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PFT1, the MED25 subunit of the plant Mediator complex, promotes flowering through CONSTANS dependent and independent mechanisms in Arabidopsis

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SUMMARY

Two aspects of light are very important for plant development: the length of the light phase or photoperiod and the quality of incoming light. Photoperiod detection allows plants to anticipate the arrival of the next season, whereas light quality, mainly the red to far-red ratio (R:FR), is an early signal of competition by neighbouring plants. phyB represses flowering by antagonising *CO* at the transcriptional and post-translational levels. A low R:FR decreases active phyB and consequently increases active CO, which in turn activates the expression of *FT*, the plant florigen. Other phytochromes like phyD and phyE seem to have redundant roles with phyB. PFT1, the MED25 subunit of the plant Mediator complex, has been proposed to act in the light-quality pathway that regulates flowering time downstream of phyB. However, whether PFT1 signals through CO and its specific mechanism are unclear. Here we show that CO-dependent and -independent mechanisms operate downstream of phyB, phyD and phyE to promote flowering, and that PFT1 is equally able to promote flowering by modulating both CO-dependent and -independent pathways. Our data are consistent with the role of PFT1 as an activator of *CO* transcription, and also of *FT* transcription, in a CO-independent manner. Our transcriptome analysis is also consistent with *CO* and *FT* genes being the most important flowering targets of PFT1. Furthermore, comparison of the *pft1* transcriptome with transcriptomes after fungal and herbivore attack strongly suggests that PFT1 acts as a hub, integrating a variety of interdependent environmental stimuli, including light quality and jasmonic acid-dependent defences.

Keywords: Arabidopsis, PFT1, flowering, CONSTANS, photoperiod, jasmonate.

INTRODUCTION

As sessile organisms, plants have to adapt their growth and development to large fluctuations in environmental conditions. Thus, plants have developed an entire machinery to monitor and integrate light and temperature information to fine-tune flowering onset, as well as several other developmental programmes (Lee *et al.*, 2008; Cerdan, 2011; Sanchez *et al.*, 2011). The length of the day, or photoperiod, is a reliable source of environmental information that can be used to anticipate the arrival of the flowering season. *Arabidopsis thaliana* is a facultative long-day (LD) plant because it flowers earlier under LD than under short-day (SD) conditions. Day-length detection is accomplished through the photoperiod pathway, which is composed of several photoreceptors and downstream components. The red and far-red light photoreceptors, the phytochromes (phyA–phyE in Arabidopsis) and the blue/UV-A photoreceptors, the cryptochromes (cry1 and cry2), regulate the activity of the transcription factor CONSTANS (CO), which is the central component of the photoperiod pathway. The expression of *CO* is tightly regulated at both mRNA and protein levels (Suarez-Lopez *et al.*, 2001; Yanovsky and Kay, 2002; Valverde *et al.*, 2004; Imaizumi *et al.*, 2005; Laubinger *et al.*, 2006; Jang *et al.*, 2008). As a result of both types of regulation, CO levels peak only under LDs to directly induce the expression of *FLOWERING LOCUS T* (*FT*) (Adrian *et al.*, 2010; Tiwari *et al.*, 2010). FT is a promoter of flowering that acts as an

integrator of different flowering pathways (Cerdan and Chory, 2003; Lee *et al.*, 2007; Turck *et al.*, 2008). After being induced, the FT protein moves to the apical meristem to induce genes required for reproductive development (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Mathieu *et al.*, 2007; Tamaki *et al.*, 2007). TWIN SISTER OF FT (TSF), the FT homologue, acts similarly to FT, downstream of the photoperiod pathway. As the phenotype of *tsf* mutants is much less evident than the phenotype of *ft* mutants, its role in the photoperiod pathway seems to be less important (Michaels *et al.*, 2005; Yamaguchi *et al.*, 2005; Jang *et al.*, 2009).

Monitoring additional light parameters, besides photoperiod, might be essential for plant survival under natural conditions. For instance, far-red light (FR) reflected by plant neighbours is an early warning of competition for sunlight (Ballare *et al.*, 1990). When plants are exposed to low red to far-red ratios (R:FRs), the phytochrome active form, Pfr, is photoconverted back to the inactive form, Pr. The lower Pfr levels trigger a series of responses known collectively as shade avoidance syndrome (SAS): the petioles and the stem elongate, leaves move upward and in some plants like Arabidopsis, flowering is accelerated (Franklin, 2008). The SAS can be induced in laboratory conditions by growing plants under light with low R:FR, usually by the addition of a FR source to a fluorescent light source, or by treating the plants with short FR pulses at the end of the photoperiod (EODFR) to decrease Pfr in the subsequent dark period. phyB is the most important photoreceptor mediating SAS, and the roles of phyD and phyE are more evident in *phyB* mutant backgrounds (Devlin *et al.*, 1998, 1999; Franklin *et al.*, 2003; Halliday and Whitelam, 2003; Halliday *et al.*, 2003). Light quality and ambient temperature signalling show an important level of interaction, suggesting that responses to low R:FR may be regulated by changing the sensitivity to ambient temperature. These interactions can be observed during all developmental stages, from germination to flowering (Franklin, 2009).

It has been recently shown that the photoperiod pathway genes *GIGANTEA* (*GI*) and *CO* play an important role in the flowering-response to light quality, downstream of phytochromes (Kim *et al.*, 2008; Wollenberg *et al.*, 2008). Plants grown under LDs of low R:FR flowered earlier. In contrast, *gi* and *co* mutants were less responsive to low R:FR, and exposure to low R:FR increased *CO* mRNA levels and stabilized the CO protein (Kim *et al.*, 2008; Wollenberg *et al.*, 2008). In stark contrast to these results, previous reports showed that *co* mutations did not affect the responsiveness to EODFR treatments in either *PHYB* or *phyB* mutant backgrounds (Devlin *et al.*, 1996, 1998, 1999). These discrepancies can be caused by different factors, including the light conditions, the background (Columbia versus Landsberg *erecta*) or the alleles used (*co-3* versus *co-9*). Nevertheless, taken together, these results suggest that light quality regulates flowering through the photoperiod pathway, and may activate both CO-dependent and -independent mechanisms.

Another candidate component of the light-quality pathway that regulates flowering time is PHYTOCHROME AND FLOWERING TIME 1 (PFT1). *pft1* mutants are hyposensitive to EODFR (Cerdan and Chory, 2003). However, the addition of FR to LD or simultaneous loss of function of phyB, phyD and phyE promote flowering, mostly independently of PFT1 (Wollenberg *et al.*, 2008). To explain these other facts, PFT1 has been proposed to act as a negative regulator of phytochrome signalling, upstream of CO (Wollenberg *et al.*, 2008). To further understand how PFT1 promotes flowering in response to light quality, we studied the behaviour of *pft1* and *co* mutations in all the *phyB*, *phyD* and *phyE* mutant combinations, and the effect of PFT1 induction in the photoperiod pathway. We conclude that PFT1 promotes flowering by CO-dependent and -independent mechanisms. Furthermore, our microarray data suggest that PFT1 is also involved in modulating other responses that are also sensitive to light quality, like the jasmonic acid (JA)-dependent defence response.

RESULTS

PFT1 seems to act as a positive regulator of flowering downstream of phyB, phyD and phyE

Three phytochromes, phyB, phyD and phyE, are regarded as the most important in regulating flowering time in response to changes in R:FR. Downstream of phytochromes, both CO and PFT1 are involved in the flowering response to light quality (Cerdan and Chory, 2003; Kim *et al.*, 2008; Wollenberg *et al.*, 2008). Recently, PFT1 has been proposed to function as a negative regulator of phytochrome signalling acting upstream of CO (Wollenberg *et al.*, 2008), which is also consistent with the short hypocotyl of *pft1* mutants under red light (Cerdan and Chory, 2003). Although the role of phyB is dominant, the relative contributions of phyB, phyD and phyE change with ambient temperature (Halliday and Whitelam, 2003). Interestingly, during early seedling development, PFT1 modulates the relative strength of signals downstream of phyA and phyB (Cerdan and Chory, 2003). Hence, we hypothesised that PFT1 could be equally involved in modulating the relative contributions of phyB, phyD and phyE in the repression of flowering. If this hypothesis were correct, we would expect phyD and phyE mutations to have a stronger phenotype in the *pft1* mutant background. We thus compared the flowering response of phytochrome and *pft1* single and higher order mutants under LDs (Figure 1; Table S1). In the wild-type (WT) background, as expected, the *phyB* mutant showed the strongest effect compared with *phyD* and *phyE*. *phyE* effects were only observed in the *phyB* mutant background, whereas *phyD* had no significant effects in either *phyB* or *PHYB* backgrounds. These results

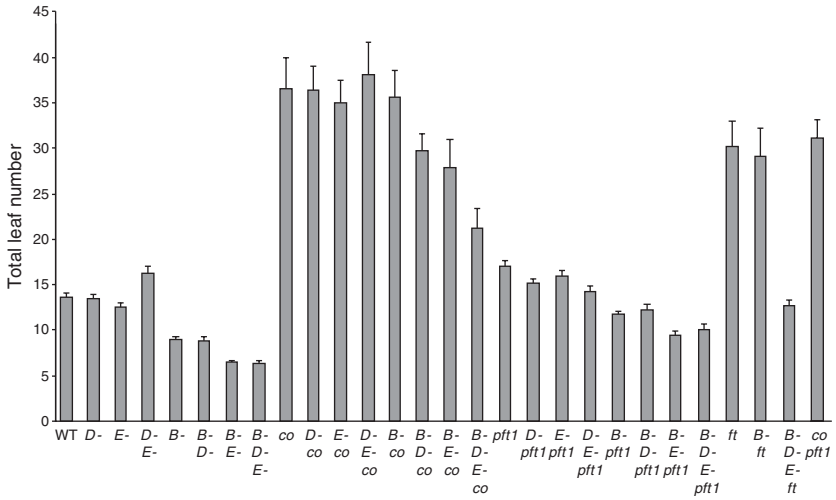

Figure 1. Flowering time of *phyB*, *phyD* and *phyE* single, double and triple mutants in *pft1* and *co* mutant backgrounds.
Plants of the indicated genotypes were grown under long days at 23°C. The total leaf number (cauline and rosette leaves) was recorded at the time of flowering. Bars represent means ± SEs of eight independent experiments including at least 48 plants for each genotype. A complete set of *P* values after one-way ANOVA and Bonferroni's post-hoc tests is given in Table S1.

suggest that at least in our conditions and in the Columbia background, phyB played the most important role, followed by phyE, whereas the role of phyD was negligible and only evident in the absence of phyE. Interestingly, the *phyD phyE* double mutant flowered slightly later than the WT ($P < 0.001$; Table S1), whereas the *phyD phyE pft1* triple mutant flowered slightly earlier than the *pft1* single mutant ($P < 0.01$; Table S1). These results suggest that PFT1 may affect to some extent the hierarchy of phytochrome action. However, the relative importance of the three phytochromes was similar in the WT and *pft1* backgrounds, strongly suggesting that PFT1 does not affect flowering time mainly by altering the relative contributions of different phytochromes. Furthermore, the quadruple mutants *phyB phyD phyE pft1* flowered later than *phyB phyD phyE* triple mutants, suggesting a positive role of PFT1 downstream of these three phytochromes. In these sets of experiments, the suppression of the *phyB* early flowering by *pft1* was not complete, contrary to previous reports (Cerdan and Chory, 2003; Wollenberg *et al.*, 2008). Several factors may account for these differences, including light sources, incubators and soil mixes.

Two genetic pathways regulate flowering downstream of phytochromes phyB, phyD and phyE

In the *co* mutant background, none of the single phytochrome mutations showed any effect on flowering, but accumulating mutations did. *phyB phyD co* and *phyB phyE co* flowered earlier than *phyB co* double mutants ($P < 0.05$; Figure 1; Table S1), and the quadruple *phyB phyD phyE co* mutants flowered even earlier. These results imply that there is more than one pathway downstream of phyB, phyD and phyE. However, these results do not discriminate whether CO and PFT1 act in the same pathway. If CO and PFT1 acted in separate pathways, we would expect the double *co pft1* mutant to flower later than each single mutant parent. The fact that the double *co pft1* mutant did not flower later than the *co* mutant alone argues against the proposition that PFT1 and CO act in different pathways. However, the double *co pft1* mutants were difficult to grow and maintain to maturity, and the mortality of plants increased as the flowering time approached. So the results of the *co pft1* double mutants might have been biased by the fact that the earlier flowering plants survived to be counted, but the later ones did not.

The FT protein plays a significant role downstream of the photoperiod and light-quality pathways. *FT* mRNA levels are increased in *phyB* mutants, and its early flowering phenotype is largely suppressed by *ft* (Figure 1; Cerdan and Chory, 2003; Halliday *et al.*, 2003; Endo *et al.*, 2005). The relatively early flowering of the quadruple *phyB phyD phyE ft* mutant, when compared with the *ft* single mutant or the *phyB phyD phyE co* quadruple mutant, strongly suggests that FT is not the sole integrator of signals downstream of phyB, phyD and phyE. On the other hand, whether FT is the only output of the photoperiod pathway sited downstream of CO has remained controversial (Yamaguchi *et al.*, 2005; Yoo *et al.*, 2005; Jang *et al.*, 2009). When we tested the effect of CO overexpression in the *ft* mutant background, the extremely early flowering time disappeared, although a residual effect remained, which was dependent on *TSF* function (Figure S1). These results also support the notion that another factor acts in parallel with CO downstream of phyB, phyD and phyE.

PFT1 affects temperature sensitivity of flowering, but it is not a global regulator of the response to ambient temperature

Phytochrome signalling, especially phyB signalling, is known to interact with temperature signalling. A few degrees below optimal growth temperatures are known to suppress the early flowering of *phyB* (Halliday *et al.*, 2003), and the responsivity of cold-regulated genes is increased in a *phyB* mutant background (Franklin and Whitelam, 2007). These facts raised the possibility that PFT1 could modulate phytochrome signalling by altering the sensitivity to temperature, so we decided to study the effects of PFT1 hyperactivity in flowering at different growth temperatures. We first generated PFT1 fusions to the rat glucocorticoid receptor domain (GR). GR chimaeric proteins have been used to study transcription factor activity because they remain in the cytoplasm unless dexamethasone (DXM) is added to the media (Simon *et al.*, 1996; Aoyama and Chua, 1997; Samach *et al.*, 2000; Wagner *et al.*, 2004; Yu *et al.*, 2004). To avoid artifacts, *PFT1:GR* chimaeras were made in the context of the full genomic clone of *PFT1*. Transgenic *pft1* mutants bearing the *PFT1:GR* construct looked like *pft1* mutants, but adding DXM to the media restored the WT leaf shape and promoted flowering (Figures 2 and S2). We grew *pft1* mutants, *PFT1* overexpressors and *PFT1:GR* transgenic lines with and without DXM, under a gradient of temperatures from 16 to 24°C. WT plants responded very well to temperature, flowering with about 15 leaves more at the lower temperatures (Figure 3a). *pft1* mutants displayed somewhat higher sensitivity. In contrast, *PFT1* overexpressors and *PFT1:GR* transgenic lines sprayed with DXM flowered earlier, regardless of temperature, indicating that high activity of PFT1 antagonises the delay in flowering time imposed by low ambient temperatures.

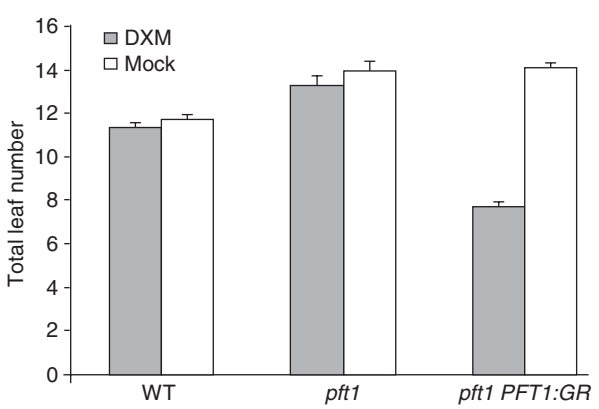

Figure 2. Flowering time of *pft1* mutants complemented with inducible versions of PFT1.
Wild-type (WT) plants, *pft1* mutants and *pft1* mutants complemented with a genomic copy of PFT1 fused to the rat glucocorticoid receptor domain (*pft1 PFT1:GR*) were grown under long days at 23°C in MS salts medium supplemented with 1 μM dexamethasone (DXM) or 0.0096% ethanol as a control (Mock). The total leaf number was recorded at the time of flowering. Bars represent means ± SEs of at least 21 plants for each genotype. Three independent transgenic lines were analysed with similar results (Figure S2).

As PFT1 is the MED25 subunit of the plant Mediator complex (Backstrom *et al.*, 2007), a complex involved in the transcription of most if not all RNA polymerase II (Pol II) transcribed genes in eukaryotes (Malik and Roeder, 2010), we asked whether PFT1 played a more general role in temperature responses. To address this issue, we carried out a microarray experiment to compare the transcriptome of *pft1* and WT plants grown under two different temperatures: 16 and 23°C.

If PFT1 played a more general role in temperature responses, the set of temperature-responsive genes should be enriched in genes affected by the *pft1* mutant background. We used gene-set enrichment analysis (GSEA) (Subramanian *et al.*, 2005; Strasser *et al.*, 2009) to test this proposition. GSEA is an unsupervised methodology specifically designed for computing the overlap between gene expression signatures, and it is an effective approach to test whether two processes produce similar effects in terms of differentially expressed genes (Subramanian *et al.*, 2005). We used an enhanced version of this algorithm, termed two-tails GSEA, which takes into account the direction of the gene expression change (Lim *et al.*, 2009). The list of genes ranked by the effect of temperature in the WT (from the most downregulated to the most upregulated by the 16°C treatment) was queried with a list of the 500 genes that were more responsive to *pft1* either at 16 or 23°C. For comparison, we used the same algorithm for the ELF3 and TFL1 regulated genes, similarly to our previous report (Strasser *et al.*, 2009). A running sum statistic (*y*-axis in Figure 3b) was calculated and plotted against the list of all genes ranked by temperature responsiveness (*x*-axis in Figure 3b). The maximum deviation from zero achieved by the running sum is the enrichment score (ES). The ES was highly significant for the *elf3* mutant at 23°C (Figure 3b, bottom left panel) and the *tfl1* mutant at 16°C (middle top panel), as previously reported (Strasser *et al.*, 2009), but it was much less significant for the *pft1* mutant at both temperatures (Figure 3b, right panels). As shown in Figure 3b, the blue vertical lines, representing downregulated genes, and the red vertical lines, representing upregulated genes, in the *pft1* background clustered to some extent with the genes that were up- or downregulated in response to temperature (*x*-axis in Figure 3b), but this clustering was much less evident compared with *elf3* at 23°C and *tlf1* at 16°C. These results strongly suggest that PFT1 is not involved in a global response to temperature, as ELF3 and TFL1 are, but that the effects of PFT1 on the temperature sensitivity of the flowering response might be caused by its effects on a narrow set of genes. When we looked at the effects of the *pft1* mutation in the expression of known flowering time genes, *FT* and *CO* appeared on the top of the list as the likely candidates (Table S2), which is consistent

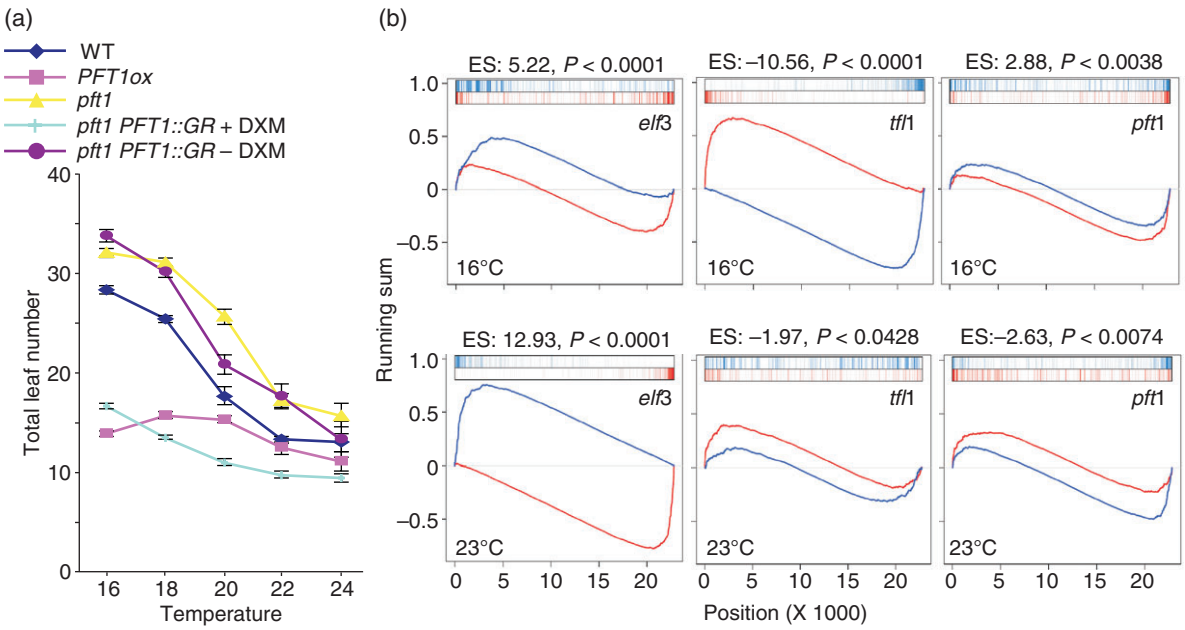

Figure 3. The effects of PFT1 in the sensitivity to ambient temperature.
(a) Plants of the indicated genotypes were grown under long days at 16, 18, 20, 22 or 24°C on soil. The *pft1 PFT1:GR* lines were sprayed every 2 days with a solution of 1 μM dexamethasone (+DXM) or 0.0096% ethanol as a control (–DXM). The total leaf number was recorded at the time of flowering. Data points represent means ± SEs of at least eight plants for each genotype and condition.
(b) GSEA of *pft1*-regulated genes on the temperature-response expression profile. The temperature expression profile was generated by sorting all genes on the microarrays by their response to temperature in wild-type (WT) plants, from the most downregulated (left) to the most upregulated (right) by the 16°C treatment, as compared with control plants grown at 23°C (abscissas). The results for the *elf3* and *tfl1* mutants (Strasser *et al.*, 2009) are shown for comparison with *pft1* mutants. The top 500 differentially expressed genes (DEGs) in the *elf3*, *tfl1* and *pft1* genotypes at 23°C and 16°C are shown in the left, middle and right panels, respectively, as indicated. Upregulated and downregulated genes in the indicated mutant genotypes are represented as red and blue vertical lines, respectively, on the temperature–response expression profile. The colour intensity of these lines is proportional to their local density. The running sums were estimated independently for the downregulated (blue line) and upregulated genes (red line), and the concordance gene-set enrichment analysis (GSEA) enrichment score (ES) and *P* values are shown in the graphs.

with the low levels of *FT* and *CO* mRNA observed in *pft1* mutants (Cerdan and Chory, 2003; Kidd *et al.*, 2009).

PFT1 induces *CO* and *FT* expression

FT has been shown to be one of the integrators of the ambient temperature pathway (Balasubramanian *et al.*, 2006; Lee *et al.*, 2007). Therefore, the low sensitivity of *PFT1:GR* lines and *PFT1* overexpressors to ambient temperature could be the result of a direct or indirect effect on *FT* transcription. An indirect effect could be mediated by CO, which targets the *FT* promoter directly (Adrian *et al.*, 2010; Tiwari *et al.*, 2010). Thus, we decided to test whether PFT1 activates *CO* and *FT* expression. By using our DXM-inducible PFT1:GR system, we found a four- and fivefold increase in the mRNA levels of *CO* and *FT*, respectively, after 3 h of exposure to DXM (Figure 4a and b). However, in these same conditions we did not observe an increase of the *FT* homologue, *TSF* (Figure 4c). Then, we asked whether PFT1 could activate *FT* expression independently of CO. We crossed the *pft1 PFT1:GR* line into *co-9* mutants to obtain the *co pft1 PFT1:GR* line. Interestingly, when DXM was added to the medium, the PFT1::GR chimaera was still able to induce *FT* mRNA by twofold in the absence of CO, indicating a CO-independent role of PFT1 on *FT* expression (Figure 5).

PFT1 can promote flowering independently of CO

Our results suggest that at least two signalling pathways operate downstream of phytochromes (Figure 1), and that PFT1 can promote *FT* expression independently of CO (Figure 5). To test whether PFT1 might also promote flowering in a CO-independent way, we measured the flowering time of *co pft1 PFT1:GR* lines in the presence or absence of DXM and compared them with *pft1*, *pft1 PFT1:GR* and *co pft1* lines under the same conditions (Figure 6). DXM did not promote flowering in WT, *pft1*, *co* or *co pft1* mutants, but promoted flowering by about 10 leaves in *co pft1 PFT1:GR* plants. Therefore, activation of PFT1 in the absence of CO not only increases *FT* expression but also promotes flowering. Once again, the *co pft1* double mutants did not flower later than *co* single mutants. Contrary to the experiments shown in Figure 1, we used axenic conditions, so it is unlikely that we had underestimated the flowering time of *co pft1* double mutants because of an increase in mortality resulting from biotic stress. These results suggest that CO

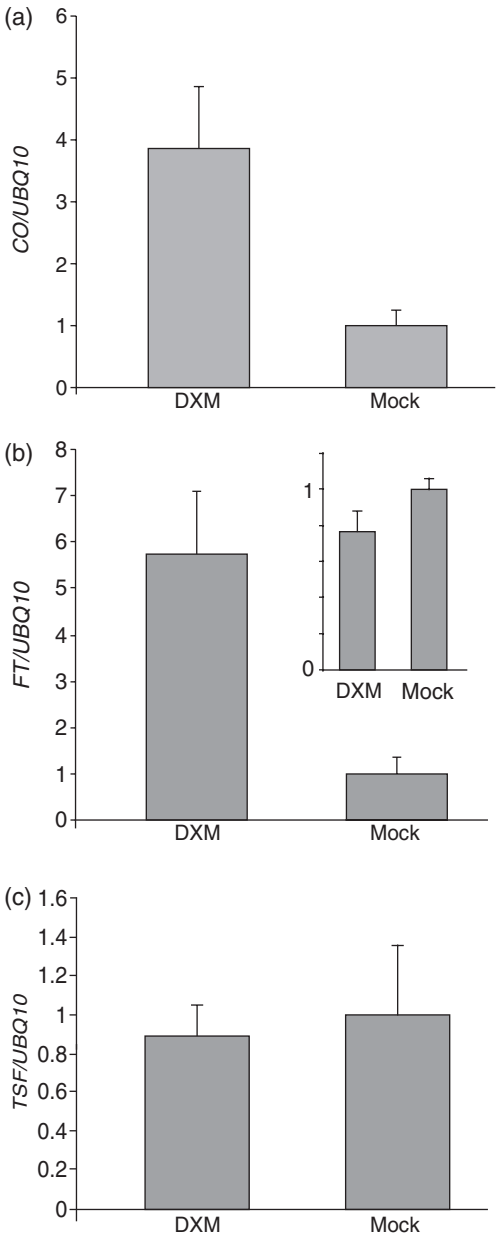

Figure 4. *CO* and *FT* mRNA levels after the induction of PFT1.
(a) *CO* mRNA expression relative to *UBQ10* mRNA in *pft1 PFT1:GR* seedlings after dexamethasone (DXM) treatment. Eight-day-old seedlings grown under continuous light were sprayed with 1 μM DXM (DXM) or with 0.0096% ethanol (Mock), and harvested 3 h later. RNA was extracted and quantitative reverse transcriptase-PCR (qRT-PCR) was performed as described in the Experimental procedures. Bars represent means ± SEs of six independent biological replicates, each analysed in triplicate ($P = 0.021$, by a Student's *t*-test). The experiment was repeated with similar results.
(b) *FT* mRNA expression relative to *UBQ10* mRNA in *pft1 PFT1:GR* seedlings after DXM treatment. Eight-day-old seedlings were treated and processed as described above in (a). Bars represent means ± SEs of six independent biological replicates, each analysed in triplicate ($P = 0.006$ by a Student's *t*-test). The experiment was repeated with similar results. The inset shows the control; DXM does not induce *FT* by itself in the wild-type background.
(c) *TSF* mRNA expression relative to *UBQ10* mRNA in *pft1 PFT1:GR* seedlings after DXM treatment. Eight-day-old seedlings were treated and processed as described above in (a). Bars represent means ± SEs of 12 independent biological replicates, each analysed in triplicate.

and PFT1 pathways indeed interact, but they also clearly show that PFT1 can promote *FT* expression and flowering in a CO-independent way.

As the response to phytochromes phyB, phyD and phyE is not completely dependent on FT (Figure 1), we decided to test whether PFT1 could promote flowering in the absence of FT. We crossed the *pft1 PFT1:GR* lines into *ft-10* mutants to obtain *ft pft1 PFT1:GR* lines. Interestingly, *ft pft1 PFT1:GR* lines flowered earlier in the presence of DXM, whereas *ft* mutants remained late flowering and insensitive to DXM (Figure 6). These results show that PFT1:GR activation induces flowering to some extent, even in the absence of FT, which is consistent with the fact that FT is not the sole integrator of flowering signals downstream of phyB, phyD and phyE (Figure 1).

PFT1 as a hub that integrates environmental signals

The eukaryotic Mediator complex is emerging as an integrator of signalling pathways at the transcriptional level (Malik and Roeder, 2010), and PFT1 was one of the first plant Mediator subunits to be characterized (Cerdan and Chory, 2003; Backstrom *et al.*, 2007). With such a role in directly mediating the effects of transcription factors on Pol-II activity, global expression profiling turns out to be a very useful approach to study the role of PFT1. Hence, we decided to use our microarray data to investigate whether PFT1 might be involved in signalling other environmental conditions where light quality plays a significant role. Temperature and light-quality signalling are well known to interact. The results of the global expression analysis did not suggest a general role for PFT1 in temperature signalling (Figure 3b). However, the dependence of the ES sign on temperature (Figure 3b, right panels), suggests an interaction between *pft1* and temperature signalling. To address this issue, we obtained a gene expression signature for temperature–*pft1* interactions by two-way ANOVA (Table S3), and examined the gene ontology biological process (GO-bp) gene sets that showed significant enrichment in this signature. Most of the GO terms were related to metabolic processes (Table S4). However, we also found the 'jasmonic acid and ethylene-dependent systemic resistance' gene set to be significantly enriched ($P = 0.037$), which is consistent with a role of PFT1 in JA signalling and defence responses to fungal infection (Kidd *et al.*, 2009). Interestingly, low R:FR decreases the sensitivity to JA and increases herbivory (Moreno *et al.*, 2009), as part of a trade-off mechanism where plants limit the allocation of resources to defence if they are shaded (Ballare, 2009). We compared our *pft1* transcriptional profiles with those obtained after JA treatment, pathogen or herbivore attack. We generated four different lists of the top 500 genes (discrete signatures) most

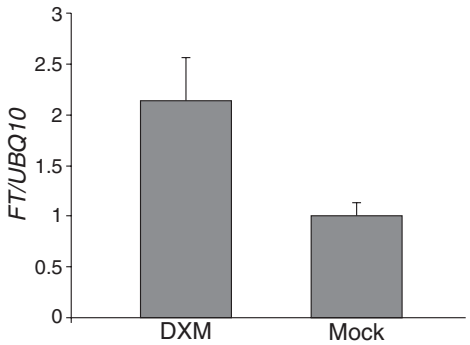

Figure 5. *FT* mRNA expression relative to *UBQ10* mRNA in *co pft1 PFT1:GR* seedlings after dexamethasone (DXM) treatment.
Eight-day-old seedlings were treated as described in Figure 4(a) and qRT-PCR was performed as described in the Experimental procedures. Bars represent means ± SEs of nine independent biological replicates, each analysed in triplicate ($P = 0.018$ by a Student's *t*-test).

affected by the *pft1* mutation: (i) the genes affected by the *pft1* mutation at 16°C (Student's *t*-tests against WT data); (ii) the genes affected by the *pft1* mutation at 23°C (Student's *t*-tests against WT data); (iii) the genes affected by the *pft1* mutation regardless of temperature (two-way ANOVA); and (iv) the genes that showed an interaction between temperature and genotype (two-way ANOVA; Table S3). These discrete signatures were queried on the genome-wide gene expression signatures (gwGES) obtained from publicly available data in response to JA (Goda *et al.*, 2008), attack by the fungi *Fusarium oxysporum* (Kidd *et al.*, 2009) and *Botrytis cinerea* (Ferrari *et al.*, 2007), and the insects *Pieris rapae* and *Frankliniella occidentalis* (De Vos *et al.*, 2005). The summary of the GSEA results, and ES and *P* values, is shown on Table 1 and the graphical displays are presented in Figure S3.

We found a highly significant and direct concordance between the *pft1* signatures and the list of genes ordered by their response to *F. oxysporum*, regardless of the temperature (Figure S3a; Table 1). In other words, the genes downregulated in response to *F. oxysporum* infection were also downregulated in the *pft1* mutant background, and the genes upregulated in response to *F. oxysporum* infection were also upregulated in the *pft1* mutant background. These results predict that *pft1* would be more resistant to *F. oxysporum* attack, and indeed this has been recently shown to be the case (Kidd *et al.*, 2009). Although the ES value was somehow lower at 23°C compared with 16°C, we did not detect a significant concordance with genes that showed a genotype–temperature interaction (Figure S3a, right panel).

We found no significant concordance between the *pft1*-responsive genes (500 top genes after a two-way ANOVA) and the JA-response gene expression signature (Figure S3b, left panel; Table 1). However, when the *pft1* signatures were obtained separately for the two temperatures, we found a weak but still significant concordance (Figure S3b, middle panels; Table 1). Interestingly, the concordance was positive at 16°C (positive ES) and negative at 23°C (negative ES). These results indicate that genes responding to JA are affected by the *pft1* mutant background in a

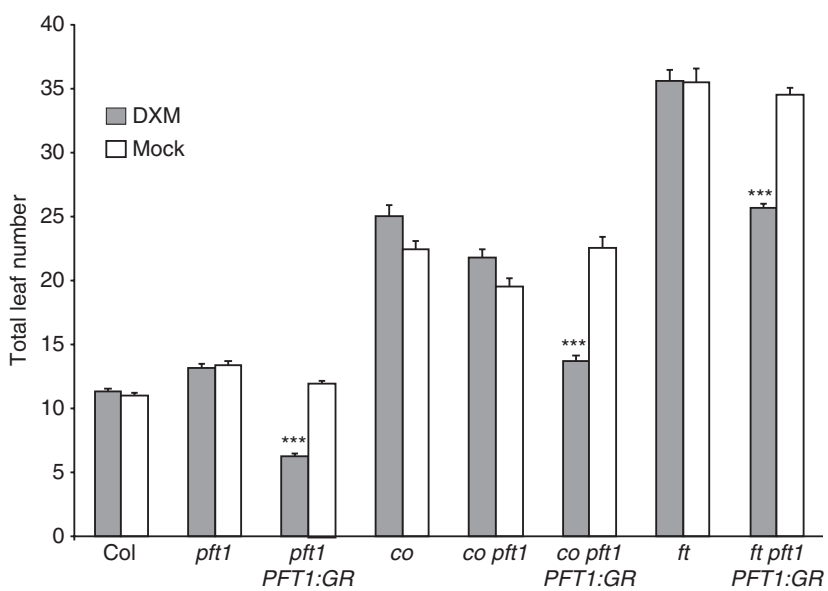

Figure 6. Flowering time of *pft1 PFT1:GR* lines in *co* and *ft* mutant backgrounds.
Plants of the indicated genotypes were grown at 23°C under continuous light in MS salts medium supplemented with 1 μM DXM or 0.0096% ethanol as a control (Mock). The total leaf number was recorded at the time of flowering. Bars represent means ± SEs of at least 35 plants for each genotype. ***Denotes statistical significance between DXM and mock-treated plants ($P < 0.001$ by a Student's *t*-test).

Table 1 Enrichment score (ES) and *P* values after two-tail gene-set enrichment analysis (GSEA) of *pft1*-regulated genes on five different genome-wide gene expression signatures (gwGES). Five gwGES (second column) were generated by sorting all genes on the genome-wide gene expression profiles by their response to the treatment. Four different discrete signatures (first column) were used to query the profiles: the top 500 differentially expressed genes (DEGs) in the *pft1* genotype after a two-way ANOVA (*pft1*), the top 500 DEGs in the *pft1* mutant grown at 16°C (*pft1*-16°C) or at 23°C (*pft1*-23°C) and the top 500 DEGs for the interaction effect (*pft1*–temperature) after a two-way ANOVA. The *P* values represent the probability of obtaining the corresponding ES values just by chance. The complete set of graphical displays is shown in Figure S3

Discrete signature	gwGES	Enrichment score	*P*
pft1	Jasmonic acid	−0.7437	0.4650
pft1–16°C	Jasmonic acid	2.7600	0.0062
pft1–23°C	Jasmonic acid	−2.3533	0.0158
pft1–temperature	Jasmonic acid	4.3491	<0.0001
pft1	*Fusarium oxysporum*	9.8875	<0.0001
pft1–16°C	*Fusarium oxysporum*	8.7097	<0.0001
pft1–23°C	*Fusarium oxysporum*	7.7414	<0.0001
pft1–temperature	*Fusarium oxysporum*	0.1085	0.9170
pft1	*Botrytis cinerea*	−1.0457	0.299
pft1–16°C	*Botrytis cinerea*	4.6385	<0.0001
pft1–23°C	*Botrytis cinerea*	−5.3997	<0.0001
pft1–temperature	*Botrytis cinerea*	7.3006	<0.0001
pft1	*Pieris rapae*	0.0822	0.9290
pft1–16°C	*Pieris rapae*	3.0580	0.0016
pft1–23°C	*Pieris rapae*	−3.5451	0.0004
pft1–temperature	*Pieris rapae*	5.6656	<0.0001
pft1	*Frankliniella occidentalis*	−0.0813	0.9440
pft1–16°C	*Frankliniella occidentalis*	2.9585	0.0024
pft1–23°C	*Frankliniella occidentalis*	−1.9178	0.0554
pft1–temperature	*Frankliniella occidentalis*	4.6264	<0.0001

temperature-dependent way. Thus, we queried the list of genes that showed genotype–temperature interaction in the microarray data (500 top genes) for enrichment among all the probes in the microarrays ordered by their response to JA (Figure S3b, right panel). Indeed, we found a highly significant direct concordance between these signatures. The graphical display (Figure S3b, right panel) shows that genes upregulated in response to JA are enriched in genes responsive to PFT1 in a temperature-dependent manner (red lines clustered to the right).

A similar result was obtained by reversing the query and the gene profile, showing a strong enrichment of the 500 most responsive genes to JA on the list of all genes ranked by *pft1*–temperature interaction (data not shown).

When the *pft1* signatures were queried against the list of genes ordered by their response to *B. cinerea*, we found similar behaviour compared with the JA gene profile, but in this case the ES values were significantly higher (Figure S3c; Table 1). Once again, the sign of the ES was reversed between the experiments performed at 16°C and 23°C (Figure S3c, middle panels), and the ES obtained by querying the genes showing *pft1*–temperature interaction was highly significant (Figure S3c, right panel); note that the red vertical lines in the right panel of Figure S3c are clustered to the right, with the genes upregulated in response to *B. cinerea* infection. The negative concordance (negative ES value) between the *pft1* gene signature (at 23°C) and the transcriptome profile after *B. cinerea* attack is consistent with the reported susceptibility of *pft1* mutants to *B. cinerea* infection (Kidd *et al.*, 2009), but the negative concordance at 16°C suggests that the susceptibility of *pft1* mutants to *B. cinerea* may decrease at low temperatures. However, the effect of temperature on plant–pathogen interactions is of too complex a nature to simply be predicted, and needs to be addressed experimentally case by case (Garrett *et al.*, 2006).

When we queried the *pft1* signatures against the gene expression profile obtained after attacks by *P. rapae* and *F. occidentalis*, we obtained qualitatively similar results to those of *B. cinerea*, although the ES values were lower. We observed again a reversion of the ES sign between 16°C and 23°C *pft1* signatures, and a very significant ES with genes showing the *pft1*–temperature interaction. Interestingly, the ES values for these responses were higher than those obtained for the JA responses, and the genes upregulated in the defence response were enriched in genes responsive to PFT1 in a temperature-dependent manner (Figure S3c–e).

These results are consistent with previous reports (Kidd *et al.*, 2009; Elfving *et al.*, 2011) and strongly suggest that PFT1 modulates the responses to other environmental factors that interact with light quality, like herbivory and temperature, and suggest that these interactions might be mediated through the regulation of JA signalling.

DISCUSSION

PFT1 was first characterized as a flowering time gene (Cerdan and Chory, 2003), and later on it was shown to be part of

the plant Mediator complex (Backstrom *et al.*, 2007) and to be involved in JA-dependent defence responses (Kidd *et al.*, 2009). However, the mechanisms by which PFT1 regulates flowering are poorly understood. In a previous report, it has been suggested that PFT1could act as a negative regulator of photostable phytochromes (phyB, phyD and phyE) (Wollenberg *et al.*, 2008). Our initial genetic approach strongly suggests that PFT1 acts positively to promote flowering downstream of phytochromes. This is supported by two facts: (i) the relationship among phyB, phyE and phyD signalling remained mostly unchanged in *pft1* and *PFT1* backgrounds; and (ii) the quadruple *phyB phyD phyE pft1* mutant flowered significantly later than the triple *phyB phyD phyE*, showing that PFT1 still promotes flowering in the absence of these three phytochromes (Figure 1). Moreover, the behaviour of *pft1* and *co* mutations also strongly suggests that at least two different pathways act downstream of phyB, phyD and phyE (Figure 1).

Given the high level of interaction between light-quality signalling (especially through phyB) and temperature signalling (Mazzella *et al.*, 2000; Halliday *et al.*, 2003; Franklin and Whitelam, 2007; Heschel *et al.*, 2007; Foreman *et al.*, 2011), PFT1 could be acting in an ambient temperature pathway to regulate signalling downstream of phytochromes. Increasing PFT1 activity led to temperature-independent flowering (Figure 3a). However, at the transcriptome level we did not find evidence of PFT1 having a general role in temperature signalling, as was evident for *elf3* and *tfl1* mutants (Figure 3b). These data taken together indicate that higher activity of PFT1 promotes flowering at lower temperatures by specifically activating flowering genes rather than changing the overall sensitivity to ambient temperature.

Our microarray analysis suggested that *FT* and *CO* could be the main flowering gene targets downstream of PFT1. This was confirmed by the fact that *FT* and *CO* mRNA increased rapidly after PFT1 induction and reached several-fold higher levels after 3 h (Figure 4). Interestingly, *FT* levels increased by twofold shortly after the induction of PFT1 in *co* mutants, showing that PFT1 was able to activate *FT* independently of CO (Figure 5). Furthermore, we found that PFT1 activation induced flowering even in the absence of CO (Figure 6). These data strongly support the notion that PFT1 promotes flowering by a feed-forward regulatory loop. First, PFT1 activates the expression of *CO* and CO is expected to activate the transcription of *FT*. Second, PFT1 activates the expression of *FT* independently of CO itself (Figure 7). Moreover, the promotion of flowering by PFT1 in the *ft* mutant background (Figure 6) strongly suggests that other flowering integrators may be regulated by PFT1. Indeed, *SUPPESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) was recently proposed to be important for the light-quality response in Arabidopsis (Hori *et al.*, 2011), and we also found *SOC1* to be upregulated after PFT1 induction (2.48-fold, $P = 0.044$). By contrast, we did not observe an increase in *TSF* mRNA under the same conditions for which we observed *FT* and *CO* induction (Figure 4c). However, we cannot rule out that PFT1 may upregulate *TSF* mRNA under other conditions, or later during development.

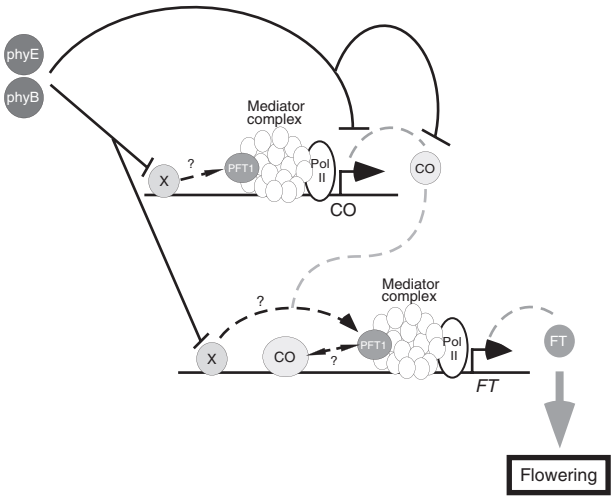

Figure 7. A model summarising the effects of PFT1 in the promotion of flowering.
In this work we show that PFT1 promotes flowering by at least two pathways: one that promotes *CO* expression, more likely at the transcriptional level; and another one that promotes *FT* expression. Factor X is a putative transcription factor, negatively regulated by phytochromes. The identity of X is currently unknown, but could be one or more of the transcription factors recently found to interact with PFT1 (Elfving *et al.*, 2011; Ou *et al.*, 2011). The other connectors represent the negative regulation imposed by phytochromes (mainly phyB) on CO, both at transcriptional and post-translational levels, as described previously (Valverde *et al.*, 2004; Wollenberg *et al.*, 2008).

The results presented above raise the question as to how phytochrome signalling interacts with PFT1 at the molecular level. We have studied the PFT1 protein levels under different light conditions, including altering R:FR, and we were unable to find any evidence that phytochrome regulates PFT1 protein levels (data not shown). As PFT1 was identified as a component of the Mediator complex and this complex is expected to be associated with most, if not all, Pol-II dependent promoters (Bjorklund and Gustafsson, 2005), it is unlikely that PFT1 binding to promoters is under regulation. However, as a Mediator complex subunit, PFT1 could be interacting with a relatively high number of transcription factors and 'mediating' their effects on Pol-II dependent transcription (Figure 7). In mammalian systems, different signalling pathways integrate with the transcription machinery through different Mediator subunits, but interacting pathways might converge on the same Mediator subunits (Malik and Roeder, 2010). In very recent papers, a set of 10 transcription factors have been found to interact with a PFT1 domain (Elfving *et al.*, 2011; Ou *et al.*, 2011). Most of them belong to the AP2-EREB family of transcription factors, but also to other transcription factor families

like Myb, HD-ZF and B-box. Some AP2-EREB transcription factors are involved in JA-mediated defence responses (Ou *et al.*, 2011) and B-Box transcription factors are involved in the response to light quality (Crocco *et al.*, 2010). These data suggest that PFT1 could aid in the integration of several environmental signals that are related to light quality, and eventually regulate the output of these signals. An emerging picture is that light quality and JA responses are connected at the molecular level. The exposure of plants to low R:FR mimicking the presence of neighbours triggers the SAS, a decrease in defence response and a subsequent increase in herbivory. These effects on defences result neither from limited resources nor from the diversion of resources to SAS, but instead are caused by a decrease in the sensitivity to JA (Izaguirre *et al.*, 2006; Moreno *et al.*, 2009). So phytochromes, mainly phyB, regulate the sensitivity to JA and avoid the diversion of resources to defence if plants are under the risk of being outcompeted by neighbours. On the other hand, JA signalling can also affect light responses (Wierstra and Kloppstech, 2000; Riemann and Takano, 2008; Riemann *et al.*, 2009; Robson *et al.*, 2010). Our microarray data analysis (Figure S3) together with previous data (Kidd *et al.*, 2009) show that PFT1 affects JA-responsive genes and, interestingly, this effect is dependent on temperature (Figure S3b). A similar pattern of interaction was observed with the transcriptomes after pathogen or herbivore attack (Figure S3c–e). Taken together, our transcriptome studies strongly suggest that PFT1 is integrating different environmental signals that are known to interact with light-quality signalling. Hence, how PFT1 regulates the transcriptional output of certain pathways is becoming a very important question. The very recent reports showing that PFT1 interacts with diverse transcription factors bring new avenues of research to deepen our understanding of how environmental signalling is integrated at the transcriptional level.

EXPERIMENTAL PROCEDURES

Plant material

All the mutants are in the *A. thaliana* Columbia background. The mutants and alleles used were: *phyB9* (Reed *et al.*, 1993), *phyD-201*, *phyE-201* (Wollenberg *et al.*, 2008; Strasser *et al.*, 2010), *co-9* (Balasubramanian *et al.*, 2006), *pft1-1* and *PFT1* overexpressors (Cerdan and Chory, 2003), *ft-10* (Yoo *et al.*, 2005), *tsf-1* (Alonso and Stepanova, 2003) and *ft-10 tsf-1* (Mathieu *et al.*, 2007).

Constructs

The complete genomic clone of *PFT1* was subcloned into binary plasmid pPZP212. An *Eco*RI site was engineered just before the TAA stop codon. The *GR*-coding region (Yu *et al.*, 2004) was subcloned into this *Eco*RI site to make the *PFT1:GR* fusion, and the constructs were introduced into *pft1-1* mutants by transformation with *Agrobacterium tumefaciens* (Clough and Bent, 1998). Only single-locus insertions were used for physiological experiments.

The 35S::CO:HA fusion construct was created by cloning *CO* cDNA into CHF5 binary vector (Hiltbrunner *et al.*, 2005).

Plant growth conditions

Seeds were sterilized with chlorine in the vapour phase and plants were grown on a 1:1:1 soil, vermiculite and perlite mix. Every 2 weeks plants were fertilized with a 0.1% solution of Hakaphos (Compo Agricultura, http://www.compo.es).

We used MS salts medium to grow plants *in vitro*. For experiments with DXM (D1756; Sigma-Aldrich, http://sigmaaldrich.com), the medium was supplemented with 1 μM DXM or 0.0096% ethanol as a mock control, or, when stated, plants were sprayed with DXM.

Plants were grown at 23°C under LDs (16-h light/8-h dark) or continuous light, with a light intensity of 80 μmol m^{-2} s^{-1} produced by cool white fluorescent tubes.

Quantitative RT-PCR

Seedlings were frozen in liquid nitrogen and total RNA was prepared using a Plant Total RNA Mini Kit (YRP50; Real Biotech Corporation, http://www.real-biotech.com), and 1 μg was used to synthesise cDNA with M-MLV reverse transcriptase (Invitrogen, http://invitrogen.com), and used to quantitate *UBQ10*, *CO*, *FT* and *TSF* expression with the Mx3005P real-time PCR system (Stratagene, http://www.genomics.agilent.com) in conjunction with SyBR Green I (Invitrogen). *UBQ10* was used as a housekeeping gene to normalize gene expression (Czechowski *et al.*, 2005). The average ratio of mock and treated sample values was used to determine the fold change in transcript level. Relative expression levels were determined using the comparative cycle threshold (C_t) method (Larionov *et al.*, 2005).

Microarray experiments

The microarray experiments were performed as described previously (Strasser *et al.*, 2009). Briefly, we used 10-day-old seedlings grown on MS salts medium as described above, and three biological replicates for each of the four conditions (two genotypes and two temperatures), synthesized the 12 cRNA samples and hybridized them to Affymetrix expression arrays (ATH1-121501; Affymetrix, http://www.affymetrix.com). The expression set was obtained after RMA normalization using the AFFY package implemented in R 2.10 (Irizarry *et al.*, 2003; Gautier *et al.*, 2004; R-Development-Core-Team, 2008). The genotype, temperature and the interaction coefficients were obtained by fitting the data to a linear model, and moderated *P* values for the null hypothesis that the coefficients are equal to zero were estimated for each gene with the limma algorithm (Smyth, 2005). Statistical significance for the overlap between signatures was computed by a modified version of Gene Set Enrichment Analysis (two-tails GSEA; Lim *et al.*, 2009). See Appendix S1 for more details.

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SUPPORTING INFORMATION

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

Figure S1. Flowering of *CO:HA* overexpressors in *ft* and *ft tsf* mutant backgrounds.

Figure S2. Flowering time of *pft1 PFT1:GR* independent transgenic lines.

Figure S3. Gene-set enrichment analysis (GSEA) of *pft1*-regulated genes on the expression profile of plants after JA treatment and fungal or herbivore attack.

Table S1. Bonferroni's post-hoc test *P* values for phytochrome mutant combinations in the WT, *pft1* and *co* mutant backgrounds.

Table S2. List of expression values of flowering-time genes in the *pft1* mutant background.

Table S3. List of genes showing an interaction between genotype and temperature in our global expression studies.

Table S4. Gene ontology analysis of genes showing interaction between genotype and temperature in our global expression studies.

Appendix S1. Full description of methods and the sequence of primers used in this study.

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