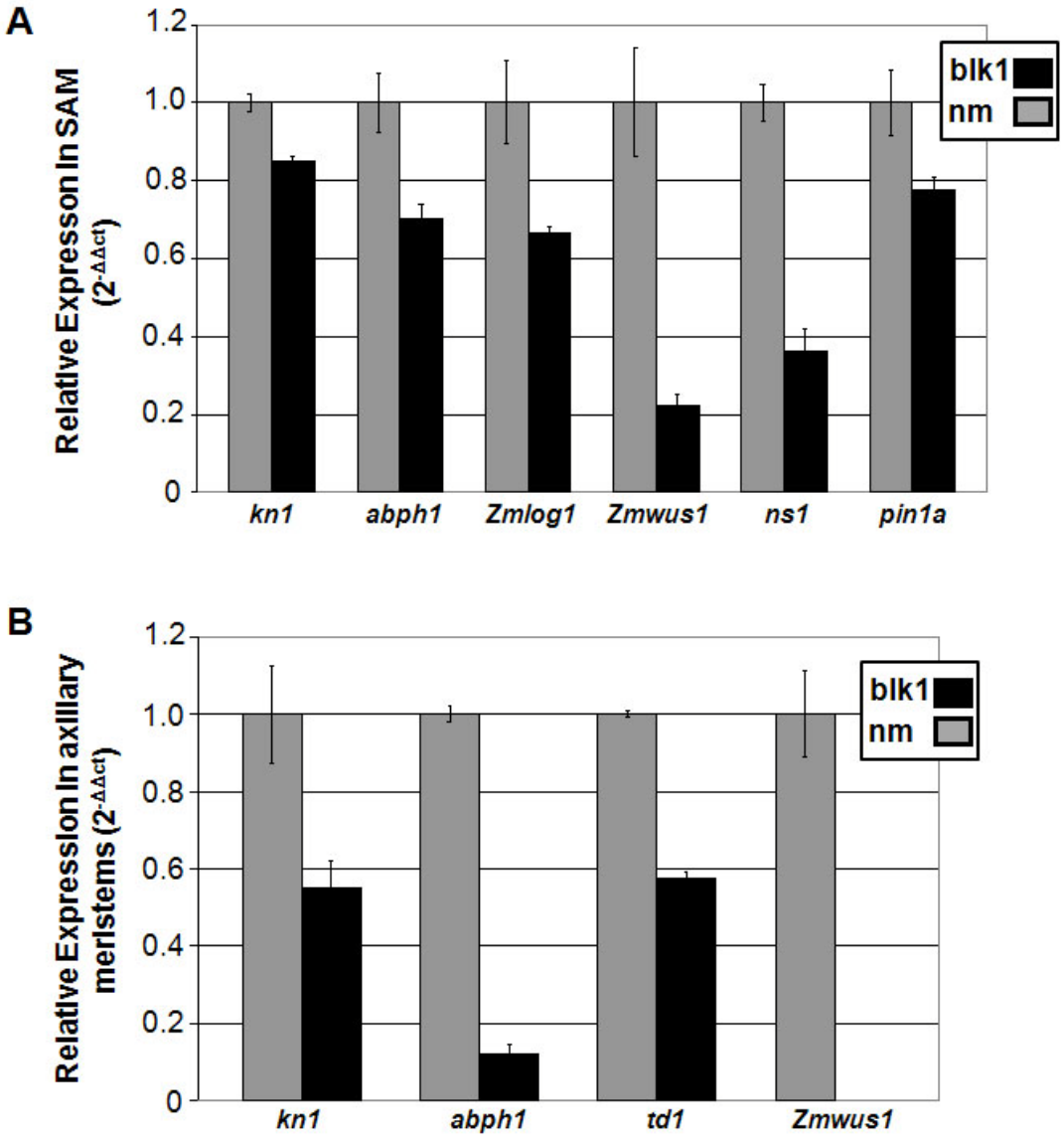


Figure 2.4: qRT-PCR analyses reveals that blk1 mutants are deficient in meristem maintenance

A. Transcript accumulation of four SAM markers and two leaf-initiation markers in blk1 laser-dissected SAM tissue (black) is significantly decreased in comparison to accumulation levels in non-mutant SAMs (grey). Threshold values were normalized with *tubulin6* and are shown relative to the non-mutant normalized value. Errors bars = ±SE (n=3).

B. Four markers of lateral meristem maintenance are down-regulated in laser-dissected blk1 mutant lateral meristems from presumptive ear nodes. Grey is non-mutant, black is blk1 mutant; errors bars = ±SE (n=3).

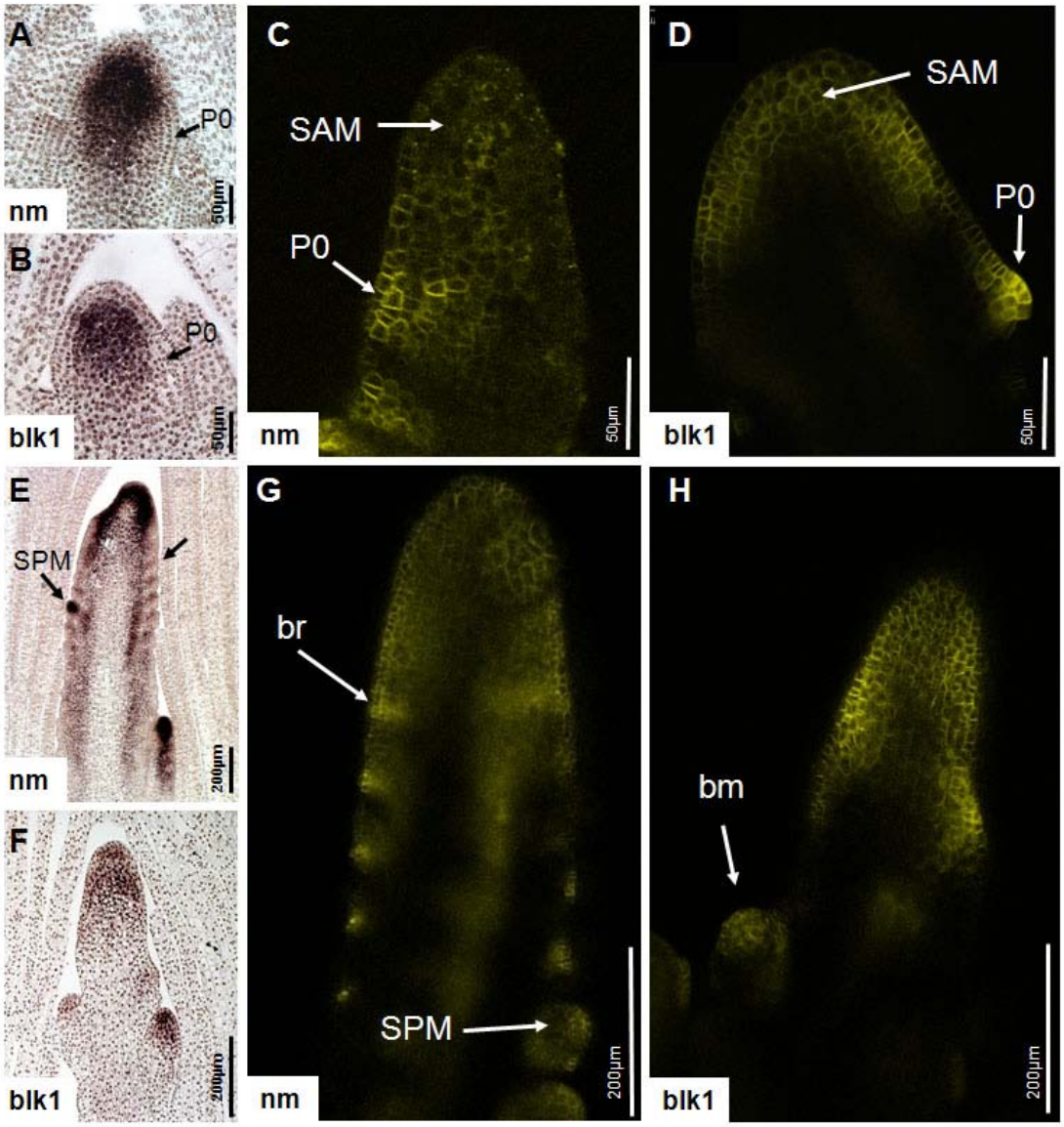
transcript abundance of these markers, ranging from a 15% reduction in the meristem maintenance gene *kn1* to a 78% reduction in the putative stem cell regulator *Zmwus1*. Accumulation of the cytokinin regulators, *abph1* and *Zmlog1* was reduced to 70% and 66% of non-mutant levels, respectively.

Lateral meristems from presumptive ear nodes were also laser-captured and used in qRT-PCR analyses. Relative to non-mutant siblings, transcript accumulation of the meristem markers *kn1, abph1, Zmwus1, and td1* were all increasingly downregulated in mutant lateral meristems as compared to vegetative SAMs (Figure 2.4B). Surprisingly, no *Zmwus1* transcripts were amplified from blk1-R mutant lateral meristems.

RNA *in situ* hybridizations were used to investigate whether reduced transcript levels of *kn1* and *abph1* in the blk1-R mutant SAM correlated with alterations in the cell/tissue specific accumulation pattern of these transcripts. In non-mutants, *kn1* transcripts accumulate in the meristem central zone and are absent at the site of leaf initiation (P0) (Figure 2.5A). Mutant SAMs exhibit a similar *kn1* accumulation pattern, but the signal intensity is consistently reduced (Figure 2.5B), corroborating the qRT-PCR data. Additionally, *abph1* transcript accumulation, which is found in a cross-sectional band slightly below the SAM apex, is greatly reduced in the blk1-R mutant SAM (Figure 2.6A-B).

The blade reduction phenotype of blk1-R mutants is evident very early in leaf development (Figure 2.2). To determine if mutants correctly accumulate markers associated with early events in maize leaf initiation, we compared the transcript accumulation of *narrow sheath1 (ns1)* and *Zmpin1a* using qRT-PCR (Figure 2.4A). The *ns1* gene is expressed in two small domains on opposite flanks of the SAM and functions to recruit cells that give rise to the lateral domains of maize leaves (Scanlon et al., 1996; Nardmann and Ji et al., 2004). The auxin efflux carrier *Zm*PIN1a exhibits

Figure 2.5: *kn1* and *Zm*PIN1a~YFP accumulation patterns are normal in the SAM, but are aberrant in blk1 mutant tassel primordia
A, B, E, F. RNA *in situ* hybridizations with *kn1* probe
C, D, G, H. Confocal images of *Zm*PIN1a~YFP fluorescence
A. *kn1* mRNA accumulates in the indeterminate cells that make up the SAM central zone, but is down-regulated at the P0 of non-mutant 21 DAG seedlings.
B. Mutant SAMs at 21-DAG have normal *kn1* expression patterns, but mRNA levels are reduced.
C. Non-mutants at 21 DAG show *Zm*PIN1a up-regulation at the P0
D. blk1 SAMs 21 DAG have normal *Zm*PIN1a up-regulation at the P0
E. In non-mutant tassel primordia, *kn1* mRNA accumulates at the apices of branch primordia, at the apex of central tassel spike, at the presumptive sites of SPM initiation (arrow), and within developing SPMs
F. *kn1* accumulation in blk1 tassel primordia is reduced and normal up-regulation does not occur along the flanks of the inflorescence
G. *Zm*PIN1a~YFP is up-regulated in SPMs and along the flanks of the non-mutant tassel primordium where it marks the initiation of bract primordia (BP).
H. *Zm*PIN1a~YFP localization is aberrant in blk1 mutant tassel primordia, which may account for the large reduction in SPM number. branch meristem (bm)


A
P0
nm
50µm
B
P0
blk1
50µm
C
SAM
P0
nm
50µm
D
SAM
P0
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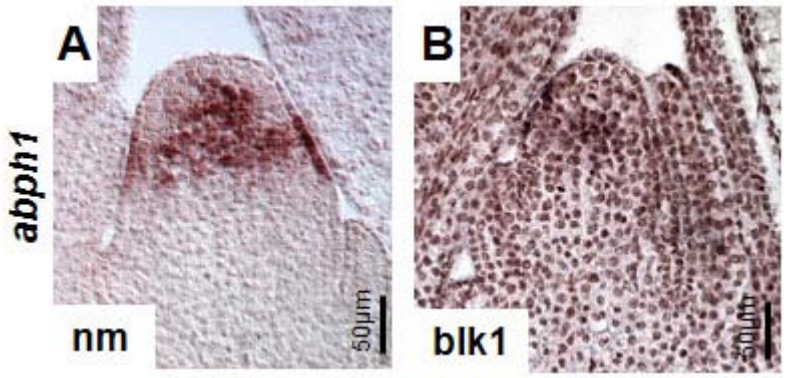


Figure 2.6: RNA *in situ* hybridizations showing *abph1* expression is reduced in blk1 mutants
A. Non-mutant 21 DAG SAMs accumulate *abph1* in a band across the meristem
B. The abph1 expression pattern is normal in blk1 21 DAG SAM, but levels are reduced.

maximum accumulation at the presumptive sites of leaf initiation (Gallavotti et al., 2008). Both *ns1* and *Zmpin1a* have decreased transcript accumulation in laser-dissected mutant SAMs (Figure 2.4A). A *Zm*PIN1a~YFP reporter was utilized to determine the cell and tissue specificity of PIN1 accumulation in non-mutant and blk1-R mutant shoot apices. As described previously (Gallavotti et al., 2008), *Zm*PIN1a~YFP accumulates throughout the SAM but is especially abundant at the site of the incipient leaf (Figure 2.5C-D). Although qRT-PCR analyses revealed decreased *Zmpin1a* transcript abundance in the blk1-R mutant SAM, no overt differences in *Zm*PIN1a~YFP localization are observed in blk1-R vegetative apices.

SEM analysis reveals that blk1-R mutants fail to correctly initiate spikelet pair meristems (Figure 2.1). To further investigate the basis of the SPM initiation defects, we analyzed two markers associated with SPM initiation. Upregulation of *kn1* occurs at sites of SPM initiation and is required for maintaining indeterminate cell populations (Vollbrecht, 2000; McSteen and Hake, 2001). As previously described, RNA *in situ* hybridizations reveal that non-mutant tassel primordia accumulate *kn1* transcripts at the apices of branch primordia, at the apex of central tassel spike, at the presumptive sites of SPM initiation, and within developing SPMs (Figure 2.5E). On the other hand, blk1-R mutant tassel primordia exhibit an attenuated and dispersed *kn1* accumulation pattern in the inflorescence and branch meristems (Figure 2.5F). Furthermore, *kn1* upregulation does not occur along the flanks of the inflorescence, which correlates with the failure to initiate SPMs (Figure 2.5F).

The upregulation and polar localization of the auxin efflux carrier *Zm*PIN1a is necessary for generating the auxin maxima that create branching events in the maize tassel (Gallavotti et al., 2008). Non-mutant inflorescences accumulate *Zm*PIN1a in bract primordia prior to the initiation of SPMs, forming regularly-spaced bands of *Zm*PIN1a~YFP signal (Figure 2.5G). Conversely, blk1-R mutant inflorescences do

not exhibit normal *Zm*PIN1a~YFP accumulation, and fail to upregulate *Zm*PIN1a in an interspaced pattern (Figure 2.5H). Curiously, some mutant tassels have abnormally wide areas of *Zm*PIN1a~YFP and *kn1* accumulation at the flanks of the inflorescence in positions where SPM should form (Figure 2.5F and 2.5H). Whether these aberrations indicate areas of occasional branch meristem or SPM formation is unknown.

Positional cloning reveals that *blk1* encodes a thiazole biosynthetic enzyme

The *blk1-R* mutation was mapped to the long arm of chromosome 3 using the B-A translocation lines (Beckett, 1978; Materials and Methods). F1 plants hypoploid for TB-3La displayed blk1 phenotypes that were equivalent to *blk1-R* homozygous plants (data not shown), suggesting that the *blk1-R* mutation is a genetic null (Muller, 1932). The *blk1-R* mutation was mapped to contig 149 of the maize agarose FPC map by linkage to SSR marker *umc2152* and the indel marker *IDP351* (Figure 2.7A). An F2 mapping population of 686 mutants was used to fine map the mutation to a 0.23 cM interval delimited by CAPS marker *CAPSK03* (2 recombinants) and *CAPSN05* (1 recombinant). This region constitutes approximately 300 kb from three contiguous sequenced BACs (Figure 2.7A). Analysis of the sequenced region revealed seven candidate open reading frames (ORF). Each ORF was evaluated for sequence polymorphisms that would disrupt the predicted gene product. In blk1-R mutants, a G to A point mutation was identified in the *thiamine biosynthesis2* (*thi2*) gene (GRMZM2G074097) resulting in a predicted missense mutation that converts Val211 to Met211 in the encoded gene product. Since *thi2* has been described previously (Belanger et al., 1995), the *blk1* gene will be renamed *thi2*, and the reference *blk1-R* mutant allele will hereafter be referred to as *thi2-blk1*. The Val211 residue is

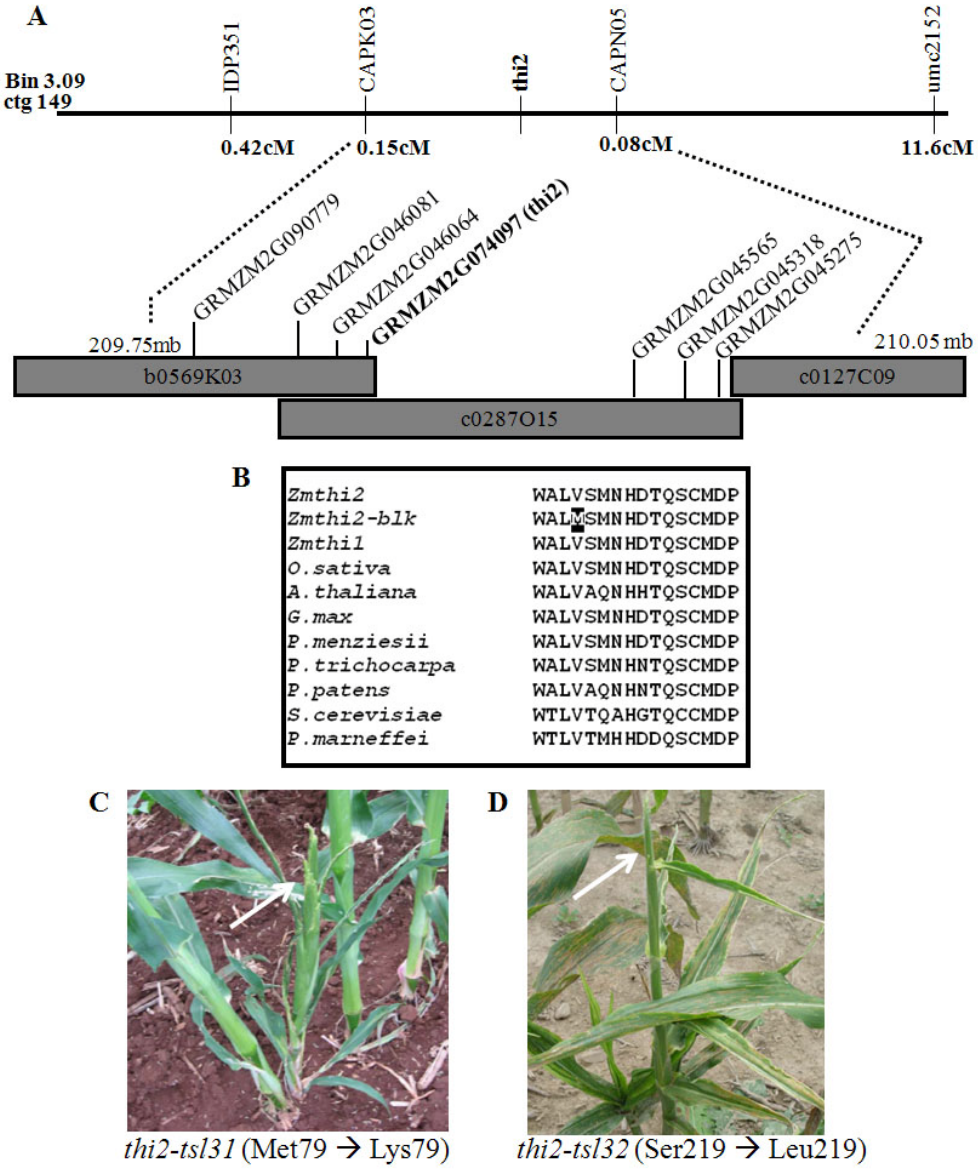


Figure 2.7: Positional cloning of the *blk1* mutation reveals a missense mutation in a conserved residue encoded by the *thiamine biosynthesis2* gene.
A. A mapping population of 686 individuals was used to map the *blk1* mutation to a small interval in Bin 3.09, ctg 149. The listed genetic markers (top) were used to delimit the interval. Recombination frequency is in centiMorgans (cM). The region spans three BACs and contains seven genes with predicted ORFs (bottom).
B. Amino acid sequence alignment of THI-homologs in various organisms. Sixteen amino acids surrounding the *blk1* mutation (black) are shown.
C-D. Two additional recessive mutant alleles of *thi2*, *thi2-tsl31***(C)** and *thi2-tsl32* **(D)**, condition equivalent shoot meristem maintenance and leaf-reduction phenotypes (arrows).

conserved in comparisons of THI2 homologs from four angiosperms (*Arabidopsis thaliana, Oryza sativa, Glycine max, Populus trichocarpa*), one gymnosperm (*Pseudotsuga Menziesii*), one bryophyte (*Physcomitrella patens*), and two fungi (*Saccharomyces cerevisiae, Penicillium marneffei*) (Figure 2.7B). Two additional mutant *thi2* alleles (*thi2-tsl31* and *thi2-tsl32*) conditioning equivalent blk1 mutant phenotypes were obtained in forward genetic screens of EMS mutagenized plants, and found to contain missense mutations in conserved THI2 residues that altered Met79 to Lys79 and Ser219 to Leu219, respectively (Figure 2.7C-D).

thi2 transcripts accumulate in rapidly dividing tissues

THI2 shares 95% amino acid similarity with the predicted gene product of *thiamine biosynethesis1* (*thi1*), a paralog located on an ancestrally duplicated region of chromosome 8 (Belanger et al., 1995). To determine the tissue specificity and timing of expression of these paralogs, qRT-PCR analyses were performed on cDNA derived from seven different tissues, including: (1) 20 day after pollination (DAP) kernels; (2) pooled P2-P6 leaves from 21 DAG seedlings; (3) roots from 21 DAG seedlings; (4) laser-microdissected SAMs from 21 DAG seedlings; (5) 2-4 mm ear primordia; (6) 2-4 mm tassel primordia; and (7) mature adult leaves (L9-L10) (Figure 2.8A). Both *thi* gene paralogs were expressed in all tissue types tested. However, relative to all other tissues examined, *thi2* is upregulated in the kernel, P2-P6 leaves, and in tassel and ear primordia. These data suggest that *thi2* expression is developmentally regulated. Similarly, *thi1* is upregulated in the kernel and mature leaf as compared to other tissues examined. When transcript accumulation of the maize *thi* paralogs is directly compared, *thi2* preferentially accumulates in the kernel (145 fold upregulated as compared to *thi1*), SAM (5 fold), tassel primordia (130 fold), ear primordia (228 fold)

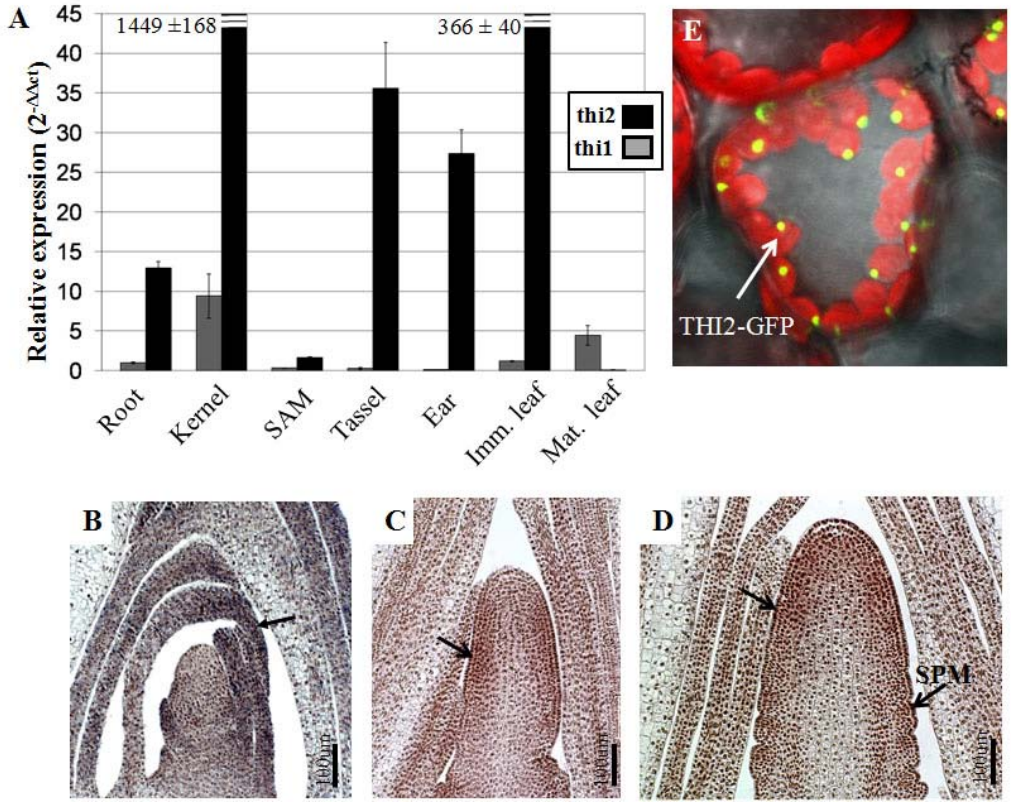


Figure 2.8: *thi2* accumulates in actively dividing tissues and encodes a plastid-targeted protein.

A. Quantitative RT-PCR reveals that the *thi1* (grey) and *thi2* (black) paralogs have distinct patterns of expression in various maize tissues. Accumulation of *thi2* in 14 DAP kernel and P2-P5 immature leaves is out of the chart range and actual values are given next to the respective bar. Threshold values were normalized with 18srRNA and are shown relative to accumulation of *thi1* in the root. Errors bars = ±SE (n=3)

B-D. RNA *in situ* hybridizations of non-mutant B73 sections using a *thi2* probe.

B. *thi* mRNA accumulates in the leaf primordia of 21 DAG SAMs. High expression is found in the distal regions (arrow) and margins of P2-P4 leaves.

C. *thi* mRNA accumulates along the flanks of the 0.5 mm tassel primordium (arrow) just prior to SPM initiation.

D. *thi* mRNA accumulates at the apex of the 1.5 mm tassel primordium and in SPMs (arrow)

E. Confocal image of THI2-GFP fusion protein after transient expression in *N. Benthamiana* leaves (Green). Chlorophyll autoflorescence is shown in red and GFP/chlorophyll fluorescence overlap is represented as yellow.

and in leaf primordia (182 fold) but is downregulated in mature leaves (74 fold decrease as compared to *thi1*).

RNA *in situ* hybridizations were used to elucidate *thi1* and *thi2* mRNA accumulation patterns in 21 DAG seedlings and in 0.5-1.5 mm tassel primordia (Figure 2.8B). Given the sequence similarity between *thi1* and *thi2*, it was not possible to generate a paralog-specific probe. Nonetheless, a 1253 base pair probe that spans the *thi2* coding region was created from a *thi2* cDNA clone. Accumulation of *thi* is high in leaf primordia, especially in the distal portions of the P2-P4 leaf primordia (Figure 2.8B). In support of qRT-PCR data, *thi* mRNA accumulation is relatively lower in the SAM. Shortly before the initiation of SPMs, a strong expression signal is found at the flanks of the tassel inflorescence meristem (Figure 2.8C). Later in tassel development, mRNA accumulation is upregulated in the developing SPM and in the inflorescence apex (Figure 2.8D).

thi2 is an indirect target of brassinosteroids

In an effort to elucidate how *thi2* expression is developmentally regulated, we treated non-mutant B73 seedlings with three phytohormones implicated during shoot development and monitored changes in *thi2* transcript accumulation by qRT-PCR. Transcript accumulation of *thi2* did not change after a four hour treatment with brassinosteroids (epi-brassinolide, BL), auxin (indole-3-acetic acid, IAA), or cytokinin (kinetin), suggesting that *thi2* is not a direct target these hormone signaling pathways (Figure 2.8). Significant differences in *abph1* transcript accumulation following BL or kinetin treatment, and in *Zmpin1a* accumulation following IAA treatment, verify that changes in gene expression occur under the experimental conditions (data not shown). Following a 24 hour BL treatment, *thi2* transcript accumulation in maize seedlings was reduced by approximately two-fold relative to *thi2* accumulation in untreated

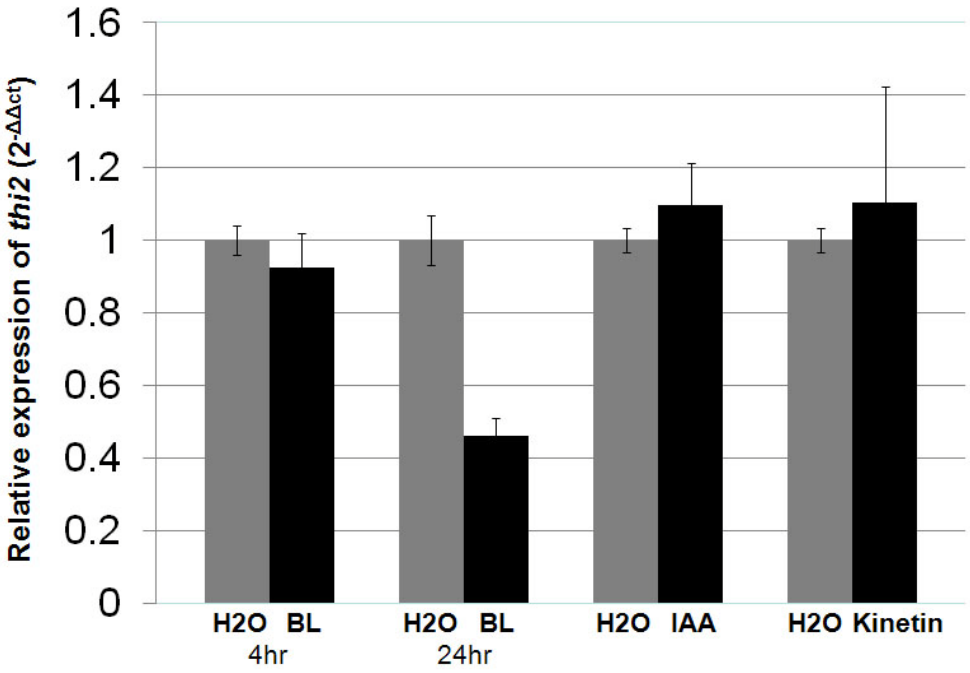


Figure 2.9: *thi2* is an indirect target of brassinosteroid signaling. qRT-PCR analyses reveal that *thi2* transcript accumulation does not change in B73 seedlings after four-hour hormone treatments (black) with auxin (IAA), cytokinin (kinetin), or brassinosteroids (epi-brassinolide, BL) when compared to control treatments without hormones (grey). *thi2* accumulation levels decrease by 2-fold, however, after a 24-hr treatment with BL. Threshold values were normalized with *tubulin6* and are shown relative to accumulation of *thi2* in control experiments. Errors bars = ±SE (n=3)

control plants (Figure 2.9), indicating that while *thi2* is not an immediate target of brassinosteroid signaling, downstream responses of BL include the downregulation of *thi2*. Similar responses to BL treatment are reported for the *Arabidopsis* ortholog *Atthi1* (Deng et al., 2007).

THI2 is a plastid-localized protein

*At*THI1 localizes to the mitochondria and chloroplasts of *Arabidopsis* (Chabregas et al., 2003). Previously, Belanger et al. (1995) utilized a THI1 antibody to show that the maize THI1 and THI2 proteins localize to plastids. To verify the subcellular localization of THI2, a THI2-GFP construct driven by the CaMV 35S promoter was transiently expressed in *Nicotiana benthamiana* leaves. Confocal imaging of infiltrated leaf sections reveals that THI2-GFP accumulates in punctate spots within the chloroplast-containing mesophyll cells (Figure 2.8E). No THI2-GFP fluorescence was observed outside of the chloroplast or in epidermal cells.

THI2 is required for normal thiamine accumulation

THI2 homologs from yeast (*Sc*THI4) and *Arabidopsis* (*At*THI1) co-purify with adenosine diphospho-5-(B-ethyl)-4-methylthiazole-2-carboxylic acid (ADT), an intermediate to the thiazole precursor of thiamine (Jurgenson et al., 2006; Godoi et al., 2006). Furthermore, the maize THI2 paralog, THI1, rescues the growth-defects of yeast thi4 mutants, suggesting a conserved function during thiamine biosynthesis (Belanger et al., 1995). To verify the function of THI2, the protein was expressed in *Escherichia coli*, purified, and analyzed for bound substrate by HPLC. Similar to *Sc*THI4, the THI2 protein co-purifies with ADT, indicating that THI2 also functions in thiazole biosynthesis (Figure 2.10A). Importantly, THI2 proteins derived from *thi2*-

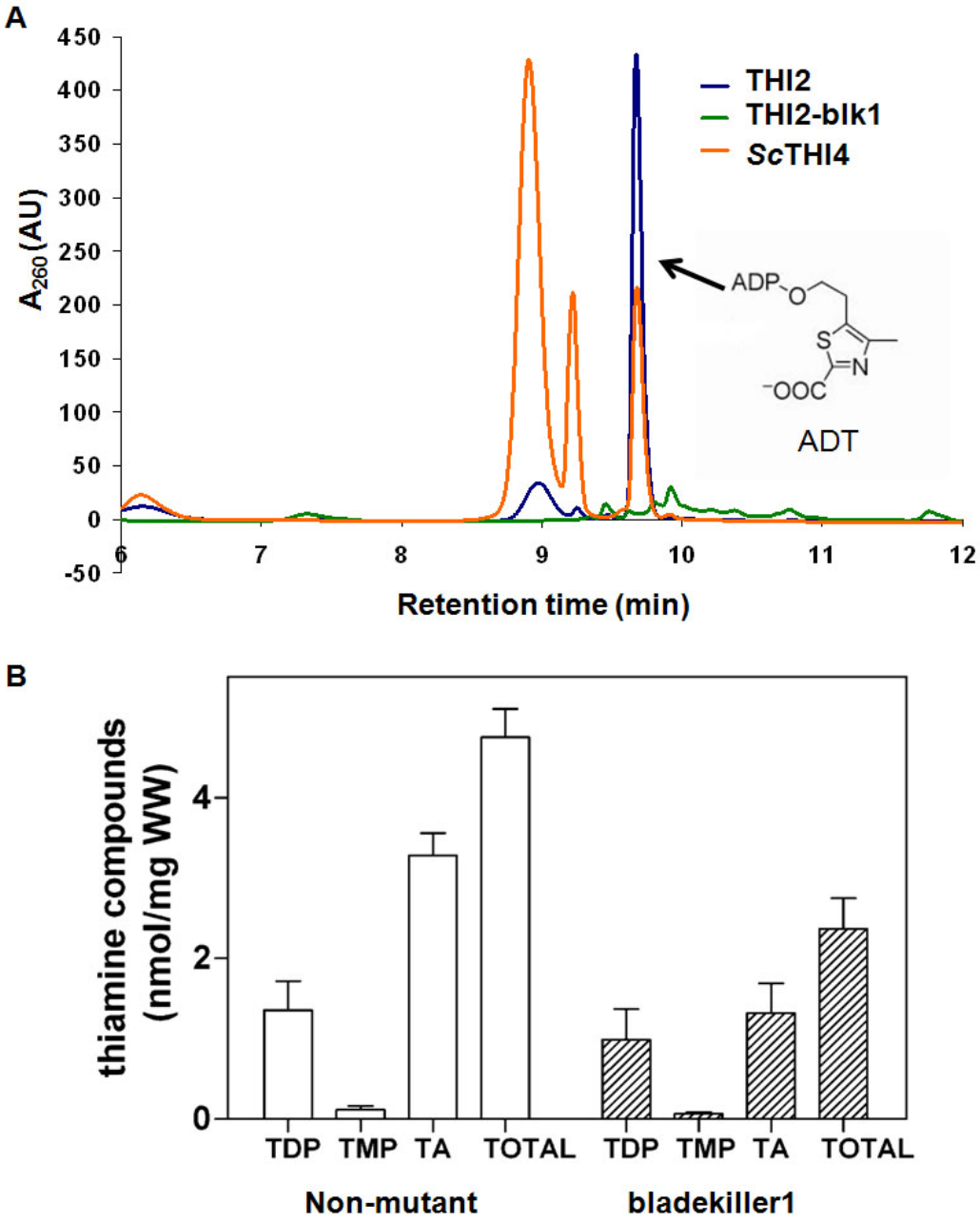


Figure 2.10: *thi2-blk1* is a null allele that conditions reduced thiamine concentrations. **A.** HPLC analysis reveals that like THI4 (orange) from *S. cerevisiae*, maize THI2 (blue) co-purifies with adenosine diphospho-5-(B-ethyl)-4-methylthiazole-2-carboxylic acid (ADT). THI2-blk1 protein (green), however, does not co-purify with bound substrate, suggesting that the enzyme is not functional.
B. HPLC analysis of thiamine-diphosphate (TDP), thiamine-monophosphate (TMP), and thiamine (TA) extracted from 12 DAG non-mutants (white) and blk1 mutants (striped) reveals that total thiamine levels are reduced in mutants. Errors bars = ±SE (n=3).

blk1 (THI2-blk1) do not purify with the ADT substrate, suggesting that the mutant protein is inactive (Figure 2.10A).

Given that THI2 functions in thiamine biosynthesis, we tested whether blk1-R mutant plants have lower thiamine content. Thiamine and its phosphorylated derivatives, thiamine-monophosphate (TMP) and thiamine-diphosphate (TDP), were extracted from 14 DAG whole seedlings and measured using HPLC. Total thiamine concentration (the sum of TMP, TDP, and thiamine) in blk1-R mutant seedlings is approximately 50% of that contained in non-mutant siblings (Figure 2.10B). The reduction in total thiamine observed in blk1-R mutant seedlings is predominantly due to decreased levels of thiamine; concentrations of TDP and TMP were not significantly reduced (Figure 2.9B).

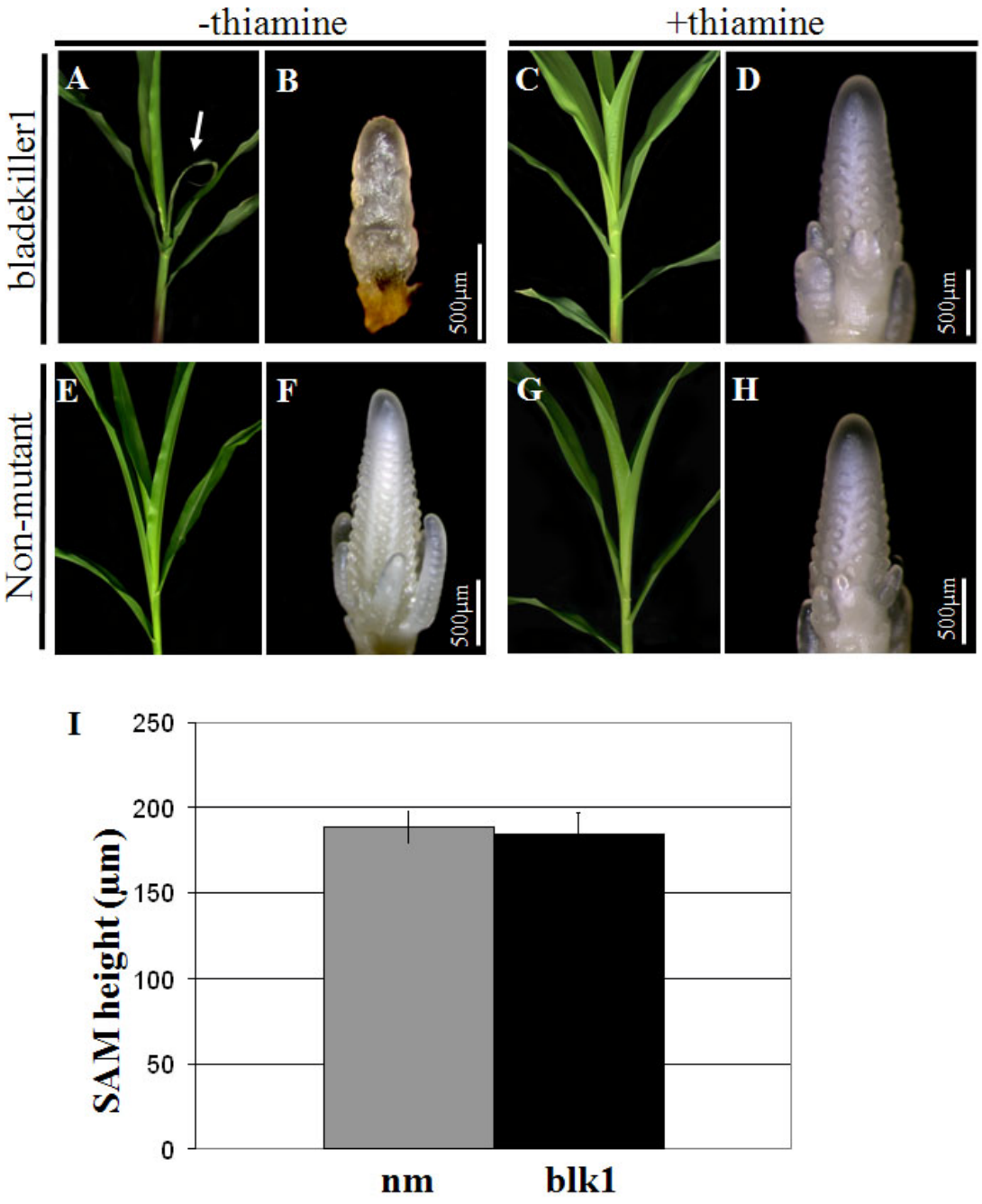
To test the hypothesis that the blk1-R mutant phenotype is caused by thiamine deficiency, plants were treated with exogenous thiamine. Thiamine treatment was sufficient to rescue all mutant phenotypes, including the defects in leaf blade development (Figure 2.11A, 2.10C), SPM initiation (Figure 2.11B, 2.11D), and SAM size maintenance (Figure 2.11I). Furthermore, 100% of the progeny derived from self-pollinated blk1-R plants treated with thiamine exhibited blk1 mutant phenotypes, verifying that the rescued plants were indeed *thi2-blk1* homozygotes. Exogenous thiamine treatment had no effect on the phenotype of non-mutant sibling plants (Figure 2.11E-I).

Discussion:

blk1-R mutants disrupt stem cell propagation outside of meristem regulatory pathways

The maize bladekiller1 (blk1) is a novel mutant, which exhibits progressive defects in meristem maintenance (Figures 2.1, 2.2, 2.7C-D). While blk1-R SAM size is not

Figure 2.11: blk1 mutants are thiamine auxotrophs and are rescued by exogenous thiamine treatment
A, B, E, and F. Untreated 32-DAG plants
C, D, G, and H. 32-DAG plants treated with 30 mM thiamine-HCL solution
A. Untreated blk1 mutant showing a reduced leaf-blade (arrow)
B. Aborted shoot apex dissected from blk1 mutant in **(A)**
C. Rescued blk1 mutant exhibiting no mutant phenotypes.
D. Rescued immature tassel primordium from blk1 mutant **(C)** showing normal SPM and branch initiation.
E. Non-mutant seedling showing normal leaf development
F. Non-mutant tassel primordia exhibiting normal development maintenance
G. Thiamine treatment has no obvious effect on the development of non-mutants.
H. Thiamine treatment has no obvious effect on the development of the non-mutant tassel.
I. Thiamine supplementation alleviates the blk1 meristem size defect found at 21-DAG. Error bars = ±SE (n=9)


-thiamine
+thiamine
bladekiller1
Non-mutant
A
B
C
D
E
F
G
H
500µm
I
SAM height (µm)
250
200
150
100
50
0
nm
blk1

changed at seven DAG, meristem height is significantly reduced at 21 DAG (Figure 2.1). Furthermore, blk1-R tassel and ear primordia are truncated and deficient in axillary meristem initiation. Several markers with known roles in meristem maintenance are misregulated in the blk1 SAM, lateral meristems, and inflorescences (Figures 2.4, 2.5, 2,6), indicating that THI2 function is necessary for maintaining shoot meristem size and indeterminacy in maize.

Meristem maintenance in *Arabidopsis* is partially regulated by negative-feedback interactions between the stem-cell promoting *wuschel* (*wus*) gene and the stem-cell repressing *clavata* (*clv1-3*) genes (Fletcher et al., 1999). The maize homologs of *clv1* and *clv2*, *tassel dwarf1* and *fasciated ear2* respectively, are required for repressing the size of maize inflorescence meristems (Bommert et al., 2005; Taguchi-Shiobara et al., 2001). Double mutant analyses revealed that *thi2* function is epistatic to *td1* and *fea2*, suggesting that THI2 is required to propagate the stem-cell niche upstream of TD1 and FEA2 function.

WUS functions during shoot meristem maintenance in *Arabidopsis* by repressing *type-A Arabidopsis response regulato*rs (*type-A arr*s), which are negative regulators of cytokinin signaling (Leibfried et al. 2005). The maize gene *aberrant phyllotaxy1* (*abph1*) is a *type-A arr* homolog that regulates shoot meristem size and contributes to phyllotaxy (Jackson and Hake, 1999). Loss of ABPH1 function conditions decussate phyllotaxy in a small proportion of abph1 single mutants (Figure 2.3B), a phenotype that is induced during embryonic development from an enlarged shoot apical meristem (Jackson and Hake 1999). The blk1-R single mutants do not exhibit SAM size phenotypes at seven DAG, which initially suggested that THI2 function is not necessary early in development. However, none of the blk1-R;abph1 double mutants exhibited decussate phyllotaxy, which may suggest that normal THI2 function is required to reach the threshold SAM size for the initiation of decussate

phyllotaxy in abph1 mutants. At three weeks after germination, blk1-R;abph1 SAMs are significantly shorter than the SAMs of non-mutant siblings and approximately the same height as blk1-single mutants, indicating that THI2 function is epistatic to ABPH1 later in development.

The progressive nature of the blk1-R phenotypes prompted the question of whether THI2 function is linked to phase change. To address this, we made double mutants with blk1-R and Teopod1, a mutant with prolonged expression of the juvenile program (Poethig, 1988). The blk1-R;Tp1 double mutants exhibit additive phenotypes with increased tillering in the basal nodes and blk1-R leaf-reduction phenotypes in more apical nodes. Since the onset of blade-reduction in double mutants occurs at the same position (L8) along the stem as in blk1-R single mutants, we conclude that THI2 function is not correlated with phase change.

blk1-R* is a null allele of *thiamine biosynthesis2

Positional cloning revealed that mutations to *thiamine biosynthesis2* cause blk1 phenotypes (Figure 2.7). The predicted THI2 amino acid sequence has homology to enzymes that generate the thiazole precursor to the B vitamin thiamine (Belanger et al., 1995). Accordingly, the THI2 protein co-purifies with an intermediate in the thiazole biosynthetic pathway (Figure 2.10A), suggesting a conserved role in thiamine biosynthesis. Both biochemical analyses and genetic dosage analyses reveal that *thi2-blk1* is a null mutation. Plants homozygous for *thi2-blk1* are phenotypically equivalent to hypoploid *thi2-blk1*/- individuals harboring a single dose of the *thi2-blk1* mutation. Moreover, the THI2-blk1 protein does not purify with a thiazole intermediate (Figure 2.10A), indicating that the mutant enzyme is not functional. Loss of THI2 function conditions lower thiamine concentrations in blk1-R mutant plants (Figure 2.10B) and exogenous thiamine application complements all blk1-R mutant

phenotypes (Figure 2.11). Taken together, these data reveal that the blk1-R mutant is a thiamine auxotroph of maize.

Non-overlapping functions of the maize THI2 and THI1 paralogs

Belanger et al. (1995) confirmed that the nearly identical THI2 paralog THI1 can restore thiamine prototrophy in yeast. Despite their shared function in thiamine biosynthesis, THI1 and THI2 are not functionally redundant, as evidenced by the blk1 mutant phenotypes and by the distinct accumulation patterns of *thi1* and *thi2* transcripts (Figure 2.8A). Accumulation of *thi2* transcripts is much higher than *thi1* in developing kernels, SAMs, immature leaves, and tassel and ear primordia but relatively lower in green leaves. These differences in transcript accumulation reflect the subfunctionalization of the maize THI paralogs, such that THI2 is heavily utilized in young, rapidly-dividing tissues and THI1 predominantly functions in mature green leaves.

Functional overlap with the *thi1* paralog during early development, however, may account for the lack of seedling phenotypes in blk1 mutants. Concordantly, transcript accumulation of *thi1* is relatively high during embryonic stages when compared to its accumulation in other tissues (Figure 2.8A). In addition, Shimamoto and Nelson (1981) have proposed that thiamine stores transferred to the developing kernel from maternal cob tissue may be utilized by the germinating seedling.

Development of the leaf-blade is progressively diminished in blk1 mutants, while the leaf-sheath is unaffected (Figure 2.2B). We speculate that during the development of the leaf, the blade may have higher thiamine demands than the sheath. In support of this model, *in situ* hybridization analyses reveal that *thi* expression is much higher in the distal portions of the maize leaf primordia than in the proximal regions (Figure 2.8B). Extending this model, the thiamine pool in blk1 mutant

embryos may be sufficient for normal development of the basal-most leaves. However as the thiamine pool is diminished in germinating seedlings due to the lack of THI2 activity, subsequent leaves would become increasingly thiamine deficient, leading to defects in blade development. Sheath development, on the other hand, may require less thiamine, and thus is not affected by the loss of THI2 function. Analyses of thi1 mutants and blk1;thi1 double mutants will help clarify the importance of thiamine during maize shoot development, and will determine the gene-specific functions of these maize *thi* paralogs.

Thiamine is a regulator of stem cell activity

A role of thiamine in maintaining meristematic tissue was first demonstrated in plant tissue culture (White, 1951, Wightman and Brown, 1952, Torrey, 1953). Excised pea root tips containing only undifferentiated tissue require thiamine for continued meristem activity (Wightman and Brown, 1953). A similar *in planta* result is first reported here, in that thiamine deficiency in blk1 mutant plants leads to defects in shoot meristem maintenance (Figure 2.1A-C). The axillary meristems that elaborate the tassel and ear are inflorescences are also sensitive to thiamine deficiency (Figure 2.1D-K). Developing mutant tassels fail to upregulate *kn1* and *Zm*PIN1a expression where spikelet pair meristems normally initiate (Figure 2.5E-H). Interestingly, *thi2* expression is upregulated at the sites of SPM formation (Figure 2.8D), suggesting that the initiation of these meristematic regions correlates with high thiamine concentrations.

Thiamine function during the maintenance of proliferative stem/initial cell growth may be universally conserved. A number of studies utilizing animal models have associated thiamine accumulation with tumor growth. Thiamine is an essential cofactor for transketolase function, and the proliferation of tumor cells requires the

non-oxidative, transketolase-dependent pentose phosphate pathway for ribose synthesis (Boros et al., 1998). The use of transketolase inhibitors such as the thiamine analog oxythiamine leads to significant decreases in tumor proliferation (Comín-Anduix et al., 2001). Moreover, supplementing tumors with thiamine leads to transketolase activation and increases in tumor growth (Comín-Anduix et al., 2001). Although it is not known whether the effects of thiamine on meristem maintenance in maize are mediated by a transketolase-based mechanism, the inhibition of thiamine function blocks the proliferative growth of the undifferentiated stem cell populations that comprise both cancer cells in animals and meristematic cells in plants.

The characterization of the thiamine auxotroph blk1 and the analysis of *thi2* present a novel approach to the understanding of meristem maintenance and expand on the otherwise minimal knowledge of the role of metabolic products in meristem function. Our data emphasize that a complete understanding of the mechanisms regulating the shoot meristem must incorporate non-classical but vital, metabolic cofactors such as thiamine.

Materials and methods:

Plant material and treatments

The *bladekiller1* allele of *thi2* was generated by ethylmethane sulfonate (EMS) treatment of maize pollen and was crossed onto non-mutant individuals in an unknown genetic background. Heterozygous *thi2-blk1/+* individuals were backcrossed at least nine times into the B73 inbred background and five times into the W22 and W23 inbred lines. Genetic analyses were conducted in Ithaca, NY or in Juana Diaz, Puerto Rico. . Double mutants were made by crossing *thi2-blk1* heterozygotes to plants homozygous for *abphl1-O* (Jackson and Hake 1999), *td1-glf* (Bommert et al., 2005), and *fea2-O* (Taguchi-Shiobara et al., 2001) or heterozygous for *Tp1* (Poethig, 1988),

and self-fertilizing the F1 progeny. For molecular studies and phenotypic analysis, plants were grown under greenhouse conditions at Cornell University in Ithaca, NY. For hormone treatments, 14 day-after-germination (DAG) plants were cut at the root/shoot junction and placed in solutions for 4 or 24 hrs as described by Lee and Johnston et al. (2009). 10 mM Kinetin (Sigma), indole-3-acetic acid (Sigma), or epi-brassinolide (Sigma) stocks were made by dissolving in 1M KOH (IAA, Kinetin) or 80% EtOH (epi-brassinolide). The hormones were diluted to 100 nM in water and each solution was brought to pH 5.8 using HCl. Control solutions were made by adding equivalent volumes of 1M KOH or 80% EtOH and pH adjustment. Following treatment, shoot apices were dissected until 8 leaves remained covering the SAM and these samples were frozen under liquid nitrogen. Thiamine rescue experiments were completed by the twice weekly application of 100 ml of a 30 mM solution of thiamine-HCL (Sigma, St. Louis) in water to the soil of growing plants.

Histological analysis and SEM

Maize apices were hand-dissected from 7 DAG or 21 DAG plants, fixed in 3.7% FAA, paraffin embedded, and 10 µm sections were stained with 0.05% toluidine blue as described (Ruzin, 1999). All micrographs were imaged on a Zeiss Z1-Apotome microscope and meristem measurements were made using Zeiss Axiovision software, release 4.6 (Thornwood, NY).

For SEM analysis, samples were hand-dissected and immediately frozen in a nitrogen slush. The samples were then moved to a BalTec cryopreparation chamber (BalTec, Technotrade, New Hampshire) attached to a Hitachi S4500 Scanning Electron Microscope (Hitachi, Pleasantville, CA), and sputter coated with 30 nm of platinum at -150 degrees C and a vacuum pressure of 2 X 10-2 Torr under argon. The coated samples were then viewed at -163 degrees C on the SEM stage using 5 KV.

Table 2.1: Primers used in this study

	Forward Primer	**Reverse Primer**
THI2 Localization		
thi2_gfp	ATGGCCACCACCGCCGCGTCCA	GGCGTCCACGACCTCGTCGTCC
qRT-PCR		
tub6	TGAGTTCGGTGTTCCCTTTC	TTAAATTCACGCCAGGAACC
18srRNA	CTGTCGGCCAAGGCTATAGACT	TCTGTGATGCCCTTAGATGTTCTG
Zmpin1a	AGGGCAAGGCGAACAAGTA	GTAGGTCCTTGCCGTCCTC
Zmlog1	GGGATCGAGGATGACAGCTA	AAAAGCCCAAAAGGAGCAGT
abphl_qrt	TGTCCTCAGAGAACGTGCCG	GACGGGCTTCAGCAGGAAAT
thi1_qrt	TGCCTTGTTGTTCAATGATGA	GTGGTGGTGCTATGAACACG
thi2_qrt	TGGGACTTTGTTGTTGTTGG	GTAGCTGTGGCATGGTGCTA
Zmwus1	AAGCTGCCACTGCTCTGAAT	GCCTTCCTTCACACATGCTT
kn1_qrt	GGCACTGGCTGAGTCTAC	CTCCTCGGATGGCTTCCAGT
td1	CCGCTAAATAGATCACAGG	CCTTGTGCAAGGTCGTCGAT
ns1	CTCCAGCTGCCGCCATG	TGGATGTGGAGCAAGAGGAGG
RNA *in situ* hybridization		
kn1_insitu	ACAAGGTGGGGGCACCA	TCGGTCTCTCCTCCGCTA
abph1_insitu	GCTCGTGTCGTTTCTGAAG	CTTGTCGGCACGTTCTCTG
Positional Cloning		
unk7	ACAACGACGAAAGATTTGCTG	CCTGCTGGGAGGTCGTACT
IDP351	CTTCCACAAATAGGGTTCGC	TAACACAGGACGACCTTCGG
BE_NO5	TGGAAGGTCTGACTGCCTTT	TCATGAGCTGTTGGAGAACG
thi2_cdna	CCCTAGCTCTCGTTGTCAGC	TGCTACACGAACACGACACA

Mapping and cloning of *thi2-blk*

B-A translocation stocks were obtained from the Maize Genetics COOP Stock Center. Plants hyperploid for TB-3La and heterozygous for the endosperm marker *a1* were crossed onto *thi2-blk1* heterozygous plants to generate individuals hypoploid for much of the long arm of chromosome 3 (described in Scanlon, 1994). An F2 mapping population was generated by crossing *thi2-blk1* heterozygotes introgressed into B73 nine times onto the Mo17 inbred. Known SSR and INDEL markers for 3L were available at maizegdb.org and used to delimit the mapping interval. Sequence available at maizesequence.org and magi.plantgenomics.iastate.edu was used identify candidate genes and build primers for amplifying genomic regions. Sequence polymorphisms between MO17 and the *thi2-blk1* progenitor were used to design CAPS markers that further narrowed the mapping interval (Table 2.1). A *thi2* (GRMZM2G074097) product was amplified by PCR using thi2-cDNA primers, which flank the predicted coding region (Table 2.1).

THI2 localization

A *thi2* PCR product was made using the thi2-gfp primers (Table 2.1) and was subsequently cloned into the pENTR vector (Invitrogen.com). Recombination with the pEarleyGate103 gateway vector (Early et al., 2006) allowed a C-terminal fusion of THI2 with GFP. The resultant vector was electroporated into agrobacterium strain C58C1 and cultures were grown overnight in LB media containing 50 ug/ml kanamycin and 50 ug/ml tetracycline. Pelleted cells were resuspended in 10 mM MgCl2 to an OD600 of 0.2. This suspension was then used to infiltrate 5 week-old *Nicotiana Benthamiana* plants that were grown under fluorescent lights. THI2-GFP was visualized in leaf discs under water approximately 3 days after infiltration.

Confocal Microscopy

To generate blk1-R mutants expressing *Zm*PIN1a-YFP, *thi2-blk1* heterozygotes were crossed with *Zm*PIN1a-YFP transgenic individuals and then self-fertilized (Galavotti et al., 2008). SAMs and immature tassels were uncovered by hand-dissection and visualized in water.

Images of fluorescent proteins were collected on a Leica TCS-SP5 confocal microscope (Leica Microsystems, Exton, PA USA) using a 10x or 20x objective (NA 0.4 or 0.7 respectively). YFP was excited with an argon-ion laser (514 nm), and emitted light was collected from 517 nm to 564 nm. GFP was excited with the argon laser (488 nm), and emitted light was collected between 498 nm and 510 nm. Chloroplasts were excited with the argon laser (488 nm), and emitted light was collected from 610 nm to 669 nm. The GFP and chloroplast signals were collected separately and later superimposed. Light images were collected simultaneously with the fluorescence images using the transmitted light detector. Images were processed using Leica LAS-AF software (version 1.8.2) and Adobe Photoshop CS2 version 9.0.2 (Adobe systems).

qRT-PCR and *in situ* hybridizations

All transcript accumulation experiments were performed in the B73 inbred background or with mutants introgressed into B73 at least nine times. Laser-capture microdissection and subsequent RNA preparation was completed as outlined in (Zhang et al., 2007 and Ohtsu et al., 2007). In brief, dissected apices from 21 DAG seedlings and lateral meristems that were still producing husk leaves at presumptive ear nodes were acetone-fixed, paraffin-embedded, sectioned at 10 µm, and laser-capture microdissected. Five meristems were captured and pooled per biological replicate before RNA extraction and T7-based amplification. Total RNA was extracted

from 20 days-after-pollination endosperm and embryo, 21 DAG roots and immature non-green leaves (P2-P6), 2-4 mm ear and tassel primordia, and fully-expand adult leaves (L12) using TRIzol (Invitrogen). For all replicates, 1 μg of RNA was treated with DNase and used in a SuperscriptIII-based cDNA synthesis (Invitrogen.com). The qRT-PCR analyses were performed with SYBR-green methodology and gene-specific primers (Table 2.1) as in Zhang et al. (2007). Three biological replicates for each genotype or tissue type were used during every experiment. Data is presented using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) with threshold values normalized to levels of *beta-6-tubulin* (LCM data) or 18srRNA (tissue panel).

Mutant and non-mutant 21 DAG or 28-35 DAG seedlings were processed for *in situ* hybridizations as described (Jackson, 1991) and with modifications (Long, 1996). A minimum of 5 replicates for each probe was used to get a consensus pattern of expression. Primers for probe construction are listed in Table 2.1.

REFERENCES

Addicott, F. (1939). Vitamin B 1 in Relation to Meristematic Activity of Isolated Pea Roots. Botanical Gazette **100,** 836-843.

Barton, M. (2009). Twenty years on: The inner workings of the shoot apical meristem, a developmental dynamo. Developmental Biology **341,** 95-113.

Bassiri, A., Irish, E., and Poethig, R. (1992). Heterochronic effects of Teopod 2 on the growth and photosensitivity of the maize shoot. The Plant Cell Online **4,** 497-504.

Bayer, E., Smith, R., Mandel, T., Nakayama, N., Sauer, M., Prusinkiewicz, P., and Kuhlemeier, C. (2009). Integration of transport-based models for phyllotaxis and midvein formation. Genes & Development **23,** 373-384.

Beckett, J. (1978). BA translocations in maize: I. Use in locating genes by chromosome arms. Journal of Heredity **69,** 27-36.

Belanger, F., Leustek, T., Chu, B., and Kriz, A. (1995). Evidence for the thiamine biosynthetic pathway in higher-plant plastids and its developmental regulation. Plant Molecular Biology **29,** 809-821.

Bennett, S., Alvarez, J., Bossinger, G., and Smyth, D. (2003). Morphogenesis in pinoid mutants of *Arabidopsis thaliana*. The Plant Journal **8,** 505-520.

Bolduc, N., and Hake, S. (2009). The maize transcription factor KNOTTED1 directly regulates the gibberellin catabolism gene ga2ox1. The Plant Cell Online **21,** 1647-1658.

Bommert, P., Lunde, C., Nardmann, J., Vollbrecht, E., Running, M., Jackson, D., Hake, S., and Werr, W. (2005). thick tassel dwarf1 encodes a putative maize ortholog of the *Arabidopsis* CLAVATA1 leucine-rich repeat receptor-like kinase. Development **132,** 1235-1245.

Boros, L., Brandes, J., Lee, W., Cascante, M., Puigjaner, J., Revesz, E., Bray, T., Schirmer, W., and Melvin, W. (1998). Thiamine supplementation to cancer patients: a double edged sword. Anticancer Research **18,** 595-602.

Bortiri, E., and Hake, S. (2007). Flowering and determinacy in maize. Journal of Experimental Botany **58,** 909-916.

Brown, R., and Wightman, F. (1952). The effect of mature tissue on division in the apical meristem of the root. Journal of Experimental Botany **3,** 253.

Byrne, M., Timmermans, M., Kidner, C., and Martienssen, R. (2001). Development of leaf shape. Current Opinion in Plant Biology **4,** 38-43.

Chabregas, S., Luche, D., Van Sluys, M., Menck, C., and Silva-Filho, M. (2003). Differential usage of two in-frame translational start codons regulates subcellular localization of *Arabidopsis thaliana* THI1. Journal of Cell Science **116,** 285-291.

Comin-Anduix, B., Boren, J., Martinez, S., Moro, C., Centelles, J., Trebukhina, R., Petushok, N., Lee, W., Boros, L., and Cascante, M. (2001). The effect of thiamine supplementation on tumour proliferation. European Journal of Biochemistry **268,** 4177-4182.

Deng, Z., Zhang, X., Tang, W., Oses-Prieto, J., Suzuki, N., Gendron, J., Chen, H., Guan, S., Chalkley, R., and Peterman, T. (2007). A proteomics study of brassinosteroid response in *Arabidopsis*. Molecular & Cellular Proteomics **6,** 2058-2071.

Earley, K., Haag, J., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. Plant Journal **45,** 616-629.

Feenstra, W. (1964). Isolation of nutritional mutants in *Arabidopsis thaliana*. Genetica **35,** 259-269.

Fleming, A. (2006). Metabolic aspects of organogenesis in the shoot apical meristem. Journal of Experimental Botany **57,** 1863-1870.

Fletcher, J., Brand, U., Running, M., Simon, R., and Meyerowitz, E. (1999). Signaling of cell fate decisions by CLAVATA3 in *Arabidopsis* shoot meristems. Science **283,** 1911-1914.

Gallavotti, A., Yang, Y., Schmidt, R., and Jackson, D. (2008). The relationship between auxin transport and maize branching. Plant physiology **147,** 1913-1923.

Giulini, A., Wang, J., and Jackson, D. (2004). Control of phyllotaxy by the cytokinin-inducible response regulator homologue ABPHYL1. Nature **430,** 1031-1034.

Godoi, P., Galhardo, R., Luche, D., Van Sluys, M., Menck, C., and Oliva, G. (2006). Structure of the thiazole biosynthetic enzyme THI1 from *Arabidopsis thaliana*. Journal of Biological Chemistry **281,** 30957-30966.

Heisler, M., Ohno, C., Das, P., Sieber, P., Reddy, G., Long, J., and Meyerowitz, E. (2005). Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. Current Biology **15,** 1899-1911.

Hudson, A. (2000). Development of symmetry in plants. Annual Review of Plant Biology **51,** 349-370.

Jackson, D. (1991). *In situ* hybridization in plants. Molecular Plant Pathology: A Practical Approach **1,** 163-174.

Jackson, D., and Hake, S. (1999). Control of phyllotaxy in maize by the abphyl1 gene. Development **126,** 315-323.

Jackson, D., Veit, B., and Hake, S. (1994). Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development **120,** 405-413.

Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P., and Tsiantis, M. (2005). KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. Current Biology **15,** 1560-1565.

Jurgenson, C., Chatterjee, A., Begley, T., and Ealick, S. (2006). Structural Insights into the Function of the Thiamin Biosynthetic Enzyme Thi4 from *Saccharomyces cerevisiae*. Biochemistry **45,** 11061-11070.

Kiesselbach, T. (1949). The structure and reproduction of corn. Univ. Nebraska Coll. Agric. Bull **161**.

Koornneef, M., and Hanhart, C. (1981). A new thiamine locus in *Arabidopsis*. *Arabidopsis* Information Server **18,** 52-58.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., and Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445,** 652-655.

Langridge, J. (1955). Biochemical Mutations in the Crucifer *Arabidopsis thaliana* (L.) Heynh. Nature **176,** 260-261

Lee, B., Johnston, R., Yang, Y., Gallavotti, A., Kojima, M., Travencolo, B., Costa, L., Sakakibara, H., and Jackson, D. (2009). Studies of aberrant phyllotaxy1 mutants of maize indicate complex interactions between auxin and cytokinin signaling in the shoot apical meristem. Plant Physiology **150,** 205-216.

Leibfried, A., To, J., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J., and Lohmann, J. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438,** 1172-1175.

Li, S., and Redei, G. (1969). Thiamine mutants of the crucifer, *Arabidopsis*. Biochemical Genetics **3,** 163-170.

Livak, K., and Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. Methods **25,** 402-408.

Long, J., Moan, E., Medford, J., and Barton, M. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. Nature **379,** 66-69

McSteen, P., and Hake, S. (2001). barren inflorescence2 regulates axillary meristem development in the maize inflorescence. Development **128,** 2881-2891.

McSteen, P., Malcomber, S., Skirpan, A., Lunde, C., Wu, X., Kellogg, E., and Hake, S. (2007). barren inflorescence2 encodes a co-ortholog of the PINOID serine/threonine kinase and is required for organogenesis during inflorescence and vegetative development in maize. Plant Physiology **144,** 1000-1011.

Muller, H. (1932). Further studies on the nature and causes of gene mutations, Proceedings of the 6th International Congress of Genetics, 213–255.

Nardmann, J., and Werr, W. (2006). The shoot stem cell niche in angiosperms: expression patterns of WUS orthologues in rice and maize imply major modifications in the course of mono-and dicot evolution. Molecular Biology and Evolution **23,** 2492-2504.

Nardmann, J., Ji, J., Werr, W., and Scanlon, M. (2004). The maize duplicate genes narrow sheath1 and narrow sheath2 encode a conserved homeobox gene function in a lateral domain of shoot apical meristems. Development **131,** 2827-2839.

Ohtsu, K., Smith, M., Emrich, S., Borsuk, L., Zhou, R., Chen, T., Zhang, X., Timmermans, M., Beck, J., and Buckner, B. (2007). Global gene expression analysis of the shoot apical meristem of maize (Zea mays L.). The Plant Journal **52,** 391-404.

Okada, K., Ueda, J., Komaki, M., Bell, C., and Shimura, Y. (1991). Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. The Plant Cell Online **3,** 677-684.

Papini-Terzi, F., Galhardo, R., Farias, L., Menck, C., and Van Sluys, M. (2003). Point mutation is responsible for *Arabidopsis* tz-201 mutant phenotype affecting thiamin biosynthesis. Plant and Cell Physiology **44,** 856-860.

Poethig, R. (1988). Heterochronic mutations affecting shoot development in maize. Genetics **119,** 959-973.

Poethig, R. (1990). Phase change and the regulation of shoot morphogenesis in plants. Science **250,** 923-930.

Poethig, S. (1984). Cellular parameters of leaf morphogenesis in maize and tobacco. White, R, A,, Dickison, W, C ed (s). Contemporary Problems in Plant Anatomy. Academic Press: Orlando, etc**,** 235-259.

Redei, G. (1965). Genetic blocks in the thiamine synthesis of the angiosperm *Arabidopsis*. American Journal of Botany **52,** 834-841.

Reinhardt, D., Mandel, T., and Kuhlemeier, C. (2000). Auxin regulates the initiation and radial position of plant lateral organs. The Plant Cell Online **12,** 507-518.

Reinhardt, D., Pesce, E., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., and Traas, J. (2003). Regulation of phyllotaxis by polar auxin transport. Nature **426,** 255-260.

Ruzin, S. (1999). Plant microtechniques and microscopy. New York. 322 pg.

Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S., and Matsuoka, M. (2001). KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. Genes & Development **15,** 581-590.

Satoh-Nagasawa, N., Nagasawa, N., Malcomber, S., Sakai, H., and Jackson, D. (2006). A trehalose metabolic enzyme controls inflorescence architecture in maize. Nature **441,** 227-230.

Scanlon, M. (2003). The polar auxin transport inhibitor N-1-naphthylphthalamic acid disrupts leaf initiation, KNOX protein regulation, and formation of leaf margins in maize. Plant Physiology **133,** 597-605.

Scanlon, M., Schneeberger, R., and Freeling, M. (1996). The maize mutant narrow sheath fails to establish leaf margin identity in a meristematic domain. Development **122,** 1683-1691.

Scanlon, M., Stinard, P., James, M., Myers, A., and Robertson, D. (1994). Genetic analysis of 63 mutations affecting maize kernel development isolated from Mutator stocks. Genetics **136,** 281-294.

Shimamoto, K., and Nelson, O. (1981). Movement of 14C-compounds from maternal tissue into maize seeds grown in vitro. Plant Physiology **67,** 429-432.

Taguchi-Shiobara, F., Yuan, Z., Hake, S., and Jackson, D. (2001). The fasciated ear2 gene encodes a leucine-rich repeat receptor-like protein that regulates shoot meristem proliferation in maize. Genes & Development **15,** 2755-2766.

Torrey, J. (1954). The Role of Vitamins and Micronutrient Elements in the Nutrition of the Apical Meristem of Pea Roots. Plant Physiology **29,** 279-287.

Veit, B., Briggs, S., Schmidt, R., Yanofsky, M., and Hake, S. (1998). Regulation of leaf initiation by the terminal ear 1 gene of maize. Nature **393,** 166-168.

Vollbrecht, E., Reiser, L., and Hake, S. (2000). Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, knotted1. Development **127,** 3161-3172.

White, P. (1951). Nutritional requirements of isolated plant tissues and organs. Annual Review of Plant Physiology **2,** 231-244.

Wightman, F., and Brown, R. (1953). The effects of thiamin and nicotinic acid on meristematic activity in pea roots. Journal of Experimental Botany **4,** 184-196.

Wu, X., and McSteen, P. (2007). The role of auxin transport during inflorescence development in maize (Zea mays, Poaceae). American Journal of Botany **94,** 1745-1755.

Wu, X., Dabi, T., and Weigel, D. (2005). Requirement of homeobox gene STIMPY/WOX9 for *Arabidopsis* meristem growth and maintenance. Current Biology **15,** 436-440.

Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A., and Ori, N. (2005). *Arabidopsis* KNOXI proteins activate cytokinin biosynthesis. Current Biology **15,** 1566-1571.

Zhang, X., Madi, S., Borsuk, L., Nettleton, D., Elshire, R., Buckner, B., Janick-Buckner, D., Beck, J., Timmermans, M., and Schnable, P. (2007). Laser microdissection of narrow sheath mutant maize uncovers novel gene expression in the shoot apical meristem. Plos Genetics **3,** 1040-1052.

CHAPTER 3

Summary

The shoot apical meristem (SAM) serves as the organogenic center for the shoot, and thus it must be maintained for continued plant development. The characterization of mutants with defects in meristem stability has elucidated multiple mechanisms contributing to SAM maintenance. In this study, the maize mutant bladekiller1 (blk1) was used to investigate the mechanisms regulating meristem maintenance in maize. Plants homozygous for the *thi2-blk1* mutant allele do not maintain shoot apical meristem (SAM) size. Additionally, blk1 mutants have reduced inflorescences due to defects in inflorescence and axillary meristem maintenance.

The blk1 mutants also exhibit a novel leaf-blade truncation phenotype, wherein successive leaves from the shoot base to the tassel exhibit a progressive truncation of leaf blade tissue. A number of genes are implicated in proximodistal patterning of the maize leaf. The liguleless1 and liguleless2 mutants and dominant Knox mutants, for example, have disrupted blade/sheath boundaries (reviewed by Foster and Timmermans, 2009). However, blk1 is the first characterized maize mutant with a specific deletion of the leaf blade.

Positional cloning revealed that mutations to *thiamine biosynthesis2* (*thi2)* confer the blk1 mutant phenotypes. The *thi2* gene is homologous to thiamine biosynthetic genes in *Arabidopsis* and yeast (Belanger et al., 1995), and biochemical analyses demonstrated that THI2 has a conserved function in the biosynthesis of the thiazole precursor to thiamine. Plants homozygous for *thi2-blk1* have lower thiamine concentrations than non-mutant siblings and the blk1 mutant phenotypes are completely rescued by exogenous thiamine application, revealing that blk1 mutants are thiamine auxotrophs. This finding is especially interesting given that few auxotrophic mutants have been described in plants. Moreover, thiamine auxotrophs in *Arabidopsis* and *Nicotiana* die after the production of several albino leaves, which highlights species specific differences in thiamine regulation.

The *thi2* gene may have partial functional redundancy with the nearly identical paralog *thiamine biosynthesis1* (*thi1*), which would account for normal development in blk1 mutants during early stages of growth. The progressive blk1 phenotypes, however, suggest that *thi2* and *thi1* have non-overlapping functions later in maize development. Concordantly, *thi1* and *thi2* have distinct accumulation levels in various maize tissues, supporting the notion that these paralogs have subfunctionalized. This developmental regulation of thi transcripts may be downstream of brassinosteroid signaling. It will be interesting to analyze thi1 mutants and blk1;thi1 double mutants to elucidate the gene-specific functions of these maize *thi* paralogs.

The analysis of *thi2* function supports a model whereby THI2-catalyzed thiamine synthesis in leaf primordia is necessary for shoot meristem perpetuation and leaf-blade outgrowth. While *thi1* and *thi2* transcripts are relatively low the SAM as measured by qRT-PCR and RNA *in situ* hybridizations, they are abundant in developing leaf primordia. Moreover, thiamine supplementation is a requirement for continued meristem activity in shoot culture, suggesting that it must be imported from external sources for meristem stability (Addicott, 1939; Brown and Wightman, 1952, 1953). Thiamine generated in a THI2-based biosynthetic pathway is likely transported from leaf primordia into the meristem, where it is utilized to sustain indeterminate meristematic growth (Figure 3.1). Loss of THI2 function in blk1 mutants conditions thiamine deficiency at the SAM and the progressive loss of meristematic cells.

This thiamine pool is also necessary for leaf-blade outgrowth as illustrated by the blk1 leaf-reduction phenotype. We speculate that the blade has higher thiamine demands than the sheath during leaf development. This is supported by the fact that *thi* mRNA accumulates in more distal regions of P2-P5 leaf primordia, time points which correlate with the clonal isolation of the blade and sheath (Poethig and Szymkowiak, 1995) and formation of the ligule at the blade/sheath boundary

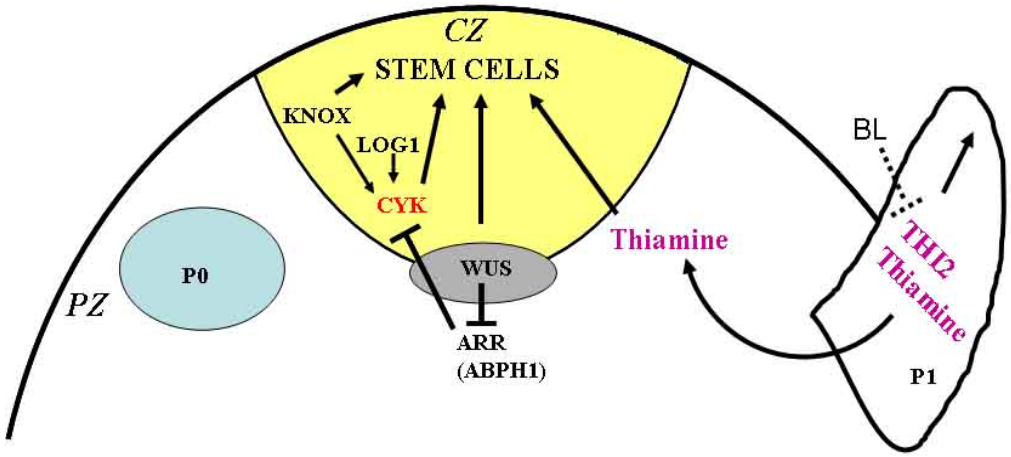


Figure 3.1: THI2 function is necessary to generate the thiamine pool that sustains the meristem and contributes to leaf blade outgrowth. The blk1 mutant has a smaller SAM with reduced expression of the meristem maintenance markers *kn1*, *abph1*, *wus1*, and *log1*, suggesting that THI2 function is required for maintaining indeterminate growth in the central zone. The expression of *thi2* may be regulated downstream of brassinosteroid signaling, as brassinolide (BL) application to maize seedlings leads to lower *thi2* accumulation levels.

(Sylvester et al., 1990). Under this model, available thiamine in the seedling is sufficient for normal leaf development at the basal nodes in blk1 mutants. However, as the thiamine concentration is decreased due to loss of THI2 function, successive leaves become increasingly thiamine deficient and blade development is compromised. Sheath development, which may require less thiamine, is not affected by the loss of THI2 function. Tissue-specific metabolite profiling may be able to help identify the thiamine loads in these tissues and provide additional support for this model (Fleming, 2005)

Little research has focused on the interplay of metabolites in the regulatory networks for SAM maintenance. As highlighted by the analysis of blk1, the nature of these processes needs to be addressed in more detail if the mechanisms regulating SAM function are to be fully understood. Additional investigation of the downstream activities of thiamine will help clarify how this vital metabolite is utilized for meristem stability.

REFERENCES

Addicott, F. (1939). Vitamin B 1 in Relation to Meristematic Activity of Isolated Pea Roots. Botanical Gazette **100,** 836-843.

Belanger, F., Leustek, T., Chu, B., and Kriz, A. (1995). Evidence for the thiamine biosynthetic pathway in higher-plant plastids and its developmental regulation. Plant Molecular Biology **29,** 809-821.

Fleming, A. (2006). Metabolic aspects of organogenesis in the shoot apical meristem. Journal of Experimental Botany **57,** 1863-1870.

Foster, T., and Timmermans, M. Axial Patterning of the Maize Leaf. Handbook of Maize: Its Biology**,** 161-178.

McHale, N., Kawata, E., and Cheung, A. (1990). Plastid disruption in a thiamine-requiring mutant of *Nicotiana sylvestris* blocks accumulation of specific nuclear and plastid mRNAs. Molecular and General Genetics MGG **221,** 203-209.

Papini-Terzi, F., Galhardo, R., Farias, L., Menck, C., and Van Sluys, M. (2003). Point mutation is responsible for *Arabidopsis* tz-201 mutant phenotype affecting thiamin biosynthesis. Plant and Cell Physiology **44,** 856-860.

Poethig, R., and Szymkowiak, E. (1995). Clonal analysis of leaf development in maize. Maydica (Italy) **40,** 67-76.

Sylvester, A., Cande, W., and Freeling, M. (1990). Division and differentiation during normal and liguleless-1 maize leaf development. Development **110,** 985-1000.

Torrey, J. (1954). The Role of Vitamins and Micronutrient Elements in the Nutrition of the Apical Meristem of Pea Roots. Plant Physiology **29,** 279-287.

Wightman, F., and Brown, R. (1953). The effects of thiamin and nicotinic acid on meristematic activity in pea roots. Journal of Experimental Botany **4,** 184-196.