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A photo-responsive F-box protein FOF2 regulates floral initiation by promoting *FLC* expression in Arabidopsis

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SUMMARY

Floral initiation is regulated by various genetic pathways in response to light, temperature, hormones and developmental status; however, the molecular mechanisms underlying the interactions between different genetic pathways are not fully understood. Here, we show that the photoresponsive gene *FOF2* (*F-box of flowering 2*) negatively regulates flowering. *FOF2* encodes a putative F-box protein that interacts specifically with ASK14, and its overexpression results in later flowering under both long-day and short-day photoperiods. Conversely, transgenic plants expressing the F-box domain deletion mutant of FOF2 (FOF2ΔF), or double loss of function mutant of *FOF2* and *FOL1* (*FOF2-LIKE 1*) present early flowering phenotypes. The late flowering phenotype of the *FOF2* overexpression lines is suppressed by the *flc-3* loss-of-function mutation. Furthermore, *FOF2* mRNA expression is regulated by autonomous pathway gene *FCA*, and the repressive effect of *FOF2* in flowering can be overcome by vernalization. Interestingly, *FOF2* expression is regulated by light. The protein level of FOF2 accumulates in response to light, whereas it is degraded under dark conditions via the *26S* proteasome pathway. Our findings suggest a possible mechanistic link between light conditions and the autonomous floral promotion pathway in Arabidopsis.

Keywords: F-box protein, FOF2, *FLOWERING LOCUS C*, flowering, light, autonomous pathway, *Arabidopsis thaliana*.

INTRODUCTION

The strict regulation of floral initiation is essential for plant reproduction because it enables the completion of seed development under favorable environmental conditions. Over the past four decades, many key regulators of flowering time have been identified in Arabidopsis by isolating and characterizing early and late flowering mutants. Flowering is affected by the photoperiod, ambient temperature, plant hormones and plant age, and approximately six genetic pathways for the promotion or repression of flowering have been identified in Arabidopsis, including photoperiod, temperature, vernalization, gibberellin (GA) biosynthesis, autonomous and aging pathways. In addition, light quality and biotic and abiotic stresses can contribute to floral induction in plants (Amasino, 2010; Song *et al.*, 2013).

The photoperiodic flowering is regulated by light signal and circadian clock. Both of these two factors converge to regulate the expression of *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*). CO is a zinc-finger transcription factor that promotes flowering through directly activating *FT* expression under long-day (LD) conditions (Samach *et al.*, 2000). Photoreceptors such as cryptochrome 2 (CRY2), phytochrome A (PhyA), phytochrome B (Phyb), and the LOV-domain F-box proteins FLAVIN-BINDING KELCH REPEAT 1 (FKF1), ZEITLUPE (ZTL) and LOV KELCH PROTEIN 2 (LKP2), mediate photoperiodic and light control of *CO* and *FT* expression and affect flowering time (Golembeski and Imaizumi, 2015). Blue light receptors CRY2 and FKF1 have been reported to promote *FT* expression by stabilizing CO protein in the late afternoon under LD and blue light

conditions (Zuo *et al.*, 2011; Song *et al.*, 2012, 2014). ZTL, opposite to that of FKF1, destabilizes CO protein in the morning under LD conditions (Zuo *et al.*, 2011; Song *et al.*, 2012, 2014). ZTL/FKF1/LKP2 family proteins also allow *CO* transcription by reducing CYCLING DOF FACTOR 2 (CDF2) abundance (Fornara *et al.*, 2009). The red/far-red light receptor phyA, antagonistic to phyB, stabilizes CO protein to facilitate the transcription of *FT* in the afternoon under LD and far-red light conditions (Valverde *et al.*, 2004).

FT is also regulated by the floral repressor FLOWERING LOCUS C (FLC; Michaels and Amasino, 1999). FLC was first identified genetically as an inhibitor of flowering that plays a central role in the timing of the transition to flowering in Arabidopsis (Koornneef *et al.*, 1991), and is negatively regulated by prolonged cold treatment (vernalization; Sheldon *et al.*, 1999).

Autonomous pathway genes promote flowering by repressing the expression of *FLC* (Simpson, 2004; Streitner *et al.*, 2008; Zhai *et al.*, 2016). For example, FCA promotes flowering by suppressing *FLC* through alternative cleavage and polyadenylation of its embedded antisense RNAs (Manzano *et al.*, 2009; Liu *et al.*, 2010). FY interacts with FCA and is required for the negative autoregulation of FCA expression and the function of FCA in the control of flowering time. FCA and FY work together to select the proximal polyadenylation site in antisense *FLC* RNAs, thereby controlling the floral transition (Henderson *et al.*, 2005). FPA represses *FLC* expression through 3′-end processing of antisense *FLC* RNAs, although the mechanism is not clear (Michaels and Amasino, 2001; Duc *et al.*, 2013). FLD is a homolog of human lysine-specific demethylase 1 (LSD1), which was found to affect the histone acetylation state of the *FLC* locus (He *et al.*, 2003; Singh *et al.*, 2014), and is required for the function of FCA and FPA in the downregulation of *FLC* (Liu *et al.*, 2007; Baurle and Dean, 2008). FVE is a component of a histone deacetylase complex, and inhibits *FLC* expression by promoting the deacetylation of *FLC* chromatin (Ausin *et al.*, 2004). *Arabidopsis thaliana* DNA-binding protein phosphatase (AtDBP1) and *ARABIDOPSIS THALIANA* GLYCINE-RICH PROTEIN 7 (AtGRP7) promote flowering at least partially through the repression of *FLC* expression, although this mechanism is not well understood (Streitner *et al.*, 2008; Zhai *et al.*, 2016).

In addition to the autonomous pathway, genes involved in pathways for temperature, abiotic stresses or other parameters also control *FLC* expression. The cold-activated INDUCER OF CBP EXPRESSION 1 (ICE1) induces *FLC* directly by binding to its promoter, thereby resulting in delayed flowering (Lee *et al.*, 2015). ABSCISIC ACID-INSENSITIVE 4 (ABI4) is a key component of the ABA signaling pathway, and it promotes *FLC* transcription by binding directly to its promoter and negatively regulates the floral transition (Shu *et al.*, 2016). AGAMOUS-LIKE 6 (AGL6) is a floral promoter that negatively regulates *FLC*, although the mechanism is unclear (Yoo *et al.*, 2011). These results suggest that FLC is a common key regulator of the flowering pathway.

As members of the SCF (Skp1/Cullin or CDC53/F-box protein) complex, F-box proteins mediate protein degradation via the *26S* proteasome by specifically identifying and combining with target proteins, and they also play essential roles in plant growth and development (Sadanandom *et al.*, 2012). For example, Arabidopsis SLEEPY1 (SLY1) and its homolog SNEEZY (SNE)/SLY2 are involved in gibberellic acid (GA) signaling and mediate the degradation of DELLA proteins (Fu *et al.*, 2004; Ariizumi *et al.*, 2011), and TIR1 target auxin/indole-3-acetic acid (AUX/IAA) factors to control the transcriptional responses to auxins (Maraschin *et al.*, 2009). In addition, F-box genes have been shown to regulate self-incompatibility (Ushijima *et al.*, 2003; Entani *et al.*, 2014) and floral development (Ni *et al.*, 2004). It has been demonstrated that ZTL, FKF1 and LKP2 play vital roles in circadian regulation and flowering time control (Han *et al.*, 2004; Baudry *et al.*, 2010; Song *et al.*, 2012). There are more than 700 F-box genes in the Arabidopsis genome (Gagne *et al.*, 2002), although it is unclear whether direct links occur between other F-box genes and flowering.

Here, we identified an F-box gene *At1 g55660* named *FOF2* (*F-box of flowering 2*) that regulates the floral transition in Arabidopsis. We demonstrate that *FOF2* is a photoresponsive gene, regulated by the autonomous pathway, and promotes *FLC* expression to inhibit flowering. Our results suggest a possible mechanistic link between light conditions and the autonomous floral promotion pathway.

RESULTS

FOF2 overexpression delays flowering

To identify novel F-box genes that regulate flowering, we conducted large-scale gain-of-function screening by cloning approximately 664 F-box genes (Gagne *et al.*, 2002) into Myc-tagged pEarleyGate203 (N-Myc) vectors under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. By surveying the phenotypes of transgenic lines, we identified two F-box genes, *FOF1* (*F-box of flowering 1*; Wang, 2012) and *FOF2*, which were named according to the order of their discovery. *FOF2* expression under the control of the CaMV *35S* promoter resulted in later flowering under both long-day (LD) and short-day (SD) conditions (Figure 1a and b). The mRNA and protein levels of *FOF2* accumulated in the transgenic lines (Figure 1c and d), demonstrating that *FOF2* was overexpressed. Hereafter, the transgenic lines are named *MycFOF2ox*. The *MycFOF2ox* lines flowered with an average of 23 leaves under LD conditions and 71 leaves under SD conditions (compared with 11 and 53 leaves under LD and SD conditions for the wild type, respectively; Table S1), and produced visible inflorescences approximately 10 and 35 days

later under LD and SD conditions, respectively, compared with that of the wild-type controls (Figure 1e; Table S1). These results indicated that *MycFOF2ox* lines flowered much later under SD conditions compared with LD conditions; thus, *FOF2* transgenic plants retain a photoperiodic response.

Mutation both of *FOF2* and its homolog *FOL1* accelerates flowering in *Arabidopsis*

The derived FOF2 protein contains at least three different domains (Figure S1a), and its N-terminal domain exhibits homology with F-box proteins (Figure S1b). The T-DNA insertion mutants *fof2-1* (*SALK_016168C*) and *fof2-2* (*SALK_061523C*), which were identified to be null mutants, exhibit no phenotypic alterations compared with wild-type

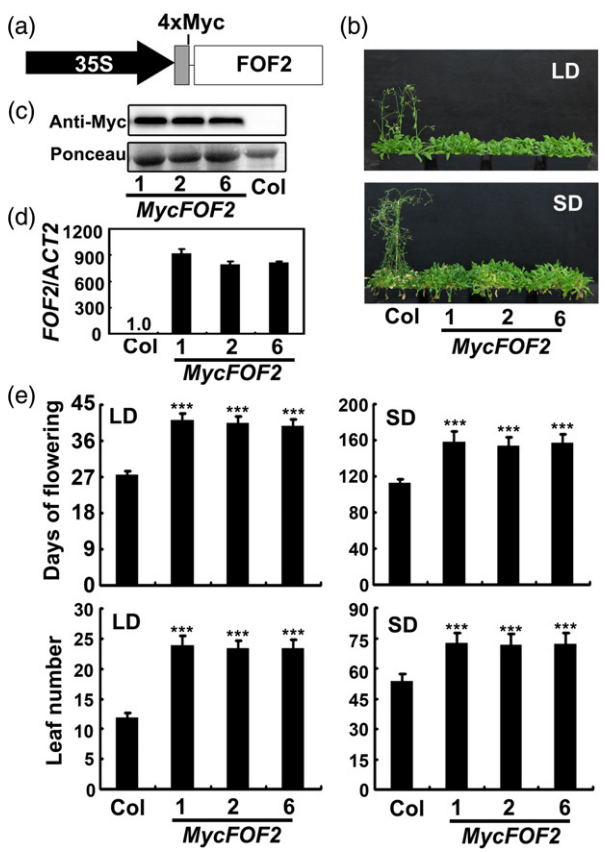

Figure 1. Overexpression of *FOF2* results in later flowering. (a) Expression construct of the *FOF2* gene under the control of the CaMV *35S* promoter. 35S, CaMV *35S* promoter; Myc, Myc tag. (b) 35- and 145-day-old *FOF2* transgenic plants grown under long days (LD; 16-h light/8-h dark) or short days (SD; 8-h light/16-h dark), respectively. (c) Immunoblots showing the expression of MycFOF2 fusion protein in *FOF2* transgenic plants and the wild type (Col); Ponceau staining was used as a loading control. (d) The mRNA expression level of *FOF2* in *FOF2* transgenic plants and the wild type (Col). *FOF2* expression was normalized to *ACTIN 2* (*ACT2*) expression. Bars represent the standard deviations of three independent experiments. (e) The days to flower and the number of rosette leaves at the day floral buds became visible. Standard deviations ($n \geq 20$) are shown. Significant differences between the wild-type and the transgenic lines are indicated: $^{***}P \leq 0.001$ (Tukey's least significant difference test).

Col-0 (Appendix S1; Figure S2). This could be because of functional redundancy with other F-box protein(s). Therefore, we used a typical dominant-negative mutation approach (Margottin *et al.*, 1998; Hart *et al.*, 1999; Risseeuw *et al.*, 2013). This strategy exploits the role of the F-box domain as a major protein–protein interaction domain of F-box proteins; therefore, the overexpression of an F-box deletion mutation could cause a dominant-negative loss-of-function phenotype. We prepared transgenic lines overexpressing the F-box deletion mutant of *FOF2*, named *MycFOF2ΔF* (Figure S3a–d). The *MycFOF2ΔF* lines flowered earlier and produced less leaves at flowering relative to the wild type under LD and SD conditions (Figure S3e; Table S2). These results suggest a role for FOF2 in regulating floral initiation, and that FOF2 might function redundantly with other F-box protein(s).

A blast search showed that Arabidopsis has two closely related homologs *At4 g00315* (= 0.0) and *At4g15060* (= $1e^{-28}$), named *FOL1* (*FOF2-LIKE 1*) and *FOL2* (Figure S4). The T-DNA insertion mutants *fol1-1* (*CS26289*) and *fol1-2* (*CS26467*), which were identified to be null mutants, did not show any phenotypic alterations compared with wild-type L*er* (Appendix S1; Figure S5). Because the null mutant of *fof2* and *fol1* are in the Col and L*er* backgrounds, respectively, we therefore generated the double mutant of *FOF2* and *FOL1* by using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (Figure 2a), which allows multiplex genome editing (Li *et al.*, 2013). Target mutations induced by Cas9 in the target regions were a one-nucleotide (1-nt) insertion in *FOL1* and *FOF2*, or a 4-nt deletion in *FOF2* and a 1-nt deletion in *FOL1* (Figure 2b). Both of these, named *CR-fof2-fol1-m1* and *CR-fof2fol1-m2*, had an early flowering phenotype, as measured by the number of leaves or by days to flowering at bolting (Figure 2c, d; Table S3). These results suggested that *FOF2* functions with *FOL1* at lest partially redundantly in regulating flowering time.

FOF2 is a nuclear protein

To determine the cellular localization pattern of the FOF2 protein, transiently transformed *Nicotiana benthamiana* (tobacco) expressing GFP-FOF2 or FOF2-GFP fusion proteins were analyzed. In transgenic tobacco, a GFP signal was detected in the nuclei of epidermal cells (Figure 3a), suggesting that FOF2 might form an Skp, Cullin, F-box containing complex (SCF complex) in which the FOF2 protein provides a binding site to regulate the protein levels of target transcription factors, which then regulate the transcription of target genes.

FOF2 interacts with the ASK14 protein

The F-box protein interacts with Skp1 in the SCF complex (Gagne *et al.*, 2002). There are 21 Skp1 homologs, ASKs, in the Arabidopsis genome. It was reported that proteins

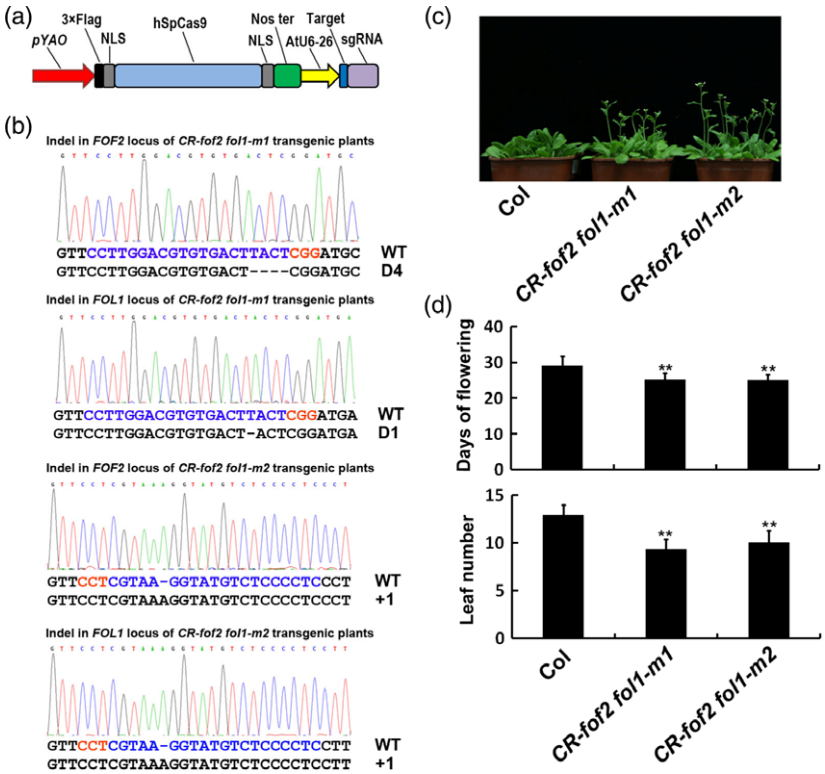

Figure 2. Double mutants with *FOF2* and *FOL1* generated by targeted gene editing showing early flowering. (a) Structure of the CRISPR/Cas9 vector. The expression cassette of hSpCas9 is driven by the *YAO* promoter, whereas sgRNA is driven by the AtU6-26 promoter. (b) Sequences of single mutant alleles of *FOF2* and *FOL1* identified from *CR-fof2 fol1-m1* and *CR-fof2 fol1-m2* transgenic plants. The wild-type sequence is shown at the top, with the PAM sequence highlighted in red and the target sequence highlighted in blue. +, insertion; D, deletion. (c) 27-day-old transgenic plants of *CR-fof2 fol1-m1* and *CR-fof2 fol1-m2* grown under long days (LD). (d) The days to flower and the number of rosette leaves at the day floral buds became visible. Standard deviations ($n \geq 40$) are shown: $^{**}P \leq 0.01$ (Tukey's least significant difference test).

carrying leucine rich repeat (LRR) and FBD domains showed a preference for ASK3 and ASK4, followed by ASK1, ASK2 and ASK11–ASK14 (Kuroda *et al.*, 2012). FOF2 contains the LRR and FBD domain (Figure S1), and we therefore checked the interaction of FOF2 with ASK1–ASK4 and ASK11–ASK14 by bimolecular fluorescence complementation (BiFC) assay. FOF2 interacts with ASK13 and ASK14 in nucleus Arabidopsis protoplasts (Figures 3b and S6). FOF2 with an F-box domain deletion can nullify these interactions (Figure 3b), suggesting that the F-box domain is required for the interaction of FOF2 with ASK13 and ASK14. Consistent with the BiFC result, FOF2 interacts with ASK14 in the coimmunoprecipitation experiment (Figure 3c); however, ASK13-Flag was not pulled down by Myc-FOF2, although ASK13 and FOF2 combined show a strong BiFC signal (Figure 3b and c). Because many factors, such as protein expression levels, protein folding efficiency and protein stability, may lead to false-positive/-negative BiFC results (Lalonde *et al.*, 2008; Kudla and Bock, 2016), further studies are needed to confirm the interaction with ASK13 and FOF2.

Expression of *FOF2* during floral transition

Because *FOF2* is involved in the control of flowering time, we examined *FOF2* expression in developing seedlings under LD conditions and found that *FOF2* expression levels decreased at day 7 after germination, and then remained at lower levels from days 7 to 19 during floral transition (Figure 4a). The *FOF2* homolog, *FOL1*, showed a similar expression pattern in developing seedlings (Figure S7a). A similar result was also observed in the *FLC* (Shen *et al.*, 2014; Figure S7b). We then examined the protein level of FOF2 in developing seedlings using transgenic plants expressing the MycFOF2 fusion protein, because none of the antibodies we prepared against FOF2 recognized the endogenous FOF2. The immunoblot results showed that the protein level of FOF2 remained unchanged during days 4–8 after germination, and then decreased during the floral transition that occurred 10–14 days after germination in our study (Figure 4b and c); however, the mRNA expression of the *MycFOF2* (*35S::Myc-FOF2*) transgene was nearly unchanged in the developing seedlings (Figure S7c), which suggests that *FOF2* is regulated by developmental stages at both the transcriptional and protein levels under the conditions tested. The expression pattern of *FOF2* in developing seedlings is well correlated with its negative role in floral transition.

FOF2 acts as a positive regulator of *FLC* mRNA expression

To determine the molecular mechanism by which *FOF2* regulates flowering, we compared the gene expression profiles of the wild-type Col4 and the *MycFOF2ox* plants using RNA-seq (Appendix S2). Expression of *FOF2* in the transgenic plants was higher than that in the wild-type plants (Table S4), which is consistent with our qRT-PCR results (Figure 1d). A number of repressors of flowering in

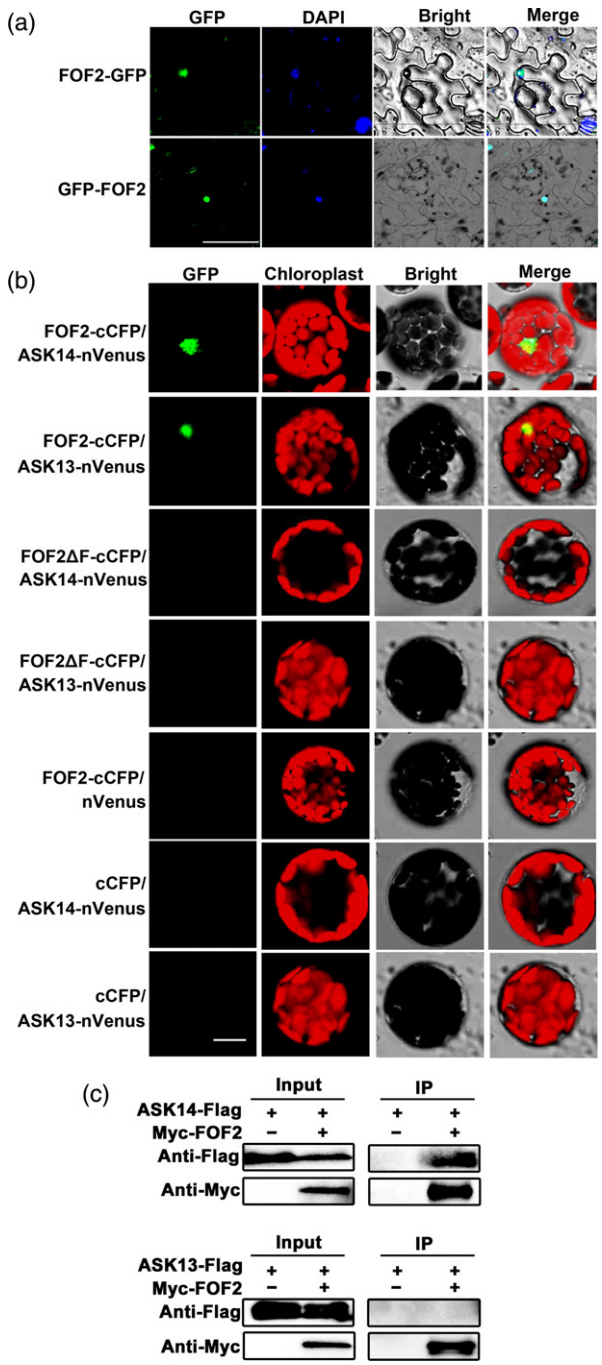

Figure 3. FOF2 expressed in nucleus and interacts with ASK14. (a) FOF2 expressed in nucleus. Subcellular localization of fused GFP-FOF2 or FOF2-GFP in *Nicotiana benthamiana* epidermal cells. The GFP and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) fluorescence images were taken from the same cell and merged by the MERGE program in PHOTOSHOP (Merge). Scale bar: 50 μm. (b) Bimolecular fluorescence complementation (BiFC) experiment showing the interactions of ASK13 and ASK14 with FOF2, but not with FOF2ΔF in Arabidopsis protoplasts. The GFP and chloroplast fluorescence images were taken from the same protoplasts and merged by the MERGE program in PHOTOSHOP (Merge). Scale bar: 10 μm. (c) Coimmunoprecipitation (Co-IP) experiment showing the interactions of FOF2 with ASK14, but not with ASK13. Co-IP was performed using tobacco. Immunoprecipitates against anti-Myc antibody (IP) or crude extracts (Input) were analyzed via immunoblots using anti-Myc or -Flag antibody.

Arabidopsis, including the key floral repressor *FLC* and its close homologs *MADS AFFECTING FLOWERING 4* (*MAF4*) and *MAF5* (Ratcliffe *et al.*, 2003) were upregulated, and integrators that promote flowering, including *FT* and *SOC1*, were downregulated in the *MycFOF2ox* plants (Table S4), and their expression levels were then confirmed by qRT-PCR. Consistent with the RNA-seq results, upregulation of *FLC* and downregulation of *FT* and *SOC1* were observed in the *MycFOF2ox* plants under LDs, and the same expression profile was also observed under SDs (Figure 5a). *MAF4* and *MAF5* were only moderately induced under LDs, and no difference was observed between the wild-type and *MycFOF2ox* plants under SDs (Figure S8a). *FT* and *SOC1* have been reported to be targets of *FLC*, and their expression was repressed by *FLC* (Helliwell *et al.*, 2006). Thus, we predicted that *FOF2* could delay flowering by inducing *FLC* expression, which suppresses the expression of *FT* and *SOC1*.

To further investigate the genetic interaction between *FOF2* and *FLC*, the effect of an *flc* mutation on the phenotype of *MycFOF2ox* was determined. Accordingly, we crossed the *flc-3* mutant, which lacks full-length *FLC* but has a functional *FRI* (Michaels and Amasino, 2001), into

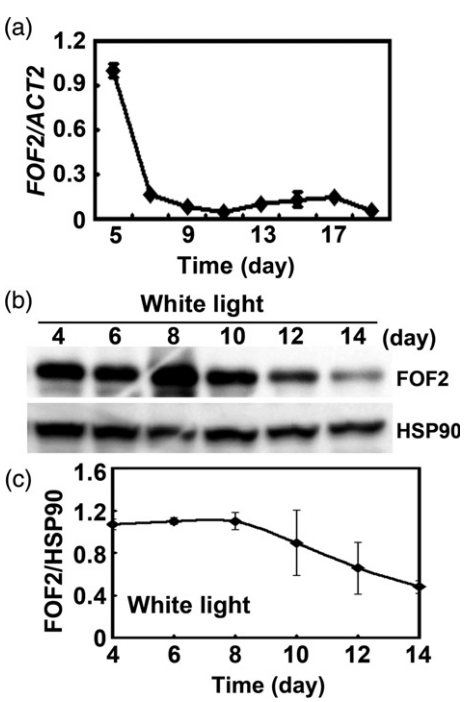

Figure 4. Expression of *FOF2* in a developing seedling of Arabidopsis. (a) Temporal expression of *FOF2* in wild-type developing seedlings grown under long days (LD). Gene expression was normalized to *ACT2* expression. Bars represent the standard deviations of three independent experiments. (b) Immunoblots showing the expression of the MycFOF2 fusion protein in Myc*FOF2ox* seedlings grown under LD conditions. The anti-HSP90 antibody was used as the loading controls. Levels of protein expression are shown as the representative immunoblots. (c) The relative protein expression level of FOF2 (mean ± SE) was calculated from triplicate independent reactions. The error bars represent SDs.

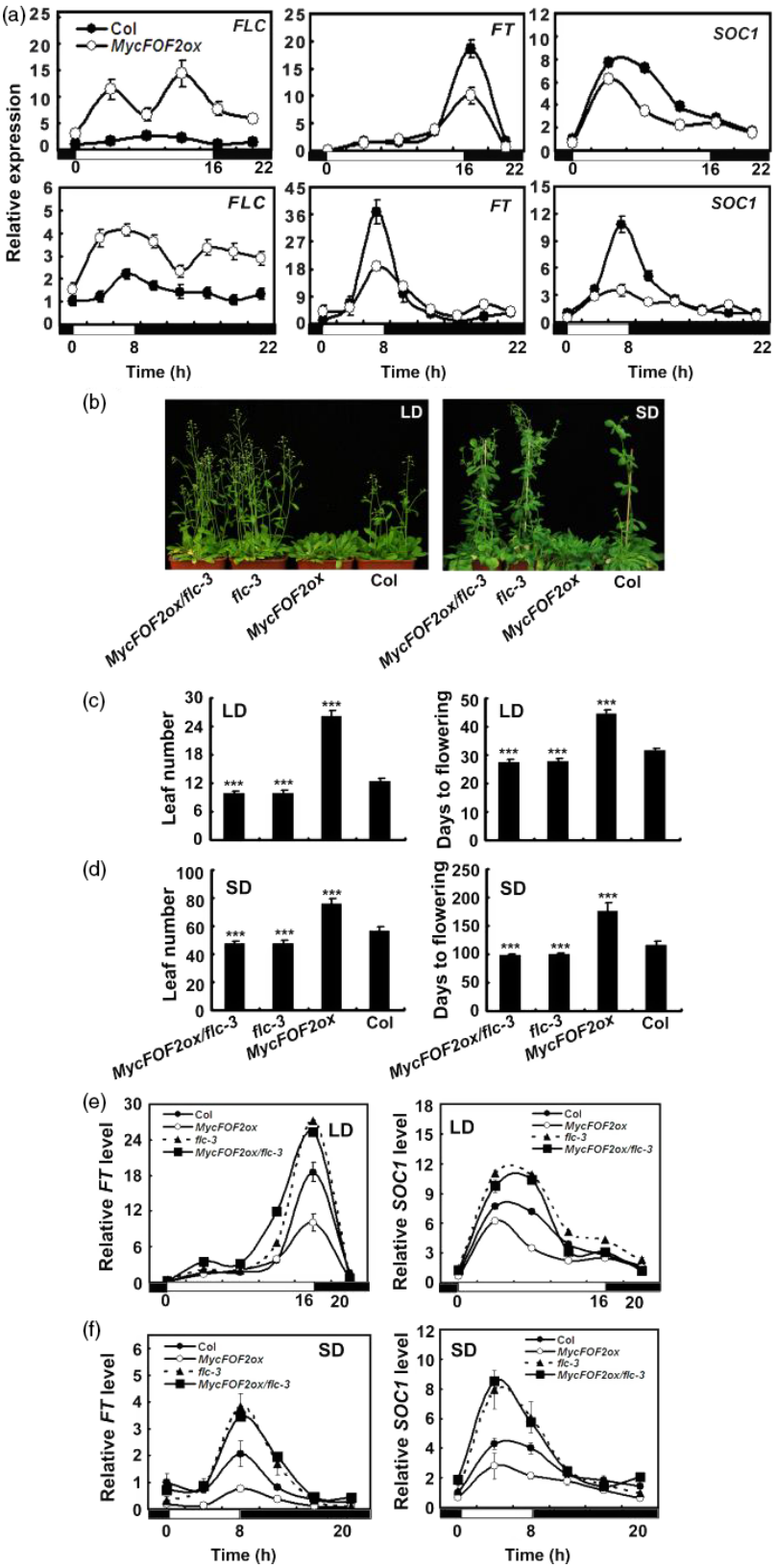

Figure 5. *flc-3* mutation can rescue the late flowering phenotype of *MycFOF2ox* plants. (a) *FLC* is upregulated and *FT* and *SOC1* are downregulated in *MycFOF2ox* plants. (b) 35- and 114-day-old *MycFOF2ox/flc-3* plants grown under long days (LD) or short days (SD). (c, d) The time to flowering and the number of rosette leaves at the time of flowering of the indicated photoperiods: LD (c) and SD (d). Significant differences are indicated, $***P \leq 0.001$ (Tukey's least significant difference test). (e, f) The mRNA level of *FT* and *SOC1* expression in 12-day-old seedlings under LD (e) or in 26-day-old seedlings under SD (f). Samples are collected every 4 h for 1 day. The white/black bars indicate light/dark phases. The time (hour) of light on at sample collection is set as zero. Bars represent the standard deviations of three independent experiments.

the *MycFOF2ox* plant background (Figure S9) and analyzed its flowering time under LD and SD conditions. The late flowering phenotype of *MycFOF2ox* was suppressed by the *flc* mutation, and *MycFOF2ox/flc-3* flowered with a similar number of rosette leaves at flowering and days to bolting as the *flc-3* mutant under both photoperiods (Figure 5b–d). These results are consistent with a model in which *FOF2* and *FLC* act in the same pathway, with *FOF2* acting upstream of *FLC* and presenting *FLC* expression-dependent flowering regulation.

To test whether *FOF2* suppresses *FT* and *SOC1* expression via *FLC*, the expression of *FT* and *SOC1* in the *MycFOF2ox/flc-3* plants were analyzed. As shown in Figure 5, the transcripts of *FT* and *SOC1* increased significantly in the *MycFOF2ox/flc-3* plants, and there were similar levels of *FT* and *SOC1* transcripts in both the *MycFOF2ox/flc-3* and *flc-3* plants under both LDs and SDs (Figure 5e and f), showing that the ability of *FOF2* to suppress the expression of *FT* and *SOC1* is mainly dependent on the promotion of *FLC* expression. Taken together, these results demonstrated that the late flowering phenotype of *MycFOF2ox* plants is genetically controlled by the promotion of *FLC* mRNA expression.

To investigate how FOF2 stimulates *FLC* expression, we first performed chromatin immunoprecipitation assays using *MycFOF2ox* seedlings (Appendix S3). The preliminary results revealed that FOF2 does not associate with the *FLC* genomic sequence (Figure S10), suggesting that FOF2 is not a transcriptional co-factor for flowering regulation. Another possibility is that the FOF2 protein stimulates *FLC* expression via proteasome-mediated degradation of its negative regulators, such as FPA, FCA, FLD, FLK, FVE, LD and FY, which promote flowering by suppressing *FLC* expression in the autonomous pathway (Simpson, 2004). To test this hypothesis, we examined the interactions with FOF2 and these negative regulators using the yeast two-hybrid (Y2H) assay (Appendix S4). FOF2 did not interact with any of these regulators (Figure S11), suggesting that FOF2 might interact with one of the regulators *in vivo* or regulate other unknown negative regulators. Because *FLC* is a potent negative integrator of flowering, multiple regulators of *FLC* have been continuously identified in recent years (Lee *et al.*, 2015; Shu *et al.*, 2016).

Regulation of *FOF2* mRNA expression by conventional flowering pathways

The expression of *FOF2* was not affected in the mutants of the important flowering regulator genes, including the photoperiodic pathway genes (*CO*, *GI* and *ELF3*), temperature pathway genes (*SVP* and *AGL24*) and genes that integrate flowering signals from various genetic pathways (*FT*, *SOC1*, *FD* and *FLC*) (Blumel *et al.*, 2015; Figure S12a–c). Interestingly, *FOF2* expression was altered in the *fca-1* mutant (Figure 6a), but not in the other autonomous pathway mutants (Figure S12d). The expression level of *FOF2* was elevated in the *fca-1* mutant, which is late flowering under both LD and SD conditions and in response to vernalization (Koornneef *et al.*, 1991). Subsequently, the transcription level of *FCA* was measured in *MycFOF2ox* plants to determine whether *FOF2* affects its expression; however, *FCA* expression was not affected in the *MycFOF2ox* plants (Figure 6b). These results suggest that *FOF2* is likely to function downstream of *FCA* in the autonomous pathway.

A vernalization response is a feature of autonomous pathway mutants (Martinez-Zapater and Somerville, 1990). Therefore, we determined whether *FOF2*-overexpressing lines respond to vernalization. As expected, after vernalization the *MycFOF2ox* lines flowered with similar leaf numbers and exhibited the same number of days to flowering as the wild-type plants (Figure 6c and d). Moreover, the expression of *FLC* decreased and the expression of *FT* and *SOC1* increased in the wild type (Col) and *MycFOF2ox* lines (Figure 6e–g), indicating that vernalization can overcome the flowering defects of *FOF2* transgenic lines. Furthermore, similar to *FLC*, the *FOF2* response to vernalization and its expression decreased after long periods of cold treatment (Figure 6h). Taken together, these results suggest that *FOF2* is regulated by the autonomous pathway.

Light regulates *FOF2* mRNA and protein expression

Interestingly, our analyses showed that although FOF2 appears to be a regulator of the major floral repressor gene *FLC*, *FOF2* itself is photoresponsive. The *FOF2* mRNA expression was approximately 5-, 20- and 80-fold higher in the blue, red and far-red light-grown seedlings than in the etiolated seedlings, and the light-induced *FOF2* expression was impaired in the *cry1 cry2*, *phyB* and *phyA* mutants, respectively (Figure 7a). Consistent with observations under continuous light conditions, the expression of *FOF2* is transiently induced and increases immediately after light treatment; however, *FOF2* showed reduced blue, red or far-red light induction in the *cry1 cry2*, *phyB* or *phyA* mutants, respectively (Figure 7b). These results indicated that cryptochromes, phyB and phyA are the major blue, red or far-red light receptors required for the light induction of *FOF2* expression. Because *FOF2* expression is higher in light than in dark conditions, it was of interest to determine the dark regulation of *FOF2* expression. As expected, the transcription levels of *FOF2* decreased dramatically after the dark treatment, and decreased 11-fold after 2 h of dark treatment (Figure 7c).

We next analyzed the protein level of FOF2 under different light conditions. Consistent with the steady-state mRNA expression of *FOF2*, the FOF2 protein level was more abundant under light than under dark conditions (Figure 7d). Interestingly, the FOF2 protein levels decreased in response to transient dark treatment and then

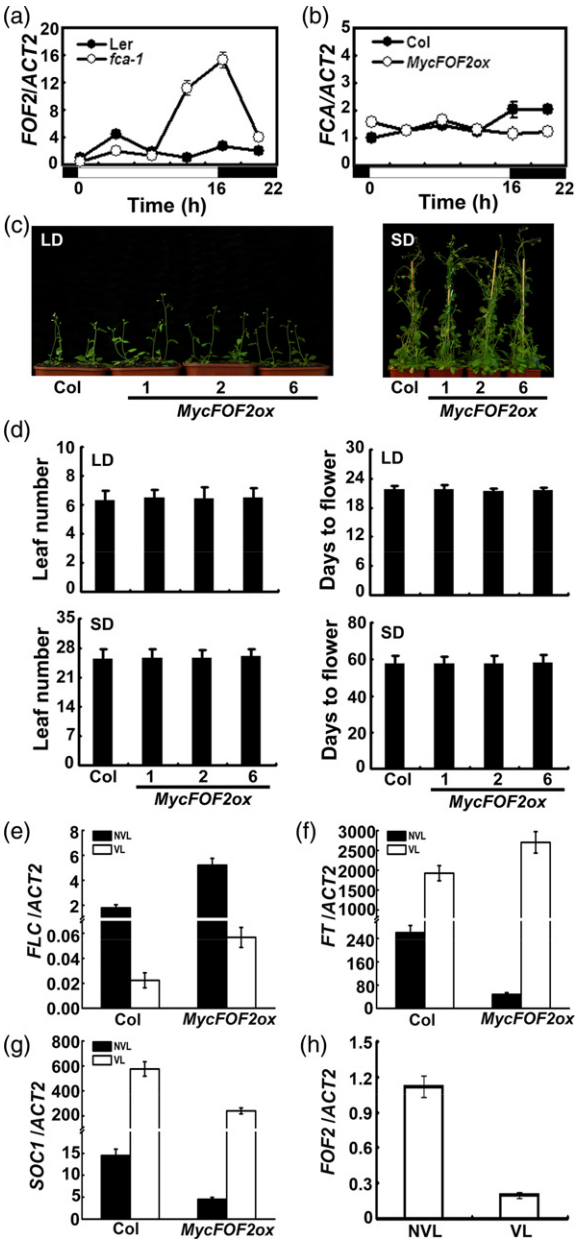

Figure 6. The mRNA expression of *FOF2* is upregulated in the *fca-1* mutant, and vernalization can overcome the late flowering phenotype of *MycFOF2ox* plants. (a, b) *FOF2* expression in 12-day-old *fca-1* plants (a) and *FCA* expression in 12-day-old *MycFOF2ox* plants (b). Samples are collected every 4 h for 1 day. The white/black bars indicate light/dark phases. The time (hour) of light on at sample collection is set as zero. Bars represent the standard deviations of three independent experiments. (c) 23- and 63-day-old plants with vernalization treatment grown under LD or SD conditions. After 7 weeks of vernalization treatment, the seedlings were transferred to soil and grown under LD or SD conditions. (d) The time to flowering and the number of rosette leaves at the time of flowering of the indicated photoperiods: LD and SD. Standard deviations ($n \geq 20$) are shown. (e–h) *FLC* (e), *FT* (f) and *SOC1* (g) mRNA expression in *MycFOF2ox* and wild-type (Col) plants, and *FOF2* (h) mRNA expression in wild-type (Col) plants with (VL) and without (NVL) vernalization treatment. Seeds were sown on MS medium and vernalized at 4°C in the dark for 7 weeks. The 12-day-old seedlings grown under LD conditions were harvested for qRT-PCR analysis. Bars represent the standard deviations of three independent experiments.

increased when exposed to light (Figure 7e and f); however, the mRNA expression of the *MycFOF2* transgene showed little change in response to light or dark treatment (Figure S13), suggesting that FOF2 protein expression is regulated at the post-transcriptional level.

Because FOF2 is regulated by light at both the mRNA and protein levels, we determined whether protein degradation or synthesis is a key regulatory step that results in the differential abundance of FOF2 under light and dark conditions. The level of FOF2 decreased within 5 h under both light and dark conditions in the presence of the cytoplasmic protein synthesis inhibitor cycloheximide, although the rate of decrease was significantly greater under dark conditions (Figure 7g). The reduced levels of FOF2 in the dark could have been caused by the degradation of pre-existing proteins. Therefore, to investigate how the FOF2 protein is degraded in darkness, we examined the FOF2 protein levels in *MycFOF2ox* plants treated with the proteasome inhibitor MG132. The levels of the FOF2 protein decreased quickly in the absence of MG132 (Figure 7h), although its degradation was inhibited to a large extent in the presence of MG132 after the transfer to dark conditions, suggesting that the FOF2 protein could be degraded via the *26S* proteasome pathway in the dark.

The light regulation pattern of FOF2 expression observed here is similar to that of transcription factors, such as HYH and HY5, which are accumulated in light and degraded in darkness at the mRNA and protein levels (Osterlund *et al.*, 2000; Holm *et al.*, 2002). It has been reported that COP1 mediates the light regulation of HYH and HY5 (Osterlund *et al.*, 2000; Holm *et al.*, 2002). Therefore, we investigated whether COP1 mediates the light regulation of FOF2. In this experiment, we introduced *MycFOF2* into the *cop1-6* mutant and examined the protein level of the *MycFOF2* transgene. The immunoblot results showed that the MycFOF2 protein was still degraded in darkness (Figure S14), suggesting that its light regulation was not mediated by COP1. Additionally, the hypocotyl length of the *MycFOF2ox* seedlings was equivalent to that of the wild-type seedlings grown under both light and dark conditions (Appendix S5; Figure S15a–c). Together, these results indicate that FOF2 could function as a flowering pathway-specific regulator.

DISCUSSION

The F-box protein FOF2 is involved in regulating flowering

Here, we characterized the flowering regulator FOF2, which was previously identified as a member of the F-box protein family in Arabidopsis (Gagne *et al.*, 2002). Transgenic lines overexpressing *FOF2* exhibited delayed transitions to flowering under both LD and SD conditions (Figure 1), whereas the transition to flowering was unaffected in the T-DNA

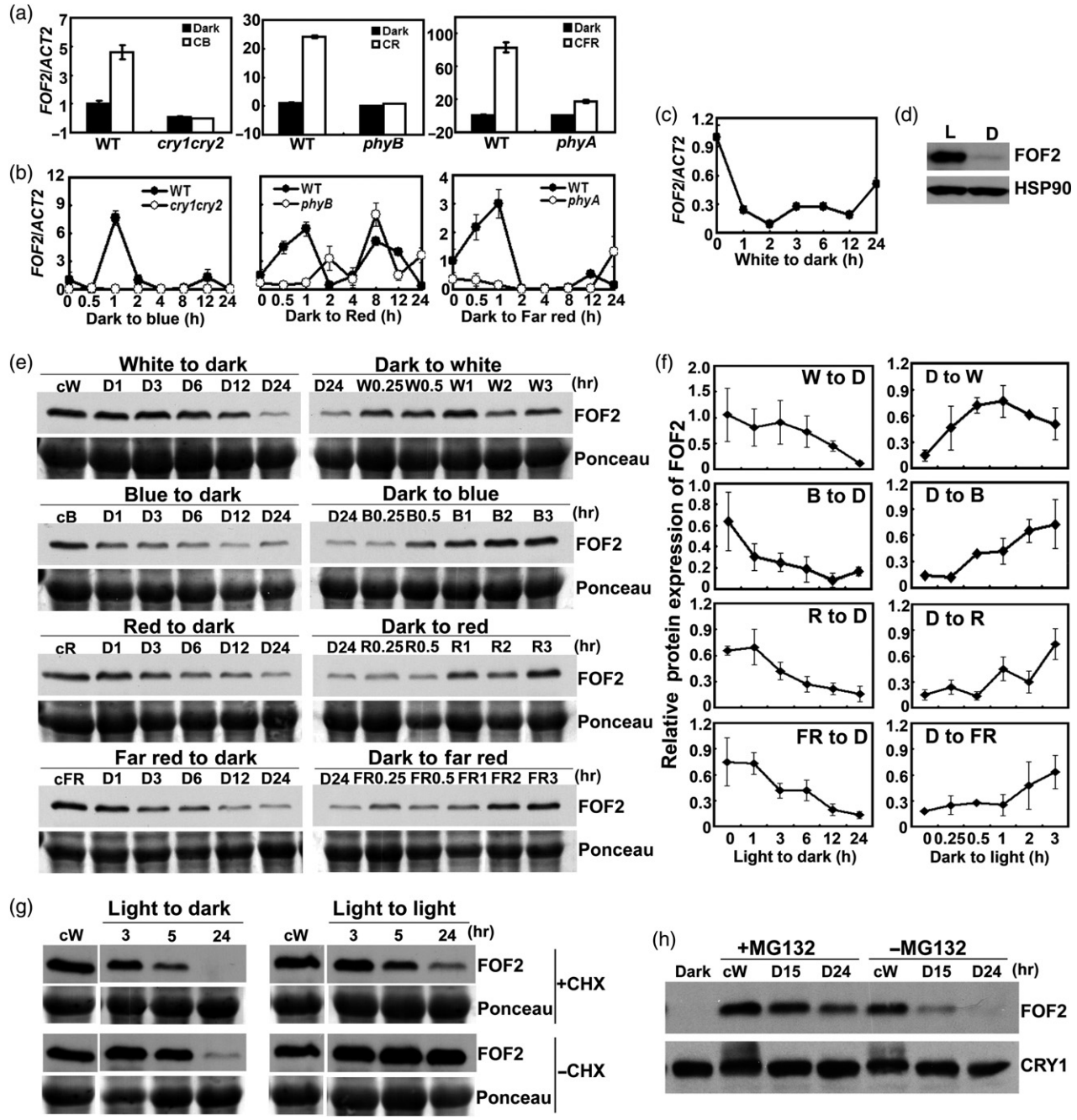

Figure 7. Light regulation of *FOF2* expression. (a–c) The mRNA expression level of *FOF2* in response to different light conditions: (a) 6-day-old seedlings grown under continuous light or darkness; (b) 6-day-old etiolated seedlings were exposed to blue, red or far-red light for the indicated times; (c) seedlings grown under white light (100 μmol m^{-2} sec^{-1}) for 6 days, and then transferred into darkness for the indicated times. Samples were collected and examined by qRT-PCR. Bars represent the standard deviations of three independent experiments. (d–f) Immunoblots showing levels of the FOF2 protein in response to different light conditions. (d) Plants were grown in continuous white light or in the dark for 6 days. (e) 6-day-old seedlings grown in continuous white (100 μmol m^{-2} sec^{-1}), blue (20 μmol m^{-2} sec^{-1}), red (30 μmol m^{-2} sec^{-1}) or far-red light (20 μmol m^{-2} sec^{-1}) were transferred to dark for the indicated times, and then exposed back to the indicated light for various time periods. Levels of protein expression are shown by representative immunoblots. (f) The relative protein expression level of FOF2 (mean ± SE) was calculated from triplicate independent reactions. The error bars represent the SDs. (g) Immunoblots showing levels of the FOF2 protein treated with or without cycloheximide. Ponceau staining was used as a loading control. The indicated cW samples are without cycloheximide treatment. (h) Immunoblots showing levels of the FOF2 protein in the absence or presence of the proteasome inhibitor MG132. anti-CRY1 antibody was used as the loading control.

insertion mutant of *FOF2* (Figure S2). These observations may have been related to the functional redundancy of *FOF2* with other homologous F-box protein(s) because the *MycFOF2ΔF* plants with an F-box deletion mutant showed early flowering under both LD and SD conditions (Figure S3). F-box proteins with deleted F-box domains cannot

interact with ASK proteins (Maldonado-Calderon *et al.*, 2012); therefore, they were unable to form the SCF ligase and failed to mediate the degradation of substrates by the *26S* proteasome, which results in a dominant-negative effect on substrate degradation, and allows for the accumulation of substrates in cells (Margottin *et al.*, 1998; Hart *et al.*, 1999; Risseeuw *et al.*, 2013; Yumimoto *et al.*, 2013). Thus, we postulated that the opposite flowering phenotypes of the *FOF2ΔF* and *FOF2* overexpression plants might have been caused by a similar mechanism. As expected, FOF2 interacted with ASK14 *in vivo*, and their interaction was dependent on the F-box domain (Figure 3b and c). Based on these results, a possible explanation for the flowering phenotype of *MycFOF2ΔF* plants is that the highly expressed FOF2ΔF protein may compete with FOF2 and its homologs to interact with substrates, which would lead to the accumulation of the substrates of FOF2 and its homologs, thereby promoting flowering. The double mutation of *FOF2* and its homolog *FOL1* accelerates flowering time in Arabidopsis to some extent (Figure 2), which confirmed that *FOF2* functions redundantly with *FOL1* at least partially in regulating flowering.

Consistent with the late flowering phenotype, the flowering gene *FLC*, which is a central flowering repressor in the vernalization and autonomous pathways (Michaels and Amasino, 1999; Sheldon *et al.*, 1999), was upregulated, and the flowering genes *SOC1* and *FT*, which are Arabidopsis flowering pathway integrators that promote flowering (Lee and Lee, 2010; Pin and Nilsson, 2012), were downregulated in plants overexpressing *FOF2* (Figure 5a). *MycFOF2ΔF* plants and the *fof2 fol1* double mutant showed decreased *FLC* and increased *SOC1* and *FT* expression, however (Figure S8b and c). According to these physiological and molecular data, we conclude that the F-box protein FOF2 plays a negative role in regulating flowering time in Arabidopsis.

FOF2 is subject to autonomous pathway regulation

Plants overexpressing *FOF2* showed a strong short-day period response and flowered much later than those under LD conditions (Figure 1b and e; Table S1), indicating that *FOF2* transgenic plants retain a photoperiod response. Additionally, *FOF2* expression was downregulated by vernalization (Figure 6h) and *FCA* (Figure 6a), which is an important regulator of the autonomous pathway (Koornneef *et al.*, 1991), although it was not regulated by *CO* (Putterill *et al.*, 1995) and *SVP* (Lee *et al.*, 2007) (figure S12a and b), which are the central regulators of the LD and thermosensory pathways, respectively. Moreover, the late flowering phenotype of the *MycFOF2ox* plant was completely abolished by vernalization (Figure 6c and d), and this effect was observed in the *fca-1* mutant (Koornneef *et al.*, 1991). We therefore hypothesized that *FOF2* is subject to autonomous pathway regulation, which needs further confirmation from additional genetic and biochemical data.

Possible mechanism for *FOF2* regulation of flowering

Our molecular and genetic data indicate that FOF2 represses flowering through the promotion of *FLC* expression. One important question is that how FOF2 stimulates *FLC* expression. It has been reported that F-box protein UNUSUAL FLORAL ORGANS (UFO) functions together with the transcriptional co-factor of LEAFY (LFY), and their interaction recruits UFO to *AP3* promoter elements, which in turn promotes *AP3* transcription (Chae *et al.*, 2008). Unlike UFO, FOF2 does not associate with the *FLC* genomic sequence (Figure S10). We therefore speculated that the FOF2 protein might promote *FLC* expression by proteasome-mediated degradation of its negative regulators; however, our Y2H assay results showed no interaction between FOF2 and the indicated negative regulators (Simpson, 2004; Figure S11). Future studies should aim to test whether FOF2 interacts with one of the regulators *in vivo* or to identify additional substrates.

Another important question is how FOF2 regulates *FT* expression. *FOF2* overexpression downregulates *FT* and *SOC1* expression (Figure 5a), indicating that *FT* and *SOC1* function downstream of *FOF2*. Because the transcription of *CO*, the major activator of *FT*, was unaffected in the *MycFOF2ox* plants (Figure S8a), we proposed that the decreased *FT* and *SOC1* expression might have been caused by the elevated *FLC* expression in the *MycFOF2ox* plants because *FT* and *SOC1* are regulated by the floral repressor FLC via direct binding (Helliwell *et al.*, 2006). As expected, the transcriptional levels of *FT* and *SOC1* were elevated in *MycFOF2ox/flc-3* plants without functional *FLC*, and were increased to similar levels in *flc-3* mutants (Figure 5e and f). These results strongly suggest that FOF2 regulates *FT* and *SOC1* expression mainly through *FLC*.

FOF2 is photoresponsive

In this study, we showed that *FOF2* is a photoresponsive gene regulated by light at both the transcriptional and post-translational levels. Although the expression pattern of FOF2 is similar to that of the transcription factors HYH and HY5 (Osterlund *et al.*, 2000; Holm *et al.*, 2002), FOF2 is neither regulated by COP1 nor involved in photomorphogenesis (Figures S14 and S15). Furthermore, COP1 was recently shown to be involved in the regulation of light input to the circadian clock via the modulation of circadian rhythms and flowering (Xu *et al.*, 2016). Therefore, we hypothesized that the light signal may control the level of the FOF2 protein to affect the autonomous pathway regulating floral initiation in Arabidopsis (Figure S15d).

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All Arabidopsis mutants used in this study were in the Columbia (Col) background, unless otherwise noted (Table S5). *fof2-1* (*salk_016168*), *fof2-2* (*salk_061523*), *fol1-1* (*CS26289*) and *fol1-2* (*CS26467*) seeds were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). *fol1-1* and *fol1-2* mutants are in the Landsberg *erecta* (L*er*) background. The *MycFOF2ox/flc-3* plant was prepared by crossing *MycFOF2ox* and *flc-3* mutants. The genotyping of the *flc-3* alleles was performed as described by Michaels and Amasino (1999).

For flowering analysis, plants were grown on soil in a culture room at 22°C. The photoperiod was 16 h of light and 8 h of dark for the LD conditions, and 8 h of light and 16 h of dark for the SD conditions. The flowering time was determined by counting the number of rosette leaves after bolting and the days from sowing to floral bud formation, as described (Mockler *et al.*, 2003).

For studies of light-regulated mRNA expression, the samples were prepared as described in our previous study (Zhao *et al.*, 2007). Seedlings were grown on MS medium in the dark for 6 days before transfer to various light conditions. For light-regulated protein expression analysis, 6-day-old seedlings grown on MS medium in various light conditions were transferred to the dark for the indicated times, and then exposed back to light conditions for various periods of time.

Plasmid construction and plant transformation

To identify F-box genes regulating flowering, the full-length CDS of about 664 F-box genes (Gagne *et al.*, 2002) were first cloned in the pDONR/ZEO Gateway donor vector, and then transferred to Myc-tagged pEarleygate203(N-Myc) vector under the control of the CaMV *35S* promoter via recombination-based cloning, as described in our previous study (Peng *et al.*, 2012). The constructs were then transformed into Col-4 wild-type Arabidopsis using the floral-dip method (Clough and Bent, 1998). The T_1 seeds were harvested, sown on compound soil submerged in the herbicide Basta, as described by Zhao *et al.* (2007), incubated in a cool room for 4 days and then transferred into LD conditions. The herbicide-resistant transgenic lines that showed earlier or later flowering than that of wild type Col-4 plants were selected as putative F-box of flowering (FOF) lines, which were subjected to further genetic analysis. The *MycFOF2ox/cop1-6* plant was prepared by introducing *MycFOF2* into the *cop1-6* mutant. The plasmid *MycFOF2ΔF* was prepared by cloning the fragments with a deletion of residues 2–100 into the pEarleygate203(N-Myc) vector.

To generate the CRISPR/CAS9 system targeting both *FOF2* and *FOL1*, the binary vector pCambia-bar plasmid was used as the backbone. The *pYAO*:hSpCas9 cassette fragments were amplified from *pYAO*:hSpCas9 plasmid (Yan *et al.*, 2015) with the primers pYAO-F and NOS-R, and cloned into *Eco*RI and *Hind*III sites in the pCambia-bar vector to generate the *pYAO*:hSpCas9-bar construct. Subsequently, the AtU6-26 promoter (Yan *et al.*, 2015) was amplified from Arabidopsis Col-4 genomic DNA using the primers AtU6-26-F and AtU6-26-R. The guide RNA and scaffold-infused fragment (target-sgRNA) was obtained by PCR with pU3-gRNA (Shan *et al.*, 2013), using the sense primer target-F, containing guide RNA sequences targeting both *FOF2* and *FOL1*, and the antisense primer scaffold-R, containing the In-fusion reaction adaptor. The AtU6-26-target-sgRNA cassette was prepared by infusing the AtU6-26 promoter and target-sgRNA fragment using the primer pairs AtU6-F and scaffold-R, and then inserted into the *Spe*I and *Mlu*I sites in the *pYAO*:hSpCas9-bar construct by using the In-Fusion cloning system (Clontech, https://www.clontech.com). The primers used are listed in Table S6.

Vernalization treatment

Seeds were grown on MS medium at 4°C for 7 weeks in the dark. Post-vernalization samples continued to grow for 7 days on plates under LD or SD conditions at 22°C. All plant samples were prepared after 16 h of light under LD conditions for RNA analyses, or transferred to normal growth conditions (22°C, LDs or SDs) on soil until flowering.

mRNA and protein expression analyses

For mRNA analysis, total RNAs were extracted using RNAiso Plus (TaKaRa, http://www.takara-bio.com), and reverse transcribed using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa), according to the manufacturer's instructions. Both the semiquantitative RT-PCR and quantitative RT-PCR (qRT-PCR) was performed as described in our previous study (Peng *et al.*, 2012). The mRNA level of *ACTIN2* was used as the internal control. The primers are listed in Table S6.

For protein analysis, total proteins were extracted and separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblots. The blots were probed by anti-MYC antibody, anti-HSP90 or anti-CRY1 antibody for the loading control. The immunoblot signals were quantified using IMAGEJ (http://rsb.info.nih.gov/ij/).

Subcellular localization analysis

The full-length coding regions of *FOF2* were cloned into pEGAD-GFP or pCAMBIA2300-GFP vectors using primers listed in Table S6 to generate GFP-FOF2 and FOF2-GFP constructs, respectively. The constructs were infiltrated into 3-week-old *N. benthamiana* (tobacco) leaves as described previously (Sparkes *et al.*, 2006; Meng *et al.*, 2013). The GFP-FOF2 or FOF2-GFP signal was observed using a confocal microscope (Nikon, http://www.nikon.com).

BiFC assay

For the BiFC assay, the *FOF2* and *FOF2ΔF* encoding sequences were inserted into pSAT1-cCFP-N to form a C-terminal in-frame fusion with cCFP, and ASK encoding sequences were introduced into pSAT1-nVenus-N to generate a C-terminal in-frame fusion with nVenus using primers listed in Table S6. BiFC was performed as described by Yoo *et al.* (2007). The fluorescence emission of GFP was observed under a confocal microscope (Nikon).

Coimmunoprecipitation (Co-IP) assay

The full-length coding regions of ASK13 and ASK14 were cloned into pCAMBIA1300-Flag using the primers listed in Table S6 to generate ASK13-Flag and ASK14-Flag constructs, respectively. Transient co-expressions of both Myc-FOF2 and ASK13-Flag or ASK14-Flag proteins in tobacco were performed as described previously (Sparkes *et al.*, 2006; Meng *et al.*, 2013). Total proteins were extracted and incubated with Red anti-*c*-Myc Affinity Gel (Sigma-Aldrich, https://www.sigmaaldrich.com) 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.

Cycloheximide and MG132 treatments

Cycloheximide and MG132 treatment experiments were carried out as described in our previous study, with minor modification (Yu *et al.*, 2007). For MG132 treatment, 6-day-old seedlings grown on MS medium in white light were excised into 2–5-mm-long sections and incubated in 50 μM MG132 (Sigma-Aldrich) or in mock solution (0.1% DMSO) for 5 h and then placed under white light or in darkness for the indicated time before sampling. For cycloheximide treatment, the seedlings were incubated in 300 μM cycloheximide (Sigma-Aldrich) or in mock solution (0.1% DMSO), and placed under white light or in darkness for the indicated time before sampling.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The amino sequence of FOF2 and alignment of F-box domains of F-box proteins known to be expressed in Arabidopsis.

Figure S2. Flowering phenotype of *FOF2* T-DNA insertion mutants.

Figure S3. Overexpression of FOF2 carrying the F-box-delete mutation results in the early flowering of Arabidopsis.

Figure S4. Amino acid sequences alignment of FOF2 with other FOL (FOF2-LIKE) proteins.

Figure S5. Flowering phenotype of *FOL1* T-DNA insertion mutants.

Figure S6. FOF2 does not interact with ASK1, ASK2, ASK3, ASK4, ASK11 or ASK12 in Arabidopsis protoplasts.

Figure S7. Expression of *FOL1*, *FLC* and *MycFOF2* in developing seedlings in Arabidopsis.

Figure S8. mRNA expression of *FLC*, *FT*, *SOC1*, *CO* and *FLC* homologs *MAF4* and *MAF5* in *MycFOF2ox* plants, *MycFOF2ΔF* mutant plants or the *fof2 fol1* double mutant.

Figure S9. Genomic PCR and western blot analysis for *MycFOF2ox/flc-3* mutant confirmation.

Figure S10. ChIP-qPCR assay showing no association between FOF2 and *FLC* genomic sequence.

Figure S11. Yeast two-hybrid assay showing no interaction with FOF2 and the indicated genes in the autonomous pathway.

Figure S12. Expression of *FOF2* in the mutants of conventional flowering pathway genes.

Figure S13. Exogenous *MycFOF2* expression in *MycFOF2ox* plants exposed to light or grown in darkness.

Figure S14. The *cop1-6* mutation has no effect on the FOF2 protein level in darkness.

Figure S15. *FOF2* has no effect on the photomorphogenesis in Arabidopsis.

Table S1. Leaf number and days to flower of *FOF2* overexpression lines under long-day (LD) and short-day (SD) conditions.

Table S2. Leaf number and days to flower of *MycFOF2ΔF* mutant lines under LD and SD conditions.

Table S3. Leaf number and days to flower of double mutant *CR-fof2 fol1-m1* and *CR-fof2 fol1-m2* under LD conditions.

Table S4. RNA-seq data for flowering-related genes that are upregulated or downregulated in *MycFOF2ox* plants.

Table S5. List of flowering time mutants used in this study.

Table S6. Primer sequences used in this study.

Appendix S1. Semiquantitative RT-PCR.

Appendix S2. RNA-seq and data analysis.

Appendix S3. ChIP-qPCR assay.

Appendix S4. Yeast two-hybrid assay.

Appendix S5. Hypocotyl elongation analysis.

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