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ORIGINAL ARTICLE

Arabidopsis inflorescence architecture requires the activities of KNOX-BELL homeodomain heterodimers

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Abstract In flowering plants, post-embryonic development is mediated by the activity of shoot and root apical meristems. Shoot architecture results from activity of the shoot apical meristem (SAM), which initiates primordia, including leaves, internodes and axillary meristems, repetitively from its flanks. Axillary meristems can develop into secondary shoots or flowers. In *Arabidopsis*, two paralogous *BEL1*-like (*BELL*) homeobox genes, *PENNYWISE* (*PNY*) and *POUND-FOOLISH* (*PNF*), expressed in the SAM, encode DNA-binding proteins that are essential for specifying floral primordia and establishing early internode patterning events during inflorescence development. Biochemical studies show that PNY associates with the knotted1-like homeobox (KNOX) proteins, SHOOT-MERISTEMLESS (STM) and BREVIPEDICELLUS (BP). PNY-BP heterodimers are essential for establishing early internode patterning events, while PNY-STM heterodimers are critical for SAM function. In this report, we examined the role of *PNY*, *PNF* and *STM* during development. First, we show that PNF interacts with STM and BP indicating that PNY and PNF are redundant functioning proteins. Inflorescence development, but not vegetative development, is sensitive to the dosage levels of PNY, PNF and STM. Characterization of *stm-10*, a weak allele in the Columbia ecotype, indicates that *STM* is also involved in floral specification and internode development. Our examination of the genetic requirements for PNY, PNF and STM demonstrates that these KNOX–BELL heterodimers control floral specification, internode patterning and the maintenance of boundaries between initiating floral primordia and the inflorescence meristem.

Keywords *Arabidopsis* · Development · Homeodomain · Inflorescence · Internode · Meristem

Abbreviations

SAM Shoot apical meristem
KNOX Knotted1-like homeobox
BELL BELL1-like homeobox
PNY PENNYWISE
PNF POUND-FOOLISH
STM SHOOTMERISTEMLESS
BP BREVIPEDICELLUS

Introduction

Formation of the plant body is dependent upon the activity of self-organizing groups of cells called meristems located at the shoot and root apices. Shoot architecture results from activity of the shoot apical meristem (SAM), which initiates primordia repetitively from its flanks. Maintenance of the SAM is essential for development of the shoot during vegetative and flowering phases (Fletcher 2002).

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One of the major developmental phase changes in higher plants is the transition from vegetative to reproductive growth (Colasanti and Sundaresan 2000; Mouradov et al. 2002; Simpson and Dean 2002). The floral transition is controlled by environmental and/or intrinsic developmental cues that converge at the SAM. Floral induction leads to the restructuring of the vegetative meristem, which results in the initiation of new patterns of growth required for inflorescence development (Bernier 1988; Lyndon 1998). Morphological changes that transform the SAM from a vegetative to an inflorescence state are achieved by changes in the rate and patterning of cell division. This process is called floral evocation (Bernier 1988; Lyndon 1998).

In *Arabidopsis*, two paralogous *BEL1*-like homeobox (*BELL*) genes, *PENNYWISE* (*PNY*; also known as *BELLRINGER*, *REPLUMLESS*, VAAMANA) and *POUND-FOOLISH* (*PNF*) encode DNA-binding proteins that are essential for inflorescence development (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003; Bao et al. 2004; Bhatt et al. 2004; Smith et al. 2004). Based on genetic studies, *PNY* is involved in regulating early internode patterning events (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003; Bao et al. 2004; Bhatt et al. 2004). In addition, *PNY* is also necessary for replum development during fruit maturation (Roeder et al. 2003). Although inflorescence development is normal in *pnf* plants, *pny pnf* double mutants display a dramatic phenotype after floral induction in which flowers are not produced and internode development is severely impaired (Smith et al. 2004). Unlike flowering time mutants, which prolong the vegetative phase, *pny pnf* plants initiate axillary branches, produce cauline leaves and show a significant increase in the rate of organ initiation at the same time that wild-type plants flower. Floral integrator genes thought to function as inflorescence meristem identity genes are expressed in the apices of *pny pnf* double mutants after floral induction. Consistent with the non-flowering phenotype, expression of floral meristem identity genes are highly reduced in *pny pnf* plants. Both PNY and PNF are expressed in vegetative, inflorescence and floral meristems (Byrne et al. 2003; Smith and Hake 2003; Bao et al. 2004; Smith et al. 2004). Examination of wild-type and *pny pnf* SAMs before and after floral induction demonstrates that *PNY* and *PNF* regulators are necessary for completion of floral evocation (Smith et al. 2004). *PNY* and *PNF* regulators also function in the allocation process required for proper meristem maintenance. Therefore, *PNY* and *PNF* are required for floral evocation and necessary for establishing new patterns of growth essential for flowering and inflorescence development (Smith et al. 2004).

BELL1-like homeobox proteins interact with the knotted1-like homeobox (KNOX) proteins in a DNA-independent manner probably forming heterodimer units (Bellaoui et al. 2001; Muller et al. 2001; Smith et al. 2002; Byrne et al. 2003; Chen et al. 2003; Smith et al. 2003; Bhatt et al. 2004; Hackbusch et al. 2005). In monocots and dicots, KNOX–BELL interactions have been detected in yeast two-hybrid systems (Bellaoui et al. 2001; Muller et al. 2001; Smith et al. 2002; Byrne et al. 2003; Chen et al. 2003; Bhatt et al. 2004; Hackbusch et al. 2005), in vitro binding (Bellaoui et al. 2001; Muller et al. 2001; Smith et al. 2002; Smith and Hake 2003; Chen et al. 2003) and in living plants cells (Bhatt et al. 2004). The MEINOX domain in KNOX proteins associates with a conserved bipartite domain in the N-terminus of BELL proteins called the MEINOX interacting domain (MID), which is composed of the SKY and BELL regions. In addition, sequences outside of the conserved MEINOX and MID domians also function in stabilizing KNOX–BELL interactions (Bellaoui et al. 2001; Chen et al. 2003). Interestingly, biochemical studies suggest that a specific KNOX protein will have a greater affinity for certain BELL proteins than others, indicating that KNOX–BELL interactions are selective (Bellaoui et al. 2001; Smith et al. 2002). The combination of selective interactions between BELL and KNOX proteins and discrete expression patterns is likely to specify unique functions during plant development.

In higher plants, class I *KNOX* genes are encoded by small gene families essential for proper meristem function (Hake et al. 2004). In *Arabidopsis*, *SHOOTMERISTEMLESS* (*STM*) and *BREVIPEDICELLUS* (*BP*) are expressed in discrete regions of the SAM (Lincoln et al. 1994; Long et al. 1996). *STM* is an essential regulator of meristem maintenance (Clark et al. 1996; Endrizzi et al. 1996; Long et al. 1996), while *BP* function is critical for establishing internode-patterning events during inflorescence development (Douglas et al. 2002; Venglat et al. 2002). The *knotted1-like from ARABIDOPSIS THALIANA 2* and *6* (*KNAT2* and *6*) genes may encode functionally redundant KNOX proteins that regulate overlapping pathways involved in carpel and lateral root development (Pautot et al. 2001; Dean et al. 2004).

Double mutant analysis of KNOX and BELL mutants have shown that PNY and BP regulate early internode patterning events in the SAM during inflorescence development, while PNY and STM are essential for meristem maintenance (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003; Bao et al. 2004; Bhatt et al. 2004). *PNY* expression overlaps with that of *BP* and *STM* in the vegetative and inflorescence meristems, but only overlaps with STM in the floral meristem (Byrne et al. 2003; Smith and Hake 2003; Bao et al.

2004). Therefore, PNY is likely to form heterodimers with STM in the floral meristem. Thus, function of PNY is dependent upon where it is localized in the meristem and the identity of the interacting KNOX protein.

To gain more insight into the role of PNY and PNF during inflorescence development, we analyzed the phenotypes of double and triple mutants between *pny*, *pnf*, *stm* and *bp*. Genetic studies show that dosage levels for PNY, PNF and STM are critical for floral specification, internode patterning, and maintaining boundaries in the SAM during inflorescence development. PNF associates with STM and BP, indicating that biochemical properties of PNY and PNF are similar. Taken together, we have defined new functions for these KNOX–BELL heterodimers during inflorescence architecture.

Materials and methods

Identification of *pny-40126*, *pnf-33879* and *bp-9* NSgenotypes

To determine the genotype for *pnf-33879* and *pny-40126*, we used the LBa primer that was complementary to the left border of the T-DNA and primers downstream of the insertion site in the genomic sequence of *PNF* and *PNY*, respectively. The *bp-9* allele is due to an insertion of the *dSpm* transposon in the first intron of the *BP* genomic sequence (Mele et al. 2003). To determine the *bp-9* genotype we used primers to the 5′ end of the transposon, dSpm5, and the genomic sequence of *BP*, BP-52. Methods for genotype determination were previously described (Smith and Hake 2003). Sequences for these primers are displayed below.

LBa: TGGTTCACGTAGTGGGCCATCG
pnf-033879: CTTCGTCCATCTCTCATTTCGTG
pny-40126: TGGAATTGGAGACAAAATGTG TTA
dspm5: CGGGATCCGACACTCTTTAATTAA CTGACACTC
BP-52: AGGATTGTTGAGGATGTGAATGGG

Genetic analysis

To determine the role of *pny* and *stm-10* during inflorescence development in Columbia, pollen was obtained from *stm-10* flowers and crossed to *pny-41026* plants. The resulting F1 plants were self-pollinated and 318 F2 plants were analyzed. Approximately 1 out of 16 plants displayed a *pny stm-10* double mutant phenotype. However, only one out of 16 plants displayed a *pny* or *stm-10* phenotype and approximately 1 out of 8 plants displayed enhanced *pny* phenotype (*pny STM/stm-10*) or an enhanced *stm-10* phenotype (*stm-10 PNY/pny*). *pny STM/stm-10* and *stm-10 PNY/pny* plants were analyzed for the T-DNA insertion in *pny*. Seed collected for *pny STM/stm-10* were planted, and after flowering we found that these plants segregated for the following genotypes: ~25% *pny*, ~50% *pny STM/stm-10*, and ~25% *pny stm-10*.

To determine the dosage effects of *pnf*, *pny* and *stm-10* during inflorescence development, pollen was obtained from *stm-10 pnf-33879* double mutants and crossed to *pny-40126*. The resulting F1 plants were self-pollinated and 262 F2 plants were analyzed. Out of 262 F2 plants, we obtained 10 (~1/32) that appeared like *pny PNF/pnf* plants and 14 (~1/16) that showed a more severe phenotype. We confirmed by PCR that all 24 plants were homozygous for *pny* and heterozygous for *pnf*. To determine if the genotype of the 14 plants with a more severe phenotype were *pny PNF/pnf STM/stm-10*, the plants were self-pollinated and 180 seedlings were germinated. Analysis of the inflorescences in the 180 plants indicates that the severe *pny PNF/pnf* phenotype is *pny PNF/pnf STM/stm-10* genotype. In addition, we performed PCR on all *pny stm* plants and found that the *pnf-33879* allele does not enhance the shootless phenotype.

Yeast two-hybrid and reverse transcriptase RT-PCR

To determine the biochemical properties of PNF and PNY, we used the HybriZAP 2.1 two-hybrid system from Stratagene. To clone *PNY/PNF* and *STM/BP* into *pAD-GAL4-2.1* and *pBD-GAL4 Cam*, respectively, each of these genes were amplified using gene specific primers with Pfu polymerase (Stratagene). Each of the primers shown below was designed with restriction enzyme sites, *Eco*RI or *Sal*I, to clone these genes in-frame with the GAL4 binding domain (BD) and activation domain (AD), respectively. After cloning, all of the genes were sequenced to verify their integrity. The restriction site at the 5′ end of each primer is underlined below.

PNFBD-F: AGAGAGAATTCCTTGATTTG ATTGGTATCTTACAG
PNFBD-R: TGAGAGTCGACTCAACCCAC AAAGTCATGAAAC
PNYBD-F: AGAGAGAATTCATGGCTGAT GCATACGAGCCTTATC
PNYBD-R: GTAGTGTCGACTCAACCTAC AAAATCATGTAGAAAC
STMAD-F: CATAGAATTCATGGAGAGT GGTTCCAACAGCAC

STMAD-R: GTGTGTGTCGACTCAAAGCA TGGTGGAGGAGATG
BPAD-F: GAGAGAATTCATGGAAGAAT ACCAGCATGACAAC
BPAD-R: AGAGAGTCGACTTATGGACCG AGACGATAAGGTC

The plasmids *pGAL4-AD*, *pGAL4BD*, *pPNY-GAL4-BD*, *pPNF-GAL4-BD*, *pSTM-GAL4-AD* and *pBP-GAL4-AD* were transformed into *AH109 cells* (James et al. 1996) using the lithium acetate method (Gietz et al. 1991), then plated on the appropriate selection media.

The reverse transcriptase (RT-PCR) was performed using gene specific primers to PNY (AT5G02030-F and AT5G02030-R) and PNF (PNF-F and PNF-R). Primer sequences and the RT-PCR protocol were previously published by Smith et al. (2002) and Smith and Hake 2003. Microarray data displayed in this figure was derived from Schmid et al. 2005 using AtGenExpress at http://www.weigelworld.org/resources/microarray/AtGenExpress/.

Results

PNF interacts with STM and BP

The *PNY* and *PNF* encode paralogous DNA-binding proteins that display redundant functions essential for establishing floral specification and internode patterning events during inflorescence development (Fig. 1a) (Smith et al. 2004). In vitro binding and yeast two-hybrid studies demonstrated that PNY physically associates with STM and BP, while little or no interaction was detected with KNAT2 (Byrne et al. 2003; Smith and Hake 2003; Bhatt et al. 2004). To understand the biochemical function of PNF, we determined if this BELL homeodomain protein could also interact with STM and BP, using the yeast two-hybrid system. In this approach, we fused PNF in-frame to the DNA BD of GAL4. As a control, we also cloned *PNY* in-frame with *BD-GAL4*. STM and BP were fused in-frame with the GAL4 AD.

In this experiment, *pSTM-GAL4-AD* and *pBP-GAL4-AD* were transformed into *AH109* yeast cells containing *pPNY-GAL4-BD* or *pPNF-GAL4-BD*. Yeast two-hybrid results showed that *AH109* cells containing *pPNY-GAL4-BD* and *pSTM-GAL4-AD* or *pPNY-GAL4-BD* and *pBP-GAL4-AD* grew on His-/Ade-selection media, indicating PNY interacts with STM and BP (Fig. 1a). The interaction displayed by PNY-STM and PNY-BP is consistent with results obtained by yeast two-hybrid and in vitro binding studies (Byrne et al. 2003; Smith and Hake 2003; Bhatt et al. 2004). When *AH109* cells were transformed with *pPNF-GAL4-BD* and *pSTM-GAL4-AD* or *pPNF-GAL4-BD* and *pBP-GAL4-AD*, yeast cells grew on His-/Ade-selection media, indicating that PNF also associates with STM and BP (Fig. 1b). As a control, *pSTM-GAL4-AD* or *pBP-GAL4-AD* was transformed into *pGAL4-BD AH109* cells while *pPNY-GAL4-BD* or *pPNF-GAL4-BD* was transformed into *pGAL4-AD AH109* cells. In these control experiments, we did not detect interaction between STM/BP and the GAL4-BD or PNY/PNF and the GAL4-AD (Fig. 1a, b). In addition, pPNY-GAL4-BD or pPNF-GAL4-BD alone did not grow on His-/Ade-selection media (data not shown), demonstrating that these fusion proteins do not autoactivate transcription of the selectable marker genes used in this screen. Thus, these results indicate that the biochemical function of PNY and PNF with regard to binding to KNOX proteins is conserved.

Genetic studies showed that *pny* mutants display defects in internode patterning while *pnf* mutants have no effect on development (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003; Bhatt et al. 2004; Smith et al. 2004). To understand the phenotypic differences between *pny* and *pnf*, we examined the expression levels of *PNY* and *PNF* by RT-PCR from isolated wild-type inflorescence apices. These dissected apices contained inflorescence meristems and developing internodes and flowers. Using gene specific primers to *PNY* and *PNF*, approximately equal amounts of PCR products were amplified from vectors containing *PNY* and *PNF* cDNAs, respectively (Fig. 1c, lane 1). RT-PCR performed on RNA isolated from inflorescence apices showed that substantially higher levels of *PNY* were amplified compared to *PNF*. These studies indicate that *PNY* is expressed at higher levels than *PNF* in the inflorescence and floral meristems (Fig. 1c, lanes 2 and 3). In addition, we examined microarray expression data obtained from microdissected vegetative and inflorescence meristems (Schmid et al. 2005). The absolute expression values derived from these microarray studies showed that *PNY* was expressed seven–eightfold higher than *PNF* in the SAM during vegetative and reproductive development (Fig. 1d). Thus, the difference in expression levels of *PNY* and *PNF* correlates with the phenotypic disparity observed in *pny* and *pnf* mutants.

PNY and STM are required for shoot development

In this study, we analyzed the genetic interactions between STM, PNY, PNF and *BP* in the Columbia ecotype. The following single mutant lines were used: *stm-10* (a weak allele of *stm*, also called *bumbershoot1-3*,

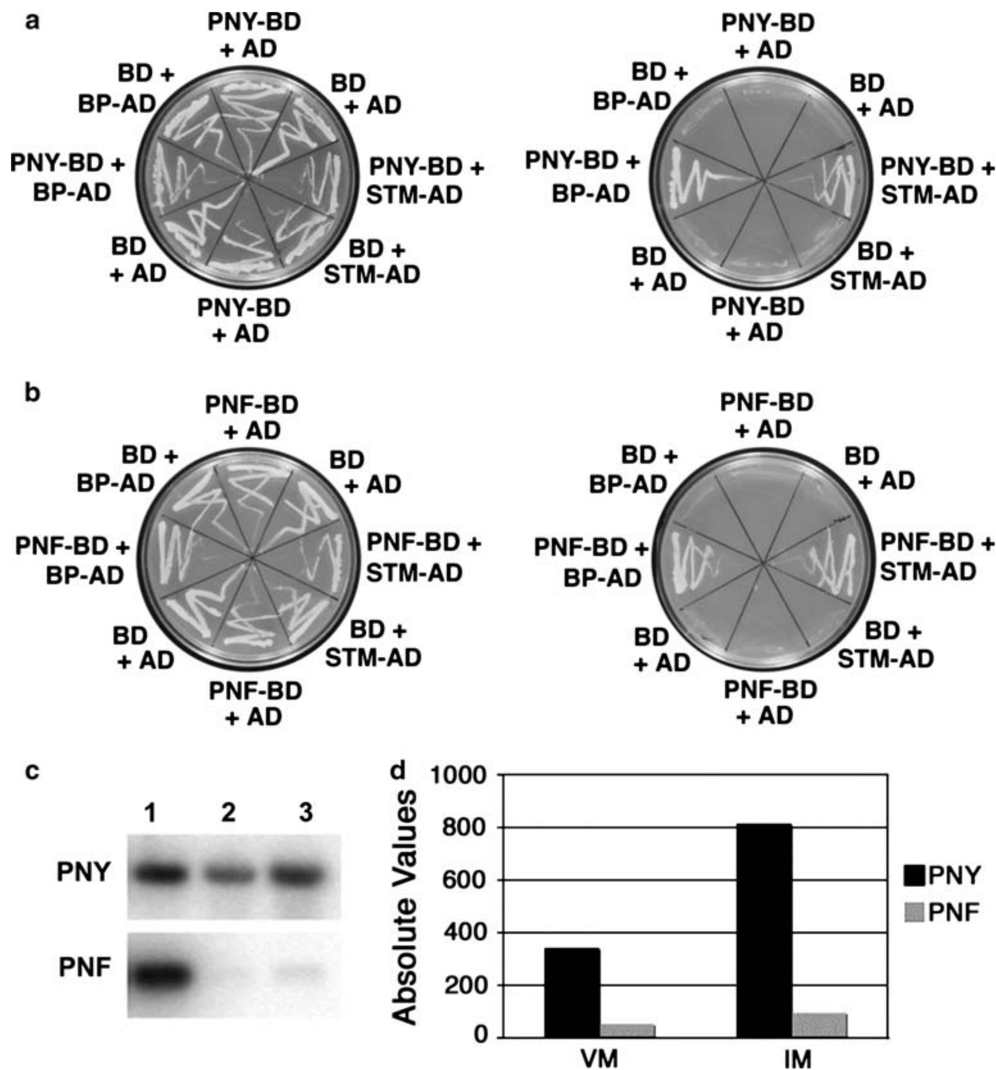

Fig. 1 POUND-FOOLISH (*PNF*) interacts with SHOOTMERISTEMLESS (*STM*) and BREVIPEDICELLUS (*BP*). **a, b** Yeast two-hybrid studies in which cells were streaked on Trp-/Leu-media that selects for the GAL4-binding domain (*BD*) and activation domain plasmids (*left plates*). To detect interaction between the KNOX/BELL proteins used in this study, cells were streaked on His-/Ade-selection media (*right plates*). **a** PENNYWISE (*PNY*) interacts with STM and BP. **b** POUND-FOOLISH (*PNF*) associates with STM and BP. **c** Reverse transcriptase (*RT*)-PCR-expression profiles of *PNY* and *PNF* in wild-type inflorescence apices. (*Lane 1*) *PNY* and *PNF* were amplified from plasmids containing the appropriate cDNA. After isolation of mRNA from isolated inflorescence apices, cDNAs were synthesized. PCR was performed with 2 µl (*Lane 2*) and 5 µl (*Lane 3*) of inflorescence cDNA's for 20 cycles. PCR products were separated by agarose electrophoresis and blotted to nylon and probed with the appropriate ^{32}P-labeled probe. **d** Microarray experiments were used to examine the expression levels of genes in the vegetative meristem (*VM*) and inflorescence meristem (*IM*) (Schmid et al. 2005). The graph in displayed the absolute expression values for *PNY* and *PNF* in the VM and IM. The expression profiling was quantile-normalized using gcRMA. The absolute expression values for *PNY* and *PNF* were linearized gcRMA values (Schmid et al. 2005)

obtained from the *Arabidopsis* Biological Resource Center), *pny-40126* (Roeder et al. 2003; Smith and Hake 2003), *pnf-33879* (Smith et al. 2004) and *bp-9* (Mele et al. 2003). *stm-10* has a stop codon at position at 906 (TGG to TGA), which truncates STM in the first helix of the homeodomain (data not shown). Note: the *stm-2* allele in Landsberg has the identical mutation as *stm-10*. Characterization of the *stm-10* inflorescences showed that *stm-10* and *stm-2* reproductive phenotypes were comparable (see supplementary Fig. 1).

Genetic studies have shown that null mutations in *PNY* enhance strong and weak alleles of *STM* in the Landsberg erecta background (Byrne et al. 2003; Bhatt et al. 2004). For example, double mutants of *pny* and the weak *stm-2* allele, terminate after production of the cotyledons (Byrne et al. 2003). To gain more insight into *PNY* and *STM* function, we analyzed *pny stm-10* double mutants. After germination, wild-type plants initiated rosette leaves in a spiral phyllotaxy on the flanks of the SAM (Fig. 2a). Mutations in *pny* displayed

a similar pattern of growth as wild-type plants (data not shown), similar to Fig. 2a. In *stm-10* plants, the SAM initiated leaves with an aberrant phyllotaxy (Fig. 2b). In addition, the SAM often terminated after the formation of a few leaves which lead to the outgrowth of secondary meristems in the axils of the rosette leaves (data not shown). In *pny stm-10* plants, shoot growth was terminated by the development of a single, trumpet shaped leaf, which initiated between the two cotyledons (Fig. 2c). During vegetative growth, wild-type leaves developed a distinct blade and petiole (Fig. 2f). After formation of the trumpet-shaped leaf, *pny stm-10* initiated leaf-like structures from the axils of the central leaf and cotyledons (Fig. 2d, e). Dissection of the leaf-like structures showed that one to two leaves initiated from the petioles of the "axillary" leaves (Fig. 2e, g, h). In addition, leaf flaps were often visible on the blades of these leaves (data not shown). *pny stm-10* plants never initiated an inflorescence and these plants eventually senescenced. Formation of the leaf-like structures in *pny stm* plants was likely due to the activity of BP, because *pny stm*-10 bp triple mutants initiated only two cotyledons and a trumpet shaped leaf (not shown), similar to Fig. 2c. Lastly, *pny stm-10 pnf* triple mutants were indistinguishable from *pny stm-10* double mutants, similar to Fig. 2d. Collectively, these genetic studies suggest that PNY function, but not PNF is necessary during vegetative development when STM activity is limiting.

Inflorescence development is sensitive to gene dosage levels of *PNY*, *STM* and *PNF*

We previously showed that loss of one copy of *PNF* enhances the defects of *pny* mutants, although *pnf* plants appear normal (Smith et al. 2004). The inflorescences of

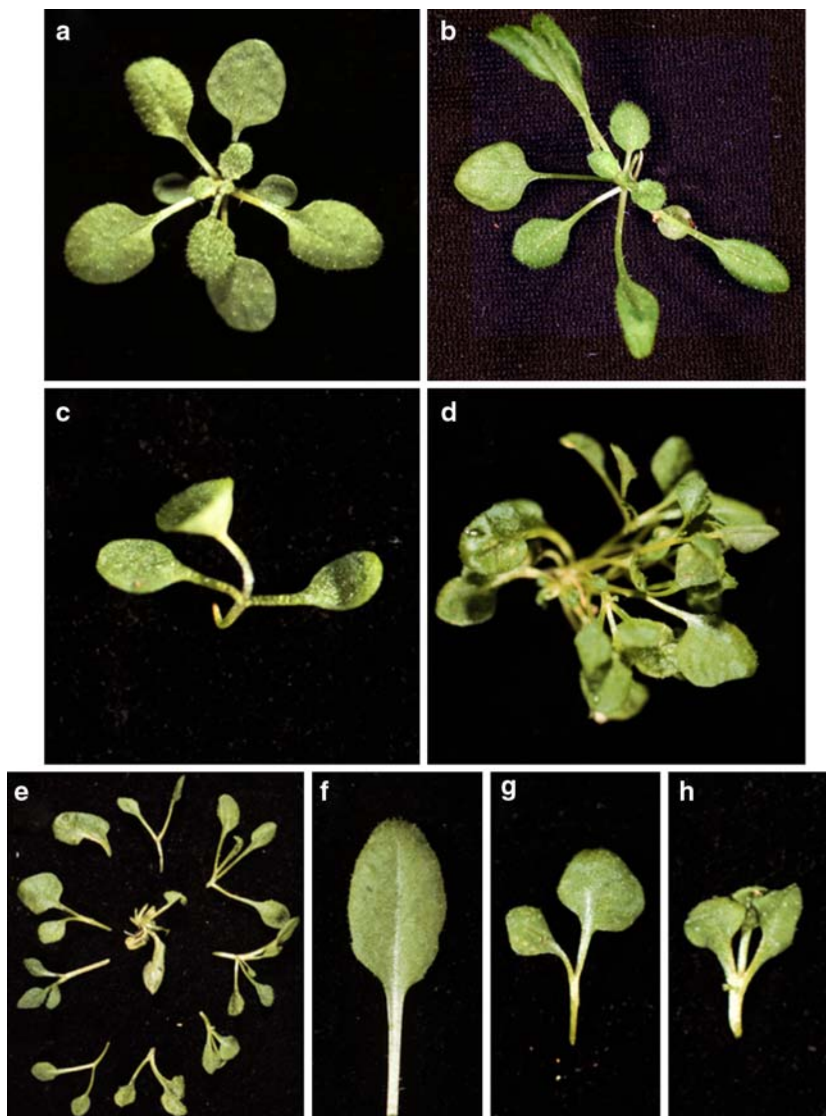

Fig. 2 Shoot development requires STM and PNY activity. Comparison of wild-type (**a**), *stm-10* (**b**) and *pny stm* plants (**c**) 14 days after germination. In *pny stm-10* plants, a trumpet shaped leaf develops between the two cotyledons. **d** *pny stm* plants initiate leaf-like structures after formation of a central tube shaped leaf. **e** Leaf-like structures dissected from *pny stm*. A wild-type rosette leaf (**f**) is compared to the leaf-like structures produced in *pny stm* plants (**g**, **h**)

pny PNF/pnf were shorter, produced fewer flowers, and displayed enhanced defects in internode development compared to *pny* plants (Fig. 3a and Supplementary Table 2a, b) (Smith et al. 2004). Here we examined the dosage effect of *STM* on the *pny* phenotype and vice versa. The inflorescences produced by *pny STM/stm-10* plants were slightly shorter and showed more severe defects in internode patterning than observed in *pny* shoots (Fig. 3a and Supplementary Table 2a, b). The inflorescences of *pny STM/stm-10* also produced slightly fewer flowers than *pny* plants (Supplementary Table 2a). In wild-type and *pny* inflorescences, pedicels develop as separate structures from the main stem (Fig. 3b). However, in *pny PNF/pnf* and *pny STM/stm-10* plants, the inflorescences contained one or two zones in which the stem was thickened due to the fusion of the pedicels to the main stem (Fig. 3c, d). Lastly, fusion of the pedicel with the main stem in both *pny PNF/pnf* and *pny STM/stm-10* was also accompanied with a curving or bending of the inflorescence (Fig. 3c, d).

Plants homozygous for *stm-10* and heterozygous for *pny* (*stm-10 PNY/pny*) were indistinguishable from *stm-10* plants during vegetative growth (data not shown). After the floral transition, *stm-10 PNY/pny* produced inflorescences that initiated cauline-like leaves and displayed severe defects in internode development (Fig. 3f, g). Compared to *stm-10* single mutants, the inflorescences of *stm-10 PNY/pny* were significantly shorter in stature and floral specification was highly reduced (Supplementary Table 1a, b). Many of the inflorescences of *stm-10 PNY/pny* plants terminated with a central flower-like structure (Fig. 3g), which lacked petals, stamens and carpels (data not shown). In addition, some of the *stm-10 PNY/pny* inflorescences terminated shoot growth without having produced a single flower (Supplementary Table 1a). Collectively, the inflorescence phenotypes displayed by *pny PNF/pnf*, *pny STM/stm-10* and *stm-10 PNY/pny* plants indicate that correct dosage levels of *PNY*, *PNF* and *STM* are essential for proper patterning events during inflorescence development.

To determine the role for *STM* and *PNF* during inflorescence development, *stm-10* was crossed to *pnf* and double mutants were analyzed. Unlike *pny*, *pnf* did not enhance the *stm-10* phenotype during either the vegetative or reproductive phases (data not shown). Results in Fig. 1c showed that *PNY* is expressed at higher levels than *PNF* in the inflorescence and floral meristems, indicating that the difference in expression levels may explain why in plants where STM activity is limiting, mutations in *PNY* have a more dramatic effect on SAM function than mutations in *PNF*.

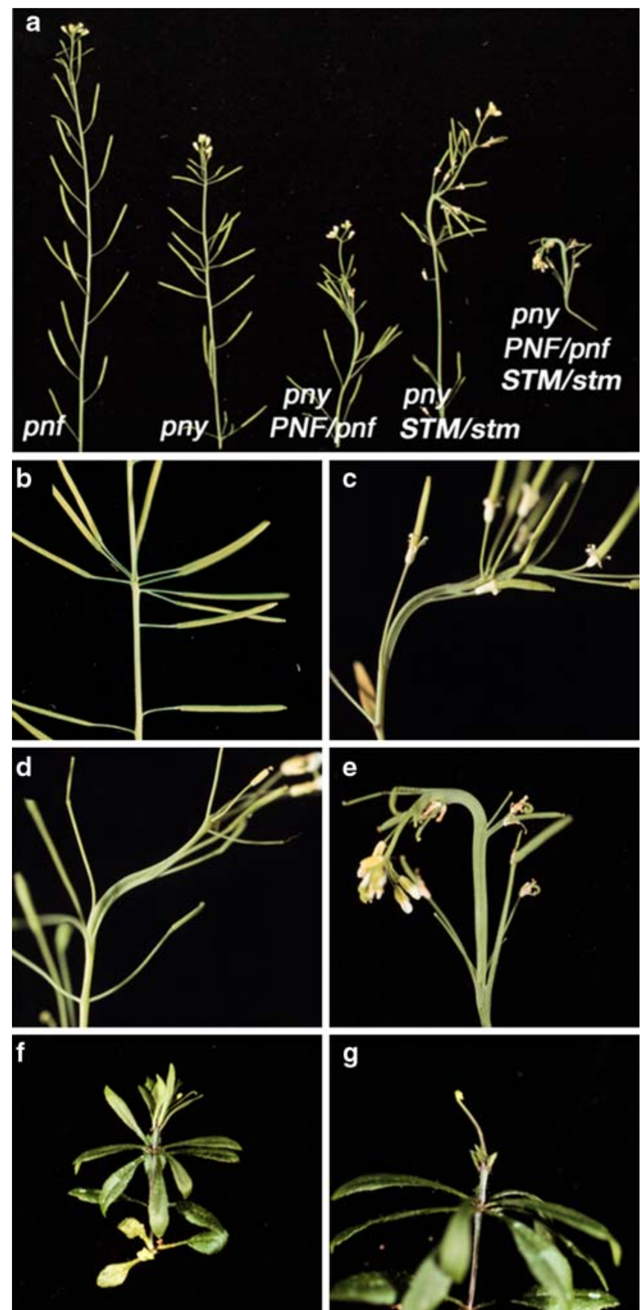

Fig. 3 Inflorescence development is dependent on dosage levels of *PNY*, *PNF* and *STM*. **a** Inflorescences of *pnf*, *pny*, *pny PNF/pnf*, *pny STM/stm-10*, and *pny PNF/pnf STM/stm-10* 18 days after floral induction. **b** Close up of *pny* stem. **c** Close up of *pny STM/stm-10* stem. **d** Close up of *pny PNF/pnf* stem. **e** Close up of *pny PNF/pnf STM/stm-10*. **f** Inflorescence of *stm-10 PNY/pny* plant. **g** Close up of *stm-10 PNY/pny* inflorescence

We also examined the dosage requirement for *STM* in *pny PNF/pnf* plants during inflorescence development. Plants homozygous for *pny* and heterozygous for *pnf* and *stm-10* (*pny PNF/pnf STM/stm-10*) were indistinguishable from wild-type plants, *pny PNF/pnf* and *pny STM/stm-10*, during vegetative development (data

not shown). After the floral transition, the inflorescences of *pny PNF/pnf STM/stm-10* were shorter, more curved and produced fewer floral primordia than *pny PNF/pnf* or *pny STM/stm-10* plants (Fig. 3a, e and Supplementary Table 2a). In *pny PNF/pnf* and *pny STM/stm-10* plants, the inflorescences contained discrete zones in which pedicels were fused to the main stem (Fig. 3c, d). However, in *pny PNF/pnf STM/stm-10* plants, the aberrant zones that contained pedicel fusion events encompassed most of the inflorescence (Fig. 3e). The fertility of *pny PNF/pnf STM/stm-10* plants was highly reduced since these plants produced fewer seed than *pny PNF/pnf* plants (data not shown). Thus, the dosage of both *PNF* and *STM* are independently and collectively important in a *pny* mutant background; reduction of either one compromises inflorescence development but reduction of both seriously limits inflorescence development.

A role for PNF in internode patterning

In *pny bp* plants, internode patterning is severely impaired and clusters of siliques are produced along the inflorescence (Fig. 4a, d and Supplementary Table 3b) (Byrne et al. 2003; Smith and Hake 2003; Bhatt et al. 2004). Inflorescences of *pny PNF/pnf* also display enhanced defects in internode patterning compared to *pny* single mutants, indicating that PNF is involved in internode development (Smith et al. 2004). However, after crossing *pnf* with *bp*, we found that *pnf bp* appeared identical to *bp* (data not shown). To determine whether the internodes produced in *pny bp* plants might be due to the activity of *PNF*, *pnf* was crossed to *pny bp* plants. Plants homozygous for *pny* and *bp* and heterozygous for *pnf* (*pny bp PNF/pnf*) produced miniature inflorescences (Fig. 4b, e), in which internode development was dramatically reduced compared to *pny bp* plants (Supplementary Table 3b). In fact, these inflorescences typically produced only 1–2 internodes that were greater than 1 mm (Supplementary Table 3b). The inflorescences of *pny bp PNF/pnf* initiated fewer flowers than *pny bp*, *pny* and *bp* inflorescences (Supplementary Table 3a). Lastly, a slight increase in axillary branch development was also observed in the *pny bp PNF/pnf* plants compared to *pny bp* double mutants (data not shown). The phenotypes produced in *pny PNF/pnf* and *pny bp PNF/pnf* indicate that PNF also regulates early internode patterning events in the inflorescence meristem.

To further examine the role of *PNY*, *PNF* and *BP* during inflorescence development, we identified *pny pnf bp* triple mutants from crosses between *pny bp* and *pnf*. The *pny pnf bp* triple mutant was indistinguishable from *pny pnf* double mutants (Fig. 4c). These results indicate that *PNY* and *PNF* may be epistatic to *BP* during inflorescence development or when internode development is severely impaired the effects of *bp* are not visibly apparent.

Discussion

The switch from vegetative to reproductive growth is a major phase change in higher plants (Colasanti and Sundaresan 2000; Mouradov et al. 2002; Simpson and Dean 2002). The floral transition transforms the fate of the SAM from vegetative to that of an inflorescence. In *Arabidopsis*, inflorescence growth is marked by

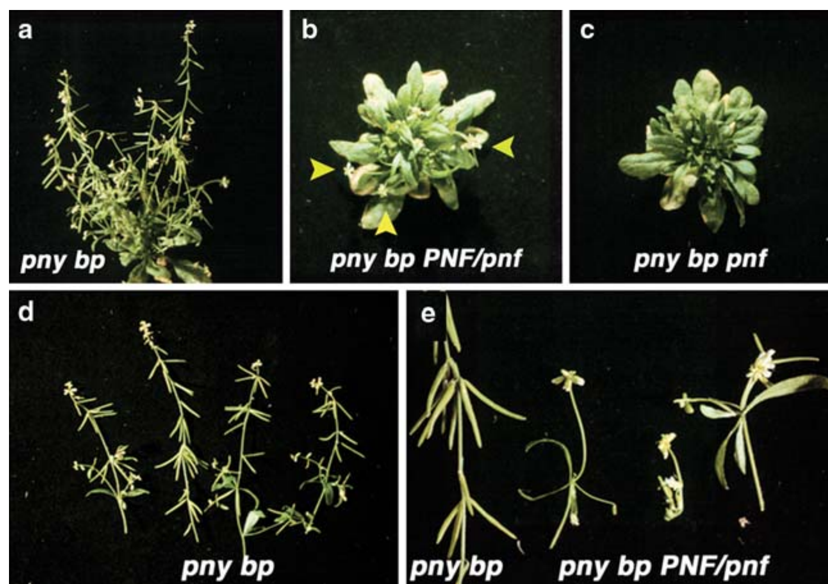

Fig. 4 POUND-FOOLISH regulates internode patterning. **a** *pny bp* plant. **b** *pny bp PNF/pnf* plants initiate short inflorescences and branches (*arrow-heads*). **c** *pny bp pnf* plant. **d** Inflorescence and axillary branches of *pny bp* plants. **e** Inflorescences of *pny bp PNF/pnf* (three inflorescences to the *right*) compared to inflorescence of *pny bp* (*left* inflorescence)

internode development, flower specification and axillary branch development (Vaughn 1955; Mikshe and Brown 1965; Hempel and Feldman 1994). Recently, we showed that homeobox regulators, PNY and PNF, are essential for establishing internode and floral patterning events during floral evocation (Smith et al. 2004). Previous studies indicate that cooperative interaction of PNY and BP mediates early internode patterning events during inflorescence development (Byrne et al. 2003; Smith and Hake 2003; Bhatt et al. 2004). Moreover, PNY also associates with STM indicating that this heterodimer is essential for SAM function (Byrne et al. 2003; Smith and Hake 2003; Bhatt et al. 2004). In this work, we assessed the genetic and biochemical requirements for PNY, PNF, STM and BP during inflorescence development. Our results indicate that inflorescence architecture is controlled by the regulatory activities of PNY-STM, PNF-STM, PNY-BP and PNF-BP heterodimers.

Molecular analysis of putatively redundant proteins

Alignment of the conserved *Arabidopsis* BELL sequences indicates that PNY and PNF are paralogous transcription factors that share 48% identity (Byrne et al. 2003; Roeder et al. 2003; Smith et al. 2004). Despite the differences in identity, we found that PNF also associates with BP and STM. In addition, *PNY* and *PNF* also display overlapping expression patterns in shoot and floral meristems (Byrne et al. 2003; Smith and Hake 2003; Bao et al. 2004; Smith et al. 2004). Taken together, these studies indicate that PNY and PNF regulate inflorescence architecture in the SAM by interacting with identical KNOX proteins. However, RT-PCR and microarray experiments demonstrate that *PNF* is expressed at substantially lower levels than *PNY*. This is supported by genetic studies, which demonstrate that *pny* plants display defects in internode patterning, while *pnf* plants are indistinguishable from wild-type plants (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003; Bhatt et al. 2004; Smith et al. 2004). The difference in expression levels between PNY and PNF may explain the phenotypic disparity between *pny* and *pnf* single mutants. Thus, shoot development is especially dependent upon PNY compared to PNF.

Our studies show that vegetative development is dependent upon PNY and STM. We also show that *bp* but not *pnf* enhances the *pny stm-10* phenotype, indicating that PNF is not required for early stages of vegetative development. Although *PNF* is expressed in the SAM during vegetative and reproductive growth (Smith et al. 2004), we failed to detect *PNF* mRNA in the embryonic SAM (H. M. S. Smith, unpublished data). Therefore, the expression levels of *PNF* in the embryonic SAM may not be sufficient to partially compensate for the loss of *PNY* in *pny stm-10* double mutants.

Dosage requirements

Previous studies have shown that *PNY* is necessary for SAM function in plants that are homozygous for weak alleles of *stm* in the Landsberg ecotype (Byrne et al. 2003; Bhatt et al. 2004). However, there was no report of dosage requirements from these studies. In addition, when strong or weak alleles of *stm* were combined with *pny* in the Landsberg ecotype, dosage requirements for these genes are not uncovered (H. M. S. Smith, unpublished data). In this report, we examined the dosage requirements of *PNY*, *PNF* and *STM* during vegetative and inflorescence development in the Columbia ecotype. In a *pny* background, defects in inflorescence architecture are enhanced in plants containing only one functional copy of either *PNF* or *STM*. Although the inflorescence phenotype is slightly more severe in *pny PNF/pnf* than in *pny STM/stm-10* plants, it is interesting to note that these genotypes produce comparable phenotypes. Compared to *pny* inflorescences, the shoots of *pny STM/stm-10* and *pny PNF/pnf* displayed zones of extremely shortened internodes and pedicels fused to the main stem. Dosage defects displayed by *STM* and *PNF* in *pny* inflorescences were further enhanced in *pny PNF/pnf STM/stm-10* plants. To understand these observations, we propose the following: (1) in *pny PNF/pnf* plants, PNY/PNF function is limiting, especially since *PNF* is expressed at significantly lower levels than *PNY*; (2) the dosage effect observed in *pny STM/stm-10* plants indicates that a reduction in STM levels limits its activity in the inflorescence meristem; (3) because STM has the potential to bind to multiple BELL partners (Hackbusch et al. 2005), the loss of a single copy of *STM* may create a condition in which BELL proteins compete for binding with this KNOX protein; and (4) the loss of *PNY* and the competition for binding to STM, combined with the low expression levels of *PNF* in *pny STM/stm-10* plants, indicates that the interaction between STM and PNF would occur less frequently. Therefore, *pny STM/stm-10* produces a similar phenotype as *pny PNF/pnf*. Moreover, we propose that the loss of one copy of *PNF* enhances the *pny STM/stm-10* phenotype because the association of PNF with STM would be even further reduced. Interestingly, dosage requirements are not observed in crosses between *pny* and *bp* (data not shown), indicating that the plant is not sensitive to doses of BP.

Our data shows that *pny* but not *pnf* enhances the *stm-10* and *bp* phenotypes. In the analysis of combining *pny* and *pnf* mutations, we found that *pnf PNY/pny* plants have no visible effect on vegetative or inflorescence development (H. M. S. Smith, unpublished data). In this study, we did not uncover any apparent phenotypes for *pnf stm-10 PNY/pny* or *pnf bp PNY/pny* (data not shown). Collectively, these results suggest that one copy of *PNY* is sufficient to mask the loss of *pnf* even when the levels of STM and BP are reduced.

Similarities between *stm-10* and *pny PNF/pnf* during inflorescence development

Characterization of *stm-10* inflorescences revealed significant similarities with *pny pnf* and *pny PNF/pnf* mutants. After the floral transition, the shoot meristem in both *stm-10* and *pny pnf* produces cauline leaves and displays defects in internode patterning. In addition, *stm-10 PNY/pny* plants terminate with a single flower like structure that lacked internal floral organs. The lack of normal flowers indicates that PNY, PNF and STM function in floral specification. Lastly, in both *pny PNF/pnf* and *stm-10* plants, inflorescence development terminates with groups of fused pedicels and flowers. The phenotypic similarities between *pny pnf*, *pny PNF/pnf* and *stm-10* plants show that these homeodomain proteins regulate overlapping functions during inflorescence development and suggests that these KNOX–BELL heterodimers regulate redundant pathways involved in floral specification and internode patterning.

Role in establishing boundaries

Boundaries play essential roles in compartmentalizing domains of gene expression during development in multi-cellular organisms (Irvine and Rauskolb 2001). In the SAM, a boundary surrounding the initiating primordia may function to prevent differentiation from consuming the meristem and terminating shoot growth. Therefore, maintenance of the boundary between the SAM and initiating primordia is an important aspect of meristem maintenance. *STM* has previously been shown to play a role in establishing the boundary between the cotyledons and the SAM (Barton and Poethig 1993; Long et al. 1996). During inflorescence development, we observed pedicels fused to the main stem in *pny PNF/pnf*, *pny STM/stm-10* and *pny STM/stm-10 PNF/pnf* mutants. These results indicate that PNY-STM and PNF-STM heterodimers are involved in maintaining the boundary between initiating floral primordia and the inflorescence meristem.

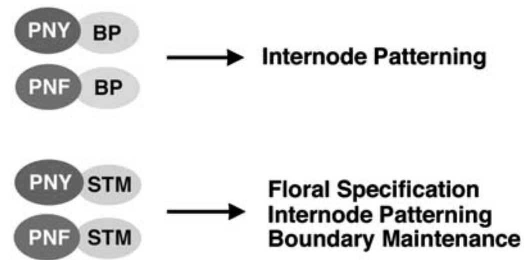

Fig. 5 Inflorescence development requires the regulatory functions of PNY-BP, PNF-BP, PNY-STM nd PNF-STM heterodimers

The fact that *pny PNF/pnf*, *stm* and *pny STM/stm-10 PNF/pnf* produce a similar terminal flower phenotype also suggests that PNY-STM and PNF-STM maintain this boundary to prevent movement of floral meristem identity into the inflorescence meristem.

In Fig. 5, we present a model for regulation of inflorescence architecture by KNOX–BELL interactions. We propose that inflorescence development requires the activities of PNY-BP, PNF-BP, PNY-STM and PNF-STM heterodimers. In our model, we propose that all four complexes regulate early internode patterning events in the inflorescence meristem. Whether or not the PNY/PNF-BP and PNY/PNF-STM heterodimers regulate identical or distinct sets of target genes essential for proper internode patterning events will be addressed in the future. We also propose that PNY-STM and PNF-STM regulate developmental programs required for floral specification and maintaining the boundary between initiating floral primordia and the inflorescence meristem. Thus, PNY-STM and PNF-STM heterodimers also regulate distinct developmental pathways that are not controlled by PNY-BP and PNF-BP heterodimers during inflorescence development.

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