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; Kardailisky 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 with flowering time regulation.
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 ($\lambda_{\text{max}}$ approximately 660 nm) are absorbed by the chlorophyll of surrounding plants, but far-red wavelengths ($\lambda_{\text{max}}$ 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 photoreact 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
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 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 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.
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),
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 gi-2 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
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 (Ler) 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 Ler (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).

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 Ler 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 Ler.

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...
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 Ler 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 promotor, while generating a 2-fold increase in protein levels, only mildly delays flowering in white light (Wester et al., 1994; Bagnall et al., 1995).

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.

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.
(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 LDentained 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 farred 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_04131), 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 cellular 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, photosynthetically active 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-40XL; 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; λmax 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; λmax 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; RFS900-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 RR010A). 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; DyNAamo 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.

Petal 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.

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