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A complementary role for ELF3 and TFL1 in the regulation of flowering time by ambient temperature

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SUMMARY

Plants regulate their time to flowering by gathering information from the environment. Photoperiod and temperature are among the most important environmental variables. Sub-optimal, but not near-freezing, temperatures regulate flowering through the thermosensory pathway, which overlaps with the autonomous pathway. Here we show that ambient temperature regulates flowering by two genetically distinguishable pathways, one requiring *TFL1* and another requiring *ELF3*. The delay in flowering time observed at lower temperatures was partially suppressed in single *elf3* and *tfl1* mutants, whereas double *elf3 tfl1* mutants were insensitive to temperature. *tfl1* mutations abolished the temperature response in *cryptochrome* mutants that are deficient in photoperiod perception, but not in *phyB* mutants, which have a constitutive photoperiodic response. In contrast to *tfl1*, *elf3* mutations were able to suppress the temperature response in *phyB* mutants, but not in *cryptochrome* mutants. Gene expression profiles revealed that the *tfl1* and *elf3* effects are due to the activation of different sets of genes, and identified *CCA1* and *SOC1/AGL20* as being important cross-talk points. Finally, genome-wide gene expression analysis strongly suggests a general and complementary role for ELF3 and TFL1 in temperature signalling.

Keywords: Arabidopsis, ELF3, TFL1, flowering, ambient temperature, microarrays.

INTRODUCTION

Plants compute variables such as light and temperature to finetune flowering onset, integrating environmental information to determine whether the appropriate flowering season is about to arrive (Boss *et al*., 2004). In *Arabidopsis thaliana*, day-length detection is accomplished through the photoperiod pathway, whereas responses to low temperatures, associated with winter time, require the vernalization pathway.

As a facultative long-day (LD) plant, Arabidopsis flowers earlier under LD conditions than under short-day (SD) conditions. CONSTANS (CO) is the central component of the photoperiod pathway, because it is essential in discriminating between LD and SD due to its regulation at different levels. First, expression of *CO* at the mRNA level is tightly regulated by the circadian clock and other components, such that maximum expression levels occur during the night under SD conditions or late in the light period under LD conditions (Suarez-Lopez *et al*., 2001; Yanovsky and Kay, 2002; Valverde *et al*., 2004; Imaizumi *et al*., 2005; Laubinger *et al*., 2006). Second, its expression is post-translationally regulated by light; photoreceptors phytochrome A (phyA) and cryptochrome 2 (cry2) promote CO stability, whereas phytochrome B (phyB) antagonizes this stabilization (Valverde *et al*., 2004; Laubinger *et al*., 2006; Jang *et al*., 2008). These results account for the late flowering of *phyA* and *cry2* mutants and the early flowering of the *phyB* mutants. As a result of both types of regulation, CO levels are only high enough under LD conditions to induce the expression of *FLOWERING LOCUS T* (*FT*), a promoter of flowering that acts as an integrator of various flowering pathways (Boss *et al*., 2004). After induction, FT protein moves to the apical meristem to switch on genes required for reproductive development (Corbesier *et al*., 2007; Jaeger and Wigge, 2007; Mathieu *et al*., 2007; Tamaki *et al*., 2007).

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In turn, the vernalization pathway present in over-wintering accessions promotes flowering by stably down-regulating a flowering repressor, the MADS box transcription factor *FLOWERING LOCUS C* (*FLC*) (Boss *et al.*, 2004; He and Amasino, 2005). FLC represses the expression of *FT* and another important flowering-time integrator, the MADS box transcription factor *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*, also known as *AGL20*) (Searle *et al.*, 2006).

Despite advances in understanding of the vernalization pathway, flowering responses to sub-optimal temperatures are mostly unknown (Samach and Wigge, 2005; Lee *et al.*, 2008; Penfield, 2008). Low temperatures can regulate development in Arabidopsis (Mazzella *et al.*, 2000), and it has been proposed that a thermosensory pathway regulates flowering time in response to ambient temperature by *FLC*-independent mechanisms (Blazquez *et al.*, 2003). *FVE* and *FCA*, two genes that were previously classified as part of the autonomous pathway, are also part of the thermosensory pathway (Blazquez *et al.*, 2003). More recently, the flowering repressor SHORT VEGETATIVE PHASE (SVP) was shown to be important for the ambient temperature response by directly regulating *FT* expression (Lee *et al.*, 2007). On the other hand, the early-flowering phenotype of *phyB* mutants was shown to be temperature-dependent (Halliday and Whitelam, 2003; Halliday *et al.*, 2003), suggesting that at least some interactions between light and temperature may be expected. These findings suggest that temperature signalling may occur by various pathways or mechanisms; however, no evidence has been presented to date to support this view. Of the known flowering repressors, only SVP was shown to be involved in temperature signalling (Lee *et al.*, 2007).

We decided to study the role of flowering repressors in the ambient temperature response. *TERMINAL FLOWER 1* (*TFL1*) and *EARLY FLOWERING 3* (*ELF3*) appeared as interesting candidates for our research. TFL1 is a member of the FT family, but acts in an antagonistic manner to FT. The antagonistic effects of FT and TFL1 have been mapped to a single amino acid position (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). *tfl1* mutants display an early-flowering phenotype that is ameliorated by growth under SD conditions (Shannon and Meeks-Wagner, 1993). *TFL1* has been placed genetically downstream of the autonomous pathway genes *FVE* and *FCA* (Ruiz-Garcia *et al.*, 1997; Page *et al.*, 1999; Soppe *et al.*, 1999), genes that are also involved in the thermosensory pathway (Blazquez *et al.*, 2003). These results suggest that *TFL1* is an interesting candidate for a temperature-signalling component.

ELF3 encodes a protein of unknown function that is involved in the photoperiodic induction of flowering (Hicks *et al.*, 2001; Liu *et al.*, 2001). ELF3 requires phyB under certain conditions, and these proteins interact *in vitro* (Liu *et al.*, 2001), but ELF3 appears to act independently of phyB in the control of several photomorphogenic responses (Reed *et al.*, 2000; Liu *et al.*, 2001). Given that the *phyB* early-flowering phenotype is temperature-dependent (Halliday and Whitelam, 2003; Halliday *et al.*, 2003), we also investigated *elf3* behaviour at lower temperatures.

Here, we present evidence that ambient temperature regulates flowering by two genetic pathways: one that is closely associated with the photoperiod pathway and requires *ELF3*, and another that requires TFL1 and is related to the autonomous pathway. Furthermore, we show by microarray analysis that both ELF3 and TFL1 play important and complementary roles in temperature signalling.

RESULTS

Ambient temperature affects the photoperiodic response

To investigate whether photoperiod signals affect the response to ambient temperature, we grew Arabidopsis plants under SD (8 h light/16 h dark) and LD (16 h light/8 h dark) conditions at either 16 or 23°C. As previously reported, growth at 16°C produced a delay in flowering time (Blazquez *et al.*, 2003; Halliday and Whitelam, 2003; Halliday *et al.*, 2003; Lee *et al.*, 2007). However, the delay was most obvious under LD conditions compared with SD conditions (Figure 1a), and a significant interaction between

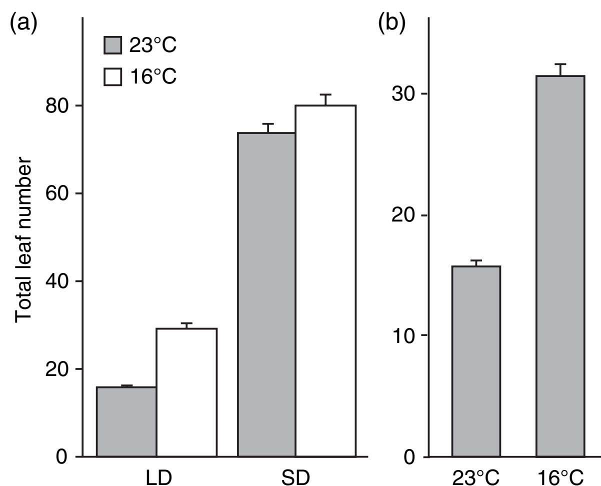

Figure 1. Temperature effects on flowering time under various photoperiods.
(a) WT plants were grown under long days (LD, 16 h light/8 h dark) or short days (SD, 8 h light/16 h dark), and at two temperatures, 23°C (grey bars) or 16°C (white bars). The total leaf number (cauline and rosette leaves) was recorded at the time of flowering. Bars represent the mean ± SE for at least 10 plants. The effects of both photoperiod and temperature as well as the interaction between these factors were statistically significant ($P < 0.01$ by two-factor ANOVA).
(b) WT plants were grown under continuous light (CL) at either 23 or 16°C. Total leaf number (cauline and rosette leaves) was recorded at the time of flowering. Bars represent the mean ± SE for two independent experiments with a total of at least 38 plants.

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photoperiod and temperature was observed (two-way ANOVA, $P < 0.01$). The effects of low temperature were also observed under continuous light (CL) (Figure 1b).

tfl1 and *elf3* mutants display a reduced response to temperature

The observed interaction between photoperiod and temperature suggests that the photoperiod pathway may be antagonized by low temperatures or promoted by higher ones. We reasoned that if low temperatures delay flowering by antagonizing the photoperiod pathway, at least some of the early-flowering mutants could have a reduced responsiveness to ambient temperature. Only a few of the known early-flowering mutants have been evaluated for flowering time at low temperatures (Blazquez *et al.*, 2003; Halliday *et al.*, 2003; Lee *et al.*, 2007). We chose two early-flowering mutants, *elf3* and *tfl1*, and compared their behaviour with that of *phyB* mutants. We measured the flowering time of *elf3-7*, *elf3-9*, *tfl1-1* and *tfl1-14* mutants and compared them side by side with the wild-type (WT) and *phyB* mutants (Figure 2). *phyB* mutants flowered at an earlier stage than the WT plants at 23°C (i.e. when they had approximately four fewer leaves), but no significant differences were observed at 16°C (Figure 2), as reported for plants grown under SD conditions (Halliday and Whitelam, 2003; Halliday *et al.*, 2003). Because *phyB* mutants flowered early at 23°C, the temperature effect was even stronger in *phyB* mutants compared with WT controls. In contrast to *phyB*, *elf3* and *tfl1* mutants showed a reduced response to temperature. In other words, low temperatures were less efficient in restraining flowering in *elf3* and *tfl1* mutants. These results were not due to allele-specific effects, because we found similar behaviours with the various alleles (Figure 2).

elf3 but not *tfl1* mutations impair the responsiveness to temperature of *phyB* mutants

The contrasting responses observed in the early-flowering mutants prompted us to study the epistatic relationships among *tfl1*, *elf3* and *phyB*. We grew *phyB*, *tfl1* and *elf3* single and double mutants under continuous light at 16 or 23°C (Figure 3). The genetic interactions among *tfl1*, *elf3* and *phyB* were strikingly different. The *elf3 phyB* double mutants flowered earlier than single mutant parents at both temperatures, which is consistent with phyB and ELF3 acting independently in flowering (Reed *et al.*, 2000). However, *elf3* was epistatic to *phyB* with respect to temperature sensitivity, a conclusion that is supported by two facts. First, loss of *ELF3* function rendered a *phyB* mutant hyposensitive to temperature (Figure 3, compare *phyB* mutants with *phyB elf3* double mutants). Second, a *phyB* mutant was early flowering at 16°C in the *elf3* genetic background (compare WT versus *phyB* and *elf3* versus *phyB elf3* at 16°C). In other words, the *elf3* mutation not only reduced the response to temperature, but also changed the behaviour of *phyB* mutants at low temperatures. In contrast to *elf3*, the *tfl1* effect was mostly additive to the *phyB* effect. *tfl1* and *elf3* mutants still showed responsiveness to temperature, but double *elf3 tfl1* plants flowered essentially at the same developmental time at either 16 or 23°C. Taken together, these results suggest that ELF3 and TFL1 regulate the flowering response to

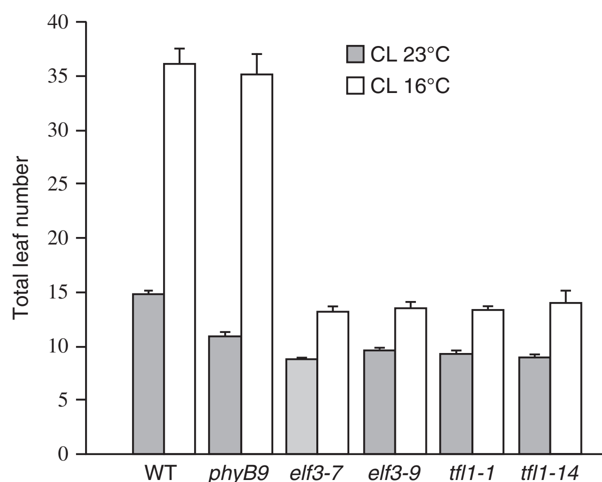

Figure 2. Effects of *tfl1* and *elf3* mutants on temperature responsiveness. Plants of the indicated genotypes were grown under continuous light (CL) at either 23°C (grey bars) or 16°C (white bars). The total leaf number (cauline and rosette leaves) was recorded at the time of flowering. Bars represent the mean ± SE for at least 22 plants for each genotype.

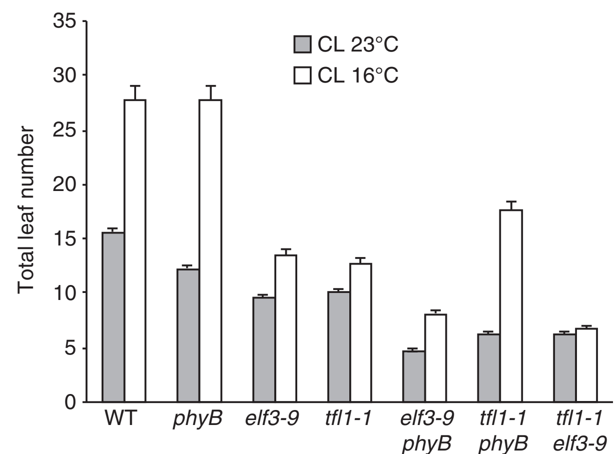

Figure 3. *tfl1 elf3* double mutants are essentially insensitive to temperature. Plants of the indicated genotypes were grown under continuous light (CL) at either 23°C (grey bars) or 16°C (white bars). Total leaf number (cauline plus rosette leaves) was recorded at the time of flowering. Bars represent the mean ± SE for at least 13 plants.

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ambient temperature by different pathways: ELF3, but not TFL1, acts in a photoperiod- and phyB-related pathway.

The *tfl1* mutation suppresses the temperature response in cryptochrome-deficient plants

The three photoreceptors that promote flowering under LD conditions in Arabidopsis are cry2, phyA and cry1, with cry2 being the most important based on the phenotype of *cry2* mutants (Mockler *et al.*, 1999, 2003). It was previously reported that, under LD conditions, the delay in flowering imposed by low temperatures was exaggerated in *cry2* mutants (Blazquez *et al.*, 2003). We decided to test whether the *tfl1* mutation was able to suppress the delayed flowering onset at 16°C reported in cryptochrome-deficient mutants. We grew WT plants and *tfl1*, c*ry2*, *tfl1 cry2*, *cry1 cry2* and *tfl1 cry1 cry2* mutants under CL at either 16 or 23°C (Figure 4). Lack of cryptochromes resulted in a delay in the time to flowering, irrespective of the presence of the *tfl1* mutation. These results imply that expression of the *tfl1* phenotype requires the presence of cryptochromes, as recently reported (Buchovsky *et al.*, 2008). However, the delay in flowering time produced by the low temperature in the *cry2* mutant disappeared in the *tfl1 cry2* double mutant. A similar but weaker effect was observed in the *tfl1 cry1 cry2* triple mutant (Figure 4). These results show that the loss of temperature sensitivity in *tfl1* mutants (Figure 2) is not due to saturation of the flowering-promoting pathways in the *tfl1* genetic background, because a similar loss of temperature responsiveness still occurs in the late-flowering *tfl1 cry2* double mutant (Figure 4).

The effect of the *elf3* mutation on the temperature response requires a functional photoperiod pathway

In *phyB* mutants, in which the photoperiod pathway is constitutively activated (Valverde *et al.*, 2004), an *elf3* mutation severely impaired the sensitivity to temperature (Figure 3). When we compared the effect of *elf3* on the temperature response in cryptochrome-deficient plants, we observed a different behaviour to that of the *tfl1* mutants. Although *elf3 cry2* double mutants flowered earlier when compared with *tfl1 cry2* mutants (Figure 4), the *elf3* mutation did not suppress the response to temperature in a *cry2* background, as observed with *tfl1*. Progressive accumulation of mutations in the other photoperiod photoreceptors, *cry1* and *phyA*, rendered an *elf3* mutation relatively ineffective at inducing early flowering at both 16 and 23°C, but even in the extreme case of the triple *phyA cry1 cry2* mutant background, *elf3* was not able to suppress the response to temperature (Figure 4). A similar effect was observed under LD conditions (Figure S1).

A *phyB* mutation accelerated flowering in the *cry2* single and *phyA cry1 cry2* triple mutant backgrounds at 23°C, consistent with previous reports (Mockler *et al.*, 1999, 2003). However, the effect of the *phyB* mutant was suppressed by low temperatures, rendering the *phyB cry2* double and *phyA phyB cry1 cry2* quadruple mutants hypersensitive to temperature (Figure S1).

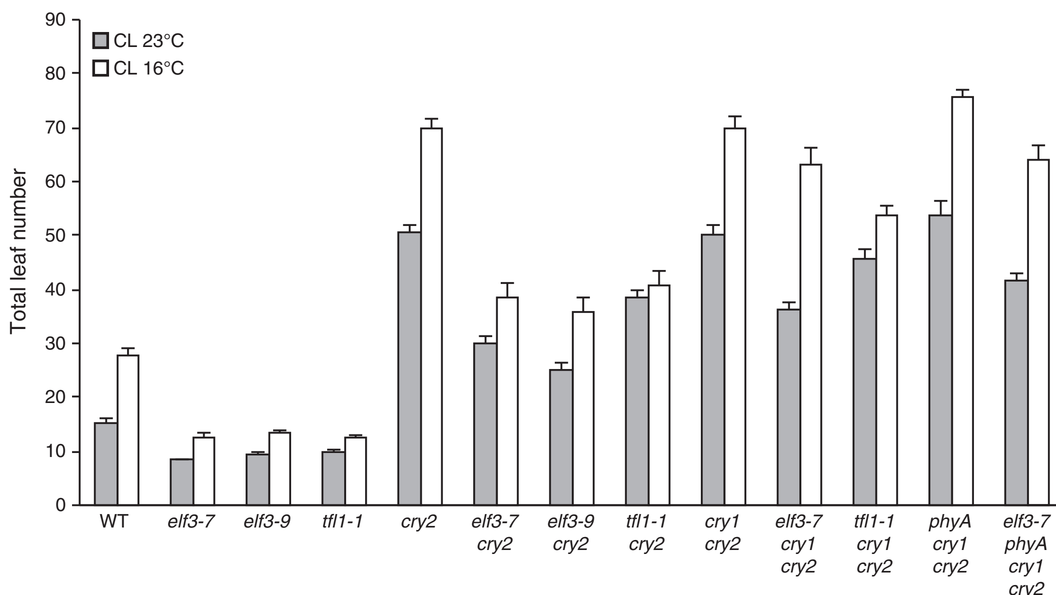

Figure 4. Epistatic interactions among *tfl1*, *elf3* and photoreceptor mutants.
Plants of the indicated genotypes were grown under continuous light (CL) at either 23°C (grey bars) or 16°C (white bars). The total leaf number (cauline and rosette leaves) was recorded at the time of flowering. Bars represent the mean ± SE for at least 10 plants. The experiment was repeated with similar results.

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***TFL1* and *ELF3* regulate the flowering response to temperature at different points**

To try to understand the molecular mechanisms underlying the involvement of TFL1 and ELF3 in modulating flowering in response to temperature, we analysed the gene expression profile of WT and the *elf3* and *tfl1* mutants. As a first approach, we focused on a list of flowering-time genes selected from the literature (Table S1). We found that *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *GIGANTEA* (*GI*), *CO*, *FT*, *SOC1* and *FLC* are differentially expressed in *elf3* mutants (Figure 5 and Figure S2; see Table S1 for *q* values for the *elf3* effect). Despite the complex behaviour of *gi* mutants, genetic evidence suggests that *GI* acts downstream of *CCA1* and *LHY* in the regulation of flowering time (Mizoguchi *et al.*, 2005; Niwa *et al.*, 2007). *GI*, *CO*, *FT* and *SOC1* appear to act in a linear pathway (Suarez-Lopez *et al.*, 2001; Yanovsky and Kay, 2002; Mizoguchi *et al.*, 2005; Yoo *et al.*, 2005). These data strongly suggest that early flowering at low temperatures in *elf3* mutants is due, at least in part, to activation of an important set of photoperiod pathway genes by the low mRNA levels of the circadian core components *CCA1* and *LHY*. The changes observed in the expression patterns of *CYCLING DOF FACTOR 1* (*CDF1*), *CRY1*, *CONSTANS-LIKE 2* (*COL2*), *ACTIN RELATED PROTEIN 2* (*ATARP4*) and *ELF4* are either relatively small or the direction of change is such that it does not account for the early-flowering phenotype of the *elf3* mutants (Ledger *et al.*, 2001; Doyle *et al.*, 2002; Imaizumi *et al.*, 2005; Kandasamy *et al.*, 2005), but we cannot rule out a small contribution from *HY5 HOMOLOG* (*HYH*) (Holm *et al.*, 2002). Interestingly, the *tfl1* expression profile showed a completely different pattern to that of *elf3*, with *SOC1* as the only flowering-time gene that was differentially expressed (Figure 5 and Figure S2; see Table S1 for *q* values for the *tfl1* effect). The slightly elevated *CDF3* levels are not expected to affect flowering time (Imaizumi *et al.*, 2005).

When *elf3* × temperature interactions were analysed, *CCA1* was at the top of the list of flowering-time genes ($q < 0.05$). Although *LHY* showed a similar expression pattern (Figure 5a and Figure S2), the interactions were not statistically significant. Interestingly, *SOC1* was down-regulated by lower temperatures ($q < 0.05$), indicating that, in *elf3* and *tfl1* mutants, the effect of temperature is partially suppressed by up-regulation of *SOC1* (Figure 5c).

The results of transcriptome profiling suggest a role for circadian clock components in temperature signalling. In

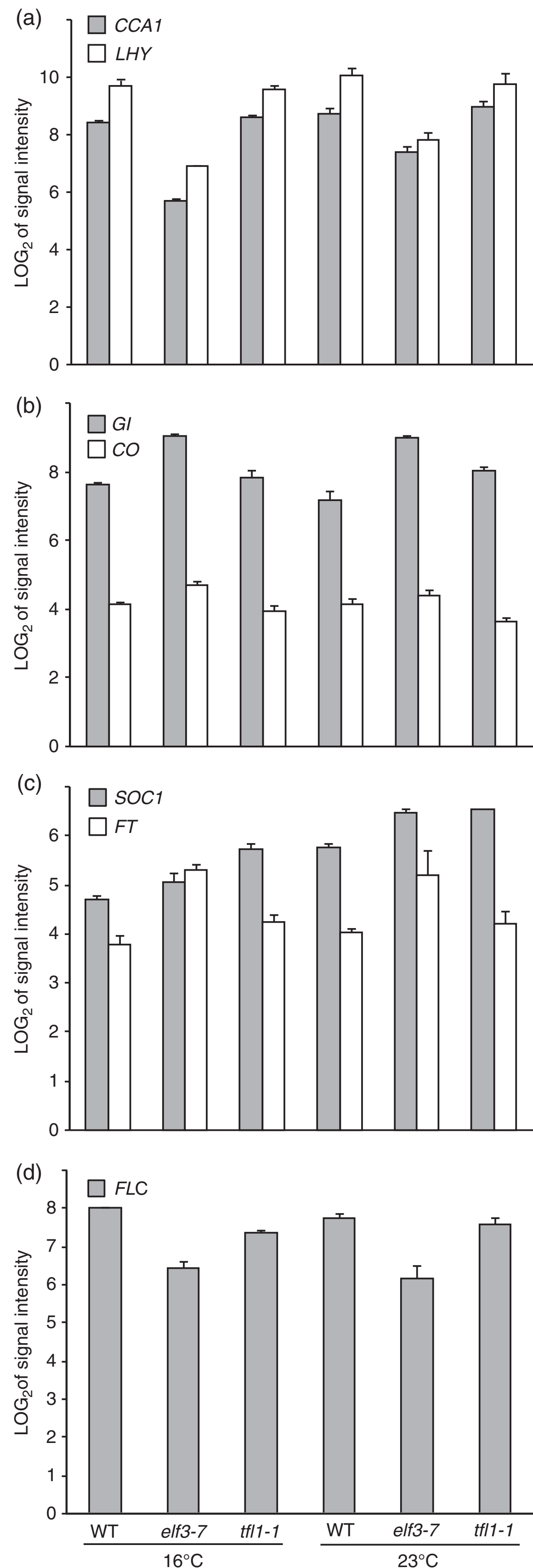

Figure 5. mRNA levels of key flowering-time genes in *elf3* and *tfl1* mutants. Seedlings of WT, *elf3-7* and *tfl1-1* genotypes were sown on MS plates and grown for 10 days in continuous light at either 16 or 23°C, as indicated. Three independent samples (biological replicates) for each treatment x genotype combination were hybridized to Affymetrix gene expression arrays. Bars represent the average of log_2-transformed data after Robust Multi-Array Average (RMA) normalization ± SE.

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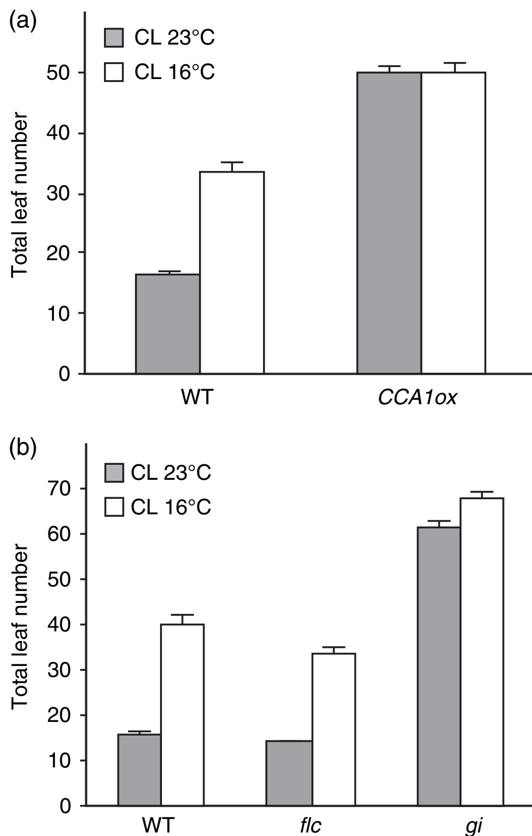

Figure 6. *CCA1* over-expressor lines and *gi* mutants are hyposensitive to temperature.
(a) WT plants and *CCA1* over-expressor lines were grown under continuous light (CL) at either 23°C (grey bars) or 16°C (white bars). The total leaf number (cauline and rosette leaves) was recorded at the time of flowering. Bars represent the mean ± SE for at least 21 plants.
(b) *flc* and *gi* mutants were grown and the flowering time scored as in (a). Bars represent the mean ± SE for at least eight plants.

addition to their early-flowering phenotype, *elf3* mutants are arrhythmic under CL (McWatters *et al.*, 2000; Covington *et al.*, 2001; Hicks *et al.*, 2001). We decided to test *CCA1* over-expressors, which are also arrhythmic, but, unlike *elf3* mutants, are late-flowering (Wang and Tobin, 1998). We grew *CCA1* over-expressor lines and *gi* mutants under CL at 16 and 23°C. As expected, both genotypes were late flowering compared with WT, but displayed reduced sensitivity to low temperatures (Figure 6).

Another important, well-known flowering gene that is mis-expressed in *efl3* mutants is *FLC*. *FLC* mRNA levels were threefold lower in *elf3* mutants (Figure S2). However, consistent with previous reports (Blazquez *et al.*, 2003; Lee *et al.*, 2007), we observed only a relatively minor loss of sensitivity to temperature in *flc* mutants (Figure 6b). The levels of both *FLC* and *FT* were not clearly regulated by temperature in the WT as previously observed (Blazquez *et al.*, 2003; Lee *et al.*, 2007). We believe that this is due to the different conditions used; unlike the previous authors, we used CL in our microarray experiments.

The data presented so far are consistent with a model in which the ambient temperature can regulate flowering by two separate pathways, one that requires TFL1 and is independent of the photoperiod pathway, and another that shows at least some degree of interaction with the photoperiod pathway and depends on ELF3 activity.

ELF3 and TFL1 play a general role in temperature signalling

We reasoned that if ELF3 or TFL1 plays a more general role in temperature responses, the set of temperature-responsive genes should be enriched in genes affected by the mutant genetic backgrounds. After filtering of the data (see Experimental procedures), we generated a list of temperature-responsive genes in WT and a list of *elf3*- and *tfl1*-responsive genes at 23°C (*t* test, $P < 0.05$). Of the 2473 temperature-regulated genes and 478 *elf3*-regulated genes, 235 were found in both groups. This intersection is highly significant ($P = 2.66 \times 10^{-16}$, Fisher exact test) (Figure S3). Furthermore, 219 of the 235 shared genes (93%) changed in the same direction after a decrease in temperature or presence of the *elf3* mutation. Of the 629 *tfl1*-regulated genes at 23°C (*t* test, $P < 0.05$), 175 were also temperature-regulated, which is significant ($P = 0.0006$, Fisher exact test); however, only 80 (46%) changed in the same direction. Twenty-six genes were found to be present in all three groups, i.e. *elf3*-, *tfl1*- and temperature-regulated ($P = 2.95 \times 10^{-5}$) (Figure S3).

Next, we compared the list of temperature-responsive genes in the WT with the list of *elf3*- and *tfl1*-responsive genes at 16°C rather than 23°C (Figure S4). Of 1263 *elf3*-regulated genes, 341 were also temperature-responsive ($P = 1.3 \times 10^{-5}$), but only 231 (68%) changed in the same direction. Of 1761 *tfl1*-regulated genes, 615 were also temperature-responsive ($P = 7.94 \times 10^{-21}$), but only 46 (7%) changed in the same direction, meaning that 93% of the shared genes changed in the opposite direction after a decrease in temperature or presence of the *tfl1* mutation at 16°C. These results show that a common set of genes is affected by temperature and *elf3* and/or *tfl1*, and also suggest that there is a concordance between the effect of lower temperatures on gene expression and the effect of the *elf3* and *tfl1* genotypes at 23 and 16°C, respectively.

These results were confirmed using gene set enrichment analysis (GSEA) (Subramanian *et al.*, 2005). This method uses a list of genes ranked by the effect of one factor (temperature in the WT), and then questions whether the genes affected by the other factor (*elf3* or *tfl1* genetic background) are randomly distributed in the former list or clustered at the top or bottom. A running sum statistic is calculated for the list of genes ranked by the temperature effect; the magnitude increases when the gene belongs to

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the group of genes affected by the genotype and decreases when it does not. The maximum deviation from zero is the enrichment score (ES). The ES was highly significant in each case (Figure S5), showing that the group of temperature-regulated genes is enriched in genes affected by the *tfl1* and *elf3* mutations at both temperatures.

The previous results confirm that ELF3 and TFL1 share common target genes with temperature signalling. However, when the targets are also similarly affected by the different treatments, a functional connection is revealed. We used a modified GSEA analysis (concordance GSEA), that has previously been used to query the Connectivity Map (Lamb *et al.*, 2006), to infer functional connections among ELF3, TFL1 and temperature signalling. The concordance GSEA takes into account both the significance and the direction of changes in gene expression. Genes in the WT were ordered from the most down-regulated to the most up-regulated by low temperature. This list was queried using the top 200 differentially expressed genes for the four treatments, i.e. *elf3* at 23°C, *elf3* at 16°C, *tfl1* at 23°C and *tfl1* at 16°C; the running sum was computed separately for the down-regulated and up-regulated genes (Figure 7). At 23°C, genes down-regulated in the *elf3* genotype (Figure 7a, blue vertical lines) were enriched among the genes down-regulated by temperature, whereas genes that were up-regulated in the *elf3* genotype (Figure 7a, red vertical lines) were enriched among the genes up-regulated by temperature. The ES was highly significant (0.870, $P < 0.0001$), confirming a direct concordance between the changes in gene expression produced by the *elf3* mutation at 23°C and those occurring at low temperatures. In other words, the *elf3* mutants grown at 23°C mimic WT plants grown at 16°C. Conversely, we did not find any significant correlation between *tfl1*-regulated genes at 23°C and the temperature-response expression profile (Figure 7c). At 16°C, the results changed dramatically. The concordance between the *elf3* genotype at 16°C and the effects of low temperature was much weaker, although significant (Figure 7b; ES = 0.276, $P < 0.001$), whereas a strong inverse concordance was found for the *tfl1* genotype (Figure 7d; ES = 0.843, $P < 0.0001$). The genes up-regulated in the *tfl1* genotype at 16°C, represented by red vertical lines (Figure 7d), were enriched among the genes down-regulated by low temperature, and genes down-regulated in the *tfl1* genotype were enriched among the genes up-regulated by low temperature (blue vertical lines, Figure 7d).

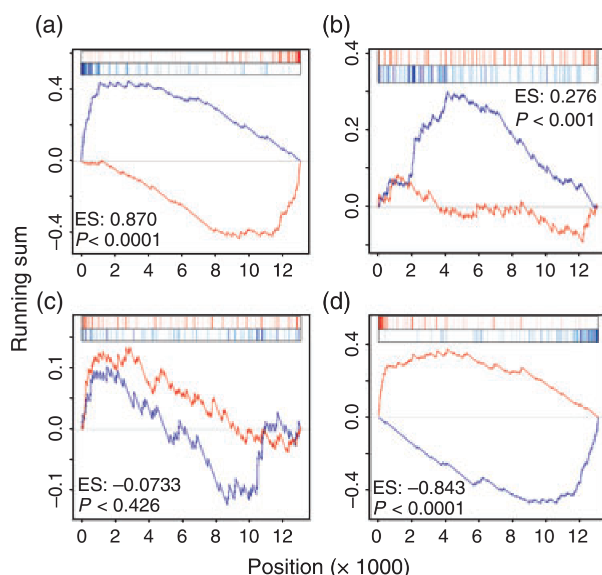

Figure 7. Gene set enrichment analysis of *tfl1*- and *elf3*-regulated genes on the temperature-response expression profile.
The expression profile was sorted from the most down-regulated to the most up-regulated genes, when WT plants grown at 16°C were compared with control plants grown at 23°C (*x* axis). Up-regulated and down-regulated genes in the mutant genotypes are represented as red and blue vertical lines, respectively, on the temperature-response expression profiles. The color intensity of these lines is proportional to their local density. The running sums were estimated independently for the down-regulated (blue line) and up-regulated genes (red line), and the concordance GSEA ES and *P* values are shown. The top 200 differentially expressed genes in the *elf3* genotype at 23 and 16°C are shown in (a) and (b), respectively. The top 200 differentially expressed genes in the *tfl1* genotype at 23 and 16°C are shown in (c) and (d), respectively.

Finally, we compared our set of data with those previously reported (Balasubramanian *et al.*, 2006). These datasets were used to generate a list of genes ranked by the responsiveness to temperature (25°C to 16°C) and the concordance with the differentially expressed genes of our data analysed as above (Figure S6). Interestingly, despite comparison of samples from plants grown under very different conditions (5-week-old SD-grown apices), we obtained similar results (compare Figure 7a with Figure S6a and Figure 7d with Figure S6d).

The inverse concordance between *tfl1* and the low-temperature response strongly suggests that TFL1 is a positive regulator of the responses to low temperature. On the other hand, the direct concordance between *elf3* and low-temperature effects suggests a negative role for ELF3. This result appears to be contradictory because *elf3* mutants are early flowering and low temperatures delay flowering. This apparent contradiction can be explained by the effects of interactions between the *elf3* genotype and temperature on gene expression. The flowering behaviour of *elf3* mutants may be explained by its effects on *CCA1* expression, which decreased in a temperature-dependent manner in *elf3* mutants (Figure 5 and Figure S2). The interactions between the *elf3* genotype and temperature are not restricted to *CCA1* expression. Seven genes from the phenlypropanoid pathway were significantly affected in the *elf3* mutant background (Figure S7 and Table S2, $P < 0.001$), including two transcription factors, AT4G09820 (*TT8*) and AT1G22640 (*MYB3*), which encode positive (Nesi *et al.*, 2000) and negative regulators of the pathway, respectively. It is noteworthy that four of the seven selected genes

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showed significant *elf3* x temperature interactions (Table S4 and Figure S7). Their expression was significantly affected at 16°C, but not at 23°C, suggesting that the pathway is more active in *elf3* mutants grown at 16°C. Furthermore, the genes that showed a significant *elf3* x temperature interaction (Table S4, uncorrected *P* value <0.01) were enriched in those that experienced higher effects of *elf3* at 16°C than at 23°C ($P = 0.0022$, Fisher exact test), which supports a global role for ELF3 in the response to ambient temperature.

DISCUSSION

In the present paper, we investigated the role of TFL1 and ELF3 in the regulation of flowering time at low ambient temperatures. Lower temperatures (16°C) delay flowering (Figure 1), and this delay is severely impaired in both *tfl1* and *elf3* single and double mutants (Figures 2 and 3). Two sets of experiments strongly suggest that TFL1 and ELF3 regulate the sensitivity to temperature by different genetic pathways. First, whereas *tfl1* and *elf3* single mutants displayed reduced sensitivity to temperature (Figure 2), the flowering time of the *elf3-9 tfl1-1* double mutant was almost unaffected by temperature (Figure 3). The *elf3-9* allele is likely to be null because of an early stop codon (Hicks *et al*., 2001), whereas the *tfl1-1* allele has undetectable TFL1 levels due to a change in a conserved amino acid (Bradley *et al*., 1997; Page *et al*., 1999; Conti and Bradley, 2007). Thus, it seems unlikely that the behaviour of single and double mutants is due to a combination of weak alleles. The second set of experiments also supports the notion of two separate pathways. Whereas *tfl1* and *phyB* effects were additive, *elf3* was mostly epistatic to *phyB* with respect to temperature sensitivity (Figure 3). On the other hand, *tfl1* was effective in reducing the temperature response in *cry2* mutants, but *elf3* was not (Figure 4).

The genome-wide gene expression data showed that *elf3* mutations affect a set of photoperiod pathway genes (Table S1, Figure 5 and Figure S2). Low levels of *CCA1* and *LHY* expression and a concomitant rise in *GI*, *CO*, *FT* and *SOCI/AGL20* levels were observed, consistent with reported effects of *elf3* and circadian clock mutations on gene expression (Suarez-Lopez *et al*., 2001; Kim *et al*., 2005b; Mizoguchi *et al*., 2005). In contrast, the *tfl1* mutation antagonized the effects of temperature on *SOC1* expression, but did not show clear mis-regulation of upstream photoperiod pathway genes (Table S1, Figure 5 and Figure S2).

Our genetic and gene expression data collectively show that ambient temperature regulates flowering time by at least two pathways. One pathway appears to work regardless of the state of the photoperiod pathway and requires TFL1. This pathway is more likely to be related to the thermosensory pathway reported previously (Blazquez *et al*., 2003). This is consistent with the proposition that *TFL1* might work genetically as a negative regulator of events that occur downstream of *FVE* and *FCA* (Ruiz-Garcia *et al*., 1997; Page *et al*., 1999; Soppe *et al*., 1999), the autonomous pathway genes that are required for the thermosensory pathway (Blazquez *et al*., 2003). Interestingly, *TFL1* appears to act genetically in the same pathway as *EARLY FLOWERING IN SHORT DAYS* (*EFS*), which was also placed downstream of *FVE* and *FCA* (Soppe *et al*., 1999). However, *EFS* is involved in histone methylation, and *efs* mutations are highly pleiotropic, leading to early flowering in several late-flowering backgrounds (Soppe *et al*., 1999; El-Assal *et al*., 2003), probably due to high levels of *SOC1* and *FT* mRNA (Zhao *et al*., 2005).

The second pathway requires ELF3 and is likely to be associated with the photoperiod pathway. Several results support this view. First, the *elf3* mutation suppressed the low-temperature response in *phyB* mutants (Figure 3), which are known to have a constitutively activated photoperiod pathway (Valverde *et al*., 2004), but not in photoperiod pathway-impaired mutants (Figure 4). Second, the *elf3* mutation affects expression of photoperiod- and clock-related genes such as *GI* and *CCA1* (Figure 5), and overexpression of *CCA1* or loss of function of *GI* impairs the response to temperature (Figure 6). These results also raise the possibility that the role of ELF3 may be exerted through the circadian clock. *elf3* mutants are arrhythmic in the light, and we confirmed that this is also the case at 16°C. After entrainment under 12 h light/12 h darkness photoperiods, we moved 8-day-old seedlings to continuous light at either 16 or 23°C. Under these free-running conditions, *CCA1* mRNA did not cycle in *elf3* mutants at either temperature (data not shown). The acclimation response to freezing temperatures, controlled by the CBF regulon, is gated by the circadian clock (Fowler *et al*., 2005). Understanding of the ambient temperature response at a similar level awaits the development of gene expression markers for the acute response to ambient temperature changes. These advances will allow the study of the interactions between ELF3 and the state of the oscillator, especially in darkness, when the *elf3* oscillator is functional.

Our data are consistent with TFL1 and ELF3 acting in different organs: ELF3 interacting with the photoperiod pathway and clock components in the leaves and TFL1 regulating *SOC1* in the apex. However, we cannot rule out the possibility that they may act in common tissues. *ELF3* is widely expressed, including in the apex (Hicks *et al*., 2001), whereas *TFL1* is mostly expressed in the apex, but also in the inflorescence (Bradley *et al*., 1997). Similarly, *SOC1* is highly expressed in the apex but is also found in leaves (Lee *et al*., 2000; Kim *et al*., 2005a), and we cannot rule out the possibility that TFL1 might regulate *SOC1* expression beyond the apex (Conti and Bradley, 2007).

Finally, GSEA analysis strongly suggests that ELF3 and TFL1 play more general roles in the responses of plants to ambient temperature. The inverse concordance between the

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effect of low temperature and the effects of *tfl1* at 16°C, but not at 23°C (Figure 7c,d), strongly suggests that TFL1 plays a positive role in the response to low ambient temperature. These results are more interesting in light of recent findings that TFL1 is associated with membranes (Sohn *et al.*, 2007) and the role of membrane processes in the perception of temperature in non-plant systems (Mansilla *et al.*, 2004). On the other hand, the direct concordance between low temperature and *elf3* effects on gene expression (Figure 7a) suggests a negative role for ELF3 in modulating the response to temperature. In principle, this proposition appears contradictory, because, if ELF3 plays a negative role in temperature signalling, we would expect *elf3* mutants to flower later than WT plants at lower temperatures. However, early flowering of *elf3* mutants at low temperatures correlates with its effects on the expression of *CCA1* and other photoperiod pathway genes (Figure 5 and Figure S2).

SVP plays an important role in the flowering response to ambient temperature (Lee *et al.*, 2007) and was recently shown to regulate *SOC1* transcription directly (Li *et al.*, 2008), which is interesting given that our microarray experiments revealed that *SOC1* is responsive to temperature. Whether SVP acts in a similar pathway to TFL1 or ELF3 is still unclear. We did not find differences in *SVP* expression in the *tfl1* or *elf3* mutants that could account for the behaviour of the *tfl1* and *elf3* mutants at lower temperatures. Conversely, we did not find significant effects of temperature on *TFL1* or *ELF3* mRNA levels in *svp* and autonomous pathway mutants (Figure S8). The NAC-family transcription factor *LONG VEGETATIVE PHASE 1* (*LOV1*) has been shown to play a dual role, repressing flowering within the photoperiod pathway and positively regulating the cold response, suggesting that LOV1 is a link between cold responses and flowering (Yoo *et al.*, 2007). In contrast to our results, *LOV1* appears to act independently of *CCA1*, *LHY* and *GI*, negatively regulating *CO* expression. We did not find relevant changes in *LOV1* expression in our microarray experiments. The fact that *GI* positively regulates *CO* in the photoperiod pathway, but that *gi* mutants are hypersensitive to cold (Cao *et al.*, 2005), whereas *co* mutants are tolerant to freezing (Yoo *et al.*, 2007), underscores the complexity of the interactions between flowering-time genes and temperature responses.

The use of Arabidopsis as a model system has elucidated the role of new players in the temperature-signalling network in plants. However, unlike several light and hormonal receptors, the nature of the thermosensor is still not well understood. Part of the difficulty is the enormous diversity of biological macromolecules whose activities are temperature-sensitive. Whatever the nature of the thermosensor(s), when spring approaches, the increase in day length induces flowering in LD plants, such as Arabidopsis. However, the variation in ambient temperature may still be drastic as seasons change. The ability of Arabidopsis to antagonize flowering promotion when temperatures are still sub-optimal ensures that seed set occurs under more benign conditions.

EXPERIMENTAL PROCEDURES

Plant material

All mutants used in this study are in the Columbia background. The mutants and alleles used were *elf3-9* and *elf3-7* (Hicks *et al.*, 2001), *tfl1-1* and *tfl1-14* (Bradley *et al.*, 1997), *phyB9* (Reed *et al.*, 2000), *gi-2*, *cry2-1*, *cry1(hy4B104) cry2-1* double mutants and *phyA412 cry1-304 cry2-1* triple mutants (Mockler *et al.*, 2003).

The *tfl1-1 cry2-1*, *elf3-7 cry2-1* and *elf3-9 cry2-1* double mutants were obtained by crossing single mutant parents, selecting for late-flowering *cry2* homozygotes in the F_2, and screening for either *tfl1-1*, *elf3-7* or *elf3-9* homozygous plants in the F_3 using dCAPs (see Appendix S1 for more details on genotyping various alleles).

The *tfl1-1 cry2-1 hy4B104* and *elf3-7 cry2-1 hy4B104* triple mutants were obtained by crossing the double mutants *tfl1-1 cry2-1* and *elf3-7 cry2-1* with the *cry2-1 hy4B104* double mutants and selecting for tall plants under blue light in the F_2. Genotypes were confirmed by PCR using dCAPs (Neff *et al.*, 1998) as described in Appendix S1.

The *elf3-7 phyA412 cry1-304 cry2-1* quadruple mutants were obtained by crossing *elf3-7* to the *phyA412 cry1-304 cry2-1* triple mutant. F_2 seedlings were grown under far-red light, and tall plants were transplanted to soil. Because *phyA* and *cry2* are linked, the selected plants were also late flowering. F_2 plants heterozygous for *elf3-7* were harvested. F_3 siblings showing consistent long hypocotyls under blue light (and therefore *cry1* homozygous candidates) were transplanted to soil and genotyped to search for *elf3-7* homozygotes.

In all cases, the double mutants were genotyped again, and the hypocotyl length was checked under far-red, blue and white light to confirm the genotypes before flowering-time experiments. The *phyA412 phyB9 cry1-304 cry2-1* quadruple mutant was a generous gift from Todd Mockler (Department of Botany and Plant Pathology, Oregon State University, OR).

Flowering experiments

Seeds were sterilized with chlorine in the vapour phase, and plants were grown on a 1:1:1 mix of peat moss, vermiculite and perlite. Every two weeks, plants were fertilized with a 0.1% solution of Hakaphos (Compo Agricultura, http://www.compo.es). Photoperiods were as indicated for each experiment, with a light intensity of 80 μmol m^{-2} sec^{-1} produced by cool white fluorescent tubes. The total leaf number, rosette plus cauline leaves, was determined at the time of flowering. Experiments were repeated at least once for consistency of the results. When a two-way ANOVA was used (Figure 1), the data were log_{10} transformed to achieve normality and homoscedasticity.

Microarray experiments

Seeds were sterilized and sown on plates with Murashige & Skoog salts and 1.2% plant agar (Duchefa Biochemie, http://www.duchefa.com). After 3 days of stratification at 4°C, plants were incubated in growth chambers under the same conditions used for flowering experiments for 10 more days. Seedlings were harvested, weighed

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and frozen in liquid N_2. Total RNA was prepared using a plant RNAeasy kit (Qiagen, http://www.qiagen.com), and 5 μg was used to prepare the cRNA that was hybridized to the Affymetrix expression arrays (ATH1-121501) as described by the manufacturer (Affymetrix, http://www.affymetrix.com). The expression set (18 chips, three biological replicates per treatment) was obtained after Robust Multi-Array Average (RMA) normalization and elimination of the probe sets with signals that were not significantly higher than the background using the affy package implemented in the R system (Irizarry *et al.*, 2003; Gautier *et al.*, 2004; R-Development Core Team, 2008). The genotype, temperature and interaction coefficients were obtained by fitting the data to the linear model: $y_i = \mu_i + \alpha_i + \beta_i + \alpha_i \times \beta_i + \varepsilon$, where μ_i is the mean for gene *i*, α_i is the genotype effect on gene *i*, β_i is the temperature effect on gene *i*, and $\alpha_i \times \beta_i$ is the interaction effect between genotype and temperature. ε is the zero mean normally distributed error. Moderated *P* values for the null hypothesis that the coefficients are equal to zero were estimated for each gene using the limma algorithm (Smyth, 2005). We used False Discovery Rate (FDR) to correct *P* values for multiple hypothesis testing, and corrected values are reported as *q* values.

The gene set enrichment analysis (GSEA) was implemented in the R system. Redundant probe sets mapping to the same gene were eliminated by keeping only the one that showed the highest dynamic range (CV) across the complete expression set. The expression set was rank-sorted using the *P* value complement: rank(1 − *P*), where *P* is the *P* value, and the enrichment score (ES) was estimated using an exponent score of 1 as previously described (Subramanian *et al.*, 2005). The *P* value for the ES was computed from a null distribution obtained by permuting the gene labels 10 000 times. For concordance GSEA, the running sums were computed independently for the up-regulated and down-regulated genes of the querying gene set using the same expression profile sorted from the most down-regulated to the most up-regulated gene. A combined running sum was computed by inverting the order of the running sum for the up-regulated genes and subtracting it from the running sum for the down-regulated genes. The maximum and minimum values of this combined running sum were added to obtain the ES.

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SUPPORTING INFORMATION

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

Figure S1. Epistatic interactions among *elf3* and photoreceptor mutants under LD conditions.
Figure S2. mRNA levels of key flowering-time genes in *elf3* and *tfl1* mutants on a linear scale.
Figure S3. Overlap between genes affected by temperature and *elf3* and *tfl1* genotypes at 23°C.
Figure S4. Overlap between genes affected by temperature and *elf3* and *tfl1* genotypes at 16°C.
Figure S5. Gene set enrichment analysis of *tfl1*- and *elf3*-regulated genes on temperature-responsive genes.
Figure S6. Gene set enrichment analysis of *tfl1*- and *elf3*-regulated genes on a temperature-response expression profile obtained from published data.
Figure S7. The effect of an *elf3* mutation on the expression of phenylpropanoid pathway genes.
Figure S8. mRNA levels of *ELF3* and *TFL1* in various genetic backgrounds.
Table S1 List of expression values for flowering-time genes.
Table S2 List of genes mis-regulated in *elf3-7* mutants.
Table S3 List of genes mis-regulated in *tfl1-1* mutants.
Table S4 List of genes showing significant genotype (*elf3-7*) by temperature interaction.
Appendix S1 Additional experimental procedures.
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