Cool nighttime temperatures induce the expression of *CONSTANS* and *FLOWERING LOCUS T* to regulate flowering in *Arabidopsis*

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Summary

- Day length and ambient temperature are major stimuli controlling flowering time. To understand flowering mechanisms in more natural conditions, we explored the effect of daily light and temperature changes on *Arabidopsis thaliana*.

- Seedlings were exposed to different day/night temperature and day-length treatments to assess expression changes in flowering genes.

- Cooler temperature treatments increased *CONSTANS* (*CO*) transcript levels at night. Nighttime *CO* induction was diminished in *flowering bhlh* (*fbh*)-quadruple mutants. *FLOWERING LOCUS T* (*FT*) transcript levels were reduced at dusk, but increased at the end of cooler nights. The dusk suppression, which was alleviated in *short vegetative phase* (*svp*) mutants, occurred particularly in younger seedlings, while the increase during the night continued over 2 wk. Cooler temperature treatments altered the levels of *FLOWERING LOCUS M-β* (*FLM-β*) and *FLM-δ* splice variants. *FT* levels correlated strongly with flowering time across treatments.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Transcript accumulation levels of *ISOPENTENYL PYROPHOSPHATE/DIMETHYLALLYL PYROPHOSPHATE ISOMERASE* (*IPP2*) remain unaltered over the day across three temperature treatments.

Fig. S2 Growth is delayed and *FLOWERING LOCUS T* (*FT*) level is depressed in seedlings exposed to constant 12°C temperatures.

Fig. S3 Growth is delayed and *FLOWERING LOCUS T* (*FT*) level is reduced in seedlings exposed to constant 17°C temperatures.

Fig. S4 CONSTANS (CO) protein stabilization is not altered during the cool night.

Fig. S5 Proposed mechanism for the transcriptional regulation of *FLOWERING LOCUS T* (*FT*) in long days with temperature changes.

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- Day/night temperature changes modulate photoperiodic flowering by changing *FT* accumulation patterns. Cooler nighttime temperatures enhance FBH-dependent induction of *CO* and consequently increase CO protein. When plants are young, cooler temperatures suppress *FT* at dusk through SVP function, perhaps to suppress precocious flowering. Our results suggest day length and diurnal temperature changes combine to modulate *FT* and flowering time.

Keywords

ambient temperature; *Arabidopsis thaliana*; CONSTANS; FLOWERING LOCUS T; flowering time; photoperiod; signal integration

Introduction

In nature, plants experience temperature fluctuations coinciding with day and night, and daily temperature fluctuations affect plant development (Myster & Moe, 1995). When exposed to day/night temperature cycles, flowering of rice and barley can be accelerated or delayed relative to constant-temperature conditions (Yin & Kropff, 1996; Karsai *et al.*, 2008). Time-of-day information is important for a range of physiological responses as sensitivity to temperature is modulated by the circadian clock throughout the day (McClung, 2006). Further, plant responses to combined environmental stimuli cannot be easily predicted from the responses to each alone (Prasch & Sonnewald, 2015). Therefore, to study the mechanisms that operate in nature, it is useful to assess the combinational influence of day length and day-to-night temperature fluctuations on seasonal flowering.

The day-length dependent (photoperiodic) flowering pathway is well studied in *Arabidopsis thaliana* (Golembeski *et al.*, 2014; Osugi & Izawa, 2014; Shrestha *et al.*, 2014). Components of this pathway are implicated in ambient-temperature-dependent flowering (Song *et al.*, 2013), indicating that light and temperature signals can be integrated into the same pathway. Photoperiod information is processed through regulation of the *CONSTANS* (*CO*) gene. *CO* peaks in the afternoon and evening in long days (LD) (Suárez-López *et al.*, 2001). CO protein production follows the same pattern during the day; however, it is degraded during the night by CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)/SUPPRESSOR OF *PHYA-105* (SPA) E3 ubiquitin ligase complex (Laubinger *et al.*, 2006; Jang *et al.*, 2008; Liu *et al.*, 2011). In LD afternoons, two types of blue-light photoreceptors CRYPTOCHROME (CRY) and FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) stabilize CO protein inducing *FLOWERING LOCUS T* (*FT*) at the end of the day to promote flowering (Zuo *et al.*, 2011; Song *et al.*, 2012, 2014; Tan *et al.*, 2013).

Temperature affects flowering by modulating *FT* levels through *CO*-dependent and -independent mechanisms (Lee *et al.*, 2007; Lazaro *et al.*, 2012; Jung *et al.*, 2012; Nomoto *et al.*, 2013). In constant lower (16°C) temperatures in LD, *FT* levels are reduced and plants flower later compared with 23°C conditions (Blazquez *et al.*, 2003; Lee *et al.*, 2007). However, in *short vegetative phase* (*svp*) mutants, the *FT* level is high even in constant 16°C (Lee *et al.*, 2007). In both 16°C and 23°C, *svp* mutants flower at similar times, indicating SVP represses *FT* and delays flowering under cool temperatures. SVP interacts with the

FLOWERING LOCUS M (FLM) splice variant, FLM-β, to bind the *FT* promoter ([Posé *et al.*, 2013]). Intermittent drops to cold (4°C) temperatures during the day may act through CO to repress flowering ([Jung *et al.*, 2012]). A temperature drop from 23°C to 4°C from Zeitgeber time (ZT) 10 to ZT16 in LD leads to *FT* repression and a delay of flowering. The decline in *FT* mRNA strongly parallels a decline in CO protein abundance.

Colder temperatures within a day do not always reduce *FT* levels. In constant light, a 22°C/12°C temperature cycle over a 24-h period causes a strong induction of *FT* during the cool periods of the cycle, and temperature cycles are likely associated with early flowering ([Schwartz *et al.*, 2009]). Under 12-h light and 12-h dark conditions, having warmer temperatures (28°C) at night but not in the day (22°C) causes up-regulation of *FT* ([Thines *et al.*, 2014]). Therefore, depending on light conditions, temperature changes occurring at different times of the day differentially regulate *FT* expression patterns and do so through several mechanisms.

In *Arabidopsis*, small numbers of consecutive LD treatments are sufficient to induce early flowering, and *FT* is immediately upregulated after plants are moved from short days (SD) to LD, but declines to basal levels shortly after plants are returned to SD ([Corbesier *et al.*, 1996, 2007]; Krzymuski *et al.*, 2015). This accumulation of a discrete amount of *FT* correlates with early flowering and suggests that environmental cues that modulate *FT* levels will affect flowering time. To better assess the mechanisms that occur in natural conditions, we analyzed the combined influence of temperature and day-length changes on photoperiodic-flowering-pathway genes and tested the extent to which *FT* gene expression levels can explain flowering times across a range of treatments.

Materials and Methods

Plant material and growth conditions

Wild-type accessions of *Arabidopsis thaliana* (L.) Heynh, Columbia-0 (Col-0), Landsberg *erecta* (L*er*), and Wassilewskija-2 (Ws-2), were used. All mutants and transgenic lines were in the Col-0 background and described previously: *co-101* and *ft-101* ([Takada & Goto, 2003]), *fbh*-quadruple mutant (*fbh-q #2*) and *FBH1:FLAG-FBH1* ([Ito *et al.*, 2012]), *CO:HA-CO and 35S:3HA-CO* ([Song *et al.*, 2012]), *svp-31* ([Lee *et al.*, 2007]), and *SVP:SVP-6HA* ([Shen *et al.*, 2011]). For flowering experiments, seeds were sown on soil (Sunshine #3 Mix; Sun Gro Horticulture) containing Osmocote Classic time-release fertilizer (Scotts) and Systemic Granules: Insect Control (Bionide). For gene, GUS activity, and protein expression analyses and the 14-d, LD-to-SD-transfer flowering experiment, seeds were placed on plates containing 1× Linsmaier and Skoog (LS) media (Caisson) and 3% sucrose. Plants were grown in LD (16-h light/8-h dark) or SD (8-h light/16-h dark) conditions for 7, 14, or 21 d to avoid temperature effects on germination and early development, then moved to constant 12°C or 22°C, or to temperature cycles of 22°C (light) and 12°C or 17°C (dark) in SD, 12-h light/12-h dark (MD) or LD conditions. HOBO Pendant temperature/light data loggers (Onset) were used to ensure temperature deviated 0.5°C or less from the target temperatures. Full-spectrum fluorescent light (Octron F032/950/48; Osram-Sylvania) intensities in the growth chambers averaged *c.* 100 µmol m^{-2} s^{-1} in SD and 60 µmol m^{-2} s^{-1} in LD. For flowering experiments, individuals from each strain were assigned random positions within

32-pot flats. The flats were repositioned at least weekly within and between chambers to avoid chamber and positional affects. For the 14-d, transfer experiment, one representative of each age (1–14 d) was randomized and put into a single flat to account for differences due to watering or fertilization. For the remaining analyses, temperature treatments were randomly assigned different incubators for each replicate. Flowering time was measured by recording the number of rosette leaves on the main stem. To assess rate of leaf production, new leaves at least 2 mm long were counted once a week.

Gene expression analysis (qPCR)

For most experiments, plants were grown for 7 d in SD conditions before being transferred to treatments and harvested every 4 h beginning at dawn (ZT0) on day 4 in treatment. For the 48-h time courses, seedlings were harvested starting at ZT12 on day three of treatment, when the seedlings were 10 d old. For the 14-d time courses, seedlings were harvested at ZT0, ZT8, and ZT16 on days 1–14 of treatment. RNA isolation, cDNA synthesis, and qPCR were described (Ito *et al.*, 2012). All genes were normalized against *ISOPENTENYL PYROPHOSPHATE/DIMETHYLALLYL PYROPHOSPHATE ISOMERASE* (*IPP2*) (Hazen *et al.*, 2005). We confirmed diurnal *IPP2* was expressed uniformly across our temperature conditions compared to other potential internal control genes, *SERINE/THREONINE PROTEIN PHOSPHATASE 2A* (*PP2A*) (Hong *et al.*, 2010) and *ACTIN 2* (*ACT2*) (Sawa *et al.* 2007) (Supporting Information Fig. S1). Primers and PCR conditions for *FT*, *CO*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*), and *FLC* (Ito *et al.*, 2012), and *ACT2* and *PP2A* were described previously (Sawa *et al.*, 2007; Hong *et al.*, 2010). The primers for *SVP* are 5′-ACGGAAGAGAACGAGCGACTTG-3′ and 5′-CTCGTACACAGCAGCGTTCTCC-3′. qPCR was done using the following program: 1 min 95°C denaturation, then 45 cycles of 10 s 95°C, 20 s 59°C, and 20 s 72°C.

Protein expression analysis (Western blot)

Extraction and detection of FLAG-FBH1 and SVP-6HA proteins, and of nuclear HA-CO were performed as described (Ito *et al.*, 2012; Song *et al.*, 2012). FLAG-FBH1 and SVP-6HA and HA-CO protein were detected by anti-FLAG antibody (Sigma) or anti-HA conjugated to horseradish-peroxidase (Roche). Actin, HSP90, and Histone H3, detected by anti-Actin, anti-HSP90, or anti-Histone H3 antibodies (Song *et al.*, 2012), respectively, were used as loading controls. Relative expression levels were normalized by the values of loading controls.

GUS activity visualization

pFT:GUS and *pCO:GUS* lines were described previously (Takada & Goto 2003). Eleven-day-old seedlings were harvested at ZT24 on day 4 of the temperature and day length treatment. Tissue preparation and staining were described previously (Ito *et al.*, 2012). Seedlings were prepared for visualization with a series of washes of 70%, 50%, and 30% ethanol, H_2O, then 25% and 50% glycerol.

Statistical analyses

Statistical analyses were done using R Statistical Computing software (v. 3.1.1 and earlier, R Core Team, 2015). Statistical significance of final rosette leaf numbers at bolt was determined using ANOVA with temperature treatment and strain as main effects. Pairwise comparisons were determined using Tukey Honest Statistical Difference (R Core Team, 2015). In some cases the replicate and/or tray was introduced as a co-variant to account for pseudo-replication. Statistical significance of leaf production over time was analyzed using linear mixed-effects regression (Bates *et al.,* 2015), which accounted for repeated measures of the same individuals, with temperature, strain, and time (weeks) included as the main effects. This was followed by Satterthwaite approximation to generate *P*-values (Satterthwaite, 1946). For leaf production, we considered only data recorded before the appearance of visible bolts. Data was transformed as needed based on Box-Cox analysis (Box & Cox, 1964) and visual comparisons of residuals to ensure equal variance. Most flowering experiments were replicated at least two times, with at least 10 individuals per strain and treatment in each replicate. The 14-d, LD-to-SD transfer experiment was replicated twice with five individuals per treatment per replicate.

Gene expression data displayed heteroskedastic residuals, so effects of temperature and time were compared using the Generalized Estimating Equation (Carey, 2015), which computes robust standard errors and test statistics, followed by pairwise Tukey comparisons using lsmeans (Lenth, 2015). To improve normality and equal variance among treatments, time (ZT) was transformed by finding the cosine, and, if necessary, day and night segregation was included as a covariate to account for oscillatory patterns. All gene and protein experiments were replicated at least three times, and we obtained similar results. Effects were considered significant when both of the following conditions were met: *P*-value significance level and the 95% Confidence Interval (CI) for the difference between a pair did not contain zero. The significance level was 0.05. For highly non-linear data, subsets (by time) were tested and the significance level was lowered using Bonferonni correction for multiple comparisons.

Results

Cool nighttime temperatures result in delay of flowering, and altered accumulation profiles of *CO* and *FT* transcripts

Outdoors, diurnal light and temperature cycles occur together. How do plants integrate these cues? Using as a guide the 21.1°C-high/11.7°C-low temperature range of the summer solstices in Seattle, Washington, USA (averages from 1971 to 2000, day length: 15 h 59 min), we overlaid a 22°C/12°C temperature cycle onto a 16-h-light, 8-h-dark LD cycle, such that the cool period occurred throughout the night (referred to as LD22/12°C) and assessed the timing of flowering. As temperature influences numerous plant processes (Parent *et al.,* 2010), we mitigated its influence on early development by growing all seedlings in non-flowering-inductive SD conditions at 22°C for 7 d before moving them to temperature treatments. Growth in cool nighttime temperatures resulted in a delay in flowering (20.84 ± 0.64 leaves) and a 40.46% increase in the number of leaves produced at bolt in Col-0 plants relative to plants grown in the constant-temperature conditions usually used in studies

of photoperiodic flowering in *Arabidopsis* (LD22/22°C) (14.96 ± 0.42 leaves, *P* 0.001; Fig. 1a; Table 1).

To determine the mechanisms responsible for this delay, we analyzed the profiles of several flowering-regulator gene transcripts (Yant *et al.*, 2009). We found significant increase of *CO* transcript accumulation at night in LD22/12°C conditions relative to plants grown in the LD22/22°C control (Fig. 1b). *FT* transcript levels in LD22/12°C were also significantly altered compared to the control. *FT* levels were reduced at dusk, but higher at dawn (Fig. 1c). A smaller (5°C) difference between day and nighttime temperatures (LD22/17°C) resulted in an intermediate response relative to the control. *CO* was induced at night in LD22/17°C conditions, but to a lesser extent than in LD22/12°C (Fig. 1b). *FT* profile in LD22/17°C conditions was similar at dusk and dawn to the control, but higher than either the control or LD22/12°C in the middle of the night (ZT20, Fig. 1c). Therefore, diurnal temperature cycles, in conjunction with day and night, influence the profiles of *CO* and *FT* transcript accumulation.

SOC1 is downstream of *FT* and acts at the shoot apex to induce *LEAFY* (*LFY*) to promote flowering (Lee *et al.*, 2008). *SOC1* level was reduced during the morning in LD22/12°C conditions relative to the control (Fig. 1d), consistent with a delay in flowering. The expression of strong *FT* repressor, *FLOWERING LOCUS C* (*FLC*) gene (Michaels & Amasino, 1999), did not respond to our temperature treatments (Fig. 1e), suggesting that if day/night temperature fluctuations affect flowering through *FLC*, it is not through altered mRNA levels. SVP represses *FT* and delays flowering time under lower temperatures (i.e. 16°C) (Lee *et al.*, 2007). The daily patterns of *SVP* transcript accumulation were not significantly changed between our two conditions (Fig. 1f).

FT and *CO* are upstream of *SOC1* and several studies suggest *FT* levels are predictive of flowering (Kobayashi *et al.*, 1999; Blazquez *et al.*, 2003; Corbesier *et al.*, 2007; Salazar *et al.*, 2009; Krzymuski *et al.*, 2015; Seaton *et al.*, 2015), therefore, we chose to explore whether *FT* and *CO* profile changes could account for the delay in flowering we observed. We tested the flowering time of *co* and *ft* mutants. The final leaf numbers at bolt of *ft* and *co* mutant plants were not significantly different between the LD22/22°C control and LD22/12°C temperature treatments (Fig. 1a; Table 1), indicating that *CO* and *FT* may be involved in the temperature-dependent delay of flowering. CO is a chief activator of daytime *FT* expression (Yanovsky & Kay, 2002). We tested whether CO is required for the cooler-night-temperature-induced *FT* changes. In the *co* mutant line, we found *FT* remained at basal levels throughout the day in both LD22/22°C and LD22/12°C conditions relative to *FT* in wild-type plants (Fig. 1g), indicating that nighttime expression of *FT* under LD22/12°C conditions still requires the *CO* gene.

To analyze whether these nighttime-temperature-induced responses are conserved in other wild-type accessions, we measured flowering time and gene expression in two common lab strains, L*er* and Ws-2. Both showed delayed flowering in LD22/12°C conditions relative to LD22/22°C that were similar to Col-0 (Fig. 2a; Table 1). As with Col-0, they both displayed nighttime induction of *CO* (Fig. 2b,c), suppression of *FT* at dusk and higher levels of *FT* at

dawn (Fig. 2d,e) in the LD22/12°C conditions relative to the LD22/22°C control, indicating that this response was not accession-specific.

We wondered whether the changes in *CO* and *FT* transcript accumulation were due to changes in the spatial accumulation patterns of these genes, which are expressed in the leaf vasculature in the LD22/22°C conditions (Takada & Goto, 2003). We examined the spatial patterns of *CO* and *FT* and found that the tissue-specific accumulation of *pFT:GUS* and *pCO:GUS* were not affected by LD22/12°C treatments relative to the control (Fig. 3a–d), indicating that temperature fluctuations only alter the temporal accumulation patterns of these genes.

FT induction is dependent on the presence of light in the afternoon in constant temperatures (Yanovsky & Kay, 2002). We asked whether nighttime *CO* induction and the altered *FT* profile were day-length dependent. We tested accumulation profiles of *CO* and *FT* transcripts in SD and 12-h-light, 12-h-dark (MD) days. In both cases, the seedlings were exposed to 12°C for the duration of the night. Induction of *CO* in response to 12°C nighttime temperatures occurred in both day lengths (Fig. 3e,f). However, *FT* was not appreciably induced in SD, while *FT* did not differ significantly between treatments in MD (Fig. 3g,h), indicating that cool nighttime temperatures do not override the requirement of *FT* for afternoon light.

Induction of *CO* and *FT* in response to cool nighttime temperatures is reproduced over 2 wk of treatment

Peak expression of *FT* increases as plants age from 5 to 15 d (Mathieu *et al*, 2009). We wondered whether the rate of increase would be slower in LD22/12°C conditions, helping to explain the delay in flowering, and whether the diurnal transcript accumulation patterns we observed remained consistent over time. We extended the LD22/22°C control and LD22/12°C treatment for 14 d (from 7- to 21-d-old plants), harvesting at dawn (ZT0), during the middle of the day (ZT8) when *CO* and *FT* transcripts are at trough levels, and at dusk (ZT16) for the full 2 wk. In LD22/12°C conditions, nighttime induction of *CO* and upregulation of *FT* at dawn were reproduced for the full 14 da (Fig. 4a,b). However, dusk suppression of *FT* in LD22/12°C disappeared over time (Fig. 4b). Could suppression of *FT* at dusk be a transient response to cool nights, perhaps acting to buffer development to short-term temperature modulations? If correct, we would expect dusk suppression of *FT* even in plants first exposed to the LD22/12°C treatment later in development. We sampled 18- and 25-d-old seedlings, each having been exposed to treatments for four days, to determine whether we observed *FT* accumulation at dusk. Interestingly, in 25-d-old plants but not in 18-d-old plants, dusk levels of *FT* were higher than those of the control (Fig. 4c,d), indicating that dusk suppression can be a transient response, dependent on developmental age, potentially suppressing flowering in young plants when temperatures are unfavorable.

FT expression correlates with flowering across treatments

A discrete number of LDs and amount of *FT* expression is required to induce flowering (Krzymuski *et al*, 2015). We confirmed that such a distinct switch occurs in our conditions. After 7 d in SD, seedlings were moved to LD22/22°C conditions. For 2 wk, after each LD

treatment, we transferred some seedlings back to SD conditions and recorded leaf number at bolt. Seedlings exposed to one to four days of LD flowered similarly to plants grown in SD (leaf number 57.7 ± 11.68 (standard deviation)) (Fig. 5a). After five consecutive LDs, most seedlings flowered early (leaf number 9.35 ± 8.03). Together with previous research, this implies that flowering occurs after *FT* reaches a threshold and that alterations in *FT* levels in response to environmental perturbations can be predictive of flowering time.

However, cool temperature affects several plant processes and slows leaf production (Parent *et al.*, 2010). It is difficult to separate temperature effects mediated through *CO* and *FT* from other factors. To assess whether leaf production slowed in LD22/12°C temperature cycle conditions relative to the LD22/22°C control, we counted the number of leaves produced each week for several wild-type and mutant strains. When considering only leaves produced before production of visible bolts, there was a slightly lower overall leaf number across strains in LD22/12°C conditions relative to the LD22/22°C control ($P < 0.001$, Fig. 5b). However, the 95% CI (–0.134, 0.1497) of the difference between treatments contained zero and so is not significant. This was true even when considering only late flowering strains, which were exposed to cool nighttime temperatures the longest, suggesting that our temperature-cycle treatments do not appreciably affect leaf production.

We then asked how well the relative *FT* levels in each strain and treatment explain final leaf numbers at bolt. Because the *FT* transcript profiles remain relatively consistent in LD (Fig. 4b), we assumed that the pattern of *FT* on day 4 of our treatments was representative of the amount of *FT* produced before plants reaching a LD threshold. We found that leaf numbers declined with increasing levels of *FT*, when accumulated day 4 *FT* transcript was found by integrating under the curve (Fig. 5c, closed circles). After the data were linearized, *FT* levels explained much of the variation in flowering across day length and temperature treatments and wild-type and mutant strains within the Col-0 background. Recent work indicated that *FT* generated around dusk (ZT12 to ZT20, Krzymuski *et al.*, 2015) is more effective for inducing flowering, and suppression of *FT* at dusk likely accounts for the delays in flowering induced by our warm-day/cool-night conditions. The correlation between *FT* amounts over this timeframe and flowering time was strong in our conditions as well, showing the same correlation as for total *FT* ($R^2 = 0.88$, RMSE = 0.25 log(leaves)). However, the correlation was strongest when daytime *FT* from samples collected at ZT0, ZT8 and ZT16 were considered (Fig. 5c inset, $R^2 = 0.92$, RMSE = 0.21 log(leaves)), indicating morning levels of *FT* may contribute to flowering time. L*er* and Ws-2 showed a similar negative correlation between *FT* and flowering between the LD22/22°C control and LD22/12°C treatments; however, the intercept was different from that of Col-0, perhaps indicating a lower requirement of *FT* for flowering in these accessions (Fig. 5c, open circles). These data imply that alterations to *FT* levels in response to environmental changes can be predictive of flowering time across multiple conditions.

Induction of *CO* requires a decrease in temperature

Once we determined *FT* transcript accumulation levels were predictive of flowering across a range of conditions, we explored the mechanisms responsible for the transcript profile we observed. As CO is a major activator of *FT*, we examined whether nighttime induction of

CO by cool temperatures showed time-dependent differences. Seedlings were exposed to cool temperatures (12°C) for 4-h durations at the beginning, middle, or end of the night in LD. Cool temperatures induced *CO* regardless of when during the night they occurred, and *CO* declined again to control levels during the warm periods of the night (Fig. 6a–c). It appeared as if a change in temperature from 22°C to 12°C induced nighttime *CO*, but it was possible that *CO* was simply high at 12°C. Would *CO* would be induced in plants grown in constant 12°C (LD12/12°C) conditions? The pattern of *CO* for plants grown in SD22/22°C then moved to LD12/12°C for 4 d was slightly higher but similar to the LD22/22°C control and did not show strong nighttime induction (Fig. 6d). These results suggested a drop in temperature during the night results in immediate upregulation of *CO*, and that there is no obvious circadian-gating effect for *CO* induction at night.

By contrast, the response of *FT* showed time-dependent differences. Cool temperatures at the beginning of the night resulted in an *FT* transcript accumulation profile that was similar to the pattern observed in LD22/12°C conditions (Fig. 6e). Cool temperatures in the middle of the night resulted in peak *FT* level at dusk (ZT16) that declined more slowly than the LD22/22°C control such that it was higher in the middle of the night (ZT20, Fig. 6f). In both cases, *FT* declined to control levels during the warm period at the end of the night (ZT24). When cool temperatures occurred at the end of the night, the *FT* profile was similar to that in the LD22/22°C conditions (Fig. 6g), suggesting that acute induction of *CO* at the beginning, but not the end, of the night is sufficient to induce nighttime *FT* expression.

As previous work showed that *FT* levels were low throughout the day in 12-h-light, 12-h-dark cycles when grown from seed in constant 16°C temperatures (Blazquez *et al.*, 2003), we anticipated that *FT* levels would be suppressed when cool temperatures were applied for 4 d throughout the day (LD12/12°C). Unexpectedly, *FT* was higher throughout the day (Fig. 6h). We wondered whether the differences in *FT* levels between our study and the previous work might be caused by growth conditions of younger seedlings, as we grew seedlings at constant SD22/22°C for a week before temperature treatments. Therefore, we grew seedlings in LD12/12°C conditions from seed and compared these to seedlings grown in LD22/22°C and in LD22/22°C for 7 d then switched to LD12/12°C for 4 d. Seedlings grown entirely in the LD12/12°C conditions had lower levels of *FT* and *CO*, and were delayed in development (Fig. S2), lacking true leaves by day 12 while plants in the other treatments had two true leaves that had begun to emerge. However, plants grown from seed in LD12/12°C conditions appeared stressed, so we repeated this experiment using constant 17°C (LD17/17°C) conditions. Plants grown from seed in LD17/17°C conditions were smaller than those grown in LD22/22°C, and had lower overall levels of *FT* similar to the previous results (Blazquez *et al.*, 2003). In the plants moved from LD22/22°C to LD17/17°C, daytime *FT* levels were higher especially in the morning (Fig. S3). Previous results also showed lower day-time temperatures upregulate *FT*. When the daytime temperature was cooler (22°C) relative to a 28°C night, *FT* levels were *c.* 15-times higher than that in constant temperature conditions (Thines *et al*, 2014), and *FT* was drastically induced during the 12°C phase under 22/12°C daily temperature cycles in constant lights (Schwartz *et al.*, 2009). Taken together, our results suggest that plants respond to cooler temperatures differently depending on how and when (including which part of the day) they are applied, and some conditions can upregulate *FT*.

FBH genes regulate the cooler night-induced *CO* transcription

We next examined which genetic components might be involved in the lower-temperature induced changes in *CO* and *FT* transcript levels. *CO* transcription is regulated by both positive and negative transcription factors (Golembeski *et al.*, 2014). CYCLING DOF FACTOR (CDF) family proteins repress *CO* during the daytime, while FLOWERING BHLH (FBH) family proteins induced *CO* especially during the night (Fornara *et al.*, 2009; Ito *et al.*, 2012). We examined whether FBHs are involved in the lower-temperature *CO* induction during night. The lower-temperature-induced accumulation of *CO* transcript was strongly attenuated in the *fbh* quadruple (*fbh-q: fbh1 fbh2 fbh3 fbh4*; Ito *et al.*, 2012) mutants when compared with the level of wild-type plants grown in LD22/12°C (Fig. 7a), indicating that FBH proteins play a role in the upregulation of *CO* during cooler nights. In the *fbh-q* mutant, nighttime *CO* levels in LD22/12°C were still slightly higher than those of the LD22/22°C control, potentially as a result of incomplete silencing of *FBH1* and *FBH4* expression in this mutant (Ito *et al.*, 2012) or due to contribution of another unknown factor. As expected, expression of *FT* was lower overall in the *fbh-q* mutant compared to the control (Fig. 7b) and the *fbh-q* flowered later than wild type under both conditions (Table 1). The patterns of *FT* were similar to those in wild type, showing suppression at dusk and upregulation at the end of the night in LD22/12°C conditions (Fig. 7b), consistent with the higher levels of *CO* in these conditions.

As FBH function seemed higher under cooler nighttime temperature conditions, we analyzed whether lower night temperature alter the expression patterns of FBH protein. Using the *FBH1:FLAG-FBH1* line (Ito *et al.*, 2012), we found similar patterns of FBH1 protein accumulated throughout the day in both LD22/22°C and LD22/12°C (Fig. 7c), indicating that FBH1-mediated induction of *CO* in response to cool nighttime temperatures may not be caused by changes in FBH1 protein abundance. Taken together, our results suggest that FBHs mediate cooler night-induced increases in *CO* transcription and this alters *FT* expression.

CO protein is higher under cool nighttime temperatures

Posttranslational regulation of CO plays an important role in the induction of *FT* (Song *et al.*, 2015). Under constant temperatures, CO protein is stable in the LD afternoon to induce *FT* (Valverde *et al.*, 2004), and is degraded at night through the activity of the COP1-SPA1/3/4 ubiquitin ligase complex (Laubinger *et al.*, 2006; Jang *et al.*, 2008; Liu *et al.*, 2011). We found that *FT* levels declined more slowly in cooler temperatures at night (Fig. 1b), and speculated that CO protein might become more stable under those conditions. We analyzed CO protein profiles in *CO:HA-CO* plants (Song *et al.*, 2012) grown in LD22/22°C or LD22/12°C conditions. We found that HA-CO protein driven by the native *CO* promoter was higher at night in LD22/12°C conditions compared to the LD22/22°C control (Fig. 8a,b). As *CO* mRNA levels are highly induced during the cooler night (Fig. 1a), it remained uncertain whether this was caused by transcriptional or posttranscriptional changes. We measured CO protein levels driven from a constitutively expressed *35S* promoter in *35S: 3HA-CO* plants and found no obvious difference in CO protein profiles in the plants grown under LD22/12°C and LD22/22°C conditions (Fig. S4). This indicates that CO protein is not particularly stabilized under cooler night conditions, and suggests that CO protein levels

become higher during cooler nights due mainly to increases in transcription rather than protein stability regulation.

Involvement of SVP in cool-nighttime-temperature induction of *FT* changes

We observed suppression of *FT* at dusk following cool nights (Fig. 4b), which may prevent precocious flowering in young plants. One possible candidate for *FT* suppression was SVP, which represses *FT* under constant 16°C LD conditions (Blazquez *et al.*, 2003). Are the proceeding cooler nights sufficient to facilitate *FT* repression by SVP at dusk the following day? We found that the dusk peak of *FT* in *svp* mutants was similar between LD22/12°C conditions and the LD22/22°C control relative to wild type (ZT16, Fig. 9a), while dawn *FT* levels still differed between the two conditions (ZT24, Fig. 9a), indicating that SVP is required to reduce the level of *FT* particularly around dusk. Consistent with a smaller difference in the amount of *FT* in the *svp* mutant between the two treatments, the *svp* mutants were delayed in flowering to a lesser degree than wild type in LD22/12°C conditions relative to LD22/22°C (Table 1). These results imply that SVP represses *FT* around dusk under our conditions and that cooler night temperatures are enough to activate SVP function.

Cooler constant temperatures stabilize SVP protein (Lee *et al.*, 2013). Do cooler nights affect daily SVP protein profiles? SVP-6HA protein levels in *SVP:SVP-6HA* plants (Shen *et al.*, 2011) in LD22/12°C conditions remained higher after dusk and into the nighttime than those in LD22/22°C (Fig. 9b,c), indicating that timing of SVP protein stability is altered when plants are exposed to lower nighttime temperatures for part of the day. Binding of SVP to the *FT* promoter is affected when temperature alters the amounts of *FLM* splicing variants (Posé *et al.*, 2013). Under cool constant temperatures, the FLM-β variant is more prevalent, promoting DNA binding of SVP, while the FLM-δ variant, which lacks a functional MADS-box domain and inhibits DNA binding of SVP by forming a complex, is present in warm temperatures (Posé et al., 2013). The increased ratio of FLM-β to FLM-δ was proposed to facilitate SVP-mediated flowering delays (Posé *et al.*, 2013); although a recent study indicated that *FLM-β* transcript levels correlate more strongly with flowering than those of *FLM-δ* (Lutz *et al.*, 2015). To analyze whether cooler night temperatures change the abundance of these splice variants, we measured the accumulated levels of *FLM-β* and *FLM-δ* under LD22/22°C, LD22/12°C, and LD12/12°C conditions. Consistent with previous results, *FLM-β* was higher under LD12/12°C than the LD22/22°C control (Fig. 9d); *FLM-β* in LD22/12°C did not significantly differ from the LD22/22°C control. However, the levels of *FLM-δ* in LD22/12°C, were lower during the day than in LD22/22°C, similar to that of LD12/12°C, causing a higher ratio of *FLM-β* to *FLM-δ* in both conditions (Fig. 9e). Together with the observation that SVP protein is slightly more stable around dusk, this result indicates that more SVP may form a heterodimer with functional FLM (FLM-β) to repress *FT* around dusk under LD22/12°C.

We observed dusk *FT* transcript levels to increase over 2 wk in LD22/12°C, becoming similar to those of LD22/22°C (Fig. 4b), and wondered whether this change might correlate with levels of *SVP* mRNA and/or protein over that period. *SVP* mRNA levels were similar between LD22/22°C and LD22/12°C treatments, although *SVP* transcript levels in

LD22/12°C were slightly lower during week 1 (Fig. 10a). SVP protein sampled before dusk (ZT13) did not differ significantly over the 2-wk period (Fig. 10b,c). We next analyzed *FLM-β* and *FLM-δ* transcript accumulation over this timeframe. *FLM-β* levels did not differ significantly between the two treatments (Fig. 10d). *FLM-δ* levels were lower in the LD22/12°C conditions relative to the LD22/22°C control (Fig. 10e). To more clearly assess the relationship between these transcripts over developmental time, we calculated the ratio of *FLM-β* relative to *FLM-δ* at each time point and then averaged across each day. The ratio of *FLM-β* to *FLM-δ* remained consistent over 2 wk in LD22/22°C (Fig. 10f). In LD22/12°C, however, the ratio of *FLM-β* to *FLM-δ* was higher than in LD22/22°C during week 1, becoming similar to the LD22/22°C control during week 2 (Fig. 10f). The decreased ratio of *FLM-β* relative to *FLM-δ* in LD22/12°C in week 2 may facilitate *FT* accumulation by affecting DNA binding ability of SVP (Posé *et al.*, 2013; Lutz *et al.*, 2015).

Discussion

Diurnal temperature changes alter flowering time and *CO* and *FT* profiles

To understand flowering time regulation under natural conditions, we studied the effects of temperature alterations coincident with light changes, and demonstrate how two environmental cues can be integrated through the *FT* gene to influence flowering. A warm-day, cool-night temperature treatment results in upregulation of the *CO* gene and protein levels at night and an altered *FT* accumulation profile in LDs. *FT* accumulates during the night and at dawn, but is suppressed at dusk through the action of SVP and FLM-β (Fig. S5). We found that *FT* levels explained much of the variance in final leaf number across several temperature and photoperiod treatments and across several mutant lines consistent with other studies (Fig. 5c and Kobayashi *et al.*, 1999; Blazquez *et al.*, 2003; Corbesier *et al.*, 2007; Salazar *et al.*, 2009; Krzymuski *et al.*, 2015; Seaton *et al.*, 2015). Therefore, while temperature influences myriad processes, the delay in flowering we observed in LD22/12°C conditions is largely explained by the amount of *FT* produced. Interestingly, the strongest correlation was observed when ZT0, 8, and 16 were considered, and all three wild-type strains tested displayed both dusk suppression and dawn induction of *FT*, indicating morning levels of *FT* may be important in the ability of plants to integrate environmental perturbations such as temperature changes.

Although this is beyond the scope of our current research, together with previous results (Schwartz *et al.*, 2009; Thines *et al.*, 2014), our results indicate that plants respond to drops in temperature differently than would be expected from studies of plants grown in constant temperature conditions (Blazquez *et al.*, 2003), and *FT* is frequently induced by lower temperatures that occur in conjunction with light (Figs 6h, S2c, S3c; Schwartz *et al.*, 2009; Thines *et al.*, 2014). The effects of cold treatment (4°C) are also more pronounced when plants are treated during the day than at night (Fowler *et al.*, 2005). Cooler temperatures occurring under light may be perceived as a signal of the changing seasons, such as a shift from summer to fall. Alterations of *FT* under these conditions may be a mechanism to accelerate flowering before unfavorable weather sets in. For instance, flowering was similarly accelerated between a 22°C-day/28°C-night treatment and a constant 28°C treatment, whereas flowering occurred later in a 28°C-day/22°C-night treatment (Thines *et*

al., 2014). Plants may have different responses to typical fluctuations (warmer day vs colder night) and changes that might associate with seasonal changes (warmer day vs colder day). If that is the case, these responses might be regulated differently for different purposes.

Roles of CO and FBH in temperature-cycle-mediated induction of *FT* at dawn

The degree of upregulation of *CO* at night is dependent upon the degree of temperature difference between day and night. The mechanism driving this phenomenon is unknown. The FBH protein family members are logical players, as we demonstrate that FBHs are required for strong nighttime induction of *CO* and *FT*. However, amounts of FLAG-FBH1 did not differ between treatments. The levels of other FBH family members may vary. Alternatively, the phosphorylation states of FBHs could be altered. FBH1 is a putative substrate of several mitogen-activated protein kinases (MPKs) (Popescu *et al.,* 2009). In addition, FBH3 is phosphorylated by SNF1-RELATED PROTEIN KINASE 2 (SnRK2) in response to ABA signaling (Takahashi *et al.,* 2013), and is a putative substrate for CALMODULIN DOMAIN PROTEIN KINASE 9 (CPK9) (Yang *et al.,* 2013). *CPK9* expression is altered by ethylene, which, along with ABA, is involved in perception of numerous environmental stresses (Qiao *et al.,* 2009; Wang *et al.,* 2013; Nakashima *et al.,* 2014). Daily temperature changes may influence amounts/activities of these kinases and, consequently *FBH* phosphorylation states, and may be a mechanism by which plant stress modulates flowering time.

Age dependent suppression of *FT* expression at dusk by SVP

Although CO induces *FT* expression in response to warm day, cool night conditions, this effect may be countered by suppression of *FT* by SVP at dusk in LD in plants less than 3 wk old. Our analysis indicated that SVP accounts for the *FT* repression at the end of the warmer day, and this repression was regulated by the cooler night in a previous day. The ratio of *FLM-β* to *FLM-δ* transcript was higher during the daytime in LD22/12°C, perhaps increasing SVP binding of the *FT* promoter and decreasing *FT* accumulation at dusk, and SVP-6HA protein levels shift from daytime to around dusk and decline more slowly at night, which may also contribute to *FT* suppression.

Dusk suppression of *FT* is alleviated over 2 wk in the cool-night treatment, and we observed dusk suppression of *FT* in 11- and 18-d-old plants, not in 25-d-old plants. The cooler temperature treatments did not drastically alter the accumulation levels of *SVP* mRNA and protein over the 2 wk, indicating that *SVP* levels likely do not cause the age-dependent alleviation of *FT* suppression. We observed the changes in both *FLM-β* and *FLM-δ* transcript levels over the 2 wk. The ratio of daily *FLM-β/FLM-δ* levels in LD22/12°C conditions decreased after 1 wk in this treatment. A recent report indicated that the *FLM-β* level, more than the *FLM-β/FLM-δ* ratio, correlates with flowering time in the accessions examined (Lutz *et al.,* 2015). However, as Lutz *et al.* also stated, and because increasing the *FLM-δ* level without altering the *FLM-β* level drastically caused early flowering (Posé *et al.,* 2013), their ratio changes may partly account for the changes in *FT* repression by altering the DNA-binding-abilities of SVP under our conditions. It is possible that developmental-age-dependent suppression by *SVP/FLM* may serve to repress precocious flowering in younger plants.

Daytime and nighttime temperatures differentially affect leaf number as well as flowering in other plant species (Yin & Kropff, 1996; Karsai *et al.*, 2008). Therefore, understanding the relationship between day and nighttime temperatures in conjunction to day length is important. Our study highlights an interface between environmental conditions and the mechanisms controlling flowering that may become more important as local weather regimes are altered with climate change.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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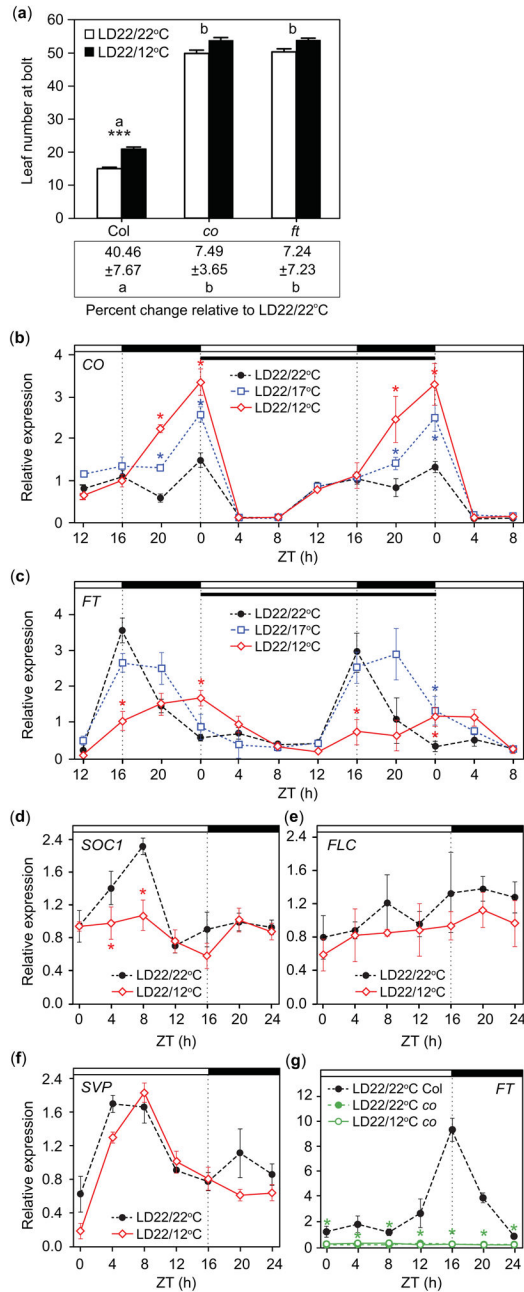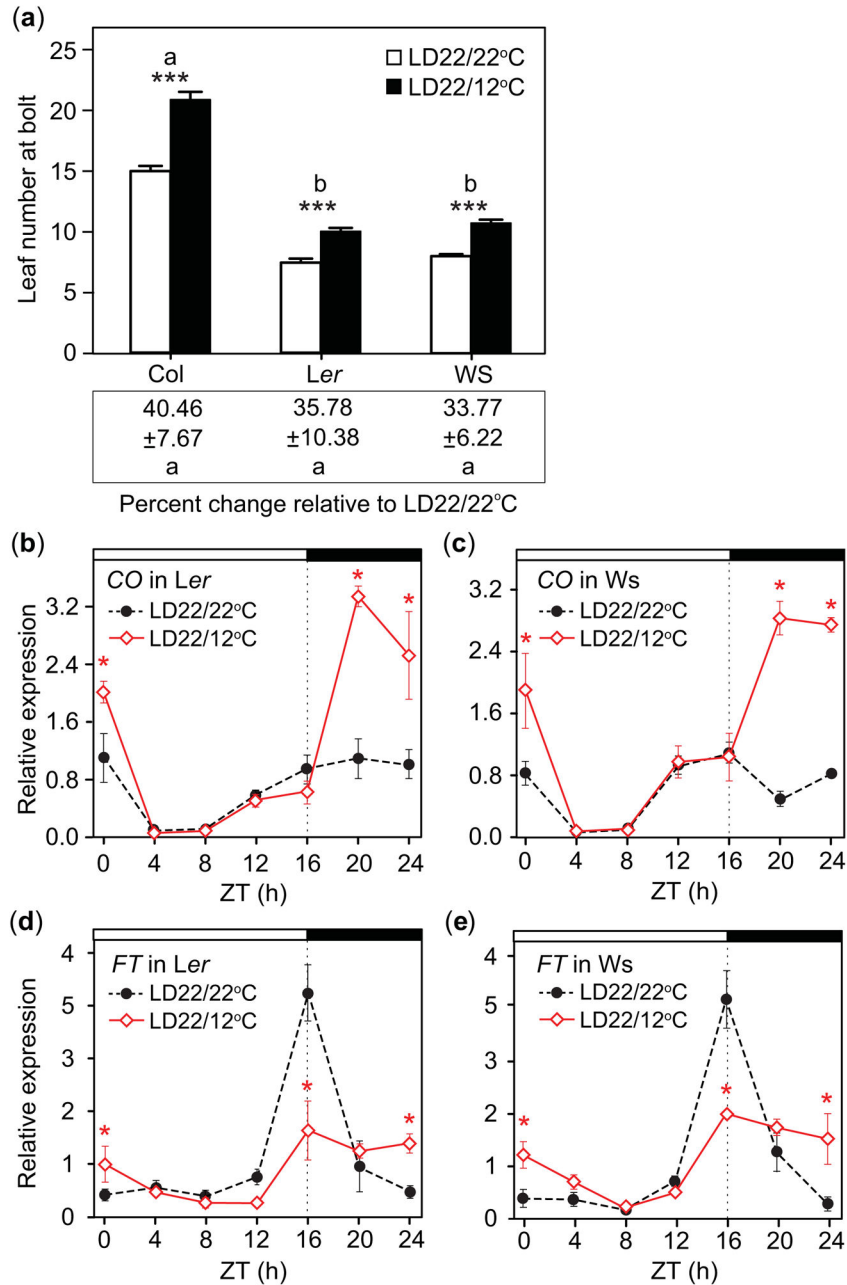
Fig. 1.

Flowering is delayed and transcript accumulation patterns of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) are altered in response to day: night temperature fluctuations. (a) Rosette leaf number of Columbia-0 (Col-0) wild-type *Arabidopsis thaliana* plants, *co-101*, and *ft-101* mutants (top) and percent change in rosette leaf number between treatment means within a replicate (bottom) in long-day treatments with 22°C daytime temperatures and 22°C or 12°C nighttime temperatures (LD22/22°C and LD22/12°C). Letters represent significant differences among strains of *P*<0.05. Asterisks (***) indicate significance between treatments within Col-0 (*P*<0.001), mean and 95% Confidence Interval

(CI, 0.329, 0.232, 0.425). Treatment differences of *co-101* and *ft-101* (top) were not significantly different (*P* = 0.199 and 0.375), mean and 95% CI (0.081, −0.015, 0.177) and (0.072, −0.025, 0.169), respectively; for differences in percent change from that of Col-0 (bottom), mean and 95% CI (−3.681, −5.095, −2.267) and (−3.489, −4.903, −2.075), respectively (*n* = 10, replicate = 5). (b, c) *CO* and *FT* transcript accumulation patterns in LD treatments with different nighttime temperatures (LD22/22°C, LD22/17°C, and LD22/12°C). Topmost white and black bars designate day and night, respectively. Dotted vertical lines are times of lights on or off. Narrow black bar designates day 4 of treatment. (d) *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*), (e) *FLOWERING LOCUS C* (*FLC*), and (f) *SHORT VEGITATIVE PHASE* (*SVP*) gene transcript accumulation patterns in Col-0, and (g) *FT* transcript accumulation in *co-101* mutant line from seedlings harvested on day 4 in LD22/22°C and LD22/12°C. Data represent means ± SEM derived from at least three biological replicates. (b–g) Asterisks (*) represent significance (*P*<0.05) and 95% CI of difference between pair did not contain zero in statistical comparisons made against the LD22/22°C control. ZT, zeitgeber time.

Fig. 2.

Flowering and transcript accumulation patterns of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) in different *Arabidopsis thaliana* ecotypes in response to day: night temperature fluctuations are similar to those in the Columbia-0 (Col-0) *Arabidopsis thaliana* accession. (a) Rosette leaf number of Col-0, Landsberg *erecta* (L*er*), and Wassilewskija-2 (Ws-2) (top) and percent change in rosette leaf number between treatment means within a replicate (bottom) in LD22/22°C (long days with day: night temperatures = 22°C: 22°C) and LD22/12°C conditions. (Top) Asterisks (***) indicate significance between treatments (*P*<0.001), mean and 95% Confidence Interval (CI, 0.329, 0.232, 0.425), (0.313, 0.206,

0.419), and (0.290, 0.183, 0.397), respectively. (Bottom) Differences in percent change from that of Col-0 for L*er* and Ws (*P* = 0.949 and 0.844, respectively), mean and 95% CI (−0.408, −1.908, 1.092) and (−0.549, −2.049, 0.951), respectively, (replicate = 5, *n* = 10). (b, c) *CO* transcript accumulation and (d, e) *FT* transcript accumulation in L*er* and Ws in LD22/22°C and LD22/12°C conditions. Seedlings were grown under the same conditions used for the experiments shown in Fig. 1. Data represent means ± SEM derived from at least three biological replicates. (b–e) Asterisks (*) indicate *P*<0.05 and 95% CI of difference between pair did not contain zero. ZT, zeitgeber time.
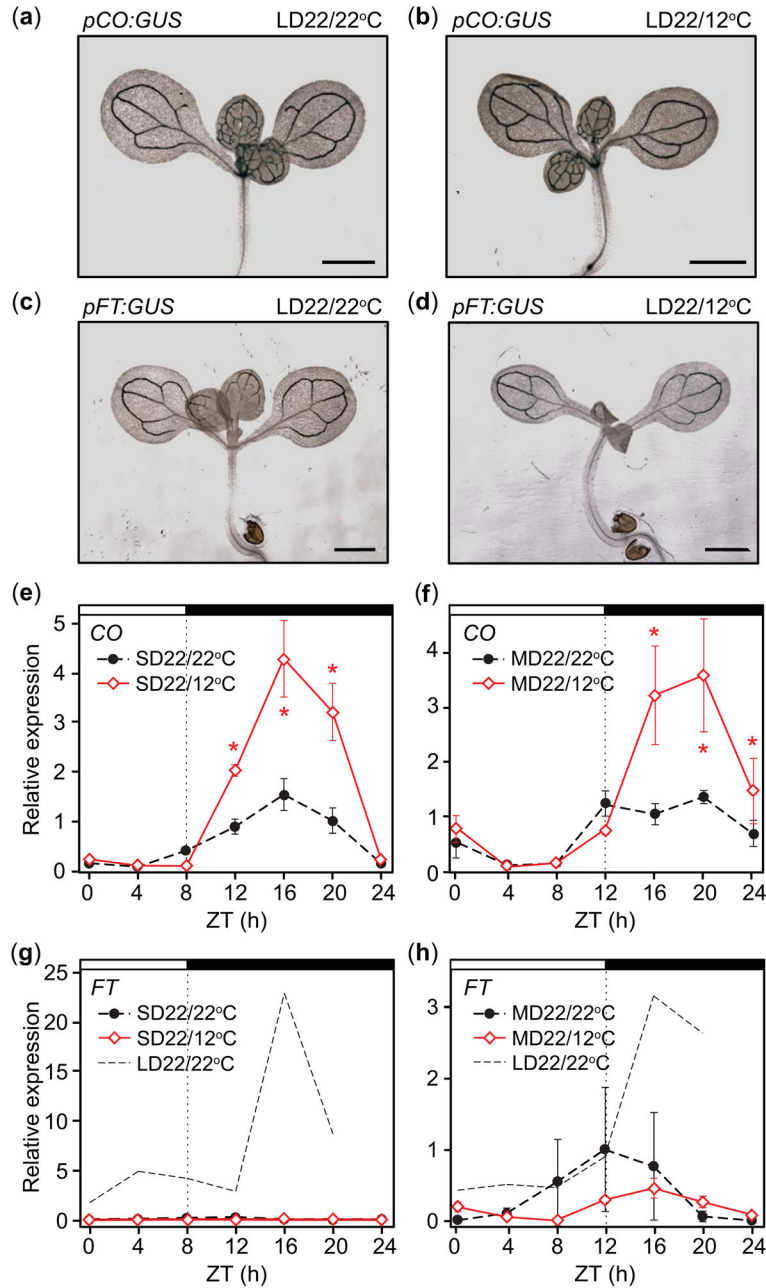
Fig. 3.

Spatial transcript accumulation patterns of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) are not altered in response to day: night temperature fluctuations in *Arabidopsis thaliana* (Columbia-0 (Col-0)). Nighttime induction of *CO* in response to cool nighttime temperatures occurs regardless of day length; *FT* induction by cool temperatures does not occur in short day (SD). (a–d) Tissue specific patterns of GUS activity in *pCO:GUS* (a, b) and *pFT:GUS* (c, d) plants grown in LD22/22°C (long days with day: night temperatures = 22°C: 22°C) and LD22/12°C conditions for 5 d. Scale bars, 1 mm. (e, f) *CO* transcript accumulation and (g, h) *FT* transcript accumulation on day 4 in SD or 12-h, mid-length-day

(MD) treatments with 22°C daytime temperatures and 22°C or 12°C nighttime temperatures. Replicate of *FT* transcript accumulation in LD22/22°C conditions is included for comparison. Data represent means ± SEM derived from at least three biological replicates. (e–h) Asterisks (*) indicate *P*<0.05 and 95% CI of difference between pair did not contain zero. ZT, zeitgeber time.
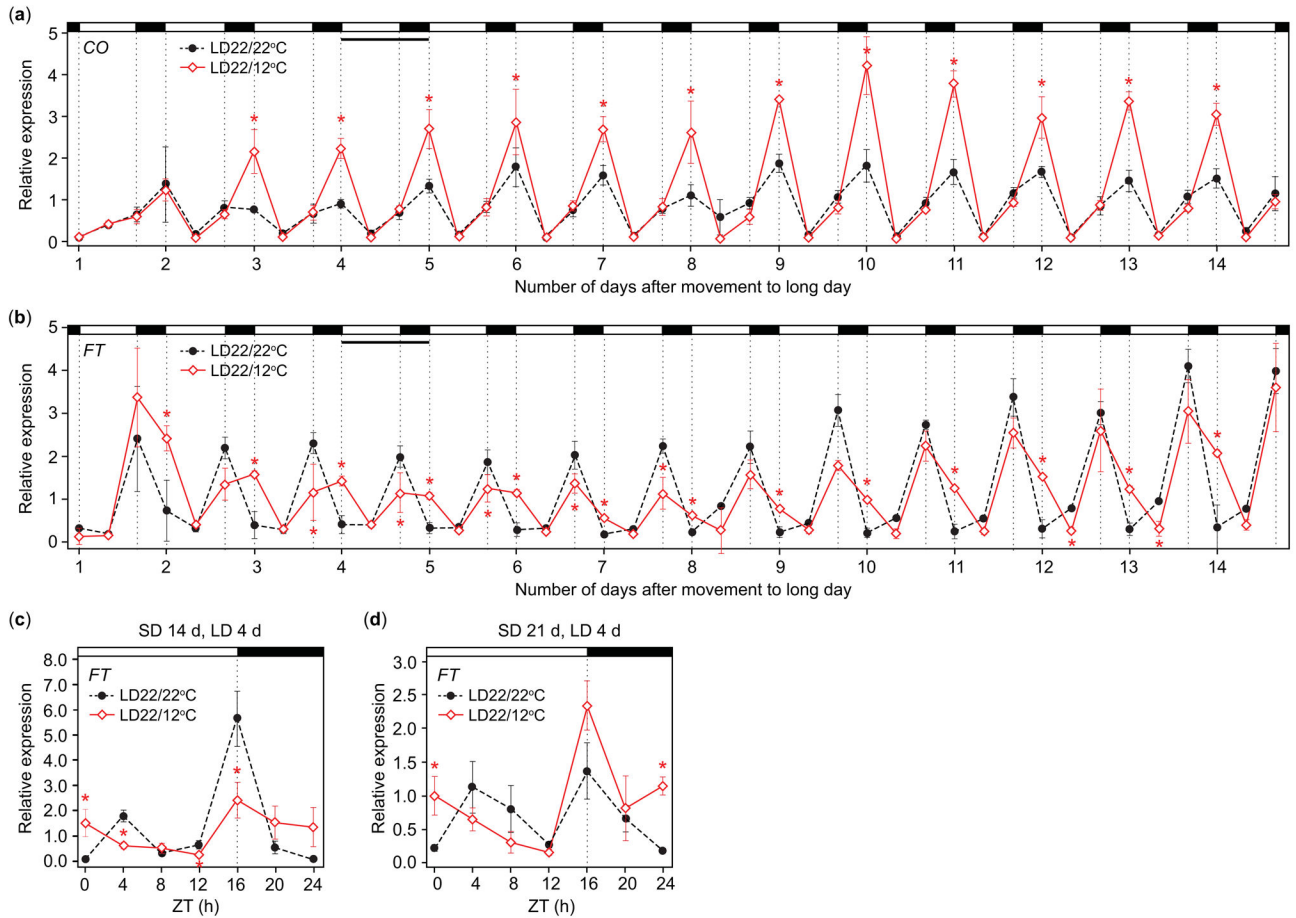
Fig. 4.

Late night/dawn inductions of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) transcript accumulation are conserved over 2 wk, dusk suppression declines as plants age in *Arabidopsis thaliana* (Columbia-0 (Col-0)). (a) *CO* and (b) *FT* transcript accumulation in LD22/22°C (long days with day: night temperatures = 22°C: 22°C) and LD22/12°C conditions for two consecutive weeks. Narrow, horizontal black bar designates day 4 of treatment. Seedlings were harvested at zeitgeber time (ZT, hours after dawn) 0, ZT8, and ZT16. Seedlings were grown for 7 d in short days (SD) before being transferred to LD, temperature treatments; harvest began on day 1 in treatments. Data represent means ± SEM derived from five biological replicates. (c, d) *FT* transcript accumulation on day 4 of LD, temperature treatments in 18-d-old seedlings (c), and in 25-d-old (d) seedlings harvested at the same time. Seedlings were kept in short days until moved to the treatments. Data represent means ± SEM derived from at least three biological replicates. (a–d) Asterisks (*) indicate $P<0.05$ and 95% CI of difference between pair did not contain zero.
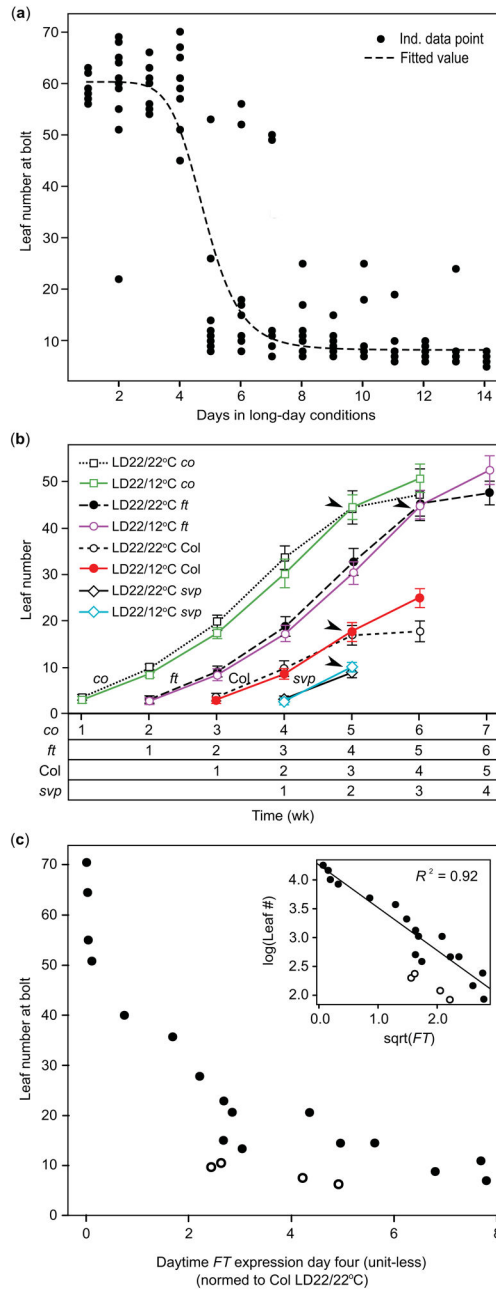
Fig. 5.

Short-term exposure to long days rapidly induces flowering in *Arabidopsis thaliana* (Columbia-0 (Col-0)), and *FLOWERING LOCUS T* (*FT*) transcript accumulation correlates with final rosette leaf number across treatments and mutant strains within the Col-0 background. (a) Seven-day-old seedlings were transferred to LD22/22°C (long days with day: night temperatures = 22°C: 22°C) and LD22/12°C for 14 d. After each LD, beginning on day 1, a subset of seedlings were transferred to short days (SD) and allowed to grow until bolts were observed (closed circles). A Hill function (dashed line) describing the final rosette leaf number (*LfNmbr*) at bolt as a function of days exposed to LD (*days*) was fitted

to the flowering data: $LfNmbr = \{(Bmax(tc\char94 n))/(tc\char94 n + days\char94 n)\} + Bmin$, where $Bmax$ is highest fitted leaf number at bolt (= 52.17 leaves), $Bmin$ is lowest fitted leaf number at bolt (= 8.23 leaves), tc is day at which transition occurs (= 4.86), n is modifier affecting abruptness of the transition (= 8.31) ($P<0.0001$ for fit of all parameters). (b) Number of rosette leaves added each week for Col-0 wild-type plants, *co-101*, *ft-101*, and *svp-31* in LD22/22°C and LD22/12°C conditions. Black arrows indicate when transition to bolting occurred. Error bars; means ± standard deviation. Overall temperature effect ($P<0.001$), mean for difference between temperature treatments and 95% Confidence Interval (CI, 0.008, −0.134, 0.1497). The strains are plotted adjacent to the others for ease of comparison; however, all were planted at the same time. At time week 1, seedlings were 7 d old and had not yet been exposed to long-day temperature treatments. LMER was used to test for a delay in leaf production, followed by Satterthwaite approximation to generate *P*-values. Leaf numbers to the right of the arrow were excluded from the test as plants in the LD22/22°C treatment had begun to bolt and stopped rosette leaf production. Note that *svp-31* plants began to bolt within the first week of treatment, so do not show the same pattern as the other strains, Landsberg *erecta* (L*er*), and Wassilewskija-2 (Ws-2) had patterns similar to *svp-31* and so are not shown. (c) Final rosette leaf number plotted against daytime *FT* transcript accumulation harvested from time points at zeitgeber time (ZT) 0, ZT8, and ZT16 across several photoperiod and temperature conditions, and strains within the Col-0 background (closed circles), and for L*er* and Ws (open circles). Accumulated *FT* at time points ZT0, ZT8, and ZT16 (dawn, trough, and dusk) was found by integrating under the curve and is normalized to Col-0 harvested at ZT16 in LD22/22°C constant temperature conditions. Data is linearized by transforming as shown in inset for linear regression analysis (RMSE = 0.208 log(leaves)).
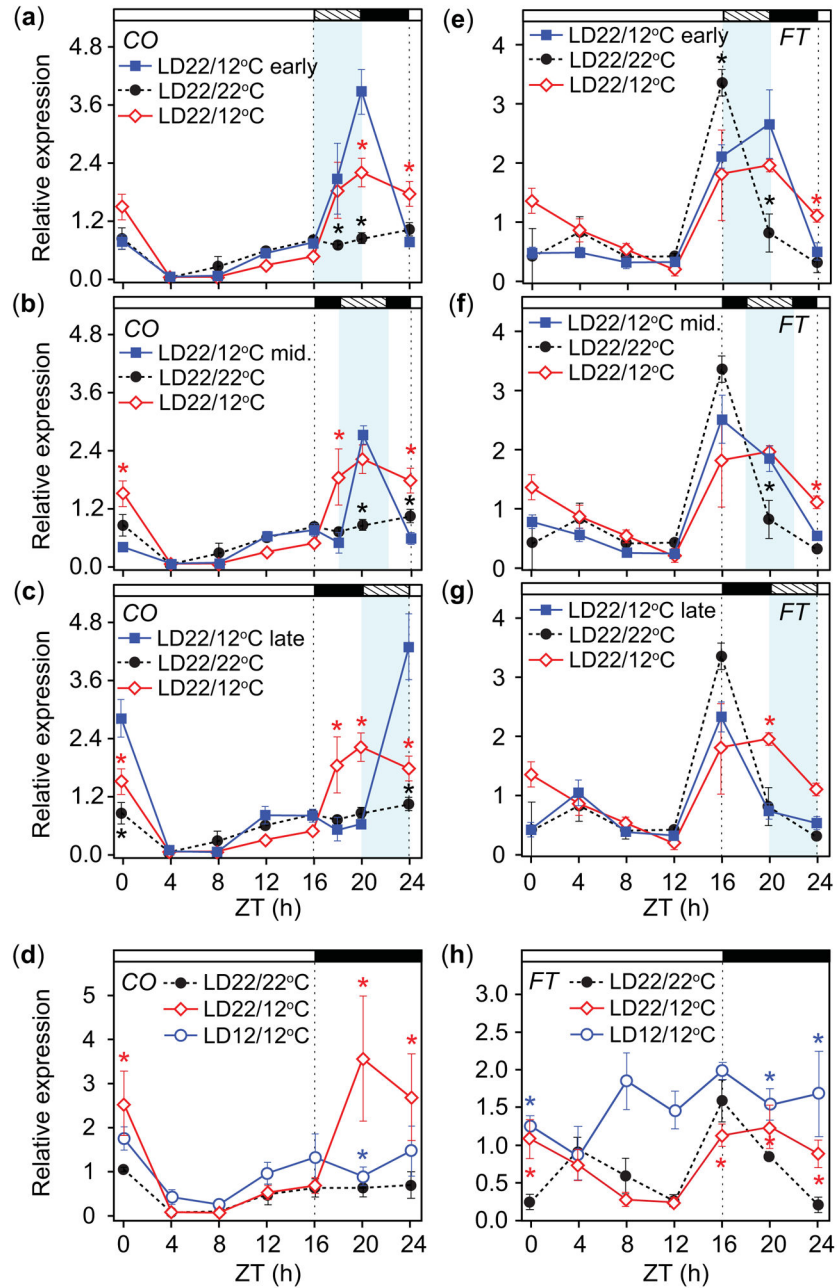
Fig. 6.

Transcript accumulation of *CONSTANS* (*CO*) is induced regardless of when temperature drops occur at night in *Arabidopsis thaliana* (Columbia-0 (Col-0)); cool temperatures at the beginning of the night induce *FLOWERING LOCUS T* (*FT*). (a–c) *CO* transcript accumulation and (e–g) *FT* transcript accumulation in response to 4-h periods of 12°C at the beginning of the night from zeitgeber time (ZT) 16 to 20 (a, e *early*), middle of the night from ZT18 to 22 (b, f *mid*), and end of the night from ZT20 to 24 (c, g *late*) in LD. (d) *CO* transcript accumulation and (h) *FT* transcript accumulation in LD with constant 12°C conditions (LD12/12°C). All panels show *CO* and *FT* transcript accumulation patterns in

LD22/22°C and LD22/12°C conditions as references. Data represent means ± SEM derived from at least three biological replicates. Topmost white and black bars designate day and night, respectively. Hashed bars on the top and light blue areas in the graphs represent 4-h cool period (a–c, e–g). Asterisks (a–c, e–g) represent significance ($P<0.05$) and 95% CI of difference between pair did not contain zero in statistical comparisons between the early, middle, or late night treatments and the LD22/22°C (black) or LD22/12°C controls (red). Asterisks (*) (d, f) represent significance ($P<0.05$) and 95% CI of difference between pair did not contain zero in statistical comparisons made against the LD22/22°C control.
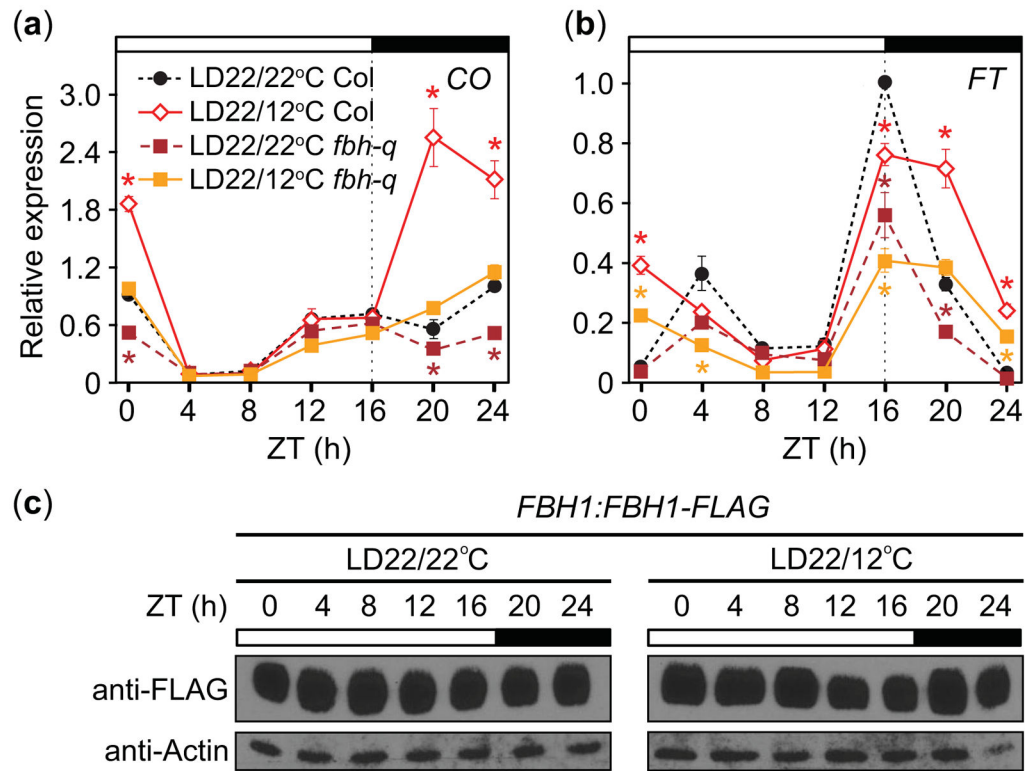
Fig. 7.

The *FLOWERING BHLH* (*FBH*) family is involved in cool-night induction of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) transcript accumulation in *Arabidopsis thaliana* (Columbia-0 (Col-0)). (a) *CO* and (b) *FT* gene transcript accumulation in *fbh*-quadruple (*fbh-q*) mutant line. Data, which are normalized to the maximum value in Col-0 wild-type control, represent means ± SEM derived from three biological replicates. Asterisks (*) represent significance (*P*<0.05) and 95% CI of difference between pair did not contain zero in statistical comparisons made within a strain against the LD22/22°C treatment. (c) Accumulation patterns of FLAG-FBH1 protein in *FBH1:FLAG-FBH1* plants grown in LD22/22°C (long days with day: night temperatures = 22°C: 22°C) and LD22/12°C conditions. Actin serves as a loading control. ZT, zeitgeber time.
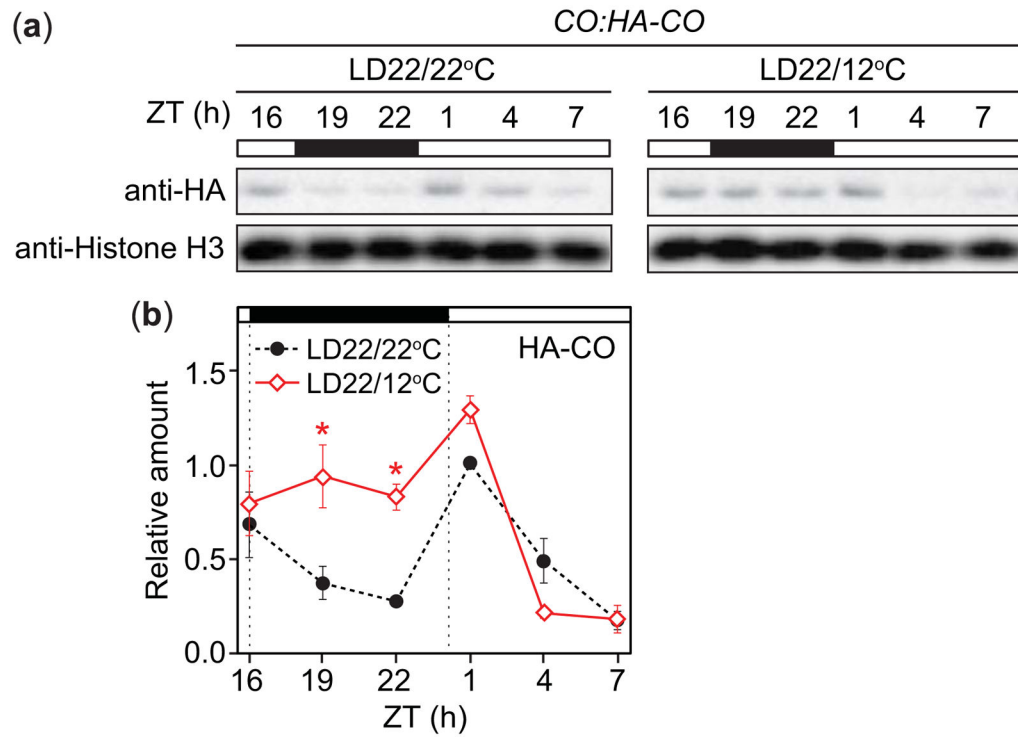
Fig. 8.
CONSTANS (CO) protein is higher during cool nights in *Arabidopsis thaliana* (Columbia (Col-0)). (a, b) Accumulation patterns (a) and quantification (b) of HA-CO protein in *CO:HA-CO* plants grown in LD22/22°C (long days with day: night temperatures = 22°C: 22°C) and LD22/12°C conditions. Histone H3 serves as a loading control. (b) Data represent means ± SEM derived from three biological replicates. Asterisks (*) indicate *P*<0.5 and 95% CI of difference between pair did not contain zero in statistical comparisons made to the LD22/22°C control. ZT, zeitgeber time.
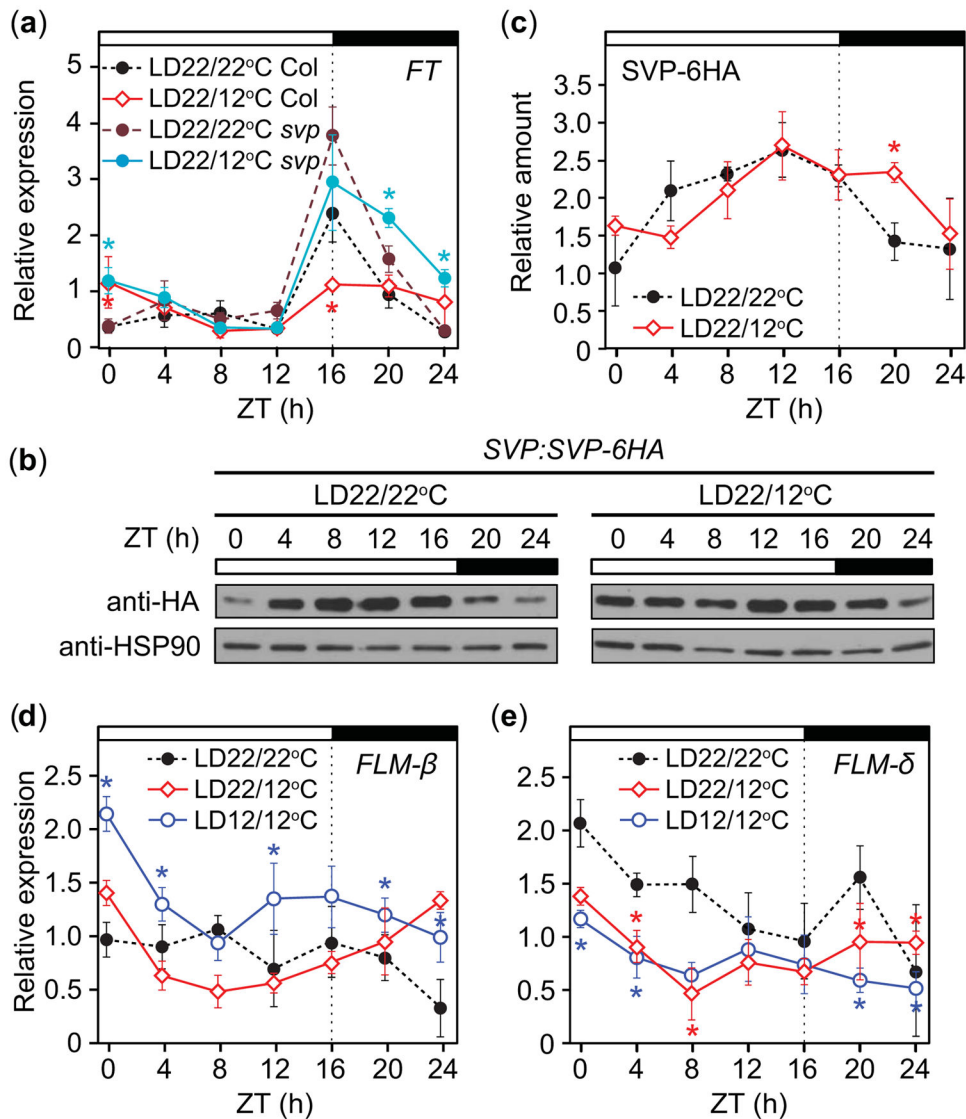
Fig. 9. SHORT VEGITATIVE PHASE

(*SVP*) is involved in cool-night induced dusk suppression of *FLOWERING LOCUS T* (*FT*) accumulation in *Arabidopsis thaliana* (Columbia-0 (Col-0)); suppression may be aided by a decrease in non-DNA-binding *FLOWERING LOCUS M* (*FLM*)-δ splice variant before dusk. (a) *FT* transcript accumulation in *svp* mutant lines. Accumulation patterns (b) and quantification (c) of SVP-6HA protein in *SVP:SVP-6HA* plants grown in LD22/22°C and LD22/12°C conditions. HEAT SHOCK PROTEIN 90 (HSP90) serves as a loading control. (d) DNA-binding *FLM-β* splice variant and (e) non-DNA-binding *FLM-δ* transcript accumulation in wild-type plants grown in LD22/22°C (long days with day: night temperatures = 22°C: 22°C), LD22/12°C, and LD12/12°C conditions. Data represent means ± SEM derived from three biological replicates. (a, c–e) Statistical comparisons are made to the LD22/22°C control; asterisks indicate $P<0.5$ and 95% CI of difference between pair did not contain zero. ZT, zeitgeber time.
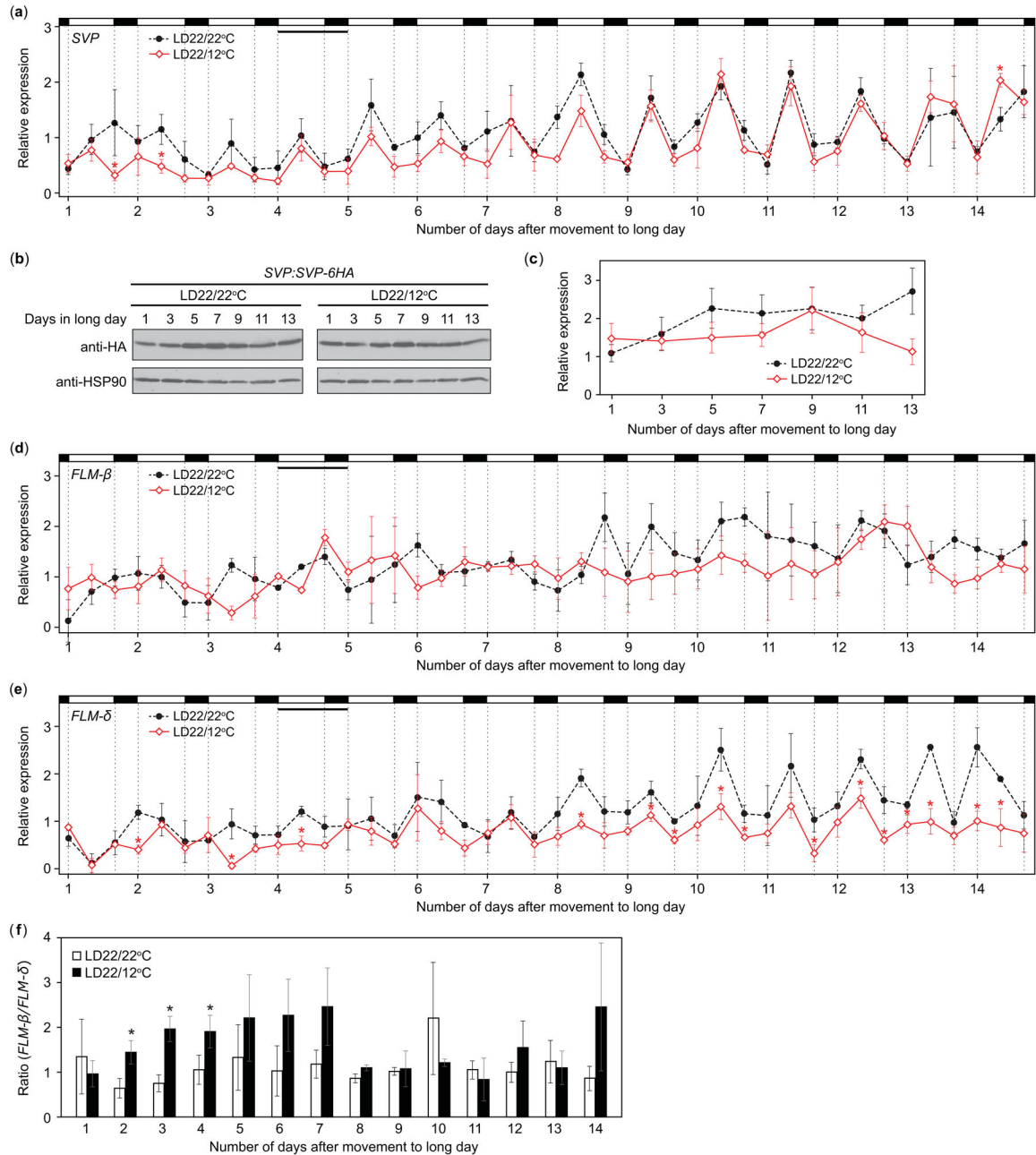
Fig. 10.
Alleviation of dusk *FLOWERING LOCUS T* (*FT*) transcript suppression over 2 wk may be caused by the change in *FLOWERING LOCUS M* (*FLM*)-*β* and *FLM*-*δ* transcripts in *Arabidopsis thaliana* (Columbia-0 (Col-0)). (a) *SVP* transcript accumulation in wild-type plants grown in LD22/22°C (long days with day: night temperatures = 22°C: 22°C) and LD22/12°C conditions in wild type for 14 d harvested at zeitgeber time (ZT) 0, ZT8, and ZT16 starting on day 1 of treatment. Accumulation patterns (b) and quantification (c) of SVP-6HA protein in *SVP:SVP-6HA* plants grown in LD22/22°C and LD22/12°C conditions over the 2 wk, harvested every other day at ZT13 starting on day 1. HSP90 serves as a

loading control. (d) *FLM-β* and (e) *FLM-δ* splice variant transcript accumulation patterns and (f) the ratio of *FLM-β* to *FLM-δ* in wild-type plants grown in LD22/22°C and LD22/12°C for 14 d harvested at ZT0, ZT8, and ZT16 starting on day 1 of treatment. The ratios (f) were calculated for each time point and then averaged over each day. In all cases plants were grown in short days (SD) for 1 wk before being transferred to treatments. Data represent means ± SEM derived from three biological replicates. (a, e, f) Asterisks (*) indicate *P*<0.05 and 95% CI of difference between pair did not contain zero.

Table 1

Means, variances, 95% Confidence Intervals, and *P*-values for final rosette leaf number at bolt of *Arabidopsis thaliana* strains in response to constant (22°C) temperatures or warm (22°C) days and cool (12°C) nights

Strain	Night temp. (°C)	Mean (leaf number)	Standard deviation	SE	Mean (log(leaf number))	95% CI		adjusted *P*[*]
						upper	lower	
Col-0[**]	22	14.96	2.91	0.42	0.3289	0.2323	0.4254	0.0000
	12	20.84	4.51	0.64				
co-101	22	49.76	6.99	1.01	0.0809	−0.0152	0.1769	0.1987
	12	53.70	5.98	0.85				
ft-101	22	50.22	6.52	0.94	0.0720	−0.0245	0.1686	0.3748
	12	53.78	4.19	0.60				
svp-31	22	7.30	1.44	0.23	0.1874	0.0784	0.2964	0.0000
	12	8.84	1.74	0.29				
Ler	22	7.40	2.07	0.33	0.3131	0.2062	0.4199	0.0000
	12	9.98	1.75	0.28				
Ws-2	22	8.00	1.04	0.17	0.2902	0.1833	0.3971	0.0000
	12	10.73	1.74	0.28				
Col-0	22	15.79	2.11	0.33	0.3144	0.2127	0.4162	0.0000
	12	22.23	5.04	0.94				
fbh-quadruple #2	22	22.80	2.99	0.55	0.2241	0.0972	0.3510	0.0000
	12	27.72	3.71	0.90				

[*] *P*-value adjusted to account for multiple comparisons.

[**] Col-0 included as a wild-type control in all experiments.