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Photoperiodic and thermosensory pathways interact through CONSTANS to promote flowering at high temperature under short days

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SUMMARY

Plants detect changes in day length to induce seasonal patterns of flowering. The photoperiodic pathway accelerates the flowering of *Arabidopsis thaliana* under long days (LDs) whereas it is inactive under short days (SDs), resulting in delayed flowering. This delay is overcome by exposure of plants to high temperature (27°C) under SDs (27°C-SD). Previously, the high-temperature flowering response was proposed to involve either the impaired activity of MADS-box transcription factor (TF) floral repressors or PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) TF-mediated activation of *FLOWERING LOCUS T* (*FT*), which encodes the output signal of the photoperiodic pathway. We integrate these observations by studying several PIFs, the MADS-box SHORT VEGETATIVE PHASE (SVP) and the photoperiodic pathway under 27°C-SD. We find that the mRNAs of *FT* and its paralogue *TWIN SISTER OF FT* (*TSF*) are increased at dusk under 27°C-SD compared with 21°C-SD, and that this requires PIF4 and PIF5 as well as CONSTANS (CO), a TF that promotes flowering under LDs. The CO and PIF4 proteins are present at dusk under 27°C-SD, and they physically interact. Although Col-0 plants flower at similar times under 27°C-SD and 21°C-LD the expression level of *FT* is approximately 10-fold higher under 21°C-LD, suggesting that responsiveness to FT is also increased under 27°C-SD, perhaps as a result of the reduced activity of SVP in the meristem. Accordingly, only *svp-41 ft-10 tsf-1* plants flowered at the same time under 21°C-SD and 27°C-SD. Thus, we propose that under non-inductive SDs, elevated temperatures increase the activity and sensitize the response to the photoperiod pathway.

Keywords: photoperiodic flowering, temperature, PHYTOCHROME-INTERACTING FACTOR 4, SHORT VEGETATIVE PHASE, FLOWERING LOCUS T, *Arabidopsis thaliana*.

INTRODUCTION

The reproductive success of plants is enhanced by the precise timing of flowering, which is tightly regulated by both internal and environmental cues. In *Arabidopsis thaliana*, endogenous factors that regulate floral initiation include the age of the plant and the levels of gibberellins, whereas day length and ambient temperature are among the major external inductive signals (Andres and Coupland, 2012; Pose *et al.*, 2012; Song *et al.*, 2013). Here, we explore the intersection between the photoperiodic and high ambient temperature pathways, and show that at high temperature the photoperiodic pathway activates flowering even under non-inductive day lengths.

Arabidopsis thaliana flowers early during the long days (LDs) of summer and late under the short days (SDs) of winter. These differences in day length are detected in the leaves by the photoperiodic flowering pathway. At the core of the regulation of this pathway is the B-box transcription factor CONSTANS (CO), which is regulated by the circadian clock at the transcriptional level and by exposure to light at the post-translational level (Suarez-Lopez *et al.*, 2001; Imaizumi *et al.*, 2003; Valverde *et al.*, 2004). Under LD conditions, a small family of *CO* transcriptional repressors called CYCLING DOF FACTORs are degraded, leading to the upregulation of *CO* mRNA (Sawa *et al.*, 2007; Fornara *et al.*, 2009). Under SDs, *CO* mRNA accumulates during the night and its protein is degraded by a ubiquitin-ligase complex containing CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and SUPPRESSOR OF

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PHYTOCHROME A (SPA) (Laubinger *et al.*, 2006; Liu *et al.*, 2008; Jang *et al.*, 2009; Zuo *et al.*, 2011). Under LDs, the peak of *CO* mRNA occurs when plants are exposed to light at the end of the day. Exposure to light also inactivates the COP1/SPA complex, allowing the accumulation of CO protein under LDs when *CO* mRNA is expressed in the light (Valverde *et al.*, 2004; Jang *et al.*, 2008; Liu *et al.*, 2008; Zuo *et al.*, 2011). In parallel, the FLAVIN KELCH F-BOX 1 protein interacts with CO to stabilize it during the day (Song *et al.*, 2012). CO then directly binds to the promoter of *FLOWERING LOCUS T* (*FT*) and activates its transcription (Kobayashi *et al.*, 1999; Samach *et al.*, 2000; Wigge *et al.*, 2005; Tiwari *et al.*, 2010; Song *et al.*, 2012; Zhang *et al.*, 2015). *FT* is a floral promoter, the expression of which is directly correlated to the timing of flowering (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). Therefore, only under LDs, when CO is stabilized by light, is *FT* transcribed. *FT* and its paralogue *TWIN SISTER OF FT* (*TSF*) encode mobile proteins that are expressed in the companion cells of the leaves and transported through the phloem to the shoot apex (Yamaguchi *et al.*, 2005; Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Mathieu *et al.*, 2007). Upon the arrival of FT in the meristem, it is believed to interact with 14-3-3 proteins and the bZIP transcription factor FD (Abe *et al.*, 2005; Wigge *et al.*, 2005; Taoka *et al.*, 2011), causing transcriptional reprogramming of the shoot meristem and activation of downstream genes such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *FRUITFULL*, *APETALA 1* and *LEAFY* (Schmid *et al.*, 2003; Abe *et al.*, 2005; Wigge *et al.*, 2005; Yoo *et al.*, 2005; Searle *et al.*, 2006; Torti *et al.*, 2012). The activation of these genes leads to the formation of an inflorescence meristem and to the production of flowers.

Other environmental signals, particularly warm ambient temperatures, also promote flowering by activating *FT* transcription. Several processes contribute to this thermosensory flowering pathway. On exposure to high temperatures, H2A.Z histone-containing nucleosomes are removed from temperature-sensitive promoters, including that of *FT* (Kumar and Wigge, 2010; Kumar *et al.*, 2012). These promoters then become more accessible to specific transcription factors that increase the expression of the cognate genes (Kumar and Wigge, 2010). For example, PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) transcription factor binds to the *FT* promoter more strongly when plants are growing under SDs at 27°C (hereafter 27°C-SD) compared with 22°C. Consistent with this observation, the *pif4* null mutant of *A. thaliana* was later flowering than the wild type under 27°C-SD, and flowered with the same number of leaves under 27°C-SD and 22°C-SD, indicating a crucial role for this transcription factor in the thermosensory induction of flowering under these conditions (Kumar *et al.*, 2012). By contrast, the *pif4* null mutant was recently described to flower at times similar to Col-0 plants under 27°C-SD (Galvao *et al.*, 2015). Similar results were observed under LDs at high temperature, where PIF4 was not required for early flowering (Koini *et al.*, 2009). *PIF4* was originally identified because of its contribution to phytochrome B (phyB)-mediated light signalling, and was shown to interact with phyB (Huq and Quail, 2002). PIF4 degradation is mediated by its interaction with active phyA and phyB, which modulates the expression of light-regulated genes (Ni *et al.*, 1999; Huq and Quail, 2002). In light-dark cycles, PIF4 promotes hypocotyl growth at dawn (Nozue *et al.*, 2007). At high temperatures and in *phyB* mutants, PIF4 promotes hypocotyl growth through the direct activation of genes such as *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*), which is involved in the auxin response (Koini *et al.*, 2009; Franklin *et al.*, 2011; Hornitschek *et al.*, 2012).

Several MADS-box transcription factors that act as floral repressors are also implicated in the thermosensory flowering pathway. One of these repressors, SHORT VEGETATIVE PHASE (SVP), delays flowering by reducing the transcription of *FT* and *TSF* in leaves and of *SOC1* in the shoot meristem (Hartmann *et al.*, 2000; Lee *et al.*, 2007; Li *et al.*, 2008; Jang *et al.*, 2009). The *svp* mutants are early flowering and insensitive to changes in ambient temperature, flowering at the same time when exposed to 16°C, 23°C or 27°C under LDs (Lee *et al.*, 2007, 2013; Pose *et al.*, 2013). Moreover, the stability of the SVP protein is reduced at high temperatures, suggesting that the reduction in SVP protein levels at 27°C contributes to early flowering under these conditions (Lee *et al.*, 2013); however, the *svp* mutant retains some responsiveness to warm temperatures under SDs (Galvao *et al.*, 2015). Two other MADS-box transcription factors that delay flowering, FLOWERING LOCUS M (FLM) and MADS AFFECTING FLOWERING 2 (MAF2), also contribute to the thermosensory pathway. The mRNA of *FLM* is alternatively spliced producing two protein varieties, FLM-β and FLM-δ. The *FLM*-β mRNA is the more abundant form at 16°C, whereas at 27°C its level is decreased (Pose *et al.*, 2013). Both protein forms interact with SVP in yeast and *in vitro* to produce the heterodimers SVP-FLM-β and SVP-FLM-δ; however, their activities differ such that SVP-FLM-β binds DNA and represses flowering, whereas SVP-FLM-δ does not bind DNA. This led to a model whereby the alternative splicing of *FLM* mRNA at 27°C increases the ratio of FLM-δ to FLM-β, inhibiting SVP activity and causing earlier flowering (Pose *et al.*, 2013). *MAF2* mRNA is also alternatively spliced at different temperatures, and the form of the protein produced at low temperature also interacts with SVP, whereas the one formed at high temperature does not (Airoldi *et al.*, 2015). Thus, these different mechanisms involving differential protein stability and splicing are proposed to lead to reduced activity of SVP at elevated temperatures. SVP binds to a large number of target genes, predominantly

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repressing their transcription (Gregis *et al.*, 2013; Hu *et al.*, 2014; Mateos *et al.*, 2015). Genetic analysis of several of these genes in *svp* mutants supports the proposal that key functions of SVP in flowering-time control are the direct repression of *FT* transcription in the leaf, of *SOC1* in the shoot apex and the reduction of gibberellin levels at the apex through the repression of *GA20ox2* transcription (Lee *et al.*, 2007; Jang *et al.*, 2009; Andres *et al.*, 2014).

Thus, several transcription factors contributing to the photoperiod and thermosensory flowering pathways converge on the transcriptional regulation of the floral integrator *FT*, but the interactions between them and their relative contributions in controlling flowering at high temperatures are poorly understood. Here, we show that although CO is stabilized by LDs and promotes flowering in response to the photoperiod, it is required under 27°C-SD for the activation of *FT*. Genetic analyses indicate that the response to 27°C-SD in the leaves depends on the coordinate functions of CO, PIF4 and PIF5, as well as SVP, and that the loss of function of the *PIF4/5* genes alone has a weak and variable effect on flowering. Furthermore, the activation of *FT* mRNA in the leaves occurs at much lower levels under 27°C-SD than under 21°C-LD, and we propose that the removal of repressors from the meristem at 27°C is also necessary for responsiveness to such low levels of *FT* expression. In support of this, genetic analysis shows that *svp-41 ft-10 tsf-1* triple mutants are insensitive to increased temperature under SDs. These data provide a genetic and molecular framework for the interaction between the photoperiod and thermosensory pathways.

RESULTS

Timing and amplitude of *FT* transcription under 27°C-SD

The transcriptional activation of *FT* in warm temperatures accelerates flowering under SDs (Balasubramanian *et al.*, 2006; Kumar *et al.*, 2012). Under LDs, *FT* activation occurs in the light 12–16 h after dawn (Suarez-Lopez *et al.*, 2001). To describe the diurnal pattern of *FT* transcription under warm SDs in Col-0 wild-type plants, *FT* mRNA was analysed through a 24-h time course in plants grown at 21°C or 27°C under SDs of 8 h light. *FT* mRNA levels peaked 8 h after dawn (Zeitgeber 8, ZT8) in the 27°C-SD time course, whereas only very low *FT* mRNA expression was detected in the seedlings grown under 21°C-SD (Figure 1a). The contribution of *FT* to flowering under 27°C-SD was then assessed using the null *ft-10* allele. Col-0 plants grown under 27°C-SD flowered much earlier than those grown under 21°C-SD, producing on average 26 and 65 leaves, respectively (Figure 1b). In comparison with Col-0, *ft-10* mutants were strongly delayed in flowering at 27°C although they were still earlier flowering than Col-0 at 21°C (Figure 1b). These results support the idea that *FT* is required for the full acceleration of flowering under these conditions, as previously shown (Balasubramanian *et al.*, 2006; Kumar *et al.*, 2012). By contrast, a null mutant of *TSF* (*tsf-1*) did not show any delay in flowering compared with Col-0 under 27°C-SD (Figure 1b). Although *TSF* mRNA abundance was increased at 27°C-SD (Figure S1), the genetic data suggest that *FT* could compensate for loss of *TSF* activity under these conditions; however, the double mutant *ft-10 tsf-1* flowered moderately later than *ft-10* under 27°C-SD (Figure 1b), indicating that *TSF* plays a significant role under these conditions in the absence of functional *FT*, as previously observed in plants grown under LDs (Yamaguchi *et al.*, 2005; Jang *et al.*, 2009). Nevertheless, even the double mutant *ft-10 tsf-1* flowered slightly earlier under 27°C-SD than under 21°C-SD, suggesting that these plants retained some responsiveness to 27°C. In summary, the acceleration of the flowering time of Col-0 plants under 27°C-SD largely depends on *FT* and *TSF*, and *FT* mRNA exhibits a diurnal pattern of expression with a peak at ZT8.

Col-0 plants growing under 21°C-LD flowered with a similar number of leaves to those growing under 27°C-SD (Figure 1c). Therefore, the levels of *FT* mRNA were directly compared in plants exposed to SDs and high temperatures with those exposed to LDs (Figure 1d). As expected, the maximum accumulation of *FT* mRNA in Col-0 seedlings growing under 21°C-LD and 27°C-SD occurred at ZT16 and ZT8, respectively (Figure 1a and d); however, under 21°C-LD the peak level of *FT* mRNA (ZT16) was at least 10-fold higher than under 27°C-SD (ZT8) (Figure 1d). Therefore, although under these two conditions the flowering time was similar, the absolute levels of *FT* mRNA differed tremendously. This suggests that, although *FT* is required for early flowering under 27°C-SD, its activation alone might not be sufficient to explain the extreme early flowering observed.

PIF4 weakly promotes flowering under 27°C-SD through the transcriptional activation of *FT* in vascular tissue

Under 27°C-SD, PIF4 induces flowering by binding to the *FT* promoter and activating transcription, whereas plants homozygous for the *pif4-101* allele growing in the same environment were strongly delayed in flowering compared with Col-0 (Kumar *et al.*, 2012). Under our 27°C-SD and 21°C-SD conditions, the flowering times of *pif4-101* and *pif4-2* mutants were measured in several experiments that included large numbers of plants of each genotype (Figure 2a). Both mutants were significantly later flowering than Col-0 controls under 27°C-SD (Figure 2a and b), but flowered much earlier than Col-0 or the *pif4* mutants grown under 21°C-SD (Figure 2a). Because of the variability of the flowering response under 27°C-SD, occasional *pif4* mutants flowered within the range of Col-0 under 21°C-SD (Figure 2a); however, under our conditions neither *pif4-101* nor *pif4-2* mutants showed as strong a suppression of the early-flowering response under 27°C-SD as

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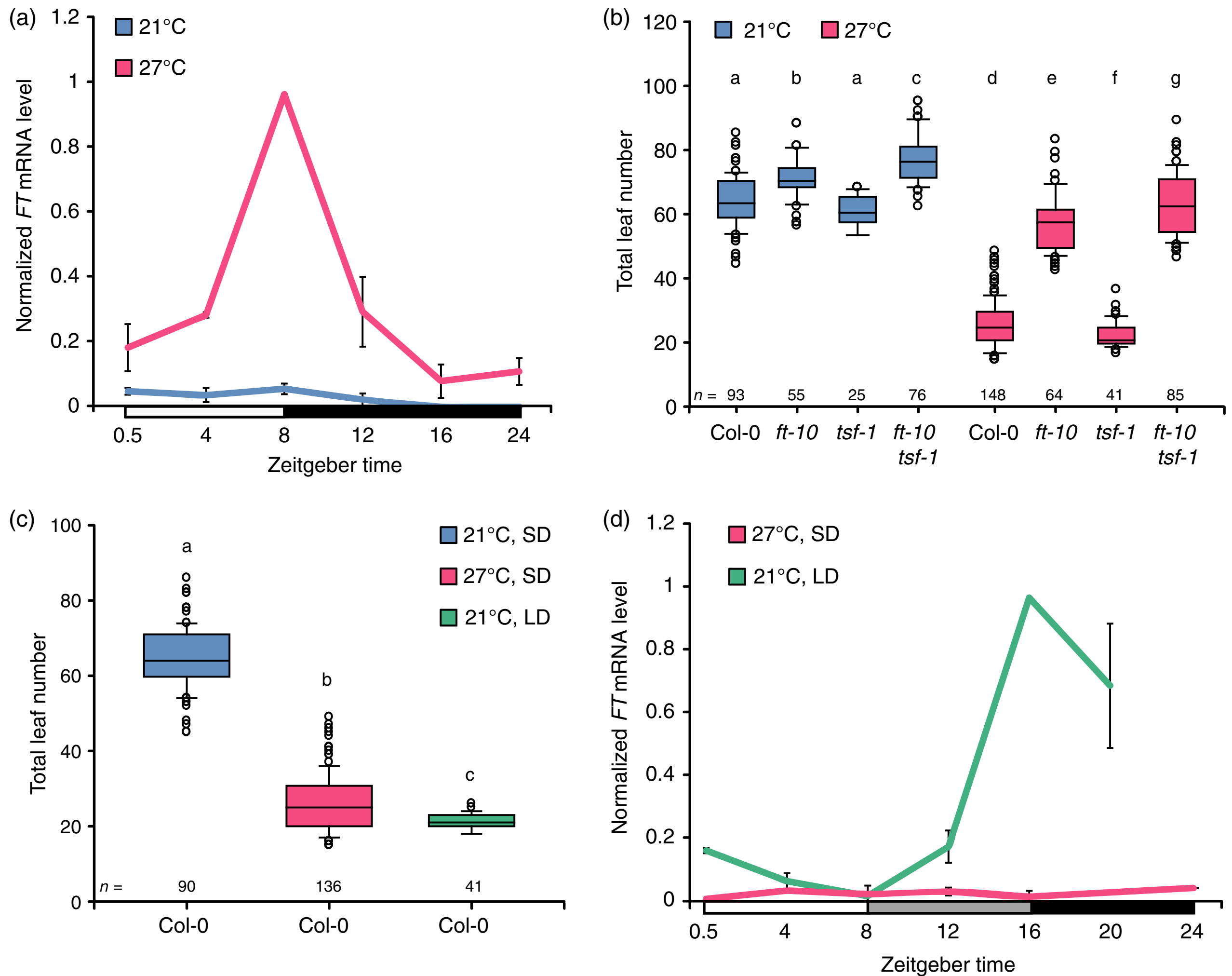

Figure 1. Role of *FT* on the flowering response to warm temperature under SDs. (a) *FT* mRNA expression time course in 14-day-old Col-0 seedlings grown under 21°C-SD and 27°C-SD. (b) Flowering time of plants grown under 21°C-SD and 27°C-SD. (c) Flowering time of Col-0 plants grown under 21°C-SD, 27°C-SD and 21°C-LD. (d) *FT* mRNA expression profile in 14- and 10-day-old Col-0 seedlings grown under 27°C-SD and at 21°C-LD, respectively. At this stage seedlings in both conditions were at the same developmental stage. In (a) and (d), seedlings were harvested every 4 h for 24 h and *FT* mRNA expression was measured by qRT-PCR; error bars are standard errors (SEs) of three and two (27°C-SD ZT16, 27°C-SD ZT24, 21°C-SD ZT16) independent biological replicates in (a) and two independent biological replicates in (d). For time points 21°C-SD ZT24 in (a) and 27°C-SD ZT24 in (d) n = 1. Data point 27°C-SD ZT20 in (d) was not determined. In (b) and (c), letters indicate statistical groups determined with a two-way analysis of variance and multiple comparisons with the Holm–Sidak method. Multiple comparisons were performed within temperatures and within genotypes. Groups were considered statistically different when $P \leq 0.05$.

previously described (Figure 2a) (Kumar *et al.*, 2012). The effect on flowering time of the loss of function of other members of the *PIF* family was tested, because of their potential functional redundancy with *PIF4*. Interestingly, the *pif5-3* single mutant flowered at similar times to the *pif4* mutants, whereas the double mutant *pif4-2 pif5-3* flowered later than the single mutant *pif4-2* (Figure 2a), suggesting that *PIF5* also has a role in the promotion of flowering under warm SDs. By contrast, *PIF1* and *PIF3*, two other members of the family, do not seem to play a role in flowering regulation under SDs at warm temperature, because flowering of the quadruple mutant *pif1-1 pif3-3 pif4-2 pif5-3* was not significantly delayed compared with *pif4-2 pif5-3* (Figure 2a).

Whether the flowering behaviour of these single and higher order *pif* mutants is correlated with changes in *FT* mRNA level was examined by quantifying the abundance of *FT* mRNA at ZT8 in seedlings grown under 21°C-SD and 27°C-SD (Figure 2c). As expected, a significant reduction in *FT* mRNA level at ZT8 was observed in *pif4-2* and *pif4-101* single mutants compared with Col-0 at 27°C, but not at 21°C. Although *pif5-3* mutants were late flowering, no reduction in *FT* mRNA levels was detected in single mutants, suggesting that PIF5 might mainly affect *FT* mRNA at other stages of development, or that it affects flowering by another route; however, the reduction in *FT* mRNA observed in *pif4-2* was enhanced in seedlings of the double mutant *pif4-2 pif5-3* (Figure 2c), and was enhanced

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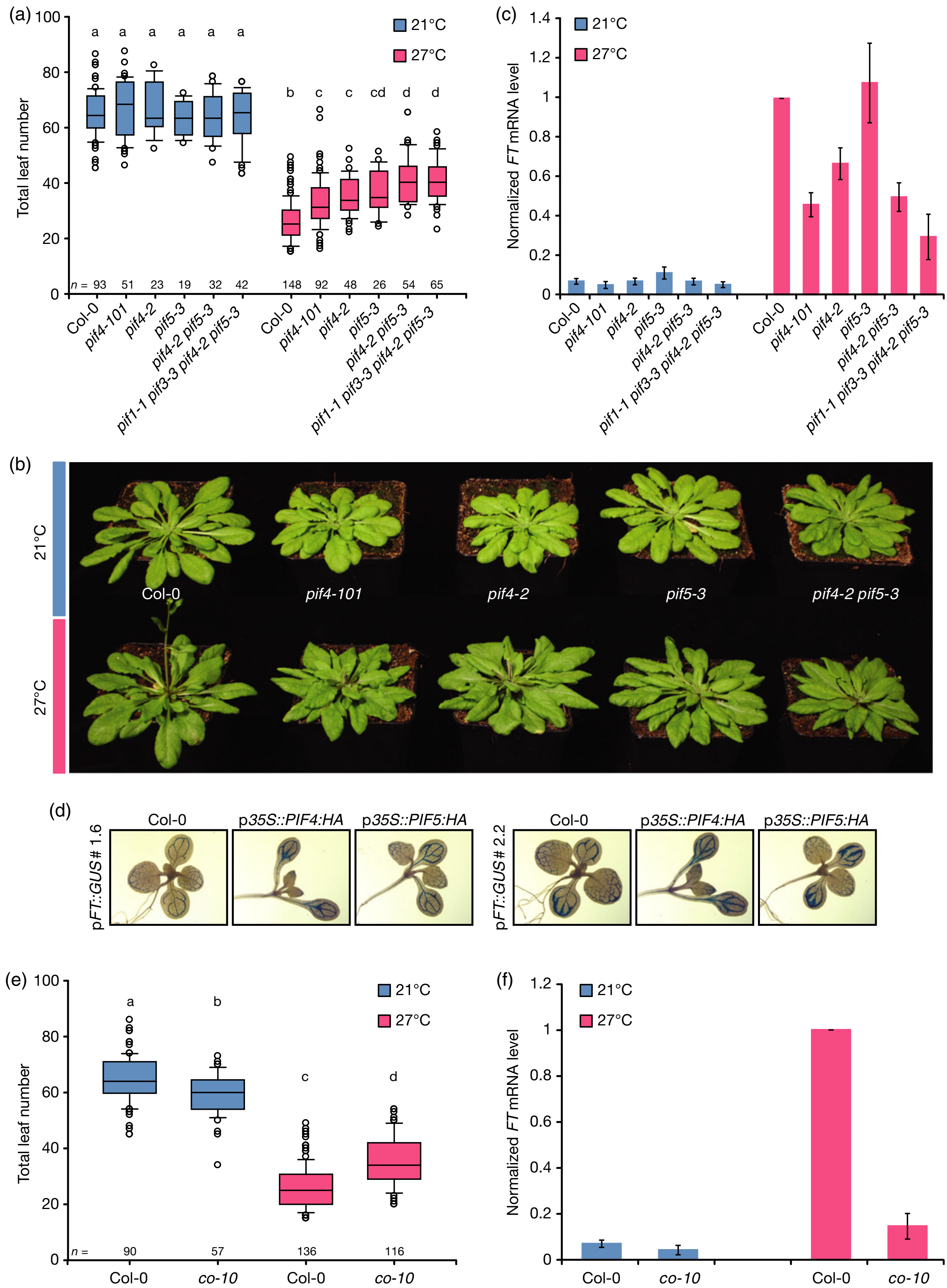
(a)
Total leaf number
21°C
27°C
a a a a a a
b c c cd d d
n = 93 51 23 19 32 42 148 92 48 26 54 65
Col-0
pif4-101
pif4-2
pif5-3
pif4-2 pif5-3
pif1-1 pif3-3 pif4-2 pif5-3
(c)
Normalized FT mRNA level
(b)
21°C
27°C
Col-0
pif4-101
pif4-2
pif5-3
pif4-2 pif5-3
(d)
Col-0
p35S::PIF4:HA
p35S::PIF5:HA
pFT::GUS # 1.6
pFT::GUS # 2.2
(e)
a b c d
n = 90 57 136 116
co-10
(f)

slightly further in the quadruple mutant *pif1-1 pif3-3 pif4-2 pif5-3* (Figure 2c), although this reduction was apparently not sufficient to cause a significant difference in flowering time (Figure 2a).

In order to define the spatial pattern of *FT* expression mediated by *PIF4*, a construct that facilitates the misexpression of *PIF4* from the constitutive promoter CaMV *35S* (p*35S::PIF4:HA*) (Lorrain *et al*., 2008) was introduced into plants harbouring the p$FT_{1.8\ kb}$::GUS (Adrian *et al*., 2010) (p*35S::PIF4:HA*; p*FT::GUS*). Under SDs and LDs, GUS expression was detected only in the vascular tissue (Figures 2d and S2). A similar GUS expression pattern was observed in *PIF5* overexpressing lines (p*35S::PIF5:HA*) (Lorrain *et al*., 2008) under LDs (Figure 2d). These results indicated that although *PIF4* is broadly expressed in the leaves of wild-type plants (Kumar *et al*., 2012), and presumably p*35S::PIF4:HA* transgenics, its effect on *FT* mRNA induction occurs only in the vascular tissue, where *FT* is normally expressed.

CO is required for the full activation of *FT* transcription and promotion of flowering under 27°C-SD

The data presented above suggest that, besides *PIF4* and *PIF5*, there must be other factors promoting early flowering and *FT* transcription under 27°C-SD. CO induces *FT* transcription specifically in the vascular tissue under LDs (An *et al*., 2004; Adrian *et al*., 2010), as was observed for p*35S::PIF4:HA* (Figure 2d). Therefore, the contribution of CO to *FT* mRNA expression under 27°C-SD was tested. To this end, the flowering times of *co-10* mutant plants and Col-0 growing under 27°C-SD were compared (Figure 2e). A significant delay in flowering of the *co-10* mutants was observed, although they still flowered much earlier than plants of the same genotype grown under 21°C-SD. Therefore, as observed for two *pif4* mutant alleles, *pif5-3* and *pif4-2 pif5-3*, the *co-10* mutation partially reduced the flowering response to 27°C-SD. Similarly, *FT* mRNA levels in *co-10* mutants were significantly reduced compared with Col-0 under 27°C-SD at ZT8, but not under 21°C-SD (Figure 2f). A similar result was observed for *TSF* mRNA levels (Figure S1). Analysis of a second *co* mutant allele confirmed the effect of CO on flowering time and *FT* mRNA expression under 27°C-SD (Figure S3a, b). Therefore, *CO* contributes to the activation of *FT* mRNA expression during the thermosensory induction of flowering under SDs.

The simultaneous accumulation of CO and PIF4 proteins overlaps with the peak in FT transcription at ZT8 under 27°C-SD

Our data support the idea that both CO and PIF4 are required for the full induction of flowering at warm temperatures under SDs. Therefore, the timing of expression of these proteins was tested under 27°C-SD and compared with the timing of the peak in *FT* transcription. Transgenic p*CO::HA:CO co-10* seedlings were used (Sarid-Krebs *et al*., 2015) and HA:CO protein abundance was followed through 24-h time courses. A diurnal peak of CO protein accumulation was observed at ZT8, both at 21°C and 27°C (Figure 3a), although this is of much lower amplitude than under LDs (Sarid-Krebs *et al*., 2015). Quantification of CO protein revealed that it is slightly more abundant (by less than twofold) at 27°C compared with 21°C (Figure 3b), suggesting that warm temperatures might favour the accumulation of CO protein at ZT8. The analysis of *CO* mRNA indicated that this increase in CO protein at ZT8 might result from the presence of more *CO* mRNA at this time under 27°C compared with 21°C (Figure 3c).

To test the accumulation of PIF4 protein under 21°C-SD and 27°C-SD, p*PIF4::PIF4:HA pif4-101* seedlings were used (Hornitschek *et al*., 2012). Figure 4(d and e) show that PIF4:HA was present during the day in a relatively unchanged and similar level at both temperatures, whereas it rapidly disappeared in darkness. At the end of the night, PIF4 protein levels increased, reaching higher levels at 27°C compared with 21°C. Analysis of *PIF4* mRNA showed that differences in the protein expression pattern between these temperatures correlated with changes in mRNA expression (Figure 3f).

The results presented above showed that CO and PIF4 proteins accumulate at the same time as the peak of *FT* mRNA under 27°C-SD. Moreover, CO and PIF4 directly bind to similar regions of the *FT* promoter (Tiwari *et al*., 2010; Kumar *et al*., 2012; Song *et al*., 2012; Zhang *et al*., 2015). Thus, we hypothesized that CO and PIF4 proteins might physically interact as a part of the mechanism promoting *FT* induction under warm SDs. In order to test this possibility, co-immunoprecipitation assays were carried out in infiltrated *Nicotiana benthamiana* leaves. PIF4:HA was co-immunoprecipitated with CO:YFP protein (Figure 3g), demonstrating the existence of a physical interaction between CO and PIF4 that might be important for the activation of *FT* transcription under 27°C-SD.

Thus, CO and PIF4 proteins are both present at ZT8 (Figure 3a and d), when the peak of *FT* mRNA under

Figure 2. Mutations in *CO* and *PIF4* affect flowering time and *FT* expression under 27°C-SD. (a) Flowering time of plants grown under 21°C-SD and 27°C-SD. (b) Pictures of representative plants of each genotype grown under 21°C-SD and 27°C-SD. (c) *FT* mRNA expression in seedlings grown under 21°C-SD and 27°C-SD. (d) GUS staining of two independent transgenic lines harbouring the p*FT::GUS* fusion construct (#1.6 and #2.2) in Col-0, p*35S::PIF4:HA* or p*35S::PIF5:HA* backgrounds. Plants were grown for 10 days under LDs and harvested at ZT16 before GUS staining (7 h staining time). (e) Flowering time of Col-0 and *co-10* plants grown under 21°C-SD and 27°C-SD. (f) *FT* mRNA expression in Col-0 and *co-10* seedlings grown under 21°C-SD and 27°C-SD. In (c) and (f), seedlings were grown for 14 days and harvested at 8 h after dawn (Zeitgeber 8, ZT8). *FT* mRNA expression was measured by qRT-PCR; error bars are standard errors (SEs) of at least six and seven independent biological replicates, respectively. Statistical analysis as described in Figure 1.

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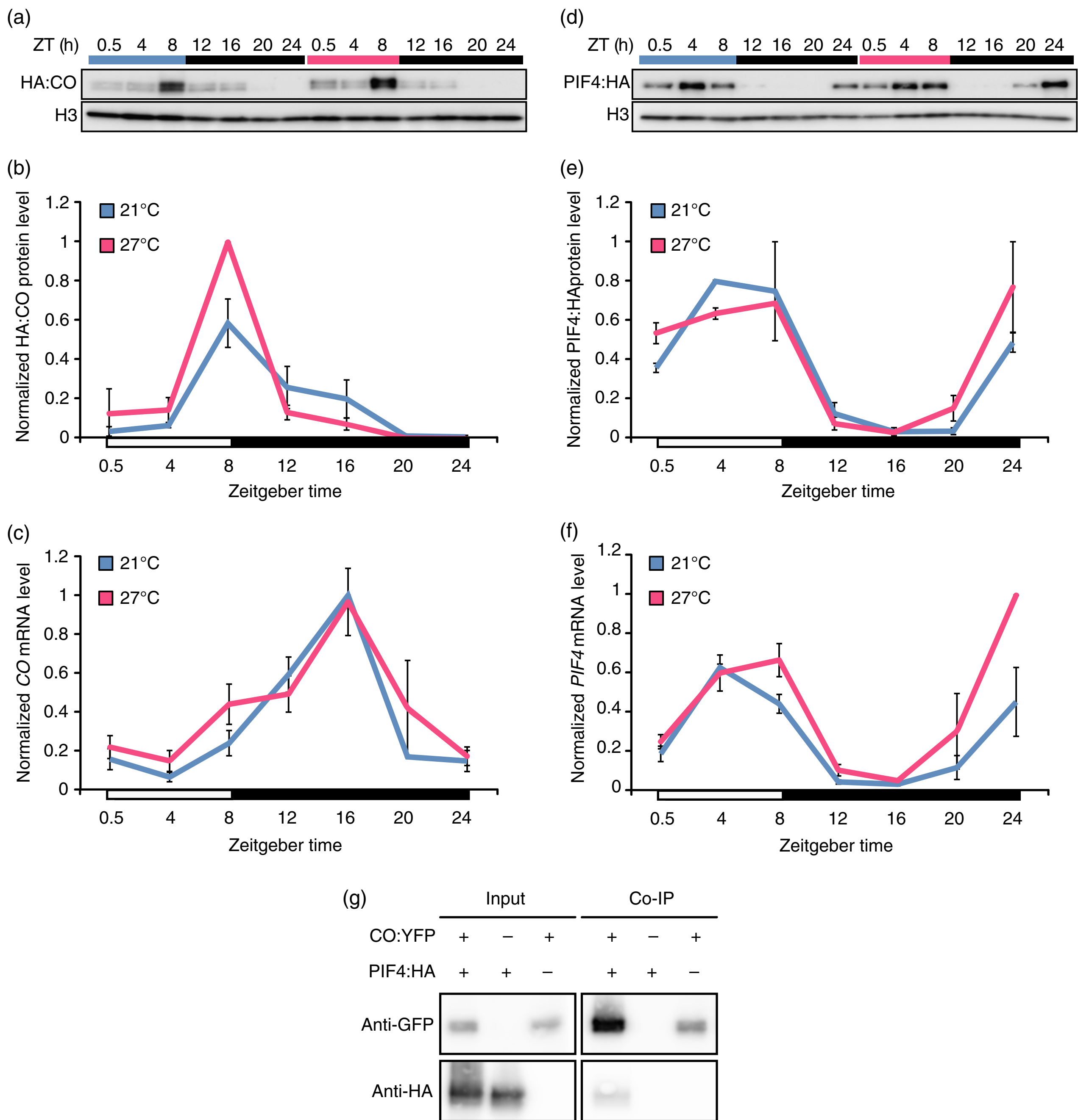

Figure 3. High levels of CO and PIF4 protein overlap 8 h after dawn (Zeitgeber 8, ZT8). (a) Western blot from nuclear protein extracts of p*CO::HA:CO co-10* plants. Time course showing HA:CO protein accumulation over 24 h. (b) HA:CO protein quantification. (c) *CO* mRNA expression in Col-0 seedlings grown under 21°C-SD and 27°C-SD. (d) Western blot from nuclear protein extracts of p*PIF4::PIF4:HA pif4-101* plants. Time course showing PIF4:HA protein accumulation over 24 h. (e) PIF4:HA protein quantification. (f) *PIF4* mRNA expression in Col-0 seedlings grown under 21°C-SD and 27°C-SD. (g) *In vivo* co-immunoprecipitation of PIF4:HA protein with CO:YFP. CO:YFP was precipitated with anti-GFP antibody and co-precipitation of PIF4:HA was detected by western blot using anti-HA antibody. In (a) and (d), nuclear proteins were extracted from seedlings grown for 14 days under 21°C-SD or 10 days under 21°C-SD, and shifted to 27°C-SD for 4 days. The effect of the shift on *FT* mRNA expression was verified as shown in Figure S4. The western blot images shown are representative results from three (a) and (d) two independent biological replicates. In (b) and (e), error bars are standard errors (SEs) of two independent biological replicates. In (c) and (f), 14-day-old seedlings were harvested every 4 h for 24 h. *CO* and *PIF4* mRNA expression was measured by qRT-PCR; error bars are SEs of three independent biological replicates for all time points, except for: 27°C-SD ZT20 and 21°C-SD ZT24 in (c) and 27°C-SD ZT20 and 21°C-SD ZT20 in (f), where two independent biological replicates were performed. For time point 21°C-SD ZT20 in (c) $n = 1$.

27°C-SD was observed (Figure 1a). In addition, CO and PIF4 protein physically interact. These results suggest that the presence of both proteins at ZT8 allow them each to contribute in activating *FT* transcription and accelerating flowering at warm ambient temperatures under SDs.

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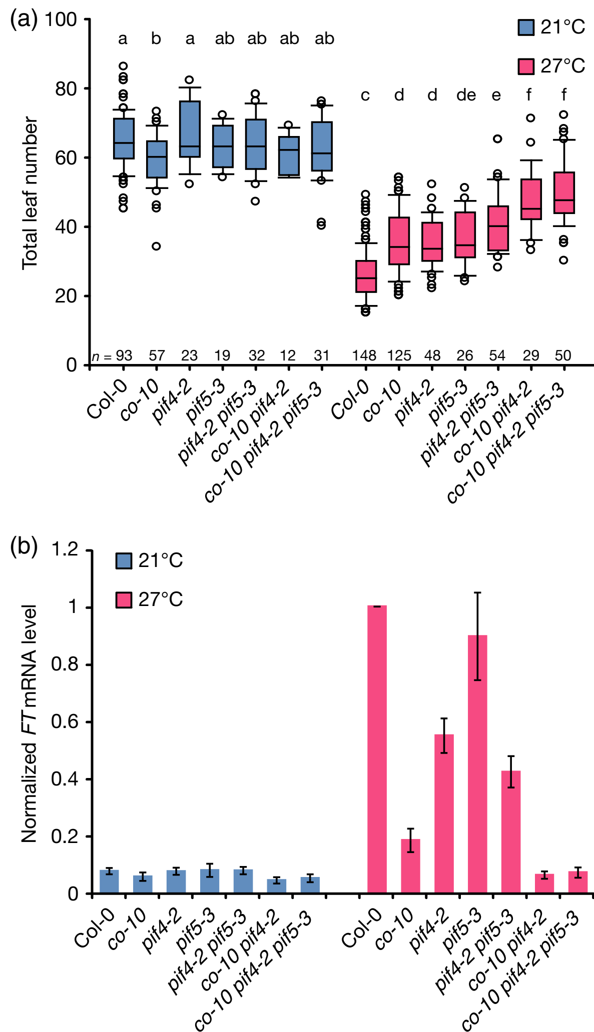

Figure 4. *CO* and *PIF4* have an additive effect on flowering time under 27°C-SD. (a) Flowering time and (b) *FT* mRNA expression of plants grown under 21°C-SD and 27°C-SD. In (b) seedlings were grown for 14 days and harvested at 8 h after dawn (Zeitgeber 8, ZT8). *FT* mRNA expression was measured by qRT-PCR; error bars are standard errors (SEs) of four independent biological replicates. Statistical analysis as described in Figure 1.

Genetic analysis demonstrates that *CO* and *PIF4* are additive in promoting flowering under 27°C-SD

CO and PIF4 bind to the *FT* promoter, both are co-expressed when *FT* is transcribed at peak levels under 27°C-SD and the two proteins physically interact. The significance of these observations was tested genetically by constructing the *co-10 pif4-2* and *co-10 pif4-2 pif5-3* double and triple mutants. Under 27°C-SD, *co-10 pif4-2* and *co-10 pif4-2 pif5-3* plants flowered later than the corresponding single mutants, so that the triple mutant flowered after producing on average 24 leaves more than Col-0 (Figure 4a). Whether the genetic interaction between *co-10* and the *pif* mutations was additive or whether there was a synergistic effect was then tested. Statistical analysis of the flowering-time data of single mutants and combinations indicated that there is not a synergistic interaction between the *co-10*, *pif4-2* and *pif5-3* mutations, but rather that the later-flowering phenotype of the triple mutant can be explained by an additive effect. Therefore, the *co-10 pif4-2 pif5-3* triple mutant is strongly impaired in its responsiveness to 27°C-SD, although it still flowered earlier under 27°C-SD than under 21°C-SD (Figure 4a).

FT mRNA levels were then tested in the *co-10 pif4-2* and *co-10 pif4-2 pif5-3* backgrounds at ZT8 under 27°C-SD. The abundance of *FT* mRNA was strongly reduced in the *co-10 pif4-2* and *co-10 pif4-2 pif5-3* plants compared with Col-0, and was similar to that observed in Col-0 growing under 21°C-SD (Figure 4b). This result indicates that the increased *FT* expression under 27°C-SD is almost entirely explained by the activity of CO, PIF4 and PIF5. Similar results were obtained for *TSF* mRNA levels in double and single mutants (Figure S1). These observations were supported by a significant correlation ($R^2 = 0.6$; $P = 0.01$) between flowering time and the levels of *FT* mRNA of plants mutated in *CO* or the *PIF* genes and the corresponding double and triple mutants (Figure S5).

Misexpression of *PIF4* in the companion cells accelerates flowering and activates the transcription of *FT*

PIF4 and *CO* act as positive regulators of flowering by inducing the transcriptional activation of *FT* under 27°C-SD. In agreement with this, transgenic plants misexpressing *PIF4* from a companion cell-specific promoter (p*SUC2*) were very early flowering under 21°C-SD as well as 27°C-SD (Figure 5a). In p*SUC2::PIF4* plants, *FT* mRNA levels were much higher than in Col-0 (Figure 5b), and this increase was stronger under 27°C-SD. The higher level of *FT* mRNA under 27°C-SD was associated with the earlier flowering of p*SUC2::PIF4* plants under these conditions. This acceleration of flowering was observed despite the very high levels of *FT* mRNA present in p*SUC2::PIF4* plants under 21°C-SD. In addition, p*SUC2::PIF4* overexpression strongly accelerates the flowering of *co-10* mutant plants (Figure 5a). This acceleration again correlates with a strong induction of *FT* mRNA levels under both 21°C-SD and 27°C-SD (Figure 5b). Therefore, *PIF4* overexpression in the vasculature can overcome the requirement for *CO* on the induction of flowering under 27°C-SD.

SVP, *FT* and *TSF* are essential for the thermosensory induction of flowering under SDs

Under LDs, *svp* mutants are insensitive to warm ambient temperatures (Lee *et al.*, 2007, 2013; Pose *et al.*, 2013). By contrast, under 27°C-SD *svp-41* flowered with around six leaves fewer than under SD-21°C (Figure 6a). The *svp-41* mutant also shows an increased expression of *FT* and *TSF* mRNA at ZT8 under 27°C-SD compared with 21°C-SD, as

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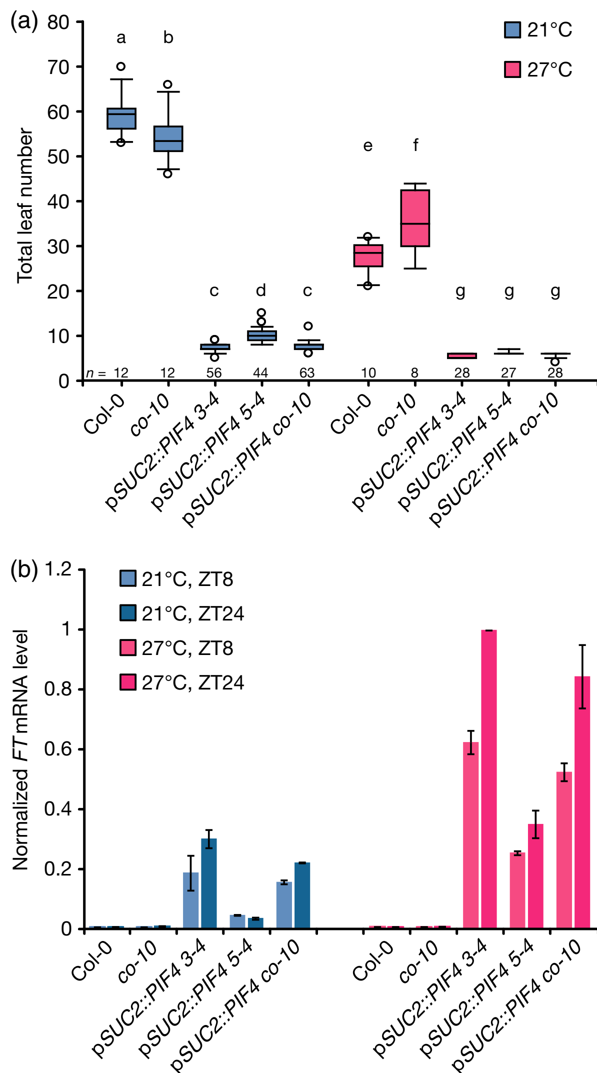

Figure 5. Misexpression of *PIF4* in the companion cells can overcome the requirement of CO for the induction of *FT* mRNA and acceleration of flowering. (a) Flowering time and (b) *FT* mRNA expression of plants grown under 21°C-SD and 27°C-SD. In (b) seedlings were grown for 10 days and harvested at 8 h after dawn (Zeitgeber 8, ZT8) and ZT24; error bars are standard errors (SEs) of two independent biological replicates. Statistical analysis as described in Figure 1.

observed in Col-0 (Figures 6b and S1). To test for a genetic interaction between the *SVP*-dependent and the *CO*- and *PIF*-dependent thermosensory flowering pathways, the flowering times of *svp-41 pif4-101* and *svp-41 co-10* double mutants were determined. Mutations in *SVP* strongly reduced the delay in flowering of *co-10* and *pif4-101* mutants grown under 27°C-SD (Figure 6a). The triple mutant *svp-41 co-10 pif4-101* was generated, and was significantly later flowering than the double mutants *svp-41 co-10* and *svp-41 pif4-101* (Figure 6a), although it retained a flowering response to 27°C-SD. Mutations in *CO* and *PIF4* also reduced the expression of *FT* mRNA in the *svp-41* mutant at 27°C-SD (Figure 6b).

Both CO and PIF4 promote the transcription of *FT* and *TSF* under 27°C-SD, and therefore the *svp-41 ft-10 tsf-1* mutant was also tested. In contrast to *svp-41 co-10 pif4-101*, the *svp-41 ft-10 tsf-1* triple mutant showed no flowering response to high temperature under SDs, and was the only genotype tested that did not flower earlier under these conditions (Figure 6c). Additionally, *svp-41 co-10 pif4-101* and *svp-41 ft-10 tsf-1* flower at similar times under 21°C-SD but *svp-41 co-10 pif4-101* flowers earlier under 27°C-SD, suggesting that in *svp-41 co-10 pif4-101* plants *FT TSF* transcription still responds to higher temperature, perhaps through PIF5 (Figure 6c). Taken together, these results demonstrate that the thermosensory induction of flowering occurs in the leaves through the promotion of *FT* and *TSF* transcription by CO and PIF4/5, as well as the inactivation of the repressor SVP, whereas the reduction of SVP activity has an additional effect in the *ft-10 tsf-1* double mutant that is likely to occur in the meristem. Additional evidence of the role of SVP in the thermosensory response in the meristem was obtained using p*KNAT1::SVP svp-41* plants (Andres *et al*., 2014), which showed delayed flowering under 27°C-SD (Figure 6c). An alternative possibility to reduced SVP activity at the meristem is that promoters of flowering expressed such as SOC1 might respond directly to higher temperatures in the meristem; however, analysis of *SOC1* mRNA under 21°C-SD and 27°C-SD detected no increase at higher temperatures (Figure S6). Therefore, a reduction of SVP activity in the meristem under 27°C-SD is proposed to increase sensitivity to the low level of FT TSF expressed under these conditions.

DISCUSSION

The photoperiodic pathway in *A. thaliana* promotes flowering specifically under LDs, so that flowering occurs rapidly under these conditions but is delayed under SDs. This delay can be overcome, however, by growing plants at high temperatures, so that they flower with similar numbers of leaves under 27°C-SD and 21°C-LD. We therefore examined the effect of high temperature on the activity of the photoperiodic pathway under SDs. Consistent with previous data, we found that *FT* and *TSF*, the output genes of the photoperiodic pathway, are essential for extreme early flowering under 27°C-SD, and that their transcript abundance is increased under these conditions. Also, we show that under 27°C-SD, CO, a central component of the photoperiodic pathway, contributes additively with PIFs to increase the expression of *FT* mRNA. Nevertheless, even this additive effect of CO and PIFs causes *FT* mRNA to accumulate to much lower levels than in plants exposed to LDs. The reduced activity of floral repressors such as SVP in the shoot meristem of plants grown at 27°C might cause a greater responsiveness to the FT signal, allowing early flowering under 27°C-SD despite the low levels of *FT*

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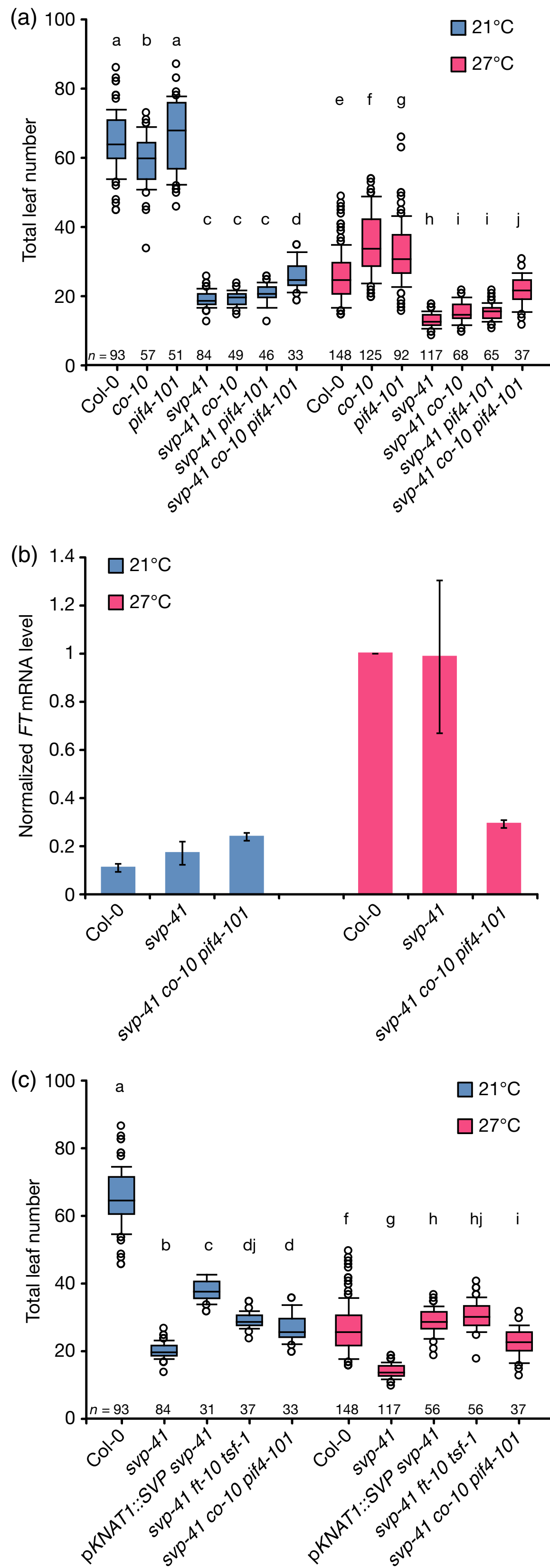

Figure 6. Convergent function of *SVP*, *CO* and *PIF4* in the regulation of *FT* and the relevance of *SVP* in the meristem on the flowering response to warm temperatures under short days (SDs). (a) Flowering time of plants grown under 21°C-SD and 27°C-SD. (b) *FT* mRNA expression in seedlings grown under 21°C-SD and 27°C-SD. (c) Flowering time of plants grown under 21°C-SD and 27°C-SD. In (b), seedlings were grown for 14 days and harvested at 8 h after dawn (Zeitgeber 8, ZT8). *FT* mRNA expression measured by qRT-PCR; error bars are standard errors (SEs) of at least two independent biological replicates. Statistical analysis as described in Figure 1.

mRNA. Consistent with this idea we show that *svp-41 ft-10 tsf-1* triple mutants show no flowering response to 27°C-SD, and indeed this was the only genotype tested that did not flower earlier when exposed to these conditions. Taken together, our results suggest that under higher temperatures the threshold level of the photoperiodic pathway components required to activate *FT TSF* transcription is lowered so that the genes are expressed under non-inductive photoperiods, and that the level of FT TSF required to induce flowering is reduced.

Low levels of *FT* mRNA under 27°C-SD are sufficient to promote early flowering

Compared with plants grown under 21°C-SD, exposure of plants to 21°C-LD or to high temperatures under SDs causes a dramatic acceleration of flowering, by approximately 43 and 39 leaves, respectively (Figure 1c). *FT* strongly contributes to both responses (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Samach *et al.*, 2000; Suarez-Lopez *et al.*, 2001; Balasubramanian *et al.*, 2006; Kumar *et al.*, 2012; Galvao *et al.*, 2015), but the level of transcriptional activation of *FT* under 27°C-SD or 21°C-LD is not proportional to the degree of acceleration of flowering time under these conditions, because we found that *FT* mRNA is over 10-fold more abundant under 21°C-LD than under 27°C-SD. This discrepancy might be explained if similar levels of FT protein accumulate under each condition despite the differences in the levels of *FT* mRNA, a possibility that remains to be tested.

In addition, exposure to high temperatures reduces the activity of floral repressors, suggesting that floral induction might be more sensitive to lower levels of FT under 27°C-SD. An alternative hypothesis that floral integrators expressed in the meristem are induced directly by high temperature was tested for *SOC1*, and no response was detected (Figure S6). By contrast, high temperature reduces the floral repressor activity of the MADS box transcription factors SVP, FLM and MAF2. The role of these repressors has been compared between 16°C and 23°C (Lee *et al.*, 2007; Gu *et al.*, 2013), a different temperature range than used here, and usually under LDs (Lee *et al.*, 2007, 2013; Gu *et al.*, 2013; Pose *et al.*, 2013). Although some studies have tested the function of these proteins under 27°C-LD (Lee *et al.*, 2013; Pose *et al.*, 2013; Airoldi

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et al., 2015), their activity under 27°C-SD has rarely been examined (Balasubramanian *et al.*, 2006; Kumar *et al.*, 2012; Galvao *et al.*, 2015). Differential splicing of *FLM* and *MAF2* mRNAs at high temperature leads to a reduction in the activity of floral repressive complexes involving SVP (Balasubramanian *et al.*, 2006; Pose *et al.*, 2013; Rosloski *et al.*, 2013; Airoldi *et al.*, 2015). The stability of SVP is also reduced at higher temperatures (Figure S7) (Lee *et al.*, 2013). These combined results suggest that floral repression by SVP is impaired at higher temperatures. In the leaves, SVP acts to repress *FT* and *TSF* transcription, whereas in the meristem it represses the transcription of *SOC1* and the accumulation of gibberellins (Lee *et al.*, 2007; Li *et al.*, 2008; Jang *et al.*, 2009; Andres *et al.*, 2014). Therefore, the reduced activity of SVP and MAF repressors at high temperatures under SDs is likely to contribute to the capacity of low levels of CO to activate the transcription of *FT* in the leaves, as well as to allow the meristem to respond when *FT* mRNA is expressed only at low levels. The importance of the role of SVP in the meristem was supported by an analysis of the *svp-41 ft-10 tsf-1* triple mutant, where *svp-41* prevented the residual flowering response to 27°C-SD detected in *ft-10 tsf-1* double mutants. That high temperatures increase the sensitivity of the meristem to FT signalling might be more directly testable using recently described *FT*-inducible systems (Krzymuski *et al.*, 2015); however, the proposed increased sensitivity of the meristem under 27°C-SD is detected when *FT* mRNA is expressed at very low levels, and whether the inducible system can be tuned to express FT at such levels remains to be established.

Alterations in *FT* chromatin structure at higher temperatures may also contribute to the increased activation of transcription by low levels of CO, and particularly the removal of H2A.Z under 27°C-SD has been proposed to increase the binding of PIF4 to the *FT* promoter and thereby the transcriptional activation of the gene (Kumar and Wigge, 2010; Kumar *et al.*, 2012). In support of the importance of *FT*, *TSF* and *SVP* for a flowering response under 27°C-SD, we observed that the *svp-41 ft-10 tsf-1* triple mutant, but not the *svp-41* single mutant or the *ft-10 tsf-1* double mutant, flowered at the same time under 21°C-SD and 27°C-SD. This differs from LDs, where the *svp* mutation alone is sufficient to cause insensitivity to ambient temperatures (Lee *et al.*, 2007; Pose *et al.*, 2013), presumably because compared with SDs the photoperiodic pathway is much more active under LDs, and therefore quantitative modulation of its activity is less likely to cause phenotypic changes under these conditions.

Roles of CO and PIFs in activation of *FT* under SDs at high temperature

Early flowering under 27°C-SD depends on *FT* activity, and the increased transcription of *FT* under 27°C-SD compared with 21°C-SD is conferred by CO and PIFs. Analysis of *co-10 pif4-2* double mutants as well as the respective single mutants (Figure 4a and b) demonstrated that CO and PIF4 act additively to promote *FT* transcription and flowering under 27°C-SD. Furthermore, both PIF4 and PIF5 contribute to this process, because mutations in either gene delayed flowering under 27°C-SD. Also, the double mutant *pif4-2 pif5-3* was later flowering than the *pif4-2* single mutant, indicating some genetic redundancy. We focused mainly on PIF4 as being representative of their activity because of the availability of tools to assay the protein when expressed from its own promoter.

Both CO and PIF4 bind to similar regions of the *FT* promoter (Kumar *et al.*, 2012; Song *et al.*, 2012; Zhang *et al.*, 2015), and we detected a physical interaction between them. The importance of the CO–PIF4 interaction for *FT* activation under 27°C-SD is uncertain. First, the two proteins can activate *FT* transcription independently of each other when expressed at high levels. So, overexpression of *PIF4* causes the earlier flowering of *co-10* mutants, whereas when CO is expressed at high levels in Col-0 under LDs, PIF4 is not required for *FT* transcription. Similarly, under 27°C-SD, the *co-10* and *pif4* single mutants express *FT* mRNA at higher levels than the double mutant, suggesting that each protein can act independently of the other in *FT* activation. This capacity to act independently presumably explains why an additive rather than an epistatic genetic interaction was detected between *pif4* and *co*, and might be explained by genetic redundancy both for *CO* and *PIF4*. The diurnal pattern of *PIF4* expression under 27°C-SD suggests that it can only activate *FT* transcription at times when CO is expressed, however. PIF4 protein was present from before dawn, when the expression of many of its target genes, such as *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2* and *IAA29* (Yamashino *et al.*, 2013), occurs throughout the photoperiod until dusk. No peak in *FT* mRNA was reproducibly detected until both CO and PIF4 were expressed at dusk, however. Therefore, the physical interaction between the proteins might contribute to the activation of *FT* transcription at dusk under 27°C-SD.

The effect of temperature on CO and PIF4 activity is striking, because in wild-type plants neither activates *FT* transcription at 21°C-SD but they do at 27°C-SD. No increase in PIF4 levels was detected at dusk, when *FT* is expressed, under 27°C-SD compared with 21°C-SD, suggesting that changes in PIF4 levels at high temperature do not contribute to the early-flowering phenotype. By contrast, CO is slightly less than twofold more abundant under 27°C-SD than under 21°C-SD, so that an increase in its level might contribute to higher *FT* induction under 27°C-SD. This appears to differ under LDs where no difference in CO abundance was observed between 27°C and 22°C (Seaton *et al.*, 2015). The much higher levels of CO protein present

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under LDs compared with SDs might prevent exposure to high temperature causing any further increase. Modulation of CO protein levels by cold is mediated by the E3 Ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1), which promotes the degradation of CO in response to short periods of cold (Jung *et al.*, 2012; Lee *et al.*, 2012; Joon Seo *et al.*, 2013). Similarly, Blazquez *et al.* (2003) showed that at 23°C p*35S::CO* induces flowering as dramatically as p*35S::FT*, whereas at 16°C p*35S::CO* plants flower later than p*35S::FT*, supporting the idea that the capacity of CO to activate *FT* is regulated by temperature at the post-transcriptional level. If the range of temperatures in which this system operates extends to 27°C, then a reduction in HOS1 activity could contribute to the increased level of CO under 27°C-SD compared with 21°C-SD. Alternatively, one of the other mechanisms that influence CO protein stability might be affected by high temperatures (Jang *et al.*, 2008; Liu *et al.*, 2008; Song *et al.*, 2012).

The activation of *FT* by CO and PIF4 under 27°C-SD but not under 21°C-SD might also depend on other aspects of *FT* regulation. For example, SVP, FLM and MAF2 are negative regulators of *FT* transcription, so impairment of their activities at high temperature might allow low levels of CO and PIF4 to additively activate *FT*. Under 27°C-SD, however, the *svp-41* mutant did not show more *FT* transcript than Col-0 at dusk (Figure 6b), suggesting that under these conditions the early flowering of *svp-41* mutants might mainly be conferred by the impairment of its role in the meristem. A similar conclusion can be drawn from the observation that the residual flowering response to 27°C-SD in *ft-10 tsf-1* mutants is abolished in *svp-41 ft-10 tsf-1*, which is probably caused by removing SVP activity in the meristem as *ft-10 tsf-1* already blocks the leaf response. Alternatively, the *svp-41* mutation might cause increases in *FT* transcript at other times, or the alteration of *FT* chromatin, for example by the removal of H2A.Z, might be critical in allowing CO and PIF4 to activate *FT* under 27°C-SD.

CONCLUSION

The delay in flowering normally observed under SDs is overcome by exposing plants to high temperatures. This depends on the photoperiodic flowering pathway components CO and FT TSF. We find that the levels of these components that induce flowering under 27°C-SD are much reduced compared with inductive LDs. We propose that the early flowering response under 27°C-SD depends on a shift in the threshold of CO and FT proteins required to activate flowering. The conclusion that the flowering response under 27°C-SD depends on threshold levels of photoperiodic flowering components can also explain the greater variability in flowering time observed under these conditions, compared with inductive LDs. Such a threshold effect might be expected to be more sensitive to natural genetic variation among accessions, as observed in other signalling pathways (Polaczyk *et al.*, 1998) and in gene expression responses to high temperatures in other systems (Chen *et al.*, 2015).

EXPERIMENTAL PROCEDURES

Growth conditions and plant material

Plants were grown on soil under controlled SD conditions (8 h light/16 h dark) at 21°C or 27°C, or under LDs (16 h light/8 h dark). For GUS staining experiments, plants were grown on Murashige and Skoog media. The photosynthetic active radiation was 100–150 μmol m^{-2} sec^{-1} in all conditions. All mutant lines are in the Col-0 background (Appendix S1) except for *co-2*, which is in the *Landsberg erecta* background (Ler-0; Koornneef *et al.*, 1991; Putterill *et al.*, 1995).

Molecular cloning

PIF4 cDNA was obtained from the REGIA collection in GATEWAY-compatible vectors (Paz-Ares and Regia, 2002) and cloned by LR recombination (Invitrogen, now ThermoFisher Scientific, http://www.thermofisher.com) into a p*SUC2::GATEWAY* destination vector under the control of a 2.1-kb fragment of the *SUCROSE TRANSPORTER 2* (*SUC2*) promoter (Imlau *et al.*, 1999). The binary vector was introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into Arabidopsis Col-0 by floral dip (Clough and Bent, 1998).

For co-immunoprecipitation experiments, *PIF4* and *CO* cDNA were inserted into p*35S::GATEWAY:HA* and p*35S::GATEWAY:YFP* destination vectors, respectively. The binary vectors were introduced into *A. tumefaciens* GV3101.

Flowering time analysis

Flowering time was determined by counting the number of rosette and cauline leaves on the main stem for the number of plants indicated for each experiment (total leaf number = rosette leaves + cauline leaves). For most flowering time plots the displayed values were generated by combining the flowering time scoring of several independent experiments. Statistical analyses were performed with the same pool of data used for flowering time graphs. The software used for the analysis was SigmaStat 3.5.

RNA extraction and quantitative real-time PCR

For gene expression analysis, total RNA was isolated from whole seedlings. The RNAeasy extraction kit was used (Qiagen, http://www.qiagen.com). Isolated RNA was treated with DNA-free DNase (Ambion, now ThermoFisher Scientific, http://www.thermofisher.com) to remove residual genomic DNA. Approximately 2–4 μg of total RNA quantified with NanoDrop (ThermoFisher Scientific) were used for reverse transcription (SuperScript III; Invitrogen). Transcript levels were quantified by quantitative PCR in a LightCycler 480 instrument (Roche, http://www.roche.com) using the *PP2AA3* gene (AT1G13320) as housekeeping (Czechowski *et al.*, 2005). iQ SYBR Green Supermix (BIO-RAD, http://www.bio-rad.com) was used to perform the reactions. For every experiment, the normalization of each expression value to one expression value in the same experiment (usually the highest) was performed. Final plots were obtained by determining the average of the normalized values from several biological replicates. Standard errors (SEs) were

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calculated from averaged values. Primers used in this study are listed in Table S1.

GUS histochemical analysis

GUS staining was performed as previously described (Adrian *et al*., 2010). Seedlings were harvested and fixed on ice with 90% acetone for 30 min, then vacuum infiltrated with GUS staining buffer (Appendix S2) and incubated until the detection of signal at 37°C in the same buffer. Clearing was performed in ethanol (30% for 1 h and then 70% until clearance of leaves). Samples were preserved in 70% ethanol.

Western blot analysis and nuclear protein quantification

Nuclear proteins were isolated from around 30 seedlings as indicated in Appendix S3. Extracted proteins were resolved in 10% SDS-PAGE and transferred and immunodetected as described in Appendix S3. Technical replicates were loaded on two independent gels. Protein quantification was performed with ImageJ using the images captured with the lowest possible exposure to avoid saturation. Each time point was normalized to the corresponding histone counterpart. Values were normalized against the highest value in each technical replicate. The averages of two technical replicates for each of two biological replicates were combined to describe the biological average and variation. SEs were calculated from averaged values.

Co-immunoprecipitation assays

For protein interaction analysis, transiently transformed *N. benthamiana* leaves were used. *Agrobacterium tumefaciens* cells harbouring p*35S::CO:YFP* or p*35S::PIF4:HA* were co-infiltrated in *N. benthamiana* leaves. Leaf material was harvested 3 days after infiltration. Co-immunoprecipitation was performed as described in Appendix S4.

ACCESSION NUMBERS

CO, *AT5G15840*; *PIF4*, *AT2G43010*; *PIF5*, *AT3G59060*; *PIF1*, AT2G20180; PIF3, *AT1G09530*; *SVP*, AT2G22540; FT, *AT1G65480*; *TSF*, *AT4G20370*.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Mutations in *CO* and *PIFs* suppress the induction of *TSF* mRNA under 27°C-SD.

Figure S2. Ectopic expression of *PIF4* induces *FT::GUS* expression in the vascular tissue.

Figure S3. Mutations in *CO* affect flowering time and *FT* expression under 27°C-SD.

Figure S4. *FT* and *TSF* mRNA expression is induced under SDs after 4 days of exposure to 27°C.

Figure S5. *FT* mRNA levels significantly correlate with the flowering time phenotype of Col-0 plants and *co-10* and *pif* mutants.

Figure S6. *SOC1* transcript level is not increased by warm temperature under SDs.

Figure S7. SVP protein stability decreases under 27°C-SD.

Table S1. List of primers used in this study.

Appendix S1. Mutant lines used in this study.

Appendix S2. GUS staining buffer composition.

Appendix S3. Nuclear protein extraction protocol and immunodetection description.

Appendix S4. *Agrobacterium tumefaciens* infiltration and co-immunoprecipitation assay protocols.

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