Transcription factor PIF4 controls the thermosensory activation of flowering

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Plant growth and development are strongly affected by small differences in temperature. Current climate change has already altered global plant phenology and distribution, and projected increases in temperature pose a significant challenge to agriculture. 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 photoperiod pathway. However, it is not required for the thermosensory induction of flowering under short days, suggesting that the photoperiod pathway also plays a role in temperature response. The flowering response to temperature is dependent on the floral pathway integrator gene CONSTANS (CO) protein, which is stabilized by light, shifting the balance of flowering to the thermosensory pathway. 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 life cycles 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. 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. The underlying mechanism is, however, unknown. The flowering response to temperature is dependent on the floral pathway integrator gene FT, indicative of a thermosensory pathway that upregulates FT expression independently of daylength. Because the bHLH transcription factor PIF4 has been shown to regulate the induction of flowering at higher temperatures, we tested if PIF4 is required for the induction of flowering at high temperature in short photoperiods. Although pif4-101 was slightly delayed in flowering at 22°C, pif4-101 mutants showed a striking loss of thermal induction of flowering at 27°C (Fig. 1a, 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. Although FT expression was strongly thermally inducible in Col-0, this response was 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, suggesting that the photoperiod pathway also interacts with the ambient temperature sensing pathway. The reduced role of PIF4 under continuous light probably reflects the instability of PIF4 in light coupled with the fact that the output of the photoperiod pathway, CONSTANS (CO) protein, is stabilized by light, shifting the balance of floral induction from PIF4 to the photoperiod pathway. Because 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 caused extremely early flowering (Fig. 1d, e), similar to the effect of overexpressing a related gene, PHYTOCHROME INTERACTING FACTOR5 (ref. 12), suggesting that PIF4 is limiting for the acceleration of flowering at low temperature in short photoperiods. Consistently, 35S::PIF4 plants showed elevated levels of FT (Fig. 1f). Furthermore, 35S::PIF4 ft-3 showed a complete suppression of the early flowering phenotype, indicating that the induction of flowering by 35S::PIF4 was dependent on FT (Fig. 1g, h). This activation of FT appears to be independent of the established...
photoperiod pathway because CO did not change in response to 35S::PIF4 (Fig. 1f). Finally, although co-9 mutants are late flowering, PIF4 acts largely independently of CO (Supplementary Information and Supplementary Fig. 1), consistent with the thermal induction of flowering being independent of the photoperiod pathway (Fig. 1f).

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. 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 increased from 12 °C to 22 °C, whereas the difference between 22 °C and 27 °C was not statistically significant. Plants at 27 °C, compared with 22 °C, showed 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 with 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. Although 35S::PIF4 plants at 22 °C were extremely early flowering, this phenotype could be largely suppressed at 12 °C (Fig. 2b and Supplementary Fig. 2), 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 destabilized by low temperature. Indeed, PIF4 protein levels have already been shown to be strongly regulated by light, and growth in red and blue phototropes destabilizes PIF4 protein at low temperatures. The PIF4 overexpression lines contain a fusion to the haemagglutinin (HA) epitope (35S::PIF4:HA). We therefore examined the levels of PIF4:HA, protein at 12, 17, 22 and 27 °C under the same light conditions used for our flowering time assays. Consistent with previous studies, we saw a strong accumulation of PIF4 at the end of the night period, which was subsequently degraded during the day. Despite the suppression of early flowering in 35S::PIF4 at 12 °C compared with 22 °C (Fig. 2b), we did not observe an appreciable difference in PIF4 protein levels at these two temperatures that was likely to account for these different phenotypes (Fig. 2c and Supplementary Fig. 3). Slightly higher levels of PIF4:HA appeared to be present at 27 °C (Fig. 2c), suggesting high-temperature stabilization 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 probably 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, and significantly PIF4 was expressed in the same domain (Fig. 3a). Because the regulation of FT by PIF4 could be either direct or indirect, we used chromatin immunopurification (ChIP) to analyse if PIF4 binds directly to the FT promoter proximal to the transcriptional start site. This region of the promoter was chosen because it has been shown to be both phylogenetically conserved and the site for light-mediated regulation of FT expression. We observed robust enrichment of PIF4 near to the transcriptional start site (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 hypothesized 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 and 27°C with primers flanking an E-box in the FT promoter (Fig. 3c). Strikingly, we observed a very strong temperature dependence for this binding, with an approximately fivefold increase in binding at 27°C compared with 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. Because 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 PIF4pro::PIF4:ProteinA (Supplementary Fig. 4). Consistent with the overexpression studies, we observed a strong increase in PIF4 binding to FT as a function of temperature. Reduced binding was observed at 17°C, consistent with the very late flowering of plants under short days at low temperature, but this binding increased at 22°C and was 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 method could be affected by the temperature at which tissues were grown. To test these possibilities, we analyzed another recently described PIF4 target locus18, CYP79B2 (At4g39950), which is upregulated in 35S::PIF4 (Supplementary Fig. 5a). We found PIF4 binding to occur constitutively at both 12 and 27°C at a region in the first exon (Supplementary Fig. 5b). Another region further upstream in the promoter showed a temperature-dependent binding of PIF4, and, in both cases, no enrichment was seen for a control locus (Supplementary Fig. 5b). This indicates that the abundant PIF4 protein we observed at 12°C is active and able to 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 studies19. 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 Arabidopsis20, suggesting that temperature may be increasing the accessibility of the PIF4-binding site at the FT promoter. Consistent with this hypothesis, we found that H2A.Z-nucleosomes were present at the PIF4-binding site in the FT promoter. Furthermore, we found that the levels of H2A.Z-nucleosomes at the FT promoter decreased 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 observed 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 and arp6-1, a background lacking incorporation of H2A.Z-nucleosomes. Interestingly, we observed 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 found that there is increased PIF4 messenger RNA in arp6-1 background (Supplementary Fig. 6). However, our results for 35S::PIF4 suppression by 12°C indicate that transcriptional upregulation 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 DNA21,22.

Consistently, plants having reduced or absent DELLA function are early flowering23. We hypothesized 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 DELLA should cause accelerated flowering at lower temperatures. In accord with this expectation, we found that a mutant lacking DELLA花 was downregulated in the absence of PIF4 expression 15-fold24. Although it was proposed more than 50 years ago that gibberellins are upstream of florigen25, the mechanism has not been clear. As DELLA proteins have been shown to be key regulators by which gibberellins influence PIF4, our findings that PIF4 is able to activate FT directly suggests a possible mechanism by which changes in gibberellin levels may influence flowering.

Climate change has already caused measurable changes in plant phenology and behaviour2, and plants that incorporate temperature information into their life cycles appear to be able to adapt to warmer conditions more effectively than those that primarily rely on photoperiod to synchronize their lifecycles3. The importance of the effects of climate change on yield are highlighted by the significant detrimental effects of increasing temperatures on yield4. 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 traits26.
METHODS SUMMARY

Detailed descriptions of the plant growth conditions, growth assays, transgenic constructs and ChIP techniques are provided in the Supplementary materials.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

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. All references to ‘35S::PIF4’ and ‘35S::PIF4::HA’ refer to this line: that is, 35S::PIF4::HA. *FT::GUS* was obtained from K. Goto. 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 complementary DNA (cDNA) transgene and the genomic DNA fragment, respectively. co-9 was genotyped with primers 3650 and 3652 for insertion, and 3291 and 3292 for the wild-type fragment. For genotyping phyb-9, DNA was amplified using oligonucleotides 2137 and 2138 followed by MnII digestion to distinguish between wild-type and mutant alleles. The global *della* mutant is in the Ler background and was described previously. PIF4::PIF4::ProteinA and PIF4::PIF4::GUS were constructed by amplifying the genomic fragment of PIF4 including the promoter with oligonucleotides 1534 and 1535. The PCR product was cloned into pENTR/D-TOPO (Invitrogen) and inserted into the binary plasmids PW889 (carboxy-terminal ProteinA) and PW395 (carboxy-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 and 27°C respectively, and grown vertically for 10 days before being imaged and the hypocotyl length measured using the ImageJ software (http://rsbweb.nih.gov/ij/). Oligonucleotide sequences are provided in Supplementary Table 1.

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

Immunoblot analysis. To analyse 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 and thereafter 30 min, 1 h and 4 h under illumination. Protein samples were separated by SDS–polyacrylamide gel electrophoresis and transferred on to nitrocellulose membrane. PIF4::HA was detected using HRP-conjugated HA antibody (Milenyi Biotech) and visualized by chemiluminescent detection using Immobilon Chemiluminescent HRP substrate (Millipore).

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 collecting. 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 de-staining in ethanol.

ChIP. ChIP was performed as described 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 collecting. Plant tissue (1.5 g) 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 4 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, 15006) 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 and 27°C. ChIP was done using anti-GFP antibody (Abcam, ab290). To analyse PIF4 binding in Col-0 and *arp6-1* backgrounds, respective genotypes with 35S::PIF4::HA were used. Seven-day-old seedlings grown on ½ MS plates in long days (16/8 h photoperiods) for 3 weeks before samples were fixed by formaldehyde cross-linking. ChIP was performed using anti-Flag M2 affinity gel (Sigma A2220). Immunocomplexes were eluted using 3× Flag peptide (Sigma F4799) according to the manufacturer’s instructions. Immunoprecipitated DNA was eluted after reverse cross-linking by boiling at 95°C for 1 min in the 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, 3604 and 3605 amplifying 35S::PIF4:HA were used for ChIP.

ft locus. As a positive control for PIF4 binding, At5g45280 was analysed using oligonucleotides 2857 and 2958. HSP70 was used as a negative control using oligonucleotides 1860 and 1861. To analyse PIF4 binding at Arp6::PIF4::HA, oligonucleotides 4240 and 4241 were used for region 1, and oligonucleotides 4246 and 4247 were used for region 2. Oligonucleotides 1860 and 1861 were used for HSP70 as a negative control. Oligonucleotide sequences are provided in Supplementary Table 1.

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