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The Role of two Homeodomain Genes *PENNYWISE(PNY)* and *POUNDFOOLISH(PNF)* in Regulating Meristem Maintenance of Shoot Apical Meristem

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DEDICATION

I dedicate this dissertation to the influences that push me moving forward, especially…

to mom for nurturing me with optimism;

to dad for shaping my perspective towards life;

to grandma for your unconditional love;

to the California sunshine, exotic food and diverse culture environment for ornamenting sparkles in me.

ABSTRACT OF THE DISSERTATION

The Role of two Homeodomain Genes *PENNYWISE(PNY)* and *POUNDFOOLISH(PNF)* in Regulating Meristem Maintenance of Shoot Apical Meristem

by

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Abstract

All aerial organs are produced from shoot meristems, which are dynamic self-sustaining structures located at the growing tips of shoots. Shoot meristems are subdivided into discrete domains with distinct functions. A population of stem cells is maintained in the central zone (CZ), while organogenesis occurs in the peripheral zone (PZ). In the core of the meristem, the rib meristem produces the pith of the stem. On the flanks of the shoot meristem a small strip of lateral organ boundary cells mediates the separation of the lateral organ from the meristem. Proper communication between these zones and the lateral organ boundary is essential for meristem integrity and function. Understanding the mechanisms that control shoot meristem fate and integrity will shed light on the mechanisms that control the patterning of the shoot. The KNOTTED1-Like HOMEOBOX (KNOX) transcription factor (TF) SHOOT MERISTEMLESS (STM) physically interacts with two related BELL1-like homeodomain (BLH) proteins called PENNYWISE (PNY) and POUND-FOOLISH (PNF) to regulate processes that maintain

meristem cell fate. Genetic studies indicate that STM-PNY/PNF also regulates boundary function and flower meristem identity during inflorescence development. Studies from the second chapter of this thesis indicate that communication between PNY/PNF and the lateral organ boundary is essential for maintaining meristem integrity as well as controlling inflorescence patterning events. Based on genetic studies, it is unclear if STM and PNY/PNF regulate developmental processes, such as stem cell homeostasis, boundary function and flower meristem identity, directly or indirectly. Experimental evidence from chapter two suggests that STM but not PNY/PNF directly regulates a gene, which encodes a protein that controls stem cell homeostasis. In the CZ and PZ of the shoot meristem, PNY-STM act to negatively regulate lateral organ boundary genes. However, in the absence of PNY/PNF function, STM functions to specify lateral organ boundary identity on the flanks of the shoot meristem. Although PNY/PNF indirectly regulates flower specification via the lateral organ boundary, studies in chapter two suggest that these BLH proteins and at least one KNOX protein directly regulates an early flower meristem identity gene. Results from this thesis, indicate that the spatial expression patterns and formation of specific KNOX-BLH complexes is essential for maintaining meristem cell fate, integrity and flower meristem identity. Furthermore, PNY/PNF and STM act to regulate these processes in an indirect and direct manner to ensure meristem integrity and function is maintained.

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INTRODUCTION

The indeterminate growth pattern displayed by shoots is mediated by the proper maintenance of the shoot meristem and the coordination among different function units at molecular level. Meristem maintenance is dependent upon the balance of stem cell perpetuation in the central zone (CZ) and organogenesis in the peripheral zone (PZ). Although the mechanisms that coordinate CZ and PZ function is not understood, meristem cell fate is likely achieved by the spatial interplay between gene regulatory networks and hormone signaling pathways. In addition, the lateral organ boundary plays essential roles in meristem maintenance, lateral organ separation and axillary meristem formation. During shoot maturation, the identity of the shoot meristem as well as the lateral organs are transformed during the vegetative and reproductive transitions. Studies in model plant systems indicate that three amino acid extension (TALE) homeodomain proteins integrate signaling events that control the integrity of CZ and Organizing Center (OC), regulate the boundary maintenance and specify floral meristem identity during shoot development. Here, the interplay among TALE homeodomain transcription factors and their communication with other key players in controlling those component developmental pathways are summarized.

Shoot development

Plants undergo extensive and elaborate programs of development post-embryonically due to the activity of shoot and root apical meristems (Lyndon 1998; Steeves and Sussex

1989). All of the above ground organs originate from shoot meristems, which are subdivided into three functional zones (Bernier 1988; Lyndon 1998; Steeves and Sussex 1989). Stem cells are maintained in the central zone (CZ), which is located at the apical region of the shoot meristem. The peripheral zone (PZ), which surrounds the CZ, acts to buffer the stem cells from the programs of differentiation, which are activated in lateral organs that emerge on the flanks of the shoot meristem. In the PZ, mechanisms that establish positional cues are essential for allocating the appropriate number of cells into leaves and axillary meristems. Cell division in the rib meristem (RM) gives rise to the pith cells of the stem. The activity of the RM plays a fundamental role in meristem morphology, as cytohistological analyses indicate that the RM plays an essential role in regulating the floral evocation (Bernier 1988; 2011) and bud dormancy and release (van der Schoot and Rinne 2011). The meristem-organ boundary is also essential for meristem maintenance as it acts to limit programs of differentiation from altering the fate of the cells in the meristem (Aida and Tasaka 2006b). In addition, the lack of cell division at the meristem-organ boundary is essential for the physical separation of leaves and axillary meristems from the SAM.

During plant development, shoots often sustain a period of indeterminate growth, which is dependent on the identity and function of the shoot apical meristem (SAM) (Lyndon 1998; Steeves and Sussex 1989). The indeterminate activity of the SAM depends upon the balance of stem cell maintenance and allocation of cells into initiating primordia (Vollbrecht et al. 2000). This hypothesis predicts that a loss of stem cell maintenance

and/or mechanism of cell allocation will eliminate and/or dramatically perturb shoot growth. Recent reviews focused on stem cell maintenance and the plant hormones that regulate meristem function and organogenesis have been published (Bleckmann and Simon 2009; Braybrook and Kuhlemeier 2010; Dodsworth 2009; Shen and Xu 2009; Vernoux et al. 2010). This introduction chapter is focused on the function of KNOTTED1-like homeobox (KNOX) and BELL1-like homeodomain (BLH) transcription factors in regulating meristem cell fate, lateral organ boundary function and meristem identity during shoot development.

The role of KNOTTED1-Like homeodomain transcription factors in meristem function

Class I KNOX transcription factors regulate meristem function as well the morphology of shoots, flowers and leaves (Hake et al. 2004) (Hamant and Pautot 2010) (Hay and Tsiantis 2010). In maize, *knotted1* (*kn1*) is expressed in CZ, PZ and RM of the shoot meristem; however, transcription of this homeobox gene is inactivated in the incipient leaf primordia (Smith et al. 1992; Jackson et al. 1994). Consistent with this expression pattern, loss of function mutations in *kn1* disrupts meristem function (Kerstetter et al. 1997; Vollbrecht et al. 2000). Interestingly, the severity of the *kn1* phenotype is related to the height of the shoot meristem (Vollbrecht et al. 2000). In this case, loss of *kn1* function in inbred lines with smaller meristems frequently gives rise to plants with a terminal shoot phenotype after the initiation of the coleoptiles. Cell divisions in the RM play a role in regulating the height of the SAM (Bernier 1988; Lyndon 1998). Therefore, shoots with

a less active RM may be more dependent upon KNOX function. Analysis of *kn1* phenotype suggested that this homeobox gene specifies meristem cell fate by maintaining meristematic cells in an undifferentiated state (Kerstetter et al. 1997) (Vollbrecht et al. 2000). In Arabidopsis, the KNOX protein SHOOT MERISTEMLESS (STM) appears to function in an analogous manner as KN1 (Barton 2010). In plants harboring *stm* null alleles, shoot growth terminates after the production of two partially fused cotyledons (Barton and Poethig 1993; Long et al. 1996). Both KN1 and STM are expressed in all shoot meristem types, which display indeterminate and determinate patterns of growth (Smith et al. 1992; Jackson et al. 1994; Long et al. 1996).

KNOX proteins such as KN1 and STM appear to maintain meristem cell fate in a number of different ways. First, KN1/ STM may act in the PZ to maintain the meristem-organ boundary, which separates the meristem from the developing leaf (Aida et al. 1999; Kerstetter and Hake 1997; Long and Barton 1998; Takada et al. 2001; Vollbrecht et al. 2000). In this light, KN1 and STM act at the meristem-organ boundary to restrict gene expression profiles that promote leaf and organ differentiation. The ectopic expression of KN1 in maize leaf margins results in the reestablishment of the proximal-distal patterning event(Ramirez et al. 2009). The reiteration of this patterning event suggests KN1 regulates the proximal identity of the leaf; therefore, in the absence of KN1 function, leaves fail to form because the proximal boundary is not defined (Ramirez et al. 2009). Experimental studies show that meristem-leaf boundaries are also crucial for the establishment and formation of axillary meristems (Aida and Tasaka 2006b). Analysis of

different *stm* alleles indicates that this homeodomain specifies axillary meristems by maintaining meristem-leaf boundaries (Barton and Poethig 1993; Kanrar et al. 2006; Takano et al. 2010). Therefore, KN1 and STM function at the meristem-leaf boundary to mediate the formation of the axillary meristems. Genetic studies indicate that KN1 and STM regulate meristem integrity and organization. For example, analysis of leaf initiation in different alleles of *stm* suggests that this homeodomain transcription factor regulates the integrity of the central region of the SAM (Endrizzi et al. 1996). Further, STM may regulate CZ function by maintaining a boundary between the CZ and PZ (Clark et al. 1996). Lastly, analysis of the hypomorphic *stm* allele, *gorgon*, suggests that *STM* also regulates the allocation of cells into initiating organ primordia (Takano et al. 2010). Taken together, KN1 and STM appear to regulate multiple pathways required for specifying meristem cell fate and maintaining meristem function.

Other class I KNOX transcription factors have been characterized and studies in Arabidopsis suggest that these homeodomain proteins also contribute to meristem maintenance but to a lesser extent (Hake et al. 2004). In Arabidopsis, mutations in *BREVIPEDICELLUS* (*BP*) and *KNOTTED1-like genes from Arabidopsis thaliana 6* (*KNAT6*) enhance weak alleles of *stm*, demonstrating role for these KNOX transcription factors in meristem maintenance (Belles-Boix et al. 2006; Byrne et al. 2002). Interestingly, recent studies indicate that *BP* regulates inflorescence architecture by repressing *KNAT6* and *KNAT2* in the internode and pedicels (Ragni et al. 2008).

KNOX regulation of gibberellin and cytokinin biosynthesis and signaling

Plant hormones play a fundamental role in meristem function (Shani et al. 2006). Gibberellin (GA) is a plant hormone that regulates seed germination, flowering and cell elongation (Itoh et al. 2008). Experimental studies in several plant species suggest that KNOX proteins regulate meristem function in part by repressing a key GA biosynthetic gene, GA20 oxidase (Chen et al. 2004; Jasinski et al. 2005; Sakamoto et al. 2001; Yanai et al. 2005). In addition, recent studies show that KN1 promotes transcription the GA2 oxidase gene, *ga2ox1*, which may serve to catabolize GA that moves into the shoot meristem via the leaves and stems (Bolduc and Hake 2009). Thus, according to current models, the repression of GA biosynthesis and activity by KNOX proteins is essential for meristem function. However, experimental studies provide evidence that GA acts as florigenic molecule that moves from the leaves to the shoot meristem to evoke flowering in plants such as Arabidopsis and Lolium (Eriksson et al. 2006; King and Evans 2003). In the shoot meristem, flower specification is controlled by inflorescence meristem identity genes, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) and *AGAMOUS-LIKE 24* (*AGL24*), which are positively regulated by multiple flowering time pathways including GA (Lee and Lee 2010). Interestingly, in the absence of the photoperiod florigenic signal, *FLOWERING LOCUS T* (*FT*), *stm-10* often initiates inflorescences that fail to form flowers despite that fact that *SOC1* and *AGL24* are expressed in the shoot meristem (Smith et al. 2011). In light of these studies, STM may function with AGL24-SOC1 to mediate flower specification in response to GA. Therefore, the interplay

between *KNOX* and GA appears to be complex and likely controlled by phase specific meristem identity transcriptional regulators.

Cytokinin (CK) plays a fundamental role in regulating meristem formation, integrity and maintenance (Kurakawa et al. 2007). Experimental evidence suggests that *KNOX* and CKs form a positive feedback loop, which is required for meristem function (Shani et al. 2006). Consistent with this model, growing a null allele of *stm* in the presence of CK partially rescues the terminal shoot phenotype (Yanai et al. 2005). In addition, a mutation in the CK receptor called *WOODEN LEG* (*WOL*) enhances a weak allele of *stm* (Jasinski et al. 2005). At the molecular level, STM positively regulates CK biosynthesis by activating *ISOPENTENYL TRANSFERASE 7* (*IPT7*) (Jasinski et al. 2005; Yanai et al. 2005). However, at the same time, STM also appears to activate *ARABIDOPSIS RESPONSE REGULATOR 5* (*ARR5*), a negative regulator of CK signaling (Jasinski et al. 2005; Yanai et al. 2005). Although the regions in the SAM in which *STM* regulates *IPT7* and *ARR5* are not known, STM and related KNOX proteins may regulate CK signaling at multiple levels to ensure meristem function is properly maintained.

Regulation of meristem function by association of KNOX proteins with BELL1-like homeodomain proteins

Molecular studies in several plant species indicate that KNOX proteins selectively associate with specific members of the BELL1-like homeodomain (BLH) proteins in plants (Hake et al. 2004; Hamant and Pautot 2010). At the molecular level, biochemical

studies indicate that the formation of KNOX-BLH complexes increases the DNA binding affinity for the TGACAGG/CT DNA-motif (Smith et al. 2002). KNOX and BLH proteins interact through large evolutionarily conserved domains in the N terminus of the proteins called the MEINOX domain and BELL or MID domain, respectively (Hake et al. 2004; Mukherjee et al. 2009).

The Arabidopsis genome contains 13 *BLH* genes and at least three of these homeodomain transcription factors regulate meristem cell fate and identity (Mukherjee et al. 2009; Roeder et al. 2003; Smith et al. 2002). Loss of function mutations in *PENNYWISE* (*PNY*: also known as *BELLRINGER*, *REPLUMLESS* and *VAAMANA*) disrupt internode and fruit patterning during reproductive development (Bhatt et al. 2004; Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003). In contrast to *pny*, shoot development is not altered in *pound-foolish* (*pnf*), the paralog of *PNY*. Both *PNY* and *PNF* are expressed in vegetative, inflorescence and floral meristems (Smith et al. 2004). Although meristem maintenance is not visibly altered in *pny* and *pnf* single mutants, the shoot meristem of *pny pnf* plants often terminates after the production of 3-4 leaves (Rutjens et al. 2009; Smith et al. 2004). In addition, the indeterminate behavior of inflorescence meristem is reduced in *pny PNF/pnf* plants (Smith et al. 2004). Recent studies indicate that *PNY* and *PNF* control the integrity of the shoot meristem by maintaining a boundary between the CZ and PZ (Ung et al. 2011). In addition, *PNY* and *PNF* regulate the meristem-leaf boundary, as there is a significant increase in the number of meristem cells allocated into leaf primordia in *pny pnf* (Smith et al. 2004; Ung et al. 2011). Finally, *PNY* and *PNF* maintain the boundary

between the inflorescence and floral meristem, since the inflorescence shoots of *pny PNF/pnf* plants display fusions of pedicels to the main stem (Smith et al. 2004). The fact that PNY and PNF associate with STM is further evidence that these homeodomain proteins regulate meristem cell fate (Bhatt et al. 2004; Byrne et al. 2003; Kanrar et al. 2006; Rutjens et al. 2009). Genetic studies show that combining mutations in *pny* with weak alleles of *stm*, including *stm-11*, results in terminal shoot phenotype after the cotyledons are produced (Bhatt et al. 2004; Byrne et al. 2003a; Kanrar et al. 2006). Lastly, the reduction in meristem indeterminacy displayed in *pny PNF/pnf* inflorescence shoots is greatly enhanced in *pny PNF/pnf STM/stm-11* shoots (Kanrar et al. 2006). Therefore, we propose that PNY-STM and PNF-STM regulate meristem integrity and function by maintaining boundaries in the shoot meristem.

The *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*) encodes a BLH homeodomain that is structurally and phylogenetically distinct from *PNY* and *PNF* (Mukherjee et al. 2009). During vegetative growth, *ATH1* is expressed in the shoot meristem and developing leaves (Proveniers et al. 2007) (Gomez-Mena and Sablowski 2008). In contrast to *PNY* and *PNF*, transcript levels for *ATH1* rapidly decline in the shoot meristem upon floral induction (Proveniers et al. 2007; Schmid et al. 2003). During vegetative growth, *ATH1* functions to maintain the basal boundary between the stem, meristem and leaf (Gomez-Mena and Sablowski 2008). Moreover, a role for *ATH1* in meristem maintenance is demonstrated through its physical interaction with STM and the fact that *stm-11 ath1* mutants produce a terminal shoot phenotype after the initiation of

cotyledons (Rutjens et al., 2009). Genetic studies show that *ath1* also enhances the *pny pnf* terminal shoot phenotype, such that vegetative shoots terminate after two leaves are produced (Rutjens et al. 2009). The fact that *pny pnf ath1* did not produce a phenotype similar to null alleles of *stm* suggests that other BLH proteins regulate meristem maintenance in association with STM and/or other KNOX partners.

Vegetative development is mediated by the interplay between MADS-box and homeodomain transcription factors

Plants maintain a vegetative growth pattern as a means to build up energy reserves needed to initiate and sustain reproductive growth. In Arabidopsis, specific members of the MADS-box transcription factors are expressed in temporal and/or spatial manner and function in a cooperative manner to regulate distinct phases of plant development (Becker and Theissen 2003). During early stages of shoot development, the MADS-box protein *FLOWERING LOCUS C* (*FLC*) regulates the vegetative phase change and represses flowering in winter annual ecotypes prior to vernalization (Amasino 2010; Crevillen and Dean 2011; Willmann and Poethig 2011). *FLC* is positively regulated by *FRIGIDA* (*FRI*) and negatively regulated by vernalization in an epigenetic manner (Amasino 2010; Crevillen and Dean 2011). *FLC* promotes vegetative growth in part by negatively regulating key flowering time genes, including the florigenic signal, *FT* and another MADS-box proteins *SOC1* (Helliwell et al. 2006; Hepworth et al. 2002; Searle et al. 2006). As stated above, *ATH1* is expressed in vegetative shoot meristems and leaf primordia but downregulated upon flowering (Proveniers et al. 2007). The temporal

expression pattern of *ATH1* displays a similar transcript profile for floral repressors such as *FLC* (Michaels and Amasino 1999; Proveniers et al. 2007). A recent study showed that ectopic *ATH1* delays flowering in the Arabidopsis C24 ecotype (Proveniers et al. 2007). However, after vernalization, the shoots of the C24 plants expressing high levels of *ATH1* are induced to flower in long-day photoinductive conditions. In contrast, expression of antisense *ATH1* transcripts results in an early flowering phenotype. At the molecular level, *ATH1* delays flowering by positively regulating *FLC* in a *FRI* dependent manner (Proveniers et al. 2007) (Fig. 1.1). Interestingly, Lolium perenne plants expressing high levels of *ATH1* displays a late flowering phenotype indicating that the function of this homeodomain protein is conserved in dicot and moncots (van der Valk et al. 2004). In monocots, such as wheat and barley, *VERNALIZATION2* (*VRN2*) encodes a zinc finger protein, which acts as a floral repressor with a similar function as *FLC* in Arabidopsis (Dubcovsky et al. 2006; Trevaskis et al. 2006; Yan et al. 2004). Although Arabidopsis and winter cereals utilize distinct floral repressors, *FLC* vs. *VRN2*, the function of *ATH1* appears to be conserved. Taken together, *ATH1* not only acts to regulate meristem maintenance, but this key vegetative specific BLH protein functions to specify vegetative meristem identity.

Networks that mediate the floral transition and flower specification in Arabidopsis

In Arabidopsis, FT acts as the long-day photoperiodic signal that moves from the leaves to the SAM to evoke flowering and promote flower specification (Amasino 2010; Fornara et al. 2010). At the molecular level, FT induces flowering in part thought its

association with the b-ZIP transcription factor, FD (Abe et al. 2005; Wigge et al. 2005). The tomato and rice FT homologs, SINGLE FLOWER TRUSS and Heading date 3a (Hda3), have been shown to associate with 14-3-3 proteins (Pnueli et al. 2001; Purwestri et al. 2009; Taoka et al. 2011). A recent study in rice suggests that 14-3-3 protein called GF14c acts as a molecular scaffold that bridges Hda3 with the Oryza sativa FD (Taoka et al., 2011). Based on this study, it is likely that the formation of FT-GF14c-FD complex, which has been termed the florigen activation complex (FAC), acts to regulate gene expression in the shoot apical meristem. Therefore, the FAC somehow mediates the floral transition by activating *SOC1* and a related MADS-box protein, *FRUITFULL* (*FUL*) in order to promote inflorescence meristem identity, in Arabidopsis (Abe et al. 2005; Schmid et al. 2003; Searle et al. 2006; Teper-Bamnolker and Samach 2005; Wang et al. 2009; Wigge et al. 2005). Experimental studies suggest that TWIN SISTER OF FT (TSF), a FT-related protein, acts redundantly with FT to regulate reproductive development (Jang et al. 2009). SOC1 forms a MADS-box complex with AGL24 which is also upregulated in the SAM upon floral induction (de Folter et al. 2005; Lee et al. 2008; Michaels et al. 2003; Yu et al. 2009). Molecular studies show that FUL interacts with SOC1 and AGL24 indicating that these three MADS-box proteins form a higher order complex to specify inflorescence meristem identity (de Folter et al. 2005) (Fig. 1.2). A subset of the *microRNA156* (*miR156*) targeted *SQUAMOSA PROMOTER BINDING PROTEINLIKE* (*SPL*) transcription factors define an endogenous flowering time pathway that appears to act both downstream and in parallel with *FT* (Wang et al. 2009). Further, *SPL*s promote the floral transition by activating inflorescence meristem identity genes,

SOC1 and *FUL* (Wang et al. 2009; Yamaguchi et al. 2009). In conclusion, both the photoperiodic and endogenous flowering time pathways converge on *SOC1* and *FUL* to mediate the floral transition and specify inflorescence meristem identity (Fig. 1.2). In a recent review, it was proposed that the floral stimulus is a two-component system that involves the synthesis and movement of FT/TSF and cytokinin (CK) from the leaves to the SAM (Bernier 2011). Recent studies have begun to shed light on the relationship between CK and the gene regulatory networks, which promote floral evocation at the SAM. In Arabidopsis and Sinapis, application of CK induces *SOC1* expression in the SAM (Bernier 2011; Bonhomme et al. 2000; D'Aloia et al. 2011). Experimental studies show that applications of CK promotes flowering by activating *TSF* in the leaves of Arabidopsis (D'Aloia et al. 2011). Further, CK promotes flowering in a TSF dependent manner indicating that activation of *SOC1* by CK may be mediated by *TSF*. Taken together, CK appears to play a fundamental role in regulating florigen in the leaves and the mitotic activation event during floral evocation (Bernier 2011; D'Aloia et al. 2011).

In Arabidopsis, flowers are specified on the flanks of the inflorescence meristem by the activity of *LEAFY* (*LFY*), an early flower meristem identity factor. Studies in Arabidopsis and Petunia, suggest that *LFY* regulates late flowering meristem identity genes in association with the F-box protein UNUSUAL FLORAL ORGANS (UFO)/DOUBLE TOP (DOT) (Chae et al. 2008; Souer et al. 2008). To date, the biochemical significance of UFO/DOT in regulating gene expression is not known. The initial upregulation of *LFY* is observed in a small group of cells in the PZ of the SAM, before any visible signs of

flower development (Weigel et al. 1992). Once established, *LFY* activates a number of late flower meristem identity genes including *APETALA1* (*AP1*), a MADS-box gene (Parcy et al. 1998; Saddic et al. 2006; Wagner et al. 1999; William et al. 2004; Winter et al. 2011). The expression of *AP1* is first detected in the cells of the floral buttress on the flanks of the inflorescence meristem (Gustafson-Brown et al. 1994). Regulation of *LFY* is quite complex as this gene is activated and maintained by inflorescence and late flower meristem identity MADS-box transcription factors, respectively. Based on recent studies, it is likely that the initial upregulation of *LFY* in the PZ of the inflorescence meristem is directly mediated by the SOC1- AGL24 complex (Lee et al. 2008; Liu et al. 2008). In addition, genetic evidence suggests that FUL also participates in the activation of *LFY* indicating that this MADs-box protein is a component of the SOC1-AGL24 complex (Ferrándiz et al. 2000). SOC1 and FUL do not appear to be involved in the maintenance of *LFY* expression, since transcripts for these MADS-box genes fail to accumulate in the cells of the floral buttress (Mandel and Yanofsky 1995; Michaels et al. 2003; Samach et al. 2000; Yu et al. 2002). In contrast to SOC1 and FUL, *AGL24* appears to play a transient role maintaining flower meristem identity together with *AP1* and *SHORT VEGETATIVE PHASE* (Gregis et al. 2008). Genetic and molecular studies indicate that a positive feedback loop between *LFY* and *AP1* maintains flower meristem identity (Bowman et al. 1993; Liljegren et al. 1999; Schultz and Haughn 1993). Therefore, *AP1* acts to sustain flower meristem identity by maintaining *LFY* expression. In addition, *AP1* represses inflorescence meristem identity by negatively regulating *SOC1*, *FUL* and *AGL24* (Gregis et al. 2008; Liu et al. 2007; Yu et al. 2004). *AP1* is also directly activated

by FT-FD complex, which acts in parallel with *LFY* to specify floral meristem identity (Abe et al. 2005; Wigge et al. 2005). A complete understanding of *FT* function appears to be incomplete as loss of function mutations in *fd*, fail to suppress the early flowering phenotype associated with Arabidopsis plants overexpressing *FT* (Abe et al. 2005; Teper-Bamnolker and Samach 2005). Therefore, GF14c may act to bridge other meristem functioning transcription factors with FT in order to mediate the floral transition and flower specification.

The role KNOX and BLH transcription factors in regulating reproductive meristem identity

KNOX proteins, such as STM, function in the SAM to specify meristem cell fate (Hake et al. 2004; Hay and Tsiantis 2009; Scofield and Murray 2006). In the absence of STM, the cells in the SAM terminally differentiate (Barton and Poethig, 1993; Long et al., 1996). During reproductive development, weak *stm* alleles, display defects in inflorescence patterning events such as axillary meristem formation, flower meristem identity and internode development (Clark et al. 1996; Endrizzi et al. 1996; Kanrar et al. 2006). Given that STM is an essential regulator of meristem cell fate, the defects in reproductive patterning events may simply result from a decrease in meristem function. However, genetic studies indicate that *FT* and *STM* function together to regulate axillary meristem formation and flower meristem identity (Smith et al. 2011). In the *stm-10 ft-2* non-flower producing inflorescences, both *SOC1* and *AGL24* are expressed in the SAM, while the levels of *LFY* are low. This observation indicates that the function of *SOC1* and

AGL24 may be partially dependent upon *STM* for specifying flower meristem identity. Taken together, *STM* may function to integrate gene regulatory networks that specify meristem formation and identity to ensure that the function of the shoot meristem is maintained during each phase of development. It should also be pointed out that the levels of FT are crucial for regulating meristem determinacy and dormancy (Shalit et al. 2009). Therefore, meristem identity proteins may also function to regulate and modulate basic meristem function. One of the intriguing features of *pny pnf* plants is the inability of the mature shoots to produce axillary meristems, which give rise to coflorescences and flowers (Smith et al., 2004; Rutjens et al., 2009). It has been postulated that *PNY* and *PNF* function to establish positional cues in subset of cells in PZ that develop into axillary meristems (Kanrar et al., 2008). Therefore, in the absence of *PNY* and *PNF* function, meristem cells allocated during organogenesis are unable to reorganize in order to form axillary meristems. During inflorescence development, it is well known that *LFY* and *FT* function to specify flower meristem identity (Kobayashi and Weigel 2007); however, analysis of *lfy ft* inflorescences also indicates that these proteins cooperatively act to specify axillary meristems that develop into coflorescence shoots (Ruiz-García et al. 1997). Interestingly, the empty cauline leaf axil phenotype is also observed in the inflorescences of *pny lfy* and *pny ft* plants (Kanrar et al., 2008). Moreover, the decrease in the specification of axillary meristems in the axils of cauline leaves is further enhanced in *pny ft PNF/pnf* and *pny lfy PNF/pnf* plants. Therefore, *FT* and *LFY* appear to perform basic function with *PNY* and *PNF* in the formation of axillary meristems that give rise to coflorescence shoots and flowers.

PNY and possibly *PNF* also specify flower meristem identity. The inflorescences of *pny lfy* plants display complete conversion of flowers into shoots, producing reproductive shoots that are phenotypically similar to *lfy ft* and *lfy fd* (Kanrar et al. 2008). The fact that *pny ft* inflorescences displayed a minor conversion of flowers into shoots suggests that these proteins function in the same pathway to specify flower meristem identity (Kanrar et al. 2008). Consistent with this hypothesis, genetic studies show that *FT* is dependent upon the function of *PNY* and *PNF*. This is supported by the fact that flower formation is severely attenuated in *pny pnf* plants ectopically expressing *FT* (Kanrar et al. 2008). A molecular understanding that describes the interplay between FT and STM-PNY/STMPNF will shed light on how these proteins regulate the specification of axillary and flower meristems, and possibly meristem determinacy. In addition to promoting the floral transition, *miR156* targeted *SPL*s also function to directly activate *LFY* and *AP1* (Yamaguchi et al. 2009). Experimental studies suggest that the ability of *FT* to regulate the *miR156* targeted *SPL*s is dependent upon *PNY* and *PNF* (Lal et al. 2011). Furthermore, *PNY* and *PNF* also are required for the downregulation of *miR156*, since the levels of this microRNA fail to decline in the mature shoots of *pny pnf*. Based on biochemical and sequence alignment studies, KNOX and BLH proteins associate with similar DNA binding motifs, TGACAG/CT (Hake et al. 2004). The TGACAG sequence is conserved in nearly all *miR156* molecules that have been identified in plants (Lal et al. 2011). Therefore, PNY and PNF may directly bind to all miR156 loci to repress their transcription of these microRNAs. As stated above, *ATH1* promotes vegetative identity

by activating *FLC* (Proveniers et al. 2007). Interestingly, plants with increased levels of *FLC* display an extended juvenile phase (Lee et al. 2000; Martínez-Zapater et al. 1995; Telfer et al. 1997; Willmann and Poethig 2011). Thus, *ATH1* may act to promote vegetative growth and juvenile identity by positively regulating *FLC* and possibly *miR156*. Interestingly, loss of *ATH1* function in *pny pnf* partially restores reproductive development (Rutjens et al. 2009). Although the levels of *miR156* have not been examined in *pny pnf ath1* shoots, reducing the activity of this *microRNA* in *pny pnf* plants results in the production of cauline leaf bearing inflorescences similar to *pny pnf ath1* (Lal, S. and Smith, H.M, unpublished data). Therefore, it would be interesting to know if the ability of *pny pnf ath1* plants to produce inflorescence shoots is due to a decrease in the levels of *miR156*. Taken together, the levels of *miR156* may be regulated by the binding of PNY/PNF and ATH1 to the TGACAG sequence in *miR156*. In this context, the ratios of PNY/PNF and ATH1 in meristem cells may coordinate the temporal levels of *miR156* during plant development.

In animals and yeast, homeodomain proteins function as cofactors for transcriptional complexes that regulate cell fate determination (Messenguy and Dubois 2003; Moens and Selleri 2006). In light of these studies, KNOX-BLH heterodimers may also function as cofactors for transcriptional complexes that control meristem cell fate and meristem identity. Recent studies indicate that *FT* controls reproductive development independent of *FD*, possibly by interacting with other meristem functioning transcription factors (Abe et al. 2005; Teper-Bamnolker and Samach 2005). Genetic analyses show that *FT*

functions with *STM* during inflorescence development (Smith et al. 2011). Further, the floral meristem specification activity of *FT* is dependent on *PNY* and *PNF* (Kanrar et al. 2008). Thus, FT may regulate floral meristem identity and meristem determinacy by directly interacting with the STM-PNY/PNF heterodimer or a transcription factor that associates with this KNOX-BLH complex. Interactions between homeodomain and MADS-box transcription factors have been described in yeast and animals (Messenguy and Dubois 2003). In Arabidopsis, experimental evidence demonstrates that the founding member of the BLH family, BELL1, associates with ovule specific MADS-box proteins, AGAMOUS and SEPALLATA3 to specify ovule identity (Brambilla et al. 2007). The association of homeodomain and MADS-box transcription factors may be evolutionarily conserved in animals, yeast and plants. Therefore, individual KNOX or BLH proteins as well as KNOX-BLH heterodimers may serve as cofactors for MADS-box complexes in plants. In accordance to this model, *PNY* and *PNF* or possibly STM-PNY/PNF heterodimers may associate with SOC1-AGL24-FUL complexes to promote the initial upregulation of *LFY* in the PZ of the inflorescence meristem. Attempts to detect interactions between PNY/PNF/STM and SOC1/FUL/AGL24 have not been successful (unpublished data). Therefore, it may be possible that the association of STM-PNY/PNF with SOC1-AGL24-FUL is more complex in that the interaction may require a molecular scaffold, such as DNA or a nucleoplasmic structure. In addition, little is known about post-translational modifications of KNOX and BLH proteins, which may also serve to stabilize the association of these transcription factors with the inflorescence meristem

identity complexes. Future studies are aimed at determining the link between PNY/PNF-STM and the regulatory proteins that control meristem identity.

Regulation of the lateral organ boundary plays a fundamental role in shoot development and architecture

As lateral organ primordia emerge from the periphery of the meristem, boundaries separating developing organs from meristem are established (Aida and Tasaka 2006a). Once established, the identity of the boundary cells are maintained to ensure that separation is completed and the identity of the meristem and lateral organ is properly determined (Aida and Tasaka 2006a; b). Two types of boundaries have been classified in plants, the Meristem-Organ (M-O) boundary, which is described above, and the organ-organ (O-O) boundary that separates developing organs such as sepals and petals (Aida et al. 1997; Aida and Tasaka 2006a; Baker et al. 2005). Based on histological analysis and molecular studies, boundaries are composed of a band of small cells, in which cell division is inactive to facilitate the separation of organ from the shoot meristem (Aida and Tasaka 2006b). Gene expression analysis demonstrates that the boundary cells express a distinct profile of genes from the surrounding cells (Aida and Tasaka 2006a). Genetic and molecular approaches have identified a diverse set of transcription factors, which appear to act synergistically to regulate boundary formation, maintenance and identity during shoot development. Moreover, genetic studies demonstrate that the function of boundaries is essential for meristem maintenance, axillary meristem formation and organ separation.

During embryogenesis, a subset of NAC transcription factors called *CUP-SHAPED COTELYDON 1,2* and *3* are crucial for the establishment and maintenance of the M-O boundary, which is essential for maintaining meristem cell fate as well as cotyledon separation (Aida et al. 1997; Barton 2010; Hibara et al. 2006). Loss of *CUC* function results in cotyledon fusion phenotype and the terminal differentiation of the SAM, which is similar to *stm*-1 phenotype (Aida et al. 1997; Hibara et al. 2006). *CUC* genes are expressed in the boundary between the SAM and cotyledon as well as leaves and axillary meristems (Takada et al. 2001). Genetic studies suggest that *CUC1* and *CUC2* function upstream of *STM* and *KNAT6* (Aida et al. 1999) (Belles-Boix et al. 2006). At the same time, STM functions to directly regulate *CUC1* (Spinelli et al. 2011). It should also be pointed out that *CUC*1 and *CUC2* are regulated by *miR164* (Baker et al. 2005; Laufs et al. 2004; Mallory et al. 2004; Nikovics et al. 2006; Sieber et al. 2007). Based on these studies, *MIR164s* functions to spatially trim the *CUC* expression domain as well as dampen transcripts accumulation via posttranscriptional regulation (Sieber et al. 2007). Genetic studies show that *STM* functions with the Myb-trancription factor *Lateral Organ Fusion1* (*LOF1*) to regulate meristem identity and cotyledon separation (Lee et al. 2009). Similar to *CUC* gene expression, *LOF1* is expressed at the lateral organ boundary throughout shoot development. The synergistic effect of *CUCs* and *LOF1* with *STM* indicate that these transcription factors likely function with other boundary regulators (see below) to facilitate meristem maintenance and organ separation (Lee et al. 2009).

Another KNOX gene implicated in boundary function is *KNAT6*, which is preferentially expressed in the lateral organ boundary (Belles-Boix et al. 2006). Genetic studies show that *KNAT6* functions together with *STM* to regulate meristem maintenance. Furthermore, *KNAT6* appears to regulate boundary function with *STM* and, in turn, they together activate *CUC3*. At the same time, experimental evidence suggests that *CUC1* and *CUC2* positively regulate *KNAT6* (Belles-Boix et al. 2006). Taken together, cross-talk between *KNOX* transcription factors, *STM* and *KNAT6* and *CUC* genes functions to establish and maintain boundary function throughout shoot development.

One of the primary differences between the M-O and O-O is that the M-O boundary is necessary for the initiation of axillary meristem (Aida and Tasaka 2006a). For example, a loss of function allele of *LATERAL SUPPRESSOR* (*LAS*) results in the decrease of axillary meristem formation in tomato and Arabidopsis (Greb et al. 2003; Schumacher et al. 1999). *LAS* is a member of the GRAS family of transcriptional regulators. Interestingly, the *las* phenotype is more penetrant under short-day growth condition than long-days in Arabidopsis (Greb et al. 2003). Another indication that *LAS* regulates organ boundaries is that *las* plants display a fusion of secondary inflorescence to the primary inflorescence, but at a low penetrance (Greb et al. 2003). Consistent with its function, *LAS* is expressed in the boundary between the shoot meristem and secondary inflorescence meristems as well as floral meristems (Greb et al. 2003). In addition, *LAS* is expressed in adaxial boundary of rosette leaf axils where axillary meristems develop. Genetic studies suggest that *LAS* specifies axillary meristem formation by positively

regulating *STM*. Activation of *STM* via *LAS* appears to require the adaxial HD-ZIP transcription factor *REVOLUTA* (Greb et al. 2003). Genetic studies show that *CUC2* and *CUC3* function to specify axillary meristems during shoot development. Moreover, *CUC2* and *CUC3* function with *LAS* to promote the formation of axillary meristems (Hibara et al. 2006; Raman et al. 2008). The R2R3 Myb transcription factor, *REGULATOR OF AXILLARY MERISTEMS1* (*RAX1*) also promotes axillary meristem formation. Similar to other *LAS*, *RAX1* is expressed at the boundary between the shoot meristem and axillary meristem (Keller et al. 2006). Molecular-genetic studies show that *RAX1* is required for the expression of *CUC2* (Keller et al. 2006). In addition, *LAS* and *RAX1* regulate axillary meristem formation via independent pathways (Keller et al. 2006). Lastly, *LOF1* not only controls accessory meristem formation, but also specifies axillary meristem formation independent of *LAS* (Lee et al. 2009).

Modular unites called phytomers are produced by the shoot meristem. Typically, a phytomer consists of a leaf, axillary bud and internode separating the two adjacent nodes (Carles and Fletcher 2003; Lyndon 1998; Steeves and Sussex 1989). The growth and development of each phytomeric components contributes to the overall architecture of the shoot. For example, the size and shape of the leaf as well as heteroblasty has a profound effect on shoot architecture. Internode development, which contributes to the height of a plant, also influences shoot morphology (Fletcher 2002; Steeves and Sussex 1989). The phyllotaxy, which can change in response to floral induction, contributes to shoot development (Kuhlemeier 2007, Barton et al. 2010). Lastly, the activity (dormant vs.

active bud) and the identity (inflorescence or floral) of the axillary bud also influences shoot architecture (Shimizu-Sato and Mori 2001). Understanding the mechanism that control shoot architecture will allow for the plant biologists to develop new cultivars and varieties with high reproductive potential (Wang and Li 2008).

Our laboratory is currently utilizing the model plant Arabidopsis to understand the mechanisms that control inflorescence architecture (Barton 2010). Arabidopsis produces an inflorescence with a raceme architecture in which the SAM maintains inflorescence identity and indeterminate growth. During inflorescence development, the fist 3-4 axillary meristems develop into coflorescences, which recapitulate the same pattern as the main shoot. The remaining axillary meristems develop into flowers, which are determinate shoots. Depending on the ecotype, after 30-40 flowers are produced the SAM becomes dormant and eventually the shoot will undergo senescence (Siriwardana and Lamb 2012; Yanofsky 1995).

Genetic studies show that *PNY* and *PNF* regulate inflorescence architecture. Both *PNY* and *PNF* regulate internode patterning as a decrease in the activity of these two homeodomain proteins severely alters internode development (Smith and Hake 2003). *PNY* and *PNF* regulate internode patterning together with *BP* and *STM* (Bao et al. 2004; Bhatt et al. 2004; Byrne et al. 2003; Kanrar et al. 2006; Roeder et al. 2003; Smith and Hake 2003). In addition, *PNY* and *PNF* are also required for the maintenance of the lateral organ boundary between the stem and pedicel. In *pny PNF/pnf* plants, pedicel-

stem fusions are apparent along the inflorescence axis. Pedicel-stem fusion displayed in *pny PNF/pnf* plants cause the inflorescence to bend and ultimately alter the architecture of the reproductive shoots (Smith et al. 2004). Genetic studies suggest that *PNY* and *PNF* regulate pedicel-stem separation with *STM* but not *BP* (Kanrar et al. 2006). Interestingly, the *KNAT6* expression domain at the pedicel-stem boundary is expanded in *pny* and *bp*. Furthermore, loss of *KNAT6* function restores internode development in *pny* and partially in *bp* (Ragni et al. 2008). The BLH transcription factor, *ATH1*, appears to be required for *KNAT6* function as *ath1 knat6 bp* produces inflorescences with normal internode patterning (Khan et al. 2012a). Whether or not loss of *KNAT6* can reestablish pedicel-stem separation in *pny PNF/pnf* inflorescence remains to be determined.

Two related BTB-ankryin transcription factors, *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* are expressed at lateral organ boundaries including the pedicel-stem boundary (Khan et al. 2012b). While loss of function mutations in *BOP1* and *BOP2* display no apparent effect on internode patterning. However, the internode development is severely impaired in plants ectopically expressing these BTB-ankryin transcription factors (Khan et al. 2012b). Molecular studies suggest that *BOP1* and *BOP2* negatively regulate *BP* (Khan et al. 2012b). Moreover, examination of *BOP1* and *BOP2* expression in *bp* and *pny* shows that the *BOP1* and *BOP2* expression domain expands into the stem and pedicel (Khan et al. 2012a; Khan et al. 2012b). In addition, internode and pedicel patterning is restored in *pny bop1 bop2* and partially *bp bop1 bop2* (Khan et al. 2012b). Experimental studies suggest that *BOP1* and *BOP2* restrict internode and pedicel growth

by activating *KNAT6* as well as *ATH1* (Khan et al. 2012a; Khan et al. 2012b). In addition, *BOP1* and *BOP2* also activate lignin biosynthesis genes, which likely contribute to the lack of internode and pedicel growth *(Khan et al. 2012b)*. Taken together, analysis of KNOX-BLH and *BOP1/BOP2* suggest that maintenance of the pedicel-stem boundary is essential for regulating internode and pedicel development, which are crucial structures that contribute to shoot architecture. Given that *PNY/PNF* also control pedicel-stem separation and the specification of axillary meristems, it would be interesting to know how the boundary contributes to other components of the phytomer and the overall architecture of the shoot.

Conclusions

In conclusion, STM-PNY/PNF complexes appear to regulate shoot meristem maintenance and integrity by controlling CZ-PZ, meristem-leaf, and inflorescence-floral meristem boundary. Experimental evidence indicates that STM-ATH1 complexes maintain the boundary between the meristem, stem and leaf. Therefore, TALE homeodomain complexes appear to play a major role in meristem maintenance and integrity by controlling boundaries within the meristem and between the meristem and lateral organs, axillary meristems and the stem. Genetic studies suggest that STM-ATH1 and STM-PNY/PNF complexes also control meristem identity by possibly interacting with and/or regulating phase specific transcriptional regulators. In addition, these TALE homeodomain complexes may act as molecular beacons that target phase specific transcriptional regulators to the appropriate loci in the genome. The vegetative phase

change may be regulated in part by PNY/PNF and ATH1, which function in an antagonistic manner to modulate the levels of miR156. Experimental evidence indicates that KNOX proteins control meristem maintenance by regulating the biosynthesis and activity of CK and GA. However, this regulatory circuit may be more complex given that CK and GA act in the shoot meristem to promote floral evocation. The genetic relationship between boundary genes and homeodomain genes reveal the crucial roles of boundary genes in organ separation, meristem maintenance and axillary meristem formation. The fusion defect between pedicel and stem caused by the genetic lesion of *PNY* and *PNF* implies the involvement of *BLH* genes in boundary function. Understanding how KNOX-BLH complexes coordinate meristem identity and boundary function can be in part addressed by determining the biochemical function KNOX-BLH complexes and identifying the target genes regulated by these complexes in different developmental pathways.

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Figure 1.1 The role of ATH1 and PNY/PNF in regulating vegetative meristem identity

In this model, we propose that *ATH1* and *PNY/PNF* act in an antagonistic manner to regulate the temporal levels of *miR156*. In addition, ATH1 promotes vegetative shoot identity by activating *FLC* in a FR1 dependent manner. Positive regulation of *miR156* and *FLC* by ATH1 results in the repression *FT* and *SPL* expression. Ultimately, inflorescence identity is repressed in part through the inactivation of *SOC1* expression. The solid line depicts known regulatory events while the white filled line indicates a proposed regulatory event.

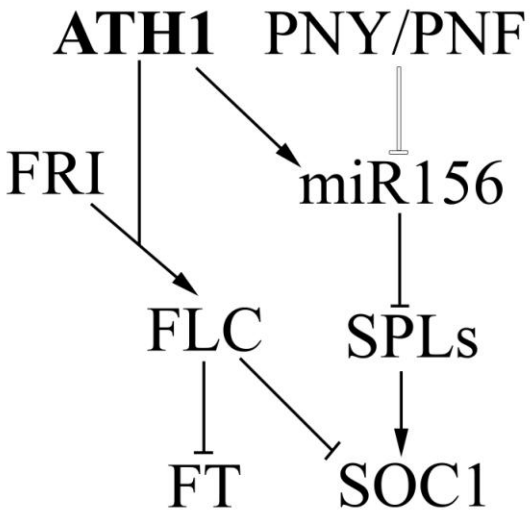
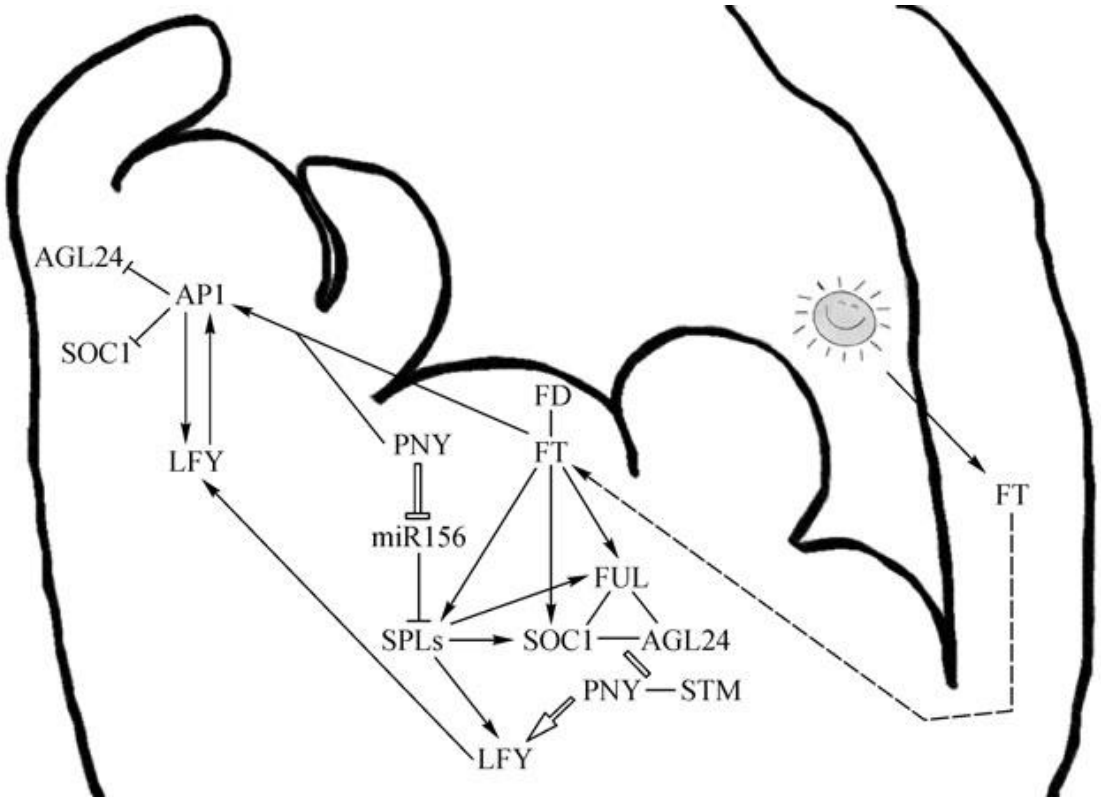

ATH1
PNY/PNF
FRI
miR156
FLC
SPLs
FT
SOC1

Figure 1.2 The role of PNYand PNF in regulating flower specification

Based on this model, we propose that PNY (as well as PNF) acts to regulate flower specification by down-regulating *miR156*, which allows for optimal *SPL* gene expression. In addition, PNY-STM (as well as PNF-STM) heterodimer may associate with SOC1-AGL24-FUL to promote the upregulation of *LFY*. Lastly, the FT activates *AP1*and *SPLs* in a PNY (as well as PNF) dependent manner. The solid lines and arrows depict known interactions and regulatory events while the white filled lines and arrows suggest possible interactions and regulatory events.


AGL24
AP1
SOC1
LFY
PNY
FD
FT
miR156
FUL
SPLs
SOC1
AGL24
PNY
STM
LFY
FT

Chapter 2

The integrity of the lateral organ boundary is essential for shoot architecture

ABSTRACT

All the aerial organs in plant are elaborated from SAM (shoot apical meristem), a dynamic self-sustained structure maintaining a constant number of stem cells and providing cells differentiating for organogenesis. The *pny pnf* SAM with a reduced meristematic activity appears disorganized and exhibits disturbed expression of *CLV3* (*CLAVATA3*) and *WUS* (*WUSCHEL*). Lateral organ boundary severs as a physical barrier to prevent meristematic cells from differentiation, in turn contributing for meristem maintenance. Lateral organ boundary gene *LOF1* (*LLATERAL ORGAN FUSION1*) and *LAS* (*LATERAL SUPPRESSOR*) play roles in lateral organ separation and axillary meristem formation, which is further confirmed with the morphologic phenotype of *lof1 las* double mutant. Loss of function in *LOF1* or *LAS* not only restores the internode patterning in *pny* but also restores the floral specification in the *pny pnf* double mutant, suggesting the role of lateral organ boundary in PNY-mediated inflorescence patterning. The GUS activity of ET4016 in *blr* and *PRO35S:STM-GR* suggest *LOF1* is downregulated by PNY but upregulated by STM. The ectopic expression of *LOF1* in *pny pnf* SAM suggests that *PNY* and *PNF* function in repressing *LOF1* expression in the meristematic cells to allow the restriction of *LOF1* within the lateral organ boundary. In addition, the ectopic expression of *CLV3* and *WUS* also get restored in *pny pnf lof1* and *pny pnf las* triple mutant. Our data implicate lateral organ boundary communicates with the central region of SAM via *PNY* and *PNF* to regulate inflorescence patterning.

INTRODUCTION

A shoot apical meristem (SAM) is a self-organizing group of cells located at the tip of shoots, which functions to maintain stem cell homeostasis and allocate cells for organogenesis (Carles and Fletcher 2003; Rieu and Laux 2009; Sablowski 2011; Wu and

Smith 2012; Xie et al. 2009). Histological and cytological studies show that the SAM is subdivided into discrete organized domains (Xie et al. 2009). Stem cells are located in the central zone (CZ), which is in the apical region of the SAM (Barton 2010). The peripheral zone (PZ) surrounds the CZ and functions in the allocation of cells into initiating primordia. The pith cells in the stem are derived from the rib meristem (RM), which also functions to mediate morphological changes required for floral evocation. During organogenesis, lateral organ boundaries mediate organ separation and restrict developmental programs to the shoot meristem or initiating primordia. Shoot meristems initiate leaves and axillary meristems as well as allocate cells for internode development. The morphological diversity of shoot architecture is mediated by patterning events established in the shoot meristem. How these patterning events are established is poorly understood. Understanding the mechanisms that control these patterning events may allow plant biologists to modify shoot architecture in order to increase crop productivity.

Stem cell homeostasis, an essential component of meristem maintenance, is regulated in part by the *CLAVATA* (*CLV*) – *WUSCHEL* (*WUS*) negative feedback loop (Perales and Reddy 2012; Schoof et al. 2000). *WUS* encodes a homeodomain transcription factor (TF) that is essential for stem cell maintenance. *WUS* is expressed in a small group of cells located in the RM and the base of the CZ. The *WUS* expression domain is referred to as the organizing center (OC). The CLV signaling pathway functions to restrict the *WUS* expression domain to the OC. *CLV3* is expressed in the CZ and encodes a small, secreted polypeptide, which acts as an extracellular ligand that associates with transmembrane

receptor kinases CLV1 and BAM in the core of the shoot meristem (Brand et al. 2002; Ogawa et al. 2008; Ohyama et al. 2009). CLV2 encodes a receptor that lacks the intracellular kinase domain (Jeong et al. 1999), yet binds to CLV3. Recent studies show that CLV2 associates with a transmembrane kinase called CRN and together this complex likely constitutes a CLV3 ligand receptor kinase complex (Muller et al. 2008). Current models predict that the CLV3-CLV1, CLV3-BAM and CLV3-CLV2-CRN complexes function in the meristem center to confine *WUS* expression to the OC (Bleckmann et al. 2010; Brand et al. 2000; Muller et al. 2008; Nimchuk et al. 2011). In turn, WUS acts in a positive manner to activate *CLV3* in the CZ (Laux et al. 1996; Mayer et al. 1998; Rieu and Laux 2009; Schoof et al. 2000; Tucker and Laux 2007). This regulation appears to be direct as WUS is capable of moving cell to cell and associates with the *CLV3* promoter in vivo (Yadav et al. 2011). Recent studies indicate that stem cell homeostasis is also mediated by a positive feedback loop between cytokinin and WUS (Busch et al. 2010; Hwang et al. 2012; Leibfried et al. 2005).

Meristem cell fate is specified by specific members of the KNOTTED1-like Homeobox (KNOX) TFs, which are members of the Three Acid Loop Extension (TALE) superclass of homeodomain proteins in plants (Hake et al. 2004; Hamant and Pautot 2010; Hay and Tsiantis 2010; Scofield and Murray 2006; Wu and Smith 2012). Maize and *Arabidopsis* plants containing null mutations in KNOX genes, *knotted1* (*kn1*) and *SHOOT MERISTEMLESS* (*STM*), respectively, display a phenotype in which the embryonic shoot meristem terminally differentiates during the formation of the embryonic leaves (Barton

and Poethig 1993; Long et al. 1996; Vollbrecht et al. 2000). *kn1*/*STM* are expressed in the shoot apical and axillary meristems throughout the growth and development of the shoot (Long and Barton 2000). Genetic and physiological studies suggest that KNOX TFs regulate meristem cell fate by establishing a positive feedback loop system with the plant hormone cytokinin, which is essential for meristem formation and maintenance (Jasinski et al. 2005; Yanai et al. 2005). At the same time, KNOX TFs function to negatively regulate a suite of genes that control differentiation. Consistent with the latter hypothesis, KNOX genes, such as *STM* and *KN1*, are expressed in the shoot meristem except at sites where leaf primordia develop. The downregulation of *KNOX* genes in the incipient leaf primordia is thought to be an essential step in leaf development that is mediated by auxin (Hay et al. 2006). Therefore, current models predict that interplay between auxin and KNOX is essential for organogenesis.

The formation and maintenance of the lateral organ boundary is predicted to play a fundamental role in regulating shoot morphology. Based on genetic and molecular studies, the lateral organ boundary is not only essential for the physical separation of the lateral organs from the shoot but this region also plays an essential role in the formation of axillary meristems. The *CUP-SHAPED COTYLEDON* (*CUC*) genes, *CUC1*, *CUC2,* and *CUC3*, are essential for the establishment and maintenance of lateral organ boundaries in *Arabidopsis* (Aida and Tasaka 2006a). For example, cotyledons are fused and meristem initiation is perturbed in *cuc1 cuc2* and *cuc2 cuc3* plants (Aida et al. 1997; Hibara et al. 2006). Moreover, recent studies also show that *CUC2* and *CUC3* are

required for the formation of tertiary axillary shoots and function in the separation of axillary shoots and flowers from the primary shoot (Hibara et al. 2006). In addition, ectopic expression of *miR164*, which targets *CUC1* and *CUC2*, in *cuc3* plants results in a severe reduction in the number of axillary meristems produced during shoot development (Baker et al. 2005; Laufs et al. 2004; Mallory et al. 2004; Nikovics et al. 2006; Raman et al. 2008; Sieber et al. 2007). Genetic studies show that *LATERAL SUPPRESSOR* (*LAS*) also functions to promote axillary meristem formation during vegetative growth, in *Arabidopsis* and tomato (Greb et al. 2003; Schumacher et al. 1999). Genetic studies in *Arabidopsis* suggest that *CUC1* and *CUC2* specify axillary meristems by upregulating *LAS* (Hibara et al. 2006; Raman et al. 2008). In contrast, *CUC3* and *LAS* appear to act in parallel to promote axillary meristem formation, as the loss of function allele of *LAS* enhances the boundary and axillary meristem phenotypes in *cuc3* (Hibara et al. 2006). Two related MYB TFs, LOF1 and LOF2, function at the lateral organ boundary between the axillary inflorescence shoot and the cauline leaf as well as pedicel and main stem in *Arabidopsis*. In addition, LOF1 acts in parallel with *CUC2* and *CUC3* to regulate axillary meristem formation during inflorescence development (Lee et al. 2009).

KNOX TFs are also implicated in the maintenance of lateral organ boundaries as the hypomorphic *stm-10* results in production of inflorescences that are reduced in axillary meristem formation and have pedicels fused to the main stem. Moreover, CUC TFs appear to act upstream to *STM* and *KNAT6* to regulate SAM formation and organ separation (Takada et al. 2001). At the same time, STM appears to regulate the spatial

expression pattern of *CUC2* (Aida et al. 1999; Spinelli et al. 2011). Current models predict that *LAS* regulates axillary meristem formation by upregulating *STM* in the axils of leaves (Greb et al. 2003). Genetic studies suggest that *LOF1* and *STM* function in parallel to regulate meristem maintenance (Lee et al. 2009). In addition, LOF1 specifies accessory meristem formation by positively regulating *STM* (Lee et al. 2009). Thus, the complex interplay between STM and boundary genes is essential for the maintenance of lateral organ boundaries and axillary meristem formation. Genetic and biochemical studies show that STM associates with specific members of the TALE-BELL1-like homeodomain (BLH) TFs, including ATH1, PNY and PNF, and together these STM-BLH complexes regulate meristem cell fate (Kanrar et al. 2006; Rutjens et al. 2009). In addition, ATH1, PNY and PNF TFs also function in maintaining boundaries in the shoot meristem. For example, ATH1 functions to maintain basal boundaries between the leaf and stem. Two related BLH TFs, PNY and PNF, appear to act redundantly to regulate axillary meristem formation in mature shoots (Smith et al. 2004). Genetic studies suggest that STM functions with PNY/PNF to regulate the separation of flowers from the main stem (Kanrar et al. 2006). Recent studies suggest that PNY and PNF regulate internode development by restricting the expression pattern of BOP1 and BOP2 to the adaxial boundary between the pedicel and main stem (Khan et al. 2012b).

In this manuscript, the genetic relationship between *LOF1* and *LAS* was examined and results indicate that these TFs regulate organ separation and axillary meristem formation in parallel. Experimental evidence shows that PNY and PNF act to maintain lateral organ

boundaries during inflorescence development (Smith et al. 2004); therefore, we examined the relationship of *LAS* and *LOF1* with PNY/PNF. Genetic studies show that loss of *LAS* or *LOF1* function in a *pny pnf* mutant background nearly restores normal inflorescence development, including axillary meristem formation, flower meristem identity and internode development. Expression patterns of *LOF1* and *LAS* were examined and results show that *LOF1* is ectopically expressed in the shoot meristem of *pny pnf* plants. In contrast, the expression pattern of *LAS* appears to be reduced in the *pny pnf* shoot meristem. Since loss of *LAS* function nearly restores inflorescence development in *pny pnf* plants, it seems reasonable that the integrity of the lateral organ boundary is compromised in *pny pnf* plants resulting in diffusion of *LAS* transcripts into the shoot meristem. The integrity of the shoot meristem is nearly restored in *pny pnf las* and *pny pnf lof1*. Therefore, we propose that PNY and PNF function to regulate shoot architecture by regulating the integrity of the lateral organ boundary during inflorescence development.

RESULTS

LAS and *LOF* function independently to regulate lateral organ boundaries and axillary meristem formation

LOF1 and LOF2 function together to mediate the separation of pedicels from the internodes of the main stem as well as cauline leaves from secondary inflorescence shoots (Lee et al. 2009). Based on genetics studies, LOF1 acts independent of the CUC TFs. To further investigate the function of *LOF1* in the lateral organ boundary gene

regulatory network, the genetic relationship between *LOF1* and *LAS* was examined. To this end, the *lof1 las* double mutant was generated and fusion events were examined during inflorescence development. In *wild-type* and *las* plants, there were no apparent fusion events between the pedicel and internode (Fig. 2.1A and 2.1B) or the cauline leaf and axillary/secondary inflorescence shoot (Fig. 2.1E and 2.1F). In *lof1* inflorescences, fusion between the pedicel and stem was not observed (Fig. 2.1C). However, *lof1* displayed fusion between the cauline leaf and axillary inflorescence stem (Fig. 2.1G). In *lof1 las* plants, 75% of the inflorescences displayed fusions between stem and pedicel (Fig. 2.1D, Table 2.1). Moreover, the fusion between axillary inflorescences and cauline leaves appeared to be more severe in *lof1 las* than in *lof1* plants (compare Fig. 2.1G with Fig. 2.1H). Axillary meristems that formed in rosette leaf axils also occasionally exhibited fusion to cauline leaves (Fig. 2.1J). In addition, 9.8% of axillary inflorescence stems were fused to the primary inflorescence in *lof1 las* double mutants (Fig. 2.1H and 2.1I). Hence, a decrease in organ separation events that are not observed in either *las* or *lof1* single mutants were displayed in *lof1 las* inflorescences shoots indicating that *LAS* and *LOF1* independently control organ boundary specification or maintenance.

Axillary meristems with inflorescence identity are typically produced in the axils of cauline leaves. To further understand the additive function of LAS and LOF1 in axillary meristem formation, the production of tertiary inflorescences that develop in the axils of secondary cauline leaves was examined in wild-type, *las*, *lof1* and *lof1 las* plants (Fig. 2.1L). Two abnormal phenotypes were observed in *lof1 las* double mutants: barren

cauline leaf axils and the formation of solitary flowers instead of inflorescences. Whereas barren cauline leaf axils and solitary flowers were produced only rarely in wild-type and *lof1* mutants, one of these abnormalities was present in approximately 50% of *las* cauline leaf axils, corresponding to a significant increase in the number of barren leaf axils and a modest increase in the formation of solitary flowers (Fig. 2.1L). In *lof1 las* secondary inflorescences, there was a higher frequency of solitary flower formation, such that nearly 80% of cauline leaf axils were abnormal. These data indicate that LOF1 and LAS both contribute to axillary meristem formation as well as organ separation.

Loss of function in *LOF1* or *LAS* suppresses the internode patterning defect in *pny* inflorescences

The organ fusion exhibited in *lof1 lof2* and *lof1 las* mutants, in which the pedicel is fused to the primary inflorescence stem is strikingly similar to the pedicel-stem fusion displayed in *pny PNF/pnf* reproductive shoots (Compare Fig. 2.1D with 2.1K) (Lee et al. 2009; Smith et al. 2004). Therefore, the genetic relationship between *PNY*/*PNF* and the boundary genes *LOF1* and *LAS* was examined. In this analysis, we first determined the phenotypes of *pny lof1* and *pny las* double mutants under long-day growth conditions. During inflorescence development, wild-type, *las* and *lof1* shoots displayed regular patterns of internode development, which separate the fruits or siliques (Fig. 2.2A-C). The majority of internodes were 6-15 mm in length in wild type, *lof1* and *las* (Fig. 2.2G). In contrast, *pny* inflorescences displayed internode patterning defects along the axis of the reproductive shoot as previously described (Smith and Hake 2003) (Fig. 2.2D).

Further, the majority of internodes between fruits were less than 10 mm in length in *pny* inflorescences (Fig. 2.2G). Interestingly, internode patterning was nearly restored in *pny lof1* and *pnf las* shoots (Fig. 2.2E and 2.2F), such that the majority of internodes were between 6-15 mm in length, similar to wild-type, *las* and *lof1* plants (Fig. 2.2G). Taken together, these results indicate that the internode patterning defects in *pny* mutants require LOF1 and LAS activity.

Inflorescence development is reestablished in *pny pnf lof1* and *pny pnf las* plants

In *pny pnf* double mutant, the SAM fails to overcome the floral transition, continuously producing leaves. In addition, internode development is severely perturbed in the mature shoots of *pny pnf* plants. To determine if the loss of *LOF1* or *LAS1* could restore inflorescence development in *pny pnf* plants, we generated *pny pnf lof1* and *pny pnf las* triple mutants. As shown in Figure 2.3, the shoots of wild type, *lof1* and *las* initiate inflorescences with similar morphology, producing similar numbers of flowers spaced at regular intervals on the inflorescence stem (Fig. 2.3A-C, Table 1 and 2). Consistent with previous studies, the mature shoots of *pny pnf* plants continuously produced leaves without forming flowers and internodes (Fig. 2.3K and 2.3L). Strikingly, *pny pny lof1* and *pny pnf las* plants produced inflorescence shoots that were similar in morphology to wild type, *las* and *lof1* (Compare Fig. 2.3A-C with 2.3D and 2.3E). Quantitative analysis showed that flower specification was completely restored in *pny pnf lof1* and *pny pnf las* plants, as these inflorescences initiated similar numbers of flowers as wild-type, *lof1* and *las* (Table 2.2). In addition, secondary inflorescence shoots were produced in the axils of

cauline leaves in *pny pnf lof1* and *pny pnf las* (data not shown). However, internode development was not completely restored in *pny pnf lof1* and *pny pnf las*, as a high percentage of internodes were reduced in length in both genotypes compared to wild type (Fig. 2.2G). In fact, *pny pnf las* plants displayed a higher percentage of internodes that were less than 1 mm in length compared to *pny pnf lof1* (Fig. 2.2G). Another lateral organ boundary phenotype observed in *pny pnf lof1* and *pny pnf las* was the fusion of pedicels to the main stem (Fig. 2.3M and 2.3N). The pedicel-stem fusion events occurred in 30% and 83.3% of the inflorescences in *pny pnf lof1* and *pny pnf las*, respectively (Table 2.1). Taken together, a reduction of LOF1 or LAS function in *pny pnf* restores the integrity of the primary SAM and axillary meristems, which form with either flower or inflorescence meristem identity. However, internode patterning defects and pedicel-stem fusion events were apparent in *pny pnf lof1* and *pny pnf las*, indicating that the lateral organ boundary is somewhat compromised in these triple mutants.

Expression of *LOF1* and *LAS* is altered in *pny pnf* shoot meristems

The fact that loss of LOF1 or LAS function reestablished internode patterning in *pny* plants and nearly restores inflorescence development in *pny pnf* plants indicates that these phenotypes are dependent on LOF1 and LAS activity. To examine the *LOF1* expression pattern in *pny* plants, the *ET4016* enhancer trap line, which reports *LOF1* expression (Lee et al. 2009) was crossed *bellringer* (*blr*), which contains an allele of *pny* in the L*er* background (Byrne et al. 2003). In 5-day-old ET4016 seedlings, GUS activity was detected in a band at the base of leaf primordia, as previously reported (Lee et al. 2009).

Compared to ET4016, an expansion and increase in the level of GUS activity was observed in *ET4016 blr* seedlings (Fig. 2.4A and 2.4B). Similar results were obtained with 7- and 10-day-old seedlings (data not shown). During inflorescence development GUS activity was expanded to the meristem in *ET4016 blr*, whereas it was restricted from the meristem in ET4016 (Fig. 2.4C and 2.4D). Taken together, these data indicate that in the absence of PNY function, the *LOF1* expression domain expands in the vegetative and inflorescence apices, consistent with PNY restricting *LOF1* expression in the SAM.

We next examined the expression pattern of *LOF1* in *pny pnf* apices using mRNA *in situ* hybridization. In wild-type and *las* vegetative apices, *LOF1* expression was detected at the boundary between the shoot meristem and the developing leaf primordia (Fig. 2.4E and 2.4G). Consistent with the expression pattern of *ET4016* in *blr* apices, *LOF1* transcripts were detected throughout the shoot apical meristem in *pny pnf* shoot apices (Fig. 2.4F). The *LOF1* mRNA localization pattern was also examined in *pny pnf las* plants. Interestingly, *LOF1* transcripts were detected in patches in the shoot meristem of *pny pnf las*, in addition to the boundary cells between the shoot meristem and leaf primordia (Fig. 2.4H). The observation that inflorescence development is nearly restored in *pny pnf las* despite ectopic *LOF1* expression in the shoot meristem indicates that ectopic LOF1 activity is not sufficient to prevent inflorescence development. Furthermore, these data suggest that LOF1 activity is dependent on LAS.

To investigate the effect of PNY and PNF on *LAS* expression, the expression pattern of *LAS* was examined in wild-type, *lof1 pny pnf* and *pny pnf lof1* shoot apices using in situ hybridization. As previously reported (Greb et al. 2003), *LAS* transcripts were detected at the lateral organ boundary between the shoot meristem and developing leaf primordia in wild-type plants, similar to *LOF1* (Fig. 2.5A). The pattern of LAS transcript accumulation was unchanged in *lof1* apices (Fig. 2.5C). Somewhat to our surprise, the expression pattern of *LAS* was perturbed in *pny pnf* apices such that the mRNA levels were difficult to detect by *in situ* hybridization (Fig. 2.5B). Expression of *LAS* could not be detected in 8 out of 12 apices examined, and in the remaining 4 apices, a faint signal was detected in the boundary domain. In contrast, *LAS* expression was easily detected at the boundary between the shoot meristem and the developing leaf in *pny pnf lof1* mutants (Fig. 2.5D). Thus, the restoration in meristem integrity in *pny pnf lof1* mutants restored normal *LAS* expression. These data suggest that ectopic LOF1 activity in the *pny pnf* SAM alters the *LAS* expression pattern. One possibility is that *LAS* transcripts accumulate in the *pny pnf* SAM at levels too low to detect. Taken together, these expression studies suggest that the integrity of the lateral organ boundary is compromised in *pny pnf* shoot meristems. Further, the cells of the *pny pnf* shoot meristems may display or possess some form of boundary identity.

Meristem organization is restored in *pny pnf lof1* and *pny pnf las* mutants

A recent study showed that the integrity of the CZ and RM is compromised in *pny pnf* shoot meristems resulting in an alteration in the expression patterns *CLV3* and *WUS* (Ung

et al. 2011). Further, genetic studies showed that increasing the meristem size in *pny pnf* plants partially restored reproductive development, which correlated with the apparent reestablishment of the *CLV3* and *WUS* expression domains. Results in Figure 3 showed that *pny pnf lof1* and *pny pnf las* produce inflorescence shoots that are similar in morphology to wild-type, indicating that loss of *LAS* or *LOF1* has a higher propensity to restore reproductive development than increasing the size of the meristem in *pny pnf* plants. To determine if the loss of *LOF1* and *LAS* can reestablish meristem integrity in *pny pnf* plants, the expression pattern of *CLV3* was examined in wild-type, *pny pnf*, *lof1*, *las*, *pny pnf lof1* and *pny pnf las* vegetative shoot apices. In wild-type, *lof1*, and *las* shoot apices, *CLV3* localized to the central apical cells that overlap with the CZ in the shoot meristem (Fig. 2.6A-C). In contrast, *CLV3* transcripts were not readily detected in *pny pnf* apices (Fig. 2.6D) as previously reported (Ung et al. 2011). However, in *pny pnf lof* and *pny pnf las* plants, the *CLV3* mRNA localization pattern was reestablished, reminiscent of the *CLV3* expression pattern displayed in wild type, *lof1* and *las* (Fig. 2.6E and 2.6F). The restoration of *CLV3* in *pny pnf lof1* and *pny pnf las* indicates that PNY and PNF regulate the integrity of the CZ by repressing boundary cell fate shoot meristem.

The next experimental goal was to examine and compare the expression of the *WUS* domain, which marks the OC of the shoot meristem. In wild-type, *lof1* and *las* apices, *WUS* expression was detected in a small group of cells in the core of the shoot meristem (Fig. 2.6G-I). As previously reported (Ung et al. 2011), the *WUS* expression domain was diffuse and the levels appeared to be reduced in the shoot meristem *pny pnf* plants (Fig.

2.6J). In contrast to the expression pattern displayed in *pny pnf* shoot meristems, the *WUS* expression domain was reestablished in *pny pnf lof1* and *pny pnf las*, indicating that a decrease in the integrity of the lateral organ boundary alters the organization of the OC or RM (Fig. 2.6K and 2.6L).

DISCUSSION

Lateral organ boundaries are essential for shoot meristem establishment, function and organogenesis (Aida and Tasaka 2006b; Rast and Simon 2008). The physical separation of the developing primordia from the shoot meristem is dependent upon the establishment and maintenance of the lateral organ boundary, which functions in part to reduce or limit cell division. Lateral organ boundaries also function to separate the identities between the two tissue types, e.g, leaf versus meristem. Lastly, the establishment of the lateral organ boundary may serve as a site for organogenesis. Genetic and molecular studies indicate that CUC TFs function upstream and in parallel with LOF1 and LAS (Hibara et al. 2006; Lee et al. 2009; Raman et al. 2008). Results from this manuscript show that LOF1 and LAS function in parallel to specify and/or maintain boundaries that are crucial for the physical separation of developing primordia and the formation of axillary meristems during inflorescence development. Experimental studies from this paper also suggest that PNY/PNF are key regulators that function to maintain the integrity of the lateral organ boundary during inflorescence development. Moreover, the maintenance of the lateral organ boundary by PNY and PNF is essential for regulating inflorescence architecture.

Interplay between LOF1 and LAS is crucial for the maintenance of the lateral organ boundary

Experimental studies show that boundary genes play an important role in regulating shoot development and architecture. In *Arabidopsis* and tomato, LAS functions to promote axillary meristem formation (Greb et al. 2003; Schumacher et al. 1999). CUC1/2/3 function redundantly to establish and maintain lateral organ boundaries during shoot development in *Arabidopsis*. In the absence of CUC function, mechanisms that regulate meristem maintenance and organ separation are severely perturbed (Aida et al. 1999; Vroemen et al. 2003). Moreover, CUC TFs also act to specify axillary meristems during shoot development (Hibara et al. 2006; Raman et al. 2008). First, CUC1 and CUC2 promote axillary meristem formation by upregulating *LAS* in the leaf axils. Second, CUC3 and LAS act together to specify axillary meristems. In addition, CUC2 and CUC3 act together with LOF1 and LOF2 to maintain lateral organ boundaries and promote axillary meristem formation during inflorescence development (Lee et al. 2009). In this study, genetic experiments show that LOF1 and LAS primarily function in parallel to mediate the separation of flowers and secondary inflorescence shoots from the internodes of the main stem. In addition, LOF1 and LAS act independently to control the separation cauline leaves from secondary inflorescence shoots. Therefore, we propose that LOF1 functions independent of or in parallel with CUC1/2 –LAS and CUC3 to regulate lateral organ boundary function during shoot development (Figure 2.7).

PNY/PNF regulate meristem cell fate in part by maintaining the integrity of the lateral organ boundary

Genetic studies show that PNY and PNF are essential for regulating the integrity of the lateral organ boundary during inflorescence development. First, the reproductive shoots of *pny PNF/pnf* displays fusions between the pedicel and internode of the main stem (Kanrar et al. 2006; Smith et al. 2004). Second, axillary meristem formation is severely impaired in the mature shoots of *pny pnf* plants. In addition, the narrow *STM* expression domain in *pny pnf* shoot meristems shows that a large proportion of meristematic cells are allocated into leaf primordia compared to wild type, further supporting the hypothesis that these homeodomain proteins regulate the integrity of the lateral organ boundary (Smith et al. 2004; Ung et al. 2011). Consistent with this hypothesis, results show that *LOF1* is ectopically expressed in the shoot meristems of *pny pnf* plants. The apparent reduction in *LAS* transcripts at the lateral organ boundary is somewhat puzzling as loss of LAS function restores inflorescence development in *pny pnf* plants. A possible explanation for this discrepancy is that *LAS* mRNA may be diffuse within the shoot meristem; as a result mRNA levels are no longer sufficient to be detected by *in situ* hybridization. A similar scenario was described for *CLV3* transcripts in pny pnf mutants (Ung et al. 2011). Ultimately, ectopic expression of boundary genes in the shoot meristem may act to not only reduce cell division in the PZ but also alter the fate of the meristem cells. Furthermore, we speculate that a larger population of meristematic cells is allocated into leaf primordia in *pny pnf* plants, because the boundary is not defined;

therefore, the mechanisms that specify different elements of the phytomer or module are severely impaired.

A previous study showed that PNY and PNF are crucial for maintaining the integrity of the CZ and RM as the expression domains for *CLV3* and *WUS* are severely altered in *pny pnf* shoot meristems (Ung et al., 2011). However, the mRNA patterns of *WUS* and *CLV3* in *pny pnf clv3* and *pny pnf clv1* are similar to the expression patterns in *clv* mutants presumably due to the increased the size of the shoot meristem. In addition, loss of CLV function in *pny pnf* plants partially restored the reproductive potential of the shoot. As a result, the increase in meristem size displayed in *pny pnf clv3* and *pny pnf clv1* plants likely increased the number of cells in the peripheral region of the shoot meristem that are responsive to floral inductive cues. In this manuscript, results showed that the *CLV3* and *WUS* expression domains as well as inflorescence development are nearly restored in *pny pnf las* and *pny pnf lof1* plants. Therefore, we propose that PNY and PNF regulate meristem cell fate and organization by maintaining the integrity of the lateral organ boundary on the flanks of the shoot meristem.

A recent study showed that STM functions with *LOF1* as a loss of function allele of *LOF1* enhances the limited shoot phenotype displayed in *stm-10* (Lee et al. 2009). Using an inducible system, results show that ectopic STM activity induces *LOF1* expression in leaves, but not in the shoot meristem (Supplemental Figure 2.1). The induction of *LOF1* by STM may indicate that this KNOX TF promotes boundary identity, which is the

opposite function of PNY and PNF. To explain these opposing functions, we propose that *STM* functions in the both the shoot meristem and the lateral organ boundary, where it has also been shown to be expressed (Lee et al. 2009). *PNY* and *PNF* expression overlaps with *STM* in the PZ but not in the boundary (Smith and Hake 2003). Thus, in the PZ, STM-PNY/PNF functions to repress boundary identity, while STM promotes boundary cell fate between the PZ and emerging lateral organ. STM may specify boundary identity as a homodimer or through the interaction with another BLH TF(s). Interestingly, genetic and molecular studies suggest that the BLH TF ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1) also interacts and functions with STM to regulate meristem function (Kanrar et al. 2006; Rutjens et al. 2009). Moreover, plants overexpressing *ATH1* display a delay flowering and produce inflorescences with internode patterning defects, similar to *pny* plants (Gomez-Mena and Sablowski 2008; Proveniers et al. 2007). In addition, loss of ATH1 function in *pny* restores a normal pattern of internode development (Rutjens et al. 2009). In contrast to PNY/PNF, *ATH1* transcript levels decrease in the shoot meristem during the floral transition (Proveniers et al. 2007). Although *ATH1* is not expressed in the inflorescence meristem, this *BLH* gene is expressed in the floral anlagen, a domain in which PNY/PNF transcripts are not detected (Cole et al. 2006; Gomez-Mena and Sablowski 2008). Therefore, the juxtaposition of *PNY*/*PNF* and *ATH1* expression domains may function to define the lateral organ boundary during inflorescence development. Further, the STM-ATH1 complex may function to specify the boundary identity during inflorescence development (Cole et al. 2006; Gomez-Mena and Sablowski 2008; Rutjens et al. 2009).

Regulation of inflorescence architecture by TALE-HD and boundary genes in the shoot meristem

The shoot meristem plays a fundamental role in regulating shoot architecture as it produces organs and structures in a reiterated modular fashion (Lyndon 1990; Steeves and Sussex 1989). Clonal analysis studies show that a subset of meristem cells is allocated to form each of the modular units called phytomers (Carles and Fletcher 2003; Lyndon 1998; Steeves and Sussex 1989). In addition, the fate of the meristem plays an essential role in determining the identity and function of the modules (Lyndon 1990; Steeves and Sussex 1989). Previous studies indicate that PNY/PNF and ATH1 control flower and vegetative meristem identity, respectively (Proveniers et al. 2007; Smith et al. 2004). Further, we propose that the spatial interplay between STM-PNY/PNF and STM-ATH1 homeodomain complexes are essential for specifying meristem cell fate and boundary identity on the flanks of the shoot meristem. Recent studies indicate that PNY and the KNOX TF BREVIPEDICELLUS (BP) function to repress KNAT6 (Ragni et al. 2008) as well as boundary expressed genes BOP1/2 (Khan et al. 2012b) during internode and pedicel development. Note, BOP1/2 and KNAT6 are not misexpressed in the *pny* inflorescence meristem. Interestingly, loss of *ATH1* function also rescues the *pny* internode phenotype, suggesting that PNY negatively regulates this BLH gene during inflorescence development. ATH1 not only interacts with KNAT6, but it may be positively regulated by BOP1(Khan et al. 2012a). Therefore, BOP1/2 appears to be key

regulators that control the antagonistic functions between PNY and ATH1 during internode development.

In this manuscript, we show that the interplay between PNY/PNF and the LOF1 and LAS boundary genes is essential for regulating inflorescence architecture including axillary meristem formation (secondary inflorescences and flowers) as well as internode patterning. Further, the results presented in this manuscript indicate that regulation between PNY/PNF and the boundary as indicated by *LOF1* and *LAS* expression occurs at the shoot meristem, the site in which patterning events are established. At this time, the relationship between LOF1 and LAS with BOP1/2 are not known. However, the altered expression patterns for *LOF1* and *LAS* in the shoot meristems of *pny pnf* plants may increases the number of cells with boundary identity that are allocated to form each module. As a result, the meristem cells expressing boundary identity are unable to respond to floral inductive cues to produce axillary meristems and internodes. The fact that either *las* or *lof1* nearly restores internode and axillary meristem formation suggests that these transcriptional regulators are somehow dependent upon each other for specifying boundary identity, which is consistent with the *las lof1* phenotype. The fact loss *pny pnf clv3* produces larger shoot meristems that are reproductively competent support our hypothesis (Ung et al., 2011). Taken together, we propose that PNY and PNF function to specify meristem cell fate in the PZ, which acts to restrict the boundary to flank of the shoot meristem. Further, the positioning of the lateral organ boundary is not only crucial for meristem integrity but also for regulating inflorescence architecture.

NOTES

Supplemental

Biochemical evidence has shown that PNY physically interacts with STM to form heterodimer, which presumably bind to KNOX-BLH binding sites to regulate target gene transcription. Genetic data suggest that STM is involved in PNY-mediated meristem maintenance. Hence, we attempted to examine whether STM could also downregulate *LOF1* expression. Pro35S:STM-GR was crossed with ET4016, which reports *LOF1* expression. Pro35S:STM-GR transgenic plants form leaf-like lateral organs with many stipules when grown on DEX. To our surprise, in the Pro35S:STM-GR background, ET4016 GUS activity was expanded to the blade of leaf-like lateral organs and was also present in junctions between the leaf-like organs and ectopic stipules when grown on DEX, indicating STM upregulates *LOF1* expression instead. We previously reported that mutation in *LOF1* enhances meristem maintenance defect in *stm-10,* which support this argument (Lee et al. 2009).

MATERIALS AND METHODS

Growth Condition

To analyze inflorescence morphology, plants were grown on soil at 23ºC under long-day condition (16hr light/ 8hr dark). For mRNA in situ hybridization, plants were grown on soil at 23 ºC under short day condition (8hr light/ 16hr dark) for 30 days. *ET4016 and*

ET4016 blr were grown on MS media in growth chamber (Percival Scientific) at 22 ºC under long-day condition. Seedlings were collected at 5, 7 and 10 days for GUS staining.

Genetics

The *lof1-1*, *las-4, pny*, and *pnf* mutants were in the Col ecotype and ET4016 and *blr* are in the L*er* ecotype (Byrne et al. 2003; Greb et al. 2003; Smith et al. 2003; Lee et al. 2009). For PCR-based genotyping of *lof1-1*, gene-specific PCR primers pLOF1-F2 (GGCAGTCCACAGCAAAATCGAATATCG) and pLOF1-R (CCCGGGTGAACCGCTACATCTCCAAGAG) were used together with LBA1 (TGGTTCACGTAGTGGGCCATCG). For *las-4*, a dCAPS marker was developed using the primers LAS-F (GTGTGAAACTGGAATTGGAGACC) and LAS-R (GCCGCTCAAAACTTACTCTCAAT), which amplify a 566 bp fragment in both wild-type and *las-4* alleles. *las-4* PCR products were digested to 191 and 375 bp by *Spe* I enzyme. *pny-40126* was identified using primers 40126-WT (CCCATCACAGCTTGTAGCTGCTCA) and 40126-1 (TGGAATTGGAGCAAAATGTGTTA) to amplify the wild-type allele of *PNY* and 40126-1 (TGGAATTGGAGCAAAATGTGTTA) and LBA-1 (TGGTTCACGTAGTGGGCCATCG) to amplify the pny mutant allele (Smith et al. 2003). For genotyping *pnf-33879*, we used primer LBA-1 and gene-specific primers 33879-1(ACGCAATCATTATTCTCTCTCAGTC) and 33879-WT (TGCATGAGTTCCATATATATAGC). For genotyping ET4016, we used gene-specific primers ET4016F (GGACTTCGGTTAAGACAGCCTCCA) and ET4016R

(TCTGCTACGGCGCAACAAGACTTA) together with Ds element primer Ds3-4 (CCGTCCCGCAAGTTAAATATG).

GUS staining

Seedlings were stained with GUS staining solution (50mM sodium phosphate (pH7.2), 0.5mM $K_4Fe(CN)_6$, 0.5mM $K_3Fe(CN)_6$ and 1mg/ml 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt) at 37ºC for 5 hr. Chlorophyll was sequentially removed with 50% and 70% ethanol. Seedlings were mounted until completely dechlorophyllized. Photographs were taken by a stero-microscope (LEICA MZ12; Leica Microsystems, Wetzlar, Germany).

mRNA *in situ* hybridization

The shoot apices were collected from plants grown under short-day condition for 30 days. The detailed procedure can be found in Appendix 2.1. The primer sets for synthesis probes were listed in Table 2.3.

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Figure 2.1: *LOF1* and *LAS* function in parallel to regulate organ separation

(A-D) Pedicel-stem junction in Col wild-type (A); *las-4* (B); *lof1-1* (C); and *lof1-1 las-4* (D). (E-I) Paraclade junction in Col (E); *las-4* (F); *lof1-1* (G); *lof1-1 las-4* (H,I). (J) Axillary stem fused to cauline leaf in *lof1-1 las-4*. (K) Pedicel stem fusion in *pny/pny PNF/pnf*. (I) Frequency of abnormal tertiary inflorescence formation in Col, *las-4, lof1-1*, and *lof1-1 las-4*.Red arrows indicate fusions.

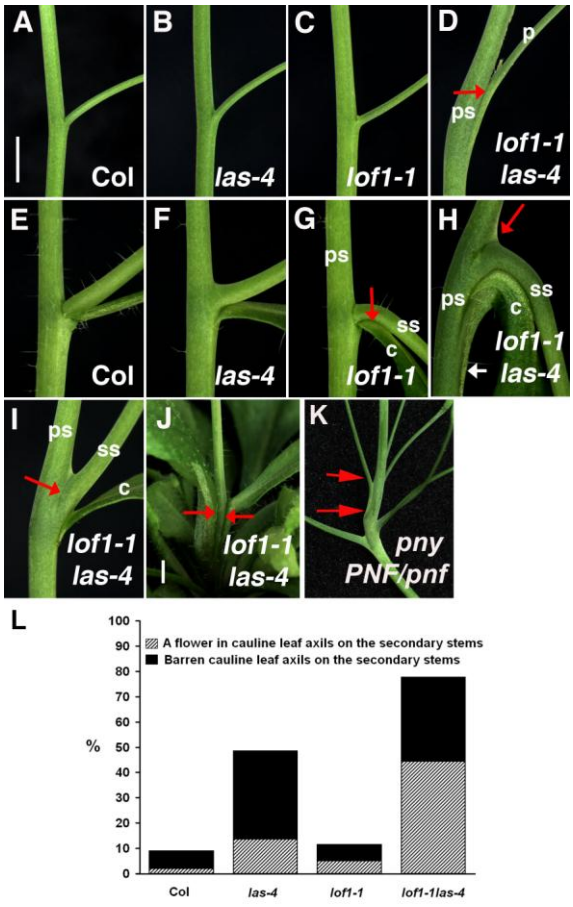
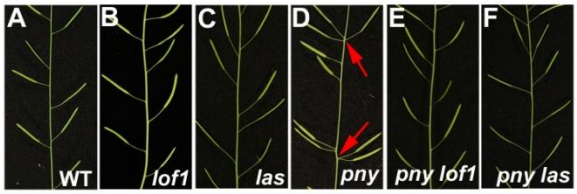

A
Col
B
las-4
C
lof1-1
D
p
ps
lof1-1
las-4
E
Col
F
las-4
G
ps
ss
c
lof1-1
H
ps
ss
c
lof1-1
las-4
I
ps
ss
c
lof1-1
las-4
J
lof1-1
las-4
K
pny
PNF/pnf
L
A flower in cauline leaf axils on the secondary stems
Barren cauline leaf axils on the secondary stems
%
100
90
80
70
60
50
40
30
20
10
0
Col
las-4
lof1-1
lof1-1las-4

Figure 2.2: The impact of LOF1 and LAS on PNY and PNF-mediated internode patterning

(A-F) Inflorescence stems of wild-type (A), *lof1* (B), *las* (C), *pny* (D), *pny lof1* (E) and *pny las* (F). (G) The frequency distribution of internode length between the 1st and 11th flowers. All the measurements were performed on 40-day-old plants grown under continuous light conditions, the primary inflorescences of which were fully developed. The red arrows point to internodes <1mm in length.


A
B
C
D
E
F
WT
lof1
las
pny
pny lof1
pny las

G

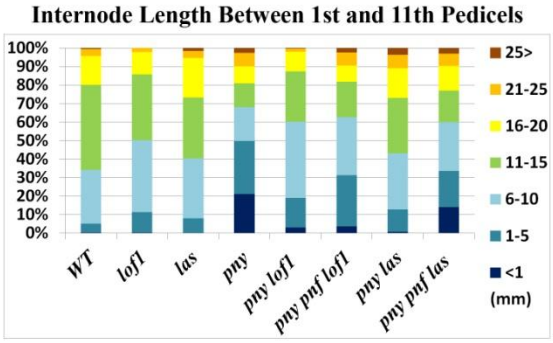

Internode Length Between 1st and 11th Pedicels
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT
lof1
las
pny
pny lof1
pny pnf lof1
pny las
pny pnf las
25>
21-25
16-20
11-15
6-10
1-5
<1
(mm)

Figure 2.3: The inflorescence of *pny pnf lof1* and *pny pnf las*

(A-E) The primary inflorescence of wild-type (A), *lof1* (B), *las* (C), *pny pnf lof1* (D) and *pny pnf las* (E). (F-J) Close-up of the internode of wild-type (F), *lof1* (G), *las* (H), *pny pnf lof1* (I) and *pny pnf las* (J). Red arrows point to the internode defect on the primary inflorescence. (K) *pny pnf* SAM continuously produce rosette leaves after perceiving floral induction. (L) Close-up of *pny pnf* SAM. Arrowhead points to initiating leaf primordia. (M-N) Fusion between primary inflorescence stem and pedicel in *pny pnf lof1* (M) and *pny pnf las* (N). White arrows point at the fusion.

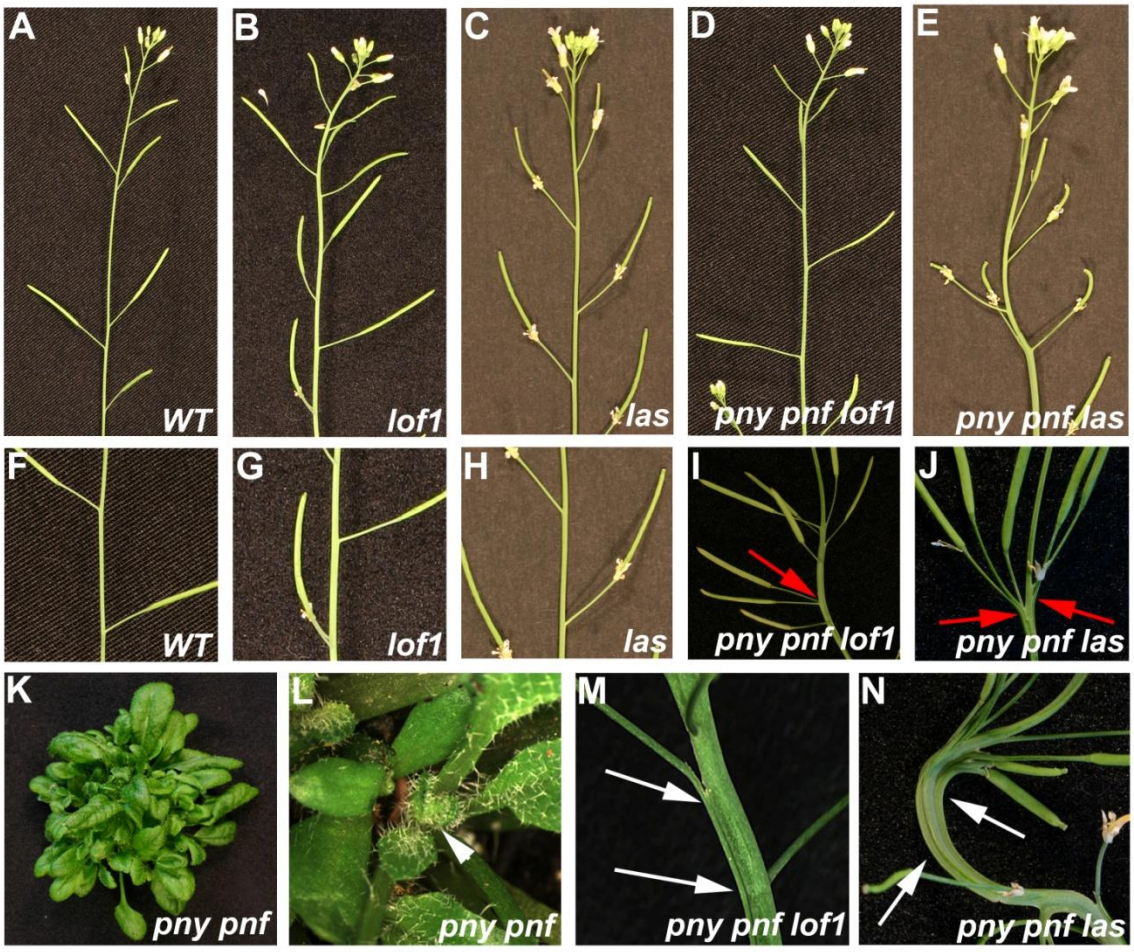

A
WT
B
lof1
C
las
D
pny pnf lof1
E
pny pnf las
F
WT
G
lof1
H
las
I
pny pnf lof1
J
pny pnf las
K
pny pnf
L
pny pnf
M
pny pnf lof1
N
pny pnf las

Figure 2.4: PNY and PNF restrict *LOF1* expression within the lateral organ boundary

(A-D) GUS activity in 5-d-old seedling (A,B) and inflorescence (C,D) of ET4016 (A,C) and ET4016 *blr* (B,D) GUS activity in boundary between SAM and leaf primordia in 5-day-old wild-type seedling. GUS activity is expanded in the *blr* background, compared to wild-type. (E-H) *LOF1* mRNA localization in vegetative apices of wild-type(E) and *pny pnf* (F); *las* (G); and *pny pnf las* (H). All plants were grown under short-day (SD) condition for 30 days when the SAM were collected and embedded.

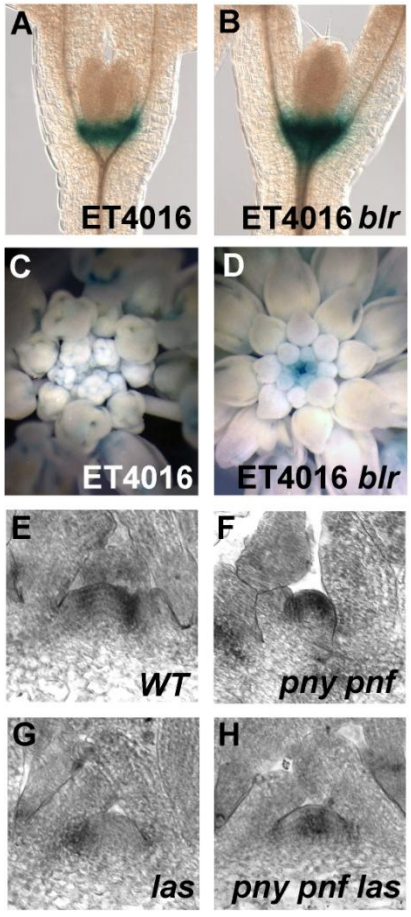

A
ET4016
B
ET4016 blr
C
ET4016
D
ET4016 blr
E
WT
F
pny pnf
G
las
H
pny pnf las

Figure 2.5: *LAS* transcript accumulation is reduced in the *pny pnf* SAM

(A-D) *LAS* mRNA localization in vegetative apices of wild-type(A); *pny pnf* (B); *lof1* (C); and *pny pnf lof1* (D). The arrows point at the lateral organ boundary between the SAM and lateral organ primordia. All the SAMs were collected from 30-d-old plants grown in short days.

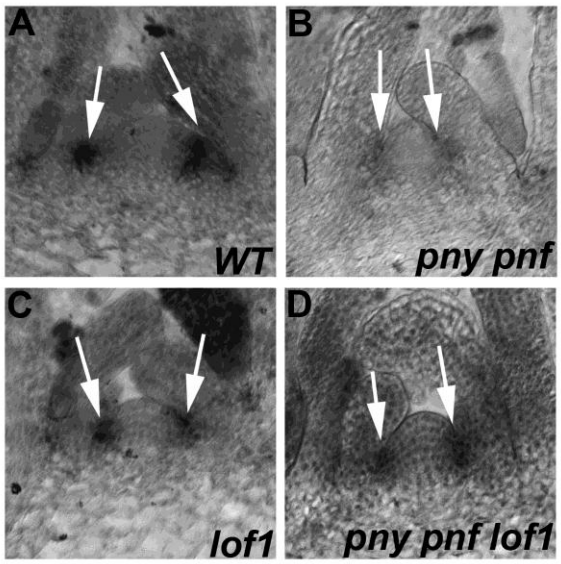

A
WT
B
pny pnf
C
lof1
D
pny pnf lof1

Figure 2.6: Loss of function in *LAS* or *LOF1* results in meristem restoration in *pny pnf*

(A-F) *CLV3* mRNA localization in vegetative apices of wild-type (A), *lof1* (B), *las* (C), *pny pnf* (D), *pny pnf lof1* (E) and *pny pnf las* (F). (G-L) *WUS* mRNA localization in vegetative apices of wild-type (G), *lof1* (H), *las* (I), *pny pnf* (J), *pny pnf lof1* (K) and *pny pnf las* (L). All the SAMs were collected from 30-d-old plants grown in short days.

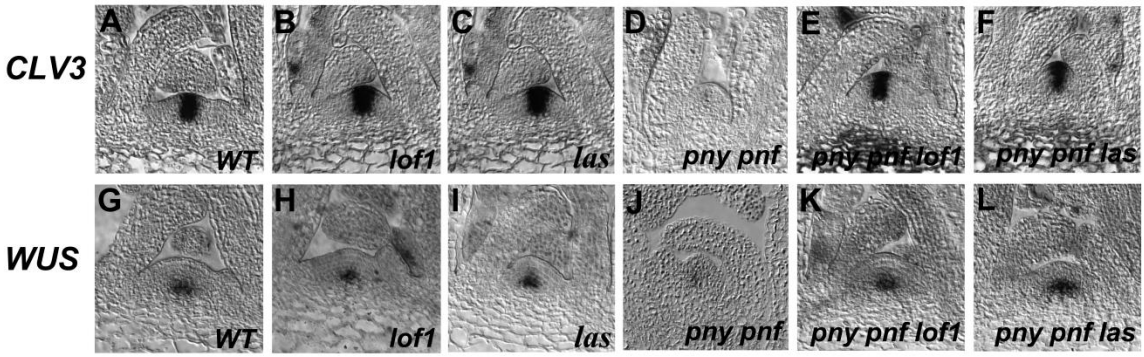
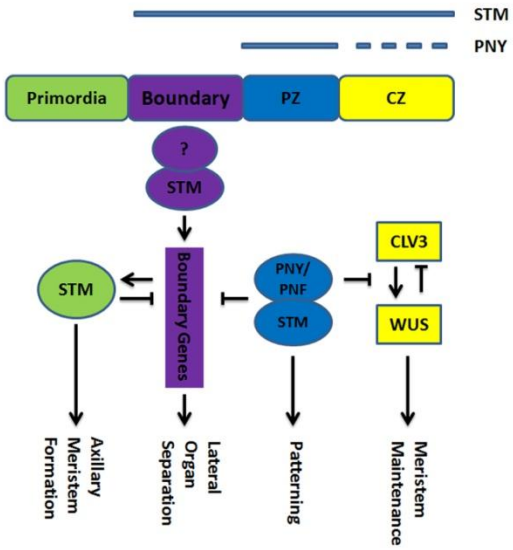

CLV3
WUS
A
WT
B
lof1
C
las
D
pny pnf
E
pny pnf lof1
F
pny pnf las
G
WT
H
lof1
I
las
J
pny pnf
K
pny pnf lof1
L
pny pnf las

Figure 2.7: Communication between the shoot apical meristem and lateral organ boundary

PNY and *PNF* are expressed and function primarily in the PZ to regulate meristem maintenance. *STM* transcripts and protein are present in both the SAM and organ boundaries (Heisler et al. 2005; Long and Barton 2000). PNY and PNF physically interact with STM to regulate meristem maintenance. We propose that boundary genes are repressed in the presence of PNY and PNF. Within the lateral organ boundary, STM is activates boundary gene expression in the absence of PNY/PNF. The activation of boundary gene may be mediated by STM homodimers or STM-ATH1 heterodimers. Genetic data suggest that PNY and PNF coordinate with boundary genes to control the integrity of CLV3-WUS negative feedback loop in the center of the SAM. Our model suggests that PNY/PNF serves as a mediator between the meristematic region and the lateral organ boundary, the communication of which is essential for meristem maintenance.


STM
PNY
Primordia
Boundary
PZ
CZ
?
STM
STM
Boundary Genes
PNY/
PNF
STM
CLV3
WUS
Axillary
Meristem
Formation
Lateral
Organ
Separation
Patterning
Meristem
Maintenance

Supplementary Figure 2.1: STM upregulates *LOF1* expression

(A) 14-day-old *Pro35S:STM-GR* germinated and grown on DEX. (B-D) GUS activity of ET4016, reporting *LOF1* expression in 14-day-old ET4016 seedlings, germinated and grown on DEX (B); 14-day-old ET4016 *Pro35S:STM-GR* seedlings, germinated and grown on DEX. (C,D). (D) shows a close-up of a leaf-like organ in (C).

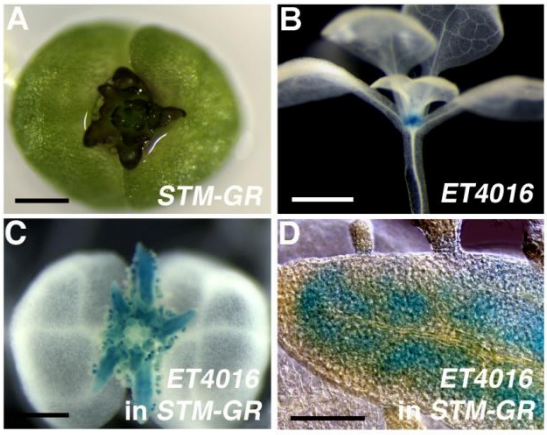

A
STM-GR
B
ET4016
C
ET4016
in STM-GR
D
ET4016
in STM-GR

Table 2.1: Fusion between the primary inflorescence stem and pedicels

Fusion Between Primary Inflorescence and Pedicel

Genotype	Average	SD	Range	Fusion (%)	n
WT	0			0	30
lof1	0			0	30
las	0			0	30
lof1 las	1	0	1	40	30
pny	0			0	30
pny PNF/pnf	2.3	1.7	1-6	70.6	18
pny lof1	0			0	30
pny pnf lof1	1.6	0.7	1-3	30	30
pny las	0			0	30
pny pnf las	2.48	1.7	1-6	83.3	30

The average number of fusion events between pedicel and primary inflorescence was determined for each genotype. The percentage of fusion is calculated by dividing the number of inflorescence carrying fusions by the total number of inflorescence examined. Average of fusion number is calculated among the inflorescences containing fusion for each genotype. SD = standard deviation. n, the total number of inflorescence examined for each genotype. All the plants are examined after 40 days of growth under long-day conditions.

Table 2.2: The number of flowers produced on the primary inflorescence

Genotype	Average	Range	SD(+/-)
WT	38.0	27-49	4.66
las	38.2	30-48	5.40
lof1	39.9	30-48	5.24
pny pnf	0	0	0
pny pnf las	40.3	30-55	5.27
pny pny lof1	46.1	33-54	5.07

The average number of flowers was determined on the primary inflorescence for each genotype. 30 individuals (n=30) were examined for each genotype after apices were dormant. All the plants were grown under continuous light conditions.

Table 2.3 Primers for synthesizing mRNA *in situ* probes

Primer	**Sequence**
CLV3-F	atggattcgaagagttttctg
CLV3-R	gaaataatacgactcactatagggactcaagggagctgaaagttgtttc
LAS-F	atgcttacttccttcaaatcctc
LAS-R	gaaataatacgactcactatagggactccttaccgaaccaccgttgctc
LOF1-F	cctcacctcaccaataatcatc
LOF1-R	gaaataatacgactcactatagggactcacgccgtccccaagccaag
WUS-F	atggagccgccacagcatcagc
WUS-R	gaaataatacgactcactatagggactctagttcagacgtagctcaagag

Appendix 2.1- mRNA *in situ* Hybridization

A. Paraformaldehyde fixation

a. Tissue Fixation

i. Prepare the 4% Paraformaldehyde fixative and incubate it on ice 1hr before tissue collection.

ii. Add 10ml fixative into 100 ml beaker. Dissect tissue and immediately place it in fixative. Swirl the beaker to let the tissue completely submerge in fixative.

iii. Add additional 20 ml fresh fixative into the breaker.

iv. Place screen firmly into the beaker near the bottom. Pour more fixative so that all the samples are submerged.

v. Apply vacuum to the mixture until it starts to bubble. Hold vacuum for 25 min.

vi. Slowly release vacuum and transfer the tissue into a 15ml tube.

vii. Empty out the solution and replace with cold fresh fixative. Incubate tissue at 4˚C overnight.

b. Dehydration

Dehydrate tissue with ethanol (EtOH) in a stepwise manner. All the steps are preformed at 4 ºC.

Solution	Incubation
1×PBS	30 min
1×PBS	30 min

0.5×PBS	30 min
30% EtOH in 0.5×PBS	45min-1.5 hr
50% EtOH in 0.5×PBS	45min-1.5 hr
70% EtOH	45min-1.5 hr
80% EtOH	40min
90% EtOH	40min
0.1% Eosin Y (Fluka, Cat#: 45240) in 95% EtOH	40min
0.1% Eosin Y in 100% EtOH	1hr, 2×
0.1% Eosin Y in 100% EtOH	overnight

c. Paraplast infusion for embedding tissue

i. Bring the tissue back into room temperature. Infuse tissue with paraplast in a stepwise manner at room temperature.

Solution	Incubation
100% EtOH	1hr
1Citrisolv: 3EtOH (Fisher scientific, Cat#: 22-143975)	1hr
1Citrisolv: 1EtOH	1hr
3Citrisolv: 1EtOH	1hr
Citrisolv	1.5hr, 2×
1Citrisolv: 1 Paraplast chips (McCormich, Cat#: 502004)	overnight

ii. Add more chips to fill the vial and melt the paraplast in 42˚C water bath for 8hr.

iii. Exchange with molten paraplast and place the vial in 60˚C oven overnight.

iv. Exchange with molten paraplast every 12hr for 5 times. (Incubate the vial in 60˚C oven.)

v. Pour the mixture in a weigh boat. Let the paraplast solidify at room temperature.

B. Section tissue and mount on slides

a. Place the "probe on plus" slides (Fisher Scientific, Cat#:22-230-900) on the slide warmer (LAB-LINE).

b. Add 1.5ml autoclaved water on the slides.

c. Section tissue on microtome (Microm, HM355) in 10 µm.

d. Mount the sectioned tissue on the "probe on plus" slides.

e. Leave them on the slide warmer (38˚C) for two nights.

C. Probe synthesis

a. Synthesis probe by PCR

Probe	50(µl)
H2O	32
10x PCR Buffer	5
2.5mM dNTP	5
10mM F+R	4

Taq (Home made taq)	1
DNA template (1:10)	3

b. Gel Extraction

   i. Run the PCR product on agarose gel.

   ii. Stain the gel with Ethidium Bromide and cut the target bands under UV light.

   iii. Isolate the PCR product with the gel extraction kit (Qiagen, Cat#: 28104) and elute the PCR product with 120µl of H_2O.

c. Phenol & Chloroform Purification

   i. Add 25ul Phenol Saturated (4˚C) (EMD, Cat#: 6710) to the PCR product tube.

   ii. Vortex the tube for 5min and spin for 10min at full speed.

   iii. Transfer the aqueous phase into a new RNA-free tube.

   iv. Add 25ul Chloroform (EMD, Cat#: 3150).

   v. Vortex the tube for 5min and spin for 10min at full speed.

   vi. Transfer the aqueous phase into a new RNA-free tube.

   vii. DNA Precipitation

DNA	100 µl
100% EtOH (RNA free)(2.5 V)	250 µl
3M NaOAc(Stock) (0.1V)	10 µl
Glycogen(Boshringer, Cat#: 901393)	1 µl
Incubate the tube in -20˚C for 2hr	

viii. Spin the DNA pellet at full speed for 10min.

ix. Wash the DNA pellet with 70% EtOH (RNase free).

x. Air dry the DNA pellet for 2hr.

xi. Dissolve the DNA in 15ul RNase-free H_2O.

d. DIG label mRNA probe

mRNA Transcription	25µl
2µg DNA Probe Template	13µl
5×Transcription Buffer (Promega, Cat#: P118B)	5µl
10x DIG label mix	2.5µl
10mM DTT(Promega, Cat#: P117C)	100mM, 2.5ul
RNasin (Promega, Cat#: N261C)	1µl
T7 RNA polymerase (Promega, Cat#: P207E)	1µl
3hr, 37˚C	
DNase (Promega, Cat#: M610A)	1µl
15 min, 37˚C	

e. mRNA Precipitation

i. Incubate DIG labeled mRNA probe (25µl) with 2.5µl 3M NaOAc and 75µl 100% EtOH at -20˚C overnight.

ii. Vortex the mix and spin down at full speed for 10min.

iii. Wash the DNA pellet with 70% EtOH (RNase free).

iv. Air dry the mRNA pellet.

v. Dissolve the DNA in 50µl RNase-free H2O.

f. Hydrolysis

mRNA probes	50µl
21 mg/mL Na2CO3 (fresh,Fluka, 71345)	30µl
16.8 mg/mL NaHCO3 (Sigma, S7277-250g)	20µl
Thydrolysis@ 60˚C Thydrolysis= (Lo-L0.15kb)/0.11* Lo*L0.15kb (Lo= Original length of the PCR product probe, L0.15kb= optimal final length= 0.15kb)	

g. mRNA Precipitation

i. Mix the hydrolyzed mRNA probe with the following reagents.

Hydrolyzed mRNA probe	100µl
10% glacial acetic acid(1/20 vol)	5µl
10 mg/mL yeast tRNA	1µl
3M NaOAc(1/10 vol)	10µl
100% EtOH(2.5 vol)	250µl

ii. Vortex the mix and spin down at full speed for 10min.

iii. Wash the DNA pellet with 70% EtOH (RNase free).

iv. Air-dry the DNA pellet.

v. Resuspend the probe in 20µl of 50% formamide.

vi. Storage at -20˚C.

D. Slide Treatments and Hybridization

a. Treat slides with the following steps.

Step	Solution (ºC)	Incubation
i. Dewax	Citrisolv	10min, 2×
ii. Hydration	100% EtOH	2min, 2×
	95% EtOH	2min
	85% EtOH	2min
	70% EtOH	2min
	50% EtOH	2min
	30% EtOH	2min
	15% EtOH	2min
	ddH2O	2min, 2×
iii. Acidification	0.2M HCl	20min
iv.Wash	ddH20	5min
	1x PBS	5min
	ddH20	5min
v. Protein Digestion	Proteinase K Solution in 37˚C water bath	30 min
vi.Glycine Block	2mg/mL Glycine Block	2 min
vii. Wash	1x PBS	2 min, 2×
	1x PBS	Quick wash
viii. Refix tissue	Refix	20 min
ix.TEA Wash	1x PBS	2 min, 2×

	0.5% Acetic Anhydride (0.1M TEA, pH8.0)	5min
	1x PBS	2 min, 2×
x. Dehydration	ddH20	2min, 2×
	15% EtOH	2min
	30% EtOH	2min
	50% EtOH	2min
	70% EtOH	2min
	85% EtOH	2min
	95% EtOH	2min
	100% EtOH	2min, 2×
xi.Dry the slides	Vacuum, 30min	

a. Pre-hybridization

   i. Preheat Hybridization Solution in 55˚C water bath.

   ii. Place one slide on the 40ºC slide warmer. Add 200ul Hybridization solution (55˚C) onto a slide and cover with another one to make a sandwich.

   iii. Pour 20ml of 50% Formamide into the Hybridization box. Place the sandwich on the rack of the Hybridization box. Cover the box with Saran wrap and incubate in 54 ºC oven for 1hr.

b. Hybridization

i. Prepare Hybridization Probe (200µl/Sandwich). Add 2ul prepared probe to 10µl 50% Formamide and heat at 75 ºC for 2min. Place the tube on ice and then add 200µl hybridization solution (55 ºC).

ii. Separate and drain the sandwiches.

iii. Apply 200µl Hybridization Probe to each sandwich on the 40ºC slide warmer.

iv. Place the sandwiches back to the hybridization box. Double-wrap the box.

v. Incubate the box in 54 ºC oven overnight.

E. Washes, RNase treatment, Antibody treatment, & Detection

a. Separate and drain the slides.

b. Treat the slides with the following steps.

Step	Solution	Incubation
i. Wash	Wash I	30min, 4×, in 55 ºC oven
	2xSSC	2min
	1x TBS	5min
ii. Blocking	BLOCK I-DIG	1.5 hr, at 4 ºC
	Block II,	30min, at 4 ºC
iii.Rinse	TBST	1min, 2×

Note: Parafilm the slide container while blocking.

a. Anti-DIG Incubation

i. Prepare anti-DIG by add 0.4µl DIG antibody into TBST containing 0.5% BSA.

ii. Apply 200ul Anti-DIG to each sandwich.

iii. Place the sandwiches into the hybridization box. Saran-wrap the box and incubate the box at 4 ºC for 3hr.

b. Rinse

i. Rinse the slides with TBST for 10min, twice.

ii. Rinse the slides with 1x TBS for 10min, twice.

iii. Rinse the slides with Wash II for 5min.

c. Developer application and exposure

i. Prepare the Development by mixing 1ul 1M TEA with 1ml Western Blue (Promega, Cat#: S3841).

ii. Place the sandwiches back into the box and double-wrap the box.

iii. Place the box in the dark and incubate overnight.

iv. Exam the staining. If no staining or not strong enough, wash the slides with WASH II, 5min and develop the slides again. Stop the reaction by the end of the day.

v. Stop the reaction by rinsing the slide in TE solution.

d. Mount

i. Dehydrate the slides with the flowing solution.

Solution	Incubation
30% EtOH	< 5s

50% EtOH	< 5s
70% EtOH	< 5s
85% EtOH	< 5s
95% EtOH	< 5s
100% EtOH	< 5s, 2×
Citrisolv	10 min, 2×

ii. Dry the slides on paper towel for 1hr.

iii. Mount the slides with Permount, (Fisher Scientific, SP15-100). Air-dry for 2 days.

Solution

Block I-DIG	50ml
0.5% Boeringer	0.25g
H2O	45ml
60˚C water bath, 1-2hr	
1×TBS	10×, 5ml
0.3% Triton X-100	10%, 1.5ml

Block II	50ml
1% BSA	500mg
1× TBS	10×, 500µl
0.3% Trition X-100	10%, 1.5ml

10x DIG label dNTP mix	20µl
5mM ATP	100 mM, 1µl
5mM CTP	100 mM, 1µl
5mM GTP	100 mM, 1µl
1.5mM UTP	100 mM, 0.3µl
2.5mM DIG-UTP	10 mM, 5µl
Depc H_2O	11.7

Glycine Block	50ml
2mg/ml Glycine	0.1g
10×PBS	5×, 5ml
H_2O	to volume

Hybridization solution	15ml
6x SSC	20×SSC, 4.5ml
1% SDS	20%, 750ul
50% Formamide (deionized)	7.5ml
100 ug/ml tRNA (sigma, 9014-25-9)	10mg/ml, 150ul
H_2O	to volume

4% Paraformaldehyde Fixative	50ml
1×PBS, pH 11, 65˚C	10×,5ml
4% Paraformaldehyde	2g
H_2O	to volume
Adjust pH to 7 with 1M H_2SO_4 and cool down the solution on ice	
0.5% Trion X-100	10%, 2.5ml
4% DMSO	2ml

Note: Paraformaldehyde needs to be dissolved in pH11 at 65˚C. After Paraformaldehyde is dissolved in solution, pH should be measure by pH strip (EMD, Cat#: 9588)

10×PBS	1L
137mM NaCl	80g
27mM KCl	2g
100mM Na_2HPO_4	11.5g
20mM KH_2PO_4	2g
Adjust pH to 7.4 with HCl	
Autoclave	

10mg/ml Proteinase K	1ml
100mM Tris-Cl pH8.0	100ul
50mm EDTA	100ul
H_2O	300ul
Proteinase K	10mg
50% Glycerol	500ul

Proteinase K Solution	50 ml
100mM Tris pH8.0	1M, 100ml
50mM EDTA	0.5M,100ml
H_2O	to volume
Preheat @37ºC	

1ug/ml Proteinase K	10mg/ml, 5ul

20x SSC	1L
3M NaCl	175g
0.3M NaCitrate (VWR, FL08-0900)	88g
H_2O	to volume

Refix	1L
1xPBS	10×, 100ml
3.7% Formaldehyde	37%, 5ml
H_2O	to volume

10x TBS	1L
1M Tris-Cl, pH7.5	121.1 g
Adjust pH to 7.5 with HCl	
4M NaCl	233.76g

TBST	1L
10×TBS	10×, 100ml
0.3% Triton X-100	10%, 30ml

H_2O	to volume

TE	200ml
10mM Tris-Cl, pH8.0	1M, 2ml
1mM EDTA	0.5m, 400ul

Wash I	1L
0.2×SSC	20X, 10ml
0.01% SDS	20%, 500ul
H_2O	to volume

WASH II	1L
10x TBS	10x, 100ml
50mM $MgCl_2$	1M, 50ml
H_2O	to volume

Chapter 2

A direct or indirect role of PENNYWISE and SHOOTMERISTEMLESS in regulating patterning during the shoot development

ABSTRACT

Shoot architecture is regulated by the maintenance, activity and identity of the shoot apical meristem (SAM). Mechanisms that control meristem cell fate, lateral organ boundary function and meristem identity are essential for understanding the processes that control shoot architecture. The KNOTTED1-LIKE HOMEOBOX (KNOX) transcription factor, SHOOT MERISTEMLESS (STM), interacts with the BELL1-like Homeodomain (BLH) transcription factor PENNYWISE (PNY) and genetic studies predict that this complex controls inflorescence architecture by directly or indirectly regulating stem cell homeostasis and lateral organ boundary function as well as identity in Arabidopsis. To define the function of STM and PNY in these processes, a chromatin immunoprecipitation (ChIP) approach was performed utilizing KNOX and PNY antibodies. Results from this study show that one or more KNOX proteins as well as PNY associate with the promoters of genes that regulate boundary function and flower meristem identity. In addition, only a KNOX protein(s) but not PNY binds to the regulatory region of a gene involved in stem-cell homeostasis. Based on this study, we propose a model to explain the role of STM in regulating stem cell homeostasis. We put forward a hypothesis to describe the dynamics that establish boundary identity based on

PNY-STM interaction and expression pattern. Finally, flower meristem identity appears to be controlled both directly and indirectly by PNY-STM interactions.

Introduction

Plant undergo extensive post-embryonic development, which is one of the fundamental differences between plant and animal. All the above-ground plant organs are originated from the shoot apical meristem (SAM), which is located at the growing tip of the shoot (Barton 2010). The SAM contains a constant number of stem cells that contribute to self-renewal and organogenesis (Besnard et al. 2011; Carles and Fletcher 2003; Tucker and Laux 2007). The SAM is histologically and functionally divided into different units (Lyndon 1998; Steeves and Sussex 1989). Stem cells are maintained in the central zone (CZ), which is located at the apical tip of the shoot meristem. The CZ is encircled by the peripheral zone (PZ), which is the site in which lateral organs are produced. Below the CZ and the PZ is the rib meristem (RM), which allocates cells that form the pith of the stem. As a lateral organ emerges from the PZ, a boundary is established between the meristem and organ primordia (Aida and Tasaka 2006b). A low level of mitotic activity is displayed in the narrow band of boundary cells, which allows the initiating organ primordia to separate from the shoot meristem (Aida and Tasaka 2006b). The identity of the lateral organ boundary must be maintained to ensure that organ separation is complete (Aida and Tasaka 2006b). The lateral organ boundary likely serves to separate meristem and organ developmental pathways required for specifying and maintaining meristem and organ identities (Aida and Tasaka 2006a). Furthermore, genetic evidence indicates that

the lateral organ boundary is essential for specifying meristem cell fate and axillary meristem formation (Aida et al. 1997; Aida and Tasaka 2006a; Barton 2010; Hibara et al. 2006).

Stem-cell homeostasis is maintained in part by a negative feedback loop between a peptide ligand expressed in the CZ termed CLAVATA3 (CLV3) and the WUSCHEL (WUS) homeobox transcription factor, which is expressed in a small number of cells in the core of the shoot meristem called the organizing center (OC) (Tucker and Laux 2007). Recent studies suggest that WUS moves out of the OC and activates *CLV3* in the CZ (Laux et al. 1996; Rieu and Laux 2009; Yadav et al. 2011). On the other hand, the CLV3 ligand moves downward to restrict *WUS* expression to the OC (Stahl and Simon 2010). The movement of WUS out of the OC occurs in a radial fashion; yet, *CLV3* is only activated in the CZ. Therefore, a key question in understanding the dynamics of the CLV3-WUS negative feedback loop is the mechanism by which WUS regulates the spatial expression domain of *CLV3*.

Meristem cell fate is specified by specific members of the KNOTTED1-LIKE HOMEOBOX (KNOX) family of transcription factors, including the Arabidopsis SHOOT MERISTEMLESS (STM) protein (Hake et al. 2004; Vollbrecht et al. 2000). KNOX transcription factors are members of the three amino acid loop extension (TALE) class of homeodomain proteins, which function to specify cell fate in animals and fungi (Burglin 2011; Hamant and Pautot 2010; Mukherjee et al. 2009). Genetic studies indicate

that in the absence of STM function, the cells in the shoot meristem terminally differentiate during cotyledon development (Barton and Poethig 1993; Long et al. 1996). Consistent with its function, *STM* is expressed throughout the shoot meristem but downregulated in the initiating organ primordia (Barton and Poethig 1993; Long et al. 1996). STM appears to regulate meristem cell fate in part through the activation of the key cytokinin biosynthetic gene *ISOPENTENYL TRANSFERASE 7* (*IPT7*) (Jasinski et al. 2005; Vanstraelen and Benkova 2012; Yanai et al. 2005). Interestingly, ectopic expression studies suggest that WUS can only activate *CLV3* in cells expressing *STM* (Brand et al. 2002; Gallois et al. 2002), suggesting WUS and STM may function cooperatively to regulate *CLV3* expression in the shoot meristem. Analysis of the *stm-10* weak allele indicates that STM regulates flower specification and internode patterning during inflorescence development (Endrizzi et al. 1996; Kanrar et al. 2006). Genetic studies suggest that STM specifies flower meristem formation in parallel with the florigen complex, which consists of (1): FD, a b-ZIP TF, (2) FLOWERING LOCUS T (FT), florigen, and (3) 14-3-3 linker protein (Abe et al. 2005; Purwestri et al. 2009; Wigge et al. 2005; Wu and Smith 2012). Chromatin immunoprecipitation (ChIP) studies suggest that this complex functions in parallel with the early flower meristem identity gene *LEAFY* (*LFY*) to activate later flower meristem identity genes including *APETALA1* (*AP1*) (Abe et al. 2005; Wigge et al. 2005).

Interestingly, study shows that STM-GFP is present in the boundary, indicating STM transcription factor moves from cell-to-cell via the plasmodesmata (Heisler et al. 2005).

Therefore, STM protein not only functions in the meristematic cells but also move into lateral organ boundary to regulate its function and identity. Mutations in *LATERAL ORGAN FUSION 1* (*LOF1*) enhance the weak *stm-10* mutant, resulting in a *stm-1*-like phenotype, indicating that STM plays roles in boundary maintenance in order to control meristem maintenance (Lee et al. 2009). In addition, loss of *CUP SHAPED COTYLEDON 1* (*CUC1*) or *CUC2* enhanced the phenotype of the weak *stm-2* allele (Aida et al. 1999). Despite the differences in their expression patterns, the relationship between the CUCs and STM is complex as these transcription factors appear to positively regulate each other (Aida et al. 1999) and function in parallel to regulate meristem cell fate. To date, little is known about how the communication between the meristem and lateral organ boundary regulates shoot morphology.

KNOX proteins interact with another family of TALE transcription factors called the BELL1-like homeodomain (BLH) proteins (Hake et al. 2004). Based on biochemical and genetic studies, it has been proposed that specific KNOX-BLH interactions regulate discrete developmental pathways during shoot development (Hake et al. 2004; Kanrar et al. 2006; Smith and Hake 2003). In addition, a common set of target genes may be regulated by more than one specific KNOX-BLH complex. Two related BLH genes called PENNYWISE (PNY) and POUND-FOOLISH (PNF) are expressed in the shoot meristem and interact with STM as well as other KNOX proteins in Arabidopsis (Kanrar et al. 2006; Smith et al. 2004). Lastly, PNY and PNF appear to control flower specification by acting upstream and in parallel with the early flower meristem identity

gene *LFY* (Kanrar et al. 2008). A recent study suggests that PNY and PNF regulate the integrity of the CZ/OC as the expression patterns for *CLV3* and *WUS* were severely altered in *pny pnf* shoot apices (Ung et al. 2011). The genetic relationship between PNY/PNF and STM as well as BREVIPEDICELLUS (BP) was examined and results indicate that PNY/PNF-STM controls flower specification, internode patterning and boundary function, while PNY/PNF-BP appears to control internode development (Kanrar et al. 2006; Smith and Hake 2003).

In vitro DNA-binding and association assays show that the KNOX and BLH proteins, KNOTTED1 (KN1) and KN1-BINDING PROTEIN (KIP) specifically associate with the TGACAG/CT motif (Smith et al. 2002). *In vitro* binding studies also show that STM and two BLH proteins, ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1) and BLH3 specifically associate with the TGACAGGT (Viola and Gonzalez 2006; 2009). In barley, the KN1 related protein called HOODED associates with a TGAC core motif (Osnato et al. 2010). Analysis of KNOX and BLH targets in tobacco, maize, potato and Arabidopsis suggest that these homeodomain transcription factors appear to associate with 'TGAC' core motifs (Bolduc et al. 2012; Chen et al. 2004; Sakamoto et al. 2001; Viola and Gonzalez 2006). Tandem TGAC sequences are often present in the regulatory regions of KNOX target genes (Bolduc et al. 2012). Furthermore, the space between TGAC motifs may be crucial, suggesting that a heterodimer (KNOX-BLH) and/or homodimer (KNOX-KNOX or BLH-BLH) associates with the tandem TGAC motif to regulate gene expression (Bolduc et al. 2012; Viola and Gonzalez 2006). A recent study utilized a

ChIP-seq approach to identify the target genes of KN1 in maize inflorescences. Results from this study indicate that KN1 controls multiple pathways required for meristem function (Bolduc et al. 2012). However, the role for most of these target genes has not been determined. Interestingly, the TGAC binding motif is only present in a small portion of KN1 target genes, suggesting that KN1 may be recruited to promoters by other transcription factors that are not BLH. Alternatively, the specificity of the KN1 binding site is altered in association with a transcription factor that is not a BLH protein.

Genetic studies support a model in which STM-PNY/PNF regulates meristem cell fate, boundary function and flower specification (Wu and Smith 2012). However, genetic studies alone cannot determine which of these processes are directly and indirectly controlled by STM-PNY/PNF. Understanding the direct and indirect function of PNY-STM (PNF-STM) is difficult as these transcription factors regulate meristem function. Alterations in meristem function may indirectly affect other processes such as internode patterning and flower meristem identity. To determine the precise function of STM-PNY/PNF will require a system to perform ChIP experiments. Although the SAM is highly dynamic, the number of cells that comprise the SAM is limited, making it difficult to determine the function of meristem specific TFs. Another obstacle that is particularly difficult for STM, PNY and PNF is the promoter and regulatory region(s) have not been clearly defined, making it difficult to express epitope tagged versions for these TFs. To investigate the direct function of STM and PNY in stem cell homeostasis, boundary maintenance and flower meristem identity, a ChIP approach was designed using purified

antibodies directed against these homeodomain proteins. We did not investigate the function of PNF, as genetic studies indicate that PNY and PNF act redundantly (Kanrar et al. 2006; Smith et al. 2004). Therefore, any targets identified for PNY will likely be regulated by PNF. To overcome the problem of isolating meristem tissues, ChIP was performed by dissecting miniature cauliflower inflorescences apices displayed in *ap1 cauliflower* (*cal*) plants (Kempin et al. 1995). Candidate genes that may be directly regulated by STM and/or PNY were analyzed in the ChIP study. Results from this study indicate that STM directly regulates *CLV3*. On the other hand, PNY appears to regulate the *CLV3* expression domain indirectly via the lateral organ boundary. Both PNY and STM associate with the promoters of two lateral organ boundary genes, *LOF1* and *LATERAL SUPPRESSOR* (*LAS*). Based on genetics and the results from ChIP, we propose that PNY-STM negatively regulate *LOF1* and *LAS* in the PZ. At the same time, STM alone or in association with another BLH protein(s) activates *LOF1* and *LAS* at the boundary. Lastly, STM and PNY appear to directly regulate flower specification via *LFY*. Taken together, these studies support a model in which STM function is modulated by BLH proteins in specific domains of the shoot meristem to regulate multiple developmental pathways.

Results

ChIP analysis and KNOX antibody purification

In order to identify target genes of STM, an antibody that recognizes the homeodomain (HD) and short C-terminal tail was generated in rabbits (Figure 3.1A). Subsequently, the

STM antibody was affinity purified from rabbit sera and used for ChIP analysis. Due to the high conservation in the HD of KNOX transcription factors, it is highly possible that this antibody will recognize other KNOX proteins expressed in the shoot apex. It should be pointed out that an attempt to generate an STM specific antibody was not successful (unpublished data). In order to characterize the affinity purified STM antibody, a western blot procedure was performed. In this analysis, recombinant STM and BP were purified to determine if the STM antibody recognizes STM as well as other KNOX proteins. 0.01µg of recombinant purified STM and BP along with the crude nuclear protein extract isolated from *ap1 cal* were separated by SDS-PAGE and transferred to nitrocellulose. After incubating the blots with 1.5ug of anti-STM, results showed that this antibody recognizes recombinant STM and BP (Figure 3.1B). In *ap1 cal* nuclear extracts, proteins similar in mass as STM (42.6kDa) and BP (45.8kDa) as well as KNAT2 (35kDa) and KNAT6 (37.2kDa) were easily detected (Figure 2.1B). Experimental attempts of using western blots comparing nuclear and total protein extracts from wild-type and *stm-10* shoot apices were unsuccessful as proteins were not detected in either sample (data not shown). In addition, the STM antibody was used to immunoprecipitate KNOX complexes from cauliflower. Subsequently, proteomics was used to identify the components of this complex. Results from this experiment showed that peptides related to Brassica KNOX transcription factors were identified, suggesting that this KNOX protein recognizes STM and other KNOX proteins (data not shown). Because the STM antibody appears to cross-react with other KNOX proteins, we refer to this tool as the KNOX antibody. Therefore, use of this antibody in ChIP experiments will not definitely determine the target genes of

each specific KNOX; however, based on genetic studies of *STM*, the target genes we analyzed are consistent with STM function.

A previous study suggests that STM directly activates *CUC1* expression at the lateral organ boundary by associating with the proximal region of *CUC1* promoter containing two TGAC core sequences (Spinelli et al. 2011). PCR primers that flank this putative KNOX DNA-binding site were designed along with a set that amplifies a 141bp product at the 3'-UTR of ACTIN8, which was used as a negative control (Table 3.1). For ChIP analysis, 0.8 grams of *ap1 cal* inflorescence apices were subjected to the cross-linking with formaldehyde. After isolating a crude nuclear extract, the chromatin was sheared to an average size of 500bp, before immunoprecipitation with KNOX and GST antibodies. Note: the cross-linked chromatin immunoprecipitated with GST antibody served as a control for the ChIP analysis. After reverse cross-linking, quantitative real-time PCR (qPCR) was employed to amplify the proximal promoter of *CUC1* and the 3'UTR of *ACTIN8* (Table 3.1). Results from the ChIP-qPCR analysis show at least one KNOX protein is bound to the proximal promoter region of *CUC1* as this region is enriched approximately 6-fold using the KNOX-antibody compare to the GST-antibody control (Figure 3.2B). In addition, selective KNOX occupancy was not detected for the 3'UTR of *ACTIN8* (Figure 3.2B). This result corroborates *CUC1* is a direct target for STM. At the same time, it shows that this KNOX-antibody is able to immunoprecipitate genomic regions bound by at least one KNOX protein, possibly STM.

Identification of KNOX targets of lateral organ boundary genes via ChIP

Genetic and molecular studies suggest STM controls meristem cell fate in part through the regulation of lateral organ boundary genes such as *CUC1* and *CUC2* (Takada et al. 2001). In plants, target genes have been successfully identified by fusing a transcription factor (TF) of interest to the Glucocorticoid Receptor (GR) domain, which acts to tether the TF in the cytoplasm of plant cells (Gallois et al. 2002; Spinelli et al. 2011). Treatment of plant tissues with a synthetic steroid hormone called dexamethasone, which binds to GR, releases the TF-GR from the cytoplasmic anchor. Subsequently, the TF-GR fusion is transported into the nucleus and regulates gene expression. Unpublished studies from Dr. Springer's laboratory (see chapter 1) showed that an inducible form of STM (STM-GR) activates *LOF1* when ectopically expressed in cotyledons and leaf tissues but not the SAM (Supplementary Figure 2.1). After inducing *STM-GR*, it takes approximately 3 hours for *LOF1* transcripts to accumulate to high levels in non-meristematic tissues. At this time, it is unknown how STM as well as other KNOX or BLH proteins control gene expression. Because *LOF1* was not rapidly transcribed after STM-GR induction, it is unclear if this KNOX protein directly activates this MYB gene. Analysis of the *LOF1* promoter showed that it contained TGAC repeats (Figure 3.2A). To investigate whether a KNOX protein associates with the genomic regions containing the KNOX DNA-binding motifs, ChIP analysis was performed. Primers were designed and used to amplify regions containing these putative binding sites, fragments 1 and 3 (Figure 3.2A). In addition, another set of PCR primers were designed to amplify a promoter region (fragment 2) in between fragments 1 and 3. Lastly, PCR primers were utilized to amplify a genomic

region in the first intron (Fragment 4) and a region downstream to the last exon (fragment 5) (Figure 3.2A and Table 3.2). ChIP q-PCR results indicate that KNOX occupied a genomic region around the start codon of *LOF1* containing two tandem TGAC sequences (Figure 3.2B). Interestingly, fragment 1 was not bound by a KNOX protein despite the presence of two tandem TGAC sequences. In addition, KNOX occupancy was not detected for the genomic fragment 2 and the regions downstream of the *LOF1* start site. Taken together, the result suggests at least one KNOX protein associates with the proximal promoter of *LOF1 in vivo.* Given that STM-GR induces transcription of *LOF1*, these results strongly suggest that STM directly regulates *LOF1 in vivo*.

CLV3 is a direct target of KNOXs TF(s) for stem cell homeostasis

Stem cell homeostasis is controlled in part by *CLV*-*WUS* negative feedback loop (Barton 2010; Tucker and Laux 2007). Understanding how these genes are regulated is important to understand the molecular mechanisms that maintain the stem cell niche in the shoot meristem. Genetic and molecular evidence demonstrate that the promoter and the 3' enhancer are necessary for maintaining the proper *CLV3* expression domain in the CZ (Brand et al. 2002; Fletcher et al. 1999). Recent studies show that *CLV3* is directly regulated by WUS (Yadav et al. 2011). Two WUS DNA-binding sites, composed of the "TAAT" core sequence, were identified in the promoter and the 3' enhancer of *CLV3* (Figure 3.3A) (Yadav et al. 2011), indicating that *WUS* not only activates *CLV3* but regulates the spatial domain for this gene. Based on the ectopic expression studies, coexpression of *WUS* and *STM* is required and sufficient to activate the *CLV3* expression

in leaf tissues (Brand et al. 2002). Therefore, the promoter and 3' regulatory region of *CLV3* were examined for potential KNOX/BLH binding sites. In the 3' enhancer, two TGAC motifs separated by a few base pairs was located in this region (Figure 3.3A). In addition, the 3' WUS DNA-binding motif was located in close proximity to these three TGAC motifs (Figure 3.3A).

To determine if KNOX TFs, such as STM, directly regulate *CLV3*, ChIP analysis was performed, using the KNOX and GST antibodies. Genomic specific primers were designed and used to amplify three regions of the *CLV3* locus (Figure 3.2). The primers used to amplify fragment 3 include the TGAC motifs in the 3' enhancer (Figure 3.3A). *ACTIN8* was used as a negative control. Result from ChIP-qPCR shows that a KNOX(s) protein bound to fragment 3 containing the three TGAC motifs with an enrichment of 8.67 fold (Figure 3.3B). In addition, at least one KNOX protein associates with fragment 1, which is adjacent to the 5' WUS DNA-binding motif (Figure 3.3B). No binding was detected with the region around fragment 2 or with the 3'UTR of the ACTIN8 control (Figure 3.3B). Taken together, these results suggest that at least one KNOX TF(s) associates with the promoter and 3' enhancer, in close proximity to the WUS DNA-binding motif. Given that WUS activates *CLV3* in cells expressing *STM* (Brand et al. 2002), the KNOX protein bound to the promoter and 3' enhancer is STM.

The association of KNOX TFs with the 3'UTR of *CLV3* suggests that TGAC sequences may be important for regulating the spatial expression domain of *CLV3*. In support of this

hypothesis, previous gene expression analyses showed that both the 1.5kb promoter and 1.2kb 3' enhancer are required to recapitulate the normal *CLV3* expression pattern (Figure 3.3C) (Brand et al. 2002; Fletcher et al. 1999). To determine if the TGAC motifs are required for the spatial regulation of *CLV3*, the sequences harboring all three "TGAC" core sequences was mutated to an *Eco*RI sequence, GAATTC (Figure 3.3C). The mutated version of *pCLV3:GUS* lacking the TGAC motifs was referred as *pCLV3m:GUS* (Brand et al. 2002; Reddy and Meyerowitz 2005). Both the *pCLV3:GUS* and *pCLV3m:GUS* binary vector were transformed into wild-type plants (Columbia). Subsequently, single insertion lines of *pCLV3:GUS* and *pCLV3m:GUS* were obtained for GUS analysis. To examine the expression pattern of *pCLV3:GUS* and *pCLV3m:GUS*, inflorescence apices were collected, stained for GUS, fixed, embedded in paraffin and sections were examined by light (Figure 3.3D-F) and dark microscopy (Figure 3.3G-I). Preliminary results showed the GUS activity was detected in the CZ of *CLV3-GUS* plants (Figure 3.3D and 3.3G). Results from this study showed that *GUS* expression displayed by light (Figure 3.3D) and dark-field (Figure 3.3G) microscopy was detected in the CZ region for plants containing the *pCLV3:GUS*. In contrast, the *GUS* expression was more broad and detected throughout the shoot meristem in plants containing *CLV3m:GUS* plants by light (Figure 3.3E and F) and dark-field microscopy (Figure 3.3H and I). In some transgenic lines, GUS activity was also expanded throughout the floral meristems in *pCLV3m:GUS* inflorescence apices (Figure 3.3H). Thus, this study suggests that the TGAC motifs in the 3' UTR are necessary for maintaining the proper *CLV3* expression domain in the inflorescence and floral meristems. These preliminary studies suggest that

at least one KNOX, possibly STM, functions to regulate the spatial expression pattern of *CLV3* in the shoot meristem. To verify the *pCLV3m:GUS* studies, more independent transgenic lines should be examined.

A direct role of KNOX TFs in regulating floral meristem identities

Characterization of the inflorescences displayed in *stm-10, fd stm-10* and *stm PNY/pny* indicates that STM functions to specify flower meristem identity (Kanrar et al. 2006; Smith et al. 2011). In fact, transcript levels for *LFY* are highly reduced in *stm-10 ft* and *stm-10 fd* inflorescence shoots (Smith et al. 2011). Given that STM associates with PNY and PNF and both of these BLH TFs function upstream of LFY, ChIP was utilized to determine if a KNOX protein associates with *LFY* locus *in vivo*. Potential KNOX binding sites were searched and we found that the promoter of *LFY* contains numerous "NGAC" tandem repeats (data not shown). Genomic specific primers were designed to amplify three fragments in the promoter and one in the second intron (Figure 3.4A). Results from this ChIP study showed that a KNOX protein(s) associated with the genomic fragments 2 and 3, as a 6- and 4-fold enrichment for these genomic regions were observed, respectively (Figure 3.4B). KNOX did not appear to bind with fragment 1 in the distal region of the *LFY* promoter or with fragment 4 in the second intron (Figure 3.4B). Thus, *LFY* appears to be a target of at least one KNOX protein. Given the genetic relationship of STM in specifying flower meristem identity, it is highly probable that STM associates with the *LFY* promoter.

ChIP system design for identifying PNY target genes

In order to complement the KNOX-ChIP study, we aimed to develop a system to directly identify potential target genes for PNY to determine if this BLH TF is directly regulates boundary, stem cell homeostasis and flower meristem identity genes. To accomplish this objective, we utilize a system that would allow us to specifically immunoprecipitate PNY with little or no cross-reactivity with other BLH proteins. The first goal was to express an epitope tagged version of PNY in the shoot meristem and utilize this system for ChIP using antibodies that specifically recognize the tag(s). The first aim was to use the *PNY* promoter to drive expression of the epitope tagged-PNY fusion protein. Because of the difficulty in characterizing the regulatory regions of the *PNY* promoter, we sought to use a characterized promoter that would drive an epitope tagged version of PNY in the meristem. Construction of *p35S:TAP-PNY* and *pSTM(3.2kb):TAP-PNY* constructs were unable to efficiently complement *pny* and *pny pnf* plants (unpublished data). To overcome this problem, we utilized the *UBIQUITIN* 10 (*UBQ10*) promoter, which expresses genes in the shoot meristem and lateral organs (Grefen et al. 2010). In this experimental aim, the promoter was fused to PNY along with a 3FLAG epitope tag located at the N-terminus (Figure 3.5A) (Grefen et al. 2010). Results from this study are shown below.

The first objective was to determine if *pUBQ10:3FLAG-PNY* complements the *pny* and *pny pnf* phenotype. The binary vector *pUBQ10:3FLAG-PNY* was transformed into *pny PNF/pnf* and single insertion lines were characterized in both *pny* and *pny pnf*

background from the T2 generation. Among 33 T2 independent lines of *pUBQ10:3FLAG-PNY pny*, the internode length distribution pattern and overall height of the primary inflorescence were examined (Figure 3.5B-D). In wild-type inflorescences, a regular pattern of internode development separated the developing flowers and fruit along the primary inflorescence (Figure 3.5B). In contrast to wild type, internode development was impaired at various intervals along the *pny* inflorescence axis (Figure 3.5C arrows). Transformation of *pny* plants with *pUBQ10:3FLAG-PNY* restored regular patterns of internode development similar to wild-type (Figure 3.5D). The frequency distribution of internode length between the first 11 flowers initiated was determined in wild-type, *pny* and *pUBQ10;3FLAG-PNY pny* was comparable; whereas a high percentage of short internodes were measured in *pny* plants (Figure 3.5J). Plant height was also determined in wild-type, *pny* and *pUBQ10:3FLAG-PNY pny* inflorescences. Results showed that *pny* inflorescences were significantly reduced compared to wild-type plants. The average height of *pUBQ10:3FLAG-PNY* plants was greater than *pny* but slightly less than wild-type (Figure 3.5K).

The next objective was to determine if *pUBQ10:3FLAG-PNY* could complement the *pny pnf* phenotype. Genetic studies show that the mature shoots of *pny pnf* fails to initiate flowers and axillary shoots (Figure 3.5B) (Smith et al. 2004). In addition, internode development is severely impaired in *pny pnf* plants (Figure 3.5B) (Smith et al. 2004). Two distinct phenotype classes were observed in *pUBQ10:3FLAG-PNY pny pnf* plants. The first class of *pUBQ10:3FLAG-PNY pny pnf* plants initiated aberrant inflorescences

that terminated with the formation of an abnormal flower(s) (Figure 3.5E), composed mainly of carpel- and stamen-like organs (Figure 3.5G and 3.5H). The *pUBQ10:3FLAG-PNY pny pnf* inflorescence often produced coflorescence shoots in the axils of cauline leaves and a partial restoration of internode development was apparent as well (Figure 3.5E). Therefore, *pUBQ10:3FLAG-PNY* partially rescues axillary meristem formation, flower specification and internode development. The inflorescences of *pUBQ10:3FLAG-PNY pny pnf* initiated small bract-like leaves (Figure 3.5I, right), which was not observed in wild-type (Figure 3.5I, left), *pny* (data not shown) or *pny pnf* (Figure 3.5F). The second class of *pUBQ10:3FLAG-PNY pny pnf* plants initiated leafy-like inflorescences shoot, which produced numerous cauline-like leaves and bracts before the shoot terminated (Figure 3.6A). In contrast to the first class of *pUBQ10:3FLAG-PNY pny pnf* plants, the second class transgenic plants never initiated flower structures, indicating that flower meristem identity was not rescued (Figure 3.6A and 3.6D). Both internode patterning and specification of coflorescences were partially rescued (Figure 3.6C, 3.6E and 3.6F). In addition aerial rosettes often formed during early stages of inflorescence development in the second class *pUBQ10-3FLAG-ONY pny pnf* plants (Figure 3.6C and 3.6E). This phenotype suggests that these shoots proceed through a transitory vegetative phase before an inflorescence growth habit is established. Results from these complementation experiments suggest that *pUBQ10:3FLAG-PNY* construct complemented *pny* function but only partially rescued the *pny pnf* phenotype.

Complementation of *pny* by *pUBQ10:3FLAG-PNY* indicates that the 3FLAG-PNY is functional. In order to identify direct targets of PNY, it is essential to enrich for inflorescence meristems. To address this objective, the *pUBQ10:3flag-PNY* binary vector was transformed into the *ap1 cal* plants. In the absence of AP1 and CAL function, axillary meristems produced by the inflorescence meristem failed to acquire and/or maintain flower meristem identity. As a result, *ap1 cal* plants initiate inflorescence shoots that display a cauliflower-like phenotype (Figure 3.7A, F and K). During inflorescence development, *ap1* shoots gave rise to flowers that initiated secondary and tertiary flowers (Figure 3.7B, G and L). In wild-type plants, the inflorescences initiated coflorescence shoots at the base and flowers along the shoot axis (Figure 3.7C, H and M). After transforming *pUBQ10:3FLAG-PNY* into *ap1 cal* plants, two major classes of inflorescence phenotypes were observed. In the first class of T1 *pUBQ10:3FLAG-PNY ap1 cal* plants, the morphology of the inflorescence was similar to wild type as the inflorescence meristem initiated flowers with normal patterning (Figure 3.7E, J and O). Single insertion lines were also examined in the T2 generation. The T2 *pUBQ10:3FLAG-PNY ap1 cal* individuals displayed altered segregation ratios between wild-type and ap1-like phenotypes, making it difficult to dissect the genetics that underlie these two classes of transgenics. It should be pointed out that all of these plants contained the 3FLAG-PNY transgene that was linked to the BASTA resistant marker. Although these results are preliminary, they suggest that increased levels of PNY can rescue the flower meristem identity *ap1 cal* phenotype. Because ectopic 3FLAG-PNY appears to rescue the *ap1 cal*

flower meristem identity phenotype, this system cannot be used to efficiently enrich for inflorescence meristem tissue.

Development of an antibody against PNY

To overcome the challenges of creating epitope tagged versions of PNY expressed in plants that overproliferate inflorescence meristems, antibodies directed against the 165 amino acid C-terminal region of PNY lacking the conserved homeodomain were produced (Figure 3.8A). Since this C-terminal region lacks a conserved domain, such as the homeodomain, we hoped to generate an antibody that would be specific for PNY. After producing the antibody in rabbits, the PNY-antibody was affinity purified and characterized by western blot analysis. Results in Figure 3.8B show that the PNY-antibody recognized recombinant PNY (62kDa) as well as lower mass protein (56 kDa), which may correspond to a degradation product. To determine the specificity of the PNY antibody, inflorescence apices were dissected and proteins were isolated from a crude nuclear extract from wild-type and *pny* plants. Unfortunately, we were unable to detect nuclear proteins in either wild-type or *pny* shoot apices (data not shown). To further characterize the PNY antibody, nuclear proteins were isolated from *ap1 cal* inflorescence apices. Western blot analysis showed that the PNY antibody recognized proteins at 62 and 56 kDa, similar to the pattern detected with the recombinant PNY. These results suggest that the PNY antibody recognizes the endogenous PNY protein in enriched meristem extracts. Thus, ChIP analysis was performed with the PNY antibody using nuclear isolated from *ap1 cal*.

The isolation of PNY target genes by ChIP analysis

Genetic analysis of PNY and PNF demonstrate a role for these TFs in regulating the separation between primary stem and pedicel (Smith et al. 2004). In addition, axillary meristem formation is severely impaired in *pny pnf* plants (Smith et al. 2004). To determine if PNY directly or indirectly regulates lateral boundary identity, ChIP analysis was performed with *CUC1*, *LOF1* and *LAS*, which function together to regulate pedicel-stem separation and axillary meristem formation. In these ChIP studies, formaldehyde cross-linked nuclei were isolated from *ap1 cal* inflorescence apices. Subsequently, the chromatin was sheared to an average size of 500bp. ChIP was performed with the affinity purified PNY antibody. Results from the ChIP study showed that PNY associated with the proximal region of the *CUC1* promoter with a 13-fold enrichment (Figure 3.9B). At the same time, PNY did not appear to bind to the genomic region in the 3'UTR for *ACTIN8*. These results suggest that PNY associates with the *CUC1* regulatory region and it is likely that PNY functions with STM (or another KNOX protein) to regulate *CUC1* expression in the shoot meristem.

To determine if PNY directly regulates *LOF1*, ChIP was performed using the PNY-antibody and the *LOF1* PCR primer sets used for the KNOX-ChIP experiments shown above. As shown in Figure 3.9C, PNY binds to the genomic region containing fragment three with 19.83-fold enrichment. In addition, PNY may also associate with the fourth fragment but at lower level (Figure 3.9C). PNY did not appear to associate with

fragments one, two and five in the *LOF1* genomic locus (Figure 3.9). Thus, PNY and at least one KNOX protein appears to regulate *LOF1* by associating with the third genomic fragment in the *LOF1* promoter.

In Chapter 1, results showed that loss of *LAS* rescues the *pny pnf* phenotype (Figure 1.2D), similar to *pny pnf lof1I* (Figure 1.3E). In addition, the expression pattern for *LAS* as altered in *pny pnf* plants (Figure 1.5a). Therefore, ChIP studies were performed to determine if PNY directly regulates *LAS*. Genomic specific primers were designed to specifically amplify the intragenic regions up and downstream of *LAS* coding region (Figure 3.9A). Amplification of fragment four overlapped with the 3' enhancer of *LAS*, which is essential for its boundary-specific expression (Figure 3.9A) (Raatz et al. 2011). In addition, the large 5' regulatory region from -2815 bp to -1352 bp also is involved with *LAS* expression (Raatz et al. 2011). The PCR designed to amplify fragments one and two overlap with the 5' regulatory region. Three TGAC sequences were detected downstream of *LAS* (data not shown). Results from the ChIP experiment indicates that PNY associated with the fragment one in the 5' regulatory region of *LAS* (Figure 3.9B). In contrast, PNY did not appear to bind to fragments two, three and four. These results indicate that PNY directly controls the expression of *LAS*.

In *pny pnf* plants, the expression pattern for *CLV3* is severely altered and suggesting that PNY may directly regulate the expression of *CLV3*. Given that STM directly associates with the promoter and enhance of *CLV3* in close proximity to the WUS DNA-binding motifs (Figure 3.3A and B), ChIP was performed using the PNY antibody. In this

experiment, we utilized the PCR primers used for KNOX-ChIP. In contrast to STM, results in Figure 3.9B showed that PNY did not appear to bind to the promoter, 3' enhancer or the intron of *CLV3*. Therefore, the PNY-ChIP studies indicate that PNY and PNF indirectly regulates *CLV3* expression domain via the lateral organ boundary.

Genetic studies suggest that PNY and PNF are required for flower specification and identity during inflorescence development (Kanrar et al. 2008). In addition, these studies show that PNY and PNF function upstream of *LFY*. Whether PNY and PNF function to directly regulate flower meristem identity is unclear. However, results from Figure 3.4 suggest that at least one KNOX protein associates with the *LFY* promoter indicating that this early flower meristem identity gene is a target of a KNOX TF(s). Therefore, ChIP analysis was performed with the PNY antibody. In this experiment, the PCR primer sets described in Figure 3.4A were used to amplify three amplicons in the *LFY* promoter as well as the fragment 4 located in the 2nd intron. Experimental results indicate that PNY is bound to the genomic region of the fragment 3 with 5.6-fold enrichment (Figure 3.9C). Little or no enrichment was detected at fragment 1, 2 and 4 as well as internal control *ACTIN8*. Given the fact that PNY physically interacts with STM and at least one KNOX proteins associates with the fragment 3 in the *LFY* promoter, these data suggest that PNY-STM and possibly PNF-STM directly regulates *LFY* and flower meristem identity (Figure 3.4 and 3.9C). A recent study suggests that PNY and PNF may directly regulate flower meristem identity by repressing *miR156*, which negatively regulates a subset of *SPL*s including *SPL3* (Lal et al. 2011). Since *SPL3* directly regulates *LFY* as well as other

flower meristem identity genes, ectopic expression of *miR156* could function to reduce flower meristem identity in *pny pnf* plants. All *miR156* genes contain the TGACAGA sequence at the 5' and 3' end of the microRNA (Lal et al. 2011). Since this sequence is similar to the KNOX DNA-binding site, we tested this hypothesis. First, genomic specific primers were designed to amplify the *MIR156a* and *MIR156b* loci as well as intergenic regions downstream of these microRNA genes: *MIR156a:3'* and *MIR156b:3'*, respectively. In this study, the ChIP results showed that PNY does not associate with these genomic regions and likely regulates *miR156* in an indirect manner.

PNY and PNF regulate their target genes expression possibly via histone modification

In eukaryotes, the activation and repression of gene transcription is orchestrated by specific sets of TFs and RNA polymerase II machinery onto *cis* elements of target genes. Chromatin remodeling proteins also function to regulate gene expression by determining whether the chromatin is accessible for TF binding (Chen and Tian 2007). In numerous cases, a TF can also recruit histone modifiers, such as histone acetyl transferase (HAT), histone deacetylase complex (HDAC) and methyltransferases, to control the accessibility of the local chromatin to transcription (Gallinari et al. 2007). Cofactors or adapter proteins, such as TOPLESS (TPL) and SAP18, are crucial for bridging the TF with the chromatin remodeling complex (Liu et al. 2009; Szemenyei et al. 2008). HD TFs function to regulate cell fate in plants, animals and fungi (Burglin 1997). KNOX and BLH TFs are members of s superclass of HD proteins, which are evolutionarily related to MYELOID

ECOTROPIC VIRAL INTEGRATION SITE (MEIS) and PBC genes in animals (Burglin 1997). Recent studies suggest one of the functions of MEIS and PBC TFs is to mark the promoters of genes for chromatin remodeling (Berkes et al. 2004; Choe et al. 2009). According to this model, the association of PBC or MEIS with promoter promoters acts to recruit a chromatin remodeling complex to open or close the chromatin.

Twelve out of thirteen BLH TFs contain at least one conserved 6aa repeat (XLXLXL), which is referred to as a ZiBEL motif (Table 3.2) (Mukherjee et al. 2009). The amino acid at the X position is usually a serine, threonine or glycine. While PNY contained two ZIBEL motifs in the N- and C-terminus, PNF only contained a C-terminal ZIBEL motif (Table 3.2). These ZiBEL motifs are similar to the Ethylene Response Factor (ERF)-associated amphiphilic repression (EAR) motif (Ikeda and Ohme-Takagi 2009). The EAR domain acts as a conserved repression domain, which functions to recruit the corepressors TPL (Kieffer et al. 2006; Szemenyei et al. 2008). Other studies showed that the LXLXL motifs in *AGAMOUS-LIKE 15* (*ALG15*) associate with SAP18, which functions to recruit the SWI-independent 3/Histone Deacetylase (SIN3/HDAC) complex to inactivate transcription (Hill et al. 2008).

In order to determine the biological function of the ZIBEL motifs in PNY and PNF, a yeast two-hybrid (Y2H) study was performed to test the interaction between these two BLH proteins with TPL and SAP18. Both SAP18 and TPL are expressed in the shoot meristem (Long et al. 2006; Song and Galbraith 2006) and therefore overlap with the

expression domain of PNY and PNF. To test association of SAP18 with PNY and PNF, these BLH TFs were fused in frame with the DNA-binding domain (BD) of GAL4. Subsequently, SAP18 was fused in frame with the GAL4 activation domain (AD). Yeast cells containing different combinations of BD and AD constructs were plated on the media to select for the plasmids (Figure 3.10A and 3.10B). Results showed that the different combination of BD and AD plasmids did not alter growth on non-selecting media (Figure 3.10A and 3.10B). To test for a possible interaction, yeast cells containing the BD and AD constructs were plated on the selective media. An interaction between the bait and prey would allow yeast cells to grow on media lacking histidine and adenine. Results showed that yeast containing BD+AD, PNY-BD+AD and BD+SAP18-AD failed to grow on selection media, while yeast cells containing PNY-BD+SAP18-AD grew on the media lacking hisitidine and adenine (Figure 3.10C). Similar results were obtained with PNF, in which yeast cells containing PNF-BD+SAP18-AD grew on the selection media (Figure 3.10D). The Y2H system was also used to determine if PNY and PNF interacted with TPL. Results from this study suggest PNY and PNF did not associate with TPL (data not shown). Based on these results, we hypothesize that the ZiBEL motifs functions to recruit SAP18, which bridges PNY and PNF with the SIN3/HDAC complex to repress gene expression. To validate these results, a co-immunoprecipitation approach is required to confirm this association between PNY/PNF and SAP18. In addition, the genetic relationship between PNY/PNF and SAP18 as well as components of the SIN3/HDAC complex should be investigated.

Discussion

Genetic and expression analyses have provided insights into the possible role of the TALE homeodomain proteins, STM, PNY and PNF. The terminal differentiation of shoot meristems displayed in *stm* alleles and *pny pnf* provide strong evidence that these TALE homeodomain proteins regulate meristem cell fate (Barton and Poethig 1993; Long et al. 1996; Rutjens et al. 2009). Genetic studies indicate that meristem cell fate is regulated in part by the positive feedback loop between cytokinin and STM (Barton 2010). In maize, genome wide analysis for targets of KNOTTED1 (KN1), the presumptive ortholog of STM, suggests that this KNOX protein directly regulates cytokinin biosynthetic and signaling genes (Bolduc et al. 2012). A positive feedback loop between CUCs and STM also function to maintain meristem cell fate and boundary identity (Aida et al. 1999). A recent study suggests that STM directly regulates *CUC1* (Spinelli et al. 2011). Despite the importance of these findings, an understanding of how STM and KN1 specify meristem cell fate is not understood. The interplay between PNY/PNF and cytokinin has not been explored. A recent study suggests that PNY and PNF regulate internode patterning by confining the expression patterns of *BLADE ON PETIOLE 1* (*BOP1*) and *BOP2* to the boundary between the pedicel and inflorescence stem (Khan et al. 2012a). In Chapter two, my research shows that communication between the PNY/PNF and lateral organ boundary genes is essential for regulating inflorescence architecture. It should also be pointed out that plants with decreased activities of *PNY/PNF* and/or STM display a significant alteration in inflorescence morphology (Kanrar et al. 2006). One of the intriguing phenotypes displayed in *stm-10*

PNY/pny and *pny pnf* plants is a significant reduction or complete loss of flower meristem specification (Kanrar et al. 2006). Whether or not these TALE homeodomain proteins directly regulate flower specification has not been determined and may be the result in a perturbation in shoot meristem function. In this chapter, the research aimed to better understand the connection between these TALE homeodomain proteins with meristem cell fate, boundary function and flower specification.

The role of STM, PNY and PNF in regulating stem cell homeostasis in shoot meristems

The CLV-WUS negative feedback loop partially contributes to stem cell homeostasis in the SAM (Sablowski 2004). Cytokinin also functions in stem cell homeostasis as this phytohormone acts to regulate to regulate the *WUS* expression domain in the OC of the shoot meristem (Bleckmann and Simon 2009). A role for STM in stem cell homeostasis is unclear. A recent phenotypic analysis of the *stm* hypomorphic allele called '*gorgon*' indicates that STM regulates stem cell homeostasis (Takano et al. 2010). Furthermore, ectopic expression studies in the leaves of Arabidopsis suggest that the ability of WUS to activate *CLV3* is dependent upon STM (Brand et al. 2002). The interplay between STM and stem-cell homeostasis is likely mediated in part through cytokinin (Barton 2010). In our study, ChIP analyses suggests that at least one KNOX protein is bound to the *CLV3* promoter and 3' enhancer in close proximity to the WUS DNA-binding motifs. Furthermore, the KNOX DNA-binding motifs in the 3' enhancer appear to be necessary for regulating the spatial aspects of the *CLV3* expression domain. Given the genetic

relationship between STM and stem cell homeostasis, and the fact that the expression pattern for this KNOX gene overlaps with *CLV3*, we propose that STM directly regulates *CLV3*. Taken together, we propose that STM specifies stem cell fate by regulating cytokinin biosynthesis and signaling as well as by directly regulating *CLV3*.

In *pny pnf* plants, the expression patterns for *CLV3* and *WUS* are severely altered (Ung et al. 2011). In our study, results from ChIP indicate that PNY does not directly regulate *CLV3*. Although little is known about the relationship between cytokinin and PNY and PNF, preliminary results from out laboratory suggests that levels of negative regulators of cytokinin signaling, *ARR4*, *ARR5*, *ARR6*, and *ARR15* are significantly higher in the apices *of pny pnf* plants than wild-type plants (S. Lal and H. M. S. Smith, unpublished). In addition, preliminary results suggest that loss of *arr4*, *arr5* or *arr6* completely rescues the *pny pnf* phenotype (S. Wu and H. M. S. Smith, unpublished). Therefore, PNY and PNF may function to regulate the *CLV3* and *WUS* expression domains via cytokinin signaling. Results in chapter two showed that PNY and PNF regulate meristem integrity and the *CLV3* and *WUS* expression domains via the lateral organ boundary. Previous studies suggest that organ specific YABBY transcription factors regulate shoot development in part by signaling non-cell autonomously from the leaf to the shoot meristem (Goldshmidt et al. 2008). The YABBY derived signals regulate the integrity of the shoot meristem and the *CLV3* and *WUS* expression domains. Interesting, the YABBY-derived signals require *LAS* suggesting that the lateral organ boundary mediates this signaling process. Due to the relationship of PNY/PNF with the lateral organ boundary and meristem

integrity, it is plausible that these BLH proteins function in the YABBY signaling network.

The coordinated regulation of PNY and KNOXs on lateral organ boundary

As stated above, the relationship between STM and PNY/PNF with the lateral organ boundary is essential for specifying meristem cell fate and maintaining the integrity of the shoot meristem (Kanrar et al. 2006; Smith et al. 2004). STM and KN1 appear to control boundary function by directly regulating *CUC* gene expression (Figure 3.2B) (Spinelli et al. 2011). In our study, the interplay between STM and PNY/PNF with the lateral organ boundary was examined further. Results from our ChIP analyses suggest that at least one KNOX protein directly regulates *LOF1*. In addition, ChIP studies suggest that PNY directly regulates *LOF1* and *LAS*. Based on genetic molecular analyses in chapter two and three, we have developed a dynamic model that describes the relationship between STM and PNF/PNF and the lateral organ boundary (Figure 4.1). First, we propose that the KNOX protein bound to *LOF1* is STM as STM-GR is able to activate transcription of this MYB gene. Second, STM functions in the shoot meristem and the lateral organ boundary, while PNY/PNF activity is restricted to the shoot meristem. Third, the recruitment of SAP18 and the HDAC by PNY/PNF to the promoters and regulatory regions of boundary genes acts to inactivate gene expression in the shoot meristem. Taken together, we propose that PNY/PNF-STM heterodimers act to down-regulate the expression of boundary genes in the shoot meristem. However, in the lateral organ boundary where PNY/PNF activity is absent, STM associates with the

promoters and regulatory regions of boundary genes to activate gene expression possibly by forming a homodimer or a heterodimer with another BLH or KNOX protein(s).

The role of PNY and STM in regulating floral meristem identity

Integration of environmental and endogenous flowering time cues in the shoot meristem regulates floral evocation and flower initiation (Andres and Coupland 2012; Huijser and Schmid 2011; Jaeger et al. 2006). The early flower meristem identity gene *LFY*, encodes a transcription factors that functions to directly regulate late flower meristem identity genes, including *AP1* and *CAL* in Arabidopsis (Siriwardana and Lamb 2012). Recent studies suggest that endogenous and photoperiodic pathways operate in part through SPL3 to directly activate *LFY* expression (Yamaguchi et al. 2009). However, programs of flower specification occur in plants in the absence of SPL function in long and short day growth conditions indicating that the mechanism controlling flower meristem identity is not fully understood.

Genetic studies suggest that PNY and PNF function are required for flower specification and the upregulation of *LFY* as well as *AP1* and *CAL* (Kanrar et al. 2008). In addition, ectopic expression of *LFY* in *pny pnf* promotes the formation of terminal flower-like structures (Kanrar et al. 2008). STM is also implicated in flower specification as flowers are often replaced by cauline leaves early in inflorescence development in *stm-10* plants (Kanrar et al. 2006). Furthermore, a complete loss of flower specification and *LFY* expression occurs in *stm-10 fd* and *stm-10 ft* (Smith et al. 2011). The role of PNY/PNF

and STM in flower specification can occur in an indirect fashion. In support of this model, inflorescence patterning including flower specification is rescued in *pny pnf lof1* and *pny pnf las*. This suggests that PNY and PNF control flower specification via the lateral organ boundary by repressing boundary genes in the shoot meristem. Therefore, in the absence of PNY and PNF function, cells in the shoot meristem partially acquire boundary identity, which alters poisitional information required for flower specification. This is supported by the fact that the florigenic activity of FT is severely diminished in *pny pnf* plants (Kanrar et al. 2008). In this study, we investigated the possibility that PNY/PNF and STM directly regulate LFY. In our analyses, ChIP suggests that PNY/PNF and at least one KNOX protein associates with the promoter of LFY, indicating that this early flower meristem identity gene is a direct target of the PNY/PNF-STM complex. Therefore, the regulation of flower specification by PNY/PNF-STM is likely complex requiring both direct and indirect mechanisms that ensure that inflorescence architecture is properly controlled.

Genetic studies suggest that PNY and PNF regulate the SPL/miR156 module (Lal et al. 2011). Our laboratory previously showed that *miR156* levels fail to decline in *pny pnf* shoots under conditions that promote flowering. Although the *miR156* molecules contain TGAC motifs (Lal et al. 2011), ChIP analyses suggest that they are not directly regulated by PNY and PNF. Experimental studies based on ChIP-seq and gene expression profiling indicates that KN1 directly regulates several *ARGONATE* (*AGO*)-like genes, which are components of the RISC complex (Bolduc et al. 2012). Based on this finding,

it is likely that the alteration in the levels of *miR156* in *pny pnf* may be due to the mis-regulation of *AGO* genes in the shoot meristem.

The roles of KNOX and BLH act as pioneer TFs for histone modification

In animals, MEIS and PBX are TALE homeodomain proteins evolutionarily related to KNOX and BLH (Burglin 1997). MEIS and PBX appear to act as 'pioneering' transcription factors, which mark target genes for transcriptional regulation by recruiting chromatin remodeling factors to 'open' or 'close' the chromatin (Berkes et al. 2004). In this way, cell fates are controlled in permissive biological cell context (Berkes et al. 2004). In animals, PBX acts to recruit the HDAC1 to the promoters of genes to repress transcription (Saleh et al. 2000). However, the repressor activity of PBX can be switched to an activator in response to Protein Kinase A signaling (Saleh et al. 2000). In addition, the repressor activity of PBX is altered by MEIS (Choe et al. 2009). Based on recent studies, the association of MEIS with PBX displaces the HDAC, allowing MEIS-PBX heterodimers to function as transcriptional activators (Choe et al. 2009). Thus, the activity of TALE homeodomain proteins is mediated by specific signaling events and protein-protein interactions.

ChIP combined with genetic and gene expression analyses are important approaches to understand the role of a transcription factor. However, understanding the biochemical events that take place on the chromatin will provide a better framework for how transcription factors and chromatin remodeling complexes orchestrate transcription

during development. To this end, our research was also aimed at understanding the biochemical function of PNY and PNF. Preliminary results from chapter three indicate that the ZiBEL motifs in the PNY/PNF mediate binding with SAP18, which may bridge these BLH TFs with the HDAC. Therefore, we propose that the repressor activity displayed by PNY and PNF is mediated by their interaction with SAP18. Further, binding PNY and PNF with the promoters of *LOF1* and possibly other boundary regulators act to recruit SAP18 and the HDAC to 'close' the chromatin in the promoters of these genes. In addition, a flowering time signaling mechanism may prevent the association of PNY/PNF with SAP18, allowing these BLH proteins to act as activators of flower meristem identity. The mobile FT florigenic signal is thought to act as growth regulator (McGarry and Ayre 2012; Shalit et al. 2009) and is good candidate that may modify the activity of PNY/PNF. In addition, kinases that function in regulating flowering time have been identified and may facilitate a switch in the repressor activity of PNY/PNF for specifying flower meristem identity (Ogiso et al. 2010).

MATERIALS AND METHODS

Plant materials and growth condition

All the plant material used in this research was in Columbia-0 (Col-0) genetic background, except for *ap1 cal,* which is in the Landsberg background (L*er*). T-DNA insertion lines *pny-40126* and *pnf-33879* were obtained from the Arabidopsis Biological Resource Center (ABRC, www.biosci.ohio-state.edu). The *pny pnf* double mutant was examined from planting out progeny derived by the selfing of *pny PNF/pnf* plants (Smith

et al. 2004). The *ap1 cal* double mutant was obtained from Dr. Martin F. Yanofsky (Kempin et al. 1995). Plants were grown in soil at 22ºC under continuous light.

Construction of *pCLV3m:GUS* transgenic plants

*p*CLV3m:GUS was modified from pCLV3::mGFP5-ER (Reddy and Meyerowitz 2005). The mGFP5-ER residing in *Bam*HI site of pCLV3::mGFP5-ER was first replaced by a *GUS* fragment. The *GUS* fragment was amplified by forward-(CACCGGATCCATGTTACGTCCTGTAGAAAC) and reverse-(CGCGGATCCTCATTGTTTGCCTCCCTG), introducing *Bam*HI site, and then ligated into pBU backbone (Brand et al. 2002) to generating *pCLV3:GUS*. The putative KNOX-BLH binding motif within the 3' enhancer was mutated to an *Eco*RI sequence, which was achieved by overlapping PCR. The 3'enhancer was divided into two fragments by the putative KNOX-BLH binding motif. The first half of 477bp DNA fragment was amplified by forward-(CACCAAAAGCGGCCGCCCTAATCTCTTGTTGCTTTAA), introducing *Not*I site, and reverse-(GGTTGTCTTGAATTCCTGCCCCAAAGTCGG), introducing *Eco*RI site and the overlapping region. The other half of 757bp was amplified by forward-(CTTTGGGGCAGGAATTCAAGACAACCATTTGTAGTCAC), introducing *Eco*RI site and the overlapping region, and reverse-(CGCGGAGCTCTATGTGTGTTTTTTCTAAACAATCA), introducing *Sac*I site. After these two fragments were connected by overlapping PCR, the resulting fragment was replaced into *Not*I-*Sac*I site of *pCLV3:GUS*, resulting in *pCLV3m:GUS*. The binary vector was transformed into *Agrobacterium tumefaciens* strain GV3101 containing *pSoup*

plasmid by electroporation (Hellens et al. 2000). Arabidopsis Col plants were used to generate the transgenic plants are generated by the floral dip method. Positive transformants were screened using Basta (Clough and Bent 1998). Single insertion lines were selected for further analysis.

Construction of *pUBC:3FLAG-PNY ap1 cal* plants

The *PNY* coding sequence was amplified from a meristem cDNA library using Taq polymerase. Briefly, the DNA sequence of 3FLAG epitope, (ATGGACTACAAAGACCATGATGGAGACTATAAGGATCACGACATCGATTAC AAGGACGATGACGATAAG), was added at the 5' end of the *PNY* CDS by overlapping PCR. The 3FLAG-PNY was cloned into PENTR/D-TOPO (Invitrogen, K240020SP). The stop codon was present at the 3' end of PNY. Next, *3FLAG-PNY* was recombined into the destination binary vector pUBC (Grefen et al. 2010) via LR reaction (Invitrogen). The binary vector was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and then transformed into *ap1 cal* double mutant by floral dip method (Clough and Bent 1998). Positive transformants were screened by Basta up to 10 day old. Single insertion lines were selected for further analysis.

GUS and Histological Analysis

Shoot apices were collected from 25-day-old *pCLV3:GUS* and *pCLV3m:GUS* transgenic lines and placed in GUS staining solution (50mM sodium phosphate (pH7.2), 0.5mM $K_4Fe(CN)_6$, 0.5mM $K_3Fe(CN)_6$ and 1mg/ml 5-Bromo-4-chloro-3-indoxyl-beta-D-

glucuronide cyclohexylammonium salt) on ice. Subsequently, a vacuum were applied to infiltrate the tissue with GUS solution at 4ºC for 20 min. Next, the tissue was incubated in GUS solution at 37ºC for 10 hours in the dark. For histological analysis, GUS-stained samples were fixed by FAA solution, containing 50% Ethanol, 3.7% Formaldehyde, 5% Glacial Acetic Acid and 0.1% Triton-100, and a vacuum was applied for 20 minutes at 4ºC. Next, the FAA solution was discarded and replaced with fresh FAA. The samples were fixed in FAA for 48 hours at 4ºC. In order to embed the samples in paraffin, the tissue was dehydrated in ethanol, transferred to 100% Citrisolve in a step wise sequential manner. Next, the tissue was embedded with paraffin, sectioned (10µm-thickness), placed on microscope slides and incubated in Citrisolve to remove the wax. After mounting in permount (Fisher Scientific Co., NJ, USA) bright field and dark field images were obtained using LEICA DMR microscope (Leica Microsystems, Wetzlar, Germany).

Yeast Two-Hybrid Assay

The yeast two-hybrid assay was performed with Matchmaker Gold Yeast Two Hybrid System (Clontech, 630489). PNY and PNF were cloned into pGAL4.2.1BD vector, in frame with GAL4 DNA-binding domain. The SAP18-AD vector, in which SAP18 was cloned in frame with the GAL4 activation domain, was provided by Dr. Sharyn E. Perry (Hill et al. 2008). The bait and prey vectors were co-transformed into PJ64A yeast straining using a standard procedure (Gietz et al. 1997), http://home.cc.umanitoba.ca/~gietz/). Transformants containing both AD and BD vectors were obtained by selecting yeast media lacking tryptophan and leucine at 28ºC. To test

for protein-protein interactions yeast cells were plated on media lacking leucine, tryptophan, histidine and adenine at 28ºC.

ChIP qPCR and data analysis

The protocol used to perform ChIP in this chapter was described in appendix 3.2. Primers used to amplify the 3' UTR of *ACTIN8* was used as an internal control to normalized the ChIP-DNA. qPCR was performed by mixing 2 µl of ChIPed DNA, 12.5 µl of SYBR Green (Bio-RAD, 170-8882), 1 µl of 5 mM forward primer, 1 µl of 5 mM reverse primer and approximately 8.5 µl of ddH_2O. Using the MyCycler (BIORAD) machine, the following qPCR program was used: denaturation at 95ºC for 15 sec, annealing at 58ºC for 15 sec and DNA synthesis at 72ºC for 45 sec. This PCR step was repeated 45 times. The primer sequences of amplicons used for ChIP analysis was shown in Table 3.1.

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Figure 3.1 Characterization of KNOX antibody

(A) A schematic diagram of the STM protein. The bracket shows the C-terminal region of STM, which was used for antibody production. Note: the C-terminal region of STM contains the conserved KNOX homeodomain. (B) Western blot analysis using the STM (KNOX) antibody. Recombinant STM (lane 1) and BP (lane 2) as well as nuclear protein extracts isolated from *apetala1 cauliflower* inflorescence apices (lane 3) were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with 1.5ug of affinity purified STM antibody. Note: the STM-antibody recognizes recombinant STM and BP as well as nuclear proteins similar in mass as STM and BP. In addition, the lower mass proteins likely correspond to KNAT2 and KNAT6, which have a mass of 37 and 32kDa. Therefore, this antibody is referred to as the KNOX antibody.

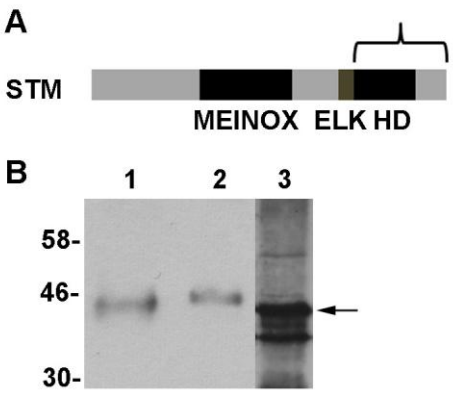

A
STM
MEINOX
ELK HD
B
1
2
3
58-
46-
30-

Figure 3.2 Identification of KNOX targets of lateral organ boundary genes via ChIP

(A) A schematic of the *LOF1* locus. In this diagram, the gray line represents the non-coding region and the black boxes represent the exons. A 1kb scale is displayed below the diagram. The black lines on top of the locus structure marked by numbers represent the amplicons tested by ChIP q-PCR. The length of the lines corresponds to the relative size of the amplified PCR product. KNOX-like DNA binding sequences (TGAC) are shown in bold. (B) Formaldehyde cross-linked chromatin from *ap1 cal* SAMs was immune-precipitated with the KNOX antibody. qPCR was used to determine the relative abundance of genomic DNA that immunoprecipitated with KNOX and GST antibodies. PCR primers were designed to *ACTIN8*, which amplifies an amplicon and was used as an internal control to normalize the ChIP-DNA samples. Shown was the mean among three technical replicates. Error bars represent SD; n=3. *$p<0.05$, **$p<0.01$, ***$p<0.001$; Student's t test. The same occupancy pattern in *LOF1* by KNOX proteins was obtained in two more independent ChIP experiments.

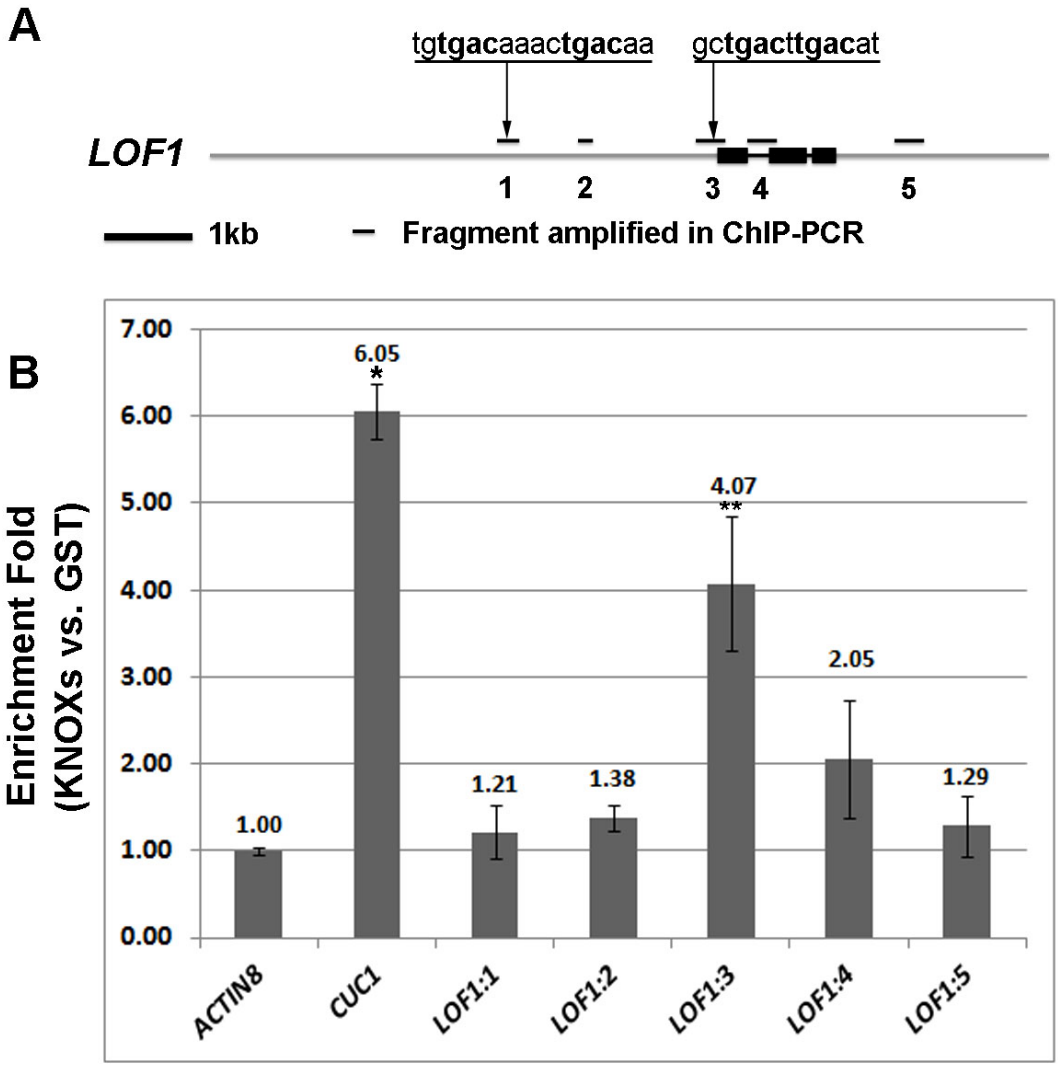
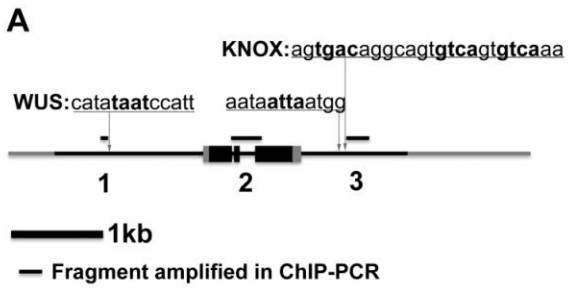
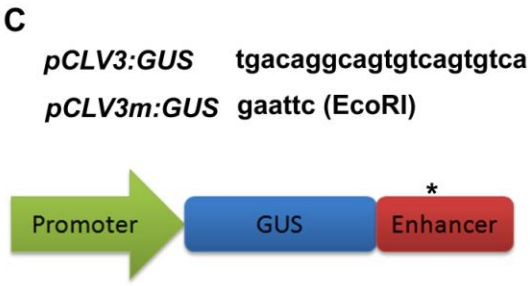
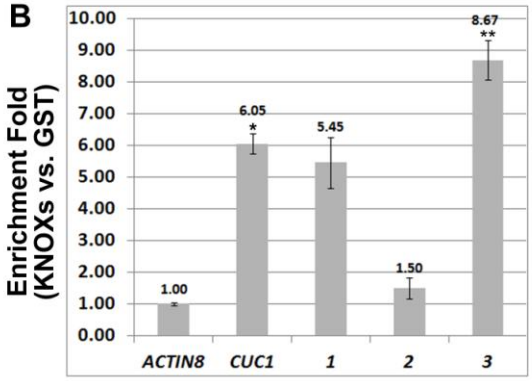

A
tgtgacaaactgacaa
gctgacttgacat
LOF1
1
2
3
4
5
1kb
Fragment amplified in ChIP-PCR
B
Enrichment Fold
(KNOXs vs. GST)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
6.05
*
4.07
**
2.05
1.21
1.38
1.29
1.00
ACTIN8
CUC1
LOF1:1
LOF1:2
LOF1:3
LOF1:4
LOF1:5

Figure 3.3 Identification of KNOX binding motifs that alter the spatial expression pattern of *CLV3:GUS*

(A) A schematic diagram of the *CLV3* locus. Gray and black boxes represent the untranslated regions and exons, respectively. The 1.5kb promoter upstream to the start condon and the 1.2kb enhancer downstream was highlighted in black. The KNOX like DNA-binding motif at the 3' enhancer of *CLV3* was shown. The "TGAC" core sequences are in bold. The WUS DNA binding motif is also shown, The "TAAT" core sequences are in bold. The numbered black lines on top of the *CLV3* locus represent the amplicons that were amplified by q-PCR. The shown scale corresponds to 1kb. (B) ChIP analysis from *ap1 cal* inflorescence meristem tissue. After immunoprecipitation with the KNOX antibody, q-PCR was used to amplify the amplicons using genomic specific primers. The enrichment fold was calculated by the abundance of ChIP-DNA immunoprecipitated by KNOX antibody divided by the abundance from the GST-ChIP, negative control. *CUC1* served as a positive control as previous studies show that this boundary gene is a direct target of STM (Spinelli et al. 2011). *ACTIN 8* was used as an internal control to normalize the ChIP-DNA samples. The numbers on each bar indicate the mean of fold change among three technical controls. Both region 1 and 3 were overrepresented. (C) The diagram of the *pCLV3:GUS* and *pCLV3m:GUS* constructs used in the GUS analysis. The asterisk marked the relative region containing the KNOX-like core sequences. Site directed mutagenesis was used to mutate the KNOX-like core sequences to the *Eco*RI sequence in the *pCLV3m:GUS*. The mutated reporter construct was transformed into wild-type and 6 individual lines were analyzed in the T2 generation. Inflorescence apices were dissected, GUS stained and fixed in FAA solution. After fixation, the tissue was embedded and sectioned (in 10 μm). The GUS staining pattern was analyzed by light (D-F) and dark-field microscopy (G-I). (D and G) show GUS staining from the same *pCLV3:GUS* line. (E and H) and (F and I) represent sections from two independent *pCLV3m:GUS* transgenic lines. Arrows were used to mark GUS domain in the apices.


A
KNOX:agtgacaggcagtgtcagtgtcaaa
WUS:catataatccatt
aataattaatgg
1
2
3
1kb
Fragment amplified in ChIP-PCR


C
pCLV3:GUS tgacaggcagtgtcagtgtca
pCLV3m:GUS gaattc (EcoRI)
Promoter
GUS
Enhancer
*


B
Enrichment Fold (KNOXs vs. GST)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1.00
6.05
*
5.45
1.50
8.67
**
ACTIN8
CUC1
1
2
3

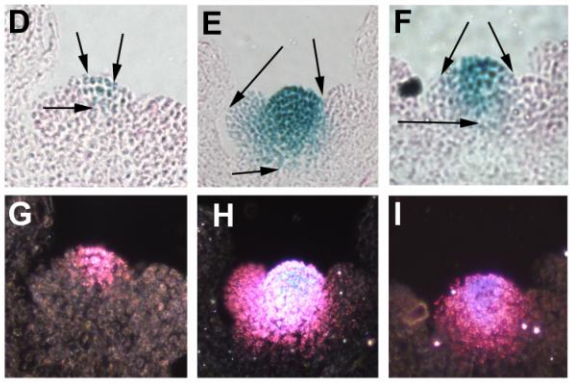

D
E
F
G
H
I

Figure 3.4: Identification of KNOX target genes in floral meristem specification via ChIP

(A) A schematic diagram of the *LFY* locus. The black boxes indicate exons and the grey boxes depict the 5'-UTR and 3'-UTR. The black lines indicate the promoter upstream to the start codon and introns. The numbered black lines represent amplicons that were amplified by q-PCR. The length of these lines indicates the size of the amplicons. A 1kb scale bar is shown below the LFY locus. (B) ChIP analysis was performed from dissected *ap1 cal* inflorescence apices. The enrichment fold of examined loci was obtained from the comparison between the amount of ChIP-DNA immunoprecipitated by KNOX and GST (negative control) antibody. *ACTIN8* was used as an internal control to normalize the ChIP-DNA samples. Shown was the mean. +/-, SD among three technical replicates. *p<0.05, **p<0.01, ***p<0.001; Student's t test. The same enrichment pattern of *LFY* was observed in two more independent ChIP experiments.

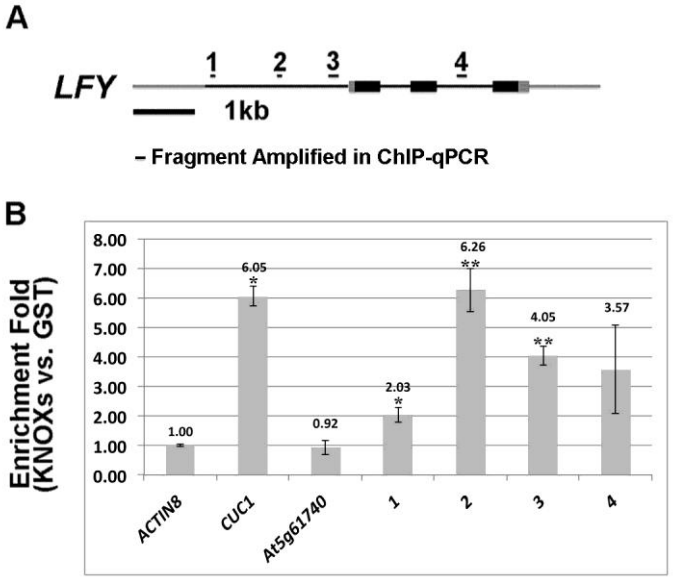

A
LFY
1
2
3
4
1kb
– Fragment Amplified in ChIP-qPCR
B
Enrichment Fold (KNOXs vs. GST)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1.00
6.05
*
0.92
2.03
*
6.26
**
4.05
**
3.57
ACTIN8
CUC1
At5g61740
1
2
3
4

Figure 3.5: Use of promoters and epitope tags to perform ChIP

(A) A schematic diagram of the *pUBQ10:3FLAG-PNY* construct. The *UBQ10* promoter was used to ectopically express proteins throughout the plant including the shoot meristem [Grefen et al. 2010]. An N-terminal 3FLAG epitope tag was fused in frame with PNY. (B-D) Complementation of *pny* using the *pUBQ10:3flag-PNY* construct. The internodes on primary inflorescence of wild-type (B), *pny* (C) and *pUBQ10:3flag-PNY pny* (D) are displayed. Arrows point at region on the *pny* inflorescence that produced shortened internodes. (E, G and H) Partial complementation of *pny pnf* with *pUBQ10:3FLAG-PNY*. The 8-week-old *pUBQ10:3flag-PNY pny pnf* transgenic plant produced branched inflorescence (arrow) terminated with the formation of aberrant flowers. (F) An 8-week-old *pny pnf* plant continuously produced leaves. (G and H) A flower-like structure produced by *pUBQ10:3flag-PNY pny pnf* was often subtended by a bract-like leave (arrow head). (H) Close-up of the aberrant flower produced by *pUBQ10:3flag-PNY pny pnf,* which initiated stamen-like (white arrow) and carpel-like (red arrow) structures. (I) A bract-like leaf produced along the inflorescence of *pUBQ10:3flag-PNY pny pnf* plant (left) and a wild-type cauline leave (right). (J) The internode length (mm) was measured between the first 11 flowers/fruits produced by each genotype and categorized into 7 groups (<1, 1-5, 6-10, 11-15, 16-20, 21-25, >25mm). The frequency distribution of internode length is shown in percentage. (K) The average height of wild-type, *pny* and *pUBQ10:3flag-PNY pny* primary inflorescences in mm. 30 individuals were measured for each genotype.

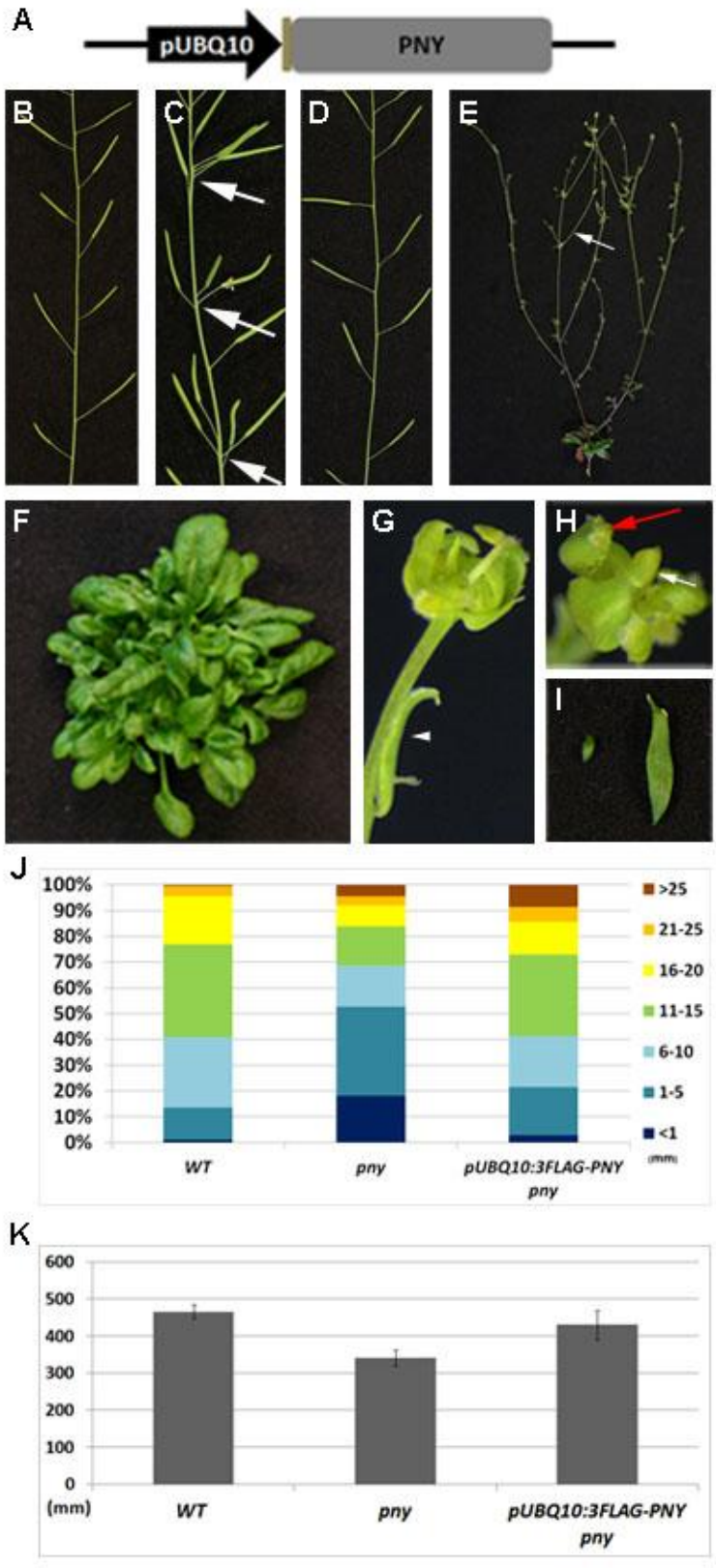

A
pUBQ10
PNY
B
C
D
E
F
G
H
I
J
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
>25
21-25
16-20
11-15
6-10
1-5
<1
(mm)
WT
pny
pUBQ10:3FLAG-PNY pny
K
600
500
400
300
200
100
0
(mm)
WT
pny
pUBQ10:3FLAG-PNY pny

Figure 3.6 Phenotype of *pUBQ10:3FLAG-PNY pny pnf* weak expression lines

(A) 8-week-old *pUBQ10:3FLAG-PNY pny pnf* plants produced clustered cauline leaves along the elongated stem. (B) 8-week-old *pny pnf* double mutant continuously produced leaves under continuous light growth conditions. During early vegetative phase, the SAM terminated and axillary meristems initiated in the axils of vegetative leaves continuously produce vegetative leaves. Upon floral induction, the shoot meristem failed to convert into inflorescence identity and internode elongation never occurred. In *pUBQ10:3FLAG-PNY pny pnf* plant, the apices (C) terminated with cauline-like leaves (arrowhead) and flower formation never occurred. The secondary axillary meristem (arrow) (D) was formed in the axil of cauline-like leave and developed into a secondary shoot without flower formation. The paraclades (E) often clustered together, resembling to *pny* and *pny PNF/pnf*. (F) The shoot branches of *pUBQ10:3FLAG-PNY pny pnf* plant shown in (A) were dissected. The primary shoot was hard to distinguish, due to the termination of SAM at the early stage of vegetative phase. The white arrow indicated internode elongation was restored. The red arrow pointed at the tertiary shoot was developed on the secondary shoot.

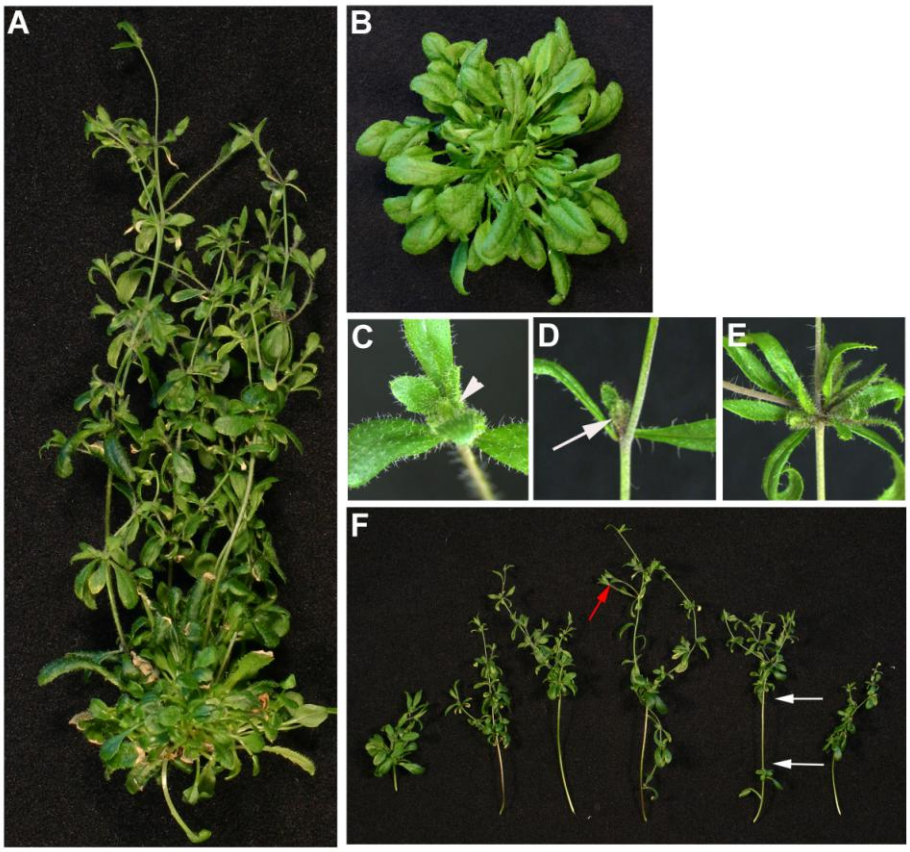

A
B
C
D
E
F

Figure 3.7 A role for *PNY* in specifying floral meristem identity

Inflorescences of (A) *ap1 cal*, (B) *ap1*, (C) wild-type, (D) *pUBQ10-PNY ap1 cal* plant displaying *ap1* phenotype and (E) *pUBQ10-PNY ap1 cal* plant displaying wild-type phenotype are displayed. Close-up of an inflorescence apex from (F) *ap1 cal*, (G) *ap1*, (H) wild-type, (I) *pUBQ10-PNY ap1 cal* plant displaying *ap1* phenotype and (J) *pUBQ10-PNY ap1 cal* plant displaying wild-type phenotype. (K) A loss of flower meristem identity in *ap1 cal* results in the proliferation of inflorescence meristems. Double flowers are produced in (L) *ap1*, and (N) *pUBQ10-PNY ap1 cal* plant displaying *ap1* phenotype. (M) a wild-type flower and a flower with similar morphology is displayed in (O) *pUBQ10-PNY ap1 cal*. Arrows point at secondary flowers initiated within flowers.

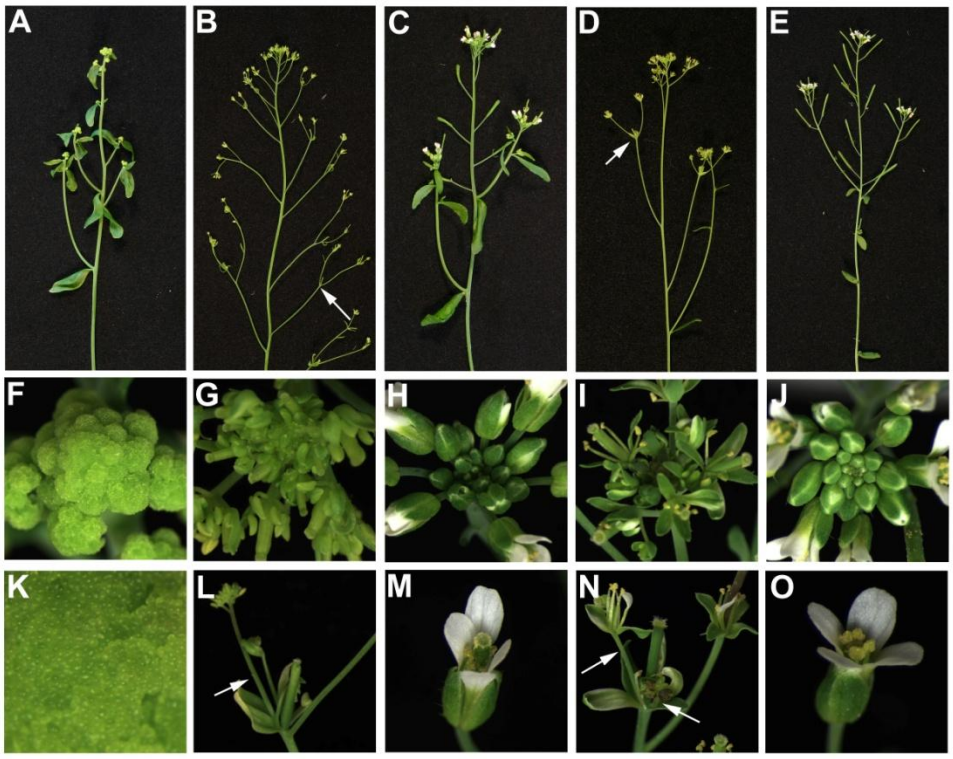

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 3.8 Characterization of PNY antibody

(A) The schematic diagram of the PNY transcription factor. The black rectangular boxes indicate the MEINOX Interaction Domain (MID) and the HOMEODOMAIN (HD). The C-terminus domain of PNY was used for antibody production (in brackets). (B) Western blot analysis was used to characterize the PNY antibody. Recombinant PNY (lane 1) and nuclear protein extracts isolated from *ap1 cal* (lane 2) were separated by SDS-PAGE and blotted to nitrocellulose membrane. Subsequently, the blot was incubated with 1.5 μg of PNY-antibody. Two bands in each sample were detected. The mass of the upper band was approximately 62kDa, while the lower band was 56kDa. In the *ap1 cal* nuclear protein extract, the 62kDa protein appears to be less abundant than the 56kDa band.

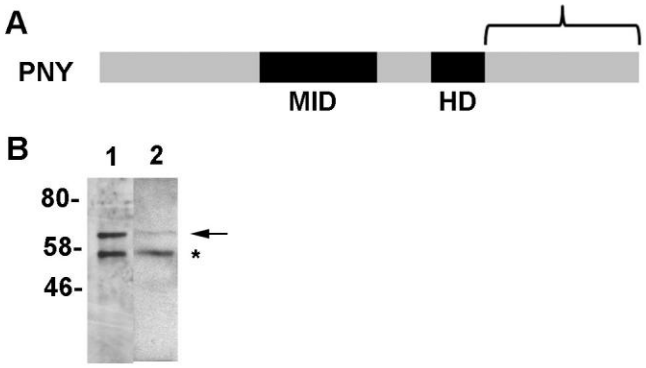

A
PNY
MID
HD
B
1
2
80-
58-
46-
*

Figure 3.9 Identification of PNY target genes via ChIP

(A) A schematic diagram of *LAS* locus. Gray and black boxes represent untranslated regions (UTR) and exons, respectively. Black lines indicate a 5' element and 3' enhancer element in the *LAS* non-coding sequence (Raatz et al. 2011). A 1kb scale bar is shown below the *LAS* locus diagram. The black lines on top of the locus structure marked by numbers represent amplicons tested by ChIP q-PCR. The length of the lines corresponds to the relative size of the amplified PCR product. KNOX-like DNA binding sequences (TGAC) in bold are shown. (B) ChIP analysis by PNY antibody. Using *ap1 cal* SAM, formaldehyde cross-linked chromatin was subjected to immunoprecipitation with PNY and GST antibodies. The abundance of ChIP-DNA by these antibodies was determined by q-PCR. The occupancy of PNY was determined in *CUC1, LAS, CLV3, MIR156a* and *MIR156b*. The same primer sets used to amplify the amplicons in *CLV3*, which was previously described in Figure3.3A, were used to detect the occupancy of PNY to the genomic regions of *CLV3*. (C) ChIP analysis by PNY antibody. Formaldehyde cross-linked chromatin isolated from *ap1 cal* apices was immunoprecipitated by PNY and treated with no antibody. The occupancy of PNY was determined in *LOF1* and *LFY*. The used primer sets for analysis was previously depicted in Figure3.2A and 3.4A. *ACTIN 8* was used as an internal control to normalize the ChIP-DNA samples in both (B) and (C). Shown was the mean. +/-, SD among three technical replicates.

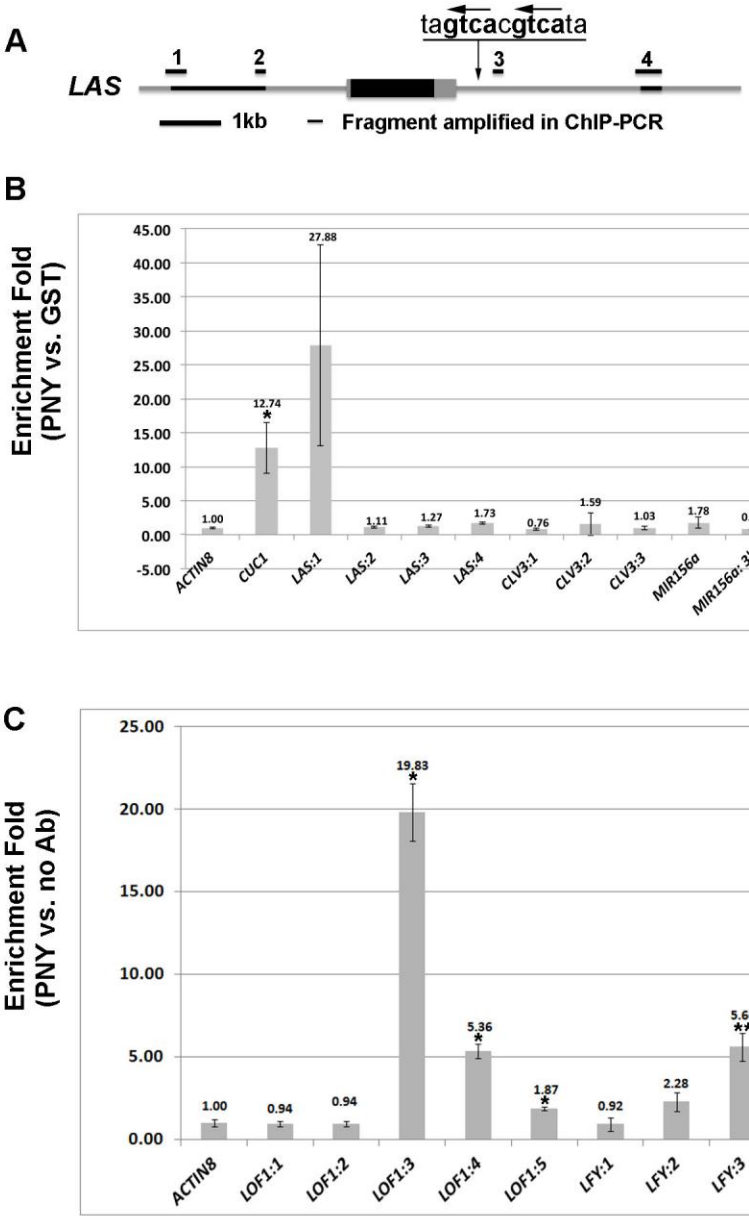

A
tagtcacgtcata
1
2
3
4
LAS
1kb
Fragment amplified in ChIP-PCR
B
Enrichment Fold (PNY vs. GST)
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
1.00
12.74
*
27.88
1.11
1.27
1.73
0.76
1.59
1.03
1.78
0.85
1.20
1.60
ACTIN8
CUC1
LAS:1
LAS:2
LAS:3
LAS:4
CLV3:1
CLV3:2
CLV3:3
MIR156a
MIR156a: 3'
MIR156b
MIR156b: 3'
C
Enrichment Fold (PNY vs. no Ab)
25.00
20.00
15.00
10.00
5.00
0.00
1.00
0.94
0.94
19.83
*
5.36
*
1.87
*
0.92
2.28
5.60
**
1.57
ACTIN8
LOF1:1
LOF1:2
LOF1:3
LOF1:4
LOF1:5
LFY:1
LFY:2
LFY:3
LFY:4

Figure 3.10 A possible interaction between PNY/PNF and SAP18

A yeast-two hybrid approach was used to determine if PNY and PNF associated with SAP18. PJ64-9A cells were co-transformed with AD and BD vectors, which were used as a control in all of the experiments. To detect association of PNY with SAP18, BD+AD, PNY-BD+AD, BD+ SAP18-AD and PNY-BD+SAP18-AD were co-transformed and plated on (A) non-selection media (-L, -W) and (C) double selection media lacking (-L, -W, -H, -Ade). To detect association of PNF with SAP18, BD+AD, PNF-BD+AD, BD+SAP18-AD and PNF-BD+SAP18-AD were co-transformed and plated on (B) non-selection media (-L, -W) and (D) double selection media lacking (-L, -W, -H, -Ade).

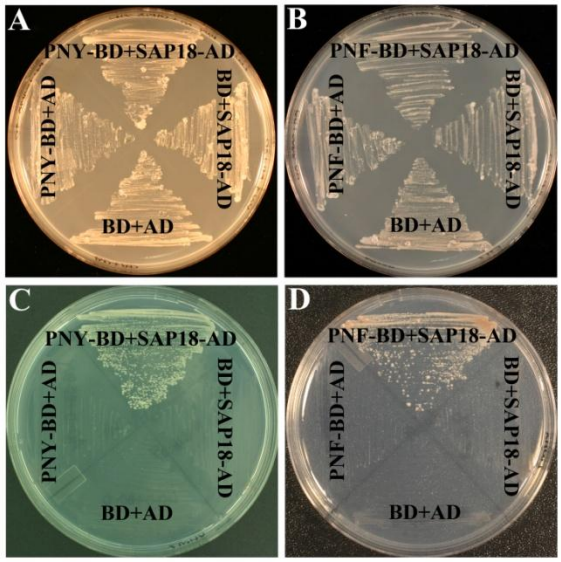

A
PNY-BD+SAP18-AD
PNY-BD+AD
BD+SAP18-AD
BD+AD
B
PNF-BD+SAP18-AD
PNF-BD+AD
BD+SAP18-AD
BD+AD
C
PNY-BD+SAP18-AD
PNY-BD+AD
BD+SAP18-AD
BD+AD
D
PNF-BD+SAP18-AD
PNF-BD+AD
BD+SAP18-AD
BD+AD

Table 3.1 Primer sequences for ChIP analysis

Note: In order to avoid non-specific amplification of target sequences, all the primer sets were checked for their specificity to targeting sequences throughout the Arabidopsis genome. In some cases, nearby regions within 500bp, the ChIP resolution limit, were chosen to ensure the specificity.

Amplicon	**Sequence**	
ACTIN8	Forward	CTAAAGAGACATCGTTTCCATGACGG
	Reverse	TCCTTAGACATCTCTCCAAACGC
CUC1	Forward	ACGGTTGGGGGAGGCCAAGA
	Reverse	ACCAGGAAGCTCCCAAGGCTCA
CLV3-1	Forward	TCGGATGGGCACGTGTCAGA
	Reverse	CGGCGCCGTATCGAGGGTA
CLV3-2	Forward	CGTTCAAGGACTTTCCAACCGCA
	Reverse	CGGGTCAGGTCCCGAAGGAA
CLV3-3	Forward	GGGGCAGTGACAGGCAGTGT
	Reverse	CGTGAGCGAAATCGCCAGTG
LOF1-1	Forward	AAATGGGCTATGGGGTCAAA
	Reverse	CTCATGGTCAATCCGTTTCG
LOF1-2	Forward	GAGAGTTTGTAGTGCGTTCG
	Reverse	ATCCCAATTGGCAGACGTAA
LOF1-3	Forward	CGACGGTCCATAACCCTTTT
	Reverse	CGTTGAAAGGGAAGTCCCAA
LOF1-4	Forward	ATTTACGGCCCACAAAACTG
	Reverse	GGTTTATCCTCGGGTCCAAT
LOF1-5	Forward	CCACACATGCCCTTCTTTCT
	Reverse	CACTCGGATGTTTGTTCCCA
LAS-1	Forward	CTCGCTAGCCAACATTTACCT
	Reverse	GCCACGTTGGATAGGACATA
LAS-2	Forward	ATAGCAAGGGCACTGCAAAT
	Reverse	ACCGTTTGGTGAGTTGTTCA
LAS-3	Forward	TTTTGCTGTGCAAGAGGGAT
	Reverse	TGGGGACGGATACGGAATAA
LAS-4	Forward	CTCTCTTCGACACACCCACTC
	Reverse	GGTTCACAAACCAAACACCCG
LFY-1	Forward	TTTCGCAAAGGAAAGTCGTTG
	Reverse	TCGGTCAGCCCATTACATTG
LFY-2	Forward	GGGAGATGACAACACGTAAGAG
	Reverse	GCAGTGTGAAAACTCCAGAAG
LFY-3	Forward	AGAGAATCACCACAGTGAAAACCCT
	Reverse	TGCTGGGAAATTGACAGTTGGGAC
LFY-4	Forward	ACGACAACGTGTCGAGTTAGGGA

	Reverse	GGACCCTCTATTATCCGCCGAGCA
miR156a	Forward	GCACTTGCTTCTCTTGCGTGC
	Reverse	GGCTAAAGGTCTCCTCCCGCA
miR156b	Forward	CTATAACTTTGCGTGTGCGTG
	Reverse	AGCACCCACTTCCACATAAC

Table 3.2 The ZiBEL motifs in BLH proteins

BLH	N-terminus	C-terminus
PNY	LSLSLS	SLTLGL
BEL1	LSLSLS	SLTLGL
BLH1	LSLTLS	SLTLGL
BLH4	LSLSLS	SLTLGL
BLH6	LSLSLG	SLTLGL
PNF		SLTLEL
BLH5		SLSLGL
ATH1	LSLSLA	
BLH2	LSLSLS	
BLH3	LSLSLG	
BLH10	LSLSLG	
BLH11		

Appendix 3.1-Antibody Purification

The truncated versions of induced recombinant proteins were injected as antigens into rabbits for immunized serum collection. Details of the injected antigen are shown in Figure 3.1A and 3.8A. The antibody produced from the collected serum was then purified with the following steps.

To affinity-purify the antibodies, the His-tagged C-terminus of target proteins (STM and PNY) were induced in *E.coli.* (BL21). Cells were first streaked out and grown on LB plates containing 100ug/ml carbenicillin overnight at 37 ºC. The plates containing single colonies were transferred to 4 ºC for 4-6 days storage for a greater induction. A single colony was picked for inoculation and grown with shaking at 250rpm in 5ml of LB liquid media, containing 100µg/ml carbenicillin, at 37 ºC overnight. The next morning, the inoculation was magnified into 2 liters and grown to a cell density of OD_{600} = 0.5-0.8. After bringing the cell culture to 28 ºC, 1mM IPTG was introduced for 4hr protein induction. The cell culture was pelleted at 5,000rpm for 10min and stored at -80 ºC overnight.

The recombinant protein was purified by Ni sepharose (GE healthcare, 17-5318-02). 800ul of Ni sepharose beads was filled in 4ml filter column (Fisher Healthcare, 11-387-50) and washed with 10ml of water and, subsequently, 10ml of guanidinium lysis buffer (pH 7.8), containing 20mM sodium phosphate (pH7.8), 6M guanidine hydrochloride and 500mM NaCl. The cell pellets were thawed in 25ml ice-cold guanidinium lysis buffer

(pH 7.8). Cells were slowly rocked for 5-10min at room temperature for full-lysis. Sonication was performed at 25% continuous (Branson, Digital Sonifier) for 30 sec followed by a rest on ice for 30sec, which were repeated three times. The suspension was centrifuged at 10,000rpm at 4ºC for 15min. The supernatant was collected and passed through the Ni sepharose column. Then the column was washed with 20ml of Denature Binding Buffer, containing 20mM sodium phosphate (pH7.8), 8M urea and 500 mM NaCl, and 20ml of Washing Buffer, containing 20mM sodium phosphate (pH7.8), 8M Urea and 200 mM NaCl. The induced protein was eventually eluted with 8-ml of Elution buffer, containing 8M urea, 20mM sodium phosphate, 200mM imidazole and 200mM NaCl. The protein elution was fractioned 800ul per tube. The distribution of protein was further analyzed and confirmed by Coomassie staining and western blot.

The induced protein was further purified and enriched by SDS-PAGE before antibody purification. The peak fraction was loaded on SDS-PAGE and separated by size. The region containing induced protein SDS-PAGE were cut out and placed in electro-separation system in order to isolate to protein. The antigen sample was dialyzed in 2L of 1xPBS with 0.1% SDS buffer twice to get rid of Tris base and Glycine.

The antibody was purified by antigen crosslinked CNBr-activated sepharose (GE healthcare, 17-0981-01). 0.5gram of CNBr sepharose was mixed in 15ml of cold sterile water, which was centrifuged by 1,000g for 2min. Most of water was poured off. 1-2mls of beads were used to generate a purification column in 4ml filter column. 10ml of HCl

was passed through the column to activate the bead, followed with 10ml of cold PBS wash. Within the column sealed by parafilm, beads were mixed with 2-3ml of antigen on a rocker for 4hr at 4ºC and then induced with 200ul of 1M ethanolamine into the mixture for an additional hour incubation. The column was washed with 20ml of washing buffer I, containing 50 mM Tris-Cl (pH 7.5) and 500mM NaCl, and 10ml of cold 100mM Glycine (pH 2.5-3). The column was re-equilibrated with 20ml of washing buffer I and then 10ml washing buffer II, containing 50mM Tris-Cl (pH 7.5) and 200mM NaCl. By this step, the antigen cross-linked CNBr-activated sepharose column was ready for antibody binding and elution.

The sera containing the target antibody was thawed and centrifuged at 20,000g to remove the debris. 40ml of sera was applied to the column, followed by washes with 50ml of washing buffer I and 10ml of washing buffer II. The antibody was eluted by 100mM Glycine (pH 2.5-3.0) and fractioned 800ul per tube. The elution was then neutralized with 1M Tris-Cl (pH 8.0).

Appendix 3.2- Chromatin Immunoprecipitation Protocol

ChIP procedure was followed by the modified version of published protocol [Kaufmann, 2010 #3570]. To perform ChIP analysis, 0.8 gram of shoot apices was collected from 4-week-old *ap1 cal*. Tissue was submerged in 25ml of ice-cold Fixation Buffer, containing 10mM sodium phosphate (pH 7.0), 1% Formaldehyde, 50mM NaCl and 0.1M sucrose. Vacuum (UVS 400, Thermo) was applied at 26Hg for 15min in vacuum oven (VWR). The fixation was stopped by 2.5ml of 1.25M glycine, followed by 2min vacuum. The fixed tissue was then washed twice with 25ml MC buffer, containing 10 mM sodium phosphate (pH 7.0), 1% Formaldehyde, 50mM NaCl and 0.1M sucrose. The fixed tissue was then dried on paper and quick-frozen in liquid nitrogen.

To isolate nuclei, tissue was ground completely in liquid nitrogen and homogenized in 20ml of M1 Buffer, containing 10mM sodium phosphate (pH 7.0), 0.1M NaCl, 1M 2-Methyl 2,4-Pentanediol, 10mM β-mercaptoethanol and protease inhibitor. The slurry was filtered through 55 µm mesh and collected. Additional 5ml of M1 buffer was added to wash the slurry residue. The total 25ml collection of filtrate was centrifuged at 1,000g at 4ºC for 20min. Then the nuclear pellet was washed with 5ml of M2 buffer, containing 10mM sodium phosphate (pH 7.0), 0.1M NaCl, 0.5% Triton X-100, 10mM MgCl2 1M 2-Methyl 2,4-Pentanediol, 10mM β-mercaptoethanol and protease inhibitor, and centrifuged down at 1,000g at 4ºC for 10min. This washing step was repeated 5 times. The nuclear pellet was further washed by 5ml of M3 buffer, containing 10mM sodium phosphate (pH 7), 0.1 M NaCl, 10 mM β-mercaptoethanol and protease inhibitor, under the same condition. The nuclei were sonicated in 1ml of Sonication Buffer, containing

10mM sodium phosphate (pH 7), 0.1M NaCl, 0.5% Sarkosyl, 10mM EDTA and protease inhibitor, with Bioruptor. The sonication was performed 15sec on and 15sec off for 30min on medium power. The suspension was centrifuged at 16,000g at 4ºC for 10min. The supernatant was then transferred a new tube and centrifuged at 16,000g at 4ºC for 8min. The supernatant was saved in a new tube for immunoprecipitation. 60ul of supernatant was set aside to serve as 'input DNA' control.

Before immunoprecipitation, the sonicated nuclei extraction was precleared with protein A beads (Santa Cruz, cat. no. sc-2001). Protein A beads were washed twice with IP buffer, containing 50mM HEPES (pH 7.5), 150mM NaCl, 5mM $MgCl_2$, 10μM $ZnSO_4$, 1% Triton X-100 and 0.05% SDS, and then blocked with IP buffer containing 5mg/ml BSA for 1 hr. The equal volume of IP buffer, was added to the nuclei extract and subsequently centrifuged at 16,000g at 4ºC for 10min, twice. 80ul of BSA-blocked protein A beads (25% slurry) was added to the nuclei-IP buffer mixture for 1hr incubation at 4ºC. To get rid of protein A bead, the precleared mixture was centrifuged at 3,800g at 4ºC for 5min and the supernatant was transferred into a new tube. Then a 10-min 16,000g centrifuge at 4ºC was applied to the supernatant. The final precleared nuclei were saved in a new 2ml microtube.

The precleared nuclei were equally divided (900ul) into two microtubes. 15-20ul of home-made KNOXs antibody and 5ul of GST (Santa cruz, sc-459; 200ug/ml), as negative control, were added into IP samples, respectively. Based on the coomassie

staining analysis, the amount of the antibodies mentioned above contained equal amount of heavy and light chain. Incubation was performed on a rotating wheel at 4ºC overnight (about 16hr). The next day morning, the IP reaction was centrifuged at 16,000g at 4ºC for 5min and the supernatant was transferred into a new tube. 40ul of BSA-blocked protein A beads were added to each tube for 50min incubation at 4ºC. The beads were collected by 3,800g centrifuge at 4ºC for 5min. 1ml of IP buffer containing no BSA was then added to the beads for 8min incubation and then centrifuge was applied again for 2min. The non-specific binding was significantly reduced after washing 5 times. This washing step was performed at room temperature. The ChIP-DNA was eluted by 100ul of Elute Buffer (pH 2.8), containing 0.1 M glycine, 0.5M NaCl and 0.05% Tween-20, with 1min incubation in 37 ºC water bath while shaking rigorously. The suspension was centrifuged at 16,000g at room temperature for 1min. The supernatant containing the ChIP-DNA was collected into a new tube and neutralized by 1M Tris-Cl (pH 9.0). This elution step was repeated twice more with a 4-min incubation for the last round. The pooled eluted ChIP-DNA was centrifuged at 16,000g for 2min to get rid of protein A bead residue. The final ChIP-DNA was saved in a 2ml microtube for reverse cross-linking.

The reverse cross-linking step was to digest proteins on the DNA with proteinase K. The total volume of ChIP-DNA was adjusted to 450ul with TE buffer, containing 10mM Tris-Cl (pH 8.0) and 1mM EDTA. 11.25ul of 20mg/ml proteinase K was added to the reaction for an overnight incubation at 37 ºC. On the next day morning, 11.25ul of 20mg/ml proteinase K was added again to the reaction tube for 6hr incubation at 65 ºC. The

digested DNA was precipitated with 2.5 vol 100 % ethanol, 1/10 vol of 3 M NaAc (pH 5.4) and 1 μl glycogen overnight at − 20 °C. DNA was centrifuged at 16,000g at 4 ºC for 30min. The DNA pellet was then resuspended in 100ul of TE, following with the second purification by PCR purification column (Qiagen, 28104). The final DNA products were eluted in 150ul for quantitative PCR analysis.

CONCLUSIONS

Genetic studies suggest that homeodomain transcription factors PENNYWISE (PNY) and POUND-FOOLISH (PNF) associate with SHOOT MERISTEMLESS (STM) to regulate key developmental pathways that control inflorescence architecture in Arabidopsis. The shoot meristem is the site of organogenesis and processes occurring in or adjacent to this structure have profound effects on shoot development. In chapter one, results show that the interplay and communication between PNY/PNF and the lateral organ boundary is essential for patterning of the inflorescence shoot. Although genetic studies provide insights into the general function of transcription factors (TFs) in plant development, it is difficult to determine whether pathways that are direct or indirectly regulated by a specific TF based on solely phenotypic analyses. In chapter two, a chromatin immunoprecipitation (ChIP) approach was developed using antibodies directed against the homeodomain of STM and the C-terminal tail of PNY. Although the STM antibody recognizes other KNOTTED1-like HOMEOBOX (KNOX) proteins, we speculate that STM is probably one of the KNOX proteins bound to the target genes regulatory sequence based on genetic studies. Through this study and genetic experiments, we were able to provide evidence that STM (and/or a related) and PNY directly or indirectly regulate key developmental processes required for establishing patterning events required for inflorescence architecture.

The mechanism of PNY and STM in regulating inflorescence development

Stem cell homeostasis is a process that is essential for maintaining meristem cell fate (Tucker and Laux 2007). If stem cell homeostasis is not maintained, the mechanism that replenishes meristem cells will be lost and organogenesis will cease after a limited number of organs are formed. Stem cell homeostasis is controlled in part by the CLAVATA (CLV)-WUSCHEL (WUS) negative feedback loop. In addition, the phytohormone cytokinin acts as a key signal, which controls the stem cell niche by regulating the spatial dynamics of *WUS*. The role of STM in maintaining the stem cell niche has not been clearly defined. Genetic and molecular studies indicate that STM regulates a key cytokinin biosynthesis gene, *ISOPENTENYL TRANSFERASE 7* as well as the *ARABIDOPSIS RESPONSE REGULATOR 5* (*ARR5*), which negatively regulates cytokinin signaling (Yani et al., 2005). Based on this study, it is not clear if the regulation is direct or indirect. Given that KNOTTED1, the presumptive ortholog of STM, appears to directly regulate genes involved in cytokinin biosynthesis and signaling (Barton 2010) suggests that the relationship between STM and this phytohormone is also direct. Therefore, cytokinin provides a link between STM and stem cell homeostasis. Results from the KNOX-ChIP study in chapter three suggest that STM may directly regulate *CLV3* by associating with the promoter and 3' enhancer. Interestingly, experimental studies suggest that the ability of WUS to activate *CLV3* is dependent upon STM (Brand et al. 2002). Further, the close proximity of the WUS and STM DNA-binding sites may indicate that these proteins form a complex that regulates *CLV3*. Animal homeodomian transcription factors evolutionarily related to STM form complexes with typical homeodomian proteins such as HOX proteins in a DNA-dependent manner (Saleh et al.

2000). Since *WUS* encodes a 'typical' homeodomain protein it would be interesting to determine if WUS and STM cooperatively associate with the regulatory regions of *CLV3*.

Role of STM and PNY/PNF in regulating boundary identity

Experimental evidence shows that PNY/PNF and STM play a fundamental role in regulating the lateral organ boundary during shoot development (Kanrar et al. 2006). At the genetic level, STM and CUP SHAPHED COTYLEDON1 (CUC1)/CUC2 appear to positively regulate each other suggesting that the TFs form a positive feedback loop that maintains meristem cell fate and boundary function (Aida et al. 1999). Moreover, a recent study suggests that STM directly regulates *CUC1* (Spinelli et al. 2011). Studies from Dr. Patricia Springer's laboratory suggest that STM positively regulates *LATERAL FUSION1* (*LOF1*) and functions in parallel with this MYB transcription factor to regulate meristem cell fate (D.K. Lee and P. Springer, unpublished).

Inflorescence development is severely limited in plants with reduced PNY and PNF function (Smith et al. 2004). For example, pedicels are often fused to the main inflorescence stem in *pny PNF/pnf* plants. In addition, mature *pny pnf* shoots fail to initiate axillary meristems and flowers. Inflorescence development is also controlled by the combined activities of lateral organ boundary TFs CUC2/CUC3, LOF1 and LATERAL SUPPRESSOR (LAS), which act to mediate pedicel-stem separation and axillary meristem initiation (Greb et al. 2003; Hibara et al. 2006; Lee et al. 2009). In chapter one, the relationship of PNY/PNF with LAS and LOF1 was investigated. Results

from chapter one show that inflorescence development is rescued in *pny pnf lof1* and *pny pnf las* triple mutants suggesting that *PNY* and *PNF* act upstream to negatively regulate *LOF1* and *LAS*. Consistent with this hypothesis, the *LOF1* expression domain expands into the shoot meristem of *pny pnf* plants. In the case of *LAS*, we were unable to detect mRNA for this boundary gene in sectioned *pny pnf* shoot apices. *LAS* expression may be controlled by the integrity of the lateral organ boundary. Therefore, in the absence of *pny pnf* function, the integrity of the lateral organ boundary may be reduced leading to a reduction in *LAS* expression. It should be emphasized that *LAS* expression is reduced but not "off" in *pny pnf* shoots, as loss of LAS function rescues inflorescence development in *pny pnf* plants.

As stated above, defects in pedicel-stem separation and axillary meristem formation is observed in plants with reduced levels of STM and/or PNY/PNF (Kanrar et al. 2006). Results in chapter two indicate that *CUC1* and *LOF1* are targets of STM. In addition, chromatin immunprecipitation experiments (ChIP) suggest that PNY directly regulate *CUC1*, *LOF1* and *LAS*. Although biochemical studies show that STM and PNY/PNF physically interact, genetic studies indicate that STM and PNF/PNF regulate boundary genes in an opposite manner. Specifically, STM appears to activate boundary genes, while PNY/PNF acts to repress these classes of genes. In order to discern these results, a model was created to explain how these homeodomian TFs function to regulate boundary genes (Figure 4.1). First, the shoot meristem is a three diemensional structure composed of specific domains with different cell types and patterns of cell divisions. In our model,

we first propose that the expression domain of PNY/PNF is limited to the peripheral zone (PZ) and central zone (CZ), but excluded from the lateral organ boundary. Experimental studies indicate that PNY does not move cell-to-cell, indicating that the mRNA expression pattern reflects the protein localization pattern for this TF, as well as PNF. The second point is that the STM mRNA and protein localizes in the CZ, PZ and the lateral boundary. The model proposes that PNY/PNF-STM complexes function in the CZ and PZ to repress boundary genes, such as *CUC1*, *LOF1* and *LAS*. As a result, boundary identity is excluded from the shoot meristem. In the boundary cells, STM acts to specify boundary identity by activating boundary specific genes in the absence of PNY/PNF. The function displayed by STM in the lateral organ boundary cells may result from the formation of a STM-STM homodimer. Alternatively, KNOX or BLH proteins that localize to the lateral organ boundary may form STM-KNOX or STM-BLH heterodimers that function to promote boundary identity.

Specification of flower meristem identity by STM and PNY/PNF

Mutations in genes that control shoot meristem integrity and fate are predicted to display severe developmental phenotypes. Identifying the primary effects from the downstream consequences that contribute to the overall phenotype resulting from a mutation in a meristematic gene is extremely challenging. For example, the loss of flower production displayed by *pny pnf* shoots could be a secondary effect that is due to decrease in meristem integrity. Given that meristem integrity and inflorescence development is reestablished in *pny pnf lof1* and *pny pnf las* would suggest that these BLH TFs control

flower specification indirectly via the lateral organ boundary. Biological systems are quite complex and results from the ChIP analysis in chapter two suggests that PNY and PNF also regulate *LFY* directly. Therefore, the ChIP analyses not only indicate the importance of lateral organ boundaries in shaping the architecture of the shoot but also diverse role of PNY and PNF as well as STM in controlling flower meristem identity. Utilization of a ChIP-Seq approach will provide a better framework of how PNY/PNF and STM functions in the gene regulatory networks that control flower meristem identity. As more ChIP studies are performed, scientists can build begin to integrate biochemical interactions, protein-protein and protein-DNA, to understand the molecular events that control flower meristem identity.

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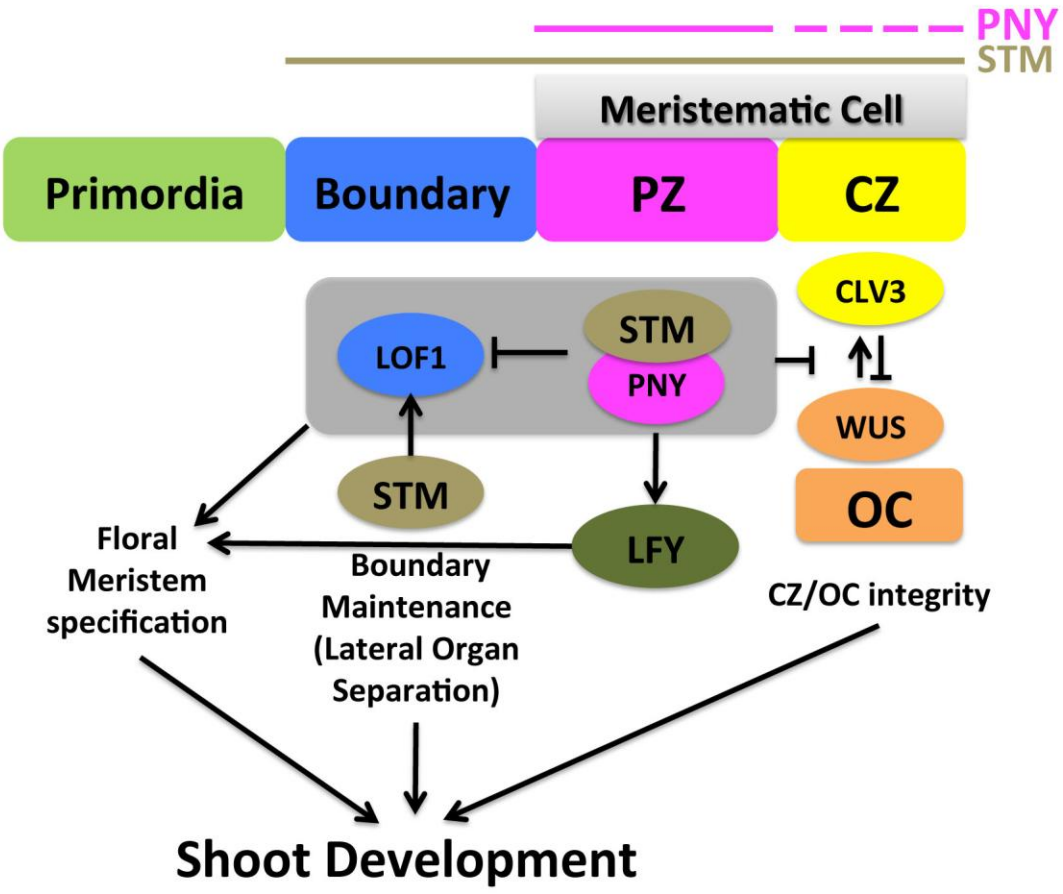
Figure 4.1 The role of PNY and STM in regulating shoot development

This model displays the diverse functions of PNY/PNF-STM in regulating meristem integrity, boundary function and floral meristem specification during inflorescence development. PNY/PNF are expressed in the central zone (CZ) and peripheral zone (PZ), while STM is expressed in the CZ, PZ and boundary. The relationship between PNY/PNF and STM with the lateral organ boundary is essential for regulating meristem integrity. First, PNY/PNF-STM control the integrity of the CZ and Organizing Center (OC) by negatively regulating the *LOF1* and possibly other boundary genes. However, in the cells of the lateral organ boundary, which lack PNY/PNF function, STM activates *LOF1* and other boundary genes. During inflorescence development, the relationship between PNY/PNF-STM and *LOF1* are also essential for indirectly regulating flower specification. At the same time, PNY/PNF-STM directly regulates *LFY* to specify flowers on the flanks of the inflorescence meristem.


PNY
STM
Meristematic Cell
Primordia
Boundary
PZ
CZ
CLV3
LOF1
STM
PNY
WUS
STM
OC
LFY
Floral
Meristem
specification
Boundary
Maintenance
(Lateral Organ
Separation)
CZ/OC integrity
Shoot Development