Acceleration of Flowering during Shade Avoidance in Arabidopsis Alters the Balance between *FLOWERING LOCUS C*-Mediated Repression and Photoperiodic Induction of Flowering[1][W][OA]

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The timing of the floral transition in Arabidopsis (*Arabidopsis thaliana*) is influenced by a number of environmental signals. Here, we have focused on acceleration of flowering in response to vegetative shade, a condition that is perceived as a decrease in the ratio of red to far-red radiation. We have investigated the contributions of several known flowering-time pathways to this acceleration. The vernalization pathway promotes flowering in response to extended cold via transcriptional repression of the floral inhibitor *FLOWERING LOCUS C* (*FLC*); we found that a low red to far-red ratio, unlike cold treatment, lessened the effects of *FLC* despite continued *FLC* expression. A low red to far-red ratio required the photoperiod-pathway genes *GIGANTEA* (*GI*) and *CONSTANS* (*CO*) to fully accelerate flowering in long days and did not promote flowering in short days. Together, these results suggest a model in which far-red enrichment can bypass *FLC*-mediated late flowering by shifting the balance between *FLC*-mediated repression and photoperiodic induction of flowering to favor the latter. The extent of this shift was dependent upon environmental parameters, such as the length of far-red exposure. At the molecular level, we found that far-red enrichment generated a phase delay in *GI* expression and enhanced *CO* expression and activity at both dawn and dusk. Finally, our analysis of the contribution of *PHYTOCHROME AND FLOWERING TIME1* (*PFT1*) to shade-mediated rapid flowering has led us to suggest a new model for the involvement of *PFT1* in light signaling.

As sessile organisms, plants cannot move away from environmental pressures. Evolution has thus favored a flexible developmental plan that allows plants to tailor their growth to particular conditions. This flexibility is reflected not only in variable patterns of vegetative growth but often in variable timing of the floral transition. By adjusting flowering time to maximize seed set in a given environment, a plant optimizes its reproductive and evolutionary success.

The effects of seasonal cues, particularly daylength and temperature, on flowering time have been well studied. Arabidopsis (*Arabidopsis thaliana*) is a facultative long-day (LD) plant, initiating flowering sooner under longer photoperiods, but eventually flowering under short days as well. Daylength is sensed through an external coincidence mechanism (for review, see Searle and Coupland, 2004; Imaizumi and Kay, 2006). In this model, the circadian clock restricts expression of the floral promoter *CONSTANS* (*CO*) to late in the day (Suarez-Lopez et al., 2001; Roden et al., 2002; Yanovsky and Kay, 2002; Imaizumi et al., 2003, 2005), and the coincidence of light with that expression (which occurs only during long days) leads to the stabilization of CO protein and the subsequent promotion of flowering (Valverde et al., 2004; Jang et al., 2008; Liu et al., 2008). *GIGANTEA* (*GI*) is required for *CO* expression (Suarez-Lopez et al., 2001; Sawa et al., 2007). *GI* expression is also regulated by the circadian clock, with a peak several hours before that of *CO* (Fowler et al., 1999; Park et al., 1999; Mizoguchi et al., 2005; David et al., 2006; Paltiel et al., 2006). Downstream of *GI* and *CO* are floral-promotion genes, including *FLOWERING LOCUS T* (*FT*; Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Takada and Goto, 2003; An et al., 2004; Yoo et al., 2005) and its relative *TWIN SISTER OF FT* (*TSF*; Michaels et al., 2005; Yamaguchi et al., 2005). *FT* and *TSF* integrate numerous environmental cues (see below) and, at least in the case of *FT*, directly link environmental perception pathways

[1] This work was supported by the College of Agricultural and Life Sciences and the Graduate School of the University of Wisconsin, the National Institutes of Health (grant no. 1R01GM079525), the National Science Foundation (grant no. 0446440), and the GRL Program from the MEST/KICOS. A.C.W. was supported by a fellowship from the National Science Foundation.

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.125468

to the induction of floral-identity genes at the meristem (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007).

Although most Arabidopsis accessions used for laboratory work do not require a long period of cold (vernalization) to promote rapid flowering, many natural accessions are late flowering even in inductive long days unless they are first vernalized (Napp-Zinn, 1961, 1964). This delay is largely due to the action of two genes: *FRIGIDA* (*FRI*) leads to up-regulation of the MADS box transcription factor *FLOWERING LOCUS C* (*FLC*; Michaels and Amasino, 2001), which is a floral repressor (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* counteracts the effects of the photoperiod pathway by repressing floral-promotion genes that CO activates (Borner et al., 2000; Lee et al., 2000; Onouchi et al., 2000; Hepworth et al., 2002; Michaels et al., 2005; Moon et al., 2005; Yamaguchi et al., 2005; Helliwell et al., 2006; Searle et al., 2006). Thus, in most accessions, flowering time in the absence of vernalization is determined by the balance between opposing activities of *FLC* and *CO* on floral-promotion genes such as *FT* and *TSF* (for review, see Simpson and Dean, 2002). During vernalization, *FLC* is permanently repressed via histone modifications; the resulting decrease in *FLC* levels makes plants competent to respond to the inductive cues provided by the photoperiod pathway (Bastow et al., 2004; Sung and Amasino, 2004; for review, see Schmitz and Amasino, 2007).

In addition to sensing seasonal cues, Arabidopsis is sensitive to the presence of competing plants. The proximity of other plants, either overhead casting direct shade or nearby forecasting future shade, is perceived as a change in light quality. Red wavelengths (λ_{max} approximately 660 nm) are absorbed by the chlorophyll of surrounding plants, but far-red wavelengths (λ_{max} approximately 730 nm) are reflected and/or transmitted, causing the ratio of red to far-red (R:FR) radiation to decrease as the density of proximal plants increases (for review, see Smith, 1982). Like many shade-intolerant plants, Arabidopsis responds with a suite of responses collectively known as the shade-avoidance syndrome (SAS). The SAS is characterized by increased elongation, decreased leaf expansion, hyponasty, decreased branching, and rapid flowering (for review, see Ballare, 1999; Franklin and Whitelam, 2005). Changes in morphology enable the plant to compete for light, and more rapid flowering may allow the plant to set seed before being outcompeted.

Red and far-red radiation are perceived by PHYTOCHROME (PHY) photoreceptors, which are encoded by a family of five genes (*PHYA–PHYE*) in Arabidopsis (Sharrock and Quail, 1989; Clack et al., 1994; for review, see Quail, 2002; Mathews, 2006; Rockwell et al., 2006). By virtue of their attached chromophore, PHYs photoconvert between the red-absorbing Pr form and the biologically active, far-red-absorbing Pfr form. The Pfr form of PHYB, PHYC, PHYD, and PHYE inhibits flowering, as indicated by the early flowering of mutants that lack these photoreceptors (Goto et al., 1991; Reed et al., 1993; Aukerman et al., 1997; Devlin et al., 1998; Monte et al., 2003; Balasubramanian et al., 2006a). Far-red enrichment promotes flowering by reducing Pfr levels of PHYB, PHYC, PHYD, and PHYE, with the removal of PHYB Pfr contributing most strongly to this acceleration at 22°C (Whitelam and Smith, 1991; Robson et al., 1993; Devlin et al., 1999; Franklin et al., 2003; Halliday and Whitelam, 2003).

Microarrays and genetic screens have advanced our understanding of the downstream pathways that lead from far-red perception to vegetative shade-avoidance phenotypes (Carabelli et al., 1993; Steindler et al., 1999; Devlin et al., 2003; Salter et al., 2003; Sessa et al., 2005; Roig-Villanova et al., 2006, 2007; Lorrain et al., 2008; Tao et al., 2008), but less is known about how far-red enrichment affects flowering-time pathways. Up-regulation of *FT* during far-red exposure is well documented (Cerdan and Chory, 2003; Devlin et al., 2003; Halliday et al., 2003; Endo et al., 2005; Sessa et al., 2005), but since this gene is a target of multiple environmental pathways and appears to be up-regulated whenever flowering is accelerated, its induction does not indicate which flowering-time pathways are affected by low R:FR conditions. There are indications that the photoperiod pathway is strengthened under a low R:FR ratio (Devlin et al., 2003; Valverde et al., 2004), but some data also suggest that the photoperiod pathway is dispensable for rapid flowering in response to shade (Goto et al., 1991; Reed et al., 1993; Halliday et al., 1994; Devlin et al., 1996; Callahan and Pigliucci, 2005). A photoperiod-independent pathway has been proposed based on the mutant phenotype of *PHYTOCHROME AND FLOWERING TIME1* (*PFT1*; Cerdan and Chory, 2003). Finally, although a significant correlation between the response to vernalization and the response to far-red enrichment was noted over a decade ago (Martinez-Zapater and Somerville, 1990; Bagnall, 1992, 1993), no molecular investigations of shade-mediated early flowering have been carried out in a background with high *FLC* expression. Thus, the mechanistic basis for this similarity remains unknown. In this study, we have clarified the involvement of various flowering-time genes and pathways in the acceleration of flowering by a low R:FR ratio.

RESULTS

A Low R:FR Ratio Bypasses *FLC*-Mediated Late Flowering without Lowering *FLC* Expression

Far-red enrichment is similar to vernalization in that it can promote rapid flowering in lines that are late flowering due to high *FLC* levels (Martinez-Zapater and Somerville, 1990; Bagnall, 1992, 1993; Lee and Amasino, 1995). Figure 1A shows far-red-mediated acceleration of flowering in a Columbia (Col) line that contains an active allele of *FRI* (Lee and Amasino, 1995). This accelerated flowering of *FLC*-expressing

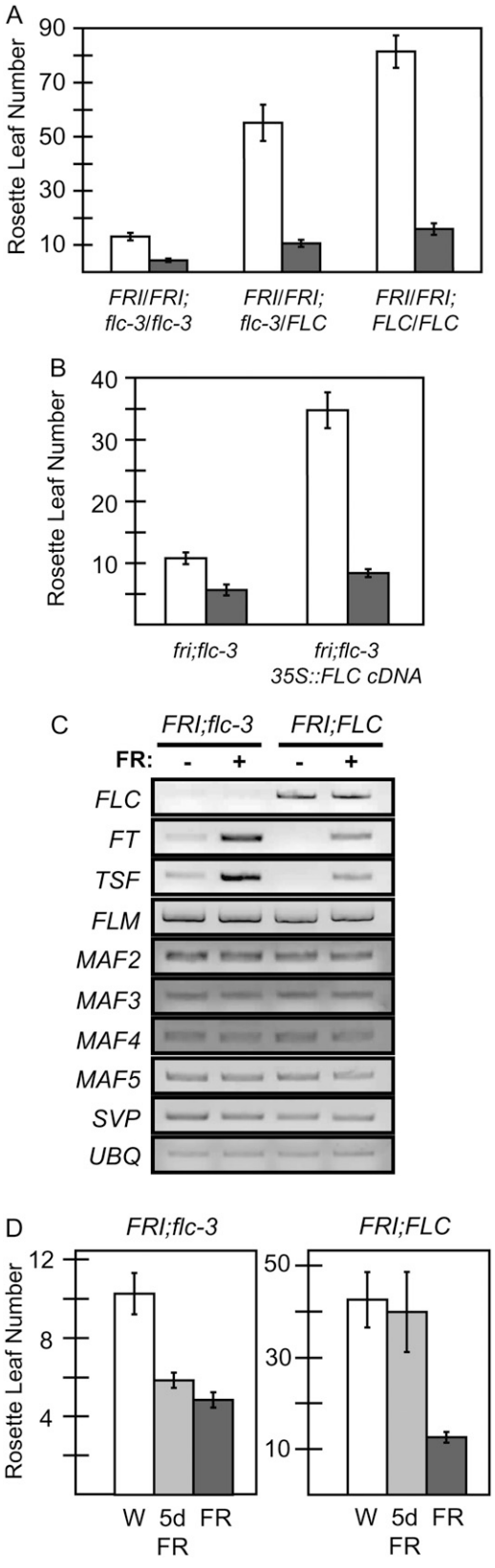

Figure 1. Prolonged exposure to a low R:FR ratio bypasses *FLC*-mediated late flowering without lowering *FLC* expression, but *FLC* blocks acceleration of flowering in response to a transient far-red exposure. A and B, Flowering time in high R:FR conditions (white bars; R:FR approximately 5) and low R:FR conditions (gray bars; R:FR approximately 0.15). Plants were grown in LD cycles (16 h of light/8 h of dark). Error bars represent SD. C, Transcript levels in 7-d-old seedlings assayed by semiquantitative RT-PCR. Seeds were germinated in continuous white light for 5 d and then either left in these high R:FR conditions for 2 d (R:FR approximately 6) or exposed to far-red-enriched light for 2 d (R:FR approximately 0.04) prior to tissue collection. *UBQ*, *UBIQUITIN* loading control. D, Flowering time under high R:FR conditions (white bars, W), low R:FR conditions (dark gray bars, FR), or conditions of 5 d of low R:FR exposure followed by a return to high R:FR exposure (light gray bars, 5 d FR). Light sources were as in A and B.

plants could occur via the repression of *FLC* during far-red exposure. We first used a genetic approach to explore this possibility. If down-regulation of *FLC* was sufficient for the rapid-flowering response, then *flc* mutants would show no additional acceleration of flowering in response to far-red enrichment. This was not the case (Fig. 1B), indicating that a low R:FR ratio does more than simply remove the FLC block on flowering. If down-regulation of *FLC* transcription was a necessary step in far-red-mediated flowering, then a line expressing the *FLC* cDNA from the constitutively expressed *35S* promoter would be insensitive to a low R:FR ratio. However, such a line retained its sensitivity to far-red enrichment (Fig. 1B), demonstrating that, unlike vernalization, a low R:FR ratio does not require down-regulation of *FLC* expression to promote rapid flowering. Consistent with the above results, no decrease in *FLC* mRNA levels was detected after 2 d of far-red enrichment, although this exposure was sufficient to induce an increase in *FT* and *TSF* levels (Fig. 1C).

FLC belongs to a clade of MADS box genes that repress the floral transition; like *FLC*, several members of this clade are down-regulated during vernalization (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001, 2003). We examined mRNA levels of these *FLC* clade members and of another MADS box transcription factor that represses flowering (*SVP*) after 2 d of far-red enrichment and found that their transcript levels were unaltered by this exposure (Fig. 1C).

FLC Buffers the Flowering Response to Transient Far-Red Exposure

Under both white light and far-red-enriched conditions, the presence of *FLC* delayed flowering in a dose-dependent manner (Fig. 1A). This indicated that the repressive effects of *FLC* compete with the promotive effects of a low R:FR ratio despite being eventually bypassed. We tested whether *FLC* might completely block the far-red-mediated acceleration of flowering during a shorter far-red enrichment, as opposed to the continuous exposure shown in Figure 1A. We grew Col-*FRI* seedlings either with or without functional *FLC* for 5 d in low R:FR conditions before shifting them to high R:FR conditions and compared this transient

treatment with continuous low or high R:FR conditions. Whereas 5 d of exposure to a low R:FR ratio accelerated flowering to the same degree as continuous far-red enrichment in *flc* null seedlings, transient exposure did not accelerate flowering in *FLC*-expressing seedlings (Fig. 1D). Although the flowering behavior of Col-*FRI* in white light sometimes varied between experiments (Fig. 1, compare A and D), we found that within each experiment, a short period of far-red exposure was insufficient to bypass *FLC*-mediated repression of flowering. The ability of Col-*FRI* seedlings to withstand a period of far-red treatment without commitment to flowering may be due to insufficient *FT/TSF* accumulation in such *FLC*-expressing plants during a short far-red exposure. Alternatively, it is possible that elevated *FLC* levels result in a more efficient reduction in *FT* expression after the removal of the far-red stimulus.

phyB;D;E Suppresses the Late Flowering of Col-*FRI* in White Light

The ability of a low R:FR ratio to promote flowering despite high *FLC* expression suggested that a reduction in Pfr activity could override *FLC*-mediated repression. Such a reduction can be generated not only through far-red enrichment but also through mutational loss of *PHY* genes. We thus introgressed *phyB*, *phyD*, and *phyE* mutations into the Col-*FRI* background. The *phyB;D;E* triple mutant suppressed the late flowering of Col-*FRI* in white light to a similar degree as did our far-red enrichment (Fig. 2, A and B). As was observed for far-red exposure (Figs. 1C and 2C), the rapid flowering of Col-*FRI* caused by loss of PHYB, PHYD, and PHYE Pfr was not associated with a decrease in *FLC* mRNA levels, nor did a combination of loss of PHYB, PHYD, and PHYE and a low R:FR ratio affect *FLC* mRNA levels (Fig. 2C). Consistent with the induction of *FT* expression during far-red exposure despite high *FLC* levels (Figs. 1C and 2C), *FT* levels were elevated by the loss of PHYB, PHYD, and PHYE despite the continued presence of *FLC* transcript (Fig. 2C). This again indicates that loss of Pfr function overrides *FLC*-mediated repression to activate *FT* expression and accelerate flowering.

GI and *CO* Are Required for a Robust Rapid-Flowering Response to a Low R:FR Ratio

As presented above, a low R:FR ratio does not appear to cause rapid flowering via the same pathway as does vernalization; rather, it overrides the block on flowering created by high FLC levels. One way to override *FLC* repression would be to strengthen the photoperiod pathway, because *FLC* and the photoperiod pathway antagonistically regulate the same downstream targets (Hepworth et al., 2002; Helliwell et al., 2006; Searle et al., 2006).

We examined the responses of the photoperiod mutants *gi-2* and *co-9* to a low R:FR ratio. With respect to vegetative shade-avoidance phenotypes such as petiole elongation (Fig. 3A) and hyponasty (Fig. 3B),

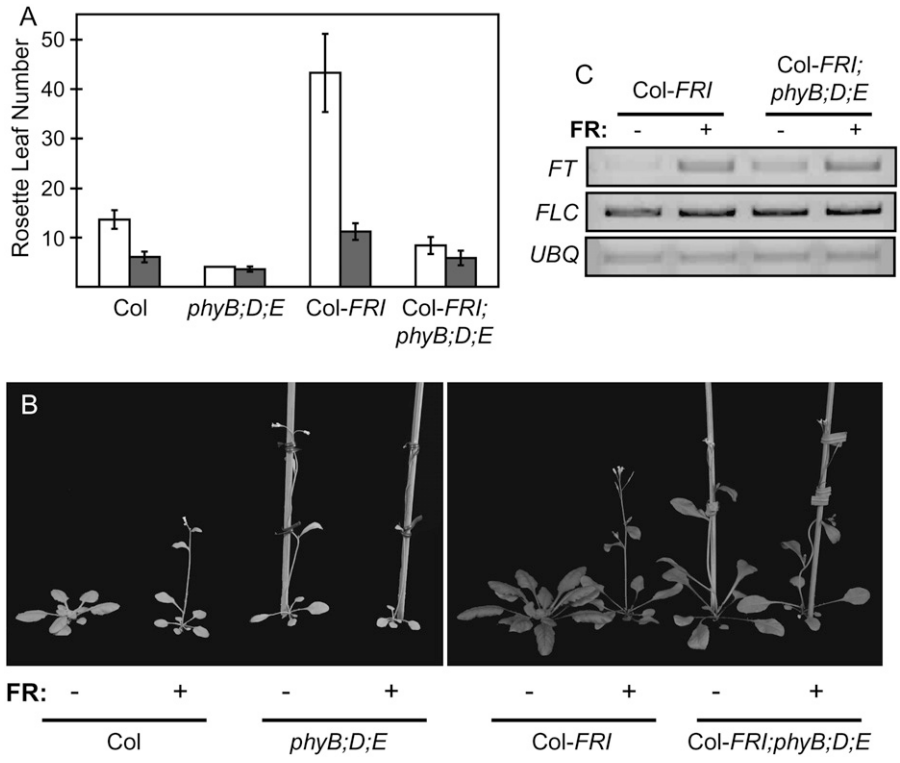

Figure 2. The triple mutant *phyB;D;E* suppresses the late flowering of Col-*FRI* without lowering *FLC* expression. A, Flowering time in high R:FR conditions (white bars; R:FR approximately 5) and low R:FR conditions (gray bars; R:FR approximately 0.15). Plants were grown in LD cycles (16 h of light/8 h of dark). Error bars represent SD. B, Representative plants from A. All four plants in each panel were photographed at the same time. C, Transcript levels in 9-d-old seedlings assayed by semiquantitative RT-PCR. Seeds were germinated in continuous white light for 5 d and then either left in these high R:FR conditions for 4 d (R:FR approximately 6) or exposed to far-red-enriched light for 4 d (R:FR approximately 0.04) prior to tissue collection. *UBQ*, *UBIQUITIN* loading control.

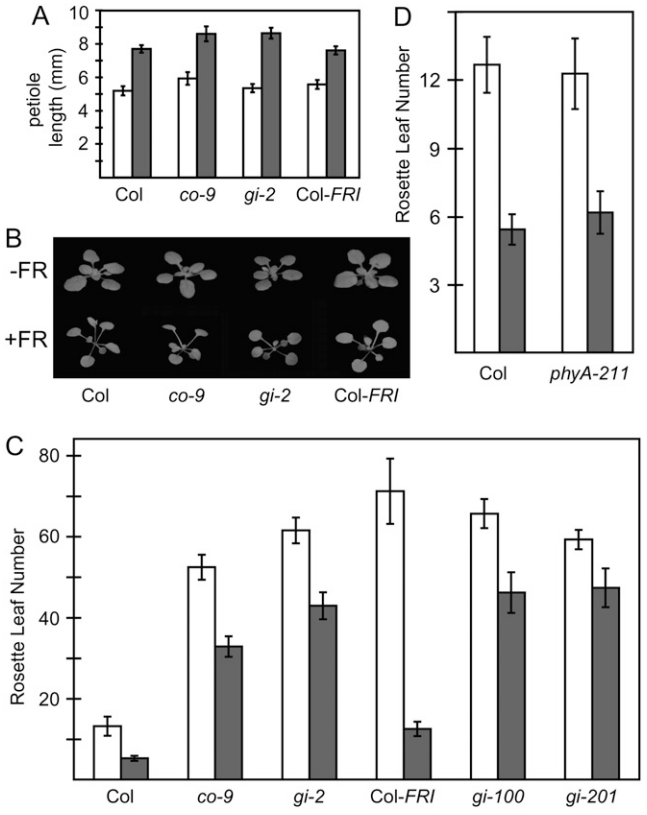

Figure 3. *co-9* and *gi-2* mutants have an attenuated flowering response to a low R:FR ratio. A, Average petiole length of the first four true leaves of 8 to 12 soil-grown plants. Seeds were germinated in continuous white light for 3 d (R:FR approximately 6), transplanted to a LD chamber (16 h of light/8 h of dark), and then either maintained in white light (white bars; R:FR approximately 5) or exposed to far-red enriched light (gray bars; R:FR approximately 0.15) for 12 d prior to petiole measurement. Error bars represent SE. B, Representative plants from A, photographed on the day of petiole measurement. C and D, Flowering time in high R:FR conditions (white bars; R:FR approximately 5) and low R:FR conditions (gray bars; R:FR approximately 0.15). Plants were grown in LD cycles (16 h of light/8 h of dark). Error bars represent SD.

the two mutants resembled Col and Col-*FRI*. However, both mutants displayed an attenuated ability to accelerate flowering in response to low R:FR exposure (Fig. 3C). This attenuation was most striking in a comparison with Col-*FRI*: whereas *gi-2* and *co-9* flowered earlier than Col-*FRI* in white light, they flowered later than Col-*FRI* in far-red-enriched light. This reversal in relative lateness indicates that the attenuated responses of the photoperiod mutants to far-red enrichment were not simply a by-product of their late-flowering phenotypes in white light but rather a demonstration of the importance of photoperiod pathway signaling in far-red-mediated acceleration of flowering. Because several *gi* mutants have allele-specific phenotypes (Park et al., 1999; Huq et al., 2000), we evaluated the responses of two additional mutants, *gi-100* and *gi-201*, and found that they also displayed an attenuated far-red flowering response (Fig. 3C).

Light-induced degradation of PHYA Pfr results in very low levels of PHYA protein in white light-grown plants (Kircher et al., 2002; Sharrock and Clack, 2002), and *phyA* mutants in Arabidopsis do not have adult phenotypes in continuous white light (Whitelam et al., 1993). Nevertheless, because PHYA may play a greater role during far-red-enriched light/dark cycles and is needed for photoperiod-mediated acceleration of flowering under certain conditions (Johnson et al., 1994; Yanovsky and Kay, 2002; Mockler et al., 2003; Valverde et al., 2004) and in certain species (Weller et al., 1997), we felt it important to assess whether PHYA was required for the CO-mediated rapid-flowering response to the low R:FR ratio in our chambers. The response of *phyA* was similar to that of the wild type (Fig. 3D), consistent with earlier genetic results showing that *PHYA* is not required for the early flowering of *phyB* mutants (Reed et al., 1994; Devlin et al., 1996). We note that the far-red-rich light sources under which *phyA* mutants are late flowering (e.g. monochromatic far-red, incandescent, or red plus far-red radiation) are also relatively poor in blue light; under such conditions, PHYA may indeed play a larger role in the acceleration of flowering.

GI, *CO*, and *FT* mRNA Accumulation Patterns Are Altered under Low R:FR Conditions

The attenuated flowering responses of *gi-2* and *co-9* mutants grown under low R:FR conditions (Fig. 3C) indicated that a low R:FR ratio accelerates flowering in part by enhancing the ability of GI and CO to promote flowering. CO protein levels are elevated in the *phyB* mutant, but little is known about the effects of a low R:FR ratio on the well-documented rhythms of *GI*, *CO*, and *FT* expression. Accordingly, we used quantitative reverse transcription (RT)-PCR to measure mRNA abundance in Col seedlings over a diurnal, LD time course both with and without far-red enrichment.

We first examined *FT* abundance over a 5-d time course (Fig. 4A). Seedlings were exposed to either 5 d of white light or 3 d of far-red-enriched light followed by 2 d of white light (Fig. 4A). *FT* expression retained a daily rhythm during far-red treatment with a peak at the end of the day, but the extent of the evening induction was much greater in low R:FR conditions. This increase was lost by the first evening after the shift back to white light.

In addition to the evening peak in *FT* abundance, we detected a morning peak that appeared on the 2nd d of far-red exposure and disappeared gradually upon return to white light. This disappearance was caused by the change in light quality: when seedlings were kept in far-red conditions through the 5th d, the morning peak in *FT* abundance was maintained (Fig. 4B). Figure 4B also demonstrates that *FT* levels continued to rise over the 5-d period: both the morning and the evening peaks were approximately 2- to 5-fold higher on the 5th d than on the 2nd d of far-red exposure. Trough *FT* levels also increased, so that by

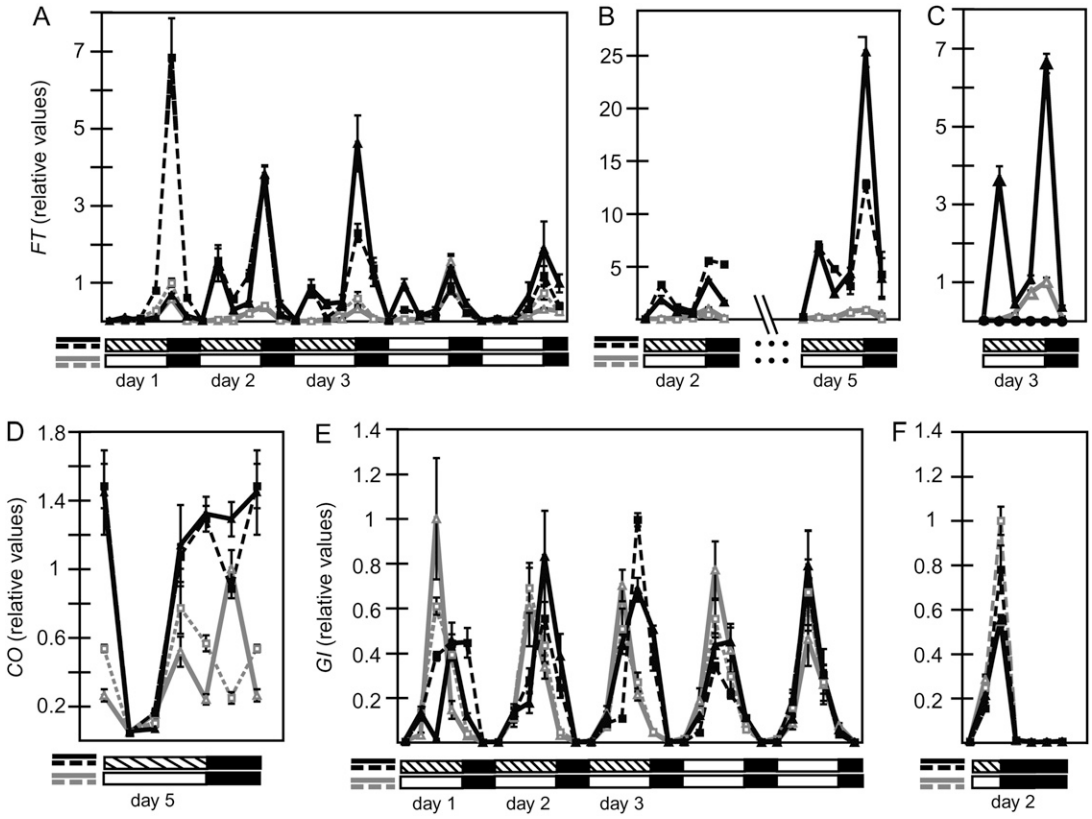

Figure 4. Expression patterns of *GI*, *CO*, and *FT* are altered during far-red exposure. Relative transcript levels in seedlings were assayed by quantitative RT-PCR. Wild-type Col seeds were germinated in white light for 5 d (A–E; 3 d of continuous light plus 2 d of LD cycles of 16 h of light/8 h of dark) or 6 d (F; 6 d of SD cycles of 8 h of light/16 h of dark) and then either left in high R:FR cycles (bottom of schematics, gray lines; R:FR approximately 5) or shifted to low R:FR cycles (top of schematics, black lines; R:FR approximately 0.15). Tissue was collected at 4-h intervals starting at dawn on the relevant day of far-red exposure. cDNA from each time point was used to amplify both *UBIQUITIN* (as a loading control) and the gene of interest, and the amount of the latter was calculated relative to the former. All values were then normalized so that peak expression of the gene of interest on the 1st d of white light conditions was equal to 1. Dotted versus solid lines represent independent biological replicates. Error bars show SE for three technical replicates. A, Time course of *FT* mRNA abundance. See text and schematic for details of light regime. B, Time course of *FT* mRNA abundance on the 2nd and 5th d of far-red exposure. C, Time course of *FT* mRNA abundance on the 3rd d of far-red exposure in Col seedlings (triangles) versus *co-9* seedlings (circles). D, As in B (day 5), but for *CO* transcript levels. The dawn time point is repeated at the end of the time course for comparative purposes. E, As in A, but for *GI* transcript levels. F, Time course of *GI* mRNA abundance on the 2nd d of far-red exposure during SD conditions. An additional time point taken 6 h after dawn is not shown for the sake of clarity, but it also showed no difference between low and high R:FR conditions.

the 5th d they were three to five times higher than peak *FT* levels in white light. Neither the morning peak nor the evening peak was present in *co-9* mutant seedlings by the 3rd d of far-red exposure (Fig. 4C, compare circles [*co-9*] with triangles [Col]), indicating that *FT* induction in response to a low R:FR ratio requires *CO* and does not occur via a recently proposed *CO*-independent microRNA pathway (Jung et al., 2007).

We examined *CO* mRNA abundance on the 5th d of far-red treatment (Fig. 4D), a length of exposure that we have shown saturates the flowering response of seedlings with low *FLC* expression (Fig. 1D). Consistent with previous reports (Suarez-Lopez et al., 2001), *CO* levels in white light were highest at dawn, at dusk, and during the night. A far-red-mediated increase in *CO* transcript was especially apparent at dawn but was also present at dusk and during the dark period. Unlike trough *FT* levels, trough *CO* levels remained low.

Over the time course first described in Figure 4A for *FT* expression, peak *GI* levels showed no increase in response to far-red enrichment; instead, peak expression occurred 4 h later in the day (Fig. 4E). This striking delay was apparent on the 1st d of far-red exposure: by 8 h after dawn, *GI* levels had peaked in white-light-grown seedlings but were still fairly low in far-red-exposed seedlings; by 12 h after dawn, *GI* levels had dropped in white-light-grown plants but had continued to increase in far-red-exposed plants. When far-red-treated seedlings were shifted back to white light conditions on the 4th d of the time course, the phase of *GI* expression was rapidly adjusted, so

that there was a decrease rather than an increase in *GI* mRNA abundance between 8 and 12 h after dawn. Seedlings that were exposed to far-red enrichment in short days (8 h of light) showed no change in *GI* expression even on the 2nd d of far-red treatment (Fig. 4F), indicating that the distinctive far-red-mediated shift in peak *GI* expression during long days was photoperiod dependent.

A Low R:FR Ratio Does Not Accelerate Flowering in Short Days

Having shown that components of the photoperiod pathway are required for a robust rapid-flowering response to far-red light (Fig. 3) and exhibit altered expression patterns under far-red-enriched conditions (Fig. 4), we wished to investigate the effect of a low R:FR ratio under conditions where the photoperiod pathway is less active: a short day of 8 h of light. Previous short-day (SD) studies have investigated the response to a monochromatic far-red pulse given at dusk, referred to as an end-of-day far-red treatment; such twilight enrichment for far-red wavelengths is thought to be a seasonal cue at high latitudes. In contrast, we examined the response to far-red enrichment provided throughout the light period, a continuous treatment that simulates vegetative shade. A low R:FR ratio that elicited a maximal response in LD conditions (Fig. 3C) did not accelerate flowering during short days in Col, Wassilewskija, or Landsberg *erecta* (L*er*) wild types (Fig. 5). The *phyB;D;E* mutant in Col (no functional *FRI* allele, low *FLC* expression) did flower early in short days, as was reported previously in L*er* (Franklin et al., 2003). However, the presence of an active *FRI* allele up-regulating *FLC* expression severely attenuated the rapid flowering of *phyB;D;E* in these noninductive SD conditions (Fig. 5); this is in contrast to the inability of *FRI*-mediated *FLC* expression to block rapid flowering of *phyB;D;E* in LD conditions (Fig. 2).

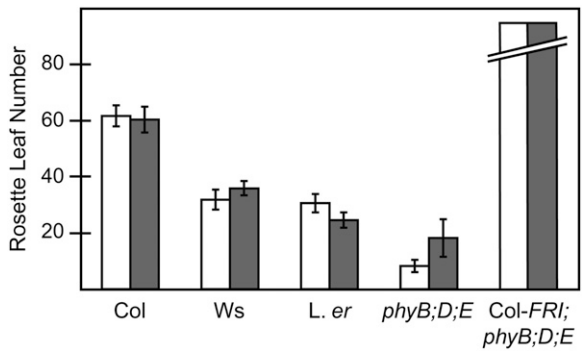

Figure 5. A low R:FR ratio that is maximally effective in long days does not accelerate flowering in SD conditions. Flowering time in high R:FR conditions (white bars; R:FR approximately 5) and low R:FR conditions (gray bars; R:FR approximately 0.15). Plants were grown in SD cycles (8 h of light/16 h of dark). Error bars represent SD. Ws, Wassilewskija.

Surprisingly, far-red enrichment during SD conditions delayed flowering of the *phyB;D;E* triple mutant (Fig. 5). This effect was background specific, occurring in the Col *phyB;D;E* triple mutant but not in the L*er* *phyB;D;E* triple mutant (A.C. Wollenberg and R.M. Amasino, unpublished data). Although we do not yet have a molecular explanation for this result, it indicates that a low R:FR ratio under SD photoperiods activates a floral repressor that is more active in Col than in L*er*.

PFT1 Is Not Required for Acceleration of Flowering in Response to Either a Low R:FR Ratio in Long Days or a Loss of PHYB, PHYD, and PHYE Pfr in the Triple Mutant

Two conditions often used to simulate shade are short days with end-of-day far-red exposure and the *phyB* mutant background. *pft1* suppresses rapid flowering in both, indicating that PFT1 may promote flowering in response to shade (Cerdan and Chory, 2003). However, the flowering response of *pft1* to continuous far-red enrichment in long days, a condition that more closely resembles vegetative shade, has not been reported. We found that the flowering time of *pft1* grown in far-red-enriched long days was indistinguishable from that of the wild type (Fig. 6A), indicating that PFT1 was not required for the acceleration of flowering under these conditions. In addition, although *pft1* suppressed the rapid flowering of *phyB* in white light (Cerdan and Chory, 2003; Fig. 6), it only weakly suppressed the rapid flowering of *phyB;D;E* in white light (Fig. 6). These data suggest that *PFT1* may negatively regulate Pfr-signaling pathways, rather than promote flowering in response to the attenuation of these pathways that occurs during shade (see Discussion for details).

DISCUSSION

A low R:FR ratio, which is indicative of shady or crowded conditions, promotes flowering in Arabidopsis (for review, see Franklin and Whitelam, 2005). We set out to characterize the relationship between far-red-mediated flowering and the pathways that promote flowering in response to vernalization or inductive photoperiods.

Our initial focus was to explore the pathway by which a low R:FR ratio promotes flowering in plants in which *FLC* levels are elevated by the presence of *FRI*. Such *FLC*-expressing plants are likely to display a winter-annual growth habit in many environments, with high *FLC* levels suppressing flowering prior to vernalization. These lines represent the ancestral genetic state in Arabidopsis; many of the constitutively rapid-flowering accessions were derived via mutations in *FRI* and/or *FLC* (Johanson et al., 2000; Le Corre et al., 2002; Gazzani et al., 2003; Michaels et al., 2003; for review, see Roux et al., 2006). In accessions with high *FLC* expression, vernalization removes this

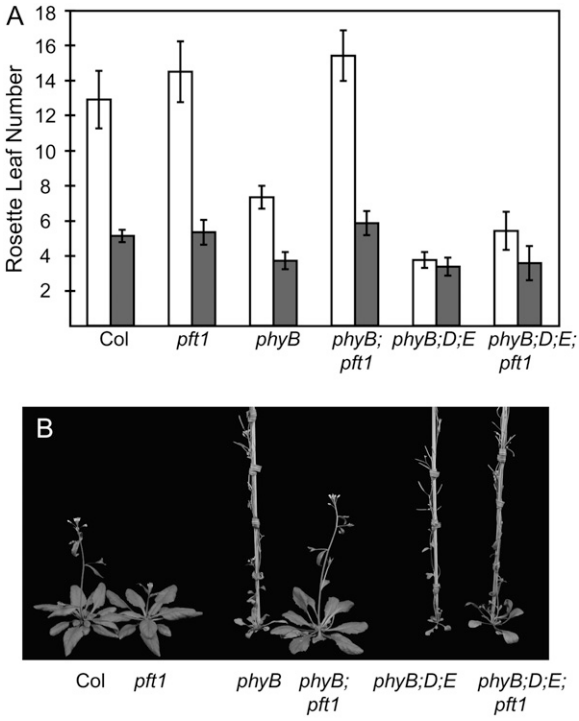

Figure 6. *pft1* suppresses the rapid flowering of *phyB* but only weakly suppresses the rapid flowering of *phyB;D;E* and does not inhibit rapid flowering in response to a low R:FR ratio. A, Flowering time in high R:FR conditions (white bars; R:FR approximately 5) and low R:FR conditions (gray bars; R:FR approximately 0.15). Plants were grown in LD cycles (16 h of light/8 h of dark). Error bars represent SD. B, Representative plants from white light-grown plants in A. All plants were photographed on the same day.

block on flowering through transcriptional repression of *FLC*. Just as they respond to vernalization, *FLC*-expressing plants flower rapidly in response to far-red enrichment (Martinez-Zapater and Somerville, 1990; Bagnall, 1992, 1993; Lee and Amasino, 1995; our results). However, we found that, unlike vernalization, a low R:FR ratio did not lower the expression of *FLC* or *FLC* clade members in a winter-annual type. Consistent with this result, a line expressing the *FLC* cDNA from a constitutive promoter showed strong acceleration of flowering under low R:FR conditions. It appears that the correlation between responsiveness to vernalization and responsiveness to far-red enrichment, while indicating that both cues can negate the repressive effects of *FLC*, does not reflect a common mechanism for floral promotion: vernalization removes *FLC* via transcriptional repression, whereas a low R:FR ratio bypasses *FLC* despite continued expression (see model below).

The ability of far-red enrichment to bypass *FLC*-mediated late flowering seems to result largely from the removal of the Pfr form of PHYB and its light-stable relatives, as the *phyB;D;E* triple mutant suppressed the late flowering of Col-*FRI* in white light to a similar extent as did our far-red enrichment in long days. As was the case with far-red enrichment, acceleration of flowering due to the loss of PHYB, PHYD, and PHYE was not associated with a decrease in *FLC* mRNA levels. A large number of loss-of-function mutants that suppress *FLC*-mediated late flowering in winter-annual types have been identified (for review, see Dennis and Peacock, 2007; Schmitz and Amasino, 2007); however, the majority of such suppressor mutants accelerate flowering by interfering with *FLC* expression. The combined loss of *PHYB*, *PHYD*, and *PHYE* thus exemplifies a new class of loss-of-function mutants in which the repressive effects of *FLC* are bypassed without a decrease in *FLC* expression.

Having established that a low R:FR ratio accelerates flowering in spite of high *FLC* levels, we turned our attention to the role of the photoperiod pathway in shade-mediated floral promotion. In our conditions, *GI* and *CO* were necessary for a robust rapid-flowering response to far-red enrichment, as indicated by the attenuated flowering responses of *gi-2* and *co-9*. These data are in good agreement with previous reports noting the relative lateness of *gi* and *co* mutants grown under incandescent light (Martinez-Zapater and Somerville, 1990; Bagnall, 1992, 1993) and with a concurrent report demonstrating late flowering of *gi* and *co* mutants grown under far-red-enriched white light (R:FR approximately 0.4; Kim et al., 2008). The latter publication has also shown genetically that floral induction in response to this far-red enrichment proceeds through the floral integrators *FT*, *TSF*, and *SOC1*. Consistent with the observation that acceleration of flowering by a low R:FR ratio requires an intact photoperiod signaling pathway, both our work and that of Kim et al. (2008) have shown that far-red enrichment does not accelerate flowering under noninductive SD photoperiods.

Although photoperiod signaling was necessary for the acceleration of flowering by far-red enrichment in this study, neither LD conditions nor CO is required for rapid flowering of Col and L*er* mutants that lack functional PHYB (Goto et al., 1991; Reed et al., 1993; Halliday et al., 1994; Devlin et al., 1996; Franklin et al., 2003; our result with *phyB;D;E* in short days). We speculate that this differing requirement for photoperiod signaling may result from differences in Pfr reduction: whereas Pfr forms are eliminated in *phy* mutants, they are only reduced during far-red enrichment (Smith, 1982). The total absence of Pfr in *phy* mutants may result in a stronger stimulation of flowering that bypasses the need for LD/CO function. Photoperiod-independent promotion of flowering in response to very low Pfr levels, or to other features of the light environment, may also explain why *co* mutants have been observed to be insensitive (Kim et al., 2008), partially insensitive (our results), or quite sensitive (Halliday et al., 1994) to a low R:FR ratio with respect to flowering time. It is interesting that introduction of an active *FRI* allele and subsequent elevation of *FLC* levels, which only mildly delayed the rapid flowering of the *phyB;D;E* triple mutant in long days, severely delayed the rapid flowering of this mutant in

short days. Thus, inductive photoperiods, while dispensable for the early flowering of *phy* mutants in a low-*FLC* rapid-cycling background, are necessary for such early flowering in a winter-annual type.

Recent models for environmental regulation of flowering time have incorporated a photoperiod-independent pathway, acting through *PFT1*, that promotes flowering in response to changes in light quality (Cerdan and Chory, 2003; Lee et al., 2006; Mathews, 2006; Backstrom et al., 2007; Kebrom and Brutnell, 2007). However, our data indicate that PFT1 is not required for rapid flowering in response to continuous far-red enrichment (mimicking canopy shade/crowding) or in response to the combined loss of *PHYB*, *PHYD*, and *PHYE* Pfr in the triple mutant. An alternative model consistent with our data is that PFT1 negatively regulates Pfr signaling pathway(s) (Fig. 7). According to this model, the ability of *pft1* to suppress the rapid flowering of *phyB* is due to stronger floral inhibition by PHYD and PHYE Pfr in the absence of PFT1. *pft1* in the *phyB;D;E* background or grown under far-red-enriched conditions is not late flowering, because lower collective levels of PHYB, PHYD, and PHYE Pfr exist in these situations, so there is minimal Pfr repression for the loss of *PFT1* to enhance. A prediction of this model might be that the *pft1* mutant would flower later than the wild type in white light, due to derepression of Pfr signaling. Indeed, *pft1* is slightly late flowering in white light (Cerdan and Chory, 2003; Fig. 6). The mildness of this delay may be due to nearly saturated Pfr repression in the wild type in white light; such saturation is indicated by the observation that overexpression of PHYB from the native promoter, while generating a 2-fold increase in protein levels, only mildly delays flowering in white light (Wester et al., 1994; Bagnall et al., 1995).

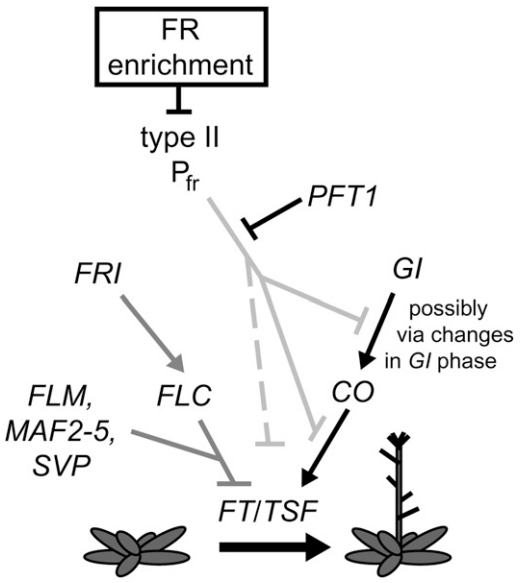

Figure 7. Model for the acceleration of flowering in response to the removal of PHY Pfr during far-red enrichment. Reduction in Pfr levels relieves repression of the photoperiod pathway at several stages (light gray lines). Regulation of *CO* transcription might involve modification of the phase of *GI* expression. Removal of Pfr may also release a photoperiod-independent floral promotion pathway (dashed line) under certain conditions (e.g. total loss of PHYB Pfr). *PFT1* negatively regulates Pfr signaling pathways, so loss of *PFT1* in the *pft1* mutant leads to more effective floral repression and later flowering only when there is at least some Pfr present (e.g. in the *phyB* mutant, which still has high PHYD and PHYE Pfr levels). Derepression of the photoperiod pathway (black lines) during far-red exposure shifts the balance toward floral promotion even in the presence of an active *FRI* allele and elevated *FLC* levels; *FLC*-mediated repression of flowering (dark gray lines) buffers the acceleration but is eventually bypassed.

Our results indicate that the photoperiod response, rather than down-regulation of *FLC* or signaling through *PFT1*, is important in generating the rapid-flowering response of the SAS. We thus examined the molecular impact of far-red enrichment on the expression of key components of the photoperiod pathway. By examining gene expression over several days of far-red treatment, we demonstrated two new features of the far-red enhancement of CO function. First, peak *CO* mRNA levels were up-regulated during far-red exposure. There was no increase at times of trough *CO* levels, which may explain the absence of increased *CO* mRNA in the *phyB* mutant at 8 h after dawn (Blazquez and Weigel, 1999). Second, we found that CO function (measured by CO-dependent *FT* induction) was not only enhanced at dusk but also derepressed at dawn. There has been one other report of a large increase in *FT* levels during the morning in a wild-type background (Corbesier et al., 2007). Intriguingly, this increase was observed after a shift from short days to long days; perhaps morning expression of *FT* is especially apparent after a sharp transition from noninductive (short days or high R:FR) to inductive (long days or low R:FR) conditions. Surprisingly, a morning peak in *FT* mRNA abundance is not observed in *phyB* mutants (Blazquez and Weigel, 1999; Cerdan and Chory, 2003) or *phyB;D;E* mutants (A.C. Wollenberg and R.M. Amasino, unpublished data) grown in white light. We are currently characterizing the window of far-red exposure necessary to generate this morning peak as well as determining its relevance to floral promotion (see discussion on gating below).

Elevated morning and evening *FT* expression was observed after a period of far-red exposure that did not generate a consistent increase in *CO* mRNA levels (A.C. Wollenberg and R.M. Amasino, unpublished data); because this increase in *FT* abundance was nevertheless dependent upon CO function (Fig. 4C), we favor a model in which enhanced CO protein stabilization occurs rapidly in response to a low R:FR ratio, whereas increased *CO* expression occurs as a response to more prolonged shade. Although the factors responsible for these far-red-mediated changes in CO regulation are not yet known, there are several candidates. The E3 ubiquitin ligase COP1 promotes the degradation of CO at dusk and during the night and may also contribute to CO degradation in the early morning

(Jang et al., 2008; Liu et al., 2008); far-red enrichment could inhibit COP1 function, leading to increased CO abundance and *FT* expression. Consistent with this possibility, *FT* levels in the white light-grown *cop1-4* mutant are elevated relative to the wild type at 4 h after dawn (Jang et al., 2008). It should be noted, however, that the *cop1-4* mutant is still able to degrade CO protein in response to red light and during certain times of the morning, indicating the existence of at least one COP1-independent mechanism for CO degradation (Jang et al., 2008) that may also be weakened by far-red enrichment.

The observed increase in *CO* transcript levels by the 5th d of far-red exposure may be related to the far-red-mediated phase delay in peak *GI* expression. A similar phase delay in *GI* expression was reported when LD-entrained seedlings were shifted into darkness for 1 d (Paltiel et al., 2006), supporting the idea that red light antagonizes an evening shift in the timing of peak *GI* expression. Consistent with this shift's having a functional role in the acceleration of flowering, it did not occur when a low R:FR ratio was provided during noninductive SD conditions. We are investigating the hypothesis that the phase delay under low R:FR conditions strengthens the interaction between GI and FKF1, enhancing FKF1-mediated degradation of the *CO* inhibitor CDF1 (Imaizumi et al., 2005; Sawa et al., 2007) and increasing *CO* transcription. Because *GI* regulates light input to the circadian clock (Huq et al., 2000; Martin-Tryon et al., 2007; Oliverio et al., 2007) and functions within the clock oscillator (Park et al., 1999; Edwards et al., 2005; Locke et al., 2005; Gould et al., 2006), changes in its phase could also alter the phase of other clock-associated factors. Not all outputs are affected (CO and *FT* phases are unchanged), but phase changes in particular rhythms in response to Pfr reduction have been reported in sorghum (*Sorghum bicolor*; Foster and Morgan, 1995) and barley (*Hordeum vulgare*; Deitzer et al., 1982), so this phenomenon may not be specific to *GI*.

The persistence of diurnal rhythms in *GI*, *CO*, and *FT* expression during far-red enrichment may indicate that a low R:FR ratio can enhance the expression of flowering-time genes only at certain times in the circadian cycle. Such gating of the SAS by the circadian clock has been reported: depending on the response being studied, far-red enrichment is most effective at dawn (Salter et al., 2003), 4 to 8 h after dawn (Franklin and Whitelam, 2007), or at dusk (Salter et al., 2003). With respect to flowering time, it was shown in barley that far-red enrichment accelerates floral initiation most effectively at dusk but accelerates the transition from initiation to floral development most effectively in the morning (Deitzer et al., 1979). In our conditions, peak *GI* levels were delayed on the 1st d of far-red exposure, implying no need for evening enrichment in generating this delay; in contrast, the morning peak in *FT* levels occurred only on the 2nd d of far-red enrichment, suggesting that evening far-red enrichment and resulting changes in CO levels/activity at dusk may be necessary for the subsequent morning expression of *FT*. Future studies will further explore whether the timing of exposure affects the influence of far-red enrichment on gene expression and flowering time.

In our model for the promotion of flowering by far-red enrichment (Fig. 7), reduction of Pfr levels under a low R:FR ratio lifts repression of the photoperiod pathway not only via stabilization of the CO protein (Valverde et al., 2004) but also through enhanced *CO* transcription (potentially via changes in the phase of *GI* expression). This strengthening of the photoperiod pathway could override *FLC*-mediated repression even if levels of active FLC protein remained high; in the absence of further data and in the interest of parsimony, we support such a model (Fig. 7), but we note that far-red-mediated inactivation of FLC protein via degradation or posttranslational modification is still a possibility.

Regardless of whether FLC protein is inactivated or simply outcompeted during far-red exposure, its repressive abilities are bypassed only when the far-red enrichment is sufficiently strong. In particular, we have demonstrated two scenarios in which a reduction in Pfr that is sufficient to accelerate flowering in the absence of *FLC* does not promote flowering in *FLC*-expressing plants. First, a short exposure to far-red enrichment, which was strong enough to elicit a maximal response in *flc* plants, had no effect on the flowering time of plants with high *FLC* levels. Second, the *phyB;D;E* triple mutant accelerated flowering in short days in the Col background (with low *FLC* expression) but could not do so in a Col-*FRI* background (with elevated *FLC* expression). It appears that the extent of rapid flowering in response to vegetative shade is determined not by the presence or absence of a single cue but rather by the cumulative effect of multiple opposing and quantitative influences, including the specific shade treatment and resulting degree of Pfr reduction, the amount of floral repression created by the particular genetic background, and the strength of other environmental cues, such as daylength. By integrating information regarding many different features of the environment, the plant may be better able to modify its rapid-flowering response to shade and thus maximize its fitness.

MATERIALS AND METHODS

Plant Material

Unless otherwise noted, all experiments were carried out in the Arabidopsis (*Arabidopsis thaliana*) Col background. Genotypes and alleles were as follows: Col-*FRI* (Lee and Amasino, 1995), *flc-3* and *FRI;flc-3* (Michaels and Amasino, 1999), *phyB-9* (EMS142 in Reed et al., 1993), *phyD-201* (SALK_027956), *phyE-201* (SALK_040131), *co-9* (Balasubramanian et al., 2006b), *gi-2* (Redei, 1962; Fowler et al., 1999), *gi-100* (Huq et al., 2000), *gi-201* (Martin-Tryon et al., 2007), *phyA-211* (Reed et al., 1994), and *pft1* (Cerdan and Chory, 2003). The *FLC* constitutive expression line was created by transformation of Col *flc* with a 35S::FLC cDNA construct in the pGREEN binary vector (BASTA resistance).

Growth Conditions

Arabidopsis seeds were plated on agar-solidified medium as described previously (Schmitz et al., 2005). Plates for RNA extraction contained a cellulose acetate membrane to prevent root penetration. Plated seeds were left at room temperature overnight, stratified at 4°C in the dark for 3 d, and then moved into the light to promote germination. For all experiments except those carried out in short days, seeds were germinated for 3 to 6 d in continuous light (General Electric F32T8-SP41 Ecolux Starcoat bulbs, R:FR approximately 6, photosynthetic photon flux density [PPFD] approximately 40–45 μmol m^{-2} s^{-1}). For experiments carried out in short days, seeds were germinated for 3 to 6 d in SD photoperiods (8 h of light/16 h of dark, Philips Lighting T12 fluorescent bulbs, R:FR approximately 5, PPFD approximately 60–70 μmol m^{-2} s^{-1}). After the germination period, seedlings were either left on plates (for expression analysis) or transplanted to soil (Sun-Gro; MetroMix360) and moved to the conditions described below. Far-red treatments were initiated the day after this shift.

Plant material for all experiments except those involving semiquantitative RT-PCR (Figs. 1C and 2C) was generated in light/dark cycling reach-in chambers (Percival Scientific I-60LX; 22°C) fitted with T12 fluorescent bulbs (Philips Lighting; R:FR approximately 5, PPFD approximately 60–70 μmol m^{-2} s^{-1}) set for long days (16 h of light/8 h of dark) or short days (8 h of light/16 h of dark). One shelf of each chamber contained arrays of far-red light-emitting diodes (Plasma Ireland; λ_{max} approximately 735–740 nm) that lowered the R:FR ratio from approximately 5 to approximately 0.15 without altering PPFD. For experiments with soil-grown plants, far-red exposure began on the day after transplanting. For semiquantitative RT-PCR, plants were kept in continuous light (see above, conditions for germination). A low R:FR ratio in these conditions was provided by an array of far-red light-emitting diodes (Plasma Ireland; λ_{max} approximately 735–740 nm) placed closer to the plants, lowering the R:FR ratio to approximately 0.04 without altering PPFD. All light measurements were made with a wideband portable spectroradiometer (International Light; RPS900-R).

RT-PCR and Quantitative RT-PCR

Approximately 20 seedlings for each genotype/treatment were frozen in liquid nitrogen. RNA extraction was as described (Abe et al., 2005). Total RNA was treated with RQ1 RNase-free DNase (Promega) according to the manufacturer's recommendations, and 2 μg of RNA was then used in a reverse transcriptase reaction (Promega; Moloney murine leukemia virus reverse transcriptase) using an oligo(T) primer. For RT-PCR, 2 μL of diluted cDNA (1:10) was used as template in 25-μL PCRs according to the manufacturer's instructions (TaKaRa; ExTaq RR001A). Primer sequences and cycle parameters are available in Supplemental Tables S1 and S2 and the accompanying text. For quantitative RT-PCR, 1.5 μL of diluted cDNA (1:8) was used as template in 20-μL PCRs according to the manufacturer's instructions (Finnzymes; DyNAmo HS SYBR Green qPCR Kit F-410) using a 384-well quantitative PCR thermocycler (Applied Biosystems; 7900-HT). Primer sequences (some as described in Mockler et al., 2004) and cycle parameters are available in Supplemental Table S3 and the accompanying text.

Petiole Length Measurements

For each genotype/treatment, 8 to 12 soil-grown plants were measured. On the 12th day after the shift to low R:FR conditions, the first four true leaves from each plant were removed, taped to paper, and scanned using a flat-bed scanner. The pixel length of each petiole was measured using ImageJ (available at http://rsb.info.nih.gov/ij/) and used to calculate length in millimeters.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Primer sequences for semiquantitative RT-PCR.

Supplemental Table S2. Gene-specific cycle parameters for semiquantitative RT-PCR.

Supplemental Table S3. Primer sequences for quantitative RT-PCR.

ACKNOWLEDGMENTS

We thank Joanne Chory, Stacy Harmer, Enamul Huq, and Jason Reed for the gift of seeds. The 35S::FLC cDNA construct was created by Yuehui He. We are grateful to Bob Gaeta, Stephanie Ellison, and Ginny Powers for reagents and helpful advice regarding the quantitative PCR experiments and to Mark Doyle for critical reading of the manuscript.

Received June 27, 2008; accepted September 2, 2008; published September 12, 2008.

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