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RUNNING HEAD:

Repression of the FT signaling pathway by *BOP1/2*

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RESEARCH AREA:

Genes, Development and Evolution

Floral induction in *Arabidopsis thaliana* by FLOWERING LOCUS T requires direct repression of *BLADE-ON-PETIOLE* genes by homeodomain protein PENNYWISE

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Summary:

Direct repression of lateral organ boundary genes in the shoot meristem is required for flowering induction.

FOOTNOTES

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ABSTRACT

Flowers form on the flanks of the shoot apical meristem (SAM) in response to environmental and endogenous cues. In *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 bZIP transcription factor FD is proposed to form in the SAM leading to activation of *APETALA1* (*AP1*) and *LEAFY* (*LFY*) 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 co-activators, in the SAM during vegetative development confers the late flowering of *pny* mutants. In wild-type plants *BOP1*/*2* are expressed in lateral organs close to boundaries with 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 *AP1* and *LFY*. 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.

INTRODUCTION

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 re-programming these pluripotent cells at the meristems, plants can readily modify their development in response to changes in environmental conditions.

Flowers develop from floral meristems (FM) 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 (Martinez-Zapater and Somerville, 1990; Lee and Amasino, 1995; Valverde et al., 2004; Andres and Coupland, 2012). On the other hand, the plant hormone gibberellin and the age of the individual 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 (PEBP)/Raf-1 Kinase Inhibitory Protein (RKIP) (Kardailsky et al., 1999; Kobayashi et al., 1999; Nakamura et al., 2014; Romera-Branchat et al., 2014). In *Arabidopsis*, *FT* is induced by long days (LDs) and has been placed at the core of the photoperiodic pathway, downstream of the *GIGANTEA* (*GI*) and *CONSTANS* (*CO*) genes (Suarez-Lopez 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 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 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 CO1 (SOC1) and FRUITFULL (FUL), which accelerate flowering, as well as APETALA1 (AP1), also a MADS-box transcription factor, and LEAFY (LFY), which promote floral meristem identity (Schmid et al., 2003; Teper-Bamnolker 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 in order 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 (BELL) 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 *BELL* family comprises 13 members (Smith et al., 2004). *PENNYWISE* (*PNY*), also known as *BELLRINGER* (*BRL*), *REPLUMLESS* (*RPL*), *VAAMANA* (*VAN*) or *LARSON* (*LSN*), encodes a BELL 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* genes 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 6* (*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 long day conditions (Smith et al., 2004). Moreover, overexpression of *FT* from the constitutive *CaMV 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 in the *FT* signaling pathway. However, the genetic and molecular mechanism underlying the effect on PNY on 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-ankyrin transcriptional co-activators, which function at lateral organ boundaries in the determination of leaf, flower, inflorescence and root nodule architecture (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005; Ha et al., 2007; Karim et al., 2009; Xu et al., 2010; Couzigou et al., 2012; Khan et al., 2012; Khan et al., 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 through *BOP* gene repression the pattern of expression of flowering-time genes in particular shoot meristem domains.

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 homologue *TWIN SISTER OF FT* (*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 p*GAS1::FT*, which is active only in phloem companion cells of the minor veins (Jang et al., 2009). Thus, p*GAS1::FT ft-10 tsf-1* plants show early flowering compared to *ft-10 tsf-1* double mutants under long days (LD) and short days (SD). These plants were used to screen for mutations that suppress promotion of flowering by FT. Seeds of p*GAS1::FT ft-10 tsf-1* were mutagenized with ethyl methanesulfonate (EMS) and late-flowering plants were screened in the M2 generation under SD. Recovered mutants are hereafter called *late flowering in pGAS::FT ft-10 tsf-1* (*lgf*). Early flowering of p*GAS::FT ft-10 tsf-1* plants grown under SD is entirely dependent on movement of FT from the leaves to the SAM, so *lgf* mutations were expected to define genes required for FT function or transport. Around 35,000 M2 plants were screened and several *lgf* mutants selected (Figure S1). The *lgf58* mutation most strongly suppressed the early flowering conferred by misexpression of FT (Figure 1 and Figure S1). This mutant also showed other phenotypic abnormalities such as short stature and lanceolate leaves (Figure S1).

The fast isogenic mapping approach was used to identify the *lgf58* mutation (Hartwig et al., 2012; Schneeberger, 2014). A mapping population was created by backcrossing (BC) *lgf58* to p*GAS::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 p*GAS::FT ft-10 tsf-1* were sequenced using Illumina technology. By applying SHOREmap (Schneeberger et al., 2009; Sun and Schneeberger, 2015), candidate loci, at which mutant alleles were strongly

over-represented in the pool, were identified on the top arm of chromosome 5 (Figure 1 and Figure S2, for more details see also "Materials and Methods"). Three loci were selected as high confidence candidates because they carried a non-synonymous mutation and showed an allele frequency equal to 1.0 and a SHORE score equal to 40 (Supplemental Table I and "Material and Methods"). Among the highest probability candidates, the gene *AT5G02030* contained a mutation in the second exon predicted to produce a premature stop codon (Figure 1 and Supplemental Table I). *AT5G02030* encodes the BEL1-like homeodomain (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 paralogue *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* (Figure 1 and Figure S1) and in Col-0 plants carrying the newly isolated mutant allele of *PNY* (hereafter called *pny-58*) segregated away from p*GAS::FT ft-10 tsf-1* (Figure 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 to p*GAS::FT ft-10 tsf-1*. However, as also described earlier for *pny pnf* double mutants, *SOC1* mRNA expression was unaltered in *lgf58* (Figure S1) (Kanrar et al., 2008). Col-0 plants carrying the *pny-58* mutant allele grown under LD also showed late flowering compared to wild-type plants and were more extreme than the previously reported mutant allele *pny-40126* (Smith and Hake, 2003; Ragni et al., 2008) (Figure S3). This delay in flowering was corrected by introducing a transgenic copy of the wild-type genomic *PNY* locus into the mutant plants (p*PNY::Venus:PNY pny-58*), confirming that the mutation *pny-58* was responsible for the late-flowering phenotype (Figure S4). Moreover, the early flowering of p*GAS::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* (Figure 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 p*GAS::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 was analyzed by *in situ* hybridization in different environments (Figure 2). Under SD *PNY* mRNA was expressed in the central zone (CZ) of the SAM and excluded from the leaf boundaries (Figure 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 p*PNY::Venus:PNY pny-40126* plants (Figure 2 and Figure 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 (Figure 2). Similarly, its pattern of expression broadened during photoperiodic induction of flowering, and was excluded from leaf boundaries and floral primordia (Figure 2). Therefore, expression of *PNY* mRNA and its translated product are 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 overlaps with that of *PNY* (Jang et al., 2009; Torti et al., 2012). A recent genome-wide study identified the *PNY* promoter as a direct target of SOC1 (Immink et al., 2012). Therefore, binding of SOC1 to the *PNY* locus was tested directly by Chromatin Immunoprecipitation followed by quantitative PCR (ChIP-qPCR). In this way, binding of SOC1 to the *PNY* promoter was confirmed. SOC1:GFP bound to the *PNY* promoter at approximately 3400bp from its ATG, in a region containing a putative CArG box. This position was very close to the observed binding peak of SOC1 by ChIP-seq (Immink et al., 2012). No binding of SOC1:GFP was found in a region comprising the 4th exon of *PNY* which was used as negative control (Figure 2). These data suggest that SOC1 might contribute to photoperiodic induction of *PNY* during flowering. Whether *PNY* expression was reduced by mutations in *FT*, *TSF* or *SOC1* was also tested. However no differences in *PNY* mRNA levels were detected in the *ft-10 tsf-1, ft-10 tsf-1 soc1-2* and *soc1-2* mutants compared to Col-0 during the floral transition (Figure 2 and Figure S6). These results suggested that *PNY* expression is increased by exposure to LDs and that SOC1

might contribute to this, although at the times tested no detectable difference in *PNY* expression could be associated with the loss of function of *SOC1*, *FT* or *TSF* by RT-qPCR.

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., 2012). 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-day old plants grown under LDs, *BOP1* and *BOP2* mRNA levels were higher in *pny-58* mutants compared to WT, as tested by RT-qPCR (Figure 3). Similarly, these genes were more highly expressed in *lgf58* plants than in p*GAS::FT ft-10 tsf-1* (Figure 3). *In situ* hybridization experiments detected broader expression of *BOP2* in the shoot apical meristem of vegetative 7-day old *pny-58* plants than in Col-0 (Figure 3 and Figure S7) and in 10-old plants that were undergoing the floral transition (Figure 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 (Figure 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* (Figure 3 and Figure 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 downregulation 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 to the wild type, the expression of *LFY* in the *bop1 bop2* double mutant was slightly increased during vegetative development (7-10 LDs), but reduced at the reproductive stage (17 LDs).

306 Taken together these data support the hypothesis that late flowering of *pny-58* mutants is due to
307 higher and ectopic expression of *BOP1/2* in the vegetative apex.

308 Since mutations in *PNY* suppress the capacity of FT to induce flowering, *BOP1/2* up-regulation
309 should also delay the floral transition promoted by this protein. To explore this further a
310 dominant activation tagging allele of *BOP1* (*bop1-6D*) was used (Norberg et al., 2005). The
311 *bop1-6D* mutants flowered later than Col-0 (Figure 4). In order to test whether this occurs
312 through the FT floral promoting pathway the double transgenic p*GAS1::FT bop1-6D* was
313 constructed. Flowering time experiments demonstrated that *bop1-6D* delayed flowering of
314 p*GAS1::FT* (Figure 4). This result supports the idea that ectopic *BOP* expression impairs activity
315 of the FT-signaling pathway. Indeed, the expression of *LFY* and *AP1*, which is activated in the
316 SAM downstream of FT (Schmid et al., 2003; Moon et al., 2005), was dramatically reduced by
317 overexpression of *BOP1* (Figure 4).

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

320 **PNY represses *BOP1* and *BOP2* transcription by directly binding to their promoters**

321 The increase of *BOP1* and *BOP2* expression in *pny-58* mutants suggested that PNY might
322 directly bind to the promoters of these genes. This possibility is further supported by proteomics
323 analysis that detected PNY and AP1 in the same transcriptional complex (Smaczniak et al.,
324 2012) and by ChIP-seq that identified *BOP1* and *BOP2* as putative direct targets of AP1
325 (Kaufmann et al., 2010). Whether PNY binds to the same *BOP1/2* promoter regions as AP1 was
326 therefore tested. ChIP-qPCR was performed on chromatin extracted from inflorescences of
327 p*PNY::Venus:PNY* transgenic plants. The chromatin was immunoprecipitated using a GFP
328 antibody, which detects Venus:PNY on Western blots (Figure S7), followed by qPCR with
329 combinations of primers spanning regions of the *BOP1* and *BOP2* promoters. Regions of the
330 *TOE1* and *LFY* promoters, which were shown to be directly bound by the AP1/PNY complex
331 (Smaczniak et al., 2012), were used as positive controls. As expected, Venus:PNY bound to the
332 *TOE1* and *LFY* loci within the same region that was reported for AP1 (Figure S7) (Kaufmann et
333 al., 2010). The binding of Venus:PNY to *BOP1* and *BOP2* was then tested. Venus:PNY bound to
334 the *BOP1* promoter at two different positions (P3 and P4). One of these positions (P3) was the

same as that reported for AP1 (Figure 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 (Figure 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 the BELL-like homeodomain protein BLH1 (Stanelonia et al., 2009). Within the region P7 the two motifs "AAATTACCA" and "AATTATCCT", which are similar to those previously identified as binding sites of BLR in the *AGAMOUS* (*AG*) intronic region ("AAATTAAAT", "AAATTAGTC" and "ACTAATTT") (Bao et al., 2004; Smaczniak et al., 2012), were also found. However, only shorter versions of these motifs ("AATTAT", "AATTT" 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 *SPL*s, *LFY* and *AP1*, that are expressed at the apex during flowering downstream of FT (Figure 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 p*GAS::FT* transgenic plants to a similar extent (Figure 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 *fd-3* mutation suppressed the expression of these genes in *pGAS1::FT* background to a similar extent as *pny-58* (Figure 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 to Col-0 wild-type plants (Figure 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 to Col-0 (Figure 5). Moreover, *in situ* hybridization experiments showed that the *FD* mRNA was reduced in the meristem-leaf boundaries of the *pny-58* (Figure 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* (Figure 5). By contrast, *FD* mRNA appeared slightly increased in the SAM of *bop1-3 bop2-1* double mutants (Figure 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 flowering. A novel allele of the homeobox gene *PNY* was the strongest effect mutation recovered. This *pny-58* allele delayed flowering both in the *pGAS1::FT ft-10 tsf-1* background used for the screen and in Col-0. Expression and genetic analyses indicated that the late-flowering of *pny* mutants was caused by ectopic expression of *BOP1* and *BOP2* in the shoot meristem during vegetative development. Thus the repression of expression of *BOP* genes is a major aspect of the contribution of *PNY* to flowering-time control. This conclusion is in agreement with previous reports that repression of *BOP* genes by PNY is necessary for wild-type inflorescence development because ectopic *BOP* gene expression causes abnormalities such as short internodes and reduced apical dominance (Norberg et al., 2005; Ha et al., 2007; Khan et al., 2012; Khan et al., 2012). 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 contributes to the reduced responsiveness to FT. We propose that restriction of *BOP* expression to the proximal regions of lateral organs and 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 (Figure 6).

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395 **Significance of defining organ boundaries for FT signaling and *FD* expression**

396 In plant meristems, lateral organ boundaries separate the meristematic zone containing
397 undifferentiated cells from the lateral organs containing differentiated cells (Rast and Simon,
398 2008; Khan et al., 2014). The *BOP1* and *BOP2* genes are expressed at the base of lateral organs,
399 adjacent to the boundary (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). They
400 contribute to define the boundary region by repressing homeobox genes, which maintain the
401 meristematic region, by directly activating *ASYMMETRIC LEAVES 2* (*AS2*) and other genes that
402 specify the boundary (Ha et al., 2007; Jun et al., 2010). We observed that in *pny* mutants the
403 *BOP* genes are ectopically expressed in the vegetative meristem, as was previously described for
404 older inflorescence meristems (Norberg et al., 2005; Ha et al., 2007; Khan et al., 2012; Khan et
405 al., 2012). Thus in wild-type plants during vegetative development PNY contributes to
406 positioning the boundary between the vegetative meristem and leaves by repressing *BOP* gene
407 expression in the meristem. Indeed, we observed that in absence of functional *PNY* (*pny-58*),
408 *BOP2* expression becomes broader in the meristem. Similarly, Khan et al (in this issue) found
409 that *BOP1* spatial pattern of expression was enlarged in the inflorescence meristem of *pny pnf*.
410 This restriction of *BOP* expression and proper localization of the boundary is required for correct
411 timing of the floral transition, because *pny* mutants are late flowering and this is suppressed in
412 the *pny bop1 bop2* triple mutant (Figure 3 and Figure S7). The sensitized screen used to identify
413 the *pny-58* mutation illustrated the importance of *PNY* and the *BOP* genes downstream of FT in
414 the photoperiodic flowering pathway, as previously shown by the capacity of *pny pnf* double
415 mutants to suppress the early-flowering phenotype caused by *35S::FT* (Kanrar et al., 2008).

416 At the meristem FT is proposed to activate downstream genes by directly interacting with the
417 bZIP FD transcription factor (Abe et al., 2005; Wigge et al., 2005). This relationship between
418 FT-related proteins and FD is highly conserved in higher plants, having also been observed in
419 rice and tomato (Pnueli et al., 2001; Taoka et al., 2011). We found that the spatial pattern of
420 expression of *FD* in the SAM is regulated by the *PNY*, *BOP1* and *BOP2* genes. Ectopic *BOP*
421 gene expression in the *pny* mutant or the gain of function *bop1-6d* mutation strongly reduced *FD*

transcription (Figure 5). Whether this repression is due to direct recruitment of BOP proteins to the *FD* gene or to an indirect effect of BOP proteins, by for example 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 *AP1* 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 (Figure 4, Figure S1 and Figure S8). These results demonstrate that ectopic expression of *BOP* genes in the vegetative meristem reduces *AP1* and *LFY* expression during the early stages of floral transition, as expected for plants with reduced FD activity, although later in the floral primordia BOP proteins have a direct role in the activation of *AP1* (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 to the wild type plants (Figure S8) (Karim et al., 2009). We interpret these data as a dual role for BOP1/2 in floral development. They might act as transcriptional repressors of *LFY* and *AP1* during the early stages of floral transition (probably mediated through *FD*) and promoting the expression of these two genes during the 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 (Figure 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 three-amino-acid-loop-extension (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 in the meristem interacts with KNOX proteins, particularly BP and STM, to form heterodimers that regulate transcription. PNF and ARABIDOPSIS THALIANA HOMEOBOX 1 (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 co-activators *BOP1* and *BOP2*.

ATH1 and KNAT6, 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 shoot apical meristem and acts as a floral repressor. A recent study reported that BOP1 regulates the expression of *ATH1* by direct binding to its promoter (Khan et al, in this issue). 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 *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 in order 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 as *bellringer* (*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 *AP1* 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 act between FT and *LFY* was supported by the observation that *pny pnf 35S: LFY* plants produced flowers but *pny pnf 35S: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 wild-type, and 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 days 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 pnf* 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 in *pny* and *pny pnf* mutants flowering is delayed, 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* that in wild-type plants are directly repressed by PNY (Figure 6).

MATERIALS AND METHODS

Plant materials and growth conditions

Wild type was the Col-0 ecotype of *Arabidopsis thaliana*. The transgenic plants p*GAS1::FT*, p*GAS1::FT ft-10 tsf-1* and p*SOC1::SOC1:GFP soc1-2* were previously described in Jang et al (2009) and Immink et al (2012). The mutants alleles used were *pny-40126* (Smith and Hake, 2003), *bop1-3 bop2-1* (Hepworth et al., 2005), *fd-3* (Abe et al., 2005), *soc1-2* (Lee et al., 2000), *ft-10 tsf-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 with the light intensity of 150 mmol m^{-2} s^{-1}, 21° C and 70% of relative humidity.

Molecular cloning of p*PNY::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 (NEB labs) following the manufacturer`s recommendations. Amplification of *PNY* coding sequence was done from genomic DNA covering from 5UTR until 3UTR (primers A1-F/A2-R) obtaining a PCR product of 3.5 Kb. The promoter was amplified from -5553 region until 78 nucleotides (nt) of Exon 1 (primers A3-F/A4-F) obtaining a PCR product of 5.7 Kb. PCR products were independently cloned into pENTR201 by BP reaction generating constructs csPNY-pENTR201 and pPNY-pENTR201, respectively. To generate *I-PIPE-1* (Insert-PIPE-1) amplification of fluorescent protein Venus (Nagai et al., 2002; Heisler et al., 2005) was done adding an overlap sequence at the 5' site to 5UTR region and at the 3' site a linker of 9 alanines (primers A5-F/A6-R) obtaining a product of 772 nt. Generation of *I-PIPE-2* was done using csPNY-pENTR201 as template producing a PCR fragment of 3.3 Kb comprising the region from Exon1 until 3UTR (primers A7-F/A8-R) containing an overlap sequence from Exon1 to Venus. Finally, *V-PIPE* (Vector-PIPE) was generated using as template pPNY-pENTR201 obtaining a PCR fragment of 7.8 Kb with overlapping sequences to both 3UTR region and to Venus (primers A9-F/A10-R). For the assembly of the different fragments equimolar amounts of each *I-PIPE* element were mixed whilst keeping a ratio 1:10 to *V-PIPE*. The mixture was cloned into chemical competent DH5-α cells. The final construct (9.5 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 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 II.

Mutagenesis and genetic screen

Around 10.000 seeds (200 mg) of p*GAS1::FT ft-10 tsf-1* were wrapped in miracloth and imbibed for 14 hours in 50 ml 0.1% KCl (50 mg KCl in 50 ml dH2O) at 4° C on a shaker. Then, the seeds were treated with 30 mM Ethyl methanesulfonate (EMS) for 12 h. After the treatment, the seeds were washed twice with 100 ml of 100 mM sodium thiosulfate followed of 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 long day 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 short days. Mutants showing late flowering compared to p*GAS1::FT ft-10 tsf-1* (*lgf* mutants) were selected and the phenotypes were confirmed in the M3 generation.

Flowering time measurements

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

Re-sequencing and mapping strategy

lgf58 homozygous mutant was crossed with p*GAS1::FT ft-10 tsf-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 one was harvested and pooled. Leaf material from the original p*GAS1::FT ft-10 tsf-1* parental was also harvested. gDNA from 1 g of the pooled and the p*GAS1::FT ft-10 tsf-1* leaf material was extracted using a DNeasy Plant Maxi Kit (Qiagen). 4 µg of gDNA was sent to the Cologne Center of Genomics (Cologne, Germany) for sequencing. Sequencing was performed on an Illumina HiSeq2000 instruments. Up to four independent gDNA samples, i.e. p*GAS1::FT ft-10 tsf-1* and various *lgf* mutants including *lgf58*, were re-sequenced 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 in total 109,948,920 reads from p*GAS::FT ft-10 tsf-1* and 87,972,438 reads from *lgf58* plants. 101,934,960 (92%) reads from p*GAS::FT ft-10 tsf-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., 2009; Schneeberger et al., 2009), and GenomeMapper (Schneeberger et al., 2009), representing on average coverage of 73x and 57x of the respective re-sequenced genome (p*GAS::FT ft-10 tsf-1* and *lgf58* population). Before identifying SNPs (Single Nucleotide Polymorphisms) from the alignment of the pooled *lgf58* plants short read data, a SNP analysis of *pGAS::FT ft-10 tsf-1* was applied to identify all fixed SNPs of this pool.

After removing these SNPs from the SNP analysis of *LGF58* we obtained in total 20,137 putative differences to the reference sequence. 1,174 of those were further removed as they were located in the mitochondria and chloroplast genome. 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 SHORE quality score of 25 and a minimum allele frequency of 0.7 for the mutated allele). The list of top candidates can be found in the Supplemental Table I. Short reads are available through the ENA (European Nucleotide Archive) under the accession number PRJEB10593.

Protein Extraction and Immunoblotting Assays

Total protein was extracted from inflorescences, ground in liquid nitrogen and homogenized in denaturing buffer (100mM Tris-HCl pH 7.5, SDS 3%, DTT 10mM and 1% protein inhibitor), mix by vortexing and rotate 10min at 4°C. Cell debris was removed by centrifugation at 14,500 rpm for 10 min at 4°C. Proteins were quantified by BCA method and 30 µg of proteins were loaded in a gel preceded by boiling for 5min. 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 blue brilliant (CBB) was used as the loading control.

Chromatin Immunoprecipitation and qPCR

ChIP was performed as previously described (Andres 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 p*PNY::Venus:PNY* and *pny-40126* plants grown under LD were collected at ZT3 (Zeitgeber Time 3) for the ChIP assays. The % of input method was employed for data normalization (Haring et al., 2007). qPCR values obtained from the immunoprecipitated samples were divided by the qPCR values of an $1:10^2$ 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 II). 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 (Andres et al., 2014). Total RNA was extracted from plant tissue by using the RNeasy 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 microgram of total RNA was used for reverse transcription (Superscript II, Invitrogen). Levels of mRNA were quantified by quantitative PCR in a LightCycler 480 instrument (Roche) using the *PEX4* gene (*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 the Supplemental Table II. Graphs were obtain from three independent technical replicates, although all RT-qPCRs were repeated at least twice and showed identical results.

Genotyping of the mutant allele *pny-58* was performed by high-resolution melting (Wittwer et al., 2003). *pny-58* allele carried a single nucleotide change (C>T). Primers flanking this mutation (K617 and K618, Supplemental Table II) were used to amplify by PCR a 79 bp amplicon from gDNA extracted from leaf material (Plant DNeasy Kit, Qiagen). The PCR products were diluted 5 times in water. 3 µL of the PCR product dilutions were used as a template for the quantitative PCR in a LightCycler 480 instrument (Roche). The primers K617 and K618 were used for the amplification. The PCR conditions were: 95° C 3 min (pre-incubation) and 22 cycles of 95° C 20 s, 60° C 20 s and 72° C 20 s. For the melting curve generation the temperature was increased from 65° C to 97° C (ramp rate 0.11° C/s and 5 acquisitions/° C). The mutant and wild-type alleles could be differentiated by analyzing the melting peaks. The *pny-58* and wild-type alleles produced a melting peak at 77.66 ± 0.03 and 78.33 ± 0.03 (° C), respectively.

In situ hybridization and microscopy techniques

In situ hybridizations were performed as described in Torti *et al* (2012). The *FD* probe was synthesized as described in Searle et al., 2006. The list of primers used to generate the other probes can be found in the Supplemental Table II. For Venus:PNY visualization in shoot meristems a method described previously (Wang et al., 2014) with smalls modifications was used. Shoot apices were collected and placed on ice-cold 2.5% paraformaldehyde (PFA; Sigma-Aldrich) prepared in phosphate-buffered saline (PBS) at pH 7.0. Samples were vacuum infiltrated for 30 min, transferred to fresh 2.5% PFA and stored at 4°C overnight. The second

day, the samples were incubated for 30 min in 1% PFA supplemented with 10%, 20% and 30% sucrose. Then, samples were embedded in OCT (Sakura Finetek) at -20°C. Sections of 35 µm were made using a cryotome (Frigocut 2800; Reichert Jung). Sections containing a visible meristem were selected and mounted with ProLong Diamond Antifade (Invitrogen). Imaging of the shoot meristems was made by confocal laser scanning microscopy (CSLM, Zeiss LSM780). The CSLM setting 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.

Accession Numbers

AT5G02030 (*PNY*), *AT3G57130* (*BOP1*), *AT2G41370* (*BOP2*), *AT1G65480* (*FT*), *AT4G35900* (*FD*), *AT2G45660* (*SOC1*), *AT1G69120* (*AP1*), *AT5G61850* (*LFY*), *AT1G53160* (*SPL4*)

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AUTHOR CONTRIBUTIONS

All author contributed to the research and/or the writing of the manuscript. F.A., M.R.B. performed most of the experiments. R.M.G. generated the p*PNY::Venus:PNY* construct. S.J. contributed to the EMS mutagenesis. J.A. and P.N. provided the genome re-sequencing data. V.P. and K.S. analyzed the NGS data. F.A. and G.C. conceived the project, designed the experiments, interpreted the data and wrote the article.

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878 **FIGURE LEGENDS**

879 **Figure 1. Identification and cloning of a functional suppressor of FT**. (A) Phenotypic
880 comparison between *lgf58*, p*GAS1::FT ft-10 tsf-1* and Col-0 wild type plants and (B) their
881 flowering time under LD (n = 10). CL: cauline leaves, RL: rosette leaves. Letters shared in
882 common between the genotypes indicate no significant difference (t-test, $P < 0.05$). (C) Graphic
883 showing the allelic frequency estimations at EMS-induced mutations (AF, *y* axis) across
884 chromosome 5 (Mb, *x* axis) of *lgf58*. AFs were calculated dividing the number of reads
885 supporting the mutant allele by the number of all reads aligning to a given marker. The color
886 code indicates the resequencing consensus (SHORE) score. EMS-mutations showing a SHORE
887 score higher than 25 were selected. AFs in chromosome 5 were higher as compared with other
888 regions in the genome (see also Figure S2). (D) Scheme of the *PNY* locus showing the position
889 of the mutation and the sequence change found in *lgf58*.

890 **Figure 2. Pattern of expression of *PNY* during photoperiod flowering.** (A) *PNY* mRNA (i-iii)
891 and protein accumulation (iv-vi). Plants were grown for 14 days under SD (i and iv; vegetative
892 stage) and then shifted to LD for 3 (ii and v; floral transition) and 5 additional days (iii and vi;
893 flower development). Scale bars: 50 μM. (B) ChIP-qPCR showing binding of SOC1:GFP on
894 *PNY* promoter. *y* axis represent the Fold Change (FC) of enrichment (percentage of input)
895 between the qPCR results using positive primers (flanking a CArG-box motif) and the negative
896 ones (flanking the coding sequence of PNY). Letters shared in common between the genotypes
897 indicate no significant difference (t-test, $P < 0.05$). (C) Localization of the primers used for
898 ChIP-qPCR of SOC1:GFP on the *PNY* promoter. Region (+) contains a CArG-box cis-motif
899 which was enriched after immunoprecipitation of SOC1:GFP. Region (-) was used as negative
900 control. (D) Study of the *PNY* expression in plants shifted from SDs to LDs. Plants were grown
901 for 2 weeks under SDs and then shifted to LDs for 3, 5 and 7 additional days. RNA was
902 extracted from dissected shoot apices. Error bars indicate s.d.

903 **Figure 3. *BOP1/2* genes are important for flowering regulation mediated by PNY**.
904 Expression of *BOP1* (A) and *BOP2* (B) of shoot apices dissected from plants grown under LDs
905 for 7 days. Letters shared in common between the genotypes indicate no significant difference (t-
906 test, $P < 0.05$). (C) In situ hybridizations with *BOP2* probe in shoot meristems of Col-0 (i) and

907 *pny-58* (ii). Plants were grown for 7 LDs and stayed at vegetative stage. Expression of *BOP2* in
908 Col-0 plants was observed at the boundaries between leaves and the SAM (black arrow). Scale
909 bars: 50 μM. (D) Flowering time of the *BOP1/2* and *PNY* mutant combinations grown under
910 LDs. CL: cauline leaves, RL: rosette leaves. In (A), (B) and (D) error bars indicate s.d.

911 **Figure 4. *BOP1/2* genes interfere with FT function and are directly bound by PNY.** (A)
912 Flowering time of plants misexpressing *BOP* genes in presence of high levels of *FT*. Letters
913 shared in common between the genotypes indicate no significant difference (t-test, $P < 0.05$).
914 (B) Expression of *LFY* of dissected shoot apices of plants grown for 7 LDs. Asterisks indicate
915 statistical differences between Col-0 and other genotypes (t-test; P = 0.0004 [**] and P = 0.006
916 [*]). (C) Expression of *AP1* of dissected shoot apices of plants grown for 7 LDs. Asterisks
917 indicate statistical differences between Col-0 and other genotypes (t-test; P = 0.001 [***], P =
918 0.006 [**] and P = 0.03 [*]). ChIP-qPCR of PNY on the promoters of *BOP1* (D) and *BOP2* (E).
919 *x* axis indicate the primers used for its qPCR. Asterisks indicate statistical differences between
920 p*PNY::Venus:PNY* and *pny-40126* (t-test; P = 0.0001 [**] and P = 0.02 [*]). (F) Localization of
921 the primers used for the ChIP-qPCR experiment on the *BOP1* (upper scheme) and *BOP2* (lower
922 scheme) loci. The asterisk (*) means AP1 binding genomic regions according to Kaufmann et al
923 (2010). Scale bars: 0.5 Kb. In (A), (B), (C), (D) and (E) error bars indicate s.d.

924 **Figure 5. *BOP1/2* genes regulate the pattern of expression of *FD*.** (A) *FD* expression levels in
925 different plants misexpressing *BOP1/2* and *PNY*. RNA was extracted from shoot apices of plants
926 grown during 7 LDs (vegetative stage), 10-13 LDs (floral transition) and 17 LDs (reproductive
927 stage). Asterisks indicate statistical differences between Col-0 and other genotypes (t-test; P =
928 0.05 [***], P = 0.01 [**] and P = 0.001 [*]). (B) In situ hybridization of plants grown for 10 LDs
929 showing the expression pattern of *FD* in Col-0 (i), *pny-58* (ii), *bop1-3 bop2-1* (iii) and *bop1-6D*
930 (iv). Bar: 50 μM. In (A), (B) and (C) error bars indicate s.d.

931 **Figure 6. Model explaining the spatial regulation of flowering-related genes by *PNY* and**
932 ***BOP1/2*.** It has been suggested that FT is delivered from the phloem to the proximity of the SAM
933 (dashed line) (Yoo et al., 2013). Once FT is in the shoot meristem it is supposed to interact with
934 FD to activate the transcription of *AP1* (yellow shade). After the floral induction, *BOP1/2*
935 activate the transcription of *AP1* mRNA in the FM. We showed that PNY directly represses

936 *BOP1/2* expression (blue shade) in the shoot meristem. So that, in absence of *PNY* (*pny-58*,
937 lower panel), *BOP1/2* pattern of expression becomes broader. We also found that *BOP1/2*
938 repress *FD* expression before floral transition. Thus, the ectopic expression of *BOP1/2* in *pny*
939 mutants leads to the reduction of the expression domain of *FD* mRNA (green shade) in the shoot
940 meristem. Consequently, FT-FD complex formation might be impaired (gray arrow) and the
941 transcriptional activation of *AP1* mRNA reduced.

942

943 **Supplemental Figures**

944 **Figure S1. *late flowering in pGAS1::FT ft-10 tsf-1* (*lgf*) mutants and characterization of**
945 ***lgf58*.** (A) *lgf* mutants identified in a sensitized genetic screen. Bar: 1 cm. (B) Comparison of
946 flowering time of identified *lgf* mutants. (C) Phenotype of *lgf58* compared to p*GAS1::FT ft-10*
947 *tsf-1*, Col-0 and *pny-40126* mutant. *lgf58* showed short stature, lanceolated leaves, phyllotactic
948 abnormalities and late flowering. (D) Quantification by RT-qPCR of expression levels of FT-
949 transcriptionally regulated genes in *lgf58* mutant. Error bars in (B) and (C) indicate s.d. Letters
950 shared in common between the genotypes indicate no significant difference (t-test, $P < 0.05$).
951 Asterisks indicate statistical differences between Col-0 and other genotypes (t-test; $P < 0.05$)

952 **Figure S2. Cloning-by-sequencing of *lgf58* mutation.** (A) Workflow employed to identify the
953 causal mutation in the *lgf58* mutant. (B) Graphics showing the allelic frequency estimations at
954 EMS-induced mutations (AF, *y* axis) across the five chromosomes (Mb, *x* axis) of *lgf58*. AFs
955 were calculated dividing the number of reads supporting the mutant allele by the number of all
956 reads aligning to a given marker. The color code indicates the resequencing consensus (SHORE)
957 score. EMS-mutations showing a SHORE score higher than 25 were selected. AFs in
958 chromosome 5 were higher as compared with other regions in the genome.

959 **Figure S3. Characterization of mutant plants carrying the *pny-58* allele.** (A) Phenotypic
960 comparison between Col-0 and *pny-58*. *pny-58* displayed late flowering, short stature and
961 phyllotactic abnormalities (B) Comparison of flowering time between *pny-40126* (Smith et al.,
962 2003) and *pny-58* mutant plants. Error bars indicate s.d. Letters shared in common between the
963 genotypes indicate no significant difference (t-test, $P < 0.05$).

Figure S4. *PNY* mutations caused suppression of FT. (A) Complementation assay of *pny-58* mutant. p*PNY::Venus:PNY* was crossed to *pny-58*. F2 *pny-58* plants carrying the transgene recapitulated early flowering. (B) The *PNY* mutant allele *pny-40126 in pGAS1::FT ft-10 tsf-1* caused late flowering. Error bars indicate s.d. Letters shared in common between the genotypes indicate no significant difference (t-test, $P < 0.05$).

Figure S5. Functional characterization of p*PNY::Venus:PNY*. Expression of p*PNY::Venus:PNY* in the distal (A) and proximal (B) fruit regions and the central region of a young floral bud (C). Complementation assay of *pny-40126* mutant with p*PNY::Venus:PNY*. p*PNY::Venus:PNY* restored the wild type phenotype in the *pny-40126* mutant. Scale bars: 100 µM.

Figure S6. Photoperiod control of *PNY* pattern of expression. (A) Expression levels of *PNY* in *soc1-2* mutant compared to Col-0. Plants were grown under SDs for two weeks. Aerial parts were used for RNA extraction. Error bars indicate s.d. (B) Pattern of expression of *PNY* in Col-0 (i and ii) and *ft-10 tsf-1* mutants (iii and iv) at vegetative (i and iii) and reproductive stages (ii and iv). Scale bars: 50 µM.

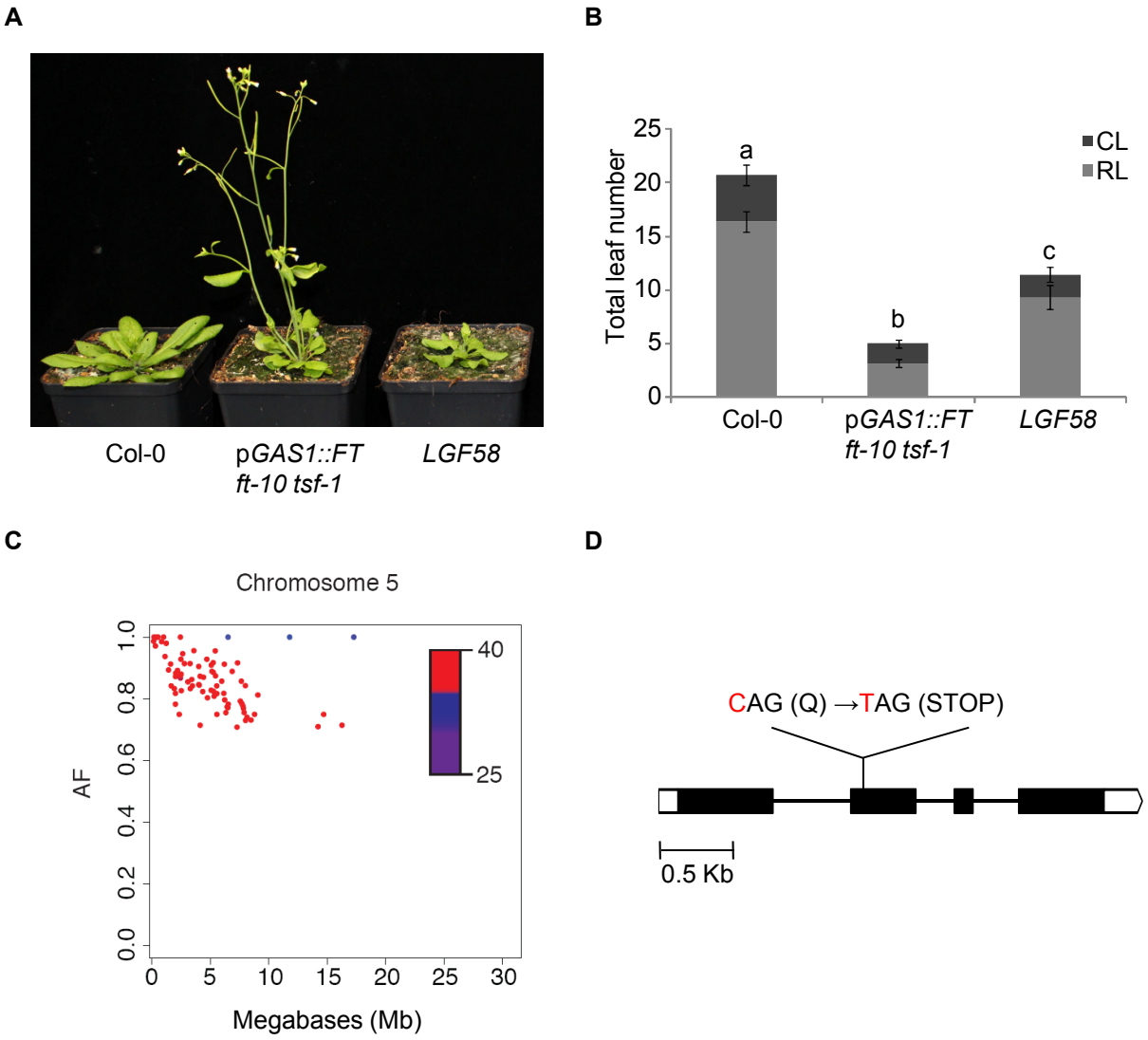
Figure S7. *PNY* controls expression of *BOP1/2* genes. (A) Expression pattern of *BOP2* in shoot meristems of Col-0 (i and iv), *pny-58* (ii and v) and *pny-40126* (iii and vi) during vegetative (I, ii and iii) and floral transition (iv, v and vi) stages. Scale bars: 50 µM. (B) Flowering time of the triple mutants *pny-58 bop1-3 bop2-1*. Letters shared in common between the genotypes indicate no significant difference in flowering time (ANOVA test, Holm-Sidak method, $P = 0.05$). (C) Picture of plants carrying various mutant combinations for *PNY, BOP1/2* and *FT*. (D) ChIP-qPCR to test PNY binding on *TOE1* and *LFY*. Error bars in (B) and (D) indicate s.d. Letters shared in common between the genotypes indicate no significant difference (t-test, $P < 0.05$). (E) Venus:PNY protein levels were detected by immunoblotting assay using an anti-GFP antibody (upper panel) in WT and pPNY:: Venus:PNY inflorescences. CBB, Coomassie Brilliant Blue was used as a loading control (lower panel).

Figure S8. *BOP1/2* genes interfere *FT* signaling pathway by affecting *FD* expression. (A) Phenotypes of plants misexpressing *BOP* genes. (B) Flowering time of different suppressors of *GAS1::FT* (C) Expression levels of FT-regulated genes in *GAS1::FT fd-3* mutant. Letters shared

in common between the genotypes indicate no significant difference (t-test, $P < 0.05$). Expression levels of *LFY* in wild type and *bop1 bop2* plants at different shoot meristem developmental stages. RNA was extracted from shoot apices of plants grown during 7 LDs (vegetative stage), 10-13 LDs (floral transition) and 17 LDs (reproductive stage). Asterisks indicate statistical differences between Col-0 and *bop1 bop2* (t-test, $P < 0.05$). Error bars in (B), (C) and (D) indicate s.d.

Supplemental Table I. Candidate loci identified by SHOREmap.

Supplemental Table II. List of primers used in this work.



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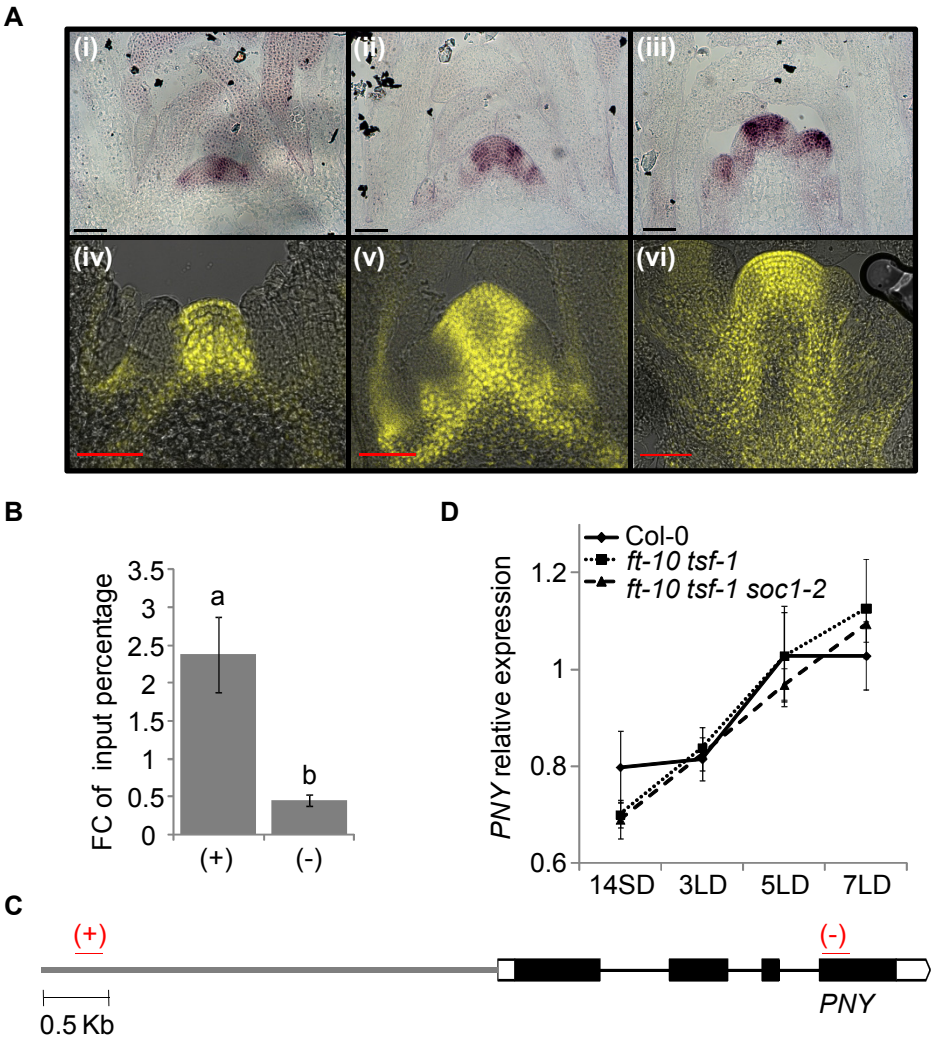

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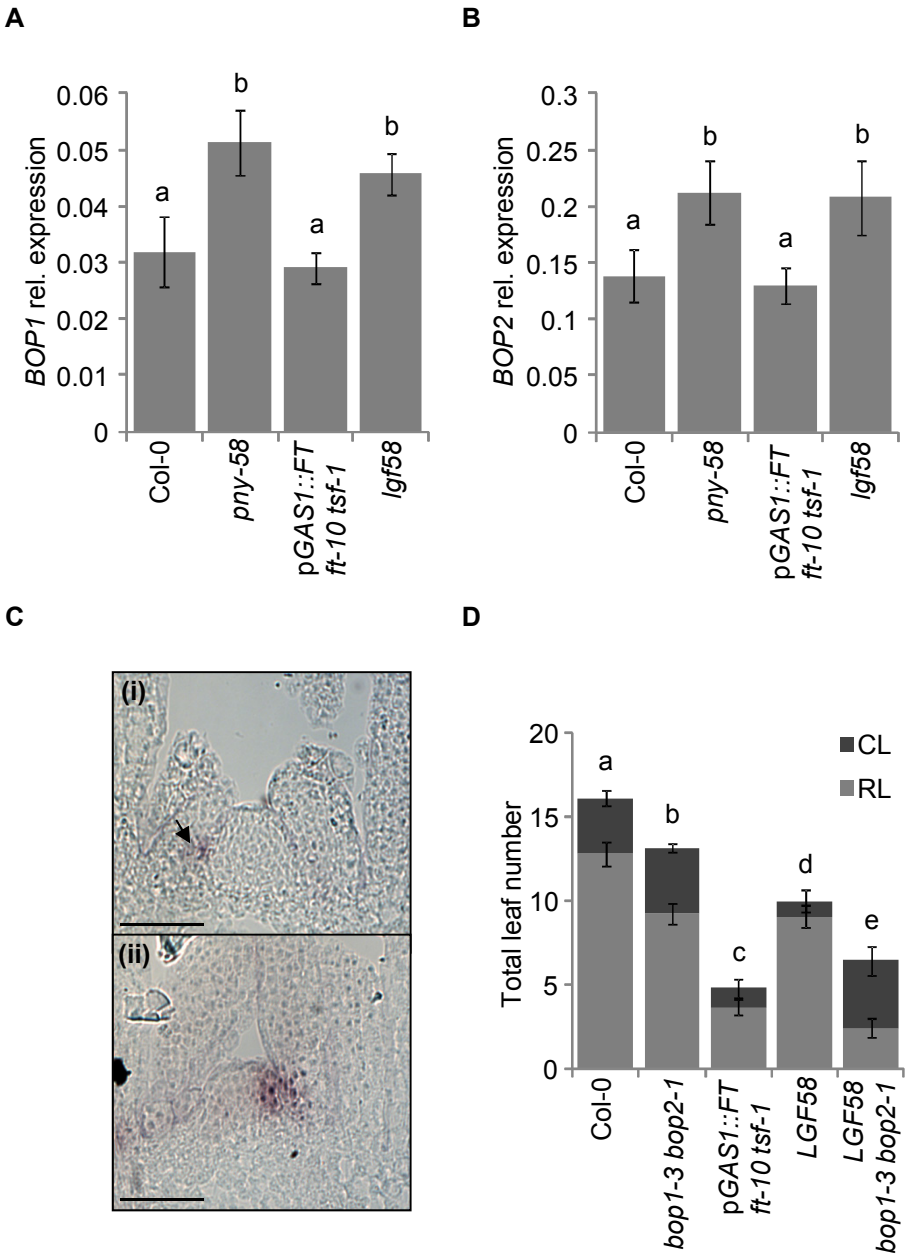
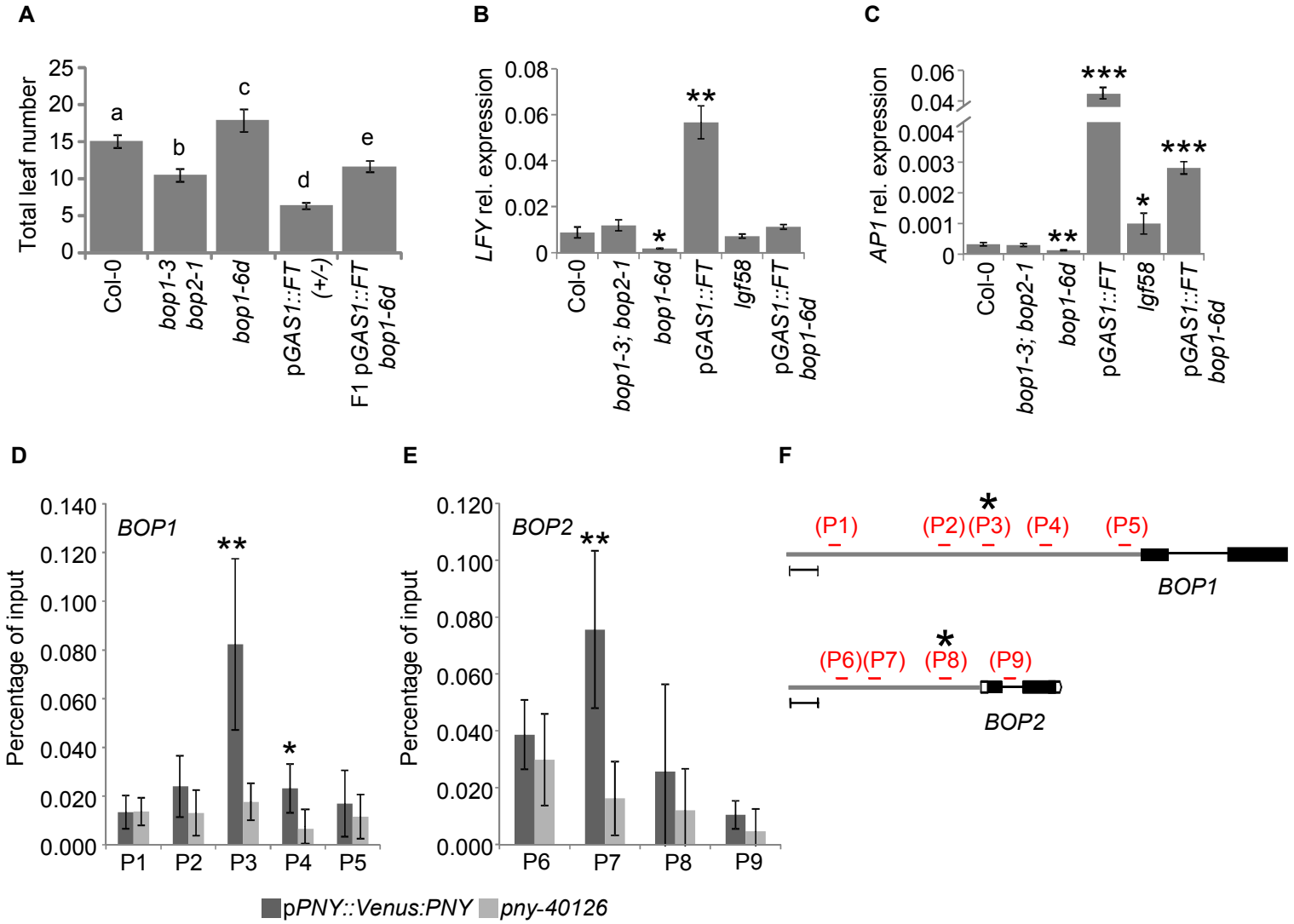


Figure 3. *BOP1/2* genes are important for flowering regulation mediated by PNY. Expression of *BOP1* (A) and *BOP2* (B) of shoot apices dissected from plants grown under LDs for 7 days. Letters shared in common between the genotypes indicate no significant difference (t-test, $P < 0.05$). (C) In situ hybridizations with *BOP2* probe in shoot meristems of Col-0 (i) and *pny-58* (ii). Plants were grown for 7 LDs and stayed at vegetative stage. Expression of *BOP2* in Col-0 plants was observed at the boundaries between leaves and the SAM (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. In (A), (B) and (D) error bars indicate s.d.



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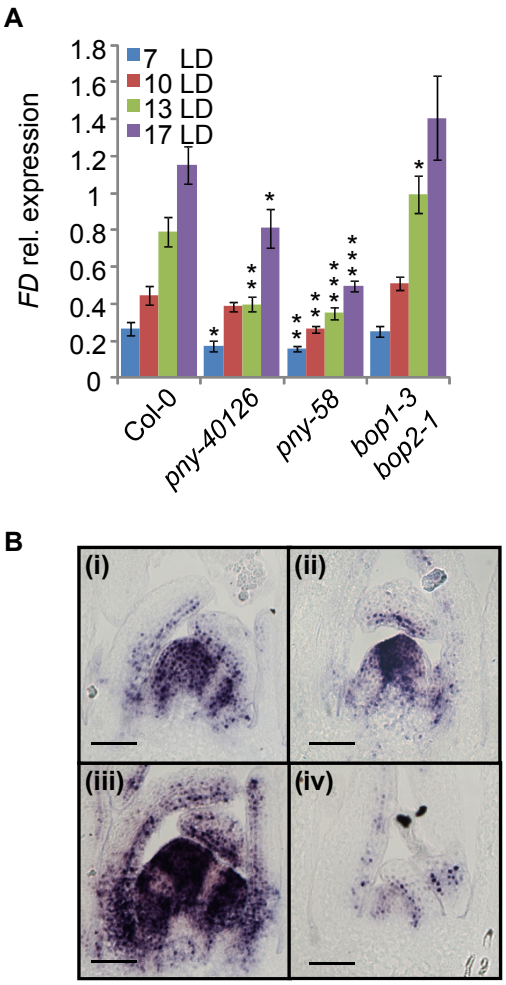

Figure 5. *BOP1/2* genes regulate the pattern of expression of *FD*. (A) *FD* expression levels in different plants misexpressing *BOP1/2* and *PNY*. RNA was extracted from shoot apices of plants grown during 7 LDs (vegetative stage), 10-13 LDs (floral transition) and 17 LDs (reproductive stage). Asterisks indicate statistical differences between Col-0 and other genotypes (t-test; P = 0.05 [***], P = 0.01 [**] and P = 0.001 [*]). (B) In situ hybridization of plants grown for 10 LDs showing the expression pattern of *FD* in Col-0 (i), *pny-58* (ii), *bop1-3 bop2-1* (iii) and *bop1-6D* (iv). Bar: 50 µM. Error bars indicate s.d.

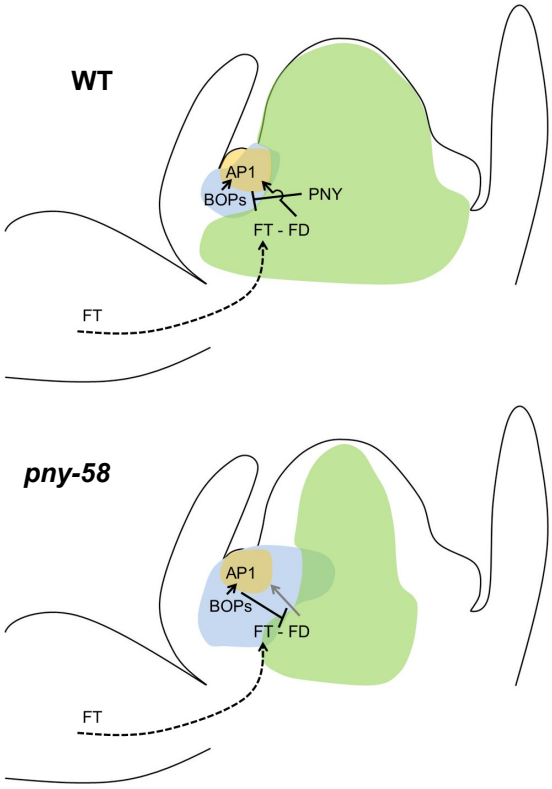

Figure 6. Model explaining the spatial regulation of flowering-related genes by *PNY* and *BOP1/2*. It has been suggested that FT is delivered from the phloem to the proximity of the SAM (dashed line) (Yoo et al., 2013). Once FT is in the shoot meristem it is supposed to interact with FD to activate the transcription of *AP1* (yellow shade). After the floral induction, *BOP1/2* activate the transcription of *AP1* mRNA in the FM. We showed that PNY directly represses *BOP1/2* expression (blue shade) in the shoot meristem. So that, in absence of *PNY* (*pny-58*, lower panel), *BOP1/2* pattern of expression becomes broader. We also found that *BOP1/2* repress *FD* expression before floral transition. Thus, the ectopic expression of *BOP1/2* in *pny* mutants leads to the reduction of the expression domain of *FD* mRNA (green shade) in the shoot meristem. Consequently, FT-FD complex formation might be impaired (gray arrow) and the transcriptional activation of *AP1* mRNA reduced.

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