Floral Induction in Arabidopsis by FLOWERING LOCUS T Requires Direct Repression of BLADE-ON-PETIOLE Genes by the Homeodomain Protein PENNYWISE

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Flowers form on the flanks of the shoot apical meristem (SAM) in response to environmental and endogenous cues. In Arabidopsis (Arabidopsis thaliana), the photoperiodic pathway acts through FLOWERING LOCUS T (FT) to promote floral induction in response to day length. A complex between FT and the basic leucine-zipper transcription factor FD is proposed to form in the SAM, leading to activation of APETALA1 and LEAFY and thereby promoting floral meristem identity. We identified mutations that suppress FT function and recovered a new allele of the homeodomain transcription factor PENNYWISE (PNY). Genetic and molecular analyses showed that ectopic expression of BLADE-ON-PETIOLE1 (BOP1) and BOP2, which encode transcriptional coactivators, in the SAM during vegetative development, confers the late flowering of pny mutants. In wild-type plants, BOP1 and BOP2 are expressed in lateral organs close to boundaries of the SAM, whereas in pny mutants, their expression occurs in the SAM. This ectopic expression lowers FD mRNA levels, reducing responsiveness to FT and impairing activation of APETALA1 and LEAFY. We show that PNY binds to the promoters of BOP1 and BOP2, repressing their transcription. These results demonstrate a direct role for PNY in defining the spatial expression patterns of boundary genes and the significance of this process for floral induction by FT.

Plants produce new organs from a population of pluripotent cells in meristems whose function is related to stem cells in animals. Meristems are located at different positions of the plant body and give rise to different organs. The shoot apical meristem (SAM) produces leaves and flowers at the tips of stems, whereas the axillary meristems give rise to lateral structures (Bowman and Eshed, 2000). By reprogramming these pluripotent cells at the meristems, plants can readily modify their development in response to changes in environmental conditions.

Flowers develop from floral meristems (FMs) that are formed on the flanks (floral primordium) of the SAM in response to environmental and endogenous cues (Pidkowich et al., 1999). Major environmental signals are the seasonal fluctuations in temperature and day length that are used by plants to anticipate optimal conditions for reproduction. Changes in temperature and day length are integrated into flowering-signaling networks by the thermosensory and vernalization/autonomous pathways and the photoperiodic pathway, respectively (Martínez-Zapater and Somerville, 1990; Lee and Amasino, 1995; Valverde et al., 2004; Andrés and Coupland, 2012). On the other hand, the plant hormone GA and the age of the plant constitute the internal signals affecting flowering in many plant species (Wilson et al., 1992; Fowler et al., 1999; Yu et al., 2012). FLOWERING LOCUS T (FT) is a key component of the photoperiodic pathway. FT encodes a small globular protein that shares high homology with mammalian phosphatidylethanolamine-binding proteins/Raf-1 kinase inhibitory protein (Kardailsky et al., 1999; Kobayashi et al., 1999; Nakamura
et al., 2014; Romera-Branchat et al., 2014). In Arabidopsis (Arabidopsis thaliana), FT is induced by long days (LDs) and has been placed at the core of the photoperiodic pathway, downstream of the GIGANTEA and CONSTANS genes (Suárez-López et al., 2001; Valverde et al., 2004; Yoo et al., 2005). FT mRNA is expressed specifically in the companion cells of the phloem, and its protein moves systemically to the shoot apex through the phloem sieve elements (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007). According to recent studies in Cucurbita moschata and Arabidopsis, FT protein is unloaded into the surrounding shoot meristem tissue from the terminal phloem (Yoo et al., 2013). Once FT is unloaded into the shoot meristem, it is thought to physically interact with two basic leucine-zipper (bZIP) transcription factors called FD and FD PARALOG (FDP), which are expressed in this tissue (Abe et al., 2005; Wigge et al., 2005). However, recent work in rice (Oryza sativa) suggested that this interaction is not direct and is mediated by 14-3-3 proteins (Taoka et al., 2011). Consistent with this model, the loss of function of FD and FDP strongly suppresses the early flowering of transgenic plants overexpressing FT (Abe et al., 2005; Wigge et al., 2005; Jaeger et al., 2013). In Arabidopsis, the FT-FD complex is believed to induce the transcription of genes encoding several floral-promoting proteins, such as the MADS-box transcription factors SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) and FRUITFULL (FUL), which accelerate flowering, as well as APETALA1 (AP1), also a MADS-box transcription factor, and LEAFY (LFY), which promote FM identity (Schmid et al., 2003; Teper-Bammolker and Samach, 2005; Wigge et al., 2005; Corbesier et al., 2007). Indeed, the FT-FD complex directly binds to the promoter of AP1, whose expression at the floral primordia is associated with FM formation (Wigge et al., 2005). Therefore the FT-FD complex is predicted to be active in the incipient floral primordia to induce the expression of AP1 and promote flowering. In addition to these genes, the FT-FD complex also promotes the transcription of the family genes encoding the SQUAMOSA BINDING PROTEIN LIKE (SPL) transcription factors. Recent studies using chromatin immunoprecipitation (ChIP) assays showed that SPL3, SPL4, and SPL5 loci are bound by FD, which transcriptionally regulates these genes (Jung et al., 2012). In turn, SPL proteins control the expression of FUL, LFY, and AP1 genes by directly binding to their promoters (Wang et al., 2009; Yamaguchi et al., 2009). These data reflect the high degree of complexity implicit to the genetic networks controlling floral induction in the SAM.

The three-amino acid-loop-extension (TALE) homeodomain superclass comprises transcription factors involved in the SAM function. The BEL1-like homeodomain (BEL) and the KNOTTED-like homeodomain (KNOX) are TALE proteins that share similar structure and function (Hamant and Pautot, 2010; Hay and Tsiantis, 2010; Arnaud and Pautot, 2014). Members of the two families can form heterodimers to regulate various developmental processes. The BEL family comprises 13 members (Smith et al., 2004). PENNYWISE (PNY), also known as BELLRINGER, REPLUMLESS (RPL), VAAMANA, or LARSON, encodes a BEL protein that plays roles in organ patterning by affecting internode length, phyllotaxis, and fruit replum development (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003). In fruits, PNY is required for the replum formation, where it acts as a transcriptional repressor of SHATTERPROOF (SHP) MADS-box genes. The repressive activity of PNY restricts SHP gene expression to the valve margin domain. In the absence of a functional PNY (in rpl mutants), SHP genes are ectopically expressed, and the replum cells take on valve margin fates (Roeder et al., 2003). PNY mutants also display dramatic defects in inflorescence development (Smith and Hake, 2003; Bao et al., 2004). Interestingly, these defects are corrected by the lack of KNOTTED-like from Arabidopsis thaliana (KNAT6) and KNAT2, two related KNOX genes. In the pny mutant, KNAT2 and KNAT6 expression domains are enlarged, indicating that PNY regulates inflorescence development at least partially by limiting their spatial pattern of expression (Ragni et al., 2008). These data suggest that a major molecular function of PNY is to maintain the spatial expression of organ patterning genes restricted to specific domains.

Recent studies elucidated that PNY is also involved in the acquisition of competence to respond to floral inductive signals. In these studies, it was shown that simultaneous loss of function of PNY and its paralog POUND-FOOLISH (PNF) completely blocks the floral transition. Indeed, the double mutant pny pnf is not able to undergo the floral transition, even under LD conditions (Smith et al., 2004). Moreover, overexpression of FT from the constitutive Cauliflower mosaic virus 35S promoter barely activates flowering of the pny pnf double mutant (Kanrar et al., 2008). At the molecular level, the concurrent loss of function of PNY and PNF affects the ability of FT to activate the transcription of AP1, LFY, and probably SPLs (Lal et al., 2011). Therefore, PNY has somehow been integrated into the FT-signaling pathway. However, the genetic and molecular mechanism underlying the effect on PNY on the FT flowering pathway remains unknown.

Here, we show that PNY operates in the FT-signaling pathway by restricting the spatial pattern of expression of BLADE-ON-PETIOLE1 (BOP1) and BOP2 genes in the SAM. PNY directly binds to BOP1 and BOP2, which encode two BTB (for Bro ad-complex, Tramtrack, Bric-à-brac)-ankyrin transcriptional coactivators, which function at lateral organ boundaries in the determination of leaf, flower, inflorescence, and root nodule architecture (Ha et al., 2004, 2007; Hepworth et al., 2005; Norberg et al., 2005; Karim et al., 2009; Xu et al., 2010; Couzigou et al., 2012; Khan et al., 2012b, 2014). We found that BOP1 and BOP2 are also involved in flowering-time regulation by repressing the expression of FD in the shoot meristem. These data indicate that PNY has an unexpected function during plant development in regulating the pattern of expression of
flowing-time genes in the shoot meristem by repressing BOP1/2 gene transcription.

RESULTS

A Sensitized Forward Genetic Screen followed by Fast Isogenic Mapping Identifies PNY as a Regulator of the FT-Signaling Pathway

A sensitized genetic screen was designed to identify genes affecting the ability of FT to activate flowering. In the double mutant ft-10 tsf-1, which carries null mutations in FT and its closest homolog TWIN SISTER OF FLOWERING LOCUS T (TSF), the floral promotion activity of FT is abolished, causing late flowering and insensitivity to photoperiod (Yamaguchi et al., 2005; Jang et al., 2009). FT function in ft-10 tsf-1 can be restored using the transgene pGAS1::FT (GALACTOL SYNTHASE1 [GAS1] promoter fused to FT), which is active only in phloem companion cells of the minor veins (Jang et al., 2009). Thus, pGAS1::FT ft-10 tsf-1 plants show early flowering compared with ft-10 tsf-1 double mutants under LDs and short days (SDs). These plants were used to screen for mutations that suppress early flowering in pGAS::FT ft-10 tsf-1 and self-fertilizing the resulting F1 plants. A total of 566 BC1F2 plants were grown, and 174 of them exhibited the late-flowering phenotype of lgf58 (an approximate ratio of 3:1), suggesting that a single recessive mutation was responsible for the effect. To construct the pool, an individual leaf from each of the 174 plants showing the mutant phenotype was collected. Genomic DNA extracted from the pooled material and the progenitor pGAS::FT ft-10 tsf-1 were sequenced using Illumina technology. By applying SHOREmap (Schneeberger et al., 2009b; Sun and Schneeberger, 2015), candidate loci, at which mutant alleles were strongly overrepresented in the pool, were identified on the top arm of chromosome 5 (Fig. 1; Supplemental Fig. S2; for more details, see also “Materials and Methods”). Three loci were selected as high-confidence candidates because they carried a nonsynonymous mutation and showed an allele frequency equal to 1.0 and a quality score equal to 40 (Supplemental Table S1; “Materials and Methods”). Among the highest probability candidates,
the gene AT5G02030 contained a mutation in the second exon predicted to produce a premature stop codon (Fig. 1; Supplemental Table S1). AT5G02030 encodes the BELL protein PNY. Mutations in PNY cause defects in plant architecture, abnormalities in the fruit replum, and late flowering (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bao et al., 2004; Smith et al., 2004). Double mutants containing mutations in PNY and its paralog PNF fail to undergo the transition from vegetative to reproductive phase, even in the presence of high levels of FT mRNA expressed from the 35S promoter (Kanrar et al., 2008). Similar phenotypes were also observed in lgf58 (Fig. 1; Supplemental Fig. S1) and in Col-0 plants carrying the newly isolated mutant allele of PNY (hereafter called pny-58) segregated away from pGAS::FT ft-10 tsf-1 (Supplemental Fig. S3). Previously, pny pnf double mutants were proposed to be blocked in the floral transition due to impairment of the ability of FT to activate the transcription of downstream target genes, such as AP1. In agreement with this idea, the mRNA levels of AP1, LFY, and SPL4, which are transcriptionally activated downstream of FT, were dramatically reduced in the lgf58 mutant compared with pGAS::FT ft-10 tsf-1. However, as also described earlier for pny pnf double mutants, SOC1 mRNA expression was unaltered in lgf58 (Supplemental Fig. S1; Kanrar et al., 2008). Col-0 plants carrying the pny-58 mutant allele grown under LD also showed late flowering compared with wild-type plants and were more extreme than the previously reported mutant allele pny-40126 (Smith and Hake, 2003; Ragni et al., 2008; Supplemental Fig. S3). This delay in flowering was corrected by introducing a transgenic copy of the wild-type genomic PNY locus into the mutant plants (pPNY:: Venus:PNY pny-58), confirming that the mutation pny-58 was responsible for the late-flowering phenotype (Supplemental Fig. S4). Moreover, the early flowering of pGAS::FT ft-10 tsf-1 was also reduced by combining it with the mutant allele pny-40126, but the effect on flowering was less severe than for lgf58 (Supplemental Fig. S4). These results demonstrate that the sensitized suppressor screen identified a novel allele of PNY that causes a stronger delay in flowering than those previously described, and which strongly reduces the ability of FT to activate flowering.

Regulation of PNY during Photoperiodic Induction of Flowering

Loss of PNY function reduced the capacity of FT to activate flowering in response to inductive LD photoperiods or in transgenic pGAS::FT plants expressing higher levels of FT mRNA. PNY mRNA is expressed in the SAM (Smith and Hake, 2003), but whether it is regulated in response to changes in day length is unknown. PNY mRNA distribution changes was analyzed by in situ hybridization in different environments (Fig. 2). Under SD, PNY mRNA was expressed in the central zone of the SAM and excluded from the leaf boundaries (Fig. 2). After transferring plants to LD, PNY mRNA was detected more broadly, but was still not detected in leaf boundaries, nor in floral primordia. The pattern of PNY protein expression was also tested by constructing pPNY::Venus:PNY pny-40126 plants (Fig. 2; Supplemental Fig. S5). The transgene complemented the defects of the pny-4026 mutant. By confocal microscopy, Venus:PNY was detected in the same domains shown by in situ hybridization to express PNY mRNA in the shoot meristem (Fig. 2). Similarly, its pattern of expression broadened during photoperiodic induction of flowering, and was excluded from leaf boundaries and floral primordia (Fig. 2). Therefore, expression of PNY mRNA and its translated product is increased by photoperiod in specific domains of the apex.

SOC1 is an important mediator of the FT-signaling pathway, and its mRNA expression pattern in the SAM
Suppression of FT Function by pny Is Caused by Ectopic Expression of BOP Genes

Mutations in PNY and other TALE transcription factors, such as BREVIPEDICELLUS (BP), impair Arabidopsis architecture, particularly shortening internodes and altering silique position and orientation (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003). These defects in plant architecture in pny mutants are associated with broader expression of BOP genes in the inflorescence stem and pedicels and are suppressed in pny bop1 bop2 triple mutants (Khan et al., 2012b). The role of BOP genes in the late flowering of pny mutants has not been examined, so we tested whether this aspect of the pny-58 phenotype was due to an increase of BOP gene expression in the SAM prior to floral induction. In 7-d-old plants grown under LDs, BOP1 and BOP2 mRNA levels were higher in pny-58 mutants compared with the wild type, as tested by RT-qPCR (Fig. 3). Similarly, these genes were more highly expressed in lgf58 plants than in pGAS1::FT ft-10 tsf-1 (Fig. 3). In situ hybridization experiments detected broader expression of BOP2 in the SAM of vegetative 7-d-old pny-58 plants than in Col-0 (Fig. 3; Supplemental Fig. S7) and in 10-d-old plants that were undergoing the floral transition (Supplemental Fig. S7). To test whether this increase in BOP1/2 expression during vegetative development contributed to the late flowering of pny-58 mutants, the pny-58 bop1-3 bop2-1 triple mutant was constructed. These plants flowered much earlier than pny-58 at a similar time to bop1-3 bop2-1, which were slightly earlier flowering than Col-0 (Supplemental Fig. S7). Therefore, BOP1 and BOP2 are required for the late flowering of pny-58 mutants. Similarly, introduction of bop1-3 bop2-1 into the pGAS1::FT pny-58 (lgf58) line restored flowering to a similar time to pGAS1::FT ft-10 tsf-1 (Fig. 3; Supplemental Fig. S7), but the pGAS1::FT bop1-3 bop2-1 pny-58 line (lgf58 bop1 bop2) produced a higher number of cauline leaves than pGAS1::FT ft-10 tsf-1, causing a slightly increased number of total leaves. This increased number of cauline leaves could be caused by the down-regulation of LFY, because lfy mutants produce more cauline leaves (Weigel et al., 1992), and BOP1/2 promote LFY expression in the meristem (Karim et al., 2009). Thus, the levels of LFY mRNA in bop1 bop2 at different developmental stages was quantified by RT-qPCR. Compared with the wild type, the expression of LFY in the bop1 bop2 double

Figure 3. BOP1/2 genes are important for flowering regulation mediated by PNY. Expression of BOP1 (A) and BOP2 (B) of shoot apices dissected from plants grown under LDs for 7 d. Letters shared in common between the genotypes indicate no significant difference (Student’s t test, P < 0.05). C, In situ hybridizations with BOP2 probe in shoot meristems of Col-0 (i) and pny-58 (ii). Plants were grown for 7 LDs and stayed at the vegetative stage. Expression of BOP2 in Col-0 plants was observed at the boundaries between leaves and the SAM (black arrow). Scale bars = 50 μm. D, Flowering time of the BOP1/2 and PNY mutant combinations grown under LDs. CL, Cauline leaves; RL, rosette leaves. Error bars in A, B, and D indicate SD.
mutant was slightly increased during vegetative development (7–10 LDs) but reduced at the reproductive stage (17 LDs, Fig. 6). Taken together these data support the hypothesis that late flowering of pny-58 mutants is due to higher and ectopic expression of BOP1/2 in the vegetative apex. Since mutations in PNY suppress the capacity of FT to induce flowering, BOP1/2 up-regulation should also delay the floral transition promoted by this protein. To explore this further, a dominant activation tagging allele of BOP1 (bop1-6D) was used (Norberg et al., 2005). The bop1-6D mutants flowered later than Col-0 (Fig. 4). To test whether this occurs through the FT floral-promoting pathway, the double transgenic pGAS1::FT bop1-6D was constructed. Flowering time experiments demonstrated that bop1-6D delayed flowering of pGAS1::FT (Fig. 4). This result supports the idea that ectopic BOP expression impairs activity of the FT-signaling pathway. Indeed, the expression of LFY and AP1, which is activated in the SAM downstream of FT (Schmid et al., 2003; Moon et al., 2005), was dramatically reduced by overexpression of BOP1 (Fig. 4).

These data together indicate that the repression of BOP genes in the shoot meristem by PNY is required for FT to efficiently promote the floral transition.

PNY Represses BOP1 and BOP2 Transcription by Directly Binding to Their Promoters

The increase of BOP1 and BOP2 expression in pny-58 mutants suggested that PNY might directly bind to the promoters of these genes. This possibility is further supported by proteomics analysis, which detected PNY and AP1 in the same transcriptional complex (Smaczniak et al., 2012), and by ChIP sequencing, which identified BOP1 and BOP2 as putative direct targets of AP1 (Kaufmann et al., 2010). Whether PNY binds to the same BOP1/2 promoter regions as AP1 was therefore tested. ChIP-qPCR was performed on chromatin extracted from inflorescences of pNY::Venus:PNY and pny-40126 (Student’s t test; **, P = 0.0001; *, P = 0.02). F, Localization of the primers used for the ChIP-qPCR experiment on the BOP1 (top scheme) and BOP2 (bottom scheme) loci. *, AP1 binding genomic regions according to Kaufmann et al. (2010). Scale bars = 0.5 kb. Error bars in A to E indicate sd.

Figure 4. BOP1/2 genes interfere with FT function and are directly bound by PNY. A, Flowering time of plants misexpressing BOP genes in the presence of high levels of FT. Letters shared in common between the genotypes indicate no significant difference (Student’s t test, P < 0.05). B, Expression of LFY of dissected shoot apices of plants grown for 7 LDs. Asterisks indicate statistical differences between Col-0 and other genotypes (Student’s t test; **, P = 0.0004; *, P = 0.006). C, Expression of AP1 of dissected shoot apices of plants grown for 7 LDs. Asterisks indicate statistical differences between Col-0 and other genotypes (Student’s t test; ***, P = 0.001; **, P = 0.006; *, P = 0.03). ChIP-qPCR of PNY on the promoters of BOP1 (D) and BOP2 (E). x axis indicates the primers used for its qPCR. Asterisks indicate statistical differences between pNY::Venus:PNY and pny-40126 (Student’s t test; **, P = 0.0001; *, P = 0.02). F, Localization of the primers used for the ChIP-qPCR experiment on the BOP1 (top scheme) and BOP2 (bottom scheme) loci. *, AP1 binding genomic regions according to Kaufmann et al. (2010). Scale bars = 0.5 kb. Error bars in A to E indicate sd.
(TOE1) and LFY promoters, which were shown to be directly bound by the AP1/PNY complex (Smaczniak et al., 2012), were used as positive controls. As expected, Venus:PNY bound to the TOE1 and LFY loci within the same region that was reported for AP1 (Supplemental Fig. S7; Kaufmann et al., 2010). The binding of Venus:PNY to BOP1 and BOP2 was then tested. Venus:PNY bound to the BOP1 promoter at two different positions (P3 and P4). One of these positions (P3) was the same as that reported for AP1 (Fig. 4; Kaufmann et al., 2010). An enrichment of chromatin immunoprecipitated by Venus:PNY was also detected within the BOP2 promoter. In this case, the enrichment was found in a region located around 1 kb upstream of the one predicted for AP1 (Fig. 4). Notably, some potential PNY binding sites within these genomic regions were identified. For example, the P3 genomic region contains a core motif (ATGGAT) reported as a binding site for BEL1-LIKE HOMEODOMAIN (BLH1; Staneloni et al., 2009). Within the region P7, the two motifs AAATTACCA and AATTATCCT, which are similar to those previously identified as binding sites of BELLRINGER (BLR) in the AGAMOUS intronic region (AAATTAAT, AATTATGC, and ACTAATTT; Bao et al., 2004; Smaczniak et al., 2012), were also found. However, only shorter versions of these motifs (AATTAT, AATTI, and AAATT) were identified within the P4 genomic region.

Collectively, these data indicate that PNY directly binds and represses the expression of BOP1/2.

**Loss of PNY Function and BOP Overexpression Reduce the mRNA of FD, a Component of the FT-Signaling Pathway, in the Shoot Meristem**

PNY loss of function caused a strong reduction in the expression of several genes, such as SPLs, LFY, and AP1, that are expressed at the apex during flowering downstream of FT (Supplemental Fig. S1). These results were in agreement with previous reports on pny pnf double mutants (Smith et al., 2004; Kanrar et al., 2008). However, the molecular mechanisms that cause the reduction of expression of these genes and a delay in flowering in pny mutants are not clear. FD directly interacts with FT (Abe et al., 2005; Moon et al., 2005), and mutations in FD and PNY were found to delay flowering of pGAS::FT transgenic plants to a similar extent (Supplemental Fig. S8), suggesting that they might influence the FT-signaling pathway at common positions. Furthermore, analysis of the mRNAs of SPLs, LFY, and AP1 by RT-qPCR showed that the fl-3 mutation suppressed the expression of these genes in a pGAS1::FT background to a similar extent as pny-58 (Supplemental Fig. S8). These observations suggested that mutations in PNY might affect the FT-signaling pathway by reducing FD expression to impair photoperiodic floral induction by FT. To test this possibility, the expression of FD mRNA in pny mutants was analyzed. FD mRNA level was tested by RT-qPCR and was reduced in shoot apices of pny-58 and pny-40126 mutants compared with Col-0 wild-type plants (Fig. 5). Because PNY regulates flowering through the transcriptional repression of BOP genes, the effect of bop mutations on FD mRNA level was tested. FD mRNA was slightly higher in bop1-3 bop2-1 double mutants compared with Col-0 (Fig. 5). Moreover, in situ hybridization experiments showed that the FD mRNA was reduced in the meristem-leaf boundaries of the pny-58 (Fig. 5). That this was due to ectopic expression of BOP genes was supported by the dramatic reduction in FD expression observed in meristems of bop1-6D (Fig. 5). By contrast, FD mRNA appeared slightly increased in the SAM of bop1-3 bop2-1 double mutants (Fig. 5). These results suggest that PNY controls flowering at least partially through repression of BOP gene expression to allow FD mRNA to increase in the meristem.

Taken together, these data indicate that the activity of the FT pathway during photoperiodic induction of flowering requires repression of BOP genes by PNY.

**DISCUSSION**

We performed a sensitized mutant screen to identify genes required for FT signaling during photoperiodic
causes abnormalities such as short internodes and reduced apical dominance (Norberg et al., 2005; Ha et al., 2007; Khan et al., 2012a, 2012b). Consistent with BOP repression being a fundamental function of PNY, we found that PNY binds directly to the promoters of both BOP genes. In the meristems of pny mutants or bop1-6d plants carrying a gain-of-function allele of BOP1, FD mRNA was strongly reduced, and this likely contributed to the reduced responsiveness to FT. We propose that restriction of BOP expression to the proximal regions of lateral organs, particularly its exclusion from the shoot meristem by PNY, is required for wild-type levels of FD expression and thus efficient floral induction in response to FT (Fig. 6).

**Significance of Defining Organ Boundaries for FT Signaling and FD Expression**

In plant meristems, lateral organ boundaries separate the meristematic zone containing undifferentiated cells from the lateral organs containing differentiated cells (Rast and Simon, 2008; Khan et al., 2014). The BOP1 and BOP2 genes are expressed at the base of lateral organs, adjacent to the boundary (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). They help define the boundary region by repressing homeobox genes, which maintain the meristematic region, by directly activating ASYMMETRIC LEAVES2 and other genes that specify the boundary (Ha et al., 2007; Jun et al., 2010). We observed that, in pny mutants, the BOP genes are ectopically expressed in the vegetative meristem, as was previously described for older inflorescence meristems (Norberg et al., 2005; Ha et al., 2007; Khan et al., 2012a, 2012b). Thus, in wild-type plants during vegetative development, PNY contributes to positioning of the boundary between the vegetative meristem and leaves by repressing BOP gene expression in the meristem. Indeed, we observed that, in the absence of functional PNY (pny-58), BOP2 expression becomes broader in the meristem. Similarly, Khan et al. (2015) found that the BOP1 spatial pattern of expression was enlarged in the inflorescence meristem of pny pnf. This restriction of BOP expression and proper localization of the boundary is required for correct timing of the floral transition, because pny mutants are late flowering, and this is suppressed in the pny bop1 bop2 triple mutant (Fig. 3; Supplemental Fig. S7). The sensitized screen used to identify the pny-58 mutation illustrated the importance of PNY and the BOP genes downstream of FT in the photoperiodic flowering pathway, as previously shown by the capacity of pny pnf double mutants to suppress the early-flowering phenotype caused by 35S::FT (Kanrar et al., 2008).

At the meristem, FT is proposed to activate downstream genes by directly interacting with the bZIP FD transcription factor (Abe et al., 2005; Wigge et al., 2005). This relationship between FT-related proteins and FD is highly conserved in higher plants, having also been observed in rice and tomato (Solanum lycopersicum; Penuel et al., 2001; Taoka et al., 2011). We found that the
spatial pattern of expression of FD in the SAM is regulated by the PNY, BOP1, and BOP2 genes. Ectopic BOP gene expression in the pny mutant or the gain-of-function bop1-6d mutation strongly reduced FD transcription (Fig. 5). Whether this repression is due to direct recruitment of BOP proteins to the FD gene or an indirect effect of BOP proteins, for example, by activating transcription of boundary genes, remains unclear. Nevertheless, the reduction in FD mRNA likely explains the impaired sensitivity of the meristem to FT. Consistent with this idea, LFY and API mRNA levels were reduced in GAS1 FT ft-10 tsf-1 pny-58 (lgf58) plants as observed for GAS1 FT fd-3 and in bop1-6d mutants (Fig. 4; Supplemental Figs. S1 and S8). These results demonstrate that ectopic expression of BOP genes in the vegetative meristem reduces API and LFY expression during the early stages of floral transition, as expected for plants with reduced FD activity, although later in the floral primordial, BOP proteins have a direct role in the activation of API (Karim et al., 2009; Xu et al., 2010). Surprisingly, the expression levels of LFY were reduced during flower development in the bop1 bop2 double mutant compared with wild-type plants (Supplemental Fig. S8; Karim et al., 2009). We interpret these data as indicating a dual role for BOP1/2 in floral development. They might act as transcriptional repressors of LFY and API during the early stages of floral transition (probably mediated through FD) and promote the expression of these two genes during floral development.

The finding that ectopic expression of BOP function represses FD in the meristem suggests that, in wild-type plants, BOP gene expression in the boundary region of lateral organs might also repress FD, thus reducing its expression in lateral organs and restricting it to the meristem. Interestingly, FD expression is excluded from a strip of cells adjacent to lateral organs that might represent the boundary domain (Fig. 5B; Wigge et al., 2005), although higher resolution analysis allowing direct comparison of boundary gene expression with FD will be required to test this suggestion. In response to FT signaling, downstream genes are expressed in specific spatial domains of the meristem. Spatial patterning of FD expression in the apex may impose spatial constraints on FT signaling by ensuring, for example, that activation of the FT pathway does not occur in boundary regions.

Genetic and Molecular Interactions between PNY and Other Homeodomain Transcription Factors in the Regulation of Flowering

PNY is a member of the TALE homeodomain transcription factor family. These proteins are divided into two classes, referred to as KNOX and BELL. PNY is a member of the BELL class and interacts in the meristem with KNOX proteins, particularly BP and SHOOT MERISTEMLESS, to form heterodimers that regulate transcription. PNF and ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) are other BELL class proteins expressed in the meristem that have been implicated in flowering-time control (Smith et al., 2004; Proveniers et al., 2007). TALE transcription factors were shown to directly regulate genes encoding components of hormonal pathways or other transcription factors (Bolduc et al., 2012; Arnaud and Pautot, 2014). We demonstrate that PNY, presumably acting as a heterodimer with KNOX proteins expressed in the meristem, acts directly to repress genes encoding the transcriptional coactivators BOP1 and BOP2.

ATH1 and KNOTENLESS6, a KNOX class protein, are expressed at lateral organ boundaries during inflorescence development, where their activation requires BOP1/2. During vegetative development, ATH1 is expressed in the SAM and acts as a floral repressor. A recent study reported that BOP1 regulates the expression of ATH1 by directly binding to its promoter (Khan et al., 2015). Therefore, the ectopic expression of BOP1/2 in the meristem of pny mutants might increase ATH1 expression, contributing to the late-flowering phenotype.

ATH1 delays flowering at least partially by activating expression of the floral repressor FLOWERING LOCUS C (FLC) in the SAM. FLC represses FD by directly binding to its promoter (Searle et al., 2006). Thus, the repression of FD by BOP1/2 could at least partially be due to increased ATH1 activity, leading to misexpression of FLC mRNA in the SAM (Proveniers et al., 2007). Alternatively, BOP1/2 might interact directly with the promoter of FD. Further studies must be done to discriminate between these possible scenarios.

PNY and the related BELL protein PNF are genetically redundant in the promotion of flowering. Nevertheless, pny-58 was clearly late flowering in the single mutant, as described for other pny alleles named blr (Byrne et al., 2003). The pny pnf double mutant did not flower in any environmental condition tested and was assumed to be impaired in the competence to flower (Smith et al., 2004). Genetic and molecular analyses of the double mutant indicated that expression of LFY and API was strongly reduced in the inflorescence apices of these plants, but FT expression in leaves was unaffected (Kanrar et al., 2008). The conclusion that PNY PNF acts between FT and LFY was supported by the observation that pny pnf 355:LFY plants produced flowers, but pny pnf 355:FT plants did not (Kanrar et al., 2008). These results are in agreement with our observation that a primary effect of pny on flowering is reduction of FD mRNA, which is required for FT to promote flowering. Similarly, Lal et al. (2011) described a reduction in SPL4 and SPL5 expression in pny pnf apices, and activation of both of these genes at the shoot meristem is dependent on FT and FD (Torti et al., 2012). However, in contrast to our data, Kanrar et al. (2008) found that FD mRNA was present in the meristem of pny pnf plants at levels similar to those found in the wild type; therefore, the mechanism by which FT activity was impaired by pny pnf was unclear. This discrepancy with our data might be due to the age of the plants examined, as we studied pny mutants during vegetative development just prior
to floral induction and found a clear decrease in FD mRNA likely due to ectopic BOP1/2 expression, whereas Kanrar et al. (2008) examined the inflorescence meristem of plants 20 d after floral induction had occurred in the wild-type controls. In support of our data, Jaeger et al. (2013) also reported a reduction of FD mRNA levels in pny pf plants grown under inductive LDs, and we found that FD was strongly repressed in bop1-6d plants that express BOP1 in the meristem. Therefore, taken together, the data suggest that flowering is delayed in pny and pny pf mutants, at least partially, by reducing FT signaling, and our data indicate that this occurs due to reduced FD expression in the vegetative meristem caused by ectopic expression of BOP1/2, are directly repressed in wild-type plants by PNY (Fig. 6).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild type was the Col-0 ecotype of Arabidopsis (Arabidopsis italiana). The transgenic plants pGAS1:FT, pGAS1::FT fl-10 tf-1, and pSOCI::SOCI::GFP soc1-2 were previously described in Jing et al. (2009) and Immink et al. (2012). The mutant alleles used were pny-40126 (Smith and Joung, 2003), bop1-3 pny-2 (Hepworth et al., 2005), fl-3 (Abe et al., 2005), soc1-2 (Lee et al., 2000), and ft-10 tf-1 soc1-2 (Torti et al., 2012). The activation-tagged overexpressing line bop1-6D was described in Norberg et al. (2005). Plants were grown in climatic chambers under LD (16-h light/8-h dark) or SD (8-h light/16-h dark) conditions (Torti et al., 2012). The activation-tagged overexpressing line bop1-6D was described in Norberg et al. (2005). Plants were grown in climatic chambers under LD (16-h light/8-h dark) or SD (8-h light/16-h dark) conditions with a light intensity of 150 mmol m$^{-2}$ s$^{-1}$, 21°C, and 70% relative humidity.

Molecular Cloning of pPNY:Venus:PNY

Cloning of locus pNY was based on polymerase incomplete primer extension (PIPE; Klock and Lesley, 2009) with modifications for large fragments and multiple inserts. All PCR amplifications were done with Phusion Enzyme (New England Biolabs) following the manufacturer’s recommendations. Amplification of the PNY coding sequence was done from genomic DNA covering from the 5’-untranslated region (SUTR) until the 3’UTR (primers A1-F/A2-R), obtaining a PCR product of 3.5 kb. The promoter was amplified from −5,535 region until 78 nucleotides of exon 1 (primers A3-F/A4-R) obtaining a PCR product of 5.7 kb. PCR products were independently cloned into pDONR201 (Invitrogen) by BP reaction generating constructs pSNY-ENTR201 and pPNY-ENTR201, respectively. Insertion of the sequence of fluorescent protein Venus (Nagai et al., 2002; Heisler et al., 2005) was done by amplifying three different PCR elements adding overlap sequences among them: I-PIPE-1 (Venus), I-PIPE-2 (coding sequence PNY), and vector-PIPE (promoter pNY-ENTR201). Elaboration of I-PIPE-1 was done by amplifying Venus and adding a linker of nine Ala to exoni1 (primers A5-F/A6-R) obtaining a product of 772 nucleotides. Generation of L-PIPE-2 was done using cpSNP-ENTR201 as template, producing a PCR fragment of 3.3 kb containing the region from exon1 until 3’UTR (primers A7-F/A8-R). Finally, vector-PIPE was generated using as template pPNY-ENTR201, obtaining a PCR fragment of 7.8 kb (primers A9-F/A10-R) comprising promoter-SUTR plus pENTR201 backbone. For the assembly of the different fragments, equimolar amounts of each I-PIPE element were mixed while keeping a ratio of 1:10 to vector-PIPE. The mixture was cloned into chemical competent DH5-α cells. The final construct (11.8 kb) was verified by digestion analysis and sequencing. Subsequently, the construct was cloned into the binary vector pEarleyGate301 (Earley et al., 2006) by LR reaction and transformed into Agrobacterium tumefaciens GV3101 cells. pny-40126 plants were transformed by the floral-dip method (Clough and Bent, 1998). The list of primers used for the molecular cloning can be found in the Supplemental Table S2.

EMS for 12 h. After the treatment, the seeds were washed twice with 100 mL of 100 mM sodium thiosulfate followed by two additional washes with 500 mL of water. The seeds were transferred to a flask containing 2 L of water and distributed in 200 pots by pipetting (50 seeds/pot). M1 generation was grown in a greenhouse under LD conditions. M2 seeds from each pot were harvested together and treated as a pool. Approximately 500 M2 seeds from each pool were used for the genetic screen. The screening of the M2 seeds was performed in climatic chambers under SDs. Mutants showing late flowering compared with pGAS1::FT ft-10 tf-1 (lgf mutants) were selected, and the phenotypes were confirmed in the M3 generation.

Flowering Time Measurements

Flowering time was scored as the number of leaves at bolting. The number of rosette leaves was determined when the shoot reached approximately 0.5-cm length. The cauline leaf number was defined when the shoot was totally elongated. At least 10 individual plants were scored by genotype. All experiments were independently repeated at least twice.

Resequencing and Mapping Strategy

lgf58 homozygous mutant was crossed with pGAS1::FT ft-10 tf-1 to generate the BC1F2 mapping population. A total of 174 late-flowering mutants out of 566 F2 plants were selected. One leaf sample of each was harvested and pooled. Leaf material from the original pGAS1::FT ft-10 tf-1 parent was also harvested. Genomic DNA (gDNA) from 1 g of the pooled and the pGAS1::FT ft-10 tf-1 leaf material was extracted using a DNeasy Plant Maxi Kit (Qiagen). Four micrograms of gDNA was sent to the Cologne Center of Genomics (Co- logne, Germany) for sequencing. Sequencing was performed on an Illumina HiSeq2000 instrument. Up to four independent gDNA samples (i.e. pGAS1::FT ft-10 tf-1 and various lgf mutants, including lgf58) were resequenced in a single HiSeq2000 flow cell lane with a read length of 100 bp (paired end) by using barcoding (multiplexing). After sequencing and applying the quality controls, we obtained a total of 10,948,820 reads from pGAS1::FT ft-10 tf-1 and 87,972,438 reads from lgf58 plants. A total of 101,934,960 (92%) reads from pGAS1::FT ft-10 tf-1 and 85,609,714 (97%) reads from lgf58 were aligned to the reference sequence TAIR10 (Arabidopsis Genome, 2000) by applying SHORE (Schneeberger et al., 2009b) and GenomeMapper (Schneeberger et al., 2009a), representing an average coverage of 73× and 57× the respective resequenced genome (pGAS1::FT ft-10 tf-1 and lgf58 population). Before identifying single nucleotide polymorphisms (SNPs) from the alignment of the pooled (lgf58 plants short read data, an SNP analysis of pGAS1::FT ft-10 tf-1 was applied to identify all fixed SNPs of this pool. After removing these SNPs from the SNP analysis of LGF58, we obtained a total of 20,137 putative differences from the reference sequence. Of those, 1,174 were further removed as they were located in the mitochrondria and plastogloben. From the remaining 18,963 putative differences, 2,212 revealed the canonical EMS mutation (G/C:A/T). From those, we obtained a final set of 137 mutations by relaxed filtering for reliable mutations (at least a 0.75 quality score and 5% or higher allele frequency of 0.7 for the mutated allele). The list of top candidates can be found in Supplemental Table S1. Short reads are available through the European Nucleotide Archive under accession number PRJEB10593.

Protein Extraction and Immunoblotting Assays

Total protein was extracted from inflorescences, ground in liquid nitrogen and homogenized in denaturing buffer (100 mM Tris-HCl [pH 7.5], SDS 3%, 10 mM dithiothreitol, and 1% protein inhibitor), mixed by vortexing, and rotated for 10 min at 4°C. Cell debris was removed by centrifugation at 14,500 rpm for 10 min at 4°C. Proteins were quantified by the bicinchoninic acid method, and 30 μg of proteins was loaded in a gel preceded by boiling for 5 min. Anti-rabbit GFP antibody (Abcam ab290) was used for the western blot. The blot was incubated with SuperSignal Femto West Substrate (Thermo Fisher Scientific) following the manufacturer's protocol and detected with a LAS-4000 Mini-image analyzer (Fujiﬁlm). Coomassie Brilliant Blue was used as the loading control.

Mutagenesis and Genetic Screen

Approximately 10,000 seeds (200 mg) of pGAS1::FT ft-10 tf-1 were wrapped in miracloth and imbibed for 14 h in 50 mL of 0.1% (w/v) KC1 (50 mg of KC1 in 50 mL of dH2O) at 4°C on a shaker. Then, the seeds were treated with 30 mM

ChIP and qPCR

ChIP was performed as previously described (Andrés et al., 2014; Mateos et al., 2015). GFP antibody from Abcam (ab290) was used to immunoprecipitate the chromatin. Inflorescences (containing flowers until stage 13) of
approximately 4-week-old pNY:Venus-pNY and pny-40126 plants grown under LD were collected at zeitgeber time 3 for the ChIP assays. The percentage of input method was used for data normalization (Haring et al., 2007). qPCR values obtained from the immunoprecipitated samples were divided by the qPCR values of a 1:10^2 dilution of the input sample. For validation of the PNY binding to BOP1 and BOP2, several pair of primers spanning the BOP1 and BOP2 promoters were tested (Supplemental Table S2). Two biological replicates were performed for each ChIP assay. Only one of the replicates is shown.

**qPCR Methods for RNA Expression Analysis and Genotyping of pny-58**

RNA expression analyses were performed as described in Andrés et al. (2014). Total RNA was extracted from plant tissue by using the RNaseasy Plant Mini Kit (Qiagen) and treated with DNA-free DNase (Ambion) to remove residual genomic DNA. The RNA was then quantified by using the Nanodrop ND-1000. One microliter of total RNA was used for RT (SuperScript III; Invitrogen). Levels of mRNAs were quantified by qPCR in a LightCycler 480 instrument (Roche) using the PEROXIN4 primer (AT5G25760) as a standard. Three biological replicates were performed for each RT-qPCR assay. The average of the three replicates is shown. The list of primers used for expression analyses can be found in Supplemental Table S2. Graphs were obtained from three independent technical replicates, all of which were optimized for the visualization of Venus fluorescent proteins (laser wavelength 514 nm; detection wavelength, 517 nm). The flower image was acquired with a visible meristem were selected and mounted with ProLong Diamond Antifade (Invitrogen). The shoot meristems were imaged by confocal laser scan microscopy techniques. Shoot apices were collected and placed on ice-cold 2.5% paraformaldehyde (PFA; Sigma-Aldrich) prepared in phosphate-buffered saline at pH 7.0. Samples were vacuum infiltrated with the same solution for 10 min and 20 min. Samples were incubated for 30 min in 1% PFA supplemented with 10%, 20%, and 30% Suc. The list of primers used in this work.

**In Situ Hybridization and Microscopy Techniques**

In situ hybridizations were performed as described in Torti et al. (2012). The Fc probe was synthesized as described in Searle et al. (2006). The list of primers used to generate the other probes can be found in Supplemental Table S2. For Venus:PNY visualization in shoot meristems, a method described previously (Wang et al., 2014) was used, with small modifications. Shoot apices were collected and placed on ice-cold 2.5% paraformaldehyde (PFA; Sigma-Aldrich) prepared in phosphate-buffered saline at pH 7.0. Samples were vacuum infiltrated for 30 min, transferred to fresh 2.5% PFA, and stored at 4°C overnight. The next day, the samples were incubated for 30 min in 1% PFA supplemented with 10%, 20%, and 30% Suc. Then, samples were embedded in OCT (Sakura Finetek). Sections of 35 μm were made using a cryostome (Frigocut 2800; Reichert Jung). Sections containing a visible meristem were selected and mounted with ProLong Diamond Antifade (Invitrogen). The shoot meristems were imaged by confocal laser scanning microscopy (Zeiss LSM780). The confocal laser scanning microscopy settings were optimized for the visualization of Venus fluorescent proteins (laser wavelength, 514 nm; detection wavelength, 517–569 nm).

**Statistical Analysis**

All of the statistical analyses were performed by using SigmaStat 3.5 software. Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT5G02030 (PNY), AT3G57130 (BOP1), AT2G41570 (BOP2), AT1G65480 (FT), AT4G35900 (FD), AT2G45660 (SOC1), AT1G69120 (API), AT5G1850 (LFY), and AT1G55160 (SPL4).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Phenotype of the mutants recovered in the genetic screen and characterization of lgf58.

**Supplemental Figure S2.** Scheme of the cloning-by-sequencing of the lgf58 mutant.

**Supplemental Figure S3.** Phenotypical characterization of mutants carrying the pny-58 allele.

**Supplemental Figure S4.** Flowering time of plants carrying mutations that suppress FT function.

**Supplemental Figure S5.** Functional characterization of pPNY:Venus-pNY transgenic plants.

**Supplemental Figure S6.** Effect of photoperiod on PNY pattern of expression.

**Supplemental Figure S7.** Interaction between PNY and BOP1/2 genes in flowering time control.

**Supplemental Figure S8.** Effect of BOP1/2 genes on the FT-signalling pathway.

**Supplemental Table S1.** Candidate loci identified by SHOREmap.

**Supplemental Table S2.** List of primers used in this work.

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