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FKF1 and GIGANTEA complex formation is required for day-length measurement in *Arabidopsis*

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Abstract

Precise timing of *CONSTANS* (*CO*) gene expression is necessary for day-length discrimination for photoperiodic flowering. The FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) and GIGANTEA (GI) proteins regulate *CO* transcription in *Arabidopsis*. We demonstrate that FKF1 and GI proteins form a complex in a blue-light dependent manner. The timing of this interaction regulates the timing of daytime *CO* expression. FKF1 function is dependent on GI, which interacts with a *CO* repressor, CYCLING DOF FACTOR 1 (CDF1), and controls CDF1 stability. GI, FKF1, and CDF1 proteins associate with *CO* chromatin. Thus, the FKF1-GI complex forms on the *CO* promoter in late afternoon to regulate *CO* expression, providing a mechanistic view of how the coincidence of light with circadian timing regulates photoperiodic flowering.

Many plants monitor seasonal changes in day-length to regulate flowering time for successful reproduction (1). In *Arabidopsis*, regulation of daytime *CO* expression is the primary process of time measurement in the photoperiodic flowering pathway (2, 3). FKF1 and GI proteins positively regulate *CO* transcription (4, 5). *FKF1* and *GI* gene expression has similar diurnal patterns (5, 6), implying that these proteins may interact to regulate *CO*. We tested their direct interaction in yeast and found that FKF1 interacts with GI (Fig. 1A). Our results obtained using truncated FKF1 proteins suggests that this interaction occurs through the FKF1 LOV (Light, Oxygen, or Voltage) domain (Fig. 1A). In addition, the GI N-terminus was sufficient to interact with FKF1 (fig. S1).

To assess whether this interaction occurs *in vivo*, and whether it is modulated by photoperiod or light conditions, we generated transgenic plants constitutively expressing both haemagglutinin (HA)-tagged FKF1 (HA-FKF1) and tandem affinity purification (TAP)-tagged GI (GI-TAP) proteins [*35S::HA-FKF1 35S::GI-TAP* lines (7)] for coimmunoprecipitation experiments. In the *35S::HA-FKF1 35S::GI-TAP* #18 / *fkf1* line, a similar amount of GI-TAP protein was precipitated at every time point in both long-day (16 hours light / 8 hours dark) and short-day (8 hours light / 16 hours dark) conditions (Fig. 1, B and C). HA-FKF1 protein was coimmunoprecipitated with GI-TAP protein (Fig. 1, B and C), demonstrating that GI-TAP and HA-FKF1 proteins form a complex *in vivo*. In both day-length conditions, the amount of coimmunoprecipitated HA-FKF1 protein increased until 4 hours after light onset, remained constant for the rest of day, and declined in the dark (Fig. 1, B and C), suggesting that light or the circadian clock modulate the FKF1 and GI interaction.

We therefore analyzed the interaction in dark-grown samples. A minimal amount of HA-FKF1 was coimmunoprecipitated with GI-TAP protein in the dark (Fig. 1D), indicating that

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this interaction is light dependent. In addition, as little as 10 minutes of light exposure resulted in a marked increase in the amount of FKF1 and GI interaction (fig. S2).

Next we analyzed how light quality (wavelength) affects this interaction. Similar amounts of FKF1 and GI interacted in blue-light irradiated samples (Fig. 1E) compared to white-light grown samples, but little interaction was observed in red-light irradiated samples (Fig. 1E), indicating that blue light induces this interaction. Further analysis revealed that the FKF1 and GI interaction is fluence rate-dependent (Fig. 1F).

Since we have shown that the FKF1 LOV domain can absorb blue light (5), we postulated that the LOV domain may function as a blue-light sensing domain for this interaction. We first tested whether FKF1 and GI proteins by themselves are sufficient to reconstitute the light-dependent interaction *in vitro* (7). FKF1-HA protein was co-purified with the glutathione S-transferase fused GI N-terminus (GST-GI-N) protein incubated under light (Fig. 1G). We then analyzed the importance of the FKF1 LOV domain for light-induced interaction with GI by using FKF1 LOV variants containing three different photochemically blind mutations [C91A, R92D, and Q163L mutations, (8–11)]. All three blind mutations attenuated the light-dependent interaction (fig. S3). These results suggest that FKF1 controls the interaction with GI by absorbing blue light through the LOV domain.

To determine more accurately when this interaction occurs *in vivo*, we performed immunoprecipitation analysis using a transgenic line [*FKF1::HA-FKF1 GI::GI-TAP / fkf1 gi-2* (7)] in which both tagged *FKF1* and *GI* expression are regulated by endogenous promoters (fig. S4). Under long-day and short-day conditions, GI-TAP protein was expressed throughout the day with an afternoon peak, while HA-FKF1 expression largely occurred in the late afternoon [Fig. 2, A, B, and (5, 12)]. In long days, the peak expression of FKF1 and GI proteins coincided (Fig. 2A). The HA-FKF1 and GI-TAP interaction was observed in the late afternoon (Fig. 2A), when daytime *CO* expression occurs [Fig. 2E and (4, 13)]. In short days, HA-FKF1 peaked about 3 hours later than the GI-TAP peak expression, and the FKF1 and GI interaction occurred only at the beginning of the FKF1 expression period (Fig. 2B).

When day-length shifts from short to long, daytime *CO* is immediately induced (5, 14), and FKF1 is involved in this induction (5). We therefore examined the FKF1 and GI interaction under day-length shift conditions. On the day when conditions were switched from short to long, the expression patterns of HA-FKF1 and GI-TAP were similar to those on short days (Fig. 2C). However, the interaction between FKF1 and GI occurred throughout the extended light period (Fig. 2C). Our results show that the duration of the FKF1 and GI interaction seems to coincide with the pattern of daytime *CO* expression.

As our results indicate that FKF1 and GI may form a complex to regulate *CO* expression, we studied the significance of this interaction. We first examined whether FKF1 regulates GI protein stability, since FKF1 mediates protein degradation (15). The *fkf1* mutation did not alter the expression patterns of GI-TAP proteins (fig. S5), indicating that FKF1 does not regulate GI protein stability. We then studied the genetic relationship between *FKF1* and *GI*. Both *fkf1* and *gi* mutants showed a late flowering phenotype in long days, and the *gi* flowering phenotype was more severe (Fig. 2D). The flowering phenotype of the *fkf1 gi* double mutant in long days and short days resembled that of the *gi* mutant (Fig. 2D). Expression of *CO* in the *fkf1 gi* mutant in long days is also similar to that in the *gi* mutant (Fig. 2E). When the *gi* mutation was introduced into the *35S::HA-FKF1* #10 / *fkf1* line, the *gi-2 35S::HA-FKF1* #10 / *fkf1* line showed a strong late flowering phenotype in long days, which is similar to the *gi* flowering phenotype (Fig. 2D). *CO* expression in the *gi-2 35S::HA-FKF1* #10 / *fkf1* line also resembled that in the *gi* mutant in long days (fig. S6).

These results indicate that FKF1 function is largely dependent on GI function. In contrast, when the *fkf1* mutation was introduced in the *35S::GI-TAP / gi-2* line, the *fkf1 35S::GI-TAP / gi-2* plant flowered slightly later than the *35S::GI-TAP / gi-2* line, but much earlier than the *fkf1* mutant (Fig. 2D), indicating that GI function is not completely dependent on FKF1 function. This suggests that GI may regulate not only FKF1 activity but also the function of other proteins that play additional roles in the photoperiodic flowering pathway.

Timing of the circadian-regulated expression of FKF1 and/or GI is thought to be important for the timing of daytime *CO* expression (5, 16). If this assumption were correct, then constitutive expression of either FKF1 or GI would abolish the day-length measurement ability of plants. However, at least in the Col wild type accession, lines overexpressing either FKF1 or GI retained the ability to discriminate differences in day length [Fig. 2D and (12)]. This result suggests that, in the Col accession, FKF1 and GI expression alone may not be sufficient for regulating photoperiodic flowering responses. Based upon our results that FKF1 and GI form a complex *in vivo* and that FKF1 function likely depends on GI function, we postulated that the timing of the FKF1-GI complex formation might constitute the time measurement mechanism itself.

To test this hypothesis, we analyzed the flowering phenotype of two independent *35S::HA-FKF1 35G::GI-TAP/ fkf1* lines (figs. S7 and S8) and the *35S::HA-FKF1 35S::HA-GI / fkf1 gi-2* line in long days and short days. All the *FKF1* and *GI* double overexpressing lines examined flowered at almost the same time in both day-length conditions (Fig. 2D and fig. S9). In the *35S::HA-FKF1 35S::GI-TAP / fkf1* lines, *CO* was expressed constantly during the day at a similar level to the daytime *CO* peak observed in wild type plants in long days (Fig. 2F and fig. S8). In short days, *CO* expression in these lines was higher than that in wild type plants in the daytime (Fig. 2G and fig. S8). These results indicate that FKF1-GI complex formation regulates the timing of daytime *CO* transcription.

Even though *CO* expression in these lines was constantly high in the daytime in both day-length conditions, *FT* expression was not constant (figs. S7 and S8). *FT* expression in the double overexpressors showed two distinct peaks in long days (figs. S7 and S8). This might be explained by the posttranscriptional regulation of CO protein (17). We observed daytime *FT* expression in the double overexpressors in short days (figs. S7 and S8). This may cause early flowering of these lines in short days.

Our results suggest that FKF1 function is mainly GI dependent and that the FKF1-GI complex regulates daytime *CO* gene expression. One of the mechanisms by which FKF1 regulates *CO* transcription is by degrading its repressor, CDF1 (15). We therefore explored the possibility that the FKF1-GI complex may be involved in this regulation. First, we tested whether the FKF1-GI complex contains CDF1. As the FKF1 and CDF1 interaction has been shown (15), we analyzed the possible interaction between CDF1 and GI. CDF1 interacted with the GI N-terminus, the same fragment that interacted with FKF1, in yeast and *in vitro* (fig. S10). In plant materials harvested in the morning, HA-CDF1 was coimmunoprecipitated with GI-TAP (Fig. 3A).

Considering FKF1 functional dependence on GI, these data led us to predict that CDF1 protein may be stable in the *gi* mutant due to loss of FKF1 activity. Therefore, we analyzed the CDF1 protein levels in *35S ‴HA-CDF1* lines (15) with or without the *gi* mutation. In the *35S ‴HA-CDF1* #17 line, the HA-CDF1 protein levels declined between 13 and 19 hours after light onset (Fig. 3B). In the *gi-2 35S ‴HA-CDF1* #17 line, HA-CDF1 expression did not change even at the end of the day (Fig. 3B), indicating that GI is involved in the regulation of FKF1-dependent CDF1 protein stability.

We also tested whether GI regulates CDF1 function using a transient expression system (15). The CDF1-VP64 (CDF1 fusion with transcriptional activation domains) increased the activity of luciferase regulated by the *CO* promoter in wild type plants and *gi-2* mutants (fig. S11), suggesting that GI does not modulate the CDF1 DNA binding ability. This implies that in the *gi* mutants endogenous CDF1 may be stable even in late afternoon and participate in *CO* repression.

As GI binds to CDF1 *in vivo*, then GI might be present at the *CO* promoter. To investigate this possibility, we performed a chromatin immunoprecipitation (ChIP) analysis using *GI*$^{\prime\prime\prime}$*GI-TAP / gi-2* plants. We analyzed the GI-TAP specific enrichment of 17 different amplicons with locations almost evenly distributed along the *CO* gene region (Fig. 3D) by quantitative polymerase reaction (Q-PCR) (7). In the *CO* promoter, the amplicon 4 region was the most highly enriched, and amplicons 3 and 9 also showed significant enrichment (Fig. 3C). This indicates that GI-TAP protein associates with these *CO* promoter regions.

We further investigated whether both FKF1 and CDF1 associate with the same *CO* regions where GI-TAP protein interacts. We utilized *FKF1* $^{\prime\prime\prime}$*FKF1-TAP / fkf1* (5) and *CDF1* $^{\prime\prime\prime}$*HA-CDF1*#19 (15) lines for the ChIP assays and analyzed the amounts of specific chromatin enrichment around amplicons 3, 4, and 9. Amplicon 4 was enriched in the *FKF1* $^{\prime\prime\prime}$*FKF1-TAP / fkf1* samples harvested at the same time as the *GI* $^{\prime\prime\prime}$*GI-TAP / gi-2* samples (Fig. 3E), indicating that both FKF1 and GI associate with this *CO* promoter region. Amplicons 3, 4, and 9 were all enriched in the *CDF1* $^{\prime\prime\prime}$*HA-CDF1* samples harvested in the morning (Fig. 3F). Together with the *in vivo* GI and CDF1 interaction results, and since CDF1 peak expression occurs before the GI peak (12, 15), GI might interact with CDF1 that has already bound to the *CO* promoter in the morning. Once FKF1 interacts with the GI-CDF1 complex in the afternoon, FKF1 might degrade CDF1 to release the repression of *CO*.

We have shown that FKF1 and GI form a complex *in vivo*, and that this interaction is induced by blue light absorbed by the LOV domain, verifying our previous proposal that FKF1 is a blue-light photoreceptor (5). In addition, our results indicate that the timing of FKF1-GI complex formation, which is controlled by both circadian regulation of FKF1 and GI expression and light induction of FKF1 and GI interaction, can regulate the timing of daytime *CO* expression (Fig. 4). Moreover, our results suggest that the FKF1-GI complex directly regulates CDF1 stability in the afternoon and that the FKF1-GI-CDF1 complex forms on the promoter region of the *CO* gene. This is likely to be a part of the molecular mechanism by which the FKF1-GI complex controls daytime *CO* transcription. Thus, we have uncovered the principal molecular mechanism that enables plants to distinguish seasonal differences in day length. In conjunction with posttranscriptional regulation of CO protein (17), this regulation could enable plants to select the most favorable season for successful flowering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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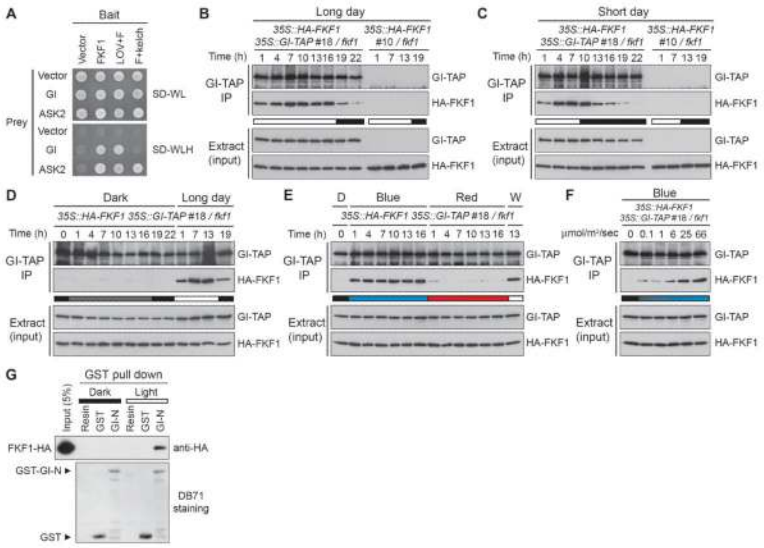

Figure 1. FKF1 interacts with GI in a blue-light dependent manner

(A) Interaction between FKF1 and GI proteins in yeast. LOV+F contains LOV and F-box domains. F+kelch contains F-box and kelch repeat domains (7). ASK2 is known to interact with F-box domain. SD-WL medium is a control; SD-WLH medium is for selection of protein interaction.

(B to **F)** GI-TAP and HA-FKF1 protein profiles in coimmunoprecipitation experiments under various light conditions. The *35S ‴HA-FKF1 35S ‴GI-TAP* line and the *35S ‴HA-FKF1* line were grown for 10 days in long days (B) or short days (C). The long-day-grown *35S ‴HA-FKF1 35S ‴GI-TAP* #18 / *fkf1* line was kept in the dark on day 10 (D). The *35S ‴HA-FKF1 35S ‴GI-TAP* #18 / *fkf1* line was incubated under blue or red light (both 25 µmol/m^2/sec) on day 10 (E). The *35S ‴HA-FKF1 35S ‴GI-TAP* #18 / *fkf1* line was incubated under different intensities of blue light for 1 hour (F). The bar color represents the light conditions.

(G) *In vitro* reconstitution of the FKF1-GI interaction. Samples were incubated in the dark or under white light (80 µmol/m^2/sec). DB71 staining showed GST and GST-GI-N proteins precipitated.

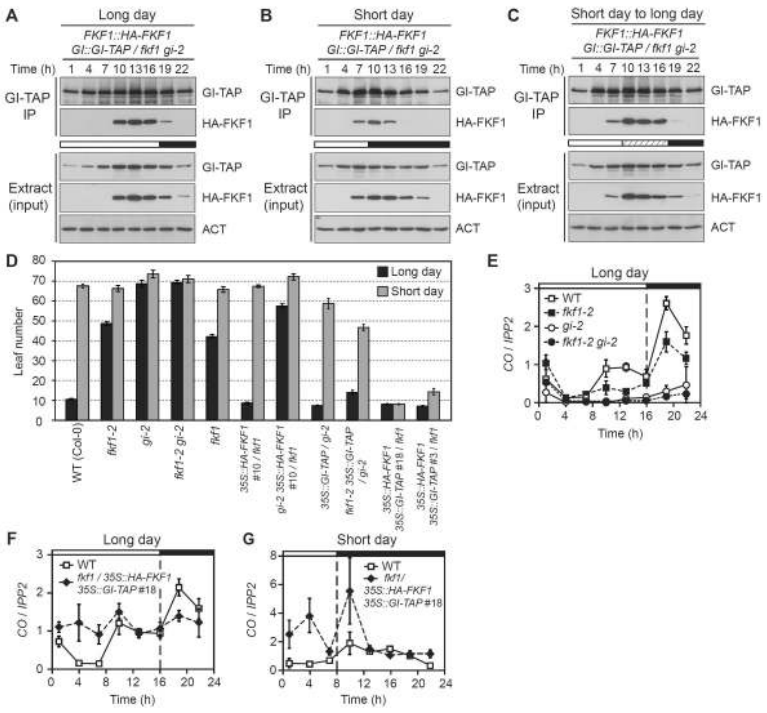

Figure 2. The FKF1-GI complex is formed in late afternoon and regulates daytime *CO* expression

(A to **C)** GI-TAP and HA-FKF1 protein profiles in coimmunoprecipitation experiments with a line expressing GI-TAP and HA-FKF1 under endogenous promoter regulation. The *FKF1* ‴*HA-FKF1 GI* ‴*GI-TAP / fkf1 gi-2* transgenic plants were grown under long day (A), short day (B) or short day to long day (C) conditions. The white and black bars represent the white light and dark conditions. The hatched bar represents extended light incubation.

(D) Flowering phenotypes of plants with various levels of *FKF1* and *GI* expression in long days and short days. Data are mean ± S.E.M. for 16 plants.

(E) *CO* expression in wild type plants, *fkf1-2*, *gi-2*, and *fkf1-2 gi-2* mutants in long days. *IPP2* expression (15) was used for normalization. *CO* expression in each panel is shown relative to the average value of wild-type plant data.

(F and **G)** *CO* expression in wild type plants and the *35S* ‴*HA-FKF1 35S* ‴*GI-TAP* #18 / *fkf1* in long days (F) and short days (G).

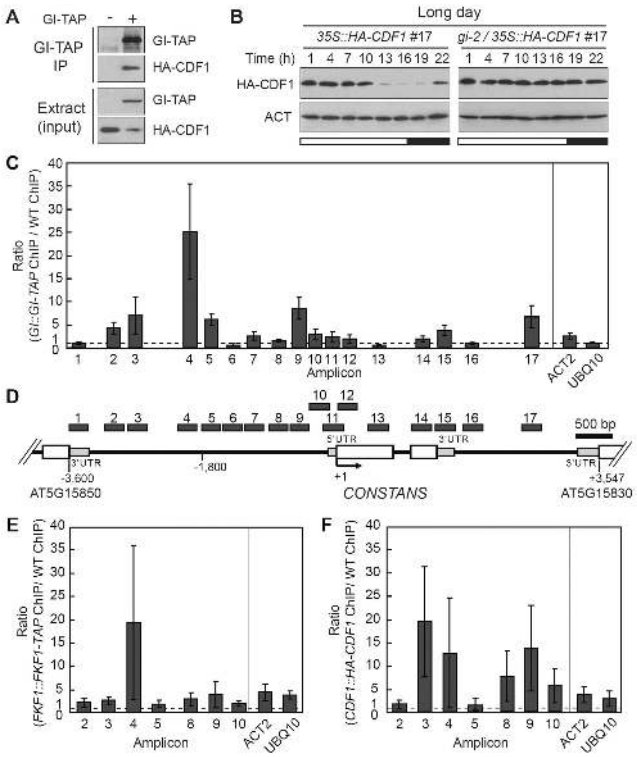

Figure 3. GI-FKF1-CDF1 complex associates with *CO* promoter *in vivo*

(A) *In vivo* interaction between HA-CDF1 and GI-TAP. The *35S ‴HA-CDF1* #17 (GI-TAP-) and *35S ‴GI-TAP 35S ‴HA-CDF1* #17 (GI-TAP+) lines were grown in long days, harvested 4 hours after light onset on day 10, and coimmunoprecipitation assays were performed (7).

(B) HA-CDF1 expression in the *35S ‴HA-CDF1* and *gi-2 35S ‴HA-CDF1* lines. Plants were harvested at day 10 in long days. ACT was used as a loading control.

(C) *CO* chromatin regions associated with GI-TAP protein. Plants were harvested 13 hours after light onset on day 10. The ratio between the specific enrichment value in the GI‴GI-TAP sample and that in the wild type sample on each amplicon was calculated from 7 independent ChIP analyses (7). *ACT2* and *UBQ10* genes were used as controls. The dotted line indicates no enrichment.

(D) Schematic drawing of the *CO* locus and the amplicon locations for ChIP analysis. The 17 amplicon locations are shown. White and light gray boxes represent exons, and 5'- and 3'-untranslated regions (UTR).

(E and **F)** *CO* promoter regions associated with FKF1-TAP and HA-CDF1 proteins. Plants were harvested 13 hours [FKF1-TAP (E)] and 4 hours [HA-CDF1 (F)] after light onset on day 10. Data were calculated from 4 independent analyses.

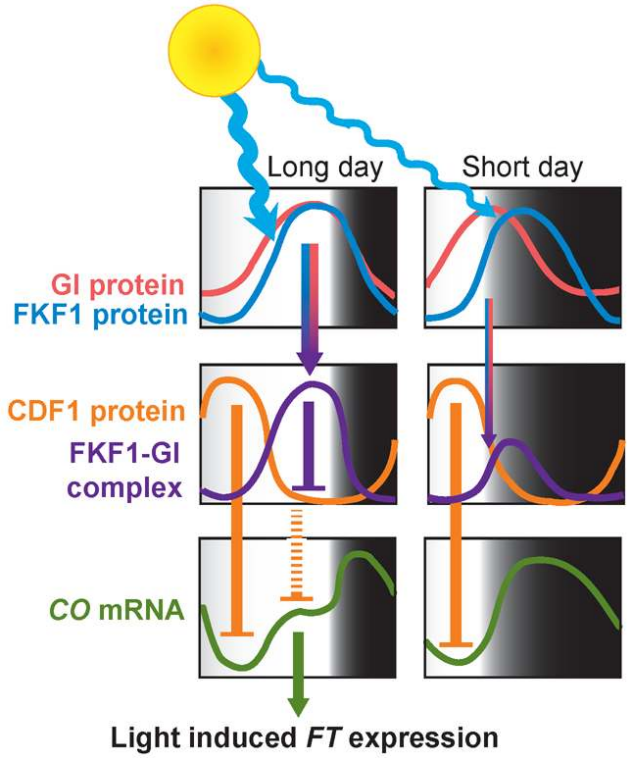

Figure 4. A model of day-length dependent *CO* transcriptional regulation

In long days, the circadian-regulated coincidence of FKF1 and GI peak expression and the light-induced FKF1 interaction with GI enable the formation of the FKF1-GI complex in late afternoon. When the complex is formed on the *CO* promoter, CDF1 associated with GI is degraded by FKF1 to facilitate the induction of daytime *CO* expression. Then CO protein is stabilized and activated by light to induce *FT* expression (13, 17). In short days, FKF1 peaks in the dark at a different time than GI, thus only a small quantity of the complex forms.