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Article type : Regular Article

BES1 regulated BEE1 controls photoperiodic flowering downstream of blue light signaling pathway in *Arabidopsis*

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Received: 27 August 2018
Accepted: 14 April 2019

Summary

- BRI1-EMS-SUPPRESSOR 1 (BES1) functions as a key regulator in the brassinosteroid (BR) pathway that promotes plant growth. However, whether BES1 is involved in photoperiodic flowering is unknown.
- Here we report that BES1 acts as a positive regulator of photoperiodic flowering, but it cannot directly bind *FLOWERING LOCUS T (FT)* promoter. *BR ENHANCED EXPRESSION 1 (BEE1)* is the direct target of BES1 and acts downstream of BES1. BEE1 is also a positive regulator of photoperiodic flowering. BEE1 binds directly to the *FT* chromatin to activate the transcription of *FT* and promote flowering initiation.
- More importantly, BEE1 promotes flowering in a blue light photoreceptor Cryptochrome 2 (cry2) partially dependent manner, since it physically interacts with cry2 under the blue light. Furthermore, BEE1 is regulated by both BRs and blue light. The transcription of BEE1 is induced by BRs, and the BEE1 protein is stabilized under the blue light.
- Our findings indicate that BEE1 is the integrator of BES1 and cry2 mediating flowering, and BES1-BEE1-FT is a new signaling pathway in regulating photoperiodic flowering.

Key Words: Cryptochrome, brassinosteroid (BR), BES1, BEE1, photoperiodic flowering.

INTRODUCTION

A major development transition in plants is the switch from the vegetative to the reproductive phase. Flowering time is essential to maximize reproductive success, there are at least five distinct pathways controlling flowering in the model plant *Arabidopsis thaliana*, including photoperiod pathway, vernalization/ thermosensory pathway, autonomous floral initiation, gibberellins pathway, and age pathway. The *CONSTANS* (*CO)* and *FT* genes are among the most important regulators that affect floral initiation in response to photoperiods (Putterill *et al.*, 1995; Kobayashi *et al.*, 1999). CO is a zinc finger transcription regulator that promotes flowering by, at least partially, activation of *FT* mRNA expression (Onouchi *et al.*, 2000; Samach *et al.*, 2000). FT is a RAF kinase inhibitor protein, which acts as a long-distance signal, migrating from leaves through the vascular system to the apical meristem (Lifschitz *et al.*, 2006; Corbesier *et al.*, 2007). *FLOWERING LOCUS C (FLC)* is a

key component in the autonomous pathway encoding a MADS box protein that represses flowering (Michaels & Amasino, 1999; Sheldon *et al.*, 1999). FLC delays flowering by blocking the transcriptions of genes in the photoperiodic pathway, such as *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *FT*.

Cryptochromes are photolyase-like photoreceptors regulating photomorphogenesis in plants and the circadian clock in plants and animals (Cashmore, 2003; Lin & Shalitin, 2003; Sancar, 2003; Liu *et al.*, 2011). The *Arabidopsis thaliana* genome encodes at least two cryptochromes, cryptochrome 1 (cry1) and cryptochrome 2 (cry2). cry2 is a major photoreceptor mediating photoperiodic control of floral initiation in *Arabidopsis* (Guo *et al.*, 1998; El-Assal *et al.*, 2001). Cryptochromes may mediate photoperiodic control of floral initiation by at least three different mechanisms: 1. Cryptochromes mediate light suppression of the CONSTITUTIVELY PHOTOMORPHOGENIC 1(COP1)-dependent degradation of CO (Yanovsky & Kay, 2002; Valverde *et al.*, 2004; Liu, LJ *et al.*, 2008; Zuo *et al.*, 2011). 2. Cryptochromes regulate the light entrainment of the circadian clock (Jang *et al.*, 2008), and then affect the expression of *CO*. 3. Cryptochromes directly modulate the transcription of *FT* through interaction with CRY2□interacting bHLH1 (CIB1), a basic-helix-loop-helix) (bHLH) transcription factor, which was isolated in a blue light differentiated yeast-two-hybrid screen (Liu, H *et al.*, 2008; Liu, H *et al.*, 2013; Liu, Y *et al.*, 2013).

Brassinosteroids (BRs) are a class of steroidal hormones essential for plant growth and development, including skotomorphogenesis, photomorphogenesis, cell elongation and flowering (Yang *et al.*, 2005; Li *et al.*, 2010; Bai *et al.*, 2012; Zhang *et al.*, 2013). BRs are perceived by the surface receptor kinases complex including BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRI1 ASSOCIATED PROTIEN KINASE 1 (BAK1) (Li *et al.*, 2002; Nam & Li, 2002; Tang *et al.*, 2010). BRI1 interacts with and presumably activates BSU1 (BR-SUPPRESSOR 1) phosphatase (Tang *et al.*, 2008; Kim *et al.*, 2009), which triggers BR-INSENSITIVE 2 (BIN2) dephosphorylation. Inactivation of BIN2 allows the accumulation of unphosphorylated transcription factors BRASSINAZOLE RESISTANT 1 (BZR1) and BES1 (BRI1-EMS-SUPPRESSOR 1) in the nucleus, which, in turn, regulates

BR target genes (Yin *et al.*, 2002; He *et al.*, 2005). *BR ENHANCED EXPRESSION 1*(*BEE1*), *BEE2* and *BEE3* are bHLH transcription factors (Toledo-Ortiz *et al.*, 2003), and their mRNA expression is regulated by brassinosteroids (Friedrichsen *et al.*, 2002). Genetic analysis demonstrates that the three BEE proteins are functionally redundant positive regulators of brassinosteroids signaling (Friedrichsen *et al.*, 2002). BR signaling regulates flowering through BRI1, BES1 and also BZR1 (Domagalska *et al.*, 2007; Yu *et al.*, 2008; Zhang *et al.*, 2013). The BRI1 promotes flowering through repressing *FLC* expression (Domagalska *et al.*, 2007). The interaction between BES1 and EARLY FLOWERING 6 (ELF6) / RELATIVE OF EARLY FLOWERING 6 (REF6) give another link between floral induction and the BR pathway (Yu *et al.*, 2008). BZR1-1D binds to the *FLOWRING LOCUS D (FLD)* promoter and suppresses the expression of *FLD*, which is a suppressor of *FLC* (Zhang *et al.*, 2013).

BES1 is involved in BR-regulated photomorphogenesis (Nolan *et al.*, 2017; Yang *et al.*, 2017), whether BES1 is involved in photoperiodic flowering is unknown. We show here that both BES1 and BEE1 are involved in photoperiodic flowering. Both BES1 and BEE1 act as positive regulators of photoperiodic flowering. BEE1 is a direct target of BES1. BEE1 interacts directly with the *FT* chromatin to activate the transcription of *FT* and promote flowering initiation. Furthermore, BEE1 promotes flowering in a CRY2 partially dependent manner, it interacts with both CRY2 and CIB1. More importantly, BEE1 is regulated by both BRs and blue light. The transcription of BEE1 is induced by BRs, while its transcription increases in the first hour of blue light treatment and then decrease afterward. BEE1 protein is stabilized under blue light. These results indicate that BES1 promotes photoperiodic flowering through BEE1. BES1 regulated BEE1 controls flowering downstream of blue light signaling in *Arabidopsis*.

MATERIALS AND METHODS

Plant Materials

Except where indicated, the Columbia ecotype of Arabidopsis was used. The *cry1cry2, bee1 bee2 bee3* mutants, *BES1-L-GFP* overexpression line, *BES1-RNAi* (Yin *et al.*, 2005)(Yin *et al.*, 2005)(Yin *et al.*, 2005)(Yin *et al.*, 2005)(Yin *et al.*, 2005)(Yin *et al.*, 2005) and *pBES1:BES1-GFP* have been previously described (Li *et al.*, 1996; Guo *et al.*, 1998; Friedrichsen *et al.*, 2002; Yin *et al.*, 2002; Jiang *et al.*, 2015). Transgenic Arabidopsis lines were prepared by floral dip transformation method (Clough & Bent, 1998; Weigel *et al.*, 2000). Phenotypes of transgenic plants were verified in at least 3 independent transgenic lines. The binary plasmids encoding the *35S:Myc-BEE1*, *35S:BEE1-CFP*, *35S:CRY2-YFP*, *35S:CIB1-RFP* were prepared by conventional and/or GATEWAY methods. pro*BEE1* represent the *BEE1* promoter (-2127 nt to -1 nt). pro*FT* represent the *FT* promoter (-2172 nt to -1 nt).

Yeast Two Hybrid assay

The CRY2, and CRY2^{W374A} were cloned in-frame with the Gal4 DNA-binding domain (BD) into the bait vector pBridge (Clontech), while BEE1 was cloned in-frame with the Gal4-AD into the pray vector pGADT7 (Clontech). To analyze CRY2-BEE1 interaction by the histidine auxotrophy assay, yeast colonies were patched in duplicate onto two His- plates and two His+ plates, grown for 2-3 days at 28^{o}C under blue light (40 μmolm^{-2}s^{-1}) illumination, with the other set wrapped in aluminum foil to block the light.

qPCR assay

For the Q-RT-PCR, total RNAs were isolated by using the RNAiso Plus (Takara). cDNA was synthesized from 500 ng of total RNA by using PrimeScript RT Reagent Kit with gDNA Eraser (Takara). SYBR Premix Ex Tag (Takara) was used for qPCR reaction, using the MX3000 System (Stratagene). The level of *ACTIN7* mRNA expression (AT5G09810) was used as the internal control. qRT-PCR data for each sample were normalized to the respective *ACT7* expression level. The cDNAs were amplified following denaturation, using the 40-cycle programs (95°C, 5 sec; 60°C, 20 sec per cycle). Biological replicates represent three

independent experiments involving about 30 seedlings per experiment. Three technical replicates were done for each experiment. The primer pairs used in the qPCR assay are listed in the Supplemental Table 1.

BiFC and BiLC assay

The BiFC assay was based on that described previously with slight modifications (Bai *et al.*, 2007; Liu, H *et al.*, 2008; Liu, H *et al.*, 2013; Liu, Y *et al.*, 2013; Ma *et al.*, 2016), CRY2 or BEE1 and CIB1 were fused to N-terminus of YFP or C-terminus of CFP, transformed to Agrobacterium strain GV3101 containing pSoup-P19 plasmid that encodes the suppressor of gene silencing (Hellens *et al.*, 2005). Overnight cultures of Agrobacteria were collected by centrifugation, re-suspended in MES buffer to 0.8 OD600, mixed, and incubated at room temperature for 2hr before infiltration. Agrobacteria suspension in a 2ml syringe (without the metal needle) was carefully press-infiltrated manually onto healthy leaves of 3-week-old *Nicotiana benthamiana*. Plants were left under continuous white light for 3 day after infiltration. For the BiLC assay, 1 mM luciferin was infiltrated before LUC activity was monitored after 3 days. The LUC signal was photograghed with a cool CCD camera (Andor DW936N-BV).

Nuclear Fractionation

Nuclear fractionation was performed as previously described (Liu, H *et al.*, 2013; Yin *et al.*, 2016; Liang *et al.*, 2018; Yang *et al.*, 2018) with modifications. 12-day-old seedlings were collected, grounded in liquid nitrogen, homogenized in extraction buffer [20 mM Tris (pH 7.4), 25% (vol/vol) glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl2, 250 mM sucrose, 1 mM DTT, 1 mM PMSF, 1×complete protease inhibitor cocktail (Roche)]. Total protein extracts were filtered through three layers of Miracloth. After centrifugation at 1,500 × g for 10 min at 4 °C, the pellet was washed twice with nuclei resuspension Triton buffer [20 mM Tris (pH 7.4), 25% glycerol, 2.5 mM MgCl2, 0.2%Triton X-100] and then went on co-immunoprecipitation.

Co-immunoprecipitation

The co-immunoprecipitatin (co-IP) procedure was described previously (Liu, H *et al.*, 2008; Liu, Y *et al.*, 2013; Ma *et al.*, 2016). For co-IP, harvested samples were grounded in liquid nitrogen, homogenized in binding buffer [20 mM Hepes (pH 7.5), 40 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF], incubated at 4 ℃ for 5 minutes, went through 1 ml syringe twice (with the metal needle) to promote the nucleus lysis, and centrifuged at 14,000 g for 10 min. The supernatant was mixed with 35 μl of anti-UVR8-IgG–coupled protein-A Sepharose, incubated at 4 ℃ for 30 min and washed twice with washing buffer [20 mM Hepes (pH 7.5), 40 mM KCl, 1 mM EDTA, 0.1% Triton X-100]. The bound proteins were eluted from the affinity beads with 4× SDS/PAGE sample buffer and analyzed by immunoblot.

ChIP

ChIP experiments for BES1 and BEE1 were performed as described (Liu, H *et al.*, 2008) using 7 day-old seedlings harboring *pBES1:BES1-GFP or MYC-BEE1* grown under long day condition. Monoclonal anti-GFP (Roche, No 11814460001) or anti-MYC (Millipore, #05-724) antibody were used in experiments. 2 g starting material were used to precipitate BES1 or BEE1. Tissue was cross-linked with 1% Formaldehyde (Sigma) for 20 min under vacuum. The primer pairs used in the ChIP experiments are listed in the Supplemental Table 1.

Transient transcription dual-luciferase (Dual-LUC) assays

Transient transcription Dual-LUC assays using *Nicotianaben thamiana* plants were done as described previously (Liu, H *et al.*, 2008; Liu, H *et al.*, 2013; Liu, Y *et al.*, 2013; Ma *et al.*, 2016). The luciferase activity of plant extract was analyzed by a luminometer (Promega 20/20), using commercial LUC reaction reagents according to the manufacturer's instruction (Promega).

EMSA

For probe, the synthetic complementary oligonucleotides of *BEE1* promoter were annealed and cloned to T-vector. The probe was then PCR amplified using Cy5 labeled M13 primer pairs. For proteins, BES1 coding sequences lacking the N-terminal 22 amino acid was cloned to pCold-TF vector (Takara), expressed and purified with Ni-NTA Agarose (Invitrogen). The binding reaction was carried out in 20 μl binding buffer [25 mM HEPES (ph7.5), 40 mM KCl, 3 mM DTT, 10% Glycerol, 0.1 mM EDTA, 0.5 mg/ml BSA, 0.5 mg/ml poly-Glutamate] with 1 ng probe and 200 ng proteins. After 30 min incubation on ice, the reactions were resolved by 6% native polyacrylamide gel at 4 □. Cy5-labeled DNA on the gel was then detected with the Starion FLA-9000 (FujiFilm, Japan).

Data availability

Sequence data from this work can be found in the Arabidopsis Information Resource or GenBank databases under the following accession numbers: *BEE1* (AT1G18400), *BEE2* (AT4G36540), *BEE3* (AT1G73830), *CRY2* (AT1G04400), *BES1* (AT1g19350.1), *L-BES1* (AT1g19350.3), *CO* (AT5G15840), *FT* (AT1G65480), *FLC* (AT5G10140), *SOC1*(AT2G45660), *CIB1*(AT4G34530), *TOE1*(AT2G28550), *SPL9*(AT2G42200), *SPL15* (AT3G57920)，*CIB2*(AT5G48560), *CIB4*(AT1G10120), *CIB5*(AT1G26260).

RESULTS

BES1 is a positive regulator of photoperiodic flowering

BES1 has two isoforms in *A. thaliana,* the BES1-L and BES1-S (Jiang *et al.*, 2015). The BES1-L is a more recently evolved and constitutively nuclear localized isoform of BES1. The overexpression of BES1-S did not exhibit flowering phenotype, but the overexpression of BES1-L promotes flowering in both Col-0 and Oystese-0 (Oy-0) (Jiang *et al.*, 2015). Then we checked the flowering phenotype of *BES1-L* overexpression lines and also the *BES1-RNAi* line in which the expression of *BES1* was significantly suppressed (Fig S1a) in both long day (LD) and short day (SD) conditions. Transgenic plants overexpressing *BES1-L* flowered significantly earlier than the wild type parents under long day conditions, but not under short

day (Fig. 1a-c). In contrast, *BES1-RNAi* displayed significant delay of flowering under the long day conditions, but not under short day conditions (Fig. 1a-c). These results indicate that BES1 is a positive regulator of photoperiodic flowering.

Transgenic plants overexpressing *BES1-L* exhibited elevated mRNA expression of the flowering-time gene *FT* in the long day, while *BES1-RNAi* exhibited decreased expression of *FT* in the long day (Fig. 1d). BEE1, the bHLH transcription factor which is induced by BR (Friedrichsen *et al.*, 2002; Toledo-Ortiz *et al.*, 2003) is reported to be up-regulated by BES1 (Yu *et al.*, 2011), we also found that expression of *BEE1* was higher in *BES1-L* but lower in *BES1-RNAi* than in the Col-0 (Fig. 1e). Expression of *CO, SOC1, CIB1, TOE1*，*SPL9* and *SPL15* were similar in *BES1-L* as in the Col-0 (Fig. 1e, S2). Expression of *SOC1* was similar in *BES1-RNAi* as in the Col-0, while expression of *CO* was lower in *BES1-RNAi* than in the Col-0 (Fig. S1a). We conclude that BES1 promotes flowering by activating *FT* mRNA expression.

BEE1 is the direct target of BES1

We then examined whether BES1 might interact with the *FT* gene to directly activate the transcription of *FT*, using the ChIP-qPCR assay. We did not detect the association of BES1 with the *FT* chromatin (Fig. S3a, b). BEE1, the bHLH transcription factor which is induced by BR (Friedrichsen *et al.*, 2002; Toledo-Ortiz *et al.*, 2003) is reported to be up-regulated by BES1 (Yu *et al.*, 2011), but whether it is a direct target of BES1 is unknown. Our ChIP-qPCR showed that *in vivo*, BES1 was associated with the chromatin of *BEE1* (Fig. 2a, b), transgenic lines expressing genomic *BES1* driven by the native *BES1* promoter (Yin *et al.*, 2002) were used in the ChIP experiment. Further evidence supporting that BES1 bound to the *BEE1* promoter came from electrophoretic mobility-shift assays (EMSA) using BES1 protein expressed *in vitro*. As shown in Fig. 2c, BES1 bound to the promoter region of *BEE1 in vitro.* We then did the qPCR to check whether BES1 could regulate the transcription of *BEE1*. Transgenic plants overexpressing *BES1-L* exhibited elevated mRNA expression of the *BEE1* (Fig. 1e). The transcription of *BEE1* was elevated in Col-0 after eBL treatment, while the transcription of *BEE1* was not induced in *BES1-RNAi* line even after eBL treatment (Fig S1b).

These results indicated the *BEE1* is a direct target of BES1. We also did qPCR in Col-0 and *bzr1-1D* to check whether BZR1 could regulate the transcription of *BEE1*, and found that the transcription of BEE1 was the same in Col-0 and *bzr1-1D* (Fig. S4), indicating that BZR1 could not regulate the transcription of BEE1. We then analyzed whether BES1 regulated the transcription of *BEE1* and *FT* using a transient transcription assay. We used a dual-LUC reporter plasmid that encodes a firefly luciferase (LUC) gene driven by the *BEE1* (-2127 bp to -1 bp) promoter or *FT* promoter (-2172 bp to -1 bp) and a Renilla luciferase (REN) gene driven by the constitutive *35S* promoter. The expression level of *BEE1* promoter-LUC was about 5-fold higher when BES1 was expressed than that without BES1, while BES1 could not promote the *FT* promoter-*LUC* transcription (Fig.2d, e; S3c, d) in this transient assay using tobacco. BEE1 is in the same clade of CIB1, the CRY2 interacting protein, they are both members of bHLH subfamily 18 (Liu, Y et al., 2013), although CIBs are not BR induced (Fig. S5) (Friedrichsen *et al.*, 2002). Then we examined the function of *BEE1* in regulating flowering.

BEEs are positive regulators of photoperiodic flowering

Transgenic plants overexpressing BEE1 (as Myc-fusion proteins) flowered significantly earlier than the wild type parents in long day conditions and long day blue light conditions (Fig. 3a, b, S6a), but not under short day conditions (Fig. S7a, b), indicating that BEE1 is involved in the photoperiodic flowering, and it is a positive regulator of photoperiodic flowering. The expression level of BEE1 in those transgenic lines was shown in Supplemental Figure 7c. Both *bee1 bee3* double mutant and *bee2* single mutant showed similar flowering phenotype and similar level of *FT* as the wild type control (Fig. S8a, b), while the *bee1 bee2 bee3* triple mutant showed a statistically significant delay of flowering under the long day conditions and also long day blue light conditions (Fig. 3c, d, S6a), but not under the short day conditions (Fig. S7a, b). These results indicate that BEEs are involved in photoperiodic flowering.

BEE1 associates with the chromatin regions of the *FT* gene

We then examined whether BEE1 affected the transcription of *CO*, *CIB1* and *FT*. Transgenic plants overexpressing BEE1 exhibited elevated mRNA expression of *FT* in long day conditions and long day blue light conditions (Fig. 4a, S6b), slightly lower expression of *CIB1* and similar level of *CO* (Fig. S7d), while the *bee1 bee2 bee3* triple mutant exhibited decreased expression of *FT* in long day conditions and long day blue light conditions (Fig. 4a, S6b) and similar level of *CO* and *CIB1* (Fig. S7d). These results indicate that BEE1 promotes flowering by activating *FT* expression.

We then examined whether BEE1 might interact with the *FT* gene to directly activate the transcription of *FT*, using the ChIP-qPCR assay. The ChIP-qPCR showed that *in vivo*, BEE1 bound the *FT* promoter under blue light, but not in dark (Fig. 4b, c), and BEE1 was associated with the same chromatin region of the *FT* promoter (region 1) as CIB1 (Fig. 4b, c) (Liu et al., 2008a), indicating that BEE1 directly interacts with the *FT* gene to activate the transcription of *FT*. We then analyzed the transcription activity of BEE1 on the *FT* promoter. A transient transcription assay in tobacco leaves was used. We used a dual-LUC reporter plasmid that encodes a firefly luciferase (LUC) driven by the *FT* promoter (-2172 bp to -1 bp) and a Renilla luciferase (REN) driven by the constitutive *35S* promoter (Fig. 4d). Our result indicates that BEE1 could activate the *FT* promoter-*LUC* transcription (Fig. 4e). These results indicate that BEE1 associates with the chromatin region of the *FT* gene to promote its transcription.

BEE1 promotes flowering in a partially CRY dependent manner

BEE1 is a CIB1 related bHLH transcription factor, BEEs and CIBs all belong to subfamily 18, although *CIBs* (*CIB1, CIB2, CIB4, CIB5*) are not BR induced (Fig. S5) (Friedrichsen *et al.*, 2002). To test whether BEE1 regulation of flowering time is directly related to the CRY2 control of floral initiation, the same as CIB1, we compared the effect of BEE1 overexpression in the wild type and the *cry1cry2* mutant background. In contrast to transgenic plants overexpressing BEE1 in the wild-type background (*35S:MycBEE1*/Col-0) that flowered significantly earlier than the WT (Fig. 5a), transgenic plants overexpressing BEE1

(*35S:MycBEE1*) in the *cry1cry2* mutant background (*35S:MycBEE1*/*cry1cry2*) flowered slightly earlier than *cry1cry2* parent, but significantly later than the WT in the long day conditions (Fig. 5a), whereas the expression of *FT* was consistent with the flower phenotype of these genotypes (Fig. 5b, S5b). The different effects of BEE1 overexpression in the two different genetic backgrounds are not due to different level of BEE1 expression, because BEE1 protein level in none of the three independent *35S:MycBEE1*/*cry1cry2* lines tested was lower than that in the *35S:MycBEE1*/Col-0 line tested (Fig. 5c). We conclude that the function of BEE1 in promoting floral initiation and *FT* transcription is at least partially dependent on CRY1 and/or CRY2.

CRY2 interacts with BEE1 to affect the binding ability of BEE1 to the *FT* promoter

BEE1 is a nuclear protein. BEE1-CFP can be detected in the nucleus in tobacco, and the blue fluorescence of BEE1-CFP co-localizes with the green fluorescence of CRY2-YFP, especially in the photobodies (Fig. 6a). We used the yeast two-hybrid assay to analyze the directly interaction between CRY2 and BEE1 in both blue light and the dark conditions. In yeast cells, BEE1 interacts with CRY2 only in blue light but not in the dark, but it interacts with $CRY2^{W374A}$ (the constitutively active form of CRY2) in both blue and dark conditions, as demonstrated by the blue light-dependent rescue of *His3* transcription and histidine auxotrophy (Fig. 6b). In contrast, yeast cells expressing CRY2 or BEE1 alone failed to rescue the histidine auxotrophy (Fig. 6b). To examine the *in vivo* interaction of BEE1 and CRY2, Bimolecular luminescence complementation (BiLC) was applied. BiLC assays indicated that BEE1 interacted directly with CRY2 in plant cells (Fig. 6c). BiFC (Bimolecular fluorescence complementation) assay (Bai *et al.*, 2007) further confirmed that CRY2 interacted with BEE1 in plant cell. In tobacco leaf epidermal cells co-expressing the C-terminal half of CFP fused to BEE1 (BEE1-cCFP) and the N-terminal half of YFP fused to CRY2 (CRY2-nYFP), strong YFP fluorescence was observed (Fig. 6d). In contrast, no YFP signal was observed when BEE1-cCFP and no-fusion nYFP, or CRY2-nYFP and no-fusion cCFP, were co-transformed (Fig. 6d). We further examined the in vivo interaction between CRY2 and BEE1 using co-immunoprecipitation (co-IP) assay under blue light condition. MycBEE1 was co-immunoprecipitated with CRY2 from the nucleus (Fig. 6e).

BEE1 promotes floral initiation and *FT* transcription in an at least partially CRY1 and/or CRY2 dependent manner and BEE1 interacts with CRY2 in a blue light dependent manner. To figure out the biological significance of the CRY2-BEE1 interaction, ChIP assay were applied to check the binding ability of BEE1 to the *FT* promoter in Col-0 and *cry1cry2* backgrounds. The ChIP-qPCR showed that BEE1 could bind *FT* promoter in Col-0 but not in *cry1cry2* background (Fig. 6f), indicating that CRYs affect the DNA binding activity of BEE1 to the *FT* promoter.

CIBs form heterodimers to bind the *FT* promoter, BEE1 binds the similar region of *FT* promoter to promote the transcription of *FT* (Liu, Y *et al.*, 2013), we examine the interaction between BEE1 and CIB1. BEE1 and CIB1 are both nucleus proteins and they co-localize in the nucleus (Fig. S9a). BiFC assay was applied to test their interaction. In tobacco leaf epidermal cells co-expressing the C-terminal half of CFP fused to BEE1 (cCFP-BEE1) and the N-terminal half of YFP fused to CIB1 (nYFP–CIB1), strong YFP fluorescence was observed (Fig. S9b). In contrast, no YFP signal was observed when cCFP-BEE1 and no-fusion nYFP (Fig. S9b). BEE1 interacts with CIB1, they may also form heterodimers to regulate the *FT* transcription.

The BEE1 protein is regulated by blue light

As we know, most of the proteins involved in light signal transduction are light regulated, such as CRY2 protein which gets degraded under blue light (Yu *et al.*, 2007), PHYA protein undergoes rapid degradation in red light (Clough *et al.*, 1999) , and PIFs get degraded in the presence of red light (Al-Sady *et al.*, 2006). CIBs are stabilized specifically under the blue light (Liu, H *et al.*, 2013; Liu, Y *et al.*, 2013). CRY2 interacts with BEE1 in a blue light dependent manner to regulate its DNA binding ability, BEE1 seems to be also a CRY2 signaling partner. Consistent with this hypothesis, BEE1 is also blue light regulated. We analyzed light responsiveness of mRNA expression of the endogenous *BEE1* gene first, the mRNA expression of the endogenous *BEE1* gene appeared to increase in the first 1 h of blue-light treatment and then decreased slightly afterward (see later Fig. S11a). Similar to CIBs, the expression of BEE1 protein is regulated by blue light. We used transgenic plants

expressing the Myc-tagged BEE1 fusion protein, which is controlled by the constitutive *35S* promoter (*35S:MycBEE1*). The immunoblot experiments showed that the BEE1 protein was much less in plants grown in dark, red light or far red light, but it started to accumulate soon after plants were exposed to blue light (Fig. 7a, c). While abundant BEE1 protein was detected in plants exposed to blue light, the BEE1 protein level decreased after plants were transferred from blue light to dark, red light or far red light (Fig. 7d-f). We also investigated the red light and far red light effect on the stability of BEE1 protein compared to dark condition. When the plants were transferred from dark to red light, the BEE1 protein was accumulated at the first hour and then decreased (Fig. S10a) and the BEE1 protein was continually decreased after plants were transferred from red light to dark (Fig. S10c). The BEE1 protein level was not significantly changed when plants were transferred from dark to far red light (Fig. S10b), but the BEE1 protein was accumulated at the first hour and then decreased when plants were transferred from far red light to dark (Fig. S10d). These results indicate that the BEE1 protein is most stable under blue light, while red light also could stabilize it.

Given that light-dependent and ubiquitin/26S-proteasome-dependent proteolysis is a common mechanism regulating light signaling proteins (Lau & Deng, 2012), we examined blue-light effects on the BEE1 protein expression in the presence or absence of the 26S proteasome inhibitor MG132. Treatment of Myc-BEE1 OX seedlings with MG132 prevented the decline of BEE1 protein abundance in the absence of blue light (Fig. 7g). These results demonstrate that BEE1 is degraded by the 26S proteasome, and that blue light suppresses their degradation.

We next investigated whether CRY1 and CRY2 mediate blue-light promotion of the BEE1 protein accumulation by examining the BEE1 protein expression in *cry1 cry2* mutant. To our surprise, although BEE1 physically interacts with CRY2 in response to blue light, the *cry1 cry2* mutants showed no discernable defect in the blue-light regulation of BEE1 protein expression (Fig. 7h, i). The BEE1 protein levels increased in response to blue light and decreased in the absence of blue light in both the wild-type and the *cry1 cry2* mutant (Fig. 7h,

i). Therefore, neither CRY1 nor CRY2 is the photoreceptor mediating blue-light suppression of BEE1 degradation.

Discussion

BES1-L regulates flowering initiation via photoperiodic pathway

Flowering time is regulated by very complex regulatory networks to ensure reproductive success. The photoperiod pathway mainly perceives external light signal from the environment to optimize seed production in specific environments. FT is one of the most important components of photoperiodic flowering pathway. Autonomous pathway is endogenous pathway that relates to the developmental stage of the plant, the autonomous pathway constitutes a heterogeneous group of genes including *FLC*, a key component in the autonomous pathway encoding a MADS box protein that represses flowering (Michaels & Amasino, 1999; Sheldon *et al.*, 1999). FLC delays flowering by blocking the transcription of genes in the photoperiodic pathway, such as *SOC1* and *FT*. *FLD*, *LD* (*LUMINIDEPENDENS*), *FCA* （*FLOWERING TIME CONTROL PROTEIN*） and *FLK* (*FLOWERING LOCUS K*) et al are also involved in autonomous pathway. BRs are a class of steroidal hormones essential for plant growth and development, including flowering (Yang *et al.*, 2005; Li *et al.*, 2010; Clouse, 2011; Bai *et al.*, 2012; Zhang *et al.*, 2013; Chaiwanon *et al.*, 2016). BRI1 deficient mutant *bri1* exhibits late flowering. it was reported that *bri1* delayed flowering of the autonomous pathway mutant *ld*, *fca* through enhancing *FLC* expression. BES1 is an important component of BR pathway and it also integrates other pathways to regulate many developmental processes. BES1 recruits two jumonji N/C (JmjN/C) domain-containing proteins, EARLY FLOWERING6 (ELF6) and RELATIVE OF EARLY FLOWERING6 (REF6), which are histone H3 Lys 27 demethylases (Yu *et al.*, 2008; Lu *et al.*, 2011), and ELF6 and REF6 genetically influence flowering (Noh *et al.*, 2004; Lu *et al.*, 2011), which gives another link between floral induction and the BR pathway. Our results indicate that BES1 and BEE1 are involved in photoperiodic flowering, and BEE1 directly activates the *FT* transcription to regulate photoperiodic flowering. BES1-BEE1-FT is a new signaling pathway in regulating photoperiodic flowering. BRs are involved in multiple pathways in regulating flowering.

BEE1 is a direct target of BES1

BEE1, *BEE2* and *BEE3* are early response genes in BR signaling, these bHLH transcription factors are induced by BR treatment, and they are positive regulators of BR signaling and plant growth (Friedrichsen *et al.*, 2002). *BEEs* are induced by BR, and BRI1 is critical for the BR induction of *BEEs* transcription, but the molecular mechanism of the *BEEs'* BR induction is unknown. Microarray experiments indicate that *BEE1* is up-regulated in the dominant mutant *bes1-D* (Yu *et al.*, 2011), but whether it is a direct target of BES1 is unknown, here we show that BES1 binds to the *BEE1* promoter in vitro and in vivo to directly regulate its transcription. BEE1, BEE2 and BEE3 are also reported to positively modulate the shade avoidance syndrome in Arabidopsis seedling (Cifuentes-Esquivel *et al.*, 2013), which gives another clue that BEEs are involved in light responses.

BES1 regulated *BEE1* controls flowering downstream of blue light signaling pathway

BR regulates numerous developmental processes, including hypocotyl elongation and flowering. BR signaling is highly integrated with the light, gibberellin and auxin pathways in controlling photomorphogenesis. Auxin and BRs play very important roles in the regulation of enhanced hypocotyl elongation of Arabidopsis seedlings in response to low-blue light shade avoidance (Keuskamp *et al.*, 2011). BZR1 represses the expression of photoreceptors phytochrome B and phototropin 1, but activates the expression of negative regulators of photomorphogenesis, such as COP1 and SPA1 (Lau & Deng, 2012). GATA 2 and GATA4 are repressed by BR and they promote photomorphogenesis downstream of both BR and light signaling pathways. GATA2 is transcriptionally repressed by BR through BZR1 and posttranslational activated by light through inhibiting COP1-mediated ubiquitination and degradation (Luo *et al.*, 2010). B-box zinc finger factor BZS1/BBX20, a positive regulator of photomorphogenesis, is also transcriptionally repressed by BZR1 and post-translationally activated by light (Sun *et al.*, 2010; Fan *et al.*, 2012). PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) is a bHLH transcription factor directly link red light photoreceptor PHYTOCHROME B (PHYB) and blue light photoreceptor CRY to light-regulated gene expression and plant development (Huq & Quail, 2002; Castillon *et al.*, 2007; Leivar & Quail, 2011; Ma *et al.*, 2016; Pedmale *et al.*, 2016). ChIP-chip and ChIP-seq data show that PIF4

and BZR1 bind the same locations, and they directly interact with each other and show synergistic and interdependent relationship in promoting gene expression and photomorphogenesis (Oh *et al.*, 2012). Photoreceptors UVR8, crys and phys also physically interact with BES1 to inhibit its DNA binding activity and BR promoted elongation (Liang *et al.*, 2018; Wang *et al.*, 2018). Endogenous BR signaling and environmental light signaling act antagonistically to regulate elongation. Here we show that BEE1 is a positive regulator of photoperiodic flowering, and it is a direct target of BES1. Furthermore, BEE1 physically interacts with CRY2 and it promotes flowering in a CRYs partially dependent manner, its binding and activation of *FT* are repressed in the absence of CRY1 and CRY2, while it still could slightly promote flowering in *cry1 cry2* mutants, this suggests it acts in another pathway of floral promotion which does not involve direct regulation of *FT*. Interestingly, BEE1 is transcriptionally activated by BR through BES1 and posttranslational activated by blue light. Endogenous BR signaling and environmental blue light signaling coordinately to regulate flowering via BEE1, BRs promote the transcription of *BEE1* so as to promote *FT* transcription and flowering, while blue light promote the stabilization of BEE1 and crys also interacts with BEE1 to promote its transcription activity.

We show that BEE1 protein accumulates when the plants are moved from dark, red light or far red light to blue light, whereas it is degraded by the 26S proteasome in the absence of blue light. Interestingly, red light also has some effects on the stabilization of BEE1 protein, BEE1 protein accumulates when the plants are moved from dark to red light, whereas it get degraded when the plants are moved from red light to dark condition. These results indicate that BEE1 protein is regulated by not only blue light but also red light, and it is most stable under the blue light. CRYs are not responsible for the blue light stabilization of BEE1 protein. It is reported before that LOV-domain proteins ZTL and LKP2 act as the photoreceptors mediating blue-light–dependent expression of CIB1 (Liu, H *et al.*, 2013), it is possible that ZTL family is responsible for the blue light stabilization of BEE1. Phytochromes may be also involved in the red-light-dependent expression of BEE1. These results support a hypothesis that BEE1 is ubiquitinated by an unknown E3 ubiquitin ligase and degraded in the absence of blue light and red light; blue light photoreceptors (exclude CRYs) or phytochromes mediates

blue-light or red-light suppression of the expression or activity of the E3 ubiquitin ligase or other proteins required for BEE1 ubiquitination and degradation.

In summary, we showed that BRs and blue light photoreceptor CRY2 could both regulate BEE1, BRs induced the transcription of *BEE1* through BES1, while CRY2 physically interacted with BEE1 in response to blue light to affect the BEE1's DNA binding ability and further activate its transcription activity (Fig. S11b). BES1 regulated BEE1 controls photoperiodic flowering downstream of blue light signaling pathway in *Arabidopsis.*

ACKNOWLEDGEMENT

The authors thank Drs. Xuelu Wang, Yanhai Yin, Joanne Chory, Zhiyong Wang, Jianming Li, Mingyi Bai for materials and technical assistances. This work is supported in part by the National Natural Science Foundation of China (31721001, 31730009, 31670282), the National Key Research and Development Program of China (2017YFA 0503800) and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB27030000).

Author contributions:

FW, YG and HL conceived the project. FW, YG performed most of the experiments, YL did the co-IP, some qPCR and aided in the performance of the ChiP assays, XZ, XG performed the genotyping, and DM, ZZ, ZY helped to prepare constructs, HX provided materials, FW, YG and HL analyzed the data, and HL and FW wrote the manuscript. FW, YG and YL contributed equally to this work.

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Supplemental Fig.1 The transcription of *BES1* and *BEE1* in Col-0 and *BES1 RNAi*.

Supplemental Fig. 2 BES1 does not affect the transcription of *TOE1, CIB1, SPL9* and *SPL15*.

Supplemental Fig. 3 BES1 can not bind *FT* promoter

Supplemental Fig. 4 *bzr1-1D* does not regulate the transcription of *BEE1*

Supplemental Fig. 5 BR specifically induce the expression of *BEE1* and *BEE3*

Supplemental Fig. 6 BEE1 can promote flowering under long day blue light conditions

Supplemental Fig. 7 BEEs do not affect flowering time in SD conditions

Supplemental Fig. 8 *bee1 bee3* double mutant and *bee2* single mutant show similar flowering phenotype with Col-0

Supplemental Fig. 9 BEE1 interacts with CIB1

Supplemental Fig. 10 The effect of red light and far red light on the stability of BEE1 protein.

Supplemental Table 1. Oligonucleotide primers used is this work

Fig. 1 BES1 is a positive regulator of photoperiodic flowering in *Arabidopsis*

(a) Representative photos of 24-d-old plants of the genotypes indicated grown in 22°C LD (long day, 16-h light/ 8-h dark) conditions. Bar = 5 cm. **(b, c)** The quantitative flowering times measured as days to flower and the number of rosette leaves at the day floral buds became visible of genotypes indicated grown in 22°C LD conditions (b) or 22°C SD (short day, 8-h light/16-h dark) conditions (c). Error bars represent standard deviation ($n \geq 20$). The asterisks indicate significant differences compared with the Col-0 under the same conditions (*$p < 0.05$,** $p < 0.01$, Student's *t*-test). **(d)** Quantitative PCR results showing mRNA expression of *FT* in genotypes indicated grown in LD conditions. Samples were collected from 7-d-old seedlings of the genotypes indicated every 4 h over one day in LD. Expression levels are normalized to the *ACT7* mRNA level. Error bars represent standard deviation of 3 biological replicates. **(e)** Quantitative PCR results showing transcription of *BEE1, CO* and *SOC1* in genotypes indicated grown in LD conditions. Samples were collected from 7-d-old seedlings of the genotypes indicated at time indicated. Error bars represent standard deviation of 3 biological replicates.

Fig. 2 BEE1 is the direct target of BES1 in *Arabidopsis*

(a) Diagram of the gene structure for BEE1. Horizontal black lines depict the DNA regions that were amplified by ChIP-qPCR using the indicated primer set. **(b)** Representative result of the ChIP-qPCR assays. ChIP-qPCR assays were performed with an anti-GFP antibody. Plants were grown under LD conditions and treated with 1uM eBL for 2 h. The GFP-IP signal was normalized with the corresponding input signal to get the relative enrichment. Error bars represents standard deviation of three biological replicates. **(c)** A competitive EMSA showing interaction of BES1 with the Cy5-labeled fragment in *BEE1* promoter (-214 bp to -263 bp, including two E-Box, one is from -232 bp to -237 bp (E-Box 1) and the other is from -240 bp to -245 bp (E-Box 2)), competition by the unlabeled wild-type *BEE1* promoter (WT), or unlabeled *BEE1* promoter with mutation in only one E-Box (m1 or m2) and lack of a strong

competition by the *BEE1* promoter with mutations in both two E-Boxes (m1;2). TF is the abbreviation of "trigger factor" and is a tag of the pCold vector. **(d)** Structure of the *BEE1* promoter-driven dual-LUC reporter gene. 35S promoter, *BEE1* promoter (-2127bp to -1bp), REN luciferase (REN), firefly luciferase (LUC), and T-DNA (LB and RB) are indicated. **(e)** Relative reporter activity (LUC/REN) in planta. Tobacco leaves were transfected with the reporter and the effector BES1. The relative LUC activities normalized to the REN activity are shown (LUC/REN, n=3). Error bars represents standard deviation of three biological replicates.

Fig. 3 BEEs are positive regulators of photoperiodic flowering in *Arabidopsis*

(a, c) Representative photos of 21-d-old plants (a) or 27-d-old plants (c) of the genotypes indicated grown in 22°C LD conditions. Bar = 5 cm. **(b, d)** The quantitative flowering times measured as days to flower and the number of rosette leaves at the day floral buds became visible of genotypes indicated grown in 22°C LD conditions. Error bars represent standard deviation ($n \geq 20$). The asterisks indicate significant differences compared with the Col-0 under the same treatment conditions (** $p < 0.01$, Student's *t*-test).

Fig. 4 BEE1 associates with the chromatin regions of the *FT* gene in *Arabidopsis*

(**a)** Quantitative PCR results showing mRNA expression of *FT* in genotypes indicated grown in LD conditions. Samples were collected from 7-d-old seedlings of the genotypes indicated every 4 h over one day in LD. Expression levels are normalized to the *ACT7* mRNA level. Error bars represent standard deviation of 3 biological replicates. **(b)** Diagram of the gene structure for *FT*. Horizontal black lines depict the DNA regions that were amplified by ChIP-qPCR using the indicated primer set. **(c)** Representative result of the ChIP-qPCR assays. ChIP-qPCR assays were performed with an anti-MYC antibody. Plants were grown under LD conditions. 10-d-old seedlings of Col-0 and transgenic plants expressing *35S::Myc-BEE1* were transferred to blue light condition at ZT0 and the seedlings of transgenic plants expressing *35S::MycBEE1* kept in dark were also used as control. All the seedlings were harvested at ZT15. The MYC-IP signal was normalized with the corresponding input signal to get the relative enrichment. Error bars represents standard deviation of three biological

replicates. **(d)** Structure of the *FT* promoter-driven dual-LUC reporter gene. FT promoter (−2172 bp to -1 bp),35S promoter, REN luciferase (REN), firefly luciferase (LUC), and T-DNA (LB and RB) are indicated. **(e)** Relative reporter activity (LUC/REN) in planta. Tobacco leaves were transfected with the reporter and the effector (BEE1). The relative LUC activities normalized to the REN activity are shown (LUC/REN, n = 3). Error bars represents standard deviation of three biological replicates.

Fig. 5 BEE1 promotes flowering in a partially CRY dependent manner in *Arabidopsis*

(a) The quantitative flowering times measured as days to flower and the number of rosette leaves at the day floral buds became visible. Error bars represent standard deviation ($n \geq 20$). (** $p < 0.01$, Student's *t*-test). **(b)** Quantitative PCR results showing mRNA expression of *FT* in genotypes indicated grown in LD conditions. Samples were collected from 7-d-old seedlings of the genotypes indicated at ZT16. Error bars represent standard deviation of 3 biological replicates. **(c)** Immunoblots showing Myc-BEE1 protein levels in the genotypes indicated. Samples were fractionated by 10% SDS/PAGE, blotted, and probed by the anti-Myc antibody (BEE1). ACTIN is shown as the loading controls.

Fig. 6 CRY2 interacts with BEE1 to affect the binding ability of BEE1 to the *FT* promoter in *Arabidopsis*

(a) Co-localization of CRY2-YFP and BEE1-CFP in the nucleus. **(b)** Histidine auxotrophy assays showing blue light-dependent interaction between CRY2 and BEE1, and the interaction between $CRY2^{W374A}$ and BEE1. Yeast cells containing plasmids encoding the indicated proteins were grown on medium in the presence (+) or absence (-) of histidine, under blue light (Blue, 40 μmol $\cdot m^{-2} \cdot s^{-1}$) or in the dark (Dark) for 3 days.

(c) BiLC assays showing that BEE1 interacts with CRY2. Leaf epidermal cells of *N. benthamiana* were co-transformed with BEE1-nLUC and cLUC-CRY2 or cLUC or nLUC with cLUC-CRY2 or cLUC. **(d)** BiFC assays of the in vivo protein interaction. Leaf epidermal cells of *N.benthamiana* were co-transformed with BEE1-cCFP and CRY2-nYFP or cCFP and CRY2-nYFP or nYFP and BEE1-cCFP. BF, bright field. Merge, overlay of the YFP and bright-field images. **(e)** Co-IP assays using isolated nuclei from 14-day-old Col-0

and *35S:MycBEE1/*Col-0 seedlings grown in long day conditions, moved to dark for one day, pretreated in MG132 then exposed to blue light (40 μmol m^{-2} s^{-1}). Total proteins (Input) or IP product of anti-CRY2 antibody (CRY2-IP) were probed, in immunoblots, by the anti-CRY1 antibody (CRY1), stripped and reprobed by the anti-MYC antibody. **(f)** Representative result of the ChIP-qPCR assays. ChIP-qPCR assays were performed with an anti-MYC antibody. Plants were grown under LD conditions and harvested at ZT15. The MYC-IP signal was normalized with the corresponding input signal to get the relative enrichment. Error bars represents standard deviation of three biological replicates. The *FT* gene structure and primers locations are the same as in Fig. 4b.

Fig. 7 The *Arabidopsis* BEE1 protein is regulated by blue light

(a, b, c) 10-d-old *35S:MycBEE1* transgene in Col-0 plants were grown in LD conditions, transferred to dark (**a)** or red light (20 μmol·m^{-2}·s^{-1}) (**b)** or far red light (5 μmol·m^{-2}·s^{-1}) (**c**), respectively, for 16 h, and then transferred to blue light (40 μmol ·m^{-2}·s^{-1}) for the indicated time before sample collection. **(d, e, f)** 10-d-old *35S:MycBEE1* transgene in Col-0 plants were grown in LD conditions, transferred to continuous blue light for 16 h, and then transferred to dark (**d**), red light (**e**), or far-red light (**f**), respectively, for the indicated time before sample collection. **(g)** Immunoblot showing the inhibition of BEE1 degradation by the proteasome inhibitor MG132. *35S:MycBEE1* transgene in Col-0 plants were grown in LD conditions for 10 days, and leaves were excised and incubated with MG132 (50 μmol/L) or mock solution (0.1% DMSO) in darkness for the indicated time before sample collection. **(h)** Transgenic plants expressing the *35S:MycBEE1* transgene in Col-0 or *cry1 cry2* were grown in LD for 10 days and exposed to blue light (40 μmol ·m^{-2}·s^{-1}) for 16 h and then transferred to dark for the indicated time before sample harvest. **(i)**The 10-day-old *35S:MycBEE1* transgene in Col-0 or *cry1 cry2* plants were treated in dark for 16 h and then transferred to blue light (40 μmol ·m^{-2}·s^{-1}) for the indicated time. Samples were fractionated by 10% SDS/PAGE, blotted, and probed by the anti-Myc antibody (BEE1). CRY1 or ACTIN are shown as the loading controls. Because of uncontrolled exposure times of ECL of different immunoblots, results of different blots are not directly comparable.