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## **PHYTOCHROME INTERACTING FACTOR4 controls the thermosensory activation of flowering**

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Plant growth and development are strongly affected by small differences in temperature<sup>1</sup>. Current climate change has already altered global plant phenology and distribution<sup>2, 3</sup>, and projected increases in temperature pose a significant challenge to agriculture<sup>4</sup>. Despite the important role of temperature on plant development, the underlying pathways are unknown. It has previously been shown that thermal acceleration of flowering is dependent on the florigen, *FLOWERING LOCUS T (FT)*<sup>5, 6</sup>. How this occurs is however not understood, since the major pathway known to upregulate *FT*, the photoperiod pathway, is not required for thermal acceleration of flowering<sup>6</sup>. Here we demonstrate a direct mechanism by which increasing temperature causes the bHLH transcription factor PHYTOCHROME INTERACTING FACTOR4 (PIF4) to activate *FT*. Our findings provide a new understanding of how plants control their timing of reproduction in response to temperature. Flowering time is an important trait in crops as well as affecting the lifecycles of pollinator species. A molecular understanding of how temperature affects flowering will be important for mitigating the effects of climate change.

*Arabidopsis thaliana*, like many higher plants, responds to warmer ambient temperatures by increasing its growth rate and accelerating the floral transition<sup>1, 5, 7</sup>. *Arabidopsis* is a facultative long day plant, and plants grown under short photoperiods are dramatically delayed in flowering. Interestingly, late flowering in short days can be overcome by growth at higher temperatures<sup>6</sup>. The underlying mechanism is however unknown. The flowering response to temperature is dependent on the floral pathway integrator gene *FT*<sup>6</sup> indicative of a thermosensory pathway that upregulates *FT* expression independently of daylength. Since the bHLH transcription factor PHYTOCHROME INTERACTING FACTOR4 (PIF4) has been shown to regulate architectural responses to high temperature<sup>8, 9</sup>, we tested if *PIF4* is required for the induction of flowering at high temperature in short photoperiods. While *pif4-101* is slightly delayed in flowering at 22 °C, *pif4-101* mutants show a striking loss of

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### AUTHOR CONTRIBUTIONS

SVK, DL, KEJ, EA performed the experiments. NPH and PAW designed the study and supervised the work. All authors discussed the results and made substantial contributions to the manuscript.

thermal induction of flowering at 27 °C (Fig. 1a and b). To test if *pif4-101* perturbed floral induction by affecting *FT* expression, we examined the thermal induction of *FT* in Col-0 and *pif4-101*. While *FT* expression is strongly thermally inducible in Col-0, this response is largely abolished in *pif4-101* at 27 °C (Fig. 1c), indicating that *PIF4* is necessary for the thermal acceleration of flowering in short days. By contrast, *PIF4* is not required for the thermosensory induction of flowering under continuous light<sup>8</sup>, suggesting that the photoperiod pathway also interacts with the ambient temperature sensing pathway. The reduced role of *PIF4* under continuous light likely reflects the instability of PIF4 in light<sup>10</sup> coupled with the fact that the output of the photoperiod pathway, CONSTANS (CO) protein, is stabilised by light<sup>11</sup>, shifting the balance of floral induction from *PIF4* to the photoperiod pathway. Since *PIF4* is necessary for the thermal induction of flowering in short days, we tested if it is sufficient to trigger flowering when overexpressed. *35S::PIF4* causes extremely early flowering (Fig. 1d and 1e), similarly to the effect of overexpressing a related gene, *PHYTOCHROME INTERACTING FACTOR5*<sup>12</sup>, suggesting that *PIF4* is limiting for the acceleration of flowering at lower temperature in short photoperiods. Consistently, *35S::PIF4* plants show elevated levels of *FT* (Fig. 1f). Furthermore, *35S::PIF4 ft-3* shows a complete suppression of the early flowering phenotype, showing that the induction of flowering by *35S::PIF4* is dependent on *FT* (Fig. 1g and h). This activation of *FT* appears to be independent of the established photoperiod pathway since *CO* does not change in response to *35S::PIF4* (Fig. 1f). Finally, while *co-9* mutants are late flowering<sup>6, 13</sup>, we find *35S::PIF4 co-9* plants are early flowering, indicating that *PIF4* acts largely independently of *CO* (supplementary information Fig. S1), consistent with the thermal induction of flowering being independent of the photoperiod pathway (Fig. 1f)<sup>6</sup>.

Although *PIF4* has been shown to be important for high temperature responses, long-term increases in either *PIF4* transcript or PIF4 protein levels in response to higher ambient temperature that can account for the observed growth responses have not been detected<sup>8, 9</sup>. To examine if variation of *PIF4* transcription under our experimental conditions might account for the increases in *PIF4* activity with temperature, we measured *PIF4* transcript levels at 12, 17, 22 and 27 °C in seedlings (Fig. 2a). *PIF4* transcript levels increase from 12 °C to 22 °C, while the difference between 22 °C and 27 °C is not statistically significant. Plants at 27 °C, compared to 22 °C, show a very large *PIF4*-dependent response, suggesting that variation in the *PIF4* transcript is not sufficient to account for the acceleration of flowering at 27 °C compared to 22 °C. To test whether temperature-mediated changes in *PIF4* transcription are rate-limiting for the biological response, we analysed the behaviour of plants constitutively expressing *PIF4*. While *35S::PIF4* plants at 22 °C are extremely early flowering, this phenotype can be largely suppressed at 12 °C (Fig. 2b and Fig. S2), indicating that even when *PIF4* transcript is abundant, lower temperatures are inhibitory for *PIF4* activity. A possible explanation for this difference is that PIF4 protein is destabilised by low temperature. Indeed, PIF4 protein levels have already been shown to be strongly regulated by light<sup>10</sup>, and growth in red and blue photocycles destabilises PIF4 protein at low temperatures<sup>14</sup>. To test this, we examined the levels of PIF4:HA protein at 12 °C, 17 °C, 22 °C and 27 °C under the same light conditions used for our flowering time assays. Consistent with previous studies<sup>10</sup> we see a strong accumulation of PIF4 at the end of the night period, which is subsequently degraded during the day. Despite the suppression of

early flowering in *35S::PIF4* at 12 °C compared to 22 ° (Fig. 2b), we do not observe an appreciable difference in PIF4 protein levels at these two temperatures that is likely to account for these different phenotypes (Fig. 2c, Fig. S3). Slightly higher levels of PIF4:HA appear to be present at 27 °C (Fig. 2c), suggesting high temperature stabilisation of PIF4 may also contribute to higher PIF4 activity at 27 °C.

Taken together, these data indicate that *PIF4* regulates *FT* in a temperature dependent manner. To determine if this is likely to be the case *in planta*, we analysed the spatial expression of *FT* and *PIF4*. *FT* has a distinctive pattern of expression in the vasculature of the leaf<sup>15, 16</sup>, and significantly *PIF4* is expressed in the same domain (Fig. 3a). Since the regulation of *FT* by *PIF4* could be either direct or indirect, we used chromatin immunoprecipitation (ChIP) to analyse if PIF4 binds directly to the *FT* promoter proximal to the transcriptional start site (TSS). This region of the promoter was chosen since it has been shown to be both phylogenetically conserved and the site for light mediated regulation of *FT* expression<sup>16, 17</sup>. We observe robust enrichment of PIF4 near to the TSS (Fig. 3b), indicating that PIF4 binds this region *in vivo* to activate *FT* expression.

Given the striking effect of ambient temperature on PIF4 activity, which occurs even when *PIF4* is constitutively expressed, we hypothesised that the ability of PIF4 to bind the *FT* promoter may be temperature dependent. To test this, we performed ChIP experiments using *35S::PIF4* plants grown at 12 °C and 27 °C with primers flanking an E-box in the *FT* promoter (Fig. 3c). Strikingly, we observe a very strong temperature dependence for this binding, with an approximately 5-fold increase in binding at 27 °C compared to 12 °C (Fig. 3d). This indicates that the later flowering of *35S::PIF4* at 12 °C is caused by a decrease in PIF4 binding to *FT*. Since the 35S promoter causes strong ectopic expression of PIF4, we sought to confirm that PIF4 protein expressed at endogenous levels displays similar temperature dependent binding to the *FT* promoter. We therefore performed ChIP experiments on a *pif4-101* line complemented with *PIF4<sub>pro</sub>::PIF4:ProteinA* (Fig. S4). Consistent with the overexpression studies, we observe a strong increase in PIF4 binding to *FT* as a function of temperature. Reduced binding is observed at 17 °C, consistent with the very late flowering of plants under short days at low temperature, but this binding increases at 22 °C and is even higher at 27 °C (Fig. 3e). The temperature dependent binding of PIF4 to *FT* could be due to growth temperature influencing the affinity of the PIF4 transcription factor for its binding site or the efficiency of the ChIP could be affected by the temperature that tissues were grown at. To test these possibilities, we analysed another recently described PIF4 target locus<sup>18</sup>, *CYP79B2* (At4g39950), which is up-regulated in *35S::PIF4* (Fig. S5a). We find PIF4 binding to occur constitutively at both 12 and 27 °C at a region in the first exon (Fig. S5b). Another region further upstream in the promoter shows a temperature dependent binding of PIF4, and in both cases, no enrichment is seen for a control locus (Fig. S5b). This indicates that the abundant PIF4 protein we observe at 12 °C is active and able to bind target sites and confirms that the ChIP method *per se* is not influenced by the temperature at which the sample is grown, consistent with other studies<sup>19</sup>. The ability of PIF4 to bind loci in a more temperature independent-manner might explain why *35S::PIF4* at 12 °C maintains hypocotyl and petiole elongation, while early flowering is strongly suppressed. We do not exclude that temperature may also influence PIF4 activity post-translationally.

Temperature signals are mediated through H2A.Z-nucleosomes in Arabidopsis<sup>20</sup>, suggesting that temperature may be increasing the accessibility of the PIF4 binding site at the *FT* promoter. Consistent with this hypothesis, we find that H2A.Z-nucleosomes are present at the PIF4 binding site in the *FT* promoter. Furthermore, we find that the levels of H2A.Z-nucleosomes at the *FT* promoter decrease with higher temperature (Fig. 3f). These results suggest that the presence of H2A.Z nucleosomes is limiting for PIF4 binding to *FT*, and that the PIF4 binding we observe at higher temperature is due to the greater accessibility of chromatin containing H2A.Z-nucleosomes at higher temperature. This suggests that in the absence of H2A.Z-nucleosomes, PIF4 should bind *FT* more strongly. We therefore compared the ability of PIF4 expressed under its own promoter to bind to the *FT* promoter in wild-type compared to *arp6-1*, a background lacking incorporation of H2A.Z-nucleosomes. Interestingly, we observe considerably greater binding of PIF4 in *arp6-1* (Fig. 3g), indicating that H2A.Z-nucleosomes are rate-limiting for PIF4 to activate *FT* expression. The eviction of H2A.Z-nucleosomes by higher temperature therefore provides a direct mechanism for the temperature regulated expression of *FT* (Fig. 4c). Consistent with our previous results and the established role of H2A.Z in regulating temperature dependent gene expression, we find that there is increased PIF4 mRNA in *arp6-1* background (Fig. S6). However, our results for *35S::PIF4* suppression by 12 °C indicate that transcriptional up-regulation of PIF4 is not the rate-limiting step in regulating PIF4 mediated flowering at higher temperatures.

Our results indicate that the temperature dependent regulation of *FT* by PIF4 is controlled at the level of chromatin accessibility of the *FT* promoter and possibly at the level of PIF4 protein activity. PIF4 activity is controlled through the repressive activity of DELLA proteins that prevent PIF4 binding DNA<sup>21, 22</sup>. Consistently, plants having reduced or absent DELLA function are early flowering<sup>23</sup>. We hypothesised that delay in flowering at lower temperatures might at least in part be due to DELLA mediated repression of PIF4 activity. If so, it would be expected that absence of DELLAs should cause accelerated flowering at lower temperatures. In accord with this expectation, we found that a mutant lacking DELLAs flowers much earlier than wild-type when grown at 12 °C (Fig. 4a and 4b). The phytohormone gibberellin (GA) triggers DELLA protein degradation, and plays a key permissive role for *FT* induction, since in a GA deficient background, GA application increases *FT* expression 15-fold<sup>24</sup>. While it was proposed more than 50 years ago that gibberellins are upstream of florigen<sup>25</sup>, the mechanism has not been clear. As DELLA proteins have been shown to be key regulators by which GA influences PIF4, our finding that PIF4 is able to directly activate *FT* suggests a possible mechanism by which changes in GA levels may influence flowering.

Climate change has already caused measurable changes in plant phenology and behaviour<sup>2</sup>, and plants that incorporate temperature information into their lifecycles appear to be able to adapt to warmer conditions more effectively than those plants that primarily rely on photoperiod to synchronise their lifestyles<sup>3</sup>. The importance of the effects of climate change on yield are highlighted by the significant detrimental effects of increasing temperatures on yield<sup>4</sup>. *PIF4* is a central integrator of environmental information in the plant and our finding that it activates *FT* at higher temperatures suggests it will be a key node for breeding crops resilient to climate change. This importance is suggested by the recent discovery that natural variation at *PIF4* plays a major role in key ecological traits<sup>26</sup>.

## ONLINE METHODS

### 1. Plant material and growth conditions

All plant lines used were in Col-0 background unless otherwise specified. *pif4-101* mutant was provided by C. Fankhauser, HA tagged *35S::PIF4* by S. Prat<sup>12</sup>. All references to “*35S::PIF*” and “*PIF:HA*” refer to this line, i.e. *35S::PIF4:HA. FT::GUS* was obtained from K.Goto<sup>15</sup>. *phyb-9 ft-10* double mutants were generated by crossing respective single mutants. *35S::PIF4:HA co-9* was obtained by crossing. The crosses were genotyped for presence of the *35S::PIF4* construct by PCR on genomic DNA using primers 2362 and 2363, resulting in two products of different size representing the cDNA transgene and the genomic DNA fragment, respectively. *ft-10* was genotyped using primers 1580 and 1582 for the insertion, or 1580 and 1581 to detect the wildtype fragment. *co-9* was genotyped with primers 3650 and 3652 for insertion, and 3291 and 3292 for the wildtype fragment. For genotyping *phyb-9*, DNA was amplified using oligos 2137 and 2138 followed by *MnlI* digestion to distinguish between wildtype and mutant alleles. The global *della* mutant is in the *Ler* background and was described previously<sup>8</sup>. *PIF4::PIF4:PROTEINA* and *PIF4::PIF4:GUS* were constructed by amplifying the genomic fragment of *PIF4* including the promoter with oligos 1534 and 1535. The PCR product was cloned into pENTR/D-TOPO (Invitrogen) and inserted into the binary plasmids PW889 (C-terminal PROTEIN A) and PW395 (C-terminal GUS), respectively, using Gateway technology (Invitrogen). Transgenic plants were obtained by transforming *pif4-101* by floral dip. For hypocotyl measurements seeds were surface sterilized, sown on ½ MS media, stratified for 2 days at 4°C in the dark and germinated for 24 h at 22°C. The plates were then transferred to short day conditions (8/16 h photoperiod) at 22°C and 27°C respectively and grown vertically for 10 days before being imaged and hypocotyl length measured using the ImageJ software (<http://rsbweb.nih.gov/ij/>). Oligonucleotide sequences are provided in Table S1.

### 2. Transcript analysis

Samples from plants grown in long days (16/8 h photoperiod) were harvested and total RNA was extracted using Trizol Reagent (Invitrogen). 2 µg of RNA were treated with DnaseI (Roche) and used for cDNA synthesis (First strand cDNA synthesis kit, Fermentas). cDNA was diluted 1:8 and used for quantitative PCR using a Roche Lightcycler 480 and the corresponding Sybr Green master mix. To detect *FT* transcript levels, oligos 3180 and 3181 were used, for *CO* oligos 2951 and 2952. *PIF4* transcript levels were analyzed using oligos 3952 and 3953. Oligos 3247 and 3408 amplifying *TUB6* (At5g12250) were used for normalization.

### 3. Immunoblot analysis

For analysing the possible effect of temperature on PIF4 protein stability, Plants overexpressing PIF4:HA (*35S::PIF4:HA*) were used. Seven day old *35S::PIF4:HA* seedlings grown in short days at 17 °C were transferred to 12, 17, 22 and 27 °C in short days for 2 days. Samples were collected at end of night (EON) and thereafter 30 min, 1 hour and 4 hours under illumination. Protein samples were separated by SDS-PAGE and transferred on to nitrocellulose membrane. PIF4:HA was detected using HRP conjugated anti-HA antibody

(Miltenyi Biotech) and visualised by chemiluminescent detection using Immobilon Chemiluminescent HRP substrate (Millipore).

#### 4. GUS histochemical assay

For GUS-staining plants were grown on ½ MS plates in long days (16/8 h photoperiods) for 10 days and kept in the dark for 24 h before harvesting. Plants were stained in buffer containing 100 mM phosphate buffer, pH 7, 10 mM EDTA, 0.1% Triton-X100, 0.5 mM K-ferrocyanide and 1 mM X-gluc at 37°C for 24 h before destaining in ethanol.

#### 5. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described<sup>20</sup> with minor modifications. *35S::PIF4:HA* seedlings were grown on ½ MS plates for 10 days and kept in the dark for 24 h at respective temperatures before harvesting. 1.5 g of plant tissue and 4 µg of antibody (HA-tag antibody ab9110 from Abcam) were used for ChIP. To analyse the dynamics of *PIF4::PIF4:PROTEINA*, plants were grown in respective temperatures under short day conditions for four weeks. Aerial parts of the plants were collected and cross-linked before being used for chromatin preparations. ChIP was done using magnetic beads (Dynabeads M-270 Epoxy, Invitrogen) coated with rabbit IgG (Sigma, I5006) as described (<http://www.ncdir.org/protocols/Rout/Conjugation%20of%20Dynabeads.pdf>). To analyse H2A.Z dynamics at the *FT* locus in response to temperature, we used 3 week old seedlings of *HTA11::HTA11:GFP* grown at 17 °C and 27 °C. ChIP was done using anti-GFP antibody (Abcam, ab290). To analyze PIF4 binding in Col-0 and *arp6-1* backgrounds, respective genotypes with *PIF4::PIF4:3XFLAG* were grown on soil at 22 °C under short photoperiods for three weeks before samples fixed by formaldehyde cross-linking. ChIP was performed using anti-FLAG M2 affinity gel (Sigma A2220). Immuno-complexes were eluted using 3X FLAG peptide (Sigma F4799) according to manufacturer's instructions. Immunoprecipitated DNA was eluted after reverse cross-linking by boiling at 95 °C for 1 min in presence of 10 % Chelex (BioRad laboratories) followed by treatment with Proteinase K. Oligonucleotides 3255 and 3256 for FT-15 region, 3613 and 3614 for FT-c1, 3607 and 3608 for FT-c and 3261 and 3262 for FT-f were used for detecting PIF4 binding to the *FT* locus. As a positive control for PIF4 binding, At5g45280 was analysed using oligos 2857 and 2958. *HSP70* was used as a negative control using oligos 1862 and 1865. For analysing PIF4 binding at At4g39950 oligonucleotides 4240 and 4241 was used for region 1; and oligonucleotides 4246 and 4247 were used for region 2. Oligos 1860 and 1861 were used for *HSP70* as a negative control. Oligonucleotide sequences are provided in Table S1.

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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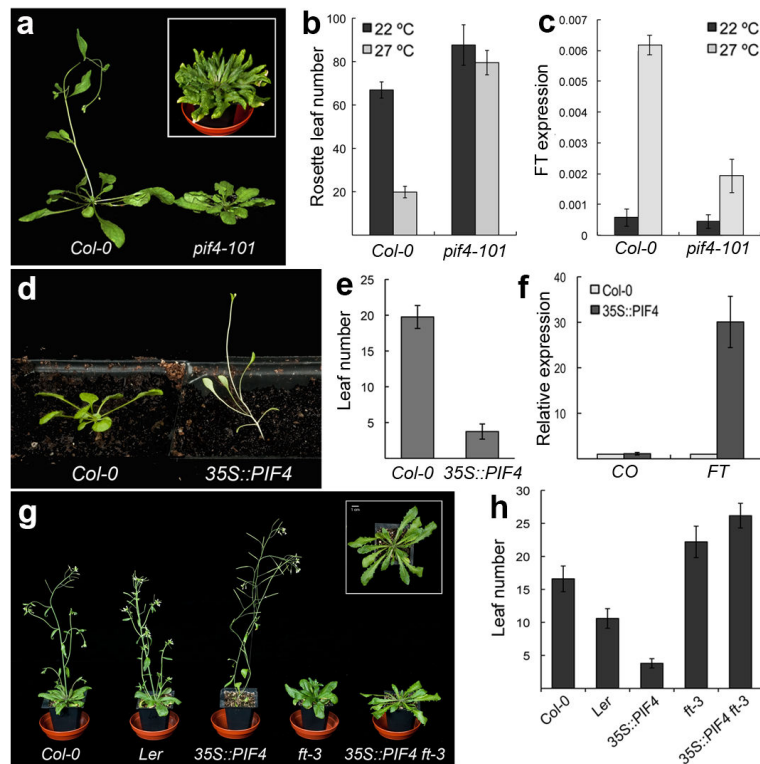
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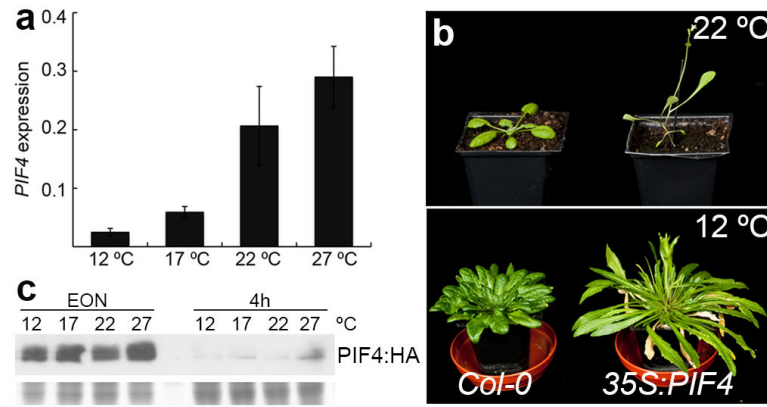
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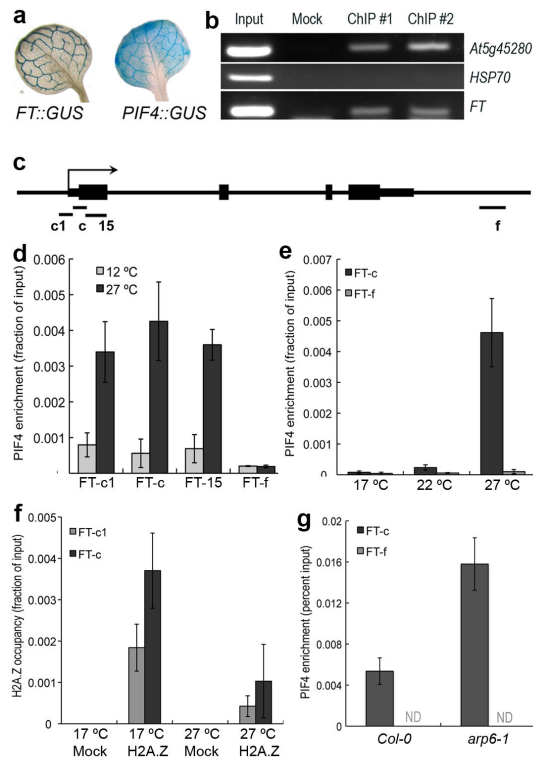


**Fig. 1. *PIF4* is necessary for the thermal induction of flowering in short photoperiods**  
 (a) *pif4-101* plants do not show acceleration of flowering at 27 °C compared to *Col-0*. Inset shows a 16 week old *pif4-101* plant grown at 27 °C. (b) rosette leaf numbers at flowering for *Col-0* and *pif4-101* grown at 22 °C and 27 °C in short photoperiod conditions (error bars are  $\pm$  SD, n=6). (c) *FT* expression as measured by Q-PCR in 4 week old plants at 22 °C and 27 °C under short photoperiods in a *PIF4* dependent manner. (data from 3 biological replicates, Error bars are  $\pm$  SD) (d) *35S::PIF4* overexpression triggers very early flowering. (e) Leaf numbers at flowering for *Col-0* and *35S::PIF4* in long photoperiods (error bars are  $\pm$  SD, n=5). (f) *CO* and *FT* gene expression data measured by Q-PCR in *Col-0* and *35S::PIF4* at 21 °C in long photoperiods (Samples taken 2 weeks after sowing; data from 3 biological replicates. All error bars are  $\pm$  SD). (g) *FT* is required for the early flowering phenotype of *35S::PIF4* plants. When crossed into the *ft-3* background, the early flowering of *35S::PIF4* is completely suppressed. Inset is a top view of the *35S::PIF4 ft-3* plant showing that petiole elongation growth is retained. (h) Flowering time data for *Col-0*, *Ler*, *35S::PIF4*, *ft-3*, and *35S::PIF4 ft-3* plants (error bars are  $\pm$  SD, n=5).



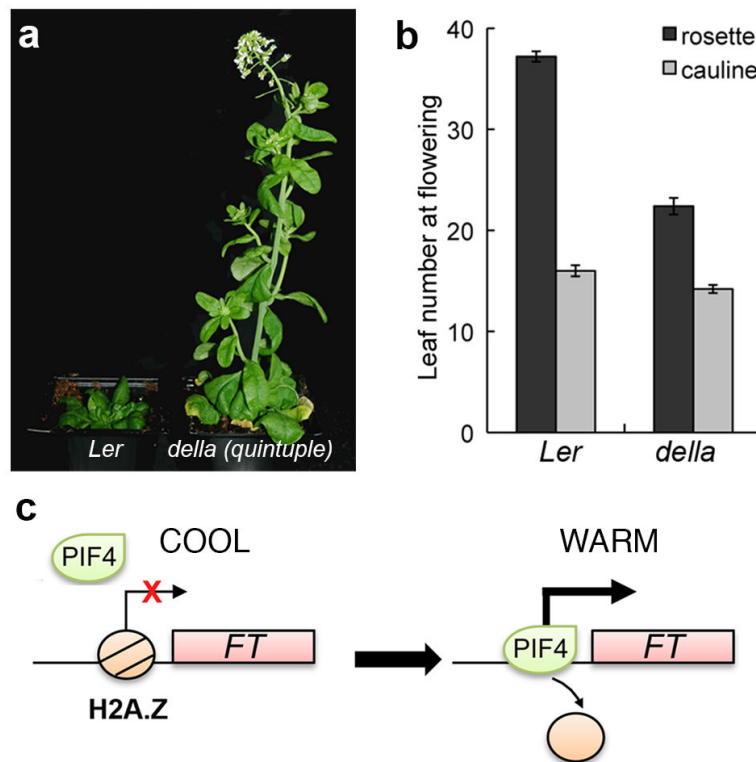
### Fig. 2. Regulation of *PIF4* by temperature

(a) Transcriptional regulation of *PIF4* by temperature. 10 day-old Col-0 seedlings grown at 12, 17, 22 and 27 °C under short photoperiods were analyzed for *PIF4* expression by Q-PCR. Data shown is from three biological replicates. Error bars are  $\pm$  SD. (b) Flowering phenotype of *35S::PIF4* is temperature dependent. While *35S::PIF4* plants (right) flower very early at 22 °C (upper panel) as compared to Col-0 (left), this phenotype is largely suppressed by growth at 12 °C (lower panel). (c) *PIF4* protein levels in *35S::PIF4* plants are not affected by growth temperature. Seven day old *35S::PIF4:HA* seedlings grown at 17 °C were transferred to 12, 17, 22 and 27 °C under short photoperiods for 2 days and samples were collected at the end of night (EON) before light and after 4 hours under illumination. While *PIF4:HA* protein levels are independent of growth temperatures, the protein is robustly degraded in presence of light. *PIF4:HA* protein was detected by HRP conjugated anti-HA antibody. Stained lower half of the gel used for immunoblot is shown as loading control.



**Fig. 3. PIF4 directly binds the *FT* promoter in a temperature dependent manner**

(a) GUS histochemical analysis of the expression domains of *FT* and *PIF4* in rosette leaves. (b) ChIP analysis shows PIF4 binding to the *FT* locus *in vivo* in seedlings. The *At5g45280* promoter is a positive control for PIF4 binding activity<sup>21</sup>, the *HSP70* promoter has been used as a negative control. (c) Schematic summarising structure of the *FT* promoter and positioning of Q-PCR amplicons for ChIP analysis. (d) ChIP analysis of *35S::PIF4:HA* at 12 °C and 27 °C (2 week old seedlings, short photoperiods). (e) ChIP analysis of *PIF4::PIF4:ProA* at 17, 22 and 27 °C (4 week old soil-grown plants, short photoperiods). (f) Analysis of H2A.Z occupancy at the *FT* locus at 17 °C and 27 °C (3 week old plate grown plants, short photoperiods). (g) ChIP analysis of PIF4 binding to *FT* in *Col-0* and *arp6-1* (3 week old soil-grown plants, 22 °C short photoperiod). (For all ChIP experiments, plant materials were collected at the end of dark period before lights come ON and were protected from light until frozen. All data presented are from two independent ChIP experiments; all error bars are +/- SD).



**Fig. 4. *PIF4* integrates environmental signals**

(a) Suppression of flowering at 12 °C is significantly repressed in the absence of *DELLA* mediated repression. (b) Leaf number at flowering for *della* global is reduced compared to *Ler* at 12 °C (error bars are +/- SD, n=6). (c) A schematic representation of temperature dependent *FT* regulation by *PIF4*. Temperature induced H2A.Z nucleosome dynamics can regulate *PIF4* binding to target loci for transcriptional activation.