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FKF1 F-box protein promotes flowering in part by negatively regulating DELLA protein stability under long-day photoperiod in *Arabidopsis*[FA]

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High-Impact Article

Abstract *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*FKF1*) encodes an F-box protein that regulates photoperiod flowering in *Arabidopsis* under long-day conditions (LDs). Gibberellin (GA) is also important for regulating flowering under LDs. However, how FKF1 and the GA pathway work in concert in regulating flowering is not fully understood. Here, we showed that the mutation of *FKF1* could cause accumulation of DELLA proteins, which are crucial repressors in GA signaling pathway, thereby reducing plant sensitivity to GA in flowering. Both *in vitro* and *in vivo* biochemical analyses demonstrated that FKF1 directly interacted with DELLA proteins. Furthermore, we showed that FKF1 promoted ubiquitination and degradation of DELLA proteins. Analysis of genetic data revealed that *FKF1* acted partially through DELLAs to regulate flowering under LDs. In addition, DELLAs exerted a negative feedback on *FKF1* expression. Collectively, these findings demonstrate that FKF1 promotes flowering partially by negatively regulating DELLA protein stability under LDs, and suggesting a potential mechanism linking the FKF1 to the GA signaling DELLA proteins.

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INTRODUCTION

The correct timing of the transition from vegetative to reproductive development is essential for plant reproduction, enabling the completion of seed development under suitable environmental conditions. Flowering is affected by environmental factors such as day length and temperature, and endogenous developmental cues such as plant hormones and age. In *Arabidopsis*, there are approximately six signaling pathways regulating flowering time, including photoperiod, temperature, vernalization, gibberellin (GA) biosynthesis, autonomous, and aging pathways (Amasino 2010; Srikanth and Schmid 2011; Song et al. 2013; Teotia and Tang 2015; Li et al. 2016b; Bao et al. 2020). These pathways converge to regulate expression of integrators, such as FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and LEAFY (LFY), which activate floral meristem identity genes such as *LFY*, *APETALA1* (*AP1*), and *FRUITFULL* (*FUL*) in the shoot apical meristem to initiate floral transition (Blázquez et al. 1998; Kardailsky et al. 1999; Blázquez and Weigel 2000; Lee et al. 2000; Lee and Lee 2010).

In the photoperiodic pathway, the light signal and circadian clock converge to regulate the expression of *CONSTANS* (*CO*) and *FT*. *CONSTANS* encodes a zinc-finger type transcription factor that promotes flowering by directly binding to the *FT* promoter and activating its transcription under long-day conditions (LDs) (Samach et al. 2000; Tiwari et al. 2010). FLAVIN-BINDING KELCH

REPEAT F-BOX 1 (FKF1), the expression of which is controlled by the circadian clock (Nelson et al. 2000; Imaizumi et al. 2003), plays important roles in modulating photoperiodic flowering (Nelson et al. 2000; Imaizumi et al. 2003; Ito et al. 2012) and regulates *CO* at both the transcriptional and post-translational levels which in turn induce *FT* expression and promote flowering (Sawa et al. 2007; Song et al. 2012; Lee et al. 2017; Hwang et al. 2019). Additionally, *FKF1* encodes an F-box protein with three domains (LOV, F-box, and Kelch repeat) that is a key component of the SKP1/CUL1/F-box (SCF)-type E3 ligase complex (Nelson et al. 2000; Imaizumi et al. 2003; Ito et al. 2012). The FKF1 Kelch repeat domain interacts with CYCLING DOF FACTOR 1 (CDF1), a Dof that represses *CO* gene transcription by directly binding to Dof binding sites in its promoter (Yanagisawa, 2002; Imaizumi et al. 2005), and mediates its poly-ubiquitination-dependent degradation to control expression of *CO* and *FT* (Imaizumi et al. 2005). Recently, FKF1 was reported to interact with CO through LOV domain and stabilize the CO protein by inhibiting CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) dependent CO degradation to activate *FT* transcription (Song et al. 2012; Lee et al. 2017).

GA was reported to be required for plant flowering under short-day conditions (SDs) (Wilson et al. 1992). However, recent studies have demonstrated that GA also functions to promote flowering under LDs (Griffiths et al. 2006; Willige et al. 2007; Hisamatsu and King 2008; Osnato et al. 2012; Porri et al. 2012). Gibberellin signal starts with binding of GA to its receptor GA-INSENSITIVE DWARF 1 (GID1) (GID1A, GID1B and GID1C in *Arabidopsis*) and stimulating formation of the GA-GID1-DELLA complex; this in turn triggers 26S proteasome-dependent DELLA protein degradation which is mediated by F-box protein sleepy1 (SLY1), relieves the growth-repressing effects (McGinnis et al. 2003; Dill et al. 2004; Willige et al. 2007; Gao et al. 2008; Murase et al. 2008; Li et al. 2016c). There are five DELLA proteins, namely, REPRESSOR OF ga1-3 (RGA), GA-INSENSITIVE (GAI), RGA-LIKE1 (RGL1), RGA-LIKE2 (RGL2), and RGA-LIKE3 (RGL3), in *Arabidopsis*; these proteins act as key repressors of the GA response (Peng et al. 1997; Silverstone et al. 2001; Lee et al. 2002; Wen and Chang 2002; Tyler et al. 2004). Under LDs, ectopic tissue-specific expression of DELLAs leads to delayed flowering by inhibiting the expression of flowering time integrator genes such as *FT* and *TWIN SISTER OF FT* (*TSF*) in leaves and *SPL* genes in both the leaves and shoot meristem (Galvão et al. 2012; Zhang et al. 2020). Expression of *gai-D*, a dominant mutant form of the GAI protein (Peng et al. 1997) in companion cells resulted in late flowering and reduced *FT* and *TSF* messenger RNA (mRNA) levels (Porri et al. 2012). DELLA was reported to interact with SQUAMOSA PROMOTER BINDING–LIKE (SPL) to attenuate SPL transcriptional activities toward *FT* in leaves and MADS box genes such as *FRUITFULL* (*FUL*) at shoot apex (Yu et al. 2012). Hou et al. reported that DELLAs interacted with nuclear factor Y (NF-Y), sequestered it from binding to *SOC1* promoter and induced RGA activity to inhibit *SOC1* expression (Hou et al. 2014). RGA-LIKE1 can interact with the transcription factors bHLH48 and bHLH60 and further inhibit activation of *FT* under LDs (Li et al. 2017). A recent study showed that RGL1 and GAI can interact with WRKY75 and repress its activation ability, attenuating expression of *FT* under LDs (Zhang et al. 2018). Additionally, DELLA proteins were reported to physically interact with CO and repress the transcriptional activity of the CO protein, thus reducing *FT* expression under LDs (Wang et al. 2016; Xu et al. 2016).

FLAVIN-BINDING KELCH REPEAT F-BOX 1 mutation strongly delays flowering under LDs (Nelson et al. 2000; Imaizumi et al. 2003; Ito et al. 2012); exogenous GA_3 treatment could rescue flowering defects of *fkf1* mutant in continuous illumination (Nelson et al. 2000). However, how FKF1 and the GA pathway work in concert in regulating flowering remains largely unknown. In this study, we found that *fkf1* mutants showed reduced sensitivity to exogenous GA_3 in flowering, while overexpression of FKF1 caused enhanced sensitivity. FLAVIN-BINDING KELCH REPEAT F-BOX 1 was found to physically interact with DELLA proteins and regulate their stability. Genetic analysis revealed that FKF1 promoted flowering under LDs in part by reducing DELLA abundance. *FKF1* expression was observed to be induced by DELLA. Thus, our findings suggest a potential mechanism linking the FKF1 to the GA signaling DELLA proteins in regulating flowering under LDs.

RESULTS

FLAVIN-BINDING KELCH REPEAT F-BOX 1 positively regulates the GA response in flowering

It has been reported that deletion of *FKF1* caused late flowering under LDs, and the later flowering of *fkf1*

mutant could be overcome by exogenous GA in continuous illumination (Nelson et al. 2000). Consistent with this, flowering of *fkf1* mutants was significantly accelerated by GA_3 treatment as measured by the days to bolting and the rosette leaf number (Figure 1A, B; Table S1). Interestingly, we observed that *fkf1* mutants displayed reduced sensitivity to GA compared with the wild-type plants. Indeed, 25.3% and 23.4% reductions in rosette leaf number and 29.9% and 24.7% reductions in the days to bolting were observed in *fkf1-1* and *fkf1-t*, respectively (compared with 35.9% and 35.8% in the wild-type, respectively) (Figure 1E). To eliminate the effect of endogenous GA, we crossed the GA-deficient mutant *ga1-3*, which has been introgressed into the Col background (Tyler et al. 2004), into the *fkf1-1* background and analyzed flowering time in response to exogenous GA_3 under LDs. Consistent with a previous study (Hisamatsu and King 2008), GA_3-treated *ga1-3* mutant plants flower significantly earlier compared to mock-treated plants under LDs (Figure S1A, B). However, the *ga1-3*/*fkf1-1* double mutant was markedly less sensitive to GA_3 during flowering than the *ga1-3* mutant as assessed by the reduction ratio of days to bolting or the rosette leaf number (Figure S1C). This result confirmed that *FKF1* mutation reduces plant response to GA in flowering. By contrast, flowering in *FKF1* overexpressing plants

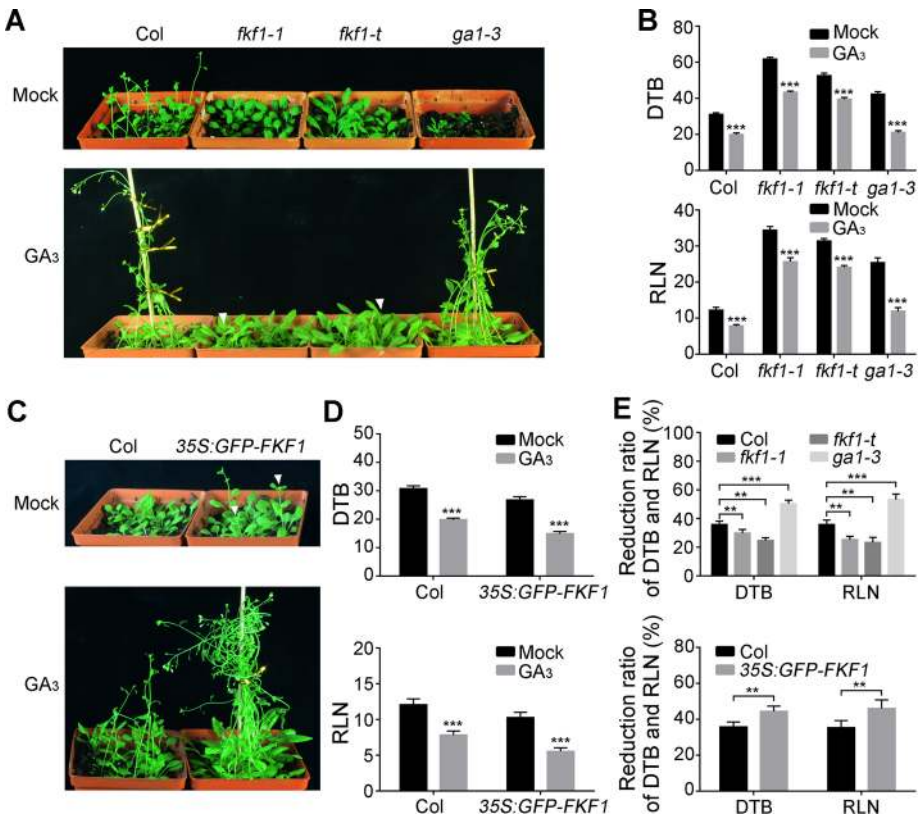

Figure 1. FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) positively regulates the gibberellin (GA) response in flowering

(**A**) Images of 30-d-old plants grown in soil under long-day conditions (LDs) treated with GA_3 or Mock. (**B**) The days to bolting (DTB) and the rosette leaf number (RLN) at flowering of the respective genotypes in response to GA_3 are shown. (**C**) Images of 22-d-old plants grown in soil under LDs treated with GA_3 or Mock. (**D**) The DTB and the rosette leaf number (RLN) at flowering of the respective genotypes in response to GA_3 are shown. (**E**) The reduction ratio of DTB and RLN of the respective genotypes in response to GA_3 are shown. The reduction ratio of DTB and RLN was calculated as (DTB (Mock)-DTB (GA)): DTB (Mock), and (number of leaves (Mock)-number of leaves (GA)): number of leaves (Mock), respectively. 7-d-old seedlings of the wild-type Col, mutant *fkf1-1*, *fkf1-t*, and *ga1-3*, and transgenic line *35S:GFP-FKF1* grown in soil were sprayed with 100 μmol GA_3 or Mock (ethanol alone) once every 2 d until bolting. Standard deviations ($n \geq 10$) are shown. The white arrows point at the position showing the flower buds. Significant differences are indicated: $^{**}P < 0.01$, $^{***}P < 0.001$ (Tukey's least significant difference test).

(*35S:GFP-FKF1*), which showed an early-flowering phenotype (Figure S2; Table S1; Lee et al. 2017), was more sensitive to GA_3 compared to the wild-type plants (Figure 1C, D). In *35S:GFP-FKF1* plants, a 46.0% reduction in rosette leaf number and a 44.4% reduction in the days to bolting were observed (compared with 35.3% and 35.7% in the wild-type plants, respectively) (Figure 1E), indicating that *FKF1* overexpressing plants were more sensitive to GA in flowering. Together, these observations suggest that FKF1 positively regulates the GA response in flowering.

To determine whether *FKF1* affected GA accumulation, we analyzed the effect of *FKF1* mutation on expression of GA biosynthetic and catabolic genes. The quantitative real-time polymerase chain reaction (qRT-PCR) analysis results showed that expression of the GA biosynthetic gene *GA3ox2* was significantly upregulated by *fkf1-1* (Figure S3A), while GA catabolic genes, including *GA2ox1*, *GA2ox2*, *GA2ox4*, and *GA2ox6*, were significantly downregulated by *fkf1-1* (Figure S3B). These results indicated that GA catabolism might be downregulated in *fkf1-1* mutant seedlings. To explore this, we measured the active GA_4 content in the *fkf1-1* mutant and wild-type seedlings. However, we did not find a significant difference (Methods S1; Figure S3C).

To further determine whether the GA signaling pathway was affected in the *fkf1* mutant, we examined the expression of GA-responsive genes *EXPANSIN A8* (*EXP8*) and *PACLUBUTRAZOL RESISTANCE 1* (*PRE1*), which are repressed by the GA synthesis inhibitor paclobutrazol (PAC) (Li et al. 2015), in response to PAC. Paclobutrazol treatment decreased the transcription levels of *EXP8* and *PRE1* in both the wild-type plants and *fkf1-1* mutant (Figure S3D, E). However, a greater reduction effect of *PRE1* expression by PAC was observed in the *fkf1-1* mutant compared with the wild-type plants (Figure S3F). Moreover, the mock-treated *fkf1-1* mutant also showed lower mRNA levels of *EXP8* and *PRE1* than the mock-treated wild-type plants (Figure S3D, E). These results suggest that GA signaling is partially blocked in the *fkf1* mutant. Thus, FKF1 is likely to play a positive role in regulating GA signaling.

FLAVIN-BINDING KELCH REPEAT F-BOX 1 negatively modulates RGA protein abundance

To unravel how *FKF1* affected GA signaling, we examined the level of the representative DELLA protein RGA, which is a repressor in GA signaling (Mutasa-Gottgens and Hedden 2009), in *fkf1-1* mutant and *35S:GFP-FKF1* transgenic plants grown under LDs using an anti-RGA antibody. The immunoblot results showed that the abundance of RGA was higher in the *fkf1-1* mutant, but lower in the *35S:GFP-FKF1* transgenic plants than in the wild-type plants (Figure 2A). To distinguish the transcriptional and post-transcriptional control of RGA by FKF1, we crossed the *35S:TAP-RGA* transgenic plant, which constitutively expresses the fusion protein TAP-RGA (Feng et al. 2008), into the *fkf1-t* and *35S:GFP-FKF1* background and analyzed the level of TAP-RGA fusion protein (Figure 2B). According to the results, the TAP-RGA protein level was more abundant in the *fkf1-t* mutant than in the wild-type plants, but less in the *35S:GFP-FKF1*, indicating that FKF1 modulated DELLA protein abundance at the post-transcriptional level. Together, these results suggest that *FKF1* negatively modulates RGA protein abundance in *Arabidopsis*.

FLAVIN-BINDING KELCH REPEAT F-BOX 1 regulates rhythmical accumulation of RGA protein

Previous studies have demonstrated that DELLA proteins are rhythmically regulated at the post-translational level (Wang et al. 2016). To determine whether FKF1 regulates cyclic accumulation of DELLA, *fkf1* mutant, and *35S:GFP-FKF1* transgenic plants were grown under LDs for 12 d, and then sampled every 4 h for 1 d for protein analysis. As previously reported (Wang et al. 2016), protein accumulation of RGA was found to be rhythmical under our growth conditions; RGA protein levels decreased at zeitgeber time (ZT)8 to ZT12 in the wild-type plants (Figure 2C, D). In the *fkf1-1* mutant, the RGA protein was more stable at ZT8 to ZT12 under LDs (Figure 2C, D). Consistently, protein abundance of RGA was lower from ZT8 to ZT12 in the *35S:GFP-FKF1* plants than that in the wild-type plants (Figure 2C, D). To distinguish transcriptional and post-transcriptional control of RGA protein levels, transgenic plants constitutively expressing TAP-RGA were used to examine RGA protein expression. As shown in Figure 2E and F, the level of TAP-RGA protein was more stable from ZT8 to ZT12 in the *fkf1* mutant background, while less stable in the *35S:GFP-FKF1* background under LDs. These results suggest that

FKF1 mediates cyclic accumulation of the RGA protein and negatively modulates RGA protein stability.

To determine whether GA-mediated protein degradation was responsible for the increased stability of RGA in the *fkf1* mutant, we measured the degradation pattern of RGA in *fkf1-1* and wild-type seedlings treated with GA_3 for varying times. Surprisingly, RGA was normally degraded with GA_3 treatment both in the wild-type plants and *fkf1-1* mutant (Figure 2G, H). Additionally, RGA proteins were more abundant in the *ga1-3*/*fkf1-1* double mutant than in the *ga1-3* single mutant (Figure 2I) which failed to synthesize GA and accumulates DELLA proteins (Sun et al. 1992; Silverstone et al. 2001; Tyler et al. 2004), indicating that *FKF1* mutation promotes over-accumulation of RGA protein in the absence of GA. This is consistent with the flowering phenotype of the *ga1-3*/*fkf1-1* double mutant, which flowered later than the *ga1-3* and the *fkf1-1* single mutant (Figure S1A, B; Table S1). Taken together, these results suggest that FKF1 might modulate RGA stability via mechanisms other than the GA pathway.

FLAVIN-BINDING KELCH REPEAT F-BOX 1 directly interacts with DELLA proteins

FLAVIN-BINDING KELCH REPEAT F-BOX 1 is involved in CDF1 protein turnover by functioning as an E3 ubiquitin ligase (Imaizumi et al. 2005), we therefore

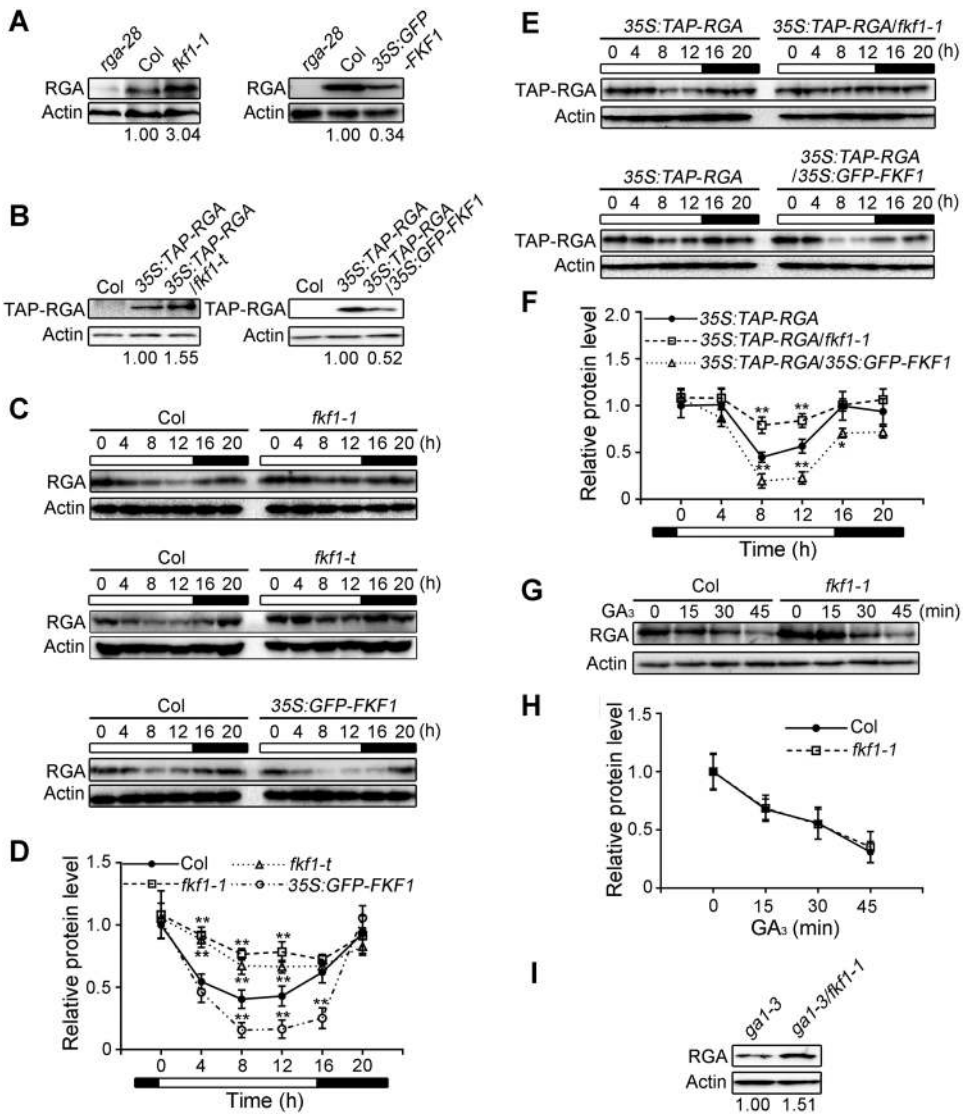

Figure 2. Continued

postulated that FKF1 might control DELLA protein abundance via a proteasome-dependent pathway. To examine this possibility, we first assessed interaction between FKF1 and DELLA proteins by bimolecular fluorescence complementation (BiFC) assay and found that FKF1 interacted with five DELLA proteins (RGA, GAI, RGL1, RGL2, and RGL3) in the nucleus of *Arabidopsis* protoplasts (Figures 3A, S4A). Consistent with the BiFC results, co-immunoprecipitation (Co-IP) analysis revealed that FKF1 interacted with three DELLA proteins (RGA, GAI, and RGL3) (Figures 3B, S4B), while RGL1-Flag and RGL2-Flag were not precipitated by FKF1-Myc, even though combinations of FKF1 and RGL1 and FKF1 and RGL2 emitted BiFC signals (Figure S4B). We next focused on FKF1-RGA and FKF1-GAI and further confirmed these direct interactions in an *in vitro* pull-down assay. The results showed that GST-RGA and GST-GAI could retain FKF1-His but GST alone could not (Figure 3C). Together, these results indicate that FKF1 physically interacts with RGA and GAI *in vitro* and *in vivo*.

FLAVIN-BINDING KELCH REPEAT F-BOX 1 has been reported to function as a blue-light receptor (Nelson et al. 2000; Imaizumi et al. 2003). We therefore detected their interaction by Co-IP assays using human embryo kidney 293T (HEK293T) cells both in the dark and in blue light. However, FKF1-RGA and FKF1-GAI interactions were not modulated by blue light (Methods S2; Figure S5). This result suggests that FKF1 is likely to interact with RGA and GAI independent of blue light.

To better understand the interaction of FKF1-DELLA, we analyzed the domains of FKF1 and DELLA protein. Our GST pull-down analysis demonstrated that the C-terminal Kelch repeat but not LOV or F-box domains of FKF1 interacted with RGA and GAI (Figure 3D, E). In addition, the GRAS domain but not DELLA domain of RGA or GAI interacted with FKF1 (Figure 3D, F). Taken together, these observations suggest that the Kelch repeat of FKF1 and the GRAS domain of DELLA are the domains for FKF1-DELLA interaction.

Figure 2. FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) negatively modulates stability of REPRESSOR OF ga1-3 (RGA) protein

(**A**) Representative immunoblots showing protein levels of RGA in the wild-type Col, *rga-28*, *fkf1-1* mutants and *35S:GFP-FKF1*. Seedlings were grown on 1/2 Murashige-Skoog (MS) medium under long-day conditions (LDs) for 12 d and sampled at zeitgeber time (ZT)12 for protein analysis. RGA protein levels were normalized to Actin. The protein level in the wild-type Col was set as 1. Three separate experiments were conducted showing similar results. (**B**) Representative immunoblots showing TAP-RGA protein levels in the wild-type Col, *35S:TAP-RGA*, *35S:TAP-RGA/fkf1-t*, and *35S:TAP-RGA/35S:GFP-FKF1* seedlings. Seedlings were grown on 1/2 MS medium under LDs for 12 d and sampled at ZT12 for protein analysis. TAP-RGA protein levels were normalized to Actin. The protein level in the *35S:TAP-RGA* was set as 1. Three separate experiments were conducted showing similar results. (**C**) Representative image of a western blot of RGA protein profiles in Col, *fkf1-1*, *fkf1-t*, and *35S:GFP-FKF1*. Plants were grown on 1/2 MS medium under LDs for 12 d, samples were collected every 4 h for 1 d for protein analysis. The white/black bars indicate light/dark phases. The time (hour) of light on at sample collection is set as zero. (**D**) Relative protein level of RGA in (**C**). RGA protein levels were normalized to Actin. Protein level in the wild-type Col at 0 h was set to 1. Bars represent the *SD* of three biological replicates. Significant differences are indicated: $^{**}P < 0.01$ (Tukey's least significant difference test). (**E**) Representative image of a western blot of TAP-RGA protein profiles in *35S:TAP-RGA*, *35S:TAP-RGA/fkf1-1* and *35S:TAP-RGA/35S:GFP-FKF1*. Plants were grown on 1/2 MS medium under LDs for 12 d, and samples were collected every 4 h for 1 d for protein analysis. The white/black bars indicate light/dark phases. The time (hour) of light on at sample collection is set as 0. (**F**) Relative protein level of TAP-RGA in (**E**). TAP-RGA protein levels were normalized to Actin. Protein level in the *35S:TAP-RGA* at 0 h was set to 1. Bars represent the *SD* of three biological replicates. Significant differences are indicated: $^{**}P < 0.05$, $^{**}P < 0.01$ (Tukey's least significant difference test). (**G**) Representative image of a western blot of RGA protein profiles in Col and *fkf1-1* in response to gibberellin (GA). 12-d-old-seedlings grown on 1/2 MS medium under LDs were treated with 100 μmol GA_3 for indicated time and sampled for protein analysis. (**H**) Relative protein level of RGA in (**G**). RGA protein levels were normalized to Actin and the value of starting point was set to 1. Bars represent the *SD* of three biological replicates. (**I**) Representative immunoblot showing RGA protein level in *ga1-3* and *ga1-3/fkf1-1* seedlings. Seedlings were grown on 1/2 MS medium under LDs for 12 d and sampled at ZT12 for protein analysis. RGA protein levels were normalized to Actin. Protein level in *ga1-3* mutant was set to 1. Three separate experiments were conducted showing similar results.

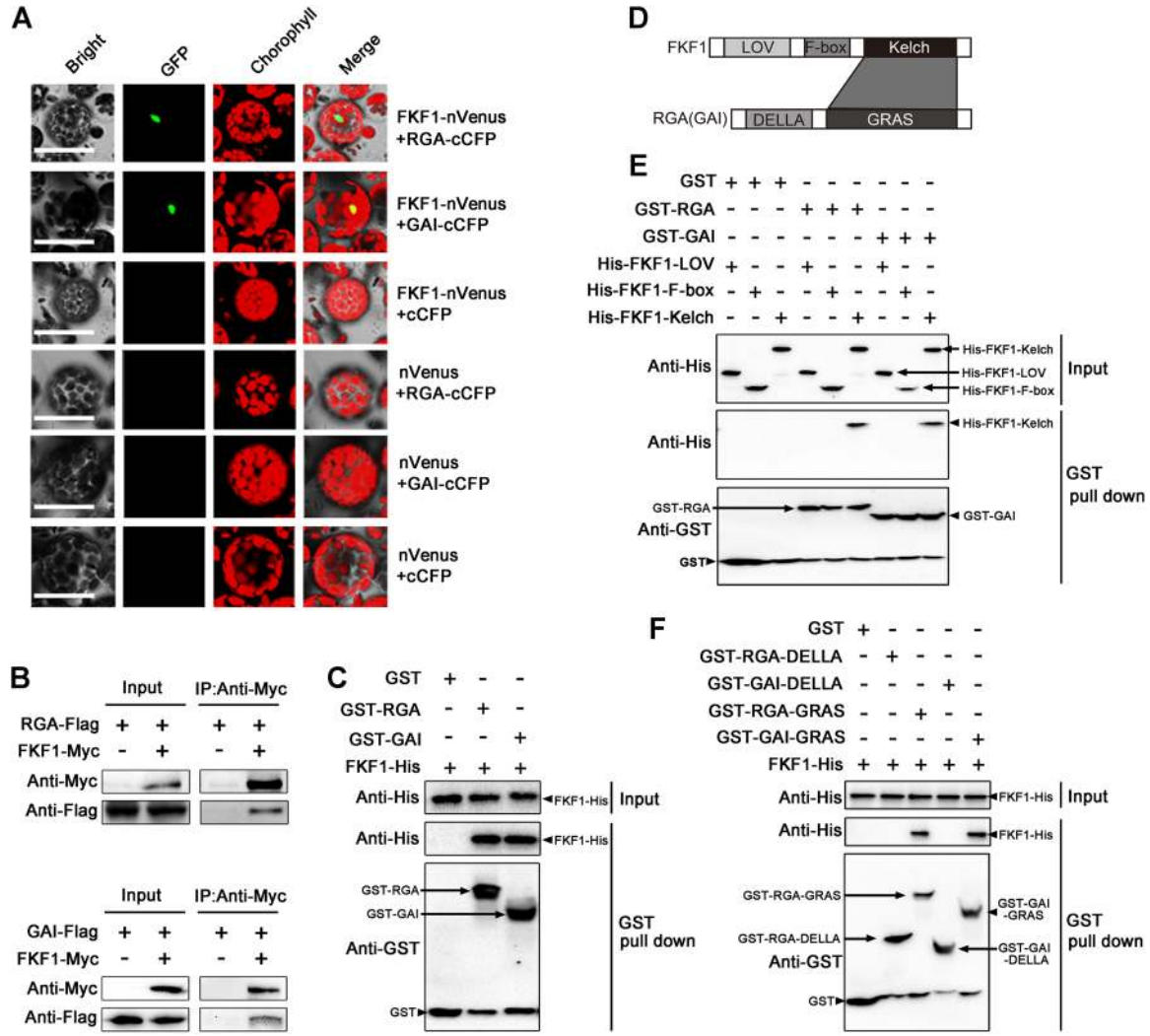

Figure 3. FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) interacts with REPRESSOR OF ga1-3 (RGA) and GA-INSENSITIVE (GAI) *in vitro* and *in vivo*

(**A**) The bimolecular fluorescence complementation (BiFC) assays showing the interaction of FKF1 with RGA and GAI in plant cell. Green fluorescent protein (GFP) images for the interaction of FKF1-nVenus with RGA-cCFP or GAI-cCFP were observed using GFP filter. Chlorophyll autofluorescence is shown as red. The negative controls failed to yield detectable green fluorescence. Bar: 50 μm. (**B**) Co-immunoprecipitation (Co-IP) experiment showing the interactions of FKF1 with RGA and GAI. Co-IP was performed using tobacco grown under long-day conditions (LDs). Immunoprecipitates against anti-Myc antibody (IP) or crude extracts (Input) were analyzed via immunoblots using anti-Myc antibody and anti-Flag antibody, respectively. (**C**) GST pull-down assay showing the direct interactions of FKF1 with RGA and GAI. Input FKF1-His were detected by immunoblot using anti-His antibody. The elution proteins were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) gel and probed with anti-His and anti-GST antibody, respectively. (**D**) Diagrams depicting the linear structures of FKF1, RGA and GAI. LOV: FKF1 N-terminal LOV domain; F-box: FKF1 F-box domain; Kelch: FKF1 C-terminal kelch repeat domain. DELLA: N-terminal DELLA domain of RGA and GAI; GRAS: C-terminal GRAS domain of RGA and GAI. (**E**) GST pull-down assay showing the direct interactions of the FKF1 Kelch repeat domain with RGA and GAI. Input His-FKF1-LOV, His-FKF1-F-box and His-FKF1-Kelch were detected by immunoblot using anti-His antibody. The elution proteins were separated by SDS-PAGE and probed with anti-His and anti-GST antibody, respectively. (**F**) GST pull-down assay showing the direct interactions of FKF1 with the GRAS domain of RGA or GAI. Input FKF1-His were detected by immunoblot using anti-His antibody. The elution proteins were separated by SDS-PAGE gel and probed with anti-His and anti-GST antibody, respectively.

FLAVIN-BINDING KELCH REPEAT F-BOX 1 promotes DELLA proteins ubiquitination and degradation

We next addressed whether the DELLA proteins RGA and GAI are ubiquitinated and degraded by FKF1. To facilitate detection of RGA or GAI ubiquitination by FKF1, the constructs *35S:RGA-Flag* and *35S:GAI-Flag* alone or together with *35S:FKF1-Myc* were transformed into tobacco leaves as described (Liu et al. 2010) to express RGA-Flag, GAI-Flag, RGA-Flag/FKF1-Myc and GAI-Flag/FKF1-Myc proteins, respectively. Total proteins were extracted and immuno-precipitated with an anti-Flag antibody and examined via immunoblot analysis with the anti-Flag antibody. As shown in Figure 4A, in addition to bands of the expected sizes for RGA-Flag or GAI-Flag, high molecular mass ubiquitinated smear ladders were observed in all the four samples. The same samples were immuno-analyzed with an anti-ubiquitin antibody, and these high molecular size bands in the RGA-Flag, GAI-Flag, RGA-Flag/FKF1-Mycand GAI-Flag/FKF1-Myc samples were recognized by an anti-ubiquitin antibody. In contrast, RGA-Flag- and GAI-Flag-specific polyubiquitinations were strongly promoted in the RGA-Flag/FKF1-Myc and GAI-Flag/FKF1-Myc samples (Figure 4A), indicating that FKF1 promoted ubiquitination of RGA and GAI. Furthermore, ubiquitination of RGA and GAI was detected in the samples without FKF1 (Figure 4A), which may be due to an ortholog of FKF1

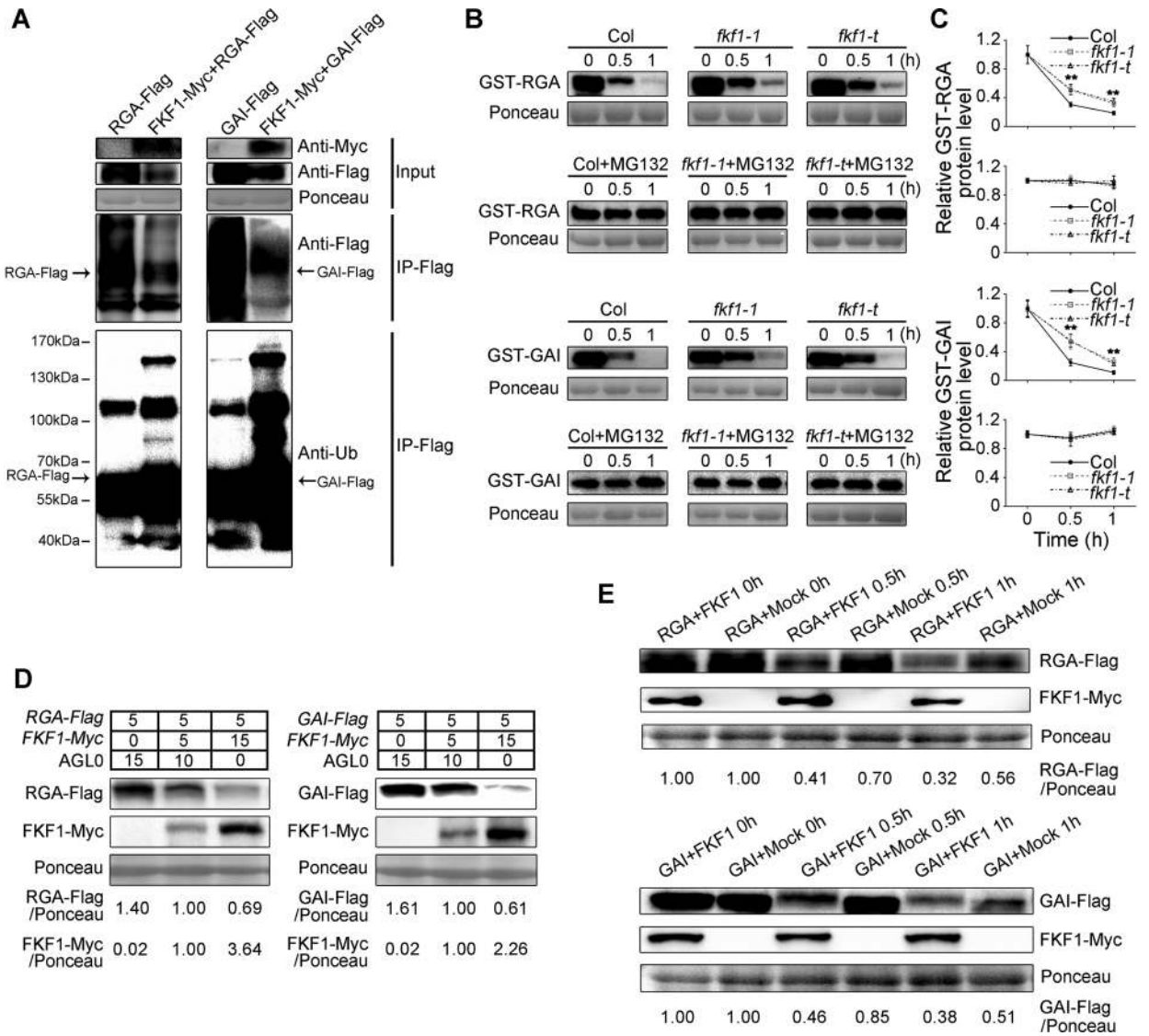

Figure 4. Continued

or another E3 ligase such as SLY1 (Dill et al. 2004) mediated ubiquitination of the RGA-Flag and GAI-Flag proteins in tobacco.

To further investigate whether DELLA proteins were degraded by FKF1, we first expressed recombinant DELLA proteins in *Escherichia coli* and examined their degradation using a cell-free system as described (Wang et al. 2009). In this experiment, GST-RGA or GST-GAI purified from *E. coli* was mixed with total protein extracts from the wild-type plants, *fkf1-1* or *fkf1-t* mutant plants and incubated for the indicated time. As shown in Figure 4B, C, the GST-RGA and GST-GAI proteins were degraded both in the wild-type plants and *fkf1-1* or *fkf1-t* mutant protein extracts, and mutation of FKF1 decreased the extent of GST-RGA and GST-GAI degradation. Moreover, GST-RGA and GST-GAI protein degradation was effectively blocked by the proteasomal inhibitor MG132. Next, we examined degradation of RGA and GAI *in vivo* as described (Liu et al. 2010) using coexpression of FKF1-Myc with RGA-Flag or GAI-Flag in the same area of tobacco leaves and found that RGA-Flag and GAI-Flag protein decreased with increasing amounts of FKF1-Myc (Figure 4D). We then separately expressed FKF1-Myc, RGA-Flag and GAI-Flag via different agroinfiltrations and mixed the samples followed by incubation for the indicated time. As shown in Figure 4E, protein signal of RGA-Flag and GAI-Flag were reduced at 0.5 h in the presence of FKF1-Myc; reductions of 68% in RGA-Flag and 62% in GAI-Flag were observed after 1 h of incubation with FKF1-Myc (compared to 44% and 49% in control samples, respectively). Degraded RGA-Flag or GAI-Flag protein was also observed in the control sample (Mock) without FKF1, possibly because tobacco contains similar E3 complexes or other E3 ligases, such as SLY1 homologs (Dill et al. 2004). Together, these results demonstrate that FKF1 likely promotes the degradation of RGA and GAI via the ubiquitin-proteasome pathway.

To determine whether the FKF1-mediated ubiquitination and degradation of DELLAs were modulated by light, we analyzed the effect of *FKF1* mutation on RGA protein accumulation and ubiquitination in response to blue light. To eliminate the effect of endogenous GA on DELLA protein stability in response to blue light, we compared protein levels of RGA in *ga1-3* versus *ga1-3/fkf1-1* plants and ubiquitination of TAP-tagged RGA in *35S:TAP-RGA* versus *35S:TAP-RGA/fkf1-1* plants treated with the GA synthesis inhibitor PAC under different blue light fluence rates. According to the results, FKF1-mediated protein degradation and ubiquitination of RGA or TAP-RGA were not modulated by blue light (Figure S6A, B). These results indicate that FKF1 likely promotes DELLA protein ubiquitination and degradation independent of blue light.

Figure 4. FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) promotes ubiquitination and degradation of REPRESSOR OF ga1-3 (RGA) and GA-INSENSITIVE (GAI)

(**A**) The ubiquitination analysis of RGA and GAI *in vivo*. *35S:RGA-Flag* and *35S:GAI-Flag* alone or together with *35S:FKF1-Myc* were transiently expressed in tobacco leaves. The leaves were infiltrated with MG132 for 12 h before being sampled. Immunoprecipitates against anti-Flag antibody (IP) or crude extracts (Input) were analyzed via immunoblots using anti-Flag antibody, anti-Myc antibody and anti-ubiquitin antibody, respectively. Ponceau staining was used as a loading control. Three separate experiments were conducted showing similar results. Ub is the abbreviation for ubiquitin. (**B**) Semi-*in vivo* degradation analysis of RGA and GAI protein. *Escherichia coli*-purified GST-RGA or GST-GAI protein mixed with total protein from the wild-type Col, *fkf1-1* or *fkf1-t* seedlings, and incubated for 0, 0.5, and 1 h. 50 μmol MG132 was used as the specific 26S proteasome degradation inhibitor. Protein levels were analyzed by immunoblot using anti-GST antibody. Ponceau staining was used as a loading control. (**C**) Relative protein level of GST-RGA and GST-GAI in (**B**). GST-RGA and GST-GAI protein levels were normalized to Ponceau. The value of the starting point was set to 1. Bars represent the *SD* of three biological replicates. Significant differences are indicated: $^{**}P < 0.01$ (Tukey's least significant difference test). (**D**) *In vivo* degradation of RGA and GAI was carried out by detecting the RGA-Flag and GAI-Flag protein levels in co-infiltration experiments with increasing amounts of FKF1-Myc. The RGA-Flag, GAI-Flag and FKF1-Myc proteins were detected using anti-Flag and anti-Myc antibody, respectively. Numbers indicate the ratio of the concentrations of *Agrobacteria* used in co-infiltration. Ponceau staining was used as a loading control. Protein level at the second lane was set to 1. Three separate experiments were conducted showing similar results. (**E**) Time-course of FKF1-promoted RGA and GAI degradation *in vivo*. RGA and GAI degradation was performed by mixing cell extracts from separately infiltrated RGA-Flag, GAI-Flag or FKF1-Myc samples. The RGA-Flag or GAI-Flag extract was mixed with FKF1-Myc extract or the wild-type control extract (Mock) and then incubated at 22°C for indicated times. Samples were collected at different time points for immunoblot assay. Ponceau staining was used as a loading control. Protein level at the first lane was set to 1. Three separate experiments were conducted showing similar results.

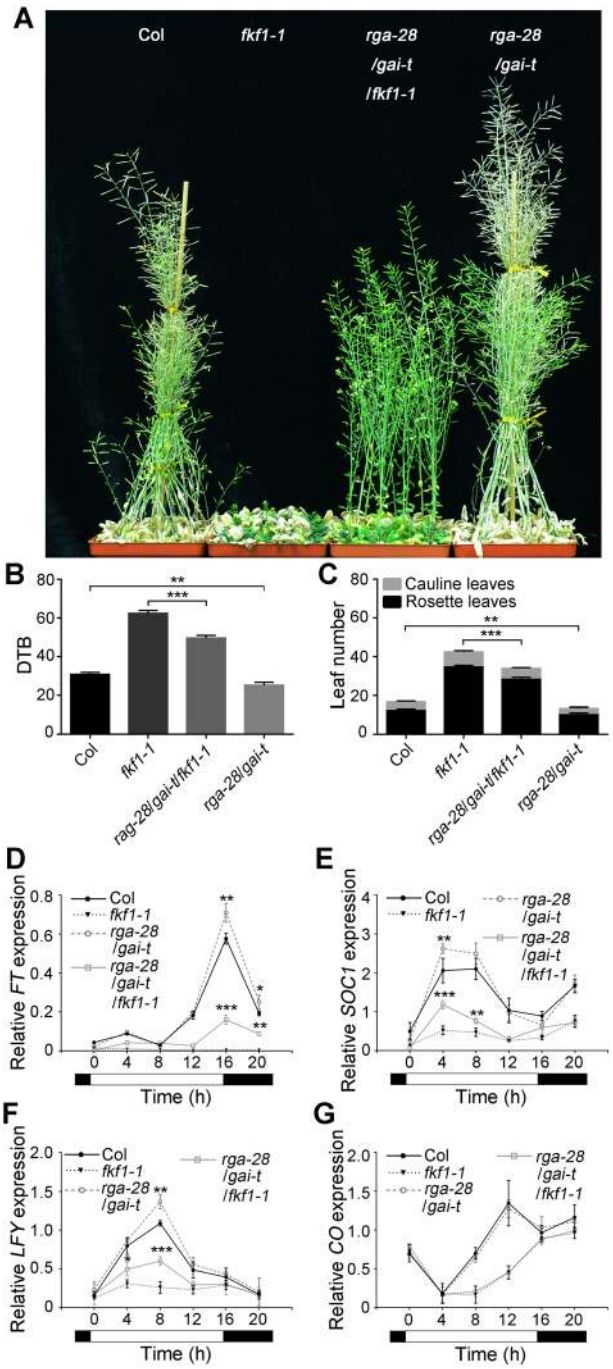

Figure 5. Mutations of *REPRESSOR OF ga1-3* (*RGA*) and *GA-INSENSITIVE* (*GAI*) can partially rescue the late-flowering phenotype of *flavin-binding kelch repeat f-bOX 1* (*fkf1-1*) plants

(**A**) Images of 60-d-old plants grown in soil under long-day conditions (LDs). (**B, C**) The days to bolting (DTB) (**B**) and the leaf number (**C**) at flowering of the respective genotypes are shown. Standard deviations ($n \geq 10$) are shown. (**D**-**G**) *FT* (**D**), *SOC1* (**E**), *LFY* (**F**), *CO* (**G**) expression levels in the wild-type Col, *fkf1-1*, *rga-28/gai-t/fkf1-1* and *rga-28/gai-t*. Plants were grown on 1/2 Murashige-Skoog (MS) medium under LDs for 12 d, samples were collected every 4 h for 1 d for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. *ACTIN2* served as the internal control. Bars represent the *SD* of three biological replicates. The white/black bars indicate light/dark phases. The time (hour) of light on at sample collection is set as 0. Significant differences are indicated: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Tukey's least significant difference test).

FLAVIN-BINDING KELCH REPEAT F-BOX 1 promotes flowering partially through DELLA under LDs

To evaluate the importance of DELLA protein accumulation in *fkf1* for the regulation of development, we crossed the *rga-28* and *gai-t* (T-DNA mutant *SALK_082622*) mutants into the *fkf1-1* background and analyzed flowering time under LDs. The late-flowering phenotype of *fkf1-1* was partially rescued by the *rga-28* and *gai-t* mutations (Figure 5A), which may be partly related to the functional redundancy of RGA and GAI with other DELLAs (Dill and Sun 2001; Gallego-Bartolome et al. 2010; Galvão et al. 2012). The triple mutant *rga-28/gai-t/fkf1-1* flowered when the plants had 28.27 ± 1.12 rosette leaves and 5.6 ± 0.49 cauline leaves (compared to the *fkf1-1* single mutant with 34.73 ± 0.85 rosette leaves and 7.8 ± 0.54 cauline leaves) and produced visible inflorescences at 12.8 d earlier under LDs than the *fkf1-1* single mutant (Figure 5B, C; Table S1). Consistent with the early-flowering phenotype, the triple mutant *rga-28/gai-t/fkf1-1* had

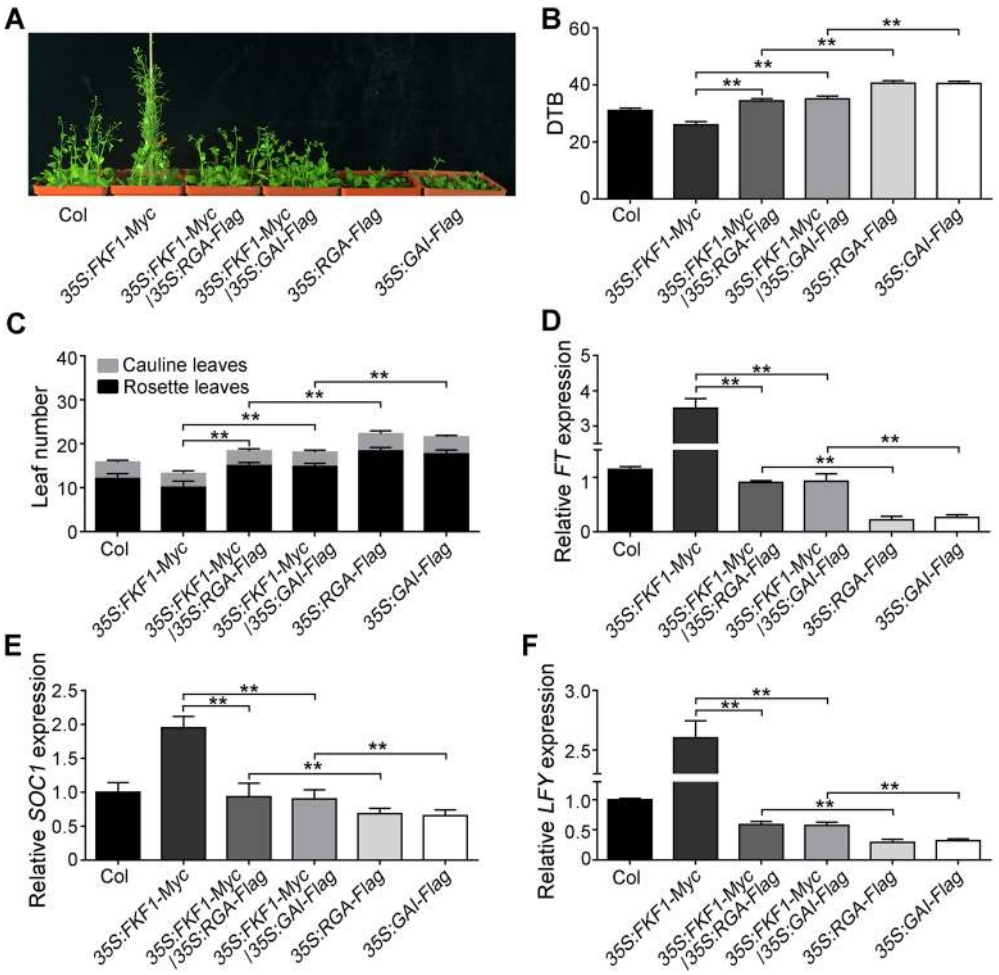

Figure 6. Overexpression of *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*FKF1*) can partially suppress the late-flowering phenotype of *REPRESSOR OF ga1-3* (*RGA*) or *GA-INSENSITIVE* (*GAI*) overexpressing plants

(**A**) Images of 36-d-old plants grown in soil under long-day conditions (LDs). (**B, C**) The days to bolting (DTB) (**B**) and the leaf number (**C**) at flowering of the respective genotypes are shown. *SD* ($n \geq 10$) are shown. Significant differences are indicated: $^{**}P < 0.01$ (Tukey's least significant difference test). (**D-F**) Relative messenger RNA (mRNA) expression of *FLOWERING LOCUS T* (*FT*) (**D**), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (**E**) and *LEAFY* (*LFY*) (**F**) expression in the respective genotypes are shown. Plants were grown in soil under LDs, 12-d-old seedlings were sampled at zeitgeber time (ZT)16, ZT4, and ZT8 for *FT*, *SOC1*, and *LFY* analysis. *ACTIN2* served as the internal control. Bars represent the *SD* of three biological replicates. Significant differences are indicated: $^{**}P < 0.01$ (Tukey's least significant difference test).

higher mRNA levels of *FT*, *SOC1* and *LFY*, which are flowering integrators (Blázquez et al. 1998; Kardailsky et al. 1999; Blázquez and Weigel 2000; Lee et al. 2000), than the *fkf1-1* single mutant (Figure 5D–F). However, the expression of *CO*, which is a crucial positive regulator for *FT* expression under LDs (Samach et al. 2000), was almost unchanged in *rga-28/gai-t/fkf1-1* compared with the *fkf1* mutant (Figure 5G). This was consistent with the notion that the DELLA proteins participate in the regulation of *FT* but not *CO* expression under LDs (Galvão et al. 2012; Wang et al. 2016). These data suggest that *FKF1* regulates flowering partially through DELLA.

To further elucidate the genetic interaction between FKF1 and DELLA, we generated transgenic plants overexpressing *FKF1*, *RGA*, or *GAI* under control of the CaMV 35S promoter (Figure S7; Table S1) and selected one line of the early-flowering transgenic plant *35S:FKF1-Myc* and late-flowering transgenic plant *35S:RGA-Flag* or *35S:GAI-Flag* for crossing to generate transgenic plants overexpressing *FKF1*, *RGA*, or *GAI*. Genetic analysis showed that *35S:FKF1-Myc* can partially suppress the late-flowering phenotype of *35S:RGA-Flag* and *35S:GAI-Flag* plants under LDs. The *35S:FKF1-Myc/35S:RGA-Flag* and *35S:FKF1-Myc/35S:GAI-Flag* plants flowered significantly earlier than the *35S:RGA-Flag* or

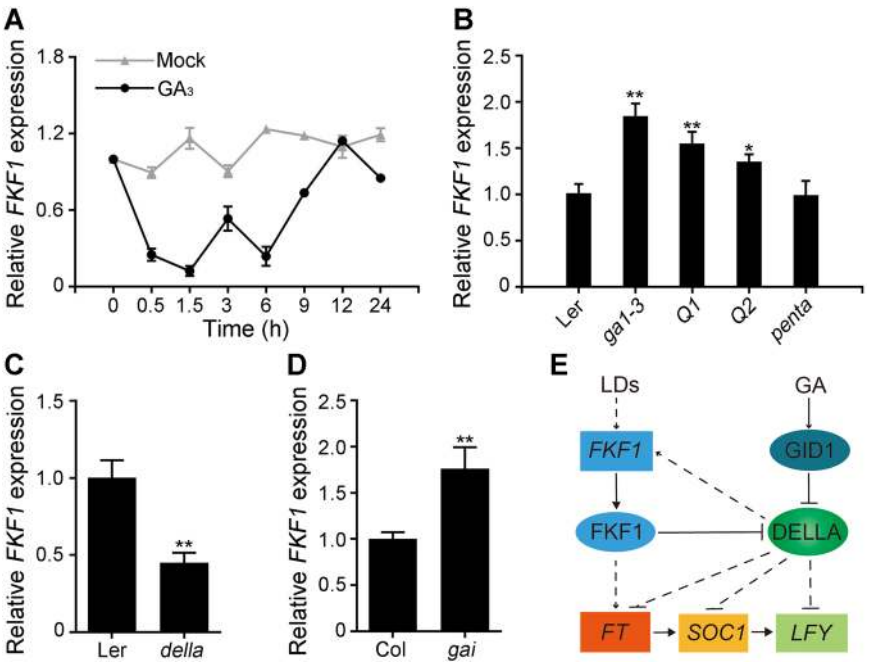

Figure 7. *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*FKF1*) messenger RNA (mRNA) expression is repressed by gibberellin (GA) and induced by DELLA

(**A**) *FKF1* mRNA expression in Col in response to GA. 12-d-old seedlings grown on 1/2 Murashige-Skoog (MS) medium under continuous white light (25 μmol/m^2/s) were treated with 100 μmol GA_3 or Mock (ethanol alone) for indicated times, and sampled for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. *ACTIN 2* served as the internal control. The value of the starting point was set to 1. Bars represent the *SD* of three biological replicates. (**B-D**) *FKF1* mRNA expression in (**B**) the wild-type *Ler*, *ga1-3* (*Ler*), Q1 (*ga1-3 gai-t6 rgl1-1 rgl2-1*), Q2 (*ga1-3 rga-t2 rgl1-1 rgl2-1*) and penta (*ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*), (**C**) the wild-type *Ler* and *della* (*gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-1*), (**D**) the wild-type Col and *gai* mutants. 12-d-old seedlings grown on 1/2 MS medium under continuous white light (25 μmol/m^2/s) were sampled for qRT-PCR analysis. *ACTIN2* served as the internal control. The mRNA level of *FKF1* in *Ler* (**B, C**) or Col (**D**) was set to 1. Bars represent the *SD* of three biological replicates. Significant differences are indicated: **$P < 0.05$, ***$P < 0.01$ (Tukey's least significant difference test). (**E**) A possible model depicts how FKF1 and DELLA coordinate to regulate flowering under long-day conditions (LDs). Photoperiod controlled FKF1 promotes flowering partially by interacting with GA signaling DELLA proteins and inducing their degradation, which inhibit *FT*, *SOC1*, and *LFY* mRNA expression. On the other hand, DELLA proteins induce *FKF1* mRNA expression. Squares and circles indicate genes and proteins, respectively. Arrows and bars represent positive and negative regulation, respectively. Dashed lines indicate that more than one step may be involved between the two points.

35S:GAI-Flag plants (Figure 6A–C; Table S1). Consistent with the flowering time, the *35S:FKF1-Myc/35S:RGA-Flag* and *35S:FKF1-Myc/35S:GAI-Flag* had higher mRNA levels of *FT*, *SOC1* and *LFY* than the *35S:RGA-Flag* and *35S:GAI-Flag* plants (Figure 6D–F). Taken together, these data suggest that *FKF1* regulates flowering partially through DELLA; FKF1 and DELLA might also act at least partially in parallel and converge on the same targets.

FLAVIN-BINDING KELCH REPEAT F-BOX 1 expression is induced by DELLA

Because FKF1 promoted flowering partially by modulating DELLA abundance, we assessed whether *FKF1* itself could be regulated by GA. Interestingly, the transcriptional level of *FKF1* was reduced by exogenous GA_3 treatment in the wild-type plants. The *FKF1* mRNA level was decreased immediately after GA_3 treatment, dropped to the lowest level within 1.5 h, and then increased to the pretreated level after 24 h (Figure 7A). Our results were consistent with a previous DNA microarray study, in which expression of *FKF1* showed a 5-fold reduction in the wild-type seedlings at 0.5 h after GA_4 treatment (GSE6150, http://www.ncbi.nlm.nih.gov/geo/) (De Grauwe et al. 2007). To determine whether FKF1 protein expression was regulated by GA, we detected protein levels following treatment with GA_3 in *FKF1* transgenic plants (*35S:GFP-FKF1*) by immunoblotting. However, we found the protein level of FKF1 was unchanged with continuous or transient GA_3 treatment under our growth conditions (Figure S8A, B). We did not detect change of the protein level of FKF1 after GA synthesis inhibitor PAC treatment (Figure S8A). These results indicate that *FKF1* is repressed by GA at the transcriptional, but not post-transcriptional, level under the conditions used.

To further confirm *FKF1* regulation by the GA pathway, we investigated *FKF1* expression in *ga1-3* mutants (wild-type for five DELLAs) in the *Ler* background (Sun et al.1992) and plants carrying various DELLA mutations, such as Q1 (*ga1-3 gai-t6 rgl1-1 rgl2-1*, wild-type for RGA and RGL3), Q2 (*ga1-3 rga-t2 rgl1-1 rgl2-1*, wild-type for GAI and RGL3), and penta (*ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*, wild-type for RGL3) (Qi et al. 2014). Consistent with GA-induced repression of *FKF1*, we noted higher levels of *FKF1* expression in the *ga1-3* mutant than that in the wild-type *Ler* (Figure 7B), and the expression of *FKF1* was rescued by the penta mutant (*ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*, wild-type for RGL3) (Figure 7B). In addition, we found that the *della* mutant (*gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-1*) showed significantly decreased *FKF1* expression level (Figure 7C), while the *gai* mutant (Figure 7D), which is a dominant DELLA mutant (Fu et al. 2004; Li et al. 2015), and *RGA* and *GAI*-overexpressing transgenic plants (*35S:RGA-Flag*; *35S:GAI-Flag*) showed increased *FKF1* expression levels (Figure S9). Furthermore, we found that *DELLA* mutation impaired the GA-induced repression of *FKF1* (Figure S10). Taken together, these results suggest that the DELLA proteins induce *FKF1* expression, and GA repress *FKF1* expression in a DELLA dependent manner.

DISCUSSION

FLAVIN-BINDING KELCH REPEAT F-BOX 1 plays a positive role in regulating GA response in flowering via controlling DELLA protein abundance

FLAVIN-BINDING KELCH REPEAT F-BOX 1 was important for regulation of photoperiodic flowering in *Arabidopsis*. The *FKF1*-overexpressing plants flowered earlier than the wild-type plants, and the *fkf1* mutants flowered later than the wild-type plants in LDs (Nelson et al. 2000; Imaizumi et al. 2003; Lee et al. 2017). The late flowering of *fkf1* mutant could be rescued by GA treatment in continuous illumination (Nelson et al. 2000). In agreement with this finding, we showed that the flowering is significantly accelerated by GA treatment under LDs in the *fkf1* mutant (Figure 1A, B). Interestingly, we have observed that FKF1 positively regulates plant flowering in response to GA (Figures 1, S1). Gibberellin signaling promotes growth by initiating the degradation of DELLA proteins (Mutasa-Gottgens and Hedden 2009). Mutation in *SLY1*, which positively regulates GA signaling, results in partial insensitivity to GA, accumulation of DELLA proteins and late-flowering phenotype in *Arabidopsis* (McGinnis et al. 2003). Similarly, consistent with the reduced sensitivity to exogenous GA, our biochemical data showed that RGA DELLA protein accumulated in the *fkf1* mutant (Figure 2A, B). By contrast, *FKF1* overexpressing plants were more sensitive to GA, and showed lower RGA DELLA protein abundance compared with the wild-type plants (Figure 2A, B). Thus, FKF1 likely plays a positive role in regulating GA response in flowering via controlling DELLA protein abundance.

Possible mechanism of FKF1 in regulating DELLA protein abundance

Previous studies showed that flowering under LDs was delayed by reducing GA biosynthesis (Porri et al. 2012), and increased GA levels were coordinated with floral transition under LDs (Andrés et al. 2014). GA has been reported to induce DELLA protein degradation to relieve *Arabidopsis* growth restraint (Dill et al. 2001; Silverstone et al. 2001). We therefore hypothesized that GA biosynthesis was altered in the *fkf1* mutant, which further regulated DELLA protein abundance. Our data showed that the transcription of some GA biosynthetic and catabolic genes changed by *fkf1-1*; however, the bioactive GA_4 level in the *fkf1-1* mutant did not show obvious change (Figure S3A–C). Although we did not observe GA content difference between *fkf1-1* and wild-type plants, we could not rule out the involvement of GA synthesis in the regulation of DELLA by FKF1. As the levels of GA content were very low in our measurements, it was possible that the difference between *fkf1-1* and wild-type plants was not detected due to technical issues.

Surprisingly, we found that the DELLA protein RGA was accumulated in the absence of GA, and normally degraded by GA treatment in the *fkf1* mutant (Figure 2G, H). We therefore speculated that FKF1, similar to DE-ETIOLATED 1(DET1) (Li et al. 2015), could modulate DELLA protein levels via mechanisms other than the GA pathway. Previous studies have demonstrated that FKF1 had E3-ubiquitin ligase activity (Imaizumi et al. 2005) which led us to think about the possibility that FKF1 might function as an E3 to degrade DELLA protein. As expected, FKF1 was found to physically interact with DELLA proteins through its C-terminal Kelch repeat domain and promote their ubiquitination and degradation (Figures 3, 4). According to these data, we conclude that FKF1 might function as an E3 ubiquitin ligase to negatively regulate DELLA protein stability.

FLAVIN-BINDING KELCH REPEAT F-BOX 1 has been identified as a blue-light photoreceptor (Nelson et al. 2000; Imaizumi et al. 2003). However, FKF1-DELLA interaction, FKF1-mediated protein degradation and ubiquitination of RGA or TAP-RGA were not modulated by blue light in our study (Figures S5, S6). The mRNA and protein level of *FKF1* were reported to be controlled by the circadian clock (Nelson et al. 2000; Imaizumi et al. 2003). The DELLA protein levels have also been shown to oscillate with a daily rhythm under LDs (Wang et al. 2016). When the FKF1 protein level increased during the day (Imaizumi et al. 2003), DELLA protein level decreased (Wang et al. 2016). We thus speculate that FKF1 might regulate the rhythmical degradation of DELLA proteins. Interestingly, the RGA and TAP-RGA protein were more stable in the *fkf1* mutant background, while less stable in the *35S:GFP-FKF1* overexpression plants during the day (Figure 2C–F). Our findings suggest that FKF1 might specifically mediate cyclic degradation of DELLA. It remains to be tested whether FKF1-DELLA interaction and FKF1 mediated ubiquitination of DELLA are modulated by day length.

Recently, it was reported that the core clock component GIGANTEA (GI), which stabilizes FKF1 especially during the day under LDs (Fornara et al. 2009), stabilized DELLA proteins during the day in SDs (Nohales and Kay 2019). A similar phenomenon was observed in the FKF1- and GI-dependent regulation of CO protein. FKF1 directly interacts and stabilizes CO protein in the afternoon (Song et al. 2012), while GI negatively regulates CO protein stability in the morning in LDs (Song et al. 2014). FLAVIN-BINDING KELCH REPEAT F-BOX 1 homolog ZEITLUPE (ZTL) was proposed to be involved in FKF1- and GI-dependent CO protein regulation, as GI stabilized ZTL protein (Kim et al. 2007) and ZTL could change the intracellular localization of FKF1 (Takase et al. 2011) and CO protein profile (Song et al. 2014). We therefore hypothesized that other factors might be involved in FKF1- and GI-dependent DELLA protein regulation under different photoperiods.

FLAVIN-BINDING KELCH REPEAT F-BOX 1 is regulated by DELLA

Although lacking a canonical DNA binding domain, DELLA can modulate gene expression by interacting with other transcription factors. DELLA was reported to interact with PHYTOCHROME INTERACTING FACTORS (PIFs) (De Lucas et al. 2008; Feng et al. 2008), BRASSINAZOLE RESISTANT1 (BZR1) (Bai et al. 2012; Gallego-Bartolome et al. 2012), SPL (Yu et al. 2012), CO (Wang et al. 2016; Xu et al.2016), bHLH48, bHLH60 (Li et al. 2017) and WRKY75 (Zhang et al. 2018) to repress their transcriptional activity. Interestingly, in addition to negative regulation of DELLA protein stability, FKF1 itself was observed to be regulated by DELLA at the transcriptional level (Figures 7B–D, S9). A transcriptional/post-translational feedback regulatory loop was formed

between FKF1 and DELLA. Similar feedback mechanism of transcription regulation was reported for the SCARECROW-LIKE 3 (SCL3), a GRAS protein but without the GA-responsive DELLA domain (Zhang et al. 2011). SCL3 is induced by DELLA and directly downregulates its own expression by interfering with DELLA (Zentella et al. 2007; Zhang et al. 2011). Whether DELLAs directly induce *FKF1* expression by interacting with regulatory factor(s) awaits further investigation.

Our western blot analysis showed that the FKF1 protein level was not significantly altered in either GA- or PAC-treated plants (Figure S8), implying that the signal transduction occurred at the transcriptional but not post-transcriptional level. However, we could not exclude that the strong 35S promoter masked the regulation at the protein level, as it was reported that if GAI accumulated to a very high level then it could not be efficiently degraded even in the presence of GA (Fleck and Harberd 2002). Thus, a weaker promoter would be used in the future to investigate whether GA regulates FKF1 at the post-transcriptional level.

FLAVIN-BINDING KELCH REPEAT F-BOX 1-DELLA interaction integrates the photoperiod and GA signaling to regulate flowering

Recently, numerous studies have demonstrated that the photoperiod and GA pathways act in concert to promote flowering in response to inductive LDs (Galvão et al. 2012; Porri et al. 2012; Wang et al. 2016), but the molecular mechanisms underlying their interactions were not fully understood. Our results suggested that photoperiod and GA pathways might work in concert partially through a direct physical interaction between FKF1 and DELLA. The binding of FKF1 to DELLA promoted DELLA ubiquitination and degradation, and subsequently induced floral initiation. However, *rga* and *gai* null allele only partially suppressed the late-flowering phenotype in the *fkf1* mutant (Figure 5A–C). This could be partially because of functional redundancy of RGA and GAI with other DELLAs (Dill and Sun 2001; Gallego-Bartolome et al. 2010; Galvão et al.2012).

The photoperiod and GA pathways converge on some common floral integrators, such as *FT*, *SOC1*, and *LFY* (Blázquez and Weigel 2000; Lee et al. 2000; Samach et al. 2000; Boss et al. 2004). It has been reported that FT protein could move from phloem cells in leaf toward the apex to start floral initiation by activating floral integrator gene *SOC1* and meristem identity genes such as *LFY* and *AP1* in the shoot apical meristem (Yu et al. 2006; Corbesier et al. 2007; Jaeger and Wigge 2007; Mathieu et al. 2007; Turck et al. 2008; Wellmer and Riechmann 2010). *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* is positively regulated by FT, and acts at least in part downstream of *FT* to promote plant flowering (Moon et al. 2005; Yoo et al. 2005). Furthermore, SOC1 has been reported to directly bind to *LFY* promoter to activate *LFY* expression, and act partially upstream of *LFY* to promote flowering (Lee et al. 2000; Moon et al. 2003, 2005). Our results showed that the expression of *FT*, *SOC1* and *LFY* were increased in the triple mutant *rga-28/gai-t/fkf1-1* compared with the *fkf1-1* single mutant (Figure 5D–F). We therefore conclude that FKF1 regulates flowering at least partially through DELLA/FT.

In addition, we found that overexpression of *FKF1* partially suppressed the late-flowering phenotype and enhanced the *FT*, *SOC1,* and *LFY* expression in *35S:RGA-Flag* and *35S:GAI-Flag* transgenic plants under LDs (Figure 6). Previously, Hisamatsu and King discussed an FT-independent role of GA application, which could rescue the late-flowering phenotype in the *ft-1* mutant under LDs (Hisamatsu and King 2008). Gibberellin and DELLA proteins were also reported to regulate the expression of *FT* in leaves and *SPLs* and MADS box gene *FUL* at the shoot apex, respectively under LDs (Galvão et al. 2012; Porri et al. 2012; Yu et al. 2012). DELLA protein also regulated the transcription of *SOC1* by interacting with NF-Y (Hou et al. 2014). Additionally, the day length and GA are reported to converge upstream of *LFY*. GA could promote flowering by activating the promoter of *LFY* (Blázquez et al. 1998; Blázquez and Weigel 2000). Therefore, FKF1 might act in parallel with DELLA and converge on the same targets, which needs to be further confirmed by additional genetic data.

Based on the previous findings and results from this study, we proposed a possible model to illustrate how FKF1 and DELLA coordinate to regulate flowering under LDs (Figure 7E). In brief, FKF1, which was controlled by the photoperiod pathway (Nelson et al. 2000; Imaizumi et al. 2003), directly interacted with DELLA proteins, which functioned as repressors in the GA signaling pathway (Mutasa-Gottgens and Hedden 2009), and induced their degradation. The

degradation of DELLA proteins induced *FT*, *SOC1*, and *LFY* expression and promoted flowering by releasing activity of transcription factors, such as CO (Wang et al. 2016; Xu et al. 2016), WRKY75 (Zhang et al. 2018), HLH48, bHLH60 (Li et al. 2017), and other unidentified factor(s). On the other hand, DELLA proteins could induce expression of *FKF1*, and GA-induced inhibition of *FKF1* expression was dependent on DELLA. Thus, our results suggest a FKF1-DELLA feedback loop. This interlocking feedback loop at least in part contributes to the fine-tuning of flowering in response to environmental cues and endogenous GA signal.

MATERIALS AND METHODS

Plant material and growth conditions

The *Arabidopsis* mutants and transgenic lines used in this study are in the Columbia (Col) or *Landsbergerecta* (*Ler*) background. The *fkf1-1* (Nelson et al. 2000; Liu et al.2013), *ga1-3* (Col) (Tyler et al. 2004), *rga-28* (Col) (Qin et al. 2014), *della* (*gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-1*; *Ler*), *gai* (Col), and *35S:TAP-RGA* transgenic plant (*Ler*) (Feng et al. 2008; Li et al. 2016a), *ga1-3* (*Ler*), Q1 (*ga1-3 gai-t6 rgl1-1 rgl2-1*; *Ler*), Q2 (*ga1-3 rga-t2 rgl1-1 rgl2-1*; *Ler*), and penta (*ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*; *Ler*) mutants (Qi et al. 2014) used in this study were described previously. *fkf1-t* (SALK_059480; Col) and *gai-t* (SALK_082622; Col) mutant seeds were obtained from *Arabidopsis* Biological Resource Center (ABRC). The *rga-28/gai-t* mutant was prepared by crossing *rga-28* with *gai-t*. The *ga1-3/fkf1-1*, *rga-28/gai-t/fkf1-1*, *35S:TAP-RGA/fkf1-1*, *35S:TAP-RGA/fkf1-t* were prepared by crossing *fkf1-1* with *ga1-3* (Col), *rga-28/gai-t*, or *35S:TAP-RGA*, and *fkf1-t* with *35S:TAP-RGA* transgenic plant. The genotyping of the *fkf1-1* and *ga1-3* alleles was performed as described (Sun et al. 1992; Nelson et al. 2000). All seeds were surface-sterilized (with 15% (v/v) bleach for 5 min) and washed five times with sterile water, then cultured in Petri dishes on 1/2 MS solid medium (1/2 MS salts, 1% Suc, and 0.8% agar) and incubated at 4 °C for 4 d. Plants were then grown in an incubator at 22 °C under continuous white light (24 h light photoperiod) or LDs (16 h light/8 h dark photoperiod), or grown in soil in a culture room at 22 °C under LDs. LED cool white fluorescent lights were used as the white light sources in all photoperiodic studies. LED-B (peak: 470 nm, half band width: 30 nm) was used for blue light source as described (Zhao et al. 2007). Fluence rates of white and blue light were measured using a Li-250 quantum photometer (LI-COR).

Plasmid construction and plant transformation

To generate *35S:GFP-FKF1* construct, the full-length coding sequences of the *FKF1* gene were cloned into the pMDC43-GFP vector under the control of the CaMV 35S promoter via recombination-based cloning as described in our previous study (Peng et al. 2012). To generate *35S:FKF1-Myc*, *35S:RGA-Flag* and *35S:GAI-Flag* constructs, full-length coding sequences of the *FKF1*, *RGA*, and *GAI* genes were cloned into pCambia1300-Myc or pCambia1300-Flag vector under the control of the CaMV 35S promoter by using the In-Fusion cloning system (Nanjing Vazyme Biotech Co., Ltd.), respectively. The primers used are listed in Table S2. The constructs were then transformed into the wild-type Col using the floral-dip method (Clough and Bent 1998). The T_0 seeds of *35S:GFP-FKF1*, *35S:FKF1-Myc*, *35S:RGA-Flag* and *35S:GAI-Flag* were selected on 1/2 MS medium containing 50 μg/mL hygromycin, and the hygromycin-resistant plants were transferred to soil 10 d after germination and grown in a culture room at 22 °C. Transgenic lines for *35S:GFP-FKF1*, *35S:FKF1-Myc* showing early-flowering phenotype, and *35S:RGA-Flag* and *35S:GAI-Flag* showing late-flowering phenotype were selected for further analysis.

Gibberellin and paclobutrazol treatment

For flowering analysis, plant seeds were sown on soil in a culture room at 22 °C. Seven d after germination, 100 μmol/L GA_3 or mock was sprayed to the seedlings once every 2 d until bolting as described (Galvão et al. 2012). Flowering time was measured by counting the days to bolting, the rosette leaves numbers and the cauline leaf number at flowering. More than 10 plants were measured for each genotype.

For mRNA or protein analysis, 12-d-old seedlings grown on 1/2 MS solid media were transferred and immersed in 1/2 MS liquid media containing 10^{-2} μmol/L PAC, 100 μmol/L GA_3 or Mock (ethanol alone) for indicated times, and sampled. Each experiment was biologically repeated three times.

Messenger RNA and protein analysis

For mRNA analysis, total RNA was isolated using TRIzol reagent (RNAiso Plus, TaKaRa). First-stand complementary DNAs were synthesized using

primeScript RT reagent Kit With gDNA Eraser (TaKaRa, Japan) following the manufacturer's instruction. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with Mx3000 Real-time PCR System (Stratagene) using SYBR green (Takara, Japan) according to the manufacturer's instructions. The primers used for qRT-PCR analysis are listed in Table S2. The relative expression levels were measured as described in our previous study (Peng et al. 2012), and the expression level of *ACTIN2* was performed as internal reference to normalize each data point, representing the average of three biological replicates.

For protein analysis, total proteins were extracted and separated on 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis gels (SDS-PAGE) and transferred to nitrocellulose membranes for immunoblots. The blots were probed by anti-Myc antibody (M20002M; Lot 294175; Abmart), anti-RGA antibody (AS111630; Agrisera), anti-GFP antibody (M0802-3a; Abicode), anti-Flag antibody (M20008M; Lot 293674; Abmart) or anti-Actin antibody (M20009M; Lot 274572; Abmart) for the loading control. The immunoblot signals were quantified using Image J (http://rsb.info.nih.gov/ij/).

Bimolecular fluorescence complementation assay

The BiFC assay was performed as described in our previous study (He et al. 2017). The encoding sequences of FKF1 were cloned into pSAT1-nVenus-N to generate a C-terminal in-frame fusion with nVenus, and DELLAs were inserted into pSAT1-cCFP-N to form a C-terminal in-frame fusion with cCFP using primers listed in Table S2. The constructs were introduced into *Arabidopsis* protoplasts via polyethylene glycol-mediated transformation (Yoo et al. 2007). After 12 h of incubation in the dark, the fluorescence emission of GFP was observed under a confocal microscope (Nikon).

GST pull-down assay

The coding regions of full-length FKF1 were cloned into the pET-28a vector, and FKF1 Kelch domain, LOV domain, or F-box domain, were cloned into pCold-TF vector, respectively. The coding sequences of full-length *RGA* and *GAI*, RGA(GAI)-DELLA domain, RGA(GAI)-GRAS domain, were cloned into pGEX-4T-1 vector, respectively. The primers used are listed in Table S2. The plasmids were then introduced into *Escherichia coli* strain BL21 (DE3) to induce protein expression. The *E.coli*-expressed FKF1-His, His-FKF1-LOV, His-FKF1-F-box and His-FKF1-Kelch fusion proteins were purified by Ni-NTA Agarose (Invitrogen; R901-01; Lot 60-0441). The *E.coli*-expressed GST-RGA, GST-GAI, GST-RGA-DELLA, GST-RGA-GRAS, GST-GAI-DELLA and GST-GAI-GRAS were purified by Pierce™ Glutathione Agarose (Thermo; 16100; Lot TL278186). The GST pull-down assay was conducted as described (Du et al. 2016).

Co-immunoprecipitation assay

For Co-IP experiments using the agro-infiltrated tobacco was conducted as previously described (Sparkes et al. 2006; Meng et al. 2013). Full-length coding regions of *FKF1* and *DELLA* genes were cloned into pCambia1300-Myc and pCambia1300-Flag vector under the control of the CaMV 35S promoter to generate *35S:FKF1-Myc* and *35S:DELLA-Flag* constructs, respectively. The construct *35S:DELLA-Flag* alone or together with the *35S:FKF1-Myc* were expressed in tobacco leaves by *Agrobacterium* infiltration. Co-immunoprecipitation assay was performed as described in our previous study (He et al. 2017). Total proteins were extracted and incubated with Red anti-c-Myc Affinity Gel (Sigma; E6654; Lot SLBQ8241V) overnight for immunoprecipitation. The beads were washed three times with wash buffer, and protein complexes were eluted from beads with 1 × SDS loading buffer and subjected to immunoblot analysis. The blots were probed by anti-Myc and anti-Flag antibody, respectively.

Cell-free degradation and ubiquitination assay

Cell-free degradation assay was performed as previously described (Wang et al. 2009). Total proteins were extracted in degradation buffer containing 25 mmol/L Tris-HCl, pH 7.5, 10 mmol/L NaCl, 10 mmol/L $MgCl_2$, 4 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L dithiothreitol, and 10 μmol/L adenosine triphosphate (ATP). The MG132 was added as indicated. The concentration of total protein extracts prepared from *fkf1-1*, *fkf1-t* and the wild-type Col control seedlings were adjusted to equal with degradation buffer. A total of 300 ng of the *E. coli*-purified GST-RGA or GST-GAI protein was added to the extracts (100 μL, 200 μg) for the individual assays, and incubated in growth chamber under continuous white light for

indicated times. Samples were taken to determine RGA and GAI protein abundance by immunoblots using anti-GST antibody (Abmart; M20007M; Lot 303959). Each experiment was biologically repeated three times.

Ubiquitination assay was conducted as previously described (Liu et al. 2010). The *35S:RGA-Flag* and *35S:GAI-Flag* constructs were infiltrated separately, or co-infiltrated with the *35S:FKF1-Myc* into the leaves of tobacco by *Agrobacterium AGL0*. The leaves were treated with 50 μmol/L MG132 for 12 h before being sampled. Total proteins were isolated and incubated with Anti-DYKDDDDK (FLAG) affinity gel (Biotool; B23102; Lot 710029) overnight for immunoprecipitation. The affinity gels were washed three times with wash buffer, and protein complexes were eluted from affinity gels with 1 × SDS loading buffer and subjected to immunoblot analysis. The blots were probed by anti-Myc antibody, anti-Flag antibody, and anti-ubiquitin antibody (Amylet Scientific; BML-PW8810-0100), respectively. Each experiment was biologically repeated three times.

In vivo and semi-*in vivo* protein degradation

The *in vivo* and semi-*in vivo* protein degradation experiments were performed as previously described (Liu et al. 2010). For *in vivo* protein degradation analysis, *Agrobacterial* strain AGL0 carrying *35S:FKF1-Myc* with *35S:RGA-Flag* or *35S:GAI-Flag* constructs were co-infiltrated into tobacco leaves at different ratios as indicated. Three days after infiltration, samples were collected for immunoblot analysis. For semi-*in vivo* protein degradation analysis, *Agrobacterial* strains carrying *35S:FKF1-Myc*, *35S:RGA-Flag* or *35S:GAI-Flag* construct were infiltrated into tobacco leaves separately. Three days after infiltration, samples were collected. Total proteins were extracted in native extraction buffer 1 (NB1) as described (Liu et al.2010). A final concentration of 10 μmol/L ATP was added to the cell lysates to preserve the function of the 26S proteasome. The RGA-Flag and GAI-Flag extracts were then mixed with FKF1-Myc or mock (the wild-type control) extract in a volume ratio of 1:1, respectively. The mixtures were incubated at 22 ℃ under white light for indicated times. Samples were taken at different time points for immunoblot analysis. The blots were probed by anti-Myc antibody and anti-Flag antibody, respectively.

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AUTHOR CONTRIBUTIONS

J.Y. and X.L. performed most of the research and J.Y. drafted the manuscript. B.Z. performed some expression analyses. M.Z. and J.Y. carried out some western bolt experiments. P.Y. and X.L. performed plant phenotype analysis. C.H. and J.L. revised the manuscript. J.Y., X.L., and X.Z. designed the experiments, supervised the study, and revised the manuscript. All authors read and approved of its content.

CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

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SUPPORTING INFORMATION

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Figure S1. *FKF1* mutation reduces the gibberellin (GA) response in flowering

(**A, B**) The days to bolting (DTB) (**A**) and the rosette leaf number (RLN) (**B**) at flowering of the respective genotypes in response to GA_3 are shown. (**C**) The reduction ratio of DTB and RLN of the respective genotypes in response to GA_3 are shown. The reduction ratio was calculated as (DTB (Mock)-DTB (GA)): DTB (Mock) and (number of leaves (Mock)-number of leaves (GA)): number of leaves (Mock), respectively. Seven-d-old seedlings of *fkf1-1*, *ga1-3* and *ga1-3/fkf1-1* mutant grown in soil under long days (LDs) were sprayed with 100 μmol GA_3 or Mock (ethanol alone) once every 2 d until bolting. Standard deviations ($n \geq 10$) are shown. Significant differences are indicated: $^{**}P < 0.01$, $^{***}P < 0.001$ (Tukey's least significant difference test).

Figure S2. The identification and phenotype of *35S:GFP-FKF1* transgenic plants

(**A**) Schematic diagram of *35S:GFP-FKF1* construct. 35S: CaMV 35S promoter, *GFP*: *GFP* tag. (**B**) Immunoblots showing the expression of GFP-FKF1 fusion protein in the wild-type Col and the representative *35S:GFP-FKF1* transgenic plants. Ponceau staining was used as a loading control. (**C**) The green fluorescent protein (GFP) fluorescence in the root of 5-d-old *35S:GFP-FKF1* seedlings grown in continuous white light. (**D, E**) The days to bolting (DTB) (**D**) and the leaf number (**E**) at flowering of the wild-type Col and *35S:GFP-FKF1* transgenic plants grown under long days (LDs). Standard deviations ($n \geq 10$) are shown. Significant differences between the wild-type and the transgenic plants are indicated: $^{*}P < 0.05$ (Tukey's least significant difference test).

Figure S3. Messenger RNA (mRNA) expression of gibberellin (GA)-metabolic, catabolic and responsive genes in *fkf1-1* mutant

(**A, B**) Relative mRNA expression of GA metabolic genes in *ga1-3* (Col) and *ga1-3/fkf1-1* mutant (**A**), and GA catabolic genes in the wild-type Col and *fkf1-1* mutant (**B**). Seedlings were grown on 1/2 Murashige-Skoog (MS) medium under long days (LDs) for 12 d and sampled at zeitgeber time (ZT) 12 for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. *ACTIN 2* served as the internal control. The mRNA expression level of each corresponding gene in *ga1-3* (**A**) or Col (**B**) was set to 1. Bars represent the *SD* of three biological replicates. (**C**) Endogenous GA_4 level in the wild-type Col and *fkf1-1* mutant seedlings. Seedlings were grown on 1/2 MS medium under LDs, and sampled at 7 d and 12 d for GA_4 analysis. Bars represent the *SD* of three biological replicates. (F.W.: fresh weight). (**D, E**) Relative mRNA expression of GA-responsive genes *EXPANSIN A8* (*EXP8*) (**D**) and *PACLUBUTRAZOL RESISTANCE 1* (*PRE1*) (**E**) in the wild-type Col, and *fkf1-1* mutant seedlings in response to paclobutrazol (PAC). 12-d-old-seedlings grown on 1/2 MS medium under LDs were transferred and immersed in 1/2 MS liquid medium containing 0 or 10^{-2} μmol PAC for 3 h, and sampled for qRT-PCR analysis. *ACTIN 2* served as the internal control. The mRNA expression level of each corresponding gene in the Mock-treated wild-type Col was set to 1. Bars represent the *SD* of three biological replicates. (**F**) The reduction ratio of *EXP8* and *PRE1*expression level in the respective genotypes in response to PAC in (**D, E**) are shown. The reduction ratio of expression level was calculated as (Relative expression level (Mock) - Relative expression level (PAC)): Relative expression level (Mock). Significant differences between the wild-type and mutant are indicated: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Tukey's least significant difference test).

Figure S4. Interaction of FKF1 with DELLA proteins RGL1, RGL2 or RGL3 *in vivo*

(**A**) The bimolecular fluorescence complementation (BiFC) assays showing the interaction of FKF1 with DELLA proteins RGL1, RGL2, and RGL3 in plant cell. Green fluorescent protein (GFP) images for the interaction of FKF1-nVenus with RGL1-cCFP, RGL2-cCFP, or RGL3-cCFP were observed using GFP filter. Chlorophyll autofluorescence is shown as red. The negative controls failed to yield detectable green fluorescence. Bar: 50 μm. (**B**) Co-immunoprecipitation (Co-IP) experiment showing FKF1 interacts with RGL3, but not with RGL1 and RGL2. Co-IP experiments were performed in tobacco. Different cell lysates or a lysate mixture (RGL1-Flag, RGL2-Flag, or RGL3-Flag with FKF1-Myc) were immunoprecipitated with anti-Myc antibodies. These immunoprecipitates were then separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis and immunoblotted with anti-Myc antibody and anti-Flag antibody, respectively.

Figure S5. FKF1 interacts with REPRESSOR OF ga1-3 (RGA) and GA-INSENSITIVE (GAI) independent of blue light

Co-immunoprecipitation (Co-IP) experiments showing the interactions of FKF1 with RGA or GAI both in the

dark and blue light. Co-IP was performed using HEK293T cells. HEK293T cells were co-transfected to express the indicated proteins, exposed to blue light (100 mmol m^2/s) for 2 h, and immunoprecipitated by green fluorescent protein antibody (GFP-IP). The IP signal (FKF1-GFP/GFP) or the Co-IP signals (Myc-RGA and Myc-GAI) were detected by immunoblots probed with anti-GFP antibody and anti-Myc antibody, respectively.

Figure S6. FKF1 regulates REPRESSOR OF ga1-3 (RGA) protein stability and ubiquitination independent of blue light

(**A**) Representative immunoblots showing protein level of RGA in *ga1-3* and *ga1-3*/*fkf1-1* mutant in response to blue light. 10-d old seedlings grown in continuous white light were dark-adapted for 3 d, and then transferred to different fluence rates of blue light (0, 30, 100 μmol/m^2/s) for 4 h, and sampled for protein analysis. Three separate experiments were conducted showing similar results. (**B**) Representative immunoblots showing ubiquitination of TAP-RGA protein in *35S:TAP-RGA* and *35S:TAP-RGA*/*fkf1-1* mutant in response to blue light. 10-d old seedlings (treated with 5 μg/mL paclobutrazol (PAC)) grown in continuous white light were dark-adapted for 3 d, and then transferred to different fluence rates of blue light (0, 30, 100 μmol/m^2/s) for 4 h, and sampled for protein analysis. Total proteins were immunoprecipitated using anti-Myc antibody, and then analyzed by western blot using antibodies to Myc, or ubiquitin. Three separate experiments were conducted showing similar result. Ub is the abbreviation for ubiquitin.

Figure S7. The identification and phenotype of *35S:FKF1-Myc*, *35S:RGA-Flag* and *35S:GAI-Flag* transgenic plants

(**A**) Schematic diagram of *35S:FKF1-Myc*, *35S:RGA-Flag* and *35S:GAI-Flag* construct. 35S: CaMV 35S promoter, *Myc*: *Myc* tag, *Flag*: *Flag* tag. (**B**) Immunoblots showing the expression of FKF1-Myc, RGA-Flag and GAI-Flag fusion protein in the wild-type Col and the representative transgenic plants. Ponceau staining was used as a loading control. (**C**) The days to bolting (DTB) and the leaf number at flowering of the wild-type Col and *35S:FKF1-Myc* transgenic plants. Standard deviations ($n \geq 10$) are shown. Significant differences between the wild-type and the transgenic plants are indicated: $*P < 0.05$ (Tukey's least significant difference test). (**D**) The DTB and the leaf number at flowering of the wild-type Col and *35S:RGA-Flag* and *35S:GAI-Flag* transgenic plants. Standard deviations ($n \geq 10$) are shown. Significant differences between the wild-type and the transgenic plants are indicated: $**P < 0.01$ (Tukey's least significant difference test).

Figure S8. The green fluorescent protein (GFP)-FKF1 protein level was not regulated by gibberellin (GA)

(**A**) Immunoblots showing GFP-FKF1 fusion protein level in 12-d-old *35S:GFP-FKF1* seedlings treated with GA_3 or paclobutrazol (PAC). Plants were grown on the 1/2 Murashige-Skoog (MS) solid medium supplemented with or without 100 μmol GA_3 or 10^2/μmol PAC under continuous white light. Total proteins were analyzed by immunoblot using anti-GFP antibody. A nonspecific band was used as a loading control. (**B**) GFP-FKF1 fusion protein level in 12-d-old *35S:GFP-FKF1* seedlings in response to GA. Seedlings were grown on the 1/2 MS solid medium for 12 d under continuous white light, and then transferred and immersed in 1/2 MS liquid medium containing 100 μmol GA_3 and incubated for indicated time. Total proteins were analyzed by immunoblot using anti-GFP antibody. A nonspecific band was used as a loading control. NC: nonspecific band.

Figure S9. *FKF1* messenger RNA (mRNA) expression was induced by REPRESSOR OF ga1-3 (RGA) and GA-INSENSITIVE (GAI)

The wild-type Col, *35S:RGA-Flag* and *35S:GAI-Flag* plants were grown on 1/2 Murashige-Skoog (MS) solid medium under continuous white light for 12 d, and sampled for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. *ACTIN2* served as the internal control. The mRNA expression level of *FKF1* in the wild-type Col was set to 1. Bars represent the *SD* of three biological replicates. Significant differences between the wild-type and transgenic plants are indicated: $*P < 0.05$ (Tukey's least significant difference test).

Figure S10. Gibberellin (GA) inhibits *FKF1* messenger RNA (mRNA) expression in a DELLA dependent manner

FKF1 mRNA expression in the wild-type *Ler* and *della* mutant in response to GA. 12-d-old seedlings grown on 1/2 Murashige-Skoog (MS) medium under continuous white light (25 μmol/m^2/s) were treated with

100 μmol GA_3 or Mock (ethanol alone) for indicated time, and sampled for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. *ACTIN 2* served as the internal control. The value of the starting point of *Ler* was set to 1. Bars represent the *SD* of three biological replicates.

Table S1. Flowering time of plants used in this study

Table S2. Primer sequences used in this study