

# Regulation of *CONSTANS* and *FLOWERING LOCUS T* Expression in Response to Changing Light Quality<sup>1[C][OA]</sup>

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In addition to pathways that regulate flowering in response to environmental signals such as photoperiod or cold temperatures (vernalization), flowering time is also regulated by light quality. In many species, far-red (FR) light is known to accelerate flowering. This is environmentally significant because leaves absorb more red light than FR light; thus, plants growing under a canopy experience light that is enriched in FR light. In this article, we have explored the promotion of flowering by FR-enriched light (FREL) in *Arabidopsis* (*Arabidopsis thaliana*). Previous work has shown that the floral promoter *CONSTANS* (*CO*) plays a critical role in day-length perception and exhibits complex regulation; *CO* mRNA is regulated by the circadian clock and *CO* protein is stabilized by light and degraded in darkness. We find that plants grown under FREL contain higher levels of *CO* mRNA in the early part of the day than plants under white light. Furthermore, transgenic plants expressing *CO* under the control of a constitutive promoter accumulate higher levels of *CO* protein under FREL, indicating that FREL can increase *CO* protein levels independently of transcription. Consistent with the model that FREL promotes flowering through *CO*, mutants for *co* or *gigantea*, which are required for *CO* transcript accumulation, are relatively insensitive to FREL. Because the red:FR ratios used in these experiments are in the range of what plants would experience under a canopy, these results indicate that the regulation of *CO* by light quality likely plays a key role in the regulation of flowering time in natural environments.

As sessile organisms, plants do not have the option of migrating from a suboptimal environment to a more favorable one. Thus, plants have evolved mechanisms that allow them to alter their growth and development in response to environmental signals, thereby increasing the likelihood of survival and reproductive success. One well-studied example of how the external environment can regulate plant development is the transition from vegetative to reproductive development. In *Arabidopsis* (*Arabidopsis thaliana*), flowering is promoted by a group of genes referred to as floral integrators. These genes, including *FLOWERING LOCUS T* (*FT*), the *FT* homolog *TWIN SISTER OF FT* (*TSF*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*)/*AGAMOUS-LIKE20*, all act as strong promoters of flowering (Kardailsky et al., 1999; Kobayashi et al., 1999; Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). Pathways that regulate flowering time in re-

sponse to environmental stimuli, such as cold or day-length, do so, in large part, by promoting or repressing the expression of these floral integrators. Thus, signals from multiple environment-sensing pathways are integrated at the levels of *FT*, *TSF*, and *SOC1* expression (Putterill et al., 2004).

In many plant species, flowering is promoted by prolonged exposure to cold temperatures, such as plants in temperate climates would experience in winter. This promotion of flowering is known as vernalization (Chouard, 1960). Winter-annual accessions of *Arabidopsis* occur naturally and are late flowering unless vernalized. This vernalization-responsive block to flowering is created by two genes, *FLOWERING LOCUS C* (*FLC*), a MADS-domain-containing transcription factor that acts as a repressor of *FT*, *TSF*, and *SOC1* (Michaels et al., 2005; Moon et al., 2005), and *FRIGIDA* (*FRI*), which is required for high levels of *FLC* expression (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). Vernalization, in turn, promotes flowering through an epigenetic shut off of *FLC* expression that is mediated by repressive histone modifications at the *FLC* locus (Bastow et al., 2004; Sung and Amasino, 2004). In contrast to winter annuals, most rapid-cycling accessions of *Arabidopsis* contain naturally occurring loss-of-function mutations in *FRI* and therefore have low levels of *FLC* expression and are early flowering even in the absence of vernalization (Johanson et al., 2000). Forward-genetic screens conducted in rapid-cycling backgrounds have identified a group of genes, collectively referred to as the autonomous floral-promotion pathway, that act to constitutively repress *FLC* expression (Koornneef et al., 1991; Michaels and Amasino, 1999; Sheldon et al.,

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1999). The phenotype of loss-of-function autonomous-pathway mutants is similar to that of *FRI*-containing winter annuals; autonomous-pathway mutants are late flowering due to elevated levels of *FLC* (Michaels and Amasino, 1999, 2001) and this late-flowering phenotype is eliminated by vernalization (Koornneef et al., 1991). It is important to note that, although vernalization removes the block to flowering created by *FLC*, vernalization alone is not sufficient to induce rapid flowering. Early flowering in Arabidopsis also requires the activation of the floral integrators by inductive daylengths.

In both winter-annual and rapid-cycling Arabidopsis, flowering occurs more rapidly in long days than in short days. The floral promoter *CONSTANS* (*CO*) is a key component in the promotion of flowering by long days. *CO* is a B-box-containing protein that acts to promote the expression of *FT*, *TSE*, and *SOC1* and is regulated at both the mRNA and protein levels (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Suarez-Lopez et al., 2001; Hepworth et al., 2002; Valverde et al., 2004). *CO* transcription is regulated by the circadian clock such that peak expression occurs late in the day under long days, but after dark in short days (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Valverde et al., 2004). This circadian expression of *CO* mRNA is dependent upon *GIGANTEA* (*GI*) because *CO* transcript levels are greatly reduced in *gi* mutants (Suarez-Lopez et al., 2001). *CO* protein is stabilized by white, blue, or far-red (FR) light by PHYTOCHROME A (*PHYA*) and CRYPTOCHROMES (*CRY1* and *CRY2*), but is degraded under red (R) light by *PHYB* or in darkness (Valverde et al., 2004). Because *CO* transcription is only coincident with light under long days, *CO* protein accumulation and subsequent activation of floral integrators provide a long-day specific flowering signal.

In addition to the duration of the light period (daylength), plants also perceive the quality of light (i.e. wavelength). Because leaves absorb more R light than FR light, plants growing under a canopy experience lower R:FR ratios than plants growing in full sun. Low R:FR ratios are perceived by the phytochrome family of photoreceptors and induce a range of responses including stem and petiole elongation, hypostatic leaves, reduced branching, and early flowering (Smith, 1995). The effect of FR-enriched light (FREL) on flowering time has been investigated in wild-type Arabidopsis, as well as late-flowering lines containing mutations in autonomous-pathway or photoperiod-pathway genes (Martinez-Zapater and Somerville, 1990; Eskins, 1992; Bagnall, 1993; Lee and Amasino, 1995; Cerdan and Chory, 2003). The molecular details of how FREL promotes flowering are not well understood, but low R:FR ratios are known to increase expression of *FT* (Cerdan and Chory, 2003; Halliday et al., 2003). Interestingly, a correlation has been observed between vernalization responsiveness and the promotion of flowering by FREL (Bagnall, 1993). *FRI*-containing winter annuals and autonomous-pathway mutants, which are late flowering due to high levels of

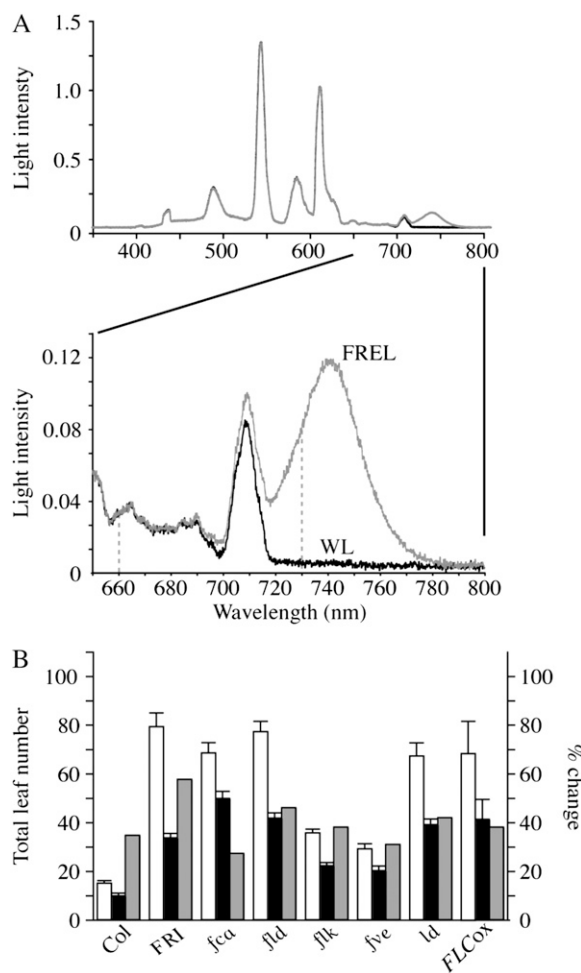
*FLC*, show a strong early-flowering phenotype when vernalized or grown under FREL. In contrast, vernalization and FREL are much less effective in promoting flowering in late-flowering photoperiod-pathway mutants, such as *gi* or *co*, in which *FLC* levels are not elevated.

Although the reason for the correlation between the effects of vernalization and FREL is unclear, at least three explanations seem plausible. Because *FLC* is required for the late-flowering phenotype of *FRI* and autonomous-pathway mutants, but not for that of photoperiod-pathway mutants (Michaels and Amasino, 2001), it is possible that, like vernalization, FREL represses *FLC* expression. Alternatively, FREL may promote flowering through activation of the photoperiod pathway, in which case photoperiod-pathway mutants would be predicted to have an attenuated response to FREL. Finally, FREL may promote flowering independently of both *FLC* and the photoperiod pathways, possibly through pathways that are responsible for the induction of other aspects of the shade-avoidance response. In this article, we provide evidence that the effect of FREL on flowering time is independent of *FLC* and genetically separable from other aspects of the shade-avoidance response (Vandenbussche et al., 2005). Furthermore, we show that FREL results in elevated levels of both *CO* mRNA and protein. Together, these data support a model in which low R:FR ratios promote flowering through activation of the photoperiod pathway.

## RESULTS

### FREL Promotes Flowering in *FRI* and Autonomous-Pathway Mutants Independently of *FLC*

A variety of light sources have been utilized to add supplemental FR light, including specialized fluorescent tubes (Eskins, 1992), incandescent bulbs (Bagnall, 1993), and, more recently, FR light-emitting diodes (LEDs; Salter et al., 2003). LEDs have several advantages over other sources of FR light, including negligible heat output and more precise control over light quality (wavelength). For our experiments, plants were grown under cool-white fluorescent lights alone (white-light [WL] conditions) or supplemented with FR LEDs (Fig. 1A). The R:FR ratio (660 nm:730 nm) was 6.0 in WL and 0.4 under FREL. A R:FR ratio of 0.4 is in the range of what plants might experience growing under a canopy (Smith, 1982). Previous work using incandescent bulbs as a source of supplemental FR light has shown a positive correlation between vernalization responsiveness and FR responsiveness in late-flowering mutants in the Landsberg *erecta* (*Ler*) background (Bagnall, 1993). Autonomous-pathway mutants, whose phenotype can be eliminated by vernalization, showed the greatest reduction in flowering time under FREL, whereas photoperiod pathway mutants are less responsive to both FREL and vernaliza-

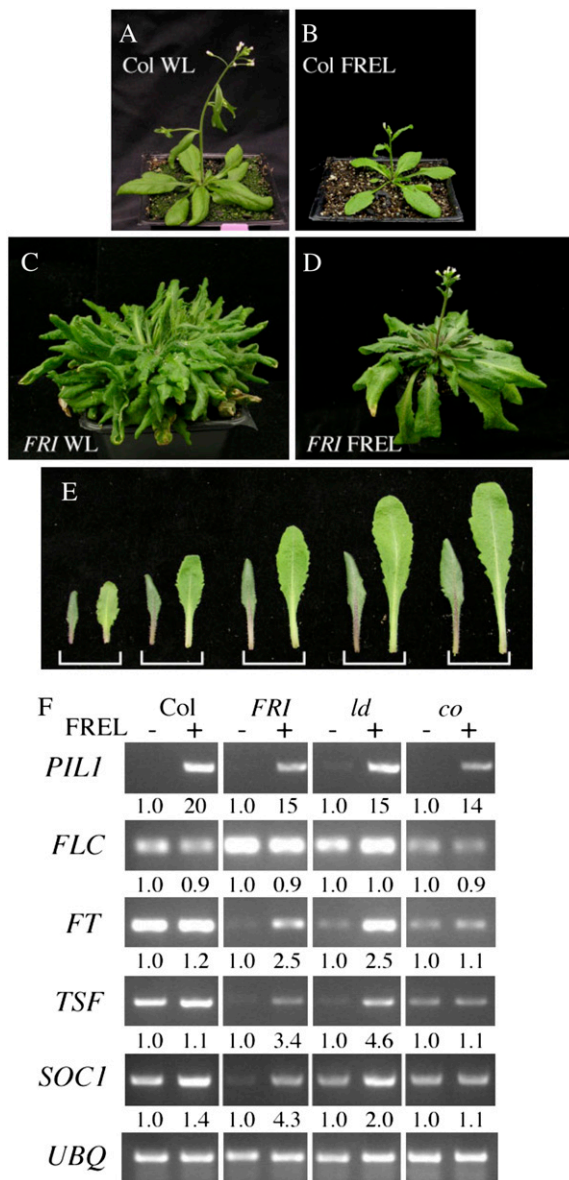


**Figure 1.** Effect of FREL on flowering time. A, Spectra of WL provided by cool-white fluorescent lamps (black line) or FREL (gray line) supplied by WL supplemented with FR LEDs. Light intensity is given in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The absorption peaks for the Pr and Pfr forms of phytochrome are indicated with vertical broken lines. B, Bars represent the total number of leaves formed prior to flowering under long days (rosette + cauline). White and black bars indicate plants grown under WL and FREL, respectively. Gray bars indicate the percentage of reduction in leaf number between WL and FREL. Error bars indicate 1 sd.

tion (Bagnall, 1993). Given this result, one possible explanation for the responsiveness of autonomous-pathway mutants to FREL is that, like vernalization, FREL promotes flowering through a reduction in *FLC* expression.

Because *Ler* contains a weak allele of *FLC* due to the insertion of a transposon in the first intron (Gazzani et al., 2003; Michaels et al., 2003), we investigated the effect of FREL on flowering in the Columbia (Col) background, which contains a more typical strong allele of *FLC*. When grown under FREL, autonomous-pathway mutants (*fca*, *fld*, *flk*, *fve*, or *ld*) or *FRI*-Col flowered significantly earlier than when grown under WL (Figs. 1B and 2, A–D); all lines showed at least a 30% reduction in total leaf number when grown under FREL (Fig. 1B). In addition to flowering early, plants

grown under FREL also exhibited other phenotypes associated with low R:FR ratios, including increased petiole elongation (Fig. 2E) and elevated expression of genes, such as *PHYTOCHROME-INTERACTING FACTOR3 (PIF3)-LIKE1 (PIL1*; Fig. 2F), whose expression is induced by low R:FR ratios (Makino et al., 2002; Salter et al., 2003). To determine whether *FLC* levels are affected by FREL, we examined *FLC* mRNA levels



**Figure 2.** Effect of FREL on *FRI* and late-flowering mutants. A to D, Col (A and B) and *FRI*-Col (C and D) plants grown under WL (A and C) or FREL (B and D). E, Leaves 5 to 9 taken from *FRI*-Col plants. In each pair of leaves shown, the leaf on the left was grown under WL and the leaf on the right was grown under FREL. F, Semiquantitative RT-PCR analysis of gene expression under WL and FREL. RNA was isolated from 14-d-old seedlings 4 h after dawn. *UBQ* was used as a control for loading. Numbers indicate the fold change in gene expression in response to FREL. All plants (A–F) were grown under long days. [See online article for color version of this figure.]

under WL or FREL. In all backgrounds tested, *FLC* mRNA levels were similar under both light conditions (Fig. 2F). Similarly, no significant change in expression was detected in an *FLC::GUS*-containing line grown under WL or FREL (Fig. 3, A and B). These results suggest that the regulation of *FLC* mRNA levels does not play a major role in the acceleration of flowering by FREL. As a further test of this hypothesis, we examined the flowering time of a line containing a constitutively expressed *35S::FLC* construct. Consistent with the result that *FLC* levels are not reduced under FREL, *35S::FLC* flowered earlier under FREL than under WL (Fig. 1B).

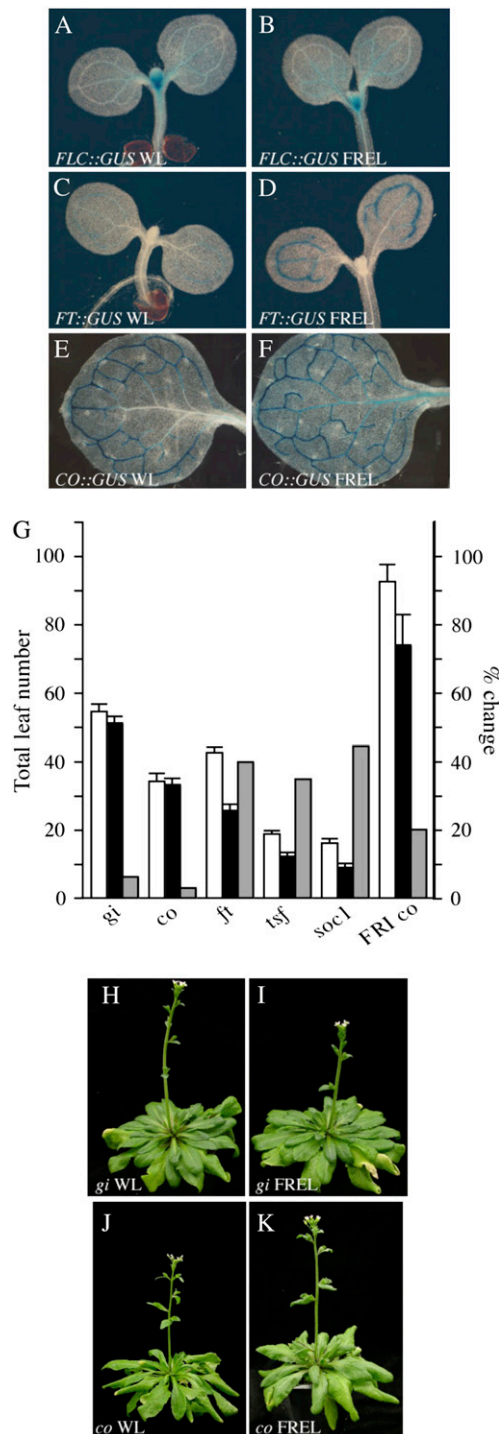
### *gi* and *co* Mutants Show an Attenuated Response to FREL

An alternative explanation for the promotion of flowering by FREL in *FRI* and autonomous-pathway mutants is that the repression of flowering conferred by *FLC* is overcome through the activation of an alternative floral-promotion pathway. To examine this possibility, we determined the flowering time of mutants in the photoperiod pathway. Interestingly, the photoperiod-pathway mutants *gi* and *co* showed little difference in flowering time when grown under WL or FREL (Fig. 3, G–K). The relative insensitivity of *gi* and *co* mutants to FREL suggests that FR light may accelerate flowering through the photoperiod pathway. If this were the case, then a photoperiod-pathway mutation would be predicted to block or reduce the response of *FRI* or autonomous-pathway mutants to FREL. To test this hypothesis, a *FRI co* line (Michaels and Amasino, 2001) was grown under WL and FREL. When grown under FREL, *FRI-Col* flowered with 57% fewer leaves than under WL (Fig. 1B). In contrast, *FRI co* showed only a 20% reduction in leaf number under FREL (Fig. 3G). Thus, an active photoperiod pathway is required for the full effect of FREL in a *FRI*-containing background.

*CO* is known to promote flowering in long days by activating the expression of the floral promoters *FT*, *TSE*, and *SOC1* (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000). Consistent with FREL promoting flowering through the photoperiod pathway, the transcript levels of *FT*, *TSE*, and *SOC1* are elevated in *FRI* or *ld* plants grown under FREL, but not in *co* mutants (Fig. 2F). The induction of *FT* by FREL was also evident in plants containing an *FT::GUS* fusion (Fig. 3, C and D). Taken together, these data are consistent with a model in which FREL acts through *GI* and *CO* to activate *FT*, *TSE*, and *SOC1* expression.

### *FT*, *TSE*, and *SOC1* Act Redundantly to Promote Flowering in Response to FREL and *CO*

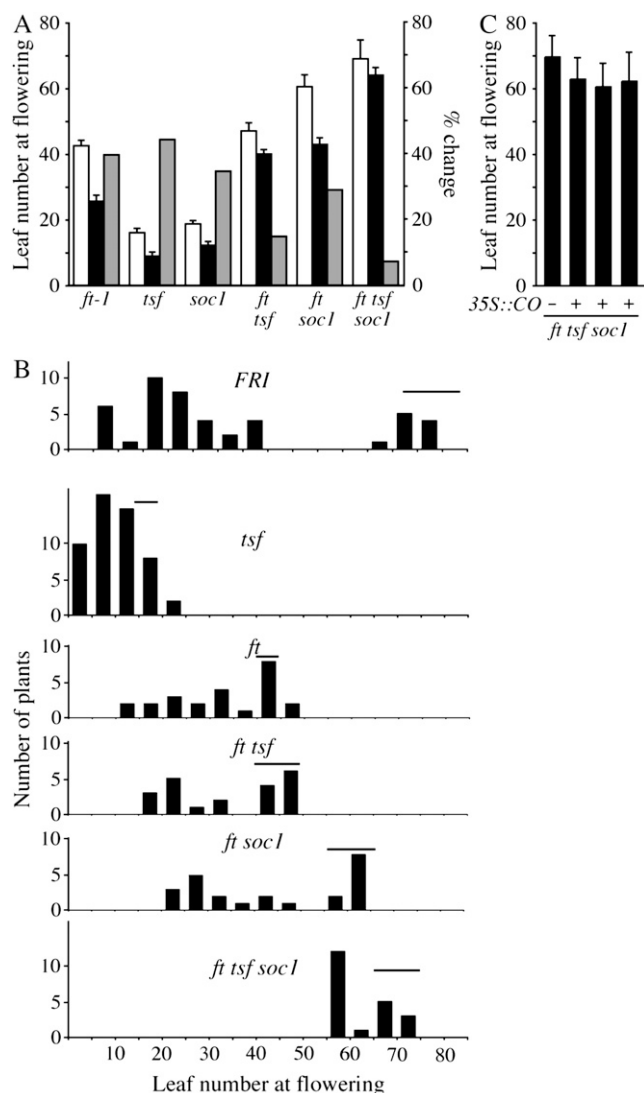
Given the fact that FREL results in increased expression of floral integrators, we investigated the effect of FREL on *ft*, *tsf*, and *soc1* mutants. All three mutants flowered significantly earlier under FREL, forming 35%



**Figure 3.** Effect of FREL on photoperiod-pathway mutants. A to D, *FLC::GUS* (A and B) and *FT::GUS* (C and D) expression in 10-d-old seedlings grown under WL (A and C) or FREL (B and D). E and F, *CO::GUS* expression in the first true leaves of 15-d-old seedlings grown under WL (E) or shifted to FREL for 8 h (F). G, Bars represent the total number of leaves formed prior to flowering (rosette + cauline). White and black bars indicate plants grown under WL and FREL, respectively. Gray bars indicate the percentage of reduction in leaf number between WL and FREL. Error bars indicate 1 s.d. H to K, *gi* (H and I) and *co* (J and K) mutants grown under WL (H and J) or FREL (I and K). All plants (A–K) were grown under long days.

to 44% fewer leaves than when grown under WL (Fig. 3G). Given that mutations in *co* render plants almost completely insensitive to FREL (Fig. 3G), it is interesting that mutations in the downstream targets of *CO* do not prevent early flowering in response to FR light. One explanation for why single mutants in the floral integrators remain sensitive to FREL is that they act redundantly. In support of this model, *FT* and *TSF* are homologous genes that have been shown to have redundant functions (Michaels et al., 2005; Yamaguchi et al., 2005). In addition, evidence suggests that *CO* directly regulates both *FT* and *SOC1* (Samach et al., 2000; Hepworth et al., 2002). To investigate the cumulative roles of *FT*, *TSF*, and *SOC1* on the promotion of flowering in response to low R:FR ratios, we examined the response of double and triple mutants to FREL. *ft* and *tsf* single mutants each formed approximately 40% fewer leaves under FREL; the *ft tsf* double mutant, however, showed a much weaker response to FREL, flowering with only a 15% reduction in leaf number (Fig. 4A). Because *CO* is thought to directly regulate *FT* and *SOC1*, we examined the flowering time of an *ft soc1* double mutant. Interestingly, the *ft soc1* double mutant showed a relatively strong response to FREL, flowering with 30% fewer leaves. The observation that the *ft tsf* is less sensitive to FREL than the *ft soc1* suggests that *TSF* may play a significant role in promoting flowering in response to FREL in *ft soc1* plants. If so, then an *ft tsf soc1* triple mutant would be predicted to be much less sensitive to FREL than the *ft soc1* double. Indeed, this is the case; the *ft tsf soc1* triple mutant was less sensitive to FREL than *ft soc1* (Fig. 4A) and flowered with only 7% fewer leaves under FREL. Thus, *FT*, *TSF*, and *SOC1* function redundantly to promote flowering in response to FREL.

Like FREL, *CO* also promotes the expression of the floral integrators. Previous work has shown that mutations in *ft* or *soc1* can partially suppress the early-flowering phenotype of *CO* overexpression (Onouchi et al., 2000; Samach et al., 2000; Yoo et al., 2005). Therefore, we investigated whether *FT*, *TSF*, and *SOC1* play redundant roles in promoting flowering in response to *CO*. Overexpression of *CO* causes a strong early-flowering phenotype regardless of photoperiod (Simon et al., 1996); therefore, we transformed single, double, and triple mutants with a construct containing *CO* under control of the 35S cauliflower mosaic virus promoter and examined the flowering time of T1 plants. To test the effectiveness of our construct, 35S::*CO* was transformed into *FRI*-Col. Most T1 plants flowered early, with many plants flowering with fewer than 10 leaves compared to approximately 75 leaves for untransformed controls (Fig. 4B). The effect of *CO* overexpression in various *ft*, *tsf*, and *soc1* mutant backgrounds was largely similar to that of FREL (Fig. 4, A and B). In *tsf* single mutants, 35S::*CO* was very effective at accelerating flowering. *CO* overexpression in *ft*, *ft tsf*, or *ft soc1* backgrounds also showed a strong early-flowering phenotype. The earliest flowering T1 plants in these backgrounds, however, were later than



**Figure 4.** Redundant roles of *FT*, *TSF*, and *SOC1* in the promotion of flowering in response to FREL and *CO*. Bars represent the total number of leaves formed prior to flowering (rosette + cauline). A, White and black bars indicate plants grown under WL and FREL, respectively. Gray bars indicate the percentage of reduction in leaf number between WL and FREL. B, Flowering time of T1 plants transformed with a 35S::*CO* construct. Horizontal lines indicate the flowering time of the untransformed parental lines. Plants were grown under WL. C, Flowering time of *ft tsf soc1* triple mutants and T2 lines transformed with a 35S::*CO*. Plants were grown under WL. All plants (A–C) were grown under long days. Error bars indicate 1 SD (A and C).

those obtained from the transformation of *tsf* or *FRI* with 35S::*CO*. As with FREL, the *ft tsf soc1* triple mutant showed the smallest change in flowering time in response to 35S::*CO*, with the earliest T1 plants flowering with greater than 55 leaves. To ensure that the early flowering observed in the transformed triple mutant was not due to stress associated with herbicide selection, seed was collected from three early-flowering T1 lines and flowering time was determined in the T2 generation without herbicide selection. Similar to the

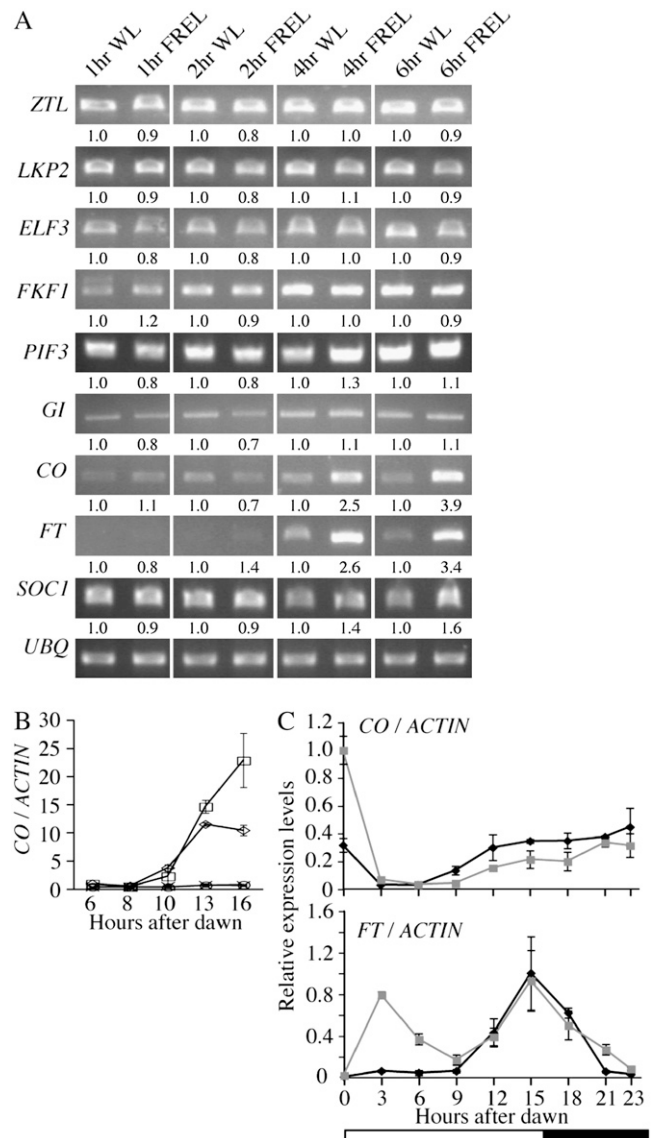
T1 results, all three lines flowered slightly earlier than the untransformed parent (Fig. 4C). Thus, *FT*, *TSF*, and *SOC1* act redundantly to promote flowering in response to both *FREL* and *CO*.

### FREL Increases *CO* Transcript Levels

The results above suggest that the promotion of flowering in *FRI* and autonomous-pathway mutants by *FREL* is due to increased expression of floral integrators, whose expression is normally repressed by high levels of *FLC* in these backgrounds. The requirement for *CO* and *GI* in the promotion of flowering by *FREL* suggests that the photoperiod pathway is involved in this activation. To determine where in the photoperiod pathway *FREL* acts, we first examined the expression of genes associated with the circadian clock. In plants shifted from WL to *FREL* for 1, 2, 4, or 6 h, *ZEITLUPE* (*ZTL*), *LOV KELCH PROTEIN2* (*LKP2*), *EARLY FLOWERING3* (*ELF3*), *FLAVIN-BINDING KELCH-REPEAT F-BOX* (*FKF1*), and *PIF3* (Zhou et al., 2007) showed no significant change in expression (Fig. 5A). This suggests that *FREL* may act downstream of the circadian clock to promote flowering. *GI*, which acts as an output of the circadian clock and regulates *CO* transcription (David et al., 2006), also showed no change in expression under WL or *FREL* (Fig. 5A). *CO*, however, showed significant increases in transcript levels after transfer to *FREL* (Fig. 5, A and B). This increase in *CO* transcription was also observed using a construct containing *GUS* fused to the *CO* promoter (Fig. 3, E and F). Consistent with the increase in *CO* expression, *FT*, and to a lesser extent *SOC1*, also showed increased expression (Fig. 5A). These results indicate that *FREL* promotes flowering, at least in part, through increased expression of *CO* mRNA.

Mutations in either *gi* or *co* lead to insensitivity to *FREL* (Fig. 3G). Previous work has shown that mutations in *gi* prevent the accumulation of *CO* transcript (Suarez-Lopez et al., 2001). Thus, a possible explanation for the insensitivity of *gi* mutants is that the *CO* transcript cannot be up-regulated by *FREL* in a *gi* mutant background. To test this hypothesis we examined the levels of *CO* mRNA in a *gi* mutant grown under WL or shifted to *FREL*. Under WL, *CO* transcript levels were much lower than that observed in Col (Fig. 5B). Moreover, no increase in *CO* transcript was observed in the *gi* mutant background after shifting to *FREL*. Thus, the inability of *gi* mutant plants to up-regulate *CO* mRNA levels in response to *FREL* may explain the similar FR-insensitive phenotypes of *co* and *gi* mutants.

In addition to the experiments described above in which plants are shifted to *FREL*, we also investigated the expression of *CO* and *FT* in plants grown continuously under WL or *FREL*. *CO* transcription is regulated by the circadian clock, with mRNA levels increasing late in the day (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). Therefore, it is possible that the increased levels of *CO* observed after shifting to *FREL* may be due



**Figure 5.** Regulation of *CO* mRNA by *FREL*. A, Col plants were grown in long days under WL for 14 d. Six hours after dawn on day 15, plants were either maintained in WL or shifted to *FREL*. After the indicated number of hours, plants were harvested and subjected to semiquantitative RT-PCR analysis. *UBQ* was used as a control for loading. Numbers indicate the fold change in gene expression in response to *FREL*. B, Quantitative RT-PCR analysis of *CO* expression. Col and *gi* plants were grown in long days under WL for 14 d. Six hours after dawn on day 15, plants were either maintained in WL or shifted to *FREL*. After the indicated number of hours, plants were harvested and subjected to quantitative RT-PCR analysis. The expression of *CO* was determined relative to *ACTIN*. Diamonds and squares indicate expression in Col under WL and *FREL*, respectively. Circles and crosses represent expression in *gi* under WL and *FREL*, respectively. Error bars indicate the  $\pm$  SE of the mean of three biological replicates. C, Quantitative RT-PCR analysis of *CO* and *FT* expression in 8-d-old Col plants grown in long-day conditions under WL (black line/diamonds) or *FREL* (gray line/squares). Plants were grown under WL or *FREL* from germination. Error bars indicate the  $\pm$  SE of the mean of three biological replicates. The horizontal bar indicates periods of light (white) and dark (black).

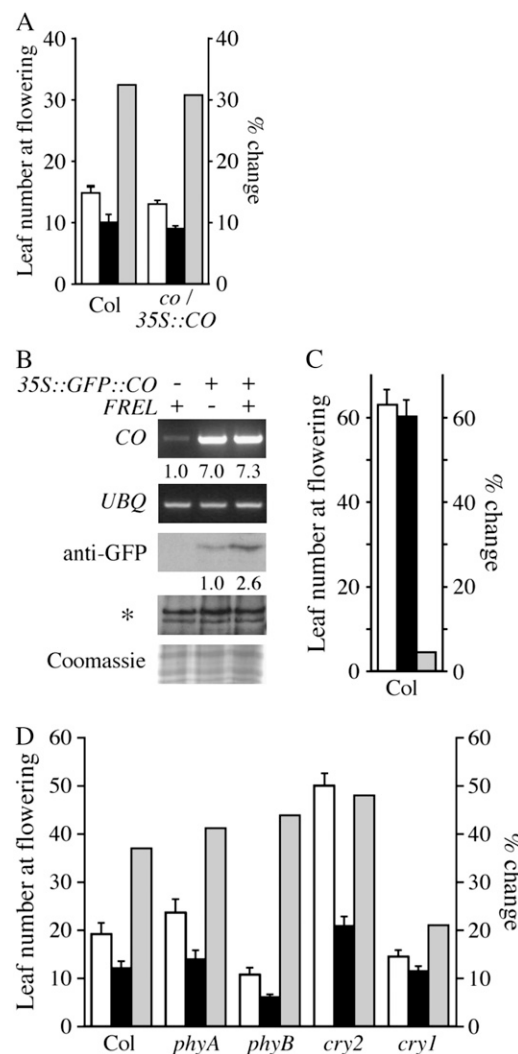
to a shift in the phase of *CO* expression, such that *CO* is expressed at higher levels earlier in the day under FREL than under WL. To investigate this possibility, we determined the expression of *CO* during a 24-h time course in plants grown continuously under WL or FREL. *CO* expression was significantly higher under FREL early in the day (near dawn; Fig. 5C). Interestingly, however, *CO* levels were similar under WL and FREL for the remainder of the day (Fig. 5C). Consistent with the expression of *CO*, *FT* levels were also significantly higher under FREL only in the early part of the day (Fig. 5C). Thus, growth under FREL does not appear to shift the phase of *CO* expression, but rather causes increased *CO* expression during the early part of the day.

### FREL Increases CO Protein Levels

If FREL acts to promote flowering solely by increasing the level of *CO* transcription, one would predict that constitutive expression of *CO* would lead to insensitivity to low R:FR ratios. To test this hypothesis, *35S::CO* was transformed into a *co* mutant. A transformed line was chosen, which flowered similarly to wild-type Col (Fig. 6A). When grown under WL and FREL, the *35S::CO* line showed approximately the same acceleration of flowering as seen in Col (Fig. 6A). Therefore, plants constitutively expressing *CO* remain sensitive to FREL.

The results above indicate that, in addition to increasing *CO* mRNA levels, FREL also accelerates flowering through mechanisms that are downstream of *CO* transcription. One possible explanation is that *CO* protein may accumulate to higher levels under FREL than under WL alone. This model is supported by previous work demonstrating that *CO* protein accumulates to higher levels under FR light than in darkness (Valverde et al., 2004). Although these data indicate that FR light is able to stabilize *CO* protein, it should be noted that *CO* accumulated to similar levels under WL or FR light (supplied by a mixture of incandescent bulbs and FR LEDs) in these experiments (Valverde et al., 2004). It remains possible, however, that WL supplemented with FR light (i.e. FREL) may be more effective at stabilizing *CO* than WL alone.

To determine whether *CO* protein levels might also play a role in accelerating flowering under FREL, we investigated the levels of *CO* protein under WL and FREL. A *35S::GFP::CO* construct was created and transformed into Col. The resulting line was early flowering, indicating that the fusion protein was functional. Plants were grown under WL for 17 d and then were either transferred to FREL or maintained in WL for 2 d. Reverse transcription (RT)-PCR analysis indicated that *CO* mRNA is indeed overexpressed and that the level of *CO* transcript is unaffected by light quality (Fig. 6B). *CO* protein levels were determined by western blot using an anti-GFP antibody to detect the GFP::*CO* fusion protein in nuclear protein extracts. In contrast to *35S::GFP::CO* mRNA levels, the level of



**Figure 6.** Regulation of *CO* protein by FREL. A, C, and D, Flowering time of plants of the indicated genotypes under WL (white bars) and FREL (black bars). Gray bars indicate the percentage of reduction in leaf number between WL and FREL. Error bars indicate 1 sd. Plants were grown under long days (A and D) or short days (C). B, Semiquantitative RT-PCR (top two images) and western-blot analysis (bottom three images) of a *35S::GFP::CO* transgenic line. *UBQ* was used as a loading control for RT-PCR. A cross-reacting band (\*) and a Coomassie-stained gel are shown as loading controls for western-blot analysis.

*CO* protein was 2.6-fold higher under FREL than under WL (Fig. 6B). Thus, *CO* protein does indeed accumulate to higher levels under FREL.

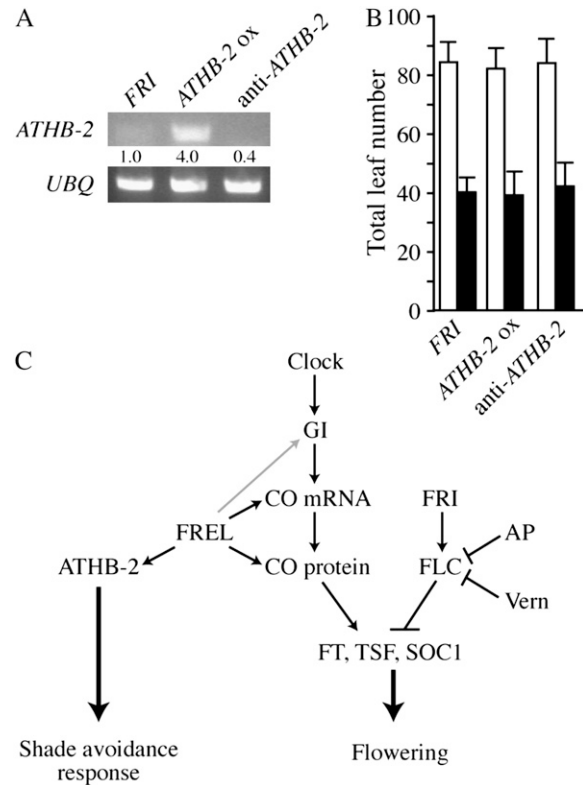
As a final experiment to test the model that FREL acts to promote flowering through *CO* protein accumulation, we investigated the effect of FREL on flowering under short days. Because of the circadian regulation of *CO* transcript, *CO* mRNA (and therefore *CO* protein) does not accumulate during the light period in short days (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Valverde et al., 2004). If this is the case, then FREL should have little effect on flowering time in short days because there is no *CO* protein to stabilize. This is

indeed the case. Col plants flowered similarly under short days regardless of light quality (Fig. 6C), supporting the model that the enhanced stabilization of CO protein by low R:FR ratios is key to the promotion of flowering by FREL.

Taken together, these results indicate that CO transcript and protein levels play an important part in the regulation of flowering time by light quality. CO expression is known to be regulated by several photoreceptors. CO transcript is increased in *phyB* mutants and decreased in *phyA* mutants (Yanovsky and Kay, 2002; Cerdan and Chory, 2003) and, at the protein level, CO is stabilized by PHYA, CRY1, and CRY2 and is destabilized by PHYB (Valverde et al., 2004). Consistent with the model that multiple photoreceptors are involved in CO regulation and previous observations (Mockler et al., 2003), we find that loss-of-function mutants in individual photoreceptors maintain a significant response to FREL under our conditions (Fig. 6D).

#### Acceleration of Flowering by FREL Is Genetically Separable from Other Shade-Avoidance Responses

In many plant species, the low R:FR ratios experienced under a canopy induce a number of responses collectively referred to as the shade-avoidance response. These include increased apical dominance and stem elongation, diminished leaf expansion, and accelerated flowering (Smith, 1995). *ATHB-2* is a homeodomain-Leu-zipper protein that is rapidly induced by low R:FR ratios (Carabelli et al., 1993). Plants overexpressing *ATHB-2* have phenotypes indicative of the shade-avoidance response in the absence of FREL, including reduced cotyledon expansion and increased hypocotyl elongation (Steindler et al., 1999). Conversely, plants with reduced *ATHB-2* expression have increased cotyledon expansion and decreased hypocotyl elongation (Steindler et al., 1999). Thus, *ATHB-2/HAT4* is a key regulator of certain aspects of the shade-avoidance response (Ruberti et al., 1991; Schena and Davis, 1992). Given its role in the regulation of other shade-avoidance-associated phenotypes, we investigated the possibility that *ATHB-2* might also be involved in the early-flowering phenotype of plants grown under low R:FR ratios. To determine whether altering *ATHB-2* expression could mimic the early-flowering phenotype observed under FREL, overexpression (*35S::ATHB-2*) and antisense (*αATHB-2*) constructs (Steindler et al., 1999) were transformed into a line containing *FRI* in the Col background (*FRI-Col*). The transgenic lines showed the previously described effects on hypocotyl elongation and cotyledon expansion (Steindler et al., 1999), indicating that the constructs were functional. RT-PCR was also performed to confirm the overexpression of *ATHB-2* in the *35S::ATHB-2* transgenic line and reduced expression in the *αATHB-2*-containing line (Fig. 7A). When grown under FREL, *FRI-Col* plants flowered significantly earlier than when grown under WL (Fig. 7B). Transgenic lines containing *35S::ATHB-2* or *αATHB-2*, however, flowered similarly to the un-



**Figure 7.** *ATHB-2* expression levels do not affect flowering time. A, Semicquantitative RT-PCR analysis of *ATHB-2* expression in 10-d-old seedlings. *UBQ* was used as a control for loading. Numbers indicated the fold change in gene expression relative to untransformed *FRI* plants. B, Bars represent the total number of leaves formed prior to flowering (rosette + cauline). White and black bars indicate plants grown under WL and FREL, respectively. Error bars indicate 1 sd. All plants (A and B) were grown under long days. C, Model for the promotion of flowering by FREL. Speculative interactions are depicted with gray arrows.

transformed controls when grown under WL or FREL (Fig. 7B). Thus, although *ATHB-2* expression is sufficient to induce other shade-avoidance phenotypes in the absence of low R:FR ratios, it is ineffective at promoting flowering. Thus, at the level of *ATHB-2* expression, the effects of FREL on flowering time and other aspects of the shade-avoidance response are genetically separable.

## DISCUSSION

A number of laboratories have examined the effect of light quality on flowering time in *Arabidopsis*. In general, this work has shown that late-flowering *FRI*-containing lines or autonomous-pathway mutants are more responsive to FREL than photoperiod-pathway mutants (Martinez-Zapater and Somerville, 1990; Bagnall, 1993; Lee and Amasino, 1995). It should be noted, however, that some reports have shown that *co* mutants exhibit strong responses to FREL (Halliday et al., 1994; Devlin et al., 1996). Although the reasons



for the discrepancies are not clear, differences in R:FR ratios, light sources, *co* alleles, and genetic backgrounds may play a role. Our results are consistent with those that have reported that photoperiod-pathway mutants are relatively insensitive to FREL (Martinez-Zapater and Somerville, 1990; Bagnall, 1993; Lee and Amasino, 1995) and can provide a molecular explanation for this observation. FREL leads to hyperactivation of the photoperiod pathway, thereby partially bypassing the block to flowering created by *FLC* (Fig. 7C). In autonomous-pathway mutants, high levels of *FLC* act to repress the expression of *FT*, *TSF*, and *SOC1*. Under FREL, however, *CO* mRNA and protein accumulate to higher levels and can increase the expression of the floral integrators. Consistent with this model, *ft tsf soc1* triple mutants show greatly reduced sensitivity to FREL and *CO* overexpression. It is interesting to note, however, that triple mutants are not completely insensitive to FREL and *CO* (Fig. 4). This suggests that FREL and *CO* may promote flowering through FT/TSF/SOC1-independent mechanisms. Another possibility, however, is that our triple mutant does not completely eliminate the function of all three genes. Although the *tsf* and *soc1* alleles used in this study are T-DNA insertional alleles and are likely to be nulls, the *ft* allele used (*ft-1* backcrossed into Col) contains a missense mutation and may not completely eliminate protein function (Kardailsky et al., 1999; Kobayashi et al., 1999). That *ft-1* is not a null is also supported by the observation that *ft-10*, a T-DNA allele, flowers later than *ft-1* (Yoo et al., 2005).

We find that FREL acts to independently promote both the accumulation of *CO* mRNA and *CO* protein. Although previous studies have shown that *CO* mRNA levels are up-regulated by pure FR light (Tepperman et al., 2001) and that *CO* transcript levels are altered in phytochrome mutants (Suarez-Lopez et al., 2001; Tepperman et al., 2001; Yanovsky and Kay, 2002; Cerdan and Chory, 2003), we believe this to be the first demonstration of the effect of environmentally relevant changes in R:FR ratios on *CO* transcript levels in wild-type plants. It is particularly interesting that the up-regulation of *CO* mRNA in plants growing under FREL occurs primarily in the early part of the day. The molecular mechanism underlying this up-regulation of *CO* mRNA by FREL is not clear, but it may involve GI. *gi* mutants have very low levels of *CO* expression and are relatively insensitive to FREL (Suarez-Lopez et al., 2001; Fig. 5B). Furthermore, *CO* mRNA levels are not up-regulated by FREL in a *gi* mutant background. Thus, GI is required for proper expression of the *CO* transcript. Interestingly, GI protein is also regulated by light. GI protein is stabilized in light (white, red, or blue) and is degraded in darkness by the 26S proteasome (David et al., 2006). Unfortunately, the effect of FR light on GI protein accumulation has not yet been determined. An interesting possibility, however, is that GI protein may accumulate to higher levels or show high activity under FREL. Given that increased expression of GI has been shown

to increase expression of *CO* mRNA (Mizoguchi et al., 2005), it seems reasonable to expect that any enhanced stabilization of GI protein by FREL would lead to increased *CO* transcription.

In addition to the up-regulation of *CO* mRNA levels, we have found that FREL also regulates *CO* protein levels. Because increased protein levels were observed using a GFP::*CO* fusion protein driven by the constitutive 35S promoter, the increase in protein under FREL cannot be attributed to increased transcription. Light quality has previously been implicated in the accumulation of *CO* protein; however, no significant difference was observed in *CO* protein accumulation under WL or pure FR light (Valverde et al., 2004). Coupled with our results, this suggests that FREL (i.e. both WL and FR light) may be more effective at promoting *CO* protein accumulation than WL or FR light separately. The increased *CO* protein levels observed under FREL could be a result of increased translation and/or decreased degradation; however, reduced protein degradation may be more likely. The addition of proteasome inhibitors to nuclear protein extracts increases *CO* protein levels in both dark- and light-treated material (Valverde et al., 2004). This result suggests that a significant amount of proteasome-dependent degradation of *CO* takes place even in the light. It is possible, then, that WL and FR light (i.e. FREL) may have an additive stabilizing effect on *CO*.

The significant changes in *CO* mRNA and protein accumulation observed under FREL illustrate the sensitivity of plants to changes in light quality in the range normally experienced in the environment. In addition to accelerating flowering, FREL also induces other phenotypes associated with the shade-avoidance response. Here, we have been able to demonstrate that accelerated flowering in response to FREL is genetically separable from other phenotypes of the shade-avoidance response; overexpression of *ATHB-2* is sufficient to induce hypocotyl elongation and reduced cotyledon expansion, but does not accelerate flowering. Thus, despite the fact that early flowering and other phenotypes associated with shade avoidance occur as a result of low R:FR ratios, they are controlled by separate outputs from the light-signaling mechanisms.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*FRI*-Col (Lee et al., 1994b), *fca-9* (Bezerra et al., 2004), *fld-3* (He et al., 2003), *fve-4* and *FRI-co* (Michaels and Amasino, 2001), *ld-1* (Lee et al., 1994a), *gi-2* (Park et al., 1999), *ft-1* in Col, *tsf*, and *soc1* (Michaels et al., 2005), *co* (SAIL24H04) (Kim and Michaels, 2006), and *phyA-211*, *phyB-9*, *cry1-304*, and *cry2-1* (Mockler et al., 2003) have been described previously. *flk* (SALK\_112850) was obtained from the Arabidopsis Biological Resource Center. The *FLC* overexpression line was created by transforming a 35S::*FLC* construct into the *flc-3* mutant. Plants were grown at 22°C under cool-white fluorescent light with a light intensity of 125  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (400–700 nm) with or without 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of supplemental FR provided by FR LEDs (L-D-735-H; Plasma Ireland Ltd.) at a density of 730 LED/m<sup>2</sup>. Light spectra were measured with a USB4000 spectrophotometer (Ocean Optics) using neutral density filters. Long and

short days consisted of 16 h light/8 h dark and 8 h light/16 h dark, respectively.

## Constructs

*ATHB-2* overexpression and antisense constructs were kindly provided by I. Ruberti and have been described previously (Steindler et al., 1999). *FLC::GUS* (Michaels et al., 2005) and *FT::GUS* and *CO::GUS* (Takada and Goto, 2003) constructs have been described previously. The *35S::CO* and *GFP::CO* fusion constructs were created by cloning *CO* cDNAs into the pEarleyGate203 (Earley et al., 2006) and pEGAD (Cutler et al., 2000) vectors, respectively. *35S::FLC* was created by placing a genomic *FLC* clone under control of the *35S* promoter in pPZP211 (Hajdukiewicz et al., 1994).

## RNA Expression Analysis

Semiquantitative PCR (Michaels et al., 2004) and quantitative PCR (Mockler et al., 2004) were performed as described previously. Primers for semiquantitative RT-PCR were as follows: *PIL1* (CAACGTAGCAATCTCTCCTGGA and GCATGAACCTGTGCTCTCCGATC), *GI* (CTGTCTTTCTCCGTTGTTTCTACTGT and TCATTCCGTTCTTCTCTGTTGTTGG), *ZTL* (GATGAAGAGG-GAGGTCTTTTCC and CCAAGAACAGGTCCAAGGTCAAT), *ELF3* (TTCC-TTCTCAGAGGTTTGGTGA and AGAGATTACAAAGCCACCTGAC), *LKP2* (AGATGAAGTGGCGGAGGATGGAT and GCTCTCCGATTGGTAAAGC-AGAA), *SOC1* (CTGAGGCATACTAAGGATCG and GAACAAGGTAACCA-CAATGAA), *FT* (AGACGTTCTTGATCCGTTTA and GTAGATCTCAGCA-AACTCGC), *CO* (AAACTCTTTCAGCTCCATGACCACTACT and CCATG-GATGAAATGTATGCGTTATGGTGA), *FKF1* (GTCTTCCGAAGTCTTCACTGG and TTCCTCACACTCTCGTCTCT), *PIF3* (GGGTTTGGGTTCAAAGAGA-AGC and CGACGATCCACAAAAGTATCAGAAAG), *ATHB-2* (TCAAGG-ATCCATGATGTTTCGAGAAAAGACGATCTGGG and GTAAGAGCTTTAG-GACCTAGGACGAAGAGCGTCA), *FLC* (TTCTCCAAACGTCGCAACGGT-CTC and GATTGTCCAGCAGGTGACATCTC), and *UBIQUITIN* (*UBQ*; GATCTTGGCCGAAAAACAATTGGAGGATGGT and CGACTTGTCAATTA-GAAAGAAAAGAGATAACAGG). For quantitative PCR, primers were as follows: *CO* (CATTAAACCATAACGCATACATTTTCATC and TCCGGCACAA-CACCAGTTT), *FT* (CAACCTCACCTCCGAGAATAT and TGCCAAAG-GTTGTTCCAGTTGT), and *ACTIN2* (GCTGAGAGATTGAGATGCCCA and GTGGATCCAGCAGTCTCCAT). All experiments were replicated at least three times with similar results.

## Protein Expression Analysis

Plants were grown in WL under long-day conditions for 17 d. Plants were then either maintained under WL or transferred to FREL for an additional 2 d. All above-ground portions of the plants from WL and FREL were harvested at dusk. Crude preparation of nuclei was conducted using the CelLytic plant nuclei isolation and extraction kit (Sigma) according to the manufacturer's instructions. Proteins were extracted from the nuclei preparation, boiled in 4× SDS sample buffer, fractioned on a 10% SDS-PAGE minigel, and then blotted to polyvinylidene difluoride membrane. The resulting membrane was probed with anti-GFP antibody, reacted with goat anti-rabbit IgG conjugated with horseradish peroxidase, and visualized using ECL western-blotting substrate (Pierce).

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