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Distinct Roles of *GIGANTEA* in Promoting Flowering and Regulating Circadian Rhythms in Arabidopsis

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The circadian clock acts as the timekeeping mechanism in photoperiodism. In *Arabidopsis thaliana*, a circadian clock–controlled flowering pathway comprising the genes *GIGANTEA* (*GI*), *CONSTANS* (*CO*), and *FLOWERING LOCUS T* (*FT*) promotes flowering specifically under long days. Within this pathway, *GI* regulates circadian rhythms and flowering and acts earlier in the hierarchy than *CO* and *FT*, suggesting that *GI* might regulate flowering indirectly by affecting the control of circadian rhythms. We studied the relationship between the roles of *GI* in flowering and the circadian clock using *late elongated hypocotyl circadian clock associated1* double mutants, which are impaired in circadian clock function, plants overexpressing *GI* (*35S*:*GI*), and *gi* mutants. These experiments demonstrated that *GI* acts between the circadian oscillator and *CO* to promote flowering by increasing *CO* and *FT* mRNA abundance. In addition, circadian rhythms in expression of genes that do not control flowering are altered in *35S*:*GI* and *gi* mutant plants under continuous light and continuous darkness, and the phase of expression of these genes is changed under diurnal cycles. Therefore, *GI* plays a general role in controlling circadian rhythms, and this is different from its effect on the amplitude of expression of *CO* and *FT*. Functional GI:green fluorescent protein is localized to the nucleus in transgenic Arabidopsis plants, supporting the idea that GI regulates flowering in the nucleus. We propose that the effect of GI on flowering is not an indirect effect of its role in circadian clock regulation, but rather that GI also acts in the nucleus to more directly promote the expression of flowering-time genes.

INTRODUCTION

Induction of flowering in response to daylength synchronizes flowering to the changing seasons and is believed to be important in adaptation of plants to growth at different latitudes (Ray and Alexander, 1966). Physiological experiments implicated the circadian clock as the timekeeping mechanism that enables the measurement of daylength (Samach and Coupland, 2000; Yanovsky and Kay, 2003). Forward genetics in *Arabidopsis thaliana* identified a genetic pathway that promotes flowering specifically on exposure to long days (LDs) (Searle and Coupland, 2004), and the role of the circadian clock in photoperiodic time measurement was confirmed by demonstrating that transcription of the genes that act in this pathway is circadian clock controlled. Mutations in one of these genes, *GIGANTEA* (*GI*), both impair circadian rhythms and delay flowering. Here, we use molecular-genetic approaches to compare the role of GI in the circadian system with its function in controlling flowering.

GI, *CONSTANS* (*CO*), and *FLOWERING LOCUS T* (*FT*) were placed in the Arabidopsis photoperiod pathway based on genetic analysis (Redei, 1962; Koornneef et al., 1991, 1998). Loss-of-function mutations in each of these genes delay flowering under LDs but have little or no effect under short days (SDs). Genetic epistasis and analysis of expression of these three genes in mutant and wild-type backgrounds placed them in the functional hierarchy *GI-CO-FT* (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Suarez-Lopez et al., 2001). *GI*, the earliest acting of these genes, encodes a protein of 1173 amino acids, which has no homology to proteins whose biochemical function is known (Fowler et al., 1999; Park et al., 1999). The Arabidopsis protein SPINDLY, an *O*-linked β-*N*-acetylglucosamine transferase implicated in gibberellin signaling, was shown to interact with GI in yeast, suggesting that the functions of these proteins might be related (Tseng et al., 2004). *GI* is highly conserved in seed plants, including monocotyledonous plants, such as rice (*Oryza sativa*) (Hayama et al., 2002), and gymnosperms, such as loblolly pine (*Pinus taeda*). By contrast, *GI* homologs appear to be absent from the genomes of the moss Physcomitrella, of Chlamydomonas, and of animals (Mittag et al., 2005). In onion epidermal cells, fusion proteins in which GI was fused to the marker proteins green fluorescent protein (GFP) and β-glucuronidase were localized to the nucleus (Huq et al., 2000). Furthermore, in *gi* mutants, the abundance of *CO* mRNA is reduced (Suarez-Lopez et al., 2001), suggesting that GI plays a role,

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which might be direct or indirect, in promoting *CO* transcription. However, the biochemical function of GI protein is unknown.

The second gene, *CO*, encodes a nuclear zinc finger–containing protein (Putterill et al., 1995; Samach et al., 2000; Robson et al., 2001). The phase of circadian clock controlled *CO* expression within the day/night cycle is such that *CO* mRNA is expressed when plants are exposed to light under LDs but not under SDs (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Imaizumi et al., 2003), and exposure to light is required to activate CO protein function (Valverde et al., 2004). In response to light, CO is proposed to directly activate expression of *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Valverde et al., 2004), which encodes a protein with homology to RAF-kinase inhibitor proteins of animals. FT strongly promotes flowering, but the biochemical function of this and related proteins in plants is not yet clear (Bradley et al., 1996; Pnueli et al., 1998, 2001; Kardailsky et al., 1999; Kobayashi et al., 1999). FT is the latest acting protein identified in this pathway and must somehow activate expression of genes involved in floral development at the apex of the plant (Schmid et al., 2003). Nevertheless, *CO* and *FT* are expressed in the phloem and will act there to promote flowering, suggesting that they may indirectly induce the floral transition at the apex (Takada and Goto, 2003; An et al., 2004).

CO and FT appear to be specific to flowering-time control, and mutations that impair the function of these proteins have no reported effect apart from delayed flowering. By contrast, *gi* mutants show several phenotypes. They are late flowering and exhibit reduced *CO* mRNA abundance (Suarez-Lopez et al., 2001), are altered in the period length of circadian rhythms (Park et al., 1999), impaired in phytochrome B (phyB) signaling in response to red light (Huq et al., 2000), are resistant to paraquat (Kurepa et al., 1998), and show increased accumulation of starch in the leaves during the photoperiod (Eimert et al., 1995). Whether there is a relationship between the role of GI in promoting flowering and the other processes that are impaired in the mutant is unclear. The circadian system (Somers et al., 1998b; McWatters et al., 2000; Covington et al., 2001; Mizoguchi et al., 2002), phytochrome signaling (Cerdan and Chory, 2003; Halliday et al., 2003), and sugar metabolism (Perilleux and Bernier, 2002) all have established roles in flowering-time control, suggesting that the effect of GI on flowering may be caused by its effect on one or all of these processes.

Because the circadian system has dramatic effects on flowering time, which it regulates through the *CO* and *FT* genes (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002), we studied possible connections between the role of *GI* in controlling circadian rhythms and its function in promoting flowering. *GI* is circadian clock regulated with a peak in mRNA abundance around 10 h after dawn (Fowler et al., 1999; Park et al., 1999; Hayama et al., 2002). The *gi-1* and *gi-2* mutations reduce the period length in circadian rhythms in leaf movements, and *gi-1* causes a similar effect in expression of the *CHLOROPHYLL a/b BINDING PROTEIN* (*CAB*) gene, whereas *gi-2* lengthens the period of the latter rhythm (Park et al., 1999). All *gi* alleles cause late flowering under LDs, in contrast with other mutations that cause short period rhythms, such as *timing of cab expression1-1* (*toc1-1*) or *late elongated hypocotyl-11* (*lhy-11*), which cause early flowering under SDs (Somers et al., 1998b; Mizoguchi et al., 2002). Some *gi* mutant alleles also cause a long hypocotyl phenotype in deetiolated seedlings, particularly under red light, indicating impaired phyB signaling (Huq et al., 2000), and this suggests that the basis of the circadian period phenotype is impaired input to the oscillator from phytochrome. Similarly, in *gi-1* mutants, circadian period length does not respond to increasing light intensity as sensitively as that of wild-type plants (Park et al., 1999). Furthermore, in *gi* mutants, the amplitude of expression of the *LHY* and *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) mRNAs is reduced (Fowler et al., 1999; Park et al., 1999; Mizoguchi et al., 2002). The proteins encoded by these genes are MYB-like transcription factors that were proposed to act in a negative feedback loop with TOC1, thereby forming part of the oscillator of the central clock mechanism (Wang et al., 1997; Schaffer et al., 1998; Alabadi et al., 2001; Mizoguchi et al., 2002). LHY/CCA1 are proposed to repress *TOC1* expression, and TOC1 in turn promotes *LHY/CCA1* expression (Alabadi et al., 2001). The promotion of *LHY/CCA1* expression by GI suggests that GI might also play a role in such a feedback mechanism (Mizoguchi et al., 2002).

Here, we describe a genetic and molecular analysis of *GI* function in transgenic plants overexpressing *GI* from the 35S promoter and in early flowering *lhy-11 cca1-1* plants in which *GI* is misexpressed due to impaired control of circadian and diurnal rhythms. We conclude that *GI* has at least two distinct functions: a general effect on circadian rhythms and a role as an activator of output pathways that promote flowering, including one that acts through *CO* and *FT*.

RESULTS

GI Is Required for Early Flowering of *lhy-11 cca1-1* Double Mutants

The *lhy-11 cca1-1* double mutant is impaired in circadian clock function under free-running conditions, flowers extremely early under SDs, and exhibits a shift in the phase of expression of circadian clock–regulated genes under diurnal cycles of light and dark (Mizoguchi et al., 2002). To test whether the photoperiodic flowering pathway is required for early flowering of *lhy-11 cca1-1*, mutations that impair the pathway and thereby cause late flowering were introduced into the double mutant. The flowering times of the triple mutants *gi-3 lhy-11 cca1-1*, *co-2 lhy-11 cca1-1*, and *ft-1 lhy-11 cca1-1* were scored under both LDs and SDs (Figure 1A). Under SDs, the *gi-3 lhy-11 cca1-1* triple mutant flowered with a similar number of leaves to the wild type and *gi-3* mutant controls and produced >30 leaves more than the *lhy-11 cca1-1* double mutant. By contrast, the *co-2 lhy-11 cca1-1* and *ft-1 lhy-11 cca1-1* triple mutants flowered under SDs with only ~10 leaves more than *lhy-11 cca1-1* double mutants and ~20 leaves fewer than the wild-type control (Figure 1A). The effect of *gi-3* on the early-flowering phenotype of *lhy-11 cca1-1* was also more severe than the effect of *co-2* and *ft-1* under LDs (Figure 1A).

With respect to flowering time, *gi-3* is therefore epistatic to *lhy-11 cca1-1*, indicating that the extreme early flowering of *lhy-11 cca1-1* double mutants is almost completely dependent on *GI* activity, particularly under SDs. Genes that act later in the photoperiod pathway, such as *CO* and *FT*, are required to a lesser extent for this phenotype.

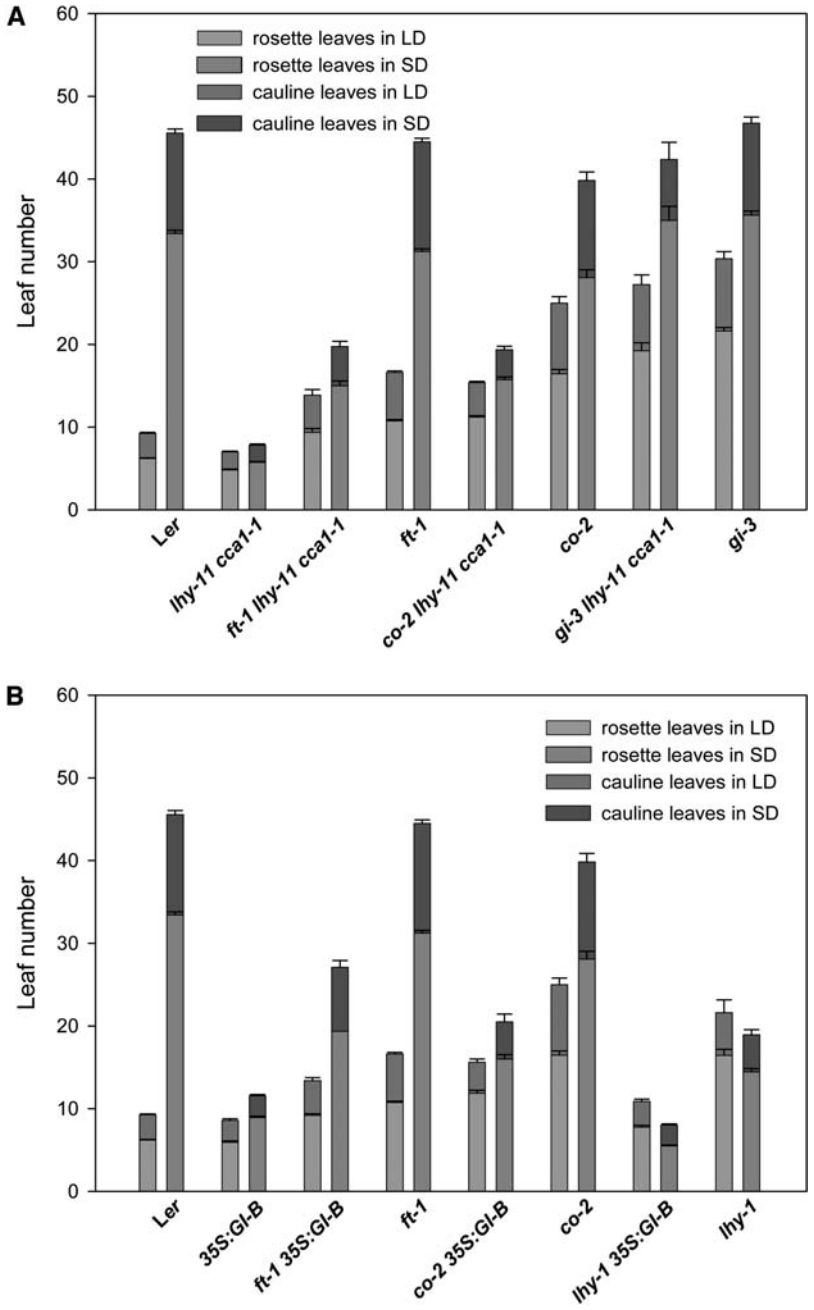

Figure 1. The Flowering Times of *lhy-11 cca1-1*, *lhy-1*, and *35S*:*GI* Plants.

The flowering time of *lhy-11 cca1-1* **(A)** or *lhy-1* and *35S*:*GI-A* **(B)** plants, with or without *gi-3*, *co-2*, and *ft-1*, was measured in LDs (left-hand column for each genotype) or in SDs (right-hand column for each genotype). Flowering time was scored by counting the number of rosette (bottom box in each column) and cauline (top box in each column) leaves on the main stem. Mean leaf number is shown ± SE. Each experiment was done at least twice with similar results.

The Expression Patterns of *GI*, *CO*, and *FT* Are Altered in the *lhy-11 cca1-1* Double Mutant

In wild-type plants grown under SDs, the *CO* mRNA only accumulates during the night, and *FT* is not expressed under these conditions. A peak in the abundance of *CO* mRNA at an earlier phase under SDs in *lhy-11 cca1-1* mutants may in part be responsible for the early flowering of these plants, as was shown for *toc1-1* mutants (Blázquez et al., 2002; Yanovsky and Kay, 2002). To test whether *CO* mRNA is expressed at an earlier phase in *lhy-11 cca1-1* double mutant plants than in the wild type, RNA was extracted at intervals of 4 h for 24 h from plants growing under SDs of 10 h light, and *CO* mRNA abundance was analyzed

by RT-PCR (Figure 2B). In wild-type plants, *CO* mRNA abundance rose 8 to 12 h after dawn and continued into the dark period with high expression also occurring 20 h after dawn. By contrast, *CO* mRNA abundance rose earlier in *lhy-11 cca1-1* plants, rising sharply between 4 and 8 h after dawn with a second peak in expression 20 h after dawn.

CO is proposed to directly activate *FT* expression in a light-dependent manner (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Valverde et al., 2004); therefore, whether the altered pattern of *CO* expression in *lhy-11 cca1-1* double mutants correlated with earlier expression of *FT* under these conditions was also tested. In the double mutant plants, the abundance of

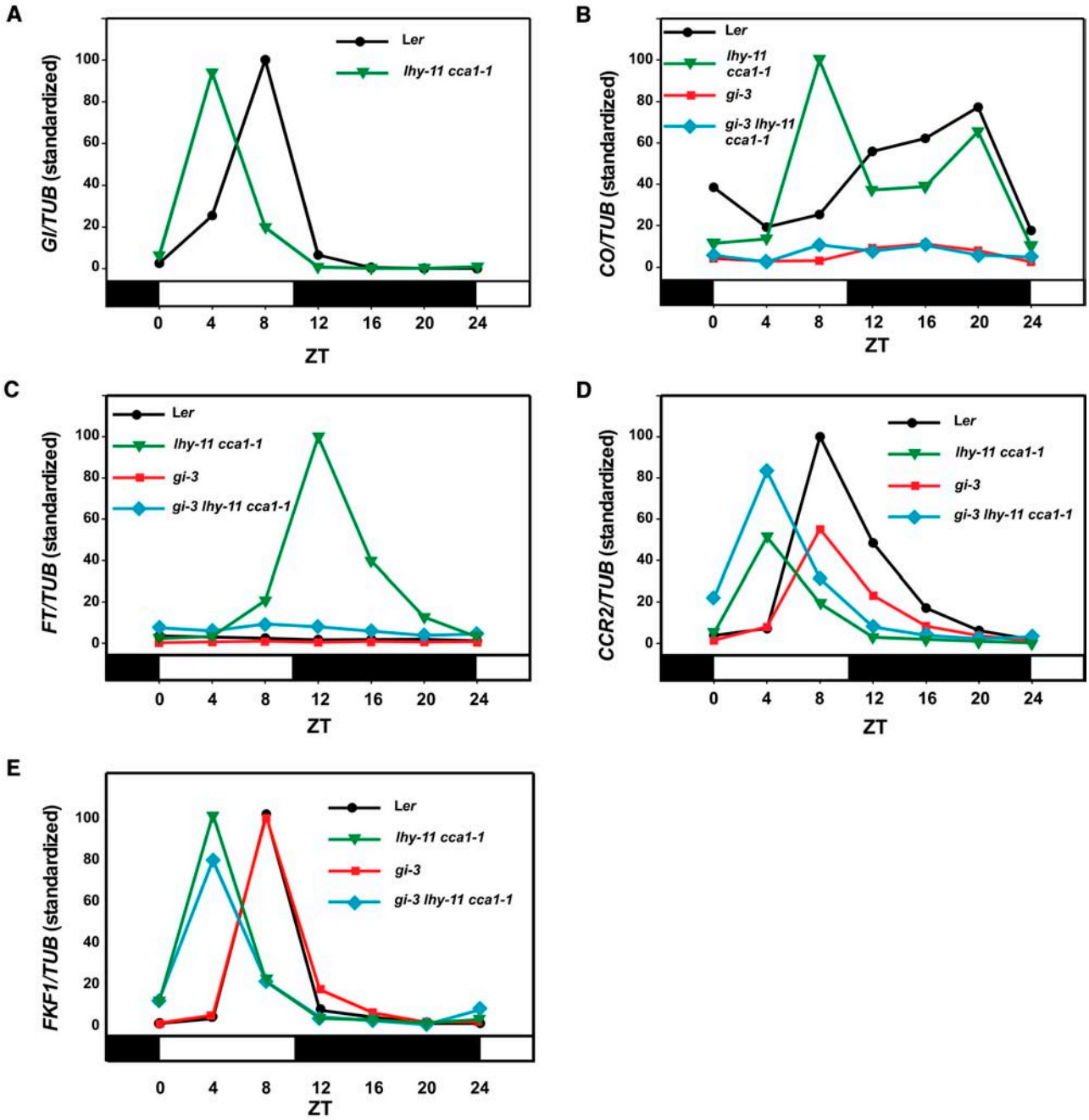

Figure 2. Abundance of the mRNAs of Flowering Time and Circadian Clock–Regulated Genes in *lhy-11 cca1-1* Plants Grown under SDs.

The expression of the *GI* **(A)**, *CO* **(B)**, *FT* **(C)**, *CCR2* **(D)**, and *FKF1* **(E)** genes was analyzed by RT-PCR in *lhy-11 cca1-1*, *gi-3 lhy-11 cca1-1*, *gi-3*, or L*er* plants grown in SDs. Results are presented as a proportion of the highest value after standardization with respect to *TUBULIN2* levels (*TUB*). Open and closed bars along the horizontal axis represent light and dark periods, respectively; these are measured in hours from dawn (zeitgeber time [ZT]). Each experiment was done at least twice with similar results.

FT mRNA rose 8 h after dawn with a strong peak at 12 h that had declined substantially by 16 h. By contrast, as expected, no expression of *FT* was detected in wild-type plants under SDs (Figure 2C). *FT* mRNA expression is therefore greatly increased in *lhy-11 cca1-1* mutants compared with wild-type plants under SDs and shows a strong peak at lights off just after the peak in *CO* mRNA (Figures 2B and 2C).

GI mRNA abundance peaks 4 h after dawn in the *lhy-11 cca1-1* double mutants grown under LDs (Mizoguchi et al., 2002) and SDs compared with 8 h after dawn in wild-type plants (Figure 2A). Therefore, in the double mutant, the *GI*, *CO*, and *FT* genes are expressed in the temporal sequence *GI-CO-FT*, as in wild-type plants. However, in *lhy-11 cca1-1* double mutants grown under SDs, the peaks of expression of *GI* and *CO* are shifted earlier, and *FT* expression occurs soon after *CO* expression at lights off.

The temporal order of expression of the *GI-CO-FT* genes and the strong suppression of the early flowering of *lhy-11 cca1-1* plants caused by *gi-3* mutations (Figure 1A) suggested that *GI* may be required for the high-amplitude, phase-shifted expression of *CO* and *FT* mRNAs in the *lhy-11 cca1-1* double mutant. Therefore, the abundance of the *CO* and *FT* mRNAs was followed in the *gi-3 lhy-11 cca1-1* triple mutant under SDs. The abundance of the *CO* and *FT* mRNAs was reduced dramatically in the triple mutant compared with *lhy-11 cca1-1* (Figures 2B and 2C), so that *gi-3* suppresses the increase in amplitude in *CO* and *FT* expression observed in *lhy-11 cca1-1*.

To test whether this reduced expression of *CO* was a general feature of circadian clock–regulated genes in the *gi-3 lhy-11 cca1-1* triple mutant, the expression of *COLD CIRCADIAN REGULATED2* (*CCR2*) was analyzed in *lhy-11 cca1-1* and *gi-3 lhy-11 cca1-1* under SDs. In wild-type plants, *CCR2* is circadian clock regulated and its mRNA accumulates 8 h after dawn under SDs (Figure 2D). The phase of expression of *CCR2* was shifted earlier in the *lhy-11 cca1-1* double mutant compared with wild-type plants, but the amplitude of expression was not reduced in the *gi-3 lhy-11 cca1-1* triple mutant (Figure 2D). Therefore, in contrast with its effect on *CO* and *FT* expression, the *gi-3* mutation did not alter the amplitude or suppress the phase shift caused by *lhy-11 cca1-1* on *CCR2*.

In addition to GI, the proposed blue light receptor FLAVIN BINDING KELCH DOMAIN F-BOX1 (FKF1), whose mRNA abundance is circadian clock regulated with a peak around 8 h after dawn under SDs (Nelson et al., 2000) (Figure 2E), promotes *CO* transcription under LDs (Imaizumi et al., 2003). *FKF1* mRNA abundance was tested in *lhy-11 cca1-1* and *gi-3 lhy-11 cca1-1* plants grown under SDs (Figure 2E). The peak in abundance of *FKF1* mRNA was shifted around 4 h earlier in *lhy-11 cca1-1* mutants compared with the wild type. However, in *gi-3* mutants and in *gi-3 lhy-11 cca1-1* plants, the pattern of *FKF1* expression was similar to that of wild-type and *lhy-11 cca1-1* plants, respectively, indicating that GI does not activate *CO* expression by promoting *FKF1* transcription and that expression of *FKF1* is not sufficient to promote *CO* transcription in the absence of GI.

These experiments are consistent with the idea that early flowering of *lhy-11 cca1-1* plants under SDs is caused by expression of *GI* at an earlier phase. GI in turn induces ectopic expression of the photoperiod pathway so that *CO* is expressed during the light phase, resulting in higher *FT* expression and early flowering.

Expression of *GI* from the *Cauliflower mosaic virus* 35S Promoter Causes Early Flowering

Analysis of *lhy-11 cca1-1* double mutants suggested that expression of *GI* early during the light phase caused early flowering under SDs by activation of expression of *CO* and *FT*. However, many circadian clock–regulated genes are expressed at an earlier phase in plants impaired in *LHY* and *CCA1* function (Alabadi et al., 2002; Mizoguchi et al., 2002). To assess whether misexpression of *GI* was sufficient to induce early flowering, a fusion of *GI* to the viral *Cauliflower mosaic virus* 35S promoter was constructed and introduced into both *gi-3* mutant and wild-type plants. In *35S*:*GI* plants, the *GI* mRNA was present throughout the daily cycle. At each time point, *GI* mRNA was more abundant in *35S*:*GI* than in wild-type plants (Figure 3A). Transgenic wild-type plants or *gi-3* mutants carrying *35S*:*GI* flowered earlier than control wild-type plants (Figure 1B; data not shown for *gi-3*). This difference was most pronounced under SDs, where *35S*:*GI* plants flowered with ~35 fewer leaves than the wild type. These data indicate that constant overexpression of *GI* is sufficient to promote early flowering, even under noninductive SD conditions.

In addition to the *lhy-11 cca1-1* double mutant, *lhy-1*, a dominant gain of function allele of *LHY*, was described as impaired in photoperiodic flowering and showed reduced *GI* expression under LDs (Schaffer et al., 1998; Fowler et al., 1999). Flowering time of the *lhy-1* mutant was measured under LDs and SDs (Figure 1B). The mutant appeared almost day-neutral, flowering later than the wild type under LDs and earlier than the wild type under SDs. *35S*:*GI* promotes early flowering in the *lhy-1* mutant (Figure 1B), consistent with *GI* playing a major role in the promotion of flowering downstream of *LHY*. *35S*:*GI lhy-1* plants were also earlier flowering than *lhy-1* under SDs and under these conditions slightly earlier flowering than *35S*:*GI*, suggesting that a part of the flowering phenotype of *lhy-1* is additive to the effect of *35S*:*GI*.

Extreme Early Flowering of *35S*:*GI* Plants Requires *CO* and *FT*

Late-flowering *gi* mutants contain lower levels of *CO* mRNA (Suarez-Lopez et al., 2001), suggesting that early flowering *35S*:*GI* plants might show increased expression of *CO* mRNA that in turn caused increased abundance of *FT* mRNA. The effect of *35S*:*GI* on the abundance of *CO* and *FT* mRNA was therefore measured in *35S*:*GI* plants grown under SDs (Figures 3B and 3C). *FT* mRNA was present at high abundance 4, 8, and 12 h after dawn, suggesting that *35S*:*GI* was sufficient to induce *FT* expression under SDs, where no *FT* expression occurs in wild-type plants. Similarly, *CO* mRNA abundance was higher in *35S*:*GI* than in wild-type plants, and this effect was most pronounced early in the day when in wild-type plants *CO* mRNA abundance is at trough levels (Figure 3B). Previously, FKF1 was proposed to activate the expression of *CO* mRNA (Imaizumi et al., 2003). However, *FKF1* mRNA levels were not affected by *35S*:*GI* (Figure 3D), indicating that GI does not regulate *CO* mRNA abundance through the regulation of *FKF1* mRNA. These data suggest that constant overexpression of *GI* from the *Cauliflower mosaic virus* 35S promoter leads to increased *CO* mRNA

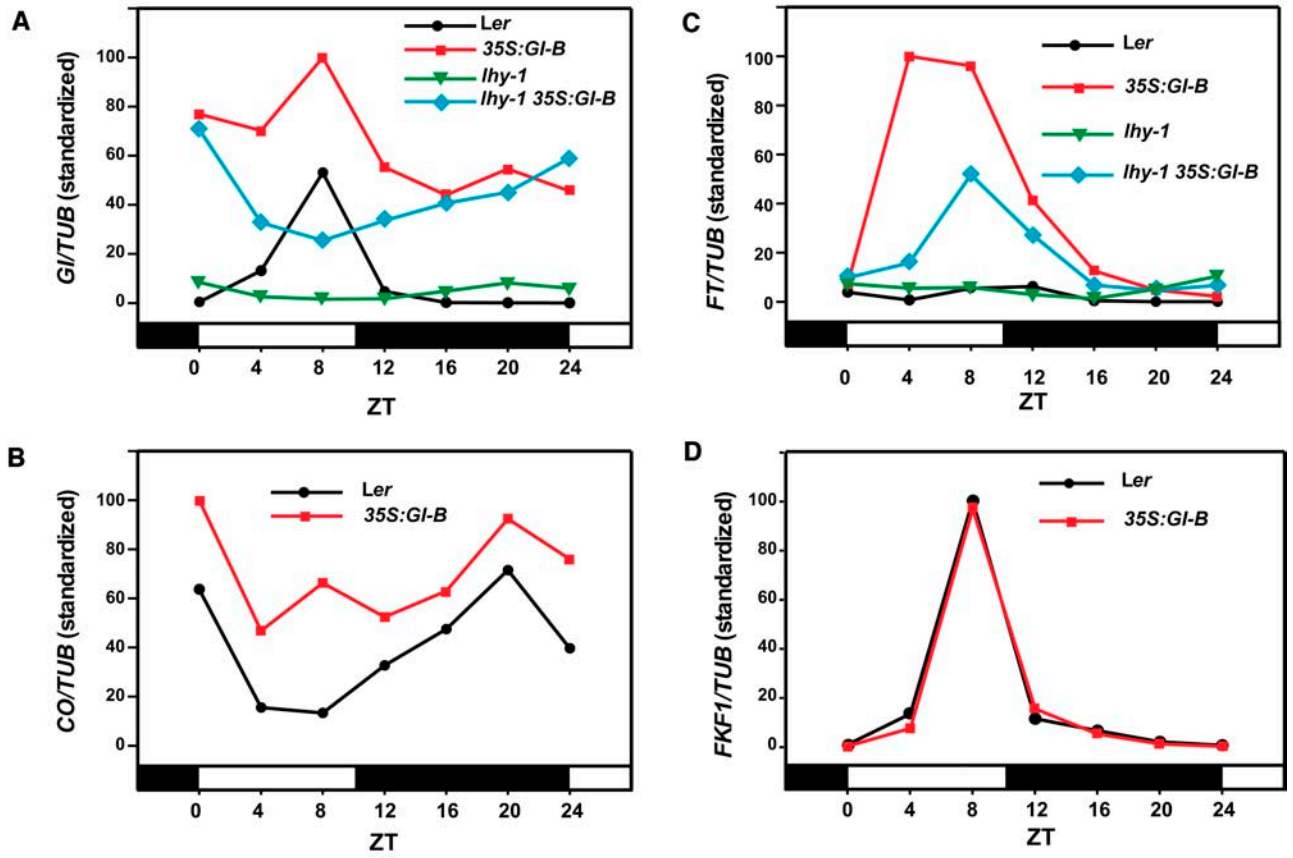

Figure 3. Abundance of the mRNAs of Flowering-Time Genes in *lhy-1* and *35S*:*GI* Plants Grown under SDs.

The expression of flowering-time gene mRNAs *GI* **(A)**, *CO* **(B)**, *FT* **(C)**, and *FKF1* **(D)** was analyzed by RT-PCR in *lhy-1*, *35S*:*GI-B*, *lhy-1 35S*:*GI-B*, and L*er* plants grown in SDs. Results are presented as a proportion of the highest value after standardization with respect to *TUB* levels. Open and closed bars along the horizontal axis represent light and dark periods, respectively. These are measured in hours from dawn (ZT). Each experiment was done at least twice with similar results.

expression during the photoperiod in SDs and that CO then activates expression of *FT*.

To test whether *CO* and *FT* are required for early flowering of *35S*:*GI* plants, mutations in these genes were introduced into the *35S*:*GI* background. The resulting lines were scored for flowering time under LDs and SDs. Under both day lengths, the *co-2 35S*:*GI* and *ft-1 35S*:*GI* plants flowered at a time intermediate between the original mutant and *35S*:*GI* (Figure 1B). Especially under SDs, the early flowering caused by *35S*:*GI* was still apparent in the mutant lines, which flowered significantly earlier than wild-type plants (Figure 1B). This suggests that the extreme early flowering of *35S*:*GI* under SDs requires functional *CO* and *FT* genes but that *GI* can also promote flowering independently of these genes.

35S:*GI* Delays the Phase of Circadian Clock–Controlled Gene Expression and Shortens Circadian Period under Continuous Light

35S:*GI* plants flower early under SDs and show increased abundance of the *CO* and *FT* mRNAs (Figures 1B, 3B, and 3C). Whether a similar effect is observed on the patterns of expression of other circadian clock–regulated genes was tested in SD-grown plants (Figures 4A and 4B). A *CCR2*:*LUCIFERASE* (*CCR2*:*LUC*) transgene was introduced into *35S*:*GI* and *gi-3* backgrounds, and five transformants in each genetic background were isolated. Luminescence of *CCR2*:*LUC* plants showed a strong diurnal rhythm, which was followed for 72 h under SDs (Figure 4B). Under these conditions, luminescence of *CCR2*:*LUC* wild-type plants reached peak levels 9.1 h after dawn, whereas luminescence of *gi-3 CCR2*:*LUC* and *35S*:*GI CCR2*:*LUC* peaked 11.6 and 12.5 h after dawn, respectively (Figure 4B). Similarly, the abundance of the *LHY* mRNA appeared to fall to trough levels more slowly in the morning and to rise in expression later in the evening, which is also consistent with a delayed phase of expression (Figure 4A). These experiments indicate that under SDs, the phase of expression of circadian clock–regulated genes is delayed by *35S*:*GI* and *gi-3*. However, these effects are much less dramatic than those observed on the amplitude of diurnal rhythms in expression of mRNAs of the flowering-time genes *CO* and *FT* (Figures 3B and 3C) and do not correlate with flowering

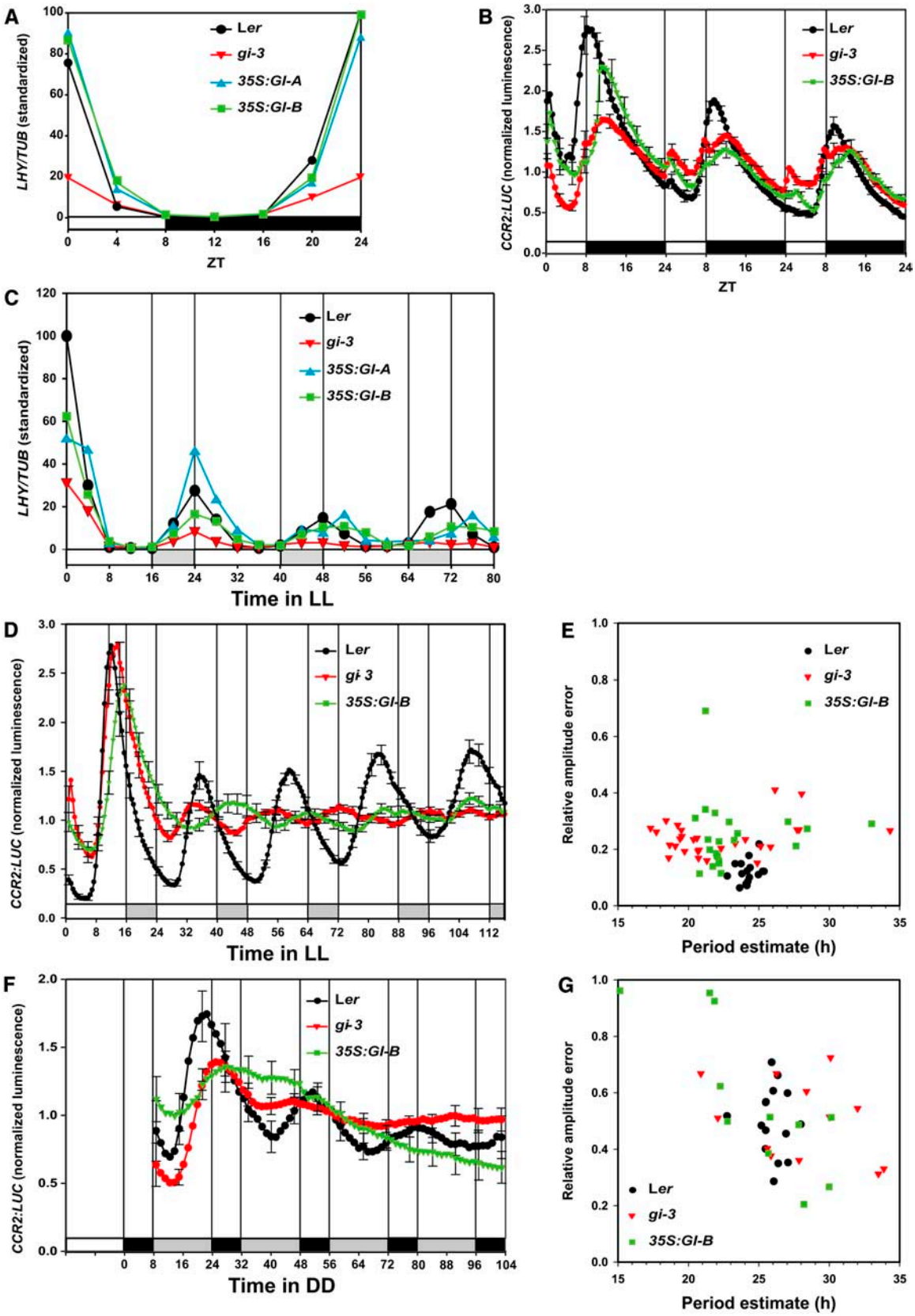

Figure 4. Circadian Clock–Regulated Gene Expression in *35S:GI* and *gi-3* Plants under SDs, LL, or DD.

time because *gi-3* and *35S*:*GI* had similar effects on phase under SDs but opposite effects on flowering time (Figures 1 and 4B).

Mutations in *GI* generally shorten free-running rhythms in gene expression under continuous light (LL), reduce the amplitude of expression of the *LHY* and *CCA1* genes, and cause late flowering (Fowler et al., 1999; Park et al., 1999; Mizoguchi et al., 2002). The early-flowering phenotype of *toc1-1* mutants can be explained by their short-period phenotype (Yanovsky and Kay, 2002). Therefore free-running rhythms in *35S*:*GI* plants were tested to assess whether alteration of these rhythms might underlie the early-flowering phenotype. *35S*:*GI*, *gi-3* mutant, and Landsberg *erecta* (L*er*) plants carrying the *CCR2*:*LUC* transgene were entrained to LD cycles of 16 h light/8 h dark and shifted to LL, and their luminescence was measured for 120 h (Figure 4D). Almost all plants of each genotype were rhythmic throughout the experiment (Figure 4E), although the rhythms dampened more rapidly in *gi-3* and *35S*:*GI* than in L*er* (Figure 4D). In *35S*:*GI*, the phase of the first peak that occurs after ZT 24 h in LL, and therefore the peak after the first subjective night, was more severely delayed compared with the wild type than under entraining conditions, indicating that the phase of the circadian rhythm in *CCR2*:*LUC* expression is delayed in *35S*:*GI*. By contrast, in *gi-3* mutants, the first peak in LL occurred earlier than that of the wild type, which is probably due to the shorter period of *gi-3* mutants under free-running conditions (see below). The period lengths of the rhythms under LL were more variable for *gi-3* and *35S*:*GI*, but mean period length was shorter for both genotypes (Figures 4D and 4E). Therefore, under LL, both *gi-3* and *35S*:*GI* mutants exhibit a shorter period length than is observed in L*er*, and *35S*:*GI* causes a significant delay in phase.

The pattern of expression of the mRNA of *LHY* was also analyzed at 4-h intervals for 80 h under LL in L*er*, *gi-3*, and *35S*:*GI* plants. The amplitude of the circadian rhythm in expression of *LHY* mRNA dampened rapidly in *gi-3* mutants as previously described (Fowler et al., 1999; Park et al., 1999; Mizoguchi et al., 2002), making the estimation of circadian period difficult (Figure 4C). However, in *35S*:*GI* plants, *LHY* mRNA showed a robust rhythm, and no increase in amplitude was observed despite the reduced amplitude observed in *gi-3*. A period length could not be extracted from these data with certainty using fast Fourier transform-nonlinear least squares (FFT-NLLS). However, visual inspection of the pattern of *LHY* mRNA abundance suggested that the effect of *35S*:*GI* on phase was similar to that observed with *CCR2*:*LUC* because, for example, at 72 to 80 h in LL, the timing of the peak in *LHY* mRNA was delayed compared with that in L*er*.

Taken together, these experiments indicate that *35S*:*GI* and *gi-3* mutations shorten circadian period and that *35S*:*GI* delays circadian phase, as measured accurately for *CCR2*:*LUC*, but that *35S*:*GI*, unlike *gi-3*, does not influence the amplitude of *LHY* expression.

Effects of *35S*:*GI* and *gi-3* on Circadian Regulation of *CCR2* in Continuous Darkness

The effect of *gi* mutations on circadian rhythms may be partly a consequence of impaired light input to the oscillator (Park et al., 1999). To follow the effect of *GI* overexpression and of impaired *GI* function on circadian rhythms in continuous dark (DD), the luminescence of *35S*:*GI*, *gi-3*, and L*er* plants carrying *CCR2*:*LUC* was compared for 4 d in DD after entrainment in LDs (Figure 4F). In contrast with wild-type plants, in *gi-3* and *35S*:*GI* plants, rhythms were of lower amplitude and the period length of the rhythms varied more widely between individuals (Figures 4F and 4G). *CCR2*:*LUC* appeared arrhythmic in both *gi-3* and *35S*:*GI* after the first 30 h in DD (Figure 4E). Therefore, in DD, *GI* is required to maintain at least a subset of circadian rhythms represented by *CCR2*:*LUC*, suggesting that the effect of GI in the circadian system is not only in light input.

The Effects of *GI* and *lhy-11 cca1-1* on Seedling Deetiolation

Mutations in *GI* were previously shown to impair phyB signaling during seedling deetiolation in red light (Huq et al., 2000), and because phyB also regulates flowering time and circadian clock entrainment, the effects of *35S*:*GI* and *lhy-11 cca1-1* on hypocotyl length were compared in a similar way as shown above for flowering time (Figure 5). To test whether the expression of *GI* is limiting for red light responsiveness, the hypocotyls of *35S*:*GI*

Figure 4. (continued).

(A) and **(C)** *LHY* and *CCR2*:*LUC* expression was analyzed in *gi-3*, *35S*:*GI-A*, *35S*:*GI-B*, or L*er* plants. The expression of the *LHY* gene was analyzed by RNA gel blotting of RNA isolated from plants grown under SDs (8 h light/16 h dark) **(A)** or LL **(C)**. Results are presented as a proportion of the highest value after standardization with respect to *TUB* levels. Numbers on the horizontal axis represent the time in hours after dawn (ZT) in SD **(A)** and after the start of the LL treatment **(C)**. Open and closed boxes on the horizontal axis indicate light and dark, respectively **(A)**, and subjective day and subjective night, respectively **(C)**.

(B), **(D)**, and **(F)** The expression of the *CCR2* gene was followed by the luminescence of transgenic plants carrying the *CCR2*:*LUC* transgene and grown under SDs (8 h light/16 h dark) **(B)**, LL **(D)**, or DD **(F)**. The results are presented as normalized luminescence. Data are the means ± SE of the luminescence of ~20 individual seedlings. Error bars are shown every fifth data point for clarity. Five independently transformed wild-type and mutant lines were analyzed under LL and DD with similar results, and under SDs two transformants were analyzed. Numbers on the horizontal axis represent the time in hours after dawn (ZT) in SD **(B)**, after the start of the LL treatment **(D)**, and in hours in darkness in DD treatment **(F)**. Open and closed boxes on the horizontal axis indicate light and dark, respectively **(B)**, subjective day and subjective night, respectively **(D)**, and light and dark boxes on horizontal axis represent subjective day and subjective night, respectively **(F)**.

(E) and **(G)** Plots showing the FFT-NLLS analysis of the *CCR2*:*LUC* data plotted in **(D)** and **(F)**, respectively. A strong circadian expression of *CCR2*:*LUC* is reflected by the clustering of data points with low relative amplitude error values, which indicate robust rhythms. Scattered data points with relative amplitude error values closer to 1 indicate weaker rhythms. All plants in **(D)** were rhythmic, whereas in **(F)**, more wild-type L*er* seedlings were rhythmic (15/18) than *gi-3* (12/19) and *35S*:*GI-B* (11/20) seedlings.

Each experiment was done at least twice with similar results.

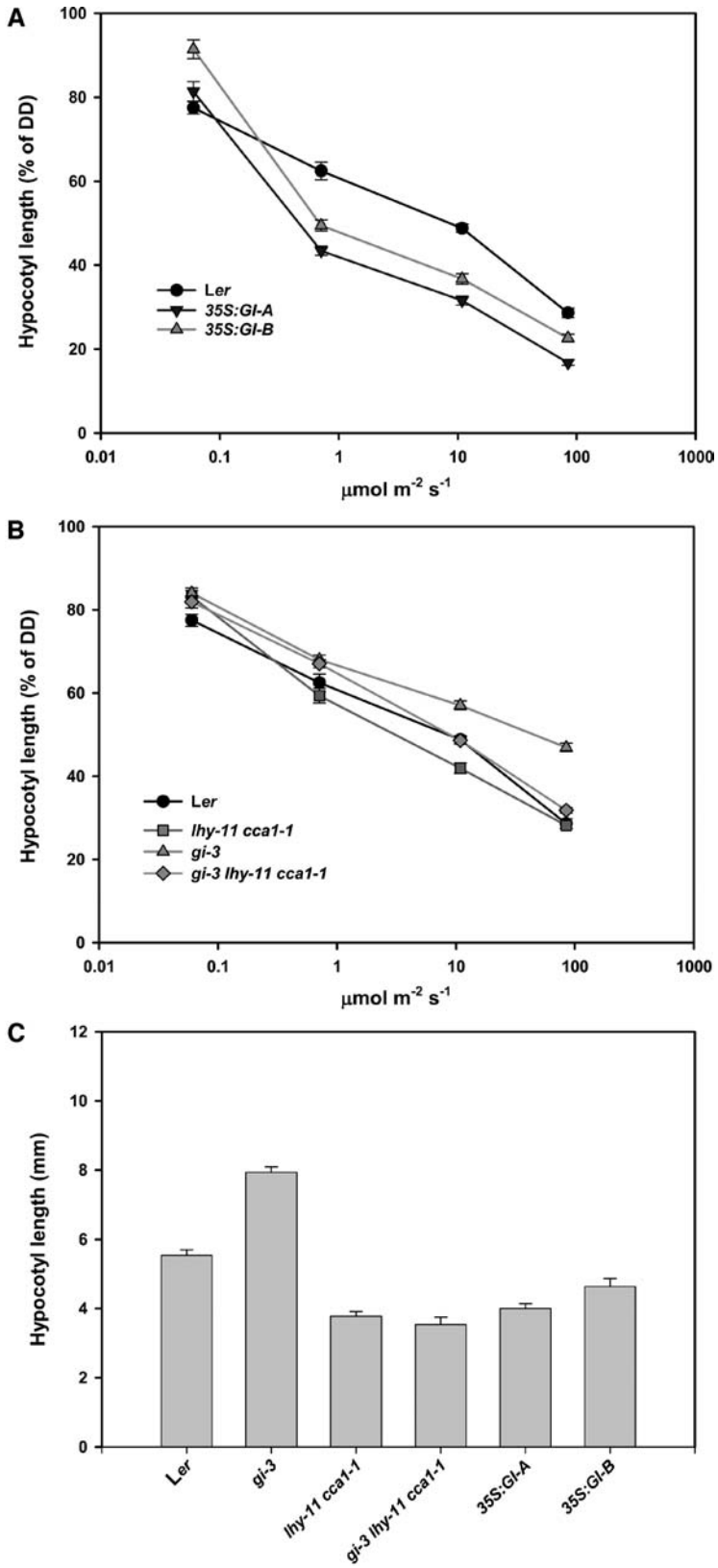

Figure 5. The Hypocotyl Length of *lhy-11 cca1-1* and *35S:GI* Plants under Different Intensities of Red Light and under SDs.

plants were measured under different intensities of red light (Figure 5A). The hypocotyls of *35S*:*GI* plants were shorter in red light than those of wild-type plants ($P < 0.05$). The difference was most pronounced at ~10 and 1 μmol m^{-2} s^{-1}. These results indicate that *35S*:*GI* plants are hypersensitive to red light and, therefore, that the expression of *GI* is limiting on red light responses, particularly at high intensities.

The early flowering of *lhy-11 cca1-1* double mutants may be explained by misexpression of *GI* (Figure 2A); therefore, the mechanism of early flowering is closely related to that of *35S*:*GI* plants. Previously, *lhy cca1* mutants were shown to have short hypocotyls under high intensity red light (Hall et al., 2003; Mas et al., 2003). To determine whether this effect requires *GI* and to compare the phenotype with that of *35S*:*GI*, fluence response curves for hypocotyl elongation of *lhy-11 cca1-1* and *gi-3 lhy-11 cca1-1* seedlings in red light were made (Figure 5B). Under these conditions, the hypocotyls of *lhy-11 cca1-1* plants, which in contrast with the previously studied *lhy cca1* lines are in the L*er* background, were shorter than the wild type at ~10 μmol m^{-2} s^{-1}, but overall, no significant difference in hypocotyl length between L*er* and *lhy-11 cca1-1* was found under red light. The *gi-3* mutant exhibited a longer hypocotyl than L*er* ($P < 0.05$), whereas the hypocotyls of *gi-3 lhy-11 cca1-1* plants were shorter than *gi-3* and slightly longer than *lhy-11 cca1-1* ($P < 0.05$). The intermediate phenotype of the triple mutant suggests a complex interaction between *gi-3* and *lhy-11 cca1-1* in regulating hypocotyl length under red light.

Hypocotyl lengths were also measured under SDs (Figure 5C). Under this condition, the hypocotyls of *lhy-11 cca1-1* plants were significantly shorter than those of the wild type. By contrast, the *gi-3* mutant exhibited a longer hypocotyl than wild-type plants. Under SDs, the hypocotyls of *gi-3 lhy-11 cca1-1* triple mutants were a similar length to those of *lhy cca1-1* mutants. The extreme short-hypocotyl phenotype of *lhy-11 cca1-1* double mutants under SDs does not therefore depend on *GI* activity, and genes that act later in or independent of the photoperiod pathway, such as *CO*, *FT*, and *FCA*, are also not required for the shorter hypocotyl phenotype (data not shown).

Taken together, our analysis indicates that in wild-type plants GI levels are limiting on seedling deetiolation under red light and SDs. In addition, in contrast with its early-flowering phenotype, the short-hypocotyl phenotype of *lhy-11 cca1-1* under SDs does not require *GI*.

GI:GFP Promotes Flowering and Is Located in Nuclei in Leaves and Hypocotyls of Arabidopsis

The nuclear location of GI protein was previously demonstrated using GUS:GI and GFP:GI fusion proteins in transient assays performed in onion epidermal cells (Huq et al., 2000). To test this in Arabidopsis and to determine the functionality of the fusion protein, *35S*:*GI*:*GFP* and *35S*:*GFP*:*GI* transgenes were made and introduced into *gi-3* mutants. Transgenic plants containing *35S*:*GFP*:*GI* were late flowering and not obviously earlier flowering than the *gi-3* progenitor. By contrast, the *gi-3* mutants containing *35S*:*GI*:*GFP* flowered early and at a similar time to *35S*:*GI* plants, indicating that the GI:GFP fusion protein was functional and promoted flowering.

Confocal microscopy was used to analyze the cellular location of GI:GFP protein in the transgenic plants (Figure 6). In epidermal cells of the hypocotyl and leaf stomatal guard cells, the fusion protein was only detected in nuclei. This demonstrates that GI:GFP, which is functionally active in promoting flowering, is localized to the nucleus, strongly suggesting that GI acts in the nucleus to control flowering time.

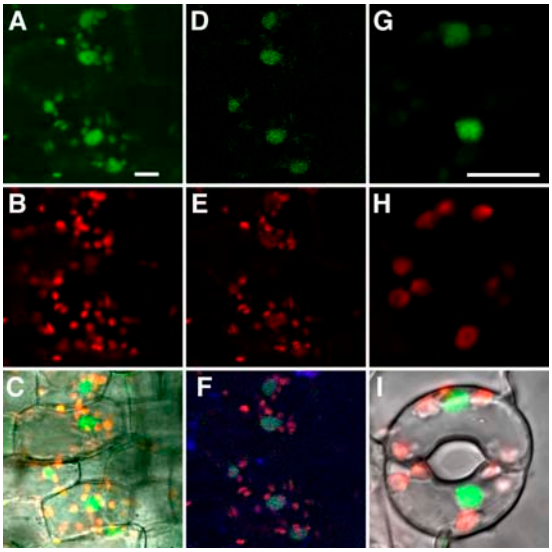

Figure 6. Cellular Localization of GI:GFP in Transgenic Arabidopsis.

Confocal microscope images of cells of *35S*:*GI*:*GFP* transgenic plants. **(A)** to **(F)** illustrate the same hypocotyl epidermal cells and **(G)** to **(I)** the same stomatal guard cells. The composite images (**[C]** and **[I]**) show the GFP fluorescence channel (**[A]** and **[G]**) overlaid with the red (**[B]** and **[H]**) and transmission channels. **(A)** shows a strong green fluorescence in the chloroplast and the nucleus; however, this is not detected by emission fingerprinting of GFP (true GFP signal; **[D]**). The signal from the red channel **(E)**, the true GFP signal **(D)**, and of the background are overlaid in the composite image **(F)**. In stomatal guard cells, strong green fluorescence was only detected in nuclei **(G)**. These data indicate that in hypocotyl epidermal and stomatal guard cells, fluorescence of GI:GFP was only detected in nuclei. Bar = 10 μm.

Figure 5. (continued).

(A) and **(B)** Red light fluence response curves of the hypocotyl length of *35S*:*GI* **(A)** or *lhy-11 cca1-1* and *gi-3 lhy-11 cca1-1* **(B)** seedlings. Hypocotyl length of seedlings grown under red light was measured and the results expressed as a percentage of the mean hypocotyl length of seedlings grown in DD. The mean value from three independent red light experiments was calculated as described in Methods and is presented ±SE. On the *x* axis, light intensity is represented on a logarithmic scale.

(C) Hypocotyl length of *35S*:*GI* seedlings and *lhy-11 cca1-1* seedlings, with or without *gi-3* grown under SDs (8 h light/16 h dark).

DISCUSSION

The phenotype of *gi* mutants suggested that GI plays important roles in red light signaling, regulation of circadian rhythms, flowering-time control, and starch accumulation in the leaves. However, it is unclear whether these effects are interrelated or represent independent functions of GI. We studied the relationship between the roles of *GI* in controlling circadian rhythms and promoting flowering. *35S*:*GI* and *gi-3* altered circadian rhythms under DD as well as LL, demonstrating that the effects of GI on the circadian system are not only due to its role in light signaling. Furthermore, under diurnal day/night cycles, *35S*:*GI* delayed the phase of expression of circadian clock–controlled genes *CCR2* and *LHY*, whereas *gi-3* delayed the phase of *CCR2* and reduced the amplitude of LHY expression. By contrast, *35S*:*GI* and *gi-3* cause early and late flowering, respectively, and their effects on the timing and amplitude of expression of the flowering-time genes *CO* and *FT* are much more dramatic than on the expression of other clock-controlled genes. We propose therefore that GI plays a significant role in controlling at least a subset of circadian rhythms in light and dark with an effect on phase in diurnal cycles but that its effect on flowering is distinct from its function in regulating these circadian rhythms. In the regulation of flowering, GI is proposed to act downstream of the putative clock components LHY/CCA1 to promote the expression of *CO* and *FT* and probably other flowering-time genes (Figure 7).

Role of *GI* in Promoting Flowering

The circadian clock regulates flowering through an output pathway that includes CO. Abundance of *CO* mRNA is reduced in *gi* mutants, and the *35S*:*CO* transgene suppresses the late flowering of *gi* mutants (Suarez-Lopez et al., 2001). We found that the *gi-3* mutation was epistatic to the early-flowering phenotype caused by *lhy-11 cca1-1* under SDs and reduced *CO* expression in this background. This supports the idea that GI triggers flowering by acting between the oscillator, which involves LHY/CCA1, and *CO*. The epistasis of *gi* to the flowering phenotype of *lhy-11 cca1-1* suggests that GI is essential for circadian clock–controlled flowering. Also, *35S*:*GI* plants show a severe early-flowering phenotype and enhanced expression of *CO* and *FT*, demonstrating that *GI* expression is limiting on flowering-time gene expression and that its misexpression is sufficient to promote early flowering. The effect of *35S*:*GI* and *lhy-11 cca1-1* on flowering was partially suppressed by *co-2* and *ft-1*

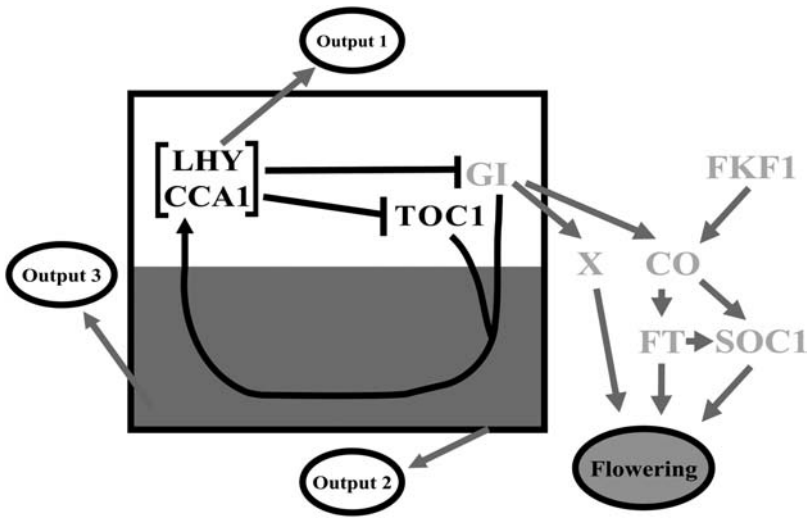

Figure 7. Dual Role for *GI* in Regulating Circadian Rhythms and Flowering Time.

The central oscillator of the Arabidopsis circadian clock was proposed to consist of a negative feedback loop comprising LHY/CCA1 and TOC1 (Alabadi et al., 2002). Within this loop, TOC1 acts in the evening to promote expression of *LHY/CCA1* in the morning, and LHY/CCA1 repress *TOC1* expression. LHY and CCA1 are also shown as negative regulators of *GI* based on the earlier phase of *GI* expression detected in a *lhy-11 cca1-1* double mutant (Mizoguchi et al., 2002); however, overexpression of *CCA1* causes an increase in *GI* expression, which may suggest a more complex pattern of regulation (Fowler et al., 1999). GI may play a role in the evening related to that of TOC1 because it is also required for high amplitude expression of *LHY/CCA1*, is expressed in a similar phase as *TOC1*, and both *gi* mutations as well as *35S*:*GI* have effects on circadian phase and period length. In the control of flowering time, GI increases the amplitude of *CO* and *FT* expression, which are both increased by *35S*:*GI* and decreased by *gi* mutations. In addition, *35S*:*GI* and *gi* mutations have opposite effects on flowering time. GI is therefore proposed to play dual roles acting within the circadian clock to regulate period length and circadian phase, while also more directly promoting expression of a circadian clock output pathway that includes *CO* and *FT* and promotes flowering. The effect of GI on flowering probably includes another pathway, indicated with an X, because *co* mutations only partially suppress the early flowering caused by *35S*:*GI* or *lhy cca1*. FT activates SOC1 downstream of CO (Schmid et al., 2003; Michaels et al., 2005). In the diagram, the square illustrates the circadian oscillator that generates circadian rhythms, white illustrates daytime, and gray shading illustrates nighttime. The flowering pathway is one of many output pathways controlled by the circadian clock, and three other pathways expressed at different times of the day are illustrated. The genes shown in gray on the right-hand side of the figure are those that promote flowering in response to LDs and delay flowering when inactivated.

mutations, supporting the idea that the mechanism by which GI promotes early flowering includes CO and its target gene *FT*. Therefore, our data support a functional hierarchy of GI-CO-FT in the flowering output pathway from the oscillator, and this also reflects the sequence with which these genes are expressed during the daily cycle.

Early flowering of the *toc1-1* mutant under SDs is caused by expression of *CO* at an earlier phase, allowing activation of CO by exposure to light under these conditions (Blázquez et al., 2002; Yanovsky and Kay, 2002). This may also explain the early flowering of *lhy-11 cca1-1* double mutants. Consistent with this model, in *lhy-11 cca1-1* double mutants, *CO* was expressed at an earlier phase under SDs, and this correlated with expression of the CO target gene *FT* (Figure 2). *GI* expression was also shifted to an earlier phase in *lhy-11 cca1-1* double mutants under SDs and was required for the expression of *CO*. This suggests that the phase shift in *GI* expression may be the primary cause of early flowering under SDs in *lhy-11 cca1-1* plants. This would be slightly different to early flowering *toc1-1* mutants, in which *CO* but not *GI* was expressed at an earlier phase under SDs (Somers et al., 1998b; Yanovsky and Kay, 2002). Furthermore, although *35S*:*GI* plants flower early, circadian phase is delayed, indicating that the early flowering of *35S*:*GI* plants could be a more direct effect of early expression of GI activating CO expression and not an indirect effect of altering circadian clock regulation. By contrast, GI probably affects *LHY/CCA1* mRNA abundance indirectly because although the amplitude of *LHY/CCA1* expression is reduced in *gi* mutants, it is not increased in *35S*:*GI* plants, and *LHY* is expressed in an earlier phase than *GI*.

GI also appears to promote flowering by a second mechanism that is independent of *CO* and *FT* (Figure 1B). The delay in flowering of *lhy-11 cca1-1* caused by *co-2* and *ft-1* was weaker than that caused by *gi-3*, suggesting that as well as promoting flowering by activating *CO* and *FT*, GI promotes flowering independently of these genes (Figure 1A). Similarly, *co-2* and *ft-1* only partially suppressed the early flowering of *35S*:*GI* plants. The late-flowering phenotype of *co-2* is similar to that of *co-8* mutants, which carry a large deletion within the *CO* gene (Robson et al., 2001), and *co-8* had a similar effect on flowering time of *lhy-12 cca1-1* to *co-2*. This supports the idea that loss of *CO* function only partially suppresses the early flowering phenotype of *lhy-12 cca1-1*. The second mechanism by which GI promotes flowering could involve *SOC1*. However, *SOC1* has an established role in promoting flowering downstream of CO (Borner et al., 2000; Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000). Therefore, we propose that in wild-type plants, GI regulates at least two circadian clock–controlled output pathways that promote flowering, one that includes *CO*, *FT*, and *SOC1* and a second that promotes flowering independently of these genes (Figure 7).

Apart from GI, the only proteins shown to promote *CO* expression are FKF1 and phyA (Tepperman et al., 2001; Imaizumi et al., 2003). The *FKF1* and *GI* genes are expressed in a similar phase (Nelson et al., 2000). However, we found that GI is not required to activate *FKF1* expression, and in *35S*:*GI* plants, *FKF1* mRNA expression is not increased (Figures 2E, 2F, 3D, and 3E). Therefore, GI does not promote *CO* expression and flowering by activating *FKF1* transcription. The role of GI in activation of *CO* is probably conserved in rice because overexpression of the rice ortholog of *GI* (*OsGI*) was shown to increase the expression of the rice ortholog of *CO* (*HEADING DATE 1*) (Hayama et al., 2003), but its effect on circadian rhythms was not tested. Although the involvement of GI in flowering-time control and in the activation of *CO* expression is likely to be widely conserved and GI protein is present in the nucleus, the mechanism by which it regulates *CO* mRNA abundance is unclear.

Role of GI in Circadian Clock Function

GI regulates the period length of circadian rhythms in expression of genes that are not involved in flowering control. Mutations in *GI* generally shorten circadian period, although the *gi-2* allele lengthens the period of *CAB*:*LUC* expression (Park et al., 1999). We supported these observations by demonstrating that under LL the *gi-3* mutation and *35S*:*GI* shorten circadian period of *CCR2*:*LUC* (Figure 4). The effect of GI on circadian rhythms in DD has not been extensively studied and was based on RNA analysis of *GI* and *LHY* expression in the *gi-1* and *gi-2* mutants (Park et al., 1999). However, these genes are not ideal markers for the effect of GI on rhythms in DD because *LHY* expression dampens rapidly in *gi* mutant backgrounds (Park et al., 1999; Mizoguchi et al., 2002), and *GI* expression can also be reduced by some *gi* alleles (Fowler et al., 1999). We therefore extended these data by following expression in *gi-3* and *35S*:*GI* backgrounds of a *CCR2*:*LUC* transgene, which shows robust circadian rhythms in wild-type plants under DD (Doyle et al., 2002; Mas et al., 2003). The *CCR2* gene is not involved in flowering and is not part of the same circadian output pathway as *CO* and *FT*. Under DD, circadian rhythm in *CCR2*:*LUC* expression dampened rapidly in *gi-3* and *35S*:*GI* plants, and these appeared arrhythmic after 30 h in DD. The altered rhythms in *gi-3* mutants and *35S*:*GI* plants indicate that the effect of GI on circadian rhythms is not limited to input from light signaling (Figure 4E). Indeed, the effect of *35S*:*GI* and *gi-3* is stronger under DD than under LL. This observation indicates that in addition to promoting expression of the output pathway controlling flowering through *CO* and *FT*, *GI* plays a general role in controlling circadian rhythms under LL and DD.

The *TOC1* and *EARLY FLOWERING4* (*ELF4*) genes, which are circadian clock regulated and expressed in a similar phase to *GI*, are also involved in the regulation of circadian rhythms in DD (Doyle et al., 2002; Mas et al., 2003). In plants overexpressing *TOC1* (*35S*:*TOC1*), circadian rhythms are strongly impaired in LL and in DD. In *35S*:*TOC1* plants grown in LL, circadian rhythms in *CAB2*, *GI*, and *CCR2* expression were undetectable, whereas *LHY* and *CCA1* mRNAs showed delayed and lower amplitude rhythms (Makino et al., 2002; Mas et al., 2003). In plants with strongly reduced *TOC1* expression, rhythms in *CCR2*:*LUC* expression were abolished under DD and showed a severe short period phenotype under LL (Mas et al., 2003). Similarly, mutations in *ELF4* strongly suppressed rhythms under LL or DD (Doyle et al., 2002). The strong effects observed by loss of *TOC1* and *ELF4* function indicate that these genes are essential for circadian rhythms under DD and that there is no redundancy in their biochemical function. Nevertheless, GI may play a related role in the circadian system to these genes because *35S*:*GI* and *gi-3*

disrupt circadian rhythms in DD, and GI, TOC1, and ELF4 all promote *LHY/CCA1* expression (Figure 7).

Role of *GI* in Light Signaling

GI was previously implicated in phyB signaling during seedling deetiolation in red light (Huq et al., 2000), and because phyB is also involved in circadian clock entrainment and the control of flowering time (Somers et al., 1998a; Cerdan and Chory, 2003), this could provide a basis for several of the phenotypes associated with *GI*. Our observation that *35S*:*GI* seedlings show shorter hypocotyls than wild-type seedlings under red light also indicates that *GI* expression is limiting on red light signaling (Figure 5B). However, we propose that this represents a function of *GI* that is largely independent of its role in circadian clock function or flowering-time control because phyB does not reduce *CO* transcription (Cerdan and Chory, 2003), as was shown for *gi-3*, and although an impairment of red light signaling might contribute to the effect of *35S*:*GI* on clock regulation in the light, it would not explain the effect of *35S*:*GI* on clock regulation in the dark.

Seedlings of *lhy-11 cca1-1* double mutants show a short hypocotyl during deetiolation under red light or after growth in SDs (Mas et al., 2003), and this may be due to impaired circadian clock function because hypocotyl growth in Arabidopsis is circadian clock regulated (Dowson-Day and Millar, 1999). To test whether misexpression of *GI* is responsible for this phenotype, as described above for the early flowering of *lhy-11 cca1-1*, the hypocotyls of *gi-3 lhy-11 cca1-1*, *lhy-11 cca1-1*, and L*er* plants were measured under four intensities of red light and under SDs (Figures 5B and 5C). Under red light, a complex interaction between *gi-3* and *lhy-11 cca1-1* was observed. However, under SDs, *lhy-11 cca1-1* was epistatic to *gi-3* with respect to hypocotyl elongation. This relationship was in contrast with the effect on flowering time, in which *gi-3* was epistatic to the early-flowering phenotype caused by *lhy-11 cca1-1*. Therefore, *GI* and *LHY/CCA1* interact differently in controlling hypocotyl length than in the regulation of flowering.

Conclusion

The biochemical function of GI is unknown, but we propose that at least some of the pleiotropic phenotypes of *gi* mutants represent separable roles for the protein in distinct processes and are not indirect effects of impairing a single process. For example, the effect of *GI* on flowering time is not a secondary consequence of its general role in controlling circadian rhythms but is more specifically associated with promoting the expression of genes in circadian output pathways that control flowering. One of these pathways contains the *CO* and *FT* genes, and there is genetic evidence for a second pathway based on the incomplete suppression of *35S*:*GI* by the *co-2* or *ft-1* mutations. Other phenotypic effects of the *gi* mutation, such as in red light signaling and starch accumulation, may represent further independent functions of the protein. Describing the biochemical function(s) of nuclear GI protein will be necessary to more clearly understand its roles in diverse processes.

METHODS

Plant Material and Growth Conditions

The *Arabidopsis thaliana* L*er* ecotype was the wild type. The *co-2*, *gi-3*, and *ft-1* mutants were kindly provided by M. Koornneef. The *lhy-1* (Schaffer et al., 1998) and *lhy-11 cca1-1* (Mizoguchi et al., 2002) mutants and *35S*:*CO* transgenic plants (Onouchi et al., 2000) were described previously. The *CCR2*:*LUC* transgenic plants were generated by introduction of the *CCR2*:*LUC* transgene (described previously in Doyle et al., 2002 and kindly provided by S. Davis) into different genotypes by *Agrobacterium tumefaciens*–mediated transformation. The *35S*:*GI*, *35S*:*GI*:*GFP*, and *35S*:*GFP*:*GI* transgenic plants in the *gi-3* and L*er* genotypes were generated by Agrobacterium-mediated transformation of constructs containing the *GI* cDNA (Fowler et al., 1999) linked to the 35S promoter, with or without translational fusions to the *GFP* coding region from the pAVA393 vector (von Arnim et al., 1998). Two independent transgenic lines, *35S*:*GI-A* and/or *35S*:*GI-B*, that were homozygous for single copy insertions of the transgene T-DNAs in L*er* were used for the experiments presented here. Plants were grown on soil in controlled environment rooms at 22°C under either LDs (10 h light/6 h day extension/8 h dark) or SDs (10 h light/14 h dark) as described (Mizoguchi et al., 2002), unless specified otherwise.

Measurement of Flowering Time

Flowering time was scored by growing plants on soil in LD and SD and counting the number of rosette and cauline leaves on the main stem. Data are presented as mean ± SE ($n = 8$ to 18). Measurement of flowering time was done at least twice with similar results.

Construction and Analysis of Double and Triple Mutants

Double and triple mutants were usually made by crossing lines homozygous for each mutation. Further information on the construction of double and triple mutants can be obtained from the authors.

RT-PCR and RNA Gel Blot Analysis of Gene Expression

For LL and DD experiments, plants were grown on GM agar plates with sucrose at 22°C under LDs (16 h light/8 h dark) for 8 d, then transferred to LL or DD at dawn, and whole plants were used for RNA preparation as described (Mizoguchi et al., 2002). For SD experiments, plants were grown on soil for 10 d, and aerial parts were used for RNA preparation. RNA gel blot analysis was performed as described by Schaffer et al. (1998). RT-PCR was performed with 1 μg of total RNA using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). cDNAs were diluted to 100 μL with TE buffer, and 1 μL of diluted cDNA was used for PCR amplification by TaKaRa Ex Taq (TaKaRa, Shiga, Japan). For RT-PCR expression studies, the following primers were used: *GI*, 5′-CTGTCTTTCTCCGTTGTTTCACTGT-3′ and 5′-TCATTCCGTTCTTCTCTGTTGTTGG-3′ (this work); *CO*, 5′-ACGCCATCAGCGAGTTCC-3′ and 5′-AAATGTATGCGTTATGGTTAATGG-3′ (Suarez-Lopez et al., 2001); *FT*, 5′-ACAACTGGAACAACCTTTGGCAATG-3′ and 5′-ACTATATAGGCATCATCACCGTTCGTTACTCG-3′ (Blázquez and Weigel, 1999); *CCR2*, 5′-CTCTTGAGCTGCCTTCG-3′ and 5′-AGAACATTCATTGGTAATCCC-3′ (Staiger et al., 2003); *FKF1*, 5′-GTCGTAACTGTCGATTCCTACA-3′ and 5′- ATCTCCAGTGTTCCAGTTATCT-3′ (this work); *TUB*, 5′-CTCAAGAGGTTCTCAGCAGTA-3′ and 5′-TCACCTTCTTCATCCGCAGTT-3′ (Kobayashi et al., 1999). Numbers of PCR cycles were as follows: 20 cycles for *GI* and *FKF1*, 25 cycles for *CO* and *FT*, 15 cycles for *CCR2*, and 18 cycles for *TUB*. Annealing temperature was 55°C for *GI*, 57°C for *FT* and *CCR2*, 60°C for *CO* and *TUB*, and 62°C for *FKF1*. Primer specificity was verified by sequencing the PCR products. The PCR

products were separated on 1.5% agarose gels and transferred to Biodyne B membranes (Nippon Genetics, Tokyo, Japan). The RT-PCR products were cloned by pGEM-T Easy Vector System I (Promega, Madison, WI), and plasmids were extracted to be templates for PCR to amplify probe DNA. The membranes were hybridized with radioactive probe DNAs in hybridization solution that contained 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS, 0.1% sarkosyl, 0.75% Blocking reagent (Boehringer Mannheim, Mannheim, Germany), and 5% dextran sulfate sodium salt at 65°C for 16 h. The blot was washed with 2× SSC and 0.1% SDS for 20 min, then 0.5× SSC and 0.1% SDS for 10 min at 65°C, and then the hybridization signal was visualized using the BioImaging Analyzer (BAS 5000; Fuji Photo Film, Tokyo, Japan); signal intensity was quantified with Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film). Values were represented relative to the highest value of the samples after standardization to the *TUB* control. All the RT-PCR analysis was performed at least twice and usually with independent RNA samples.

Luminescence Measurement and Analysis of Circadian Period Length Using *CCR2*:*LUC* Transgenic Plants

T2 generation plants from independent *CCR2*:*LUC* transformant lines were used for the analysis of luminescence and period length. Seven-day-old plants grown on agar in SD and LD were transferred into agar-filled wells of 96-well opaque microtiter plates and treated with 20 μL 5 mM D-luciferin per plant ($n \geq 24$). From the next day, the luminescence of individual seedlings was measured by counting in a Packard Topcount (Packard, Meriden, CT). The average luminescence for each genotype at each time point was calculated from the luminescence normalized for each emitting individual. The period length of free running cycles was estimated from at least 96 h of luminescence measurements starting 24 h after transfer into LL or 12 h into DD using FFT-NLLS software (Plautz et al., 1997).

Analysis of Hypocotyl Length

In the red light fluence response experiments, seeds were placed on GM agar plates without sucrose, kept 5 d in the dark at 4°C, exposed to 6 h of white light then 18 h of darkness, and then seedlings were grown either in DD or in E30-LED cabinets (Percival Scientific, Perry, Iowa) with red LED light (670 nm) filtered through layers of neutral density filters (Filter 299; Lee Filters, Andover, UK) to obtain different red light intensities (0.06, 0.71, 10.88, and 84.68 μmol m^{-2} s^{-1}). After 5 d, the seeds were placed flat on agar plates, and their hypocotyl lengths measured with MetaMorph imaging software (Universal Imaging, Downington, PA). The hypocotyl length of each red light–grown seedling was divided by the mean hypocotyl length of the seedlings grown in DD, to avoid difficulties with uneven germination, as described (Fankhauser and Casal, 2004). The data presented are the mean ± SE of the pooled standardized measurements ($n = 60$) from three independent experiments. In the SD experiments, seeds were placed on agar plates in SD conditions (8 h light/16 h dark). Light was provided by Philips TL741 tubes (Eindhoven, The Netherlands) giving photosynthetically active radiation of 48 μmol m^{-2} s^{-1}. Hypocotyl length was measured after 10 d, and the mean value ± SE was calculated ($n = 80$ for each genotype).

Effects of light intensity and genotype were analyzed using two-way analysis of variance as part of the Sigmastat 3.0 software (SPSS ASC, Erkrath, Germany).

Confocal Microscopy of GFP Fluorescence

The hypocotyls of 8-d-old transgenic *35S*:*GI*:*GFP gi-3* seedlings grown on GM agar plates under LD conditions were analyzed using a Zeiss LSM 510 Meta confocal laser scanning microscope (Jena, Germany). Confocal images were collected using the 63× oil-immersion lens. Excitation of fluorescence was at 488 nm with an argon laser, and the GFP fluorescence was detected at 500 to 530 nm and the autofluorescence of mainly chlorophyll from 602 to 709 nm. Simultaneously, transmission images were taken using the 543-nm HeNe laser. For emission spectral fingerprinting at 488 nm excitation (argon laser), lambda stacks were recorded with the Meta detector (Zeiss) between 494.7 and 591 nm in 10.7-nm steps. Emission spectra of GFP, of the green fluorescent chloroplast component(s), and of the background were measured in respective regions of the lambda stacks and selected for the linear unmixing calculation.

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Distinct Roles of *GIGANTEA* in Promoting Flowering and Regulating Circadian Rhythms in Arabidopsis

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