Brassinosteroids Regulate Circadian Oscillation via the BES1/TPL-CCA1/LHY Module in Arabidopsis thaliana

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HIGHLIGHTS
Circadian rhythm is shortened in the presence of phytohormone BR
BR signaling is relayed to circadian clock through BES1
BES1/TPL complex binds to CCA1/LHY promoter in the dark, repressing expression
The BES1/TPL-CCA1/LHY module shapes circadian gating of BR signaling

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Brassinosteroids Regulate Circadian Oscillation via the BES1/TPL-CCA1/LHY Module in Arabidopsis thaliana

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SUMMARY
Brassinosteroids (BRs) regulate a variety of physiological processes in plants via extensive crosstalk with diverse biological signaling networks. Although BRs are known to reciprocally regulate circadian oscillation, the molecular mechanism underlying BR-mediated regulation of circadian clock remains unknown. Here, we demonstrate that the BR-activated transcription factor bri1-EMS-SUPPRESSOR 1 (BES1) integrates BR signaling into the circadian network in Arabidopsis. BES1 repressed expression of CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCHOTYL (LHY) at night by binding to their promoters, together with TOPLESS (TPL). The repression of CCA1 and LHY by BR treatment, which occurred during the night, was compromised in bes1-ko and tpl-8 mutants. Consistently, long-term treatment with BR shortened the circadian period, and BR-induced rhythmic shortening was impaired in bes1-ko and tpl-8 single mutants and in the cca1-1 lhy-21 double mutant. Overall, BR signaling is conveyed to the circadian oscillator via the BES1/TPL-CCA1/LHY module, contributing to gating diurnal BR responses in plants.

INTRODUCTION
Steroids are important for biological development in animals and plants. While animal steroid hormones are perceived by nuclear receptors (He et al., 2010; Sever and Glass, 2013), the plant steroid hormone brassinosteroid (BR) binds to the extracellular domain of the membrane-bound receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Kinoshita et al., 2005; Hothorn et al., 2011; Sun et al., 2013). The plasma-membrane-anchored co-receptor of BRI1, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), activates intracellular signaling pathways comprising the serine/threonine protein kinase BRASSINOSTEROID-SIGNALING KINASE 1 (BSK1), the protein phosphatase bri1-SUPPRESSOR 1 (BSU1), the GLYCOCEN SYNTHASE KINASE 3 (GSK3)-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2), PROTEIN PHOSPHATASE 2A (PP2A) phosphatases, and BRASSINAZOLE RESISTANT 1 (BZR1) family transcription factors (Ye et al., 2011; Wang et al., 2012).

In the absence of BRs, BIN2 inactivates BES1/BZR2 and BZR1 via phosphorylation (He et al., 2002; Wang et al., 2002; Yin et al., 2002). The phosphorylated BES1 and BZR1 not only retain their cytoplasmic localization but also exhibit reduced affinity for DNA (Gampala et al., 2007; Ryu et al., 2007, 2010). In the presence of BRs, BRI1 inactivates BIN2 and stimulates PP2A activity to catalyze the dephosphorylation of BES1 and BZR1 (Tang et al., 2011). Dephosphorylated BES1 and BZR1 then enter the nucleus, where they regulate the expression of BR-responsive genes by binding directly to their promoters (He et al., 2005; Yin et al., 2005; Sun et al., 2010).

BRs regulate a variety of developmental and physiological processes in plants including cell division, photomorphogenesis, reproductive organ development, stomata development, leaf senescence, and biotic and abiotic stress responses (Wang et al., 2012; Gruszka, 2013; Zhiponova et al., 2013; Wang et al., 2001; Planas-Riverola et al., 2019; Yu et al., 2018; Ackerman-Lavert and Savaldi-Goldstein, 2020). Consistently, the molecular crosstalk between the BR signaling pathway and other hormonal and developmental signaling pathways is starting to emerge.

In plants, the circadian clock maintains an endogenous rhythm with a period of ~24 h (Covington et al., 2008; Mizuno and Yamashino, 2008; Hsu and Harmer, 2012). Extensive transcriptional feedback loops...
form the basic framework of the clock oscillator. Intricate connections among several transcriptional regulators, including CCA1, LHY, TIMING OF CAB EXPRESSION 1 (TOC1), PSEUDO-RESPONSE REGULATORS (PRRs), EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX), are involved in the maintenance of circadian homeostasis (Alabadi et al., 2001; Nakamichi et al., 2010; Nusinow et al., 2011; Huang et al., 2012). The circadian oscillator diurnally regulates a variety of output pathways in plants, including primary and secondary metabolism, hormone biosynthesis and signaling, and responses to environmental challenges to induce relevant physiological changes at a specific time of the day (Dodd et al., 2005; Legnaioli et al., 2009; Singh and Mas, 2018). In addition, it is noteworthy that some circadian-controlled phytohormones reciprocally regulate core clock genes to ensure an accurate circadian gating of hormone metabolism and signaling (Covington and Harmer, 2007; Lee et al., 2016; Singh and Mas, 2018).

A bidirectional regulation has been suggested between the circadian clock and BR signaling. The majority of BR biosynthesis and signaling genes are clock-controlled (Bancos et al., 2006). Moreover, exogenous BR application also influences clock activity (Hanano et al., 2006). However, molecular factors and mechanisms underlying the link between BR signaling and the circadian clock remain unclear. In this study, we demonstrate that BES1 regulates CCA1 and LHY expression to relay BR signals to the clock oscillator. In the presence of BRs, BES1 binds to CCA1 and LHY promoters and represses their expression, especially at night. This BES1-CCA1/LHY module delicately regulates circadian oscillation to ensure acute activation of BR signaling at a specific time of day.

RESULTS

BES1 Specifically Relays BR-Regulated Clock Activity

Although circadian clock activity exerts diurnal control over hormone signaling, it is also reciprocally regulated by hormone signaling (Covington and Harmer, 2007; Lee et al., 2016; Singh and Mas, 2018; Nohales and Kay, 2019). Plant hormones, specifically abscisic acid (ABA) and BRs, have a considerable impact on circadian oscillation (Hanano et al., 2006; Lee et al., 2016), although the underlying molecular mechanisms remain unknown. Therefore, we investigated the link between BR signaling and circadian networks. To test whether BRs influence clock oscillation in our growth conditions, we examined the effects of exogenous BR treatment on circadian oscillation by monitoring luciferase (LUC) activity in pCCA1:LUC transgenic plants. Exogenous treatment with epi-brassinolide (epi-BL) significantly shortened the circadian period (Figure 1A). Quantitative analysis revealed that mock-treated wild-type plants displayed circadian periods of approximately 24.6 h under free-running conditions, whereas epi-BL-treated wild-type plants showed a circadian period of approximately 23.7 h (Figure 1A).

To understand the molecular basis of BR-mediated regulation of the circadian clock, we interfered with BR signaling and examined circadian oscillation. Notably, a potent chemical inhibitor of GSK proteins, bikinin (Uehara et al., 2019), shortened the rhythmic period in pCCA1:LUC transgenic plants compared with mock treatment (Figure 1B), similar to the epi-BL treatment (Figure 1A). These observations suggest that the effect of BRs on circadian oscillation could be mediated by GSK kinases or by downstream components of the BR signaling pathway, such as BES1 and BZR1. Because circadian components are largely under transcriptional control, we focused on the potential role of BES1 and BZR1 in circadian oscillation.

We analyzed circadian oscillation in bes1-1D and bZR1-1D mutants, which constitutively activate BES1 and BZR1, respectively (Wang et al., 2002, 2012; Li et al., 2018). Quantitative real-time RT-PCR (RT-qPCR) analysis of the CHLOROPHYLL A/B-BINDING PROTEIN 2 (CAB2) circadian marker gene revealed that bes1-1D plants exhibited a shortened circadian period (Figure 1C), whereas bZR1-1D plants exhibited normal circadian oscillation (Figure S1), similar to wild-type plants under free-running conditions (Figure S1). However, bes1-ko single mutant did not display alterations in circadian oscillation, probably because the BR signaling was below the threshold required for circadian control under normal growth conditions (Figure S1). In support, the brr1-5 mutant also exhibited no alteration in circadian oscillation compared with wild-type under normal growth conditions (Figure S2). These results suggest that BES1 is responsible for controlling circadian oscillation especially in the presence of BRs.

BES1 Binds to CCA1 and LHY Promoters

Next, we aimed to decipher how BES1 regulates circadian oscillation. To identify the target genes of BES1 in the circadian pathway, we analyzed cis-elements in the core clock gene promoters, which may be targeted by the
atypical basic-helix-loop-helix (bHLH) DNA-binding motifs of BES1 (Nosaki et al., 2018). Promoter analysis revealed the presence of the conserved BR-response element (BRRE) (CGTG(T/C)G) and/or E-box (CAnnTG) motif in the promoter regions of several core clock genes (Li et al., 2018) (Figure 2A).

Figure 1. BES1 Is Responsible for BR-Dependent Circadian Control

(A) Effects of exogenous BR treatment on circadian period. pCCA1:LUC transgenic plants (Col-0 background) grown and entrained for 7 days on MS-solid medium with 3% sucrose under neutral day (ND) conditions were transferred to continuous dark (DD) conditions. During the free-running conditions, seedlings were incubated in MS-liquid medium supplemented with or without 100 μM epi-brassinolide (BL). Circadian period estimates of pCCA1:LUC activity in the presence or absence of 100 μM epi-BL was shown (right panel). Data represented as mean ± SEM were analyzed with using the Fast Fourier Transform Non-linear Least Squares (FFT-NLLS) suite of programs available in the Biodare2 software (n > 15). Significant differences between Mock and BL are indicated using an asterisk (*p < 0.05; Student’s t test).

(B) Effects of bikinin on circadian oscillation. Seven-day-old pCCA1:LUC transgenic plants grown under ND conditions were transferred to DD conditions. LUC activity was examined in the presence or absence of 40 μM bikinin (left panel). Circadian period estimates of pCCA1:LUC activity in the presence or absence of 40 μM bikinin was shown (right panel). Data represent mean ± SEM (n > 15) and were analyzed by FFT-NLLS. Significant differences between Mock and Bikinin are indicated using an asterisk (*p < 0.05; Student’s t test).

(C) Expression of CAB2 in bes1-D mutant. Seedlings grown under ND conditions for 2 weeks were transferred to continuous light (LL) conditions. Transcript levels of genes were determined by quantitative real-time RT-PCR (RT-qPCR). Gene expression values were normalized relative to the expression of EUKARYOTIC TRANSLATION INITIATION FACTOR 4a1 (eIF4a) and represented as n-fold relative to the value of the sample at ZT24. Data represent mean ± SEM.
To determine whether BES1 binds to core clock gene promoters to control circadian oscillation, we employed \textit{pBES1:bes1-D-HA} plants and performed chromatin immunoprecipitation (ChIP) analysis using an anti-HA antibody. Fragmented DNA was eluted from protein–DNA complexes and subjected to quantitative PCR (qPCR) analysis. DNA enrichment indicated that BES1 binds to \textit{CCA1} and \textit{LHY} promoters (Figure 2B) but not to the promoters of other clock genes, including \textit{ELF3}, \textit{LUX}, \textit{TOC1}, \textit{PRR5}, \textit{PRR7}, and \textit{PRR9} (Figures 2B and S3). In addition, coding regions of \textit{CCA1} and \textit{LHY} loci were not targeted by BES1 (Figure S4), indicating that the promoter regions of \textit{CCA1} and \textit{LHY} are the primary binding sites of BES1. In addition, BZR1 did not bind to any of the core clock gene promoters (Figure S5) and was independent of circadian regulation. Overall, our results indicate that BES1 is specifically associated with \textit{CCA1} and \textit{LHY} genes.

To confirm the results, we performed transient \(\beta\)-glucuronidase (GUS) expression assays using \textit{Arabidopsis} protoplasts. We designed two reporter constructs for each gene: one was a longer promoter sequence containing BES1-binding site (BS) (B regions in Figure 2A) and the other was a shorter promoter sequence without BES1-BS (Figure S6). The promoter sequences were transcriptionally fused to a cauliflower mosaic...
These observations indicate that BES1 directly regulates the expression of CCA1 at zeitgeber time 40 (ZT40) (Figure 2C), consistent with the circadian accumulation of BES1 protein.

BR signaling components were predicted to be circadian-regulated (Figure S7). However, the BES1 gene did not exhibit rhythmic expression (Figure S7). Nonetheless, the stability of BES1 protein is known to be diurnally regulated by posttranslational modifications (Martinez et al., 2018). Notably, an active dephosphorylated form of BES1 is enriched during night time (Yang et al., 2017; Zhang et al., 2017; Martinez et al., 2018). Based on these observations, we speculated that BES1 regulation of the circadian clock may be relevant at night. To test this possibility, we examined the time of day when BES1 binds to CCA1 and LHY promoters. ChIP-qPCR analysis showed that BES1 bound CCA1 and LHY promoters primarily at zeitgeber time 40 (ZT40) (Figure 2C), consistent with the circadian accumulation of BES1 protein. These observations indicate that BES1 directly regulates the expression of CCA1 and LHY, especially during the night.

BR Shortens the Circadian Period via the BES1-CCA1/LHY Module

BES1 mediates the effects of BRs on the circadian clock by controlling the expression of CCA1 and LHY genes, in the presence of BR. To provide further support, we examined the transcript accumulation of CCA1 and LHY in wild-type seedlings subjected to a short-term (4 h) BR treatment at different times during the day. RT-qPCR analysis showed that BR suppressed CCA1 and LHY expression in wild type (Figure 3A), and this suppression occurred only from ZT40 to ZT44 (Figure 3A). Furthermore, this BR-dependent suppression of CCA1 and LHY was impaired in bes1-ko mutant plants (Figure 3A), whereas the expression of other clock genes was uninfluenced from ZT40 to ZT44 (Figure 3B). This result is consistent with the binding of BES1 to CCA1 and LHY promoters at night (Figure 2C), suggesting that the BES1-CCA1/LHY module is most likely responsible for the BR-regulated circadian rhythm.

Furthermore, this result raised the possibility that BR-induced period shortening (Figure 1A) is also mediated by BES1 and CCA1/LHY. To test this possibility, we monitored the bioluminescence of protoplasts transiently expressing pCCA1:LUC. In wild-type protoplasts expressing pCCA1:LUC, LUC activity oscillation was similar to that observed in wild-type seedlings (Figures 1A and 4A), and long-term treatment with epi-BL reduced circadian period (Figures 4A and 4B). Notably, BR-dependent period shortening disappeared in bes1-ko single mutant (Figure 4A) and cca1-1lhy-21 double mutant protoplasts (Figure 4B). These results indicate that the BES1-CCA1/LHY module is important for reciprocal clock resetting in response to BR treatment.

TPL Is Required for BES1-Dependent Repression of CCA1 and LHY

The BES1 protein acts either as a transcriptional activator or as a transcriptional repressor (Yin et al., 2002, 2005; Li et al., 2009; Espinosa-Ruiz et al., 2017). In this study, BES1 suppressed CCA1 and LHY expression, in the presence of BRs. We thus postulated that BES1 recruits additional trans-factors to repress gene expression. Previous studies showed that BES1 usually accompanies TPL and TPL-RELATED (TPR) proteins in processes requiring predominantly repressive activities (Ryu et al., 2014; Espinosa-Ruiz et al., 2017; Kim et al., 2019). Yeast-two-hybrid (Y2H) assays performed in this study supported the BES1-TPL interaction (Figure S8). However, none of the other clock components showed physical interaction with BES1 (Figure S8). Based on these observations, we speculated that the BES1-TPL transcriptional co-repressor complex primarily represses CCA1 and LHY expression to control circadian oscillation in response to BRs.

To support our claim, we examined the binding of TPL to CCA1 and LHY promoters using pTPL:TPL-HA transgenic plants. ChIP-qPCR analysis revealed that TPL directly associated with CCA1 and LHY loci (Figure 5A). The binding sites of TPL on CCA1 and LHY promoters overlapped with those of BES1 (Figures 5A and 2B, see also Figures S3, S4, S9, and S10). Furthermore, the binding of TPL to CCA1 and LHY promoters was observed specifically at night (Figure 5B), consistent with the timing of BES1 binding (Figure 2C).
We also performed transient coexpression analyses using *Arabidopsis* protoplasts. The core binding regions of BES1 in *CCA1* and *LHY* promoters were cloned upstream of the β-glucuronidase (GUS) reporter gene. Recombinant reporter plasmids (*pCCA1*:GUS and *pLHY*:GUS) were cotransformed with effector plasmids (*p35S*:bes1-D and *p35S*:TPL) into *Arabidopsis* protoplasts isolated from wild-type and tpl-8 mutant leaves. In wild-type protoplasts, cotransfection of reporter plasmids with the bes1-D construct repressed *CCA1* and *LHY* expression (Figure 5C). In addition, gene repression was synergistic when coexpressed with the TPL construct (Figure 5C). In tpl-8 mutant protoplasts, the repression of *CCA1* and *LHY* by BES1 was impaired, but TPL complementation restored BES1-dependent repression of *CCA1* and *LHY* (Figure 5C).

These results were supported by the short-term treatment of wild-type and tpl-8 mutant plants with epi-BL; although short-term epi-BL treatment reduced the expression of *CCA1* and *LHY* genes from ZT40-44 in wild-type seedlings, this effect was compromised in tpl-8 mutant seedlings (Figure 6A), similar to that observed in bes1-ko mutant plants (Figure 3A). Furthermore, the effect of long-term treatment with BR
on circadian oscillation was also mediated by TPL. BR-induced period shortening was impaired in tpl-8 protoplasts expressing pCCA1:LUC (Figure 6B), indicating that BES1 requires TPL to suppress CCA1 and LHY expression and thereby shorten the circadian period, in the presence of BR.

Figure 4. BR-Regulated Circadian Oscillation Is Compromised in bes1-ko and cca1-1lhy21 Mutants
(A and B) Bioluminescence circadian rhythms of bes1-ko (Col-0 background) (A) and cca1-1 lhy-21 (Ws-2 background) (B) mesophyll protoplasts transiently expressing the pCCA1:LUC construct in the presence or absence of 100 μM epi-BL. Mesophyll protoplasts of bes1-ko, cca1-1 lhy-21, and wild type were isolated from leaves of 3-week-old seedlings entrained MS-solid medium under ND cycles. Isolated protoplasts were transiently PEG-transfected with the pCCA1-LUC plasmid and then subjected to DD conditions to monitor bioluminescence traces. Data represent mean ± SEM (*p < 0.05; Student’s t test). Period estimates of pCCA1:LUC activity in the presence or absence of 100 μM epi-BL analyzed by FFT-NLLS in Biodare2 were shown (right panel). NS, not significant.
Taken together, our results suggest that the BES1/TPL-CCA1/LHY module reciprocally regulates circadian oscillation. In the presence of BRs, BES1 is activated at night and binds to CCA1 and LHY promoters, together with TPL, to repress their expression. Consequently, BES1 delays the rising phase of CCA1 and LHY (Figure 7), which attenuate BR responses (Figure S11). This circadian phase resetting allows precise gating of BR signaling as well as plant growth and development at a specific time of day. In addition, the BES1/TPL-CCA1/LHY-module-dependent circadian control is likely relevant in tissues or cells with high concentrations of BRs.

Figure 5. Repression of CCA1 and LHY by BES1 Is Dependent on TPL
(A) Binding of TPL to CCA1 and LHY promoters.
(B) Timing of TPL binding to CCA1 and LHY promoters at ZT40. In (A) and (B), 2-week-old plants grown under ND cycles were subjected to LL conditions and harvested at ZT28 and ZT40 for ChIP analysis with anti-HA antibody. Enrichment of putative TPL binding regions in clock gene promoters was analyzed by ChIP-qPCR. Data represent mean ± SEM (*p < 0.05; Student’s t test).
(C) Transient expression analysis using Arabidopsis protoplasts. Core elements containing BES1-binding sites (BS) of CCA1 and LHY promoters were cloned into the reporter plasmid. The effector and reporter constructs were transiently coexpressed in Col-0 and tpl-8 protoplasts. GUS activity in protoplasts was measured using a fluorometer. LUC gene expression was used to normalize GUS activity. Data represent mean ± SEM of biological triplicates. Different letters represent a significant difference at p < 0.05 (one-way ANOVA with Fisher’s post hoc test).
Figure 6. TPL Mediates BL-Regulated Circadian Oscillation

(A) Impaired repression of CCA1 and LHY by BL in tpl-8. Two-week-old seedlings grown under ND conditions were transferred to MS-liquid medium supplemented with or without 1 μM epi-BL at the indicated ZT points and incubated for 4 h under LL conditions. Transcript accumulation was analyzed by RT-qPCR. Gene expression values were normalized relative to eIF4a expression and represented as n-fold relative to the value of the mock-treated wild-type sample. Data represent mean ± SEM (*p < 0.05; Student’s t test).

(B) Bioluminescence circadian expression of protoplasts isolated from Col-0 and tpl-8 leaves in the presence of 100 μM epi-BL. Mesophyll protoplasts of tpl-8 and wild type were isolated from leaves of 3-week-old seedlings entrained on MS-solid medium under ND cycles. Isolated protoplasts were transiently PEG-transfected with the pCCA1-LUC plasmid and
DISCUSSION
Circadian Gating of Hormone Signaling

The circadian clock is a biological timekeeper that ensures an endogenous rhythm with a period of approximately 24 h. Correct matching of the endogenous rhythm with environmental cycles maximizes plant fitness and survival (Nozue et al., 2007; Nomoto et al., 2012; Lee et al., 2016). Approximately 50% of the Arabidopsis transcriptome is under the control of the circadian clock (Mizuno and Yamashino, 2008; Doherty and Kay, 2010); thus many biological processes are diurnally gated (Zhou et al., 2015; Lee et al., 2016; Nohales and Kay, 2019). For example, stress response and hormone-related pathways are under robust circadian regulation (Covington and Harmer, 2007; Grundy et al., 2015; Singh and Mas, 2018; Thines et al., 2019), and consequently many biological processes related to stress responses and hormone-mediated plant growth and development are gated at a specific time of day (Zhou et al., 2015; Lee et al., 2016; Nohales and Kay, 2019).

Accumulating evidence shows that circadian gating is shaped by bidirectional regulation. Not only the circadian clock regulate output pathways but also components of output pathways reciprocally regulate the clock oscillator (Lee et al., 2016). This bidirectional regulation readjusts circadian oscillation, thus allowing optimum coordination of output signaling with environmental cycles (Lee et al., 2016). For instance, bidirectional regulation has been demonstrated between circadian and ABA signaling. Plants are usually exposed to drought stress during the day, and the water-deficit crisis is generally at its peak in the afternoon (Legnaioli et al., 2009; Seo and Mas, 2015; Lee et al., 2016). Consistently, ABA metabolism and responses are gated primarily from midday to dusk. The majority of ABA signaling genes is diurnally activated and displays peak expression in the afternoon (Michael et al., 2008; Legnaioli et al., 2009; Lee et al., 2016). The CCA1 and Evening Complex transcriptional repressors are putative upstream regulators of stress signaling genes and allow their expression during the day (Legnaioli et al., 2009; Lee et al., 2016). In addition, ABA signaling reciprocally regulates circadian activity. The ABA-inducible MYB96 transcription factor activates the TOC1 gene by binding directly to its promoter (Lee et al., 2016). This reciprocal regulation of the circadian oscillator by output pathways most likely facilitates the readjustment of circadian oscillation in a sophisticated manner, ensuring near-perfect matching between diurnal physiological processes and environmental fluctuations to maximize plant performance under a given condition.

Bidirectional Regulation between BRs and the Circadian Clock

BR metabolism and signaling are intensively regulated by the circadian control. The BR biosynthesis gene CONSTANATIVE PHOTOMORPHOGENIC DWARF (CPD) exhibits rhythmic expression, with peak expression at dusk (Bancos et al., 2006). Furthermore, BR signaling components are circadian controlled and show diurnal changes in protein accumulation (Bancos et al., 2006; Martinez et al., 2018; Kim et al., 2019). For example, E3 ligases regulate the levels of phosphorylated and dephosphorylated BES1 proteins. During the light period, SINA OF ARABIDOPSIS THALIANAs (SINATs) accumulate to mediate the degradation of dephosphorylated BES1; thus a low ratio of dephosphorylated BES1 to phosphorylated BES1 (BES1/pBES1) is maintained to attenuate BR signaling (Yang et al., 2017). During the dark period, SINATs are degraded in a self-dependent manner, thus increasing the level of dephosphorylated BES1. In addition, pBES1 is degraded by the CONSTANTIVE PHOMORPHOGENIC 1 (COP1) protein, leading to a high ratio of BES1/pBES1 and activation of BR signaling (Kim et al., 2014; Yang et al., 2017).

We showed that BR signaling reciprocally influences circadian oscillation. BRs activated BES1, which subsequently repressed the expression of CCA1 and LHY genes during the night, together with TPL. Consistently, circadian oscillation in bes1-ko and tpi-8 single mutants and cca1-1lhy-21 double mutant was insensitive to BR treatment. These results indicate that the BES1-CCA1/LHY module plays a central role in relaying BR signaling to the circadian oscillator, establishing a bidirectional regulation between BR and circadian signaling.

BR regulation of the circadian clock likely strengthens BR responses at night. For instance, circadian gating of BR signaling is particularly important for cell elongation (Zhang et al., 2017). Consistently, the growth rate
of hypocotyls in Arabidopsis is the highest at night (Nusinow et al., 2011; Soy et al., 2012; Pham et al., 2018). Reciprocal regulation of the circadian clock by BR-activated BES1 ensures circadian gating. BES1 suppresses CCA1 and LHY expression, which attenuate BR signaling, and delays the rising phase of the morning genes during the night, ensuring acute BR signaling at a specific time of day. Notably, this function is exaggerated by long-term exogenous BR treatment, which shortens the circadian period. Given that the circadian clock is globally linked to vast cellular networks, this signaling scheme is not limited to BR responses but is widely applicable to a variety of biological processes in plants.

Limitations of the Study
In this study, we demonstrated that the phytohormone BR regulates circadian clock through direct binding of BES1 to the promoters of CCA1 and LHY. We would like to note that the effects of BR on circadian rhythm is observed upon the treatment with high concentrations of epi-BL. We suspect that additional internal and external cues should be further considered to account for the physiological impact of BR in circadian clock.

In addition, we have demonstrated the bidirectional regulation between circadian clock and BR response, but the detailed molecular mechanism of how CCA1/LHY attenuate BR signaling should be elucidated in the future studies.

Resource Availability
Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pil Joon Seo (pjseo1@snu.ac.kr).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
The data used and analyzed during this study are available from the corresponding author on reasonable request.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101528.
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AUTHOR CONTRIBUTIONS

P.J.S. participated in the design of the study and wrote the article. H.G.L., J.H.W., Y.R.C., and K.L. performed the molecular experiments. H.G.L. and J.H.W. performed large-scale circadian time course experiments; all authors read and approved the final article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Ackerman-Lavert, M., and Savaldi-Goldstein, S. (2020). Growth models from a brassinosteroid perspective. Curr. Opin. Plant Biol. 53, 90–97.

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Mas, P., and Kay, S.A. (2001). Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science 293, 880–883.

Bancos, S., Szatmari, A.M., Castle, J., Koza-Bognar, L., Shibata, K., Yokota, T., Bishop, G.J., Nagy, F., and Szekeres, M. (2004). Diurnal regulation of the brassinosteroid-biosynthetic CPD gene in Arabidopsis. Plant Physiol. 141, 299–309.

Covington, M.F., and Harmer, S.L. (2007). The circadian clock regulates auxin signaling and responses in Arabidopsis. PLoS Biol. 5, e222.

Covington, M.F.; Maloof, J.N.; Straume, M., Kay, S.A.; and Harmer, S.L. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol. 9, R130.

Dodd, A.N., Salathia, N., Hall, A., Kevei, E., Toth, R., Nagy, F., Holdber, J.M., Millar, A.J., and Webb, A.A. (2005). Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309, 630–633.

Doherty, C.J., and Kay, S.A. (2010). Circadian control of global gene expression patterns. Annu. Rev. Genet. 44, 419–444.

Espinosa-Ruiz, A., Martinez, C., de Lucas, M., Fabregas, N., Bosch, N., Cano-Delgado, A.I., and Prat, S. (2017). TOPLESS mediates brassinosteroid control of shoot boundaries and root meristem development in Arabidopsis thaliana. Development 144, 1619–1628.

Gampala, S.S.; Kim, T.W.; He, J.X.; Tang, W.; Deng, Z.; Bai, M.Y.; Guan, S.; Lalande, S.; Sun, Y.; Gendron, J.M., et al. (2007). An essential role for 14-3-3 proteins in brassinosteroid signal transduction in Arabidopsis. Dev. Cell 13, 177–189.

Grundy, J., Stoker, C., and Carre, I.A. (2015). Circadian regulation of abiotic stress tolerance in plants. Front. Plant Sci. 6, 648.

Gruszka, D. (2013). The brassinosteroid signaling pathway—new key players and interconnections with other signaling networks crucial for plant development and stress tolerance. Int. J. Mol. Sci. 14, 8740–8774.

Hanano, S.; Domagalska, M.A.; Nagy, F.; and Davis, S.J. (2006). Multiple phytohormones influence distinct parameters of the plant circadian clock. Genes Cells 11, 1381–1392.

He, J., Cheng, Q., and Xie, W. (2010). Minireview: nuclear receptor-controlled steroid hormone synthesis and metabolism. Mol. Endocrinol. 24, 11–21.

He, J.X.; Gendron, J.M.; Sun, Y.; Gampala, S.S.; Gendron, N.; Sun, C.Q.; and Wang, Z.Y. (2005). BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. Science 307, 1634–1638.

He, J.X.; Gendron, J.M.; Yang, Y.; Li, J.; and Wang, Z.Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid-signaling pathway in Arabidopsis. Proc. Natl. Acad. Sci. U S A 99, 10185–10190.

Hothorn, M.; Belkhadir, Y.; Dreux, M.; Dabi, T.; Noel, J.P.; Wilson, I.A.; and Chory, J. (2011). Structural basis of steroid hormone perception by the receptor kinase BRI1. Nature 474, 467–471.

Hsu, P.Y., and Harmer, S.L. (2012). Circadian phase has profound effects on differential expression analysis. PLoS One 7, e49853.

Huang, W.; Perez-Garcia, P.; Pokhilko, A.; Millar, A.J.; Antoshechkin, I.; Riechmann, J., and Mas, P. (2012). Mapping the core of the Arabidopsis circadian clock defines the network structure of the oscillator. Science 336, 75–79.

Kim, B.; Jeong, Y.J.; Corvalan, C.; Fujoka, S.; Cho, S.; Park, T.; and Choe, S. (2014). Darkness and gulliver2/phyB mutation decrease the abundance of phosphorylated BZR1 to activate brassinosteroid signaling in Arabidopsis. Plant J. 77, 737–747.

Kim, H.; Shim, D.; Moon, S.; Lee, J.; Bae, W.; Choi, H.; Kim, K.; and Ryu, H. (2019). Transcriptional network regulation of the brassinosteroid signaling pathway by the BES1-TPL-HDA19 corepressor complex. Planta 250, 1371–1377.

Kinoshita, T.; Cano-Delgado, A.; Seto, H.; Hirunuma, S.; Fujikawa, S.; Yoshida, S.; and Chory, J. (2005). Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. Nature 433, 167–171.

Lee, H.G., Mas, P., and Seo, P.J. (2016). MYB96 shapes the circadian gating of ABA signaling in Arabidopsis. Sci. Rep. 6, 17754.

Legnaoui, T.; Cuevas, J.; and Mas, P. (2009). TOC1 functions as a molecular switch connecting the circadian clock with plant responses to drought. EMBO J. 28, 3745–3757.

Li, L.; Yu, X.; Thompson, A.; Guo, M.; Yoshida, S.; Asami, T.; Chory, J.; and Yin, Y. (2009). Arabidopsis MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. Plant J. 58, 275–286.

Li, Q.F.; Lu, J.; Yu, J.-W.; Zhang, C.-Q.; He, J.-X.; and Liu, Q.-Q. (2018). The brassinosteroid-regulated transcription factors BZR1/BES1 function as a coordinator in multisignal-regulated plant growth. Biochim. Biophys. Acta. Gene Regul. Mech. 1861, 561–571.

Martinez, C.; Espinosa-Ruiz, A.; de Lucas, M.; Bernardo-Garcia, S.; Franco-Zorrilla, J.M.; and Prat, S. (2018). FIFA4-induced BR synthesis is critical to diurnal and thermomorphogenic growth. EMBO J. 37, e99552.

Michael, T.P.; Breton, G.; Hazen, S.P.; Priest, H.; Mockler, T.C.; Kay, S.A.; and Chory, J. (2008). A morning-specific phytohormone gene
expression program underlying rhythmic plant growth. PLoS Biol. 6, e225.

Mizuno, T., and Yamashino, T. (2008). Comparative transcriptome of diurnally oscillating genes and hormone-responsive genes in Arabidopsis thaliana: insight into circadian clock-controlled daily responses to common ambient stresses in plants. Plant Cell Physiol 49, 481–487.

Nakamichi, N., Kiba, T., Henriques, R., Mizuno, T., Chua, N.H., and Sakakibara, H. (2010). PSEUDO-RESPONSE REGULATOR9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. Plant Cell 22, 594–605.

Nohales, M.A., and Kay, S.A. (2019). GIGANTEA gates gibberellin signaling through stabilization of the DELLA proteins in Arabidopsis. Proc. Natl. Acad. Sci. U S A 116, 21893–21899.

Nomoto, T., Kubozono, S., Miyachi, M., Yamashino, T., Nakamichi, N., and Mizuno, T. (2012). A circadian clock- and PIF4-mediated double coincidence mechanism is implicated in the thermosensitive photoperiodic control of plant architectures in Arabidopsis thaliana. Plant Cell Physiol 53, 1965–1973.

Nosaki, S., Miyakawa, T., Xu, Y., Nakamura, A., Hirabayashi, K., Asami, T., Nakano, T., and Tanokura, M. (2018). Structural basis for brassinosteroid response by BIL1/BZR1. Nat. Plants 4, 771–776.

Nozue, K., Covington, M.F., Duek, P.D., Lorrain, S., Fankhauser, C., Harmer, S.L., and Maloof, J.N. (2007). Rhythmic growth explained by coincidence between internal and external cues. Nature 448, 358–361.

Nusinow, D.A., Helfer, A., Hamilton, E.E., King, J.J., Imazumi, T., Schultz, T.F., Farre, E.M., and Kay, S.A. (2011). The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature 472, 396–402.

Pham, V.N., Kathare, P.K., and Huq, E. (2018). Dynamic regulation of PIFs by COP1-SPA complex to optimize morphogenesis in Arabidopsis. Plant J. 96, 260–273.

Planas-Riverola, A., Gupta, A., Betegen-Putze, I., Bosch, N., Ibarra, M., and Cano-Delgado, A.I. (2019). Brassinosteroid signaling in plant development and adaptation to stress. Development 146, dev151894.

Ryu, H., Cho, H., Bae, W., and Hwang, I. (2014). Control of early seedling development by BES1/TPF/HD19-mediated epigenetic regulation of ABI3. Nat. Commun. 5, 4138.

Ryu, H., Cho, H., Kim, K., and Hwang, I. (2010). Phosphorylation dependent nucleocytoplasmic shuttling of BES1 is a key regulatory event in brassinosteroid signaling. Mol. Cells 29, 283–290.

Ryu, H., Kim, K., Cho, H., Park, J., Choe, S., and Hwang, I. (2007). Nucleocytoplasmic shuttling of BZR1 mediated by phosphorylation is essential in Arabidopsis brassinosteroid signaling. Plant Cell 19, 2749–2762.

Seo, P.J., and Mas, P. (2015). STRESSing the role of the plant circadian clock. Trends Plant Sci. 20, 230–237.

Sever, R., and Glass, C.K. (2013). Signaling through nuclear receptors. Cold Spring Harb. Perspect. Biol. 5, a016709.

Singh, M., and Mas, P. (2018). A functional connection between the circadian clock and hormonal timing in Arabidopsis. Genes (Basel) 9, 167.

Soy, J., Leivar, P., Gonzalez-Schain, N., Sentandreu, M., Prat, S., Quai!, P.H., and Monte, E. (2012). Phytochrome-terminated oscillations in PIF3 protein abundance regulate hypocotyl growth under diurnal light/dark conditions in Arabidopsis. Plant J. 77, 390–401.

Sun, Y., Fan, X.Y., Cao, D.M., Tang, W., He, K., Zhu, J.Y., He, J.X., Bai, M.Y., Zhu, S., Oh, E., et al. (2010). Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. Dev. Cell 19, 765–777.

Sun, Y., Han, Z., Tang, J., Hu, Z., Chai, C., Zhou, B., and Chai, J. (2013). Structure reveals that BAK1 as a co-receptor recognizes the BRI1-bound brassinolide. Cell Res. 23, 1326–1329.

Tang, W., Yuan, M., Wang, R., Yang, Y., Wang, C., Oses-Prieto, J.A., Kim, T.W., Zhou, H.W., Deng, Z., Gampala, S.S., et al. (2011). PP2A activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating BZR1. Nat. Cell Biol. 13, 124–131.

Thines, B., Parlan, E.V., and Fulton, E.C. (2019). Circadian network interactions with jasmine signaling and defense. Plants (Basel) 8, 252.

Uehara, T.N., Mizutani, Y., Kuwata, K., Hirota, T., Sato, A., Mizoi, J., Takao, S., Matsuo, H., Suzuki, T., Ito, S., et al. (2019). Casein kinase 1 family regulates PRR5 and TOC1 in the Arabidopsis circadian clock. Proc. Natl. Acad. Sci. U S A 116, 11528–11536.

Wang, Z.Y., Bai, M.Y., Oh, E., and Zhu, J.Y. (2012). Brassinosteroid signaling network and regulation of morphomorphogenesis. Annu. Rev. Genet. 46, 701–724.

Wang, Z.Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., et al. (2002). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev. Cell 2, 505–513.

Wang, Z.Y., Seto, H., Fujisawa, S., Yoshida, S., and Chory, J. (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature 410, 380–383.

Yang, M., Li, C., Cai, Z., Hu, Y., Nolan, T., Yu, F., Yin, Y., Xie, Q., Tang, G., and Wang, X. (2017). SINAT E3 ligases control the light-mediated stability of the brassinosteroid-activated transcription factor BES1 in Arabidopsis. Dev. Cell 41, 47–58 e4.

Ye, H., Li, L., and Yin, Y. (2011). Recent advances in the regulation of brassinosteroid signaling and biosynthesis pathways. J. Integr. Plant Biol. 53, 455–466.

Yin, Y., Vafeados, D., Tao, Y., Yoshida, S., Asami, T., and Chory, J. (2005). A new class of transcription factors mediates brassinosteroid-regulated gene expression in Arabidopsis. Cell 120, 249–259.

Yin, Y., Wang, Z.Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., and Chory, J. (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell 109, 181–191.

Yu, M.-H., Zhao, Z.-Z., and He, J.-X. (2018). Brassinosteroid signaling in plant–microbe interactions. Int. J. Mol. Sci. 19, 4091.

Zhang, T., Xu, P., Wang, W., Wang, S., Caruana, J.C., Yang, H.Q., and Lia, H. (2017). Arabidopsis G-protein beta subunit AG81 interacts with BES1 to regulate brassinosteroid signaling and cell elongation. Front. Plant Sci. 8, 2225.

Zhiponova, M.K., Vanhoutte, J., Boudolf, V., Betti, C., Dhondt, S., Coppens, F., Myle, E., Maes, S., Gonzalez-Garcia, M.P., Cano-Delgado, A.I., et al. (2013). Brassinosteroid production and signaling differentially control cell division and expansion in the leaf. New Phytol. 197, 490–502.

Zhou, M., Wang, W., Karapetyan, S., Mwimba, M., Marques, J., Buchler, N.E., and Dong, X. (2015). Redox rhythm reinforces the circadian clock to gate immune response. Nature 523, 472–476.
Supplemental Information

Brassinosteroids Regulate Circadian Oscillation
via the BES1/TPL-CCA1/LHY
Module in Arabidopsis thaliana

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Transparent Methods

Plant Materials and Growth Conditions

*Arabidopsis thaliana* ecotypes Columbia (Col-0), Wassilewskija (Ws-2), and Enkheim (En-2) were used as wild-type controls. Plants were grown in a growth chamber (Vision Scientific) under neutral day (ND) conditions (12-h light/12-h dark cycles) using cool white fluorescent lamps (120 μmol photons m⁻² s⁻¹) at 22-23°C, otherwise specified.

The *bri1-5* (Ws-2), *bes1-D* (En-2 background), *bzr1-1D* (Col-0 background), *bes1-ko* (Col-0 background), *bzr1-2* (Col-0 background), *pBES1::bes1-D-HA* (Col-0 background), and *pTPL-TPL-HA* (Col-0 background) plants were previously described (Noguchi et al., 1999; Wang et al., 2002; Ryu et al., 2014; Espinosa-Ruiz et al., 2017; Galstyan and Nemhauser, 2019). The T-DNA insertion mutant *tpl-8* (SALK_036566), *bzr1-2* mutant (GK-857E04), and *cca1-1 lhy-21* (Ws-2 background, CS9809) were obtained from the *Arabidopsis* Biological Resource Center (ABRC), Ohio, USA. The *tpl-8* mutant (Col-0 background) had a T-DNA insertion at 10th exon of *TPL* gene, which resulted in a complete null allele (Long, 2006). *bri1-5 x pCCA1:LUC* plants were generated by crossing *bri1-5* with *pCCA1:LUC* transgenic plant and further backcrossed to *pCCA1:LUC* plants (Col-0 background) (Salome and McClung, 2005), which was obtained from Dr. C Robertson McClung (Dartmouth College, USA).

Short-Term BL Treatment

Seedlings were grown on 1/2 MS-solid medium supplemented with 0.8% (w/v) agar and 1% (w/v) sucrose under ND conditions for 2 weeks. Plants were transferred to 1/2 MS-liquid medium (without sucrose) supplemented with 1 μM epi-BL (E1641; Sigma, St Louis, MO,
USA) or 0.1% (v/v) DMSO (mock control) at the indicated time points and incubated for 4 h. The concentration was determined based on the previous report (Ren et al., 2009).

**Quantitative Real-Time RT-PCR Analysis**

To extract total RNA, the harvested samples were ground in liquid nitrogen, and 700 μL TRI reagent (TAKARA Bio, Singa, Japan) was mixed thoroughly with the ground tissue. The samples were centrifuged at 4°C for 10 min. Then, 250 μL chloroform was added to the supernatant, and RNA in the aqueous phase was precipitated with isopropanol. The RNA pellet was washed with 70% ethanol and dissolved in RNase-free deionized water. Subsequently, 2 μg RNA pretreated with DNAsel (New England Biolabs, Beverly, MA, USA) was reverse transcribed using the Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Dr. Protein, Seoul, Korea).

Then, qPCR was performed in 96-well blocks on the Step-One Plus Real-Time PCR System (Applied Biosystems) using sequence-specific primers (Table S1). The expression of each gene was normalized relative to the *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*eIF4A*) gene (At3g13920). All RT-qPCR reactions were performed using total RNA extracted from independent replicates. The comparative threshold cycle (CT) method was employed to evaluate relative quantities of each amplified product. The CT value of each reaction was determined automatically by the system using default parameters. The specificity of RT-qPCR reactions was determined by melt curve analysis of the amplified products using the standard method installed in the system.

**Bioluminescence Assays**

*pCCA1:LUC* (Salome and McClung, 2005) or *pCCA1:LUC x bri1-5* seeds (backcrossed to
Col-0 background) were sown in 1/2 MS-solid medium, supplemented with 0.72% (w/v) agar and 3% (w/v) sucrose, and stratified at 4°C for 3 days (Davis et al., 2018; Chen et al., 2020). Germinated seedlings grown and entrained for 7 days under ND conditions at 22-23°C were transferred to 96-well microplates containing 1/2 MS-liquid medium supplemented with 3% sucrose, 0.24 mM D-luciferin (BioThema, Handen, Sweden), and either 100 µM epi-BL or 40 µM bikinin. For epi-BL treatment, 4 mM epi-BL stock solution dissolved in EtOH were used for final concentration of 100 µM epi-BL and 2.5% (v/v) EtOH was used as a mock treatment. Long-term treatment with BL required higher concentrations possibly due to signaling attenuation by intrinsic feedback regulation (Tanaka et al., 2005). DMSO was used as a vehicle for bikinin (SML0094; Sigma, St Louis, MO, USA) treatment, 10 mM bikinin stock solution was diluted to make the final concentration of 40 µM (Uehara et al., 2019) and 0.4% (v/v) DMSO was used as a mock treatment.

Luminescence rhythms were monitored using the Tristar2 LB 942 Multimode Microplate Reader (Berthold Technologies, Wildbad, Germany) and analyzed as previously described (Lee et al., 2016). The circadian period was estimated using the Fast Fourier Transform-Nonlinear Least Squares (FFT-NLLS) suite of programs available in the Biodare2 software (Zielinski et al., 2014).

To examine circadian oscillations in protoplasts in the presence of epi-BL, mesophyll protoplasts were isolated from the lower epidermal layer of leaves of 3-week-old Arabidopsis seedlings. The detached leaves were rinsed twice using sterile water. Leaf surfaces were soaked in 10 mL of an enzyme solution (400 mM mannitol, 20 mM KCl, 20 mM MES-KOH [pH 5.7], 10 mM CaCl₂, 1% Cellulase R10, 0.5% Macerozyme R10, and 0.1% bovine serum albumin) in a petri dish for 4 h in the dark. The released protoplasts were filtered through sterile 100 mm stainless mesh, and resuspended in W5 solution as previously described (Kim
and Somers, 2010; Hansen and van Ooijen, 2016). The pCCA1:LUC plasmids were prepared by CsCl gradient purification. Then, the pCCA1:LUC recombinant construct was transiently introduced into Arabidopsis protoplasts via polyethylene glycol (PEG)-mediated transformation (Kim and Somers, 2010; Hansen and van Ooijen, 2016).

**Chromatin Immunoprecipitation (ChIP) Assays**

ChIP assays were conducted using pBES1:bes1-D-HA, pBZR1:bzr1-1D-HA, and pTPL:TPL-HA transgenic plants. Seeds sown on 1/2 MS-solid medium supplemented with 1% (w/v) sucrose, 0.75% (w/v) agar were stratified for 3 days, then grown and entrained in a growth chamber for 2 weeks under ND conditions at 22-23°C. Whole plants were harvested, fixed in 1% formaldehyde, and ground in liquid nitrogen. Chromatin solubilized by the nuclei lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM ethylene diamine tetraacetic acid [EDTA; pH 8.0], 1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1X PIs) was sonicated at 4°C to approximately 500 bp fragments using a Bioruptor. After fragmentation, the extract was precleared with 100 μL protein A/G agarose beads (sc2003; Santa Cruz, CA, USA) for 2 h. The precleared supernatant was incubated overnight with 1 μg corresponding antibodies, including anti-HA antibody (ab9110; Abcam, Cambridge, UK), and the supernatant was mixed with 100 μL protein A/G agarose beads for 16 h at 4°C on a rotator. Then, the DNA fragments were purified using the DNA elution kit and quantified by quantitative real-time PCR (qRT-PCR) using sequence-specific primers (Table S2). The amplified PCR products were normalized relative to the input DNA.

**Y2H Assays**

Y2H assays were performed using the BD Matchmaker system (Clontech, Mountain View,
PCR products were subcloned into the pGBK7 and pGADT7 vectors to generate GAL4 activation domain (AD) and GAL4 DNA-binding domain (BD) fusion constructs, respectively. The GAL4 AD and BD constructs were cotransformed into the yeast strain pJG69-4A harboring the LacZ and His reporter genes, and the transformed cells were selected by growth on synthetic defined (SD) medium lacking leucine and tryptophan (SD/-Leu/-Trp), and SD medium lacking Leu, Trp, histidine, and adenine (SD/-Leu/-Trp/-His/-Ade). Protein–protein interactions were analyzed by measuring β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside as the substrate.

**Hypocotyl Length Measurement**

35S:CCA1α-MYC (Col-0 background) (Seo et al., 2012) and cca1-1 lhy-21 (Ws-2 background) seeds were sown on 1/2 MS medium containing 1 µM epi-BL or 0.1% DMSO (mock treatment). After stratification at 4°C for 3 days, plants were vertically grown under ND conditions at 22-23°C. Seedlings were photographed at the indicated time and hypocotyl lengths were measured using the Image J software (http://rsb.info.nih.gov/ij).

**Transient Gene Expression Assays**

Transient gene expression assays were conducted in Arabidopsis protoplasts using reporter and effector plasmids. The core element in CCA1 and LHY promoters was subcloned into the reporter plasmid containing a minimal 35S (Min 35S) promoter sequence and the GUS gene. To construct p35S:bes1-D, p35S:brz1-1D, and p35S:TPL effector plasmids, coding sequences of bes1-D, brz1-1D, and TPL genes were cloned downstream of the cauliflower mosaic virus (CaMV) 35S promoter. Recombinant reporter and effector plasmids were cotransformed into Arabidopsis protoplasts along with the p35S:LUC construct (internal control) via PEG-
mediated transformation. GUS activity was measured using a fluorometric method, and LUC activity was measured using the Luciferase Assay System Kit (Promega, Madison, WI, USA).

REFERENCES

Chen, W.W., Takahashi, N., Hirata, Y., Ronald, J., Porco, S., Davis, S.J., Nusinow, D.A., Kay, S.A., and Mas, P. (2020). A mobile ELF4 delivers circadian temperature information from shoots to roots. Nature Plants 6:416-426.

Davis, A.M., Ronald, J., Ma, Z., Wilkinson, A.J., Philippou, K., Shindo, T., Queitsch, C., and Davis, S.J. (2018). HSP90 contributes to entrainment of the Arabidopsis circadian clock via the morning loop. Genetics 210:1383-1390.

Espinosa-Ruiz, A., Martinez, C., de Lucas, M., Fabregas, N., Bosch, N., Cano-Delgado, A.I., and Prat, S. (2017). TOPELESS mediates brassinosteroid control of shoot boundaries and root meristem development in Arabidopsis thaliana. Development 144:1619-1628.

Galstyan, A., and Nemhauser, J.L. (2019). Auxin promotion of seedling growth via ARF5 is dependent on the brassinosteroid-regulated transcription factors BES1 and BEH4. Plant Direct 3:e00166.

Hansen, L.L., and van Ooijen, G. (2016). Rapid analysis of circadian phenotypes in Arabidopsis protoplasts transfected with a luminescent clock reporter. J. Vis. Exp. doi: 10.3791/54586.

Kim, J., and Somers, D.E. (2010). Rapid assessment of gene function in the circadian clock using artificial microRNA in Arabidopsis mesophyll protoplasts. Plant Physiol. 154:611-621.

Lee, H.G., Mas, P., and Seo, P.J. (2016). MYB96 shapes the circadian gating of ABA
signaling in Arabidopsis. Sci Rep 6:17754.

Long, J.A. (2006). TOPLESS Regulates Apical Embryonic Fate in Arabidopsis. Science 312:1520-1523.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K.A., and Tax, F.E. (1999). Brassinosteroid-insensitive dwarf mutants of Arabidopsis accumulate brassinosteroids. Plant Physiol. 121:743-752.

Ren, C., Han, C., Peng, W., Huang, Y., Peng, Z., Xiong, X., Zhu, Q., Gao, B., and Xie, D. (2009). A leaky mutation in DWARF4 reveals an antagonistic role of brassinosteroid in the inhibition of root growth by jasmonate in Arabidopsis. Plant Physiol. 151:1412-1420.

Ryu, H., Cho, H., Bae, W., and Hwang, I. (2014). Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of ABI3. Nat. Commun. 5:4138.

Salome, P.A. and McClung, C.R. (2005). PSEUDO-RESPONSE REGULATOR 7 and 9 are partially redundant genes essential for the temperature responsiveness of the Arabidopsis circadian clock. 17:791-803.

Seo, P.J., Park, M.-J., Lim, M.-H., Kim, S.-G., Lee, M., Baldwin, I.T., and Park, C.-M. (2012). A self-regulatory circuit of CIRCADIAN CLOCK-ASSOCIATED1 underlies the circadian clock regulation of temperature responses in Arabidopsis. The Plant Cell 24:2427-2442.

Tanaka, K., Asami, T., Yoshida, S., Nakamura, Y., Matsuo, T., and Okamoto, S. (2005). Brassinosteroid homeostasis in Arabidopsis is ensured by feedback expressions of multiple genes involved in its metabolism. Plant Physiol. 138:1117-1125.

Uehara, T.N., Mizutani, Y., Kuwata, K., Hirota, T., Sato, A., Mizoi, J., Takao, S., Matsuo, H., Suzuki, T., Ito, S., et al. (2019). Casein kinase 1 family regulates PRR5 and TOC1 in the Arabidopsis circadian clock. Proc. Natl. Acad. Sci. U S A 116:11528-11536.
Wang, Z.Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., et al. (2002). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev Cell 2:505-513.

Zielinski, T., Moore, A.M., Troup, E., Halliday, K.J., and Millar, A.J. (2014). Strengths and limitations of period estimation methods for circadian data. PLoS One 9:e96462.
Supplemental Figure

Figure S1. Circadian expression of CCA1 in bes1-ko, bzr1-1D, and bzr1-2, Related to Figure 1. Two-week-old seedlings grown under neutral day (ND) conditions were transferred to continuous light (LL) conditions at ZT0. Whole seedlings were harvested from ZT72 to ZT116 to analyze transcript accumulation. Bars indicate the standard error of the mean. The white and grey boxes indicate the subjective day and night, respectively.
Figure S2. Circadian expression of *CCA1* in *bri1-5* under normal conditions, Related to Figure 1.
Seven-day-old seedlings grown under neutral day (ND) conditions were transferred to continuous dark (DD) conditions at ZT0 (left panel). Circadian period estimates of *pCCA1:LUC* activity were shown (right panel). Data represented as mean ± SEM were analyzed with using the Fast Fourier Transform Non-linear Least Squares (FFT-NLLS) suite of programs available in the Biodare2 software (*n* > 14). NS, not significant.
Figure S3. Positive and negative controls for BES1 binding, Related to Figure 2.

Two-week-old seedlings entrained under ND cycles were subjected to LL conditions. Plants were harvested for ChIP analysis with anti-HA antibody. Enrichment of fragmented genomic regions of positive control genes (CPD and DWF4) and negative control genes (CNX5 and UBC) was analyzed by ChIP-qPCR (Huang et al., 2013; Wang et al., 2018). Biological triplicates were averaged, and statistical significance was determined by Student’s $t$-test ($P < 0.05$). Bars indicate standard error of the mean.

- **Huang, H.Y., Jiang, W.B., Hu, Y.W., Wu, P., Zhu, J.Y., Liang, W.Q., Wang, Z.Y., and Lin, W.H.** (2013). BR signal influences Arabidopsis ovule and seed number through regulating related genes expression by BZR1. Mol. Plant 6:456-469.

- **Wang, W., Lu, X., Li, L., Lian, H., Mao, Z., Xu, P., Guo, T., Xu, F., Du, S., Cao, X., et al.** (2018). Photoexcited CRYPTOCHROME1 interacts with dephosphorylated BES1 to regulate brassinosteroid signaling and photomorphogenesis in Arabidopsis. Plant Cell 30:1989-2005.
Figure S4. Binding of BES1 to coding regions of CCA1 and LHY, Related to Figure 2.
Two-week-old plants entrained with ND cycles were subjected to LL conditions. Plants were harvested at ZT28 and ZT40 for ChIP analysis with anti-HA antibody. Enrichment of fragmented genomic regions was analyzed by ChIP-qPCR. Biological triplicates were averaged, and statistical significance was determined by Student’s t-test (*P < 0.05). Bars indicate standard error of the mean. TSS, transcriptional start site.
Figure S5. Binding of BZR1 to core clock genes, Related to Figure 2.

(A) Genomic structures of core clock genes. Underbars indicate the regions amplified by quantitative PCR (qPCR) following chromatin immunoprecipitation (ChIP). Red lines indicate E-boxes, and blue lines indicate BR-response elements (BRREs). TSS, transcription start site.

(B) Binding of BZR1 to clock gene promoters at ZT40. Two-week-old seedlings entrained under ND cycles were subjected to LL conditions. Plants were harvested at ZT40 for ChIP analysis with anti-HA antibody. Biological triplicates were averaged. Data represent mean ± SEM.
The recombinant reporter and effector constructs were coexpressed transiently in *Arabidopsis* protoplasts, and GUS activity was determined. Luciferase gene expression was used to normalize GUS activity. The normalized values in control protoplasts were set to 1 and represented as relative values. Three independent measurements were averaged. Different letters represent a significant difference at $P < 0.05$ (one-way ANOVA with Fisher’s post hoc test). Bars indicate the standard error of the mean. Note that two promoter constructs for each gene were designed to examine the relevance of BES1-binding sites (BS) (see Figures 2A and 2B) in gene regulation. w/o BS, without binding site; w BS, with binding site.

### Figure S6. Transient expression assays using *Arabidopsis* protoplasts, Related to Figure 2.

| Reporter | Effector |
|----------|----------|
| pMin35S  | Min 35S  | GUS | Nos-T |
| pCCA1 (w/o BS) | pCCA1 (+112 ~ +414) | Min 35S | GUS | Nos-T |
| pCCA1 (w BS) | pCCA1 (-1198 ~ +414) | Min 35S | GUS | Nos-T |
| pLHY (w/o BS) | pLHY (+378 ~ +762) | Min 35S | GUS | Nos-T |
| pLHY (w BS) | pLHY (-1774 ~ -34) | Min 35S | GUS | Nos-T |

### Diagram

![Diagram showing relative GUS activity for different constructs](image)
Figure S7. Expression of BR signaling components in circadian cycles, Related to Figure 2.

(A) *BES1* expression. Two-week-old seedlings grown under ND conditions were transferred to LL conditions at ZT0. Transcript levels were determined by RT-qPCR. Bars indicate the standard error of the mean. The white and grey boxes indicate the subjective day and night, respectively.

(B) Circadian expression of BR signaling genes. Data from the DIURNAL project (http://diurnal.mocklerlab.org) microarray repository were used to compare RNA levels. Raw data from the experimental set LL_LDHC were investigated.
Figure S8. Yeast-two-hybrid (Y2H) assays, Related to Figure 5.

Y2H assays were performed with the BES1 protein fused with the DNA binding domain (BD) of GAL4 and circadian clock proteins fused with the transcriptional activation domain (AD) of GAL4 for analysis of their interactions. Interactions were examined by cell growth on selective media. -LWHA indicates Leu, Trp, His, and Ade drop-out plates. -LW indicates Leu and Trp drop-out plates. GAL4 was used as a positive control (P).
Two-week-old seedlings entrained under ND cycles were subjected to LL conditions. Plants were harvested for ChIP analysis with anti-HA antibody. Enrichment of fragmented genomic regions of positive control genes (CPD and DWF4) and negative control genes (CNX5 and UBC) was analyzed by ChIP-qPCR (Huang et al., 2013; Wang et al., 2018). Biological triplicates were averaged, and statistical significance was determined by Student’s t-test ($P < 0.05$). Bars indicate standard error of the mean.

- **Huang, H.Y., Jiang, W.B., Hu, Y.W., Wu, P., Zhu, J.Y., Liang, W.Q., Wang, Z.Y., and Lin, W.H.** (2013). BR signal influences Arabidopsis ovule and seed number through regulating related genes expression by BZR1. Mol. Plant 6:456-469.
- **Wang, W., Lu, X., Li, L., Lian, H., Mao, Z., Xu, P., Guo, T., Xu, F., Du, S., Cao, X., et al.** (2018). Photoexcited CRYPTOCHROME1 interacts with dephosphorylated BES1 to regulate brassinosteroid signaling and photomorphogenesis in Arabidopsis. Plant Cell 30:1989-2005.
Figure S10. Binding of TPL to coding regions of CCA1 and LHY, Related to Figure 5.
Two-week-old plants entrained with ND cycles were subjected to LL conditions. Plants were harvested at ZT28 and ZT40 for ChIP analysis with anti-HA antibody. Enrichment of fragmented genomic regions was analyzed by ChIP-qPCR. Biological triplicates were averaged, and statistical significance was determined by Student’s t-test (*P < 0.05). Bars indicate standard error of the mean. TSS, transcriptional start site.
Figure S11. Reduced BR responses in CCA1-ox plants, Related to Figure 7.

(A) Hypocotyl length. Ten-day-old seedlings were grown on MS-medium supplemented with mock solution (0.1% DMSO) or 1 µM epi-BL under ND conditions. The numbers above the bars indicate the ratio of hypocotyl length in mock and BL-treated seedlings (BL/mock). Data represent mean ± SEM (n > 8).

(B) ACS5 expression at ZT40. Two-week-old seedlings grown under ND conditions were used to analyze transcript accumulation of a BR-responsive marker gene (Xiong et al. 2019). The elf4a gene was used as an internal control. Biological triplicates were averaged, and statistical significance was determined by Student’s t-test (P < 0.05). Bars indicate standard error of the mean.

- Xiong, F., Zhuo, F., Reiter, R.J., Wang, L., Wei, Z., Deng, K., Song, Y., Qanmber, G., Feng, L., Yang, Z., et al. (2019). Hypocotyl elongation inhibition of melatonin is involved in repressing brassinosteroid biosynthesis in Arabidopsis. Front Plant Sci. 10:1082.
### Table S1. Primers used in this study, Related to Figure 1, 3 and 6.

| Primer       | Usage         | Sequence                              |
|--------------|---------------|---------------------------------------|
| eIF4a-F      | RT-qPCR       | 5’-TGACCACACAGTCTCTGCAA               |
| eIF4a-R      | RT-qPCR       | 5’-ACCAAGGGAGACTGTGTTGAG              |
| CAB2-F       | RT-qPCR       | 5’-TTCCCAAGSTAATGCCAGCC               |
| CAB2-R       | RT-qPCR       | 3’-CTTTACCGGAGAGCTTC                 |
| CCA1-F       | RT-qPCR       | 5’-GATCTTGTTATTAAGCTGGAAGCCATATAC     |
| CCA1-R       | RT-qPCR       | 5’-GCTCTTTTCTCTACACTGCC               |
| LHY-F        | RT-qPCR       | 5’-CTTATCCCTGAAAAGGCTG               |
| LHY-R        | RT-qPCR       | 5’-GAACTCTTGGACCCGCTTGT              |
| LUX-F        | RT-qPCR       | 5’-TACCGTGAGGAGAGAGGCTGGA            |
| LUX-R        | RT-qPCR       | 5’-TCCATCACCCTTGTGAGTCTT            |
| ELF3-F       | RT-qPCR       | 5’-AGAAAGASGAGCAGGGAGAG           |
| ELF3-R       | RT-qPCR       | 5’-CCTCCCCCATTCTCTGTTT               |
| TOC1-F       | RT-qPCR       | 5’-CTCTGGAGAATCCCTGTG               |
| TOC1-R       | RT-qPCR       | 5’-GCTGCACTGCTCTGAGGCTGGA           |
| PRR5-F       | RT-qPCR       | 5’-GCTGCACTGCTCTGAGGCTGGA           |
| PRR5-R       | RT-qPCR       | 5’-TCTTCTCTGAGCTGAGGTGAGGG         |
| PRR7-F       | RT-qPCR       | 5’-GAGGCACTATGGAAGGACGAG            |
| PRR7-R       | RT-qPCR       | 5’-TTTACCGCAAAAAATTGATG               |
| PRR9-F       | RT-qPCR       | 5’-TGTTCTCTGAGCTGAGGTGAGG          |
| ACS5-F       | RT-qPCR       | 5’-GCTATGCTGGAGTGTAGG               |
| ACS5-R       | RT-qPCR       | 5’-TTGAGAACTGAGAGAGTC                |
| SAUR15-F     | RT-qPCR       | 5’-GAGGAGATCTCTCTGAGTCT            |
| SAUR15-R     | RT-qPCR       | 5’-GAACTGAGAGGCTGAGGCTG             |
| bes1-D-F(pBA002) | cloning     | 5’-GAGGCCGCGCATGAAAAGATCTCTCTATAATTCAGAC  |
| bes1-D-R(pBA002) | cloning     | 5’-GAACCTAGTACTAAGCCTTACCTTCCATATCCCA |
| bZR1-1 D-F(pBA002) | cloning     | 5’-GAGGCCGCGCATGACTTGCTGAGGATACG     |
| bZR1-1 D-R(pBA002) | cloning     | 5’-GAACCTAGTACTAAGCCTTACCTTCCATATCCCA |
| TPL-F(myc-pBA) | cloning     | 5’-GAGGCGTCGGTATTTAAAGAGAAGATCTCTCTGAGGTTTGGAGGATACG |
| TPL-R(myc-pBA) | cloning     | 5’-GAGGCGTCGGTATTTAAAGAGAAGATCTCTCTGAGGTTTGGAGGATACG |
| pCCA1 (+3198~+414) - F(pMin35S) | cloning     | 5’-GAGGATCCGAGCACTCTTGAGGTGACTTACAC   |
| pCCA1 (+3198~+414) - R(pMin35S) | cloning     | 5’-GAGGATCCGAGCACTCTTGAGGTGACTTACAC   |
| pCCA1 (+5074~+611) - F(pMin35S) | cloning     | 5’-GAGGATCCGAGCACTCTTGAGGTGACTTACAC   |
| pCCA1 (+5074~+611) - R(pMin35S) | cloning     | 5’-GAGGATCCGAGCACTCTTGAGGTGACTTACAC   |
| pLLH (+1774~+34) - F(pMin35S) | cloning     | 5’-GAGGATCCGAGCACTCTTGAGGTGACTTACAC   |
| pLLH (+1774~+34) - R(pMin35S) | cloning     | 5’-GAGGATCCGAGCACTCTTGAGGTGACTTACAC   |

RT-qPCR primers were designed using the Primer Express Software installed into the Applied Biosystems PCR System. The sizes of PCR products ranged from 80 to 300 nucleotides in length. F, forward primer; R, reverse primer.
Table S2. Primers used in chromatin immunoprecipitation (ChIP) assays, Related to Figure 2 and 5.

| Primer   | Sequence                        |
|----------|---------------------------------|
| CCA1 (A) | F CTTCCTTTGTGAATCCTGAACCAA      |
|          | R GAATTGAGTTTCTCCCAACTACGTTTT   |
| CCA1 (B) | F ATATACAAACTAGGGCCCAAAATAAGTTTT |
|          | R ATTTGATCTAGTGGGGACCTAC        |
| CCA1 (C) | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| CCA1 (D) | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| LHY (A)  | F GATGGAGGAAGTCCTGCTGAATC        |
|          | R GGGTTGACTGTCCAGTTCGGAATC      |
| LHY (B)  | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| LHY (C)  | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| LHY (D)  | F GATGGAGGAAGTCCTGCTGAATC        |
|          | R GGGTTGACTGTCCAGTTCGGAATC      |
| LUX (A)  | F GATGGAGGAAGTCCTGCTGAATC        |
|          | R GGGTTGACTGTCCAGTTCGGAATC      |
| LUX (B)  | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| LUX (C)  | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| LUX (D)  | F GATGGAGGAAGTCCTGCTGAATC        |
|          | R GGGTTGACTGTCCAGTTCGGAATC      |
| ELF3 (A) | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| ELF3 (B) | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| ELF3 (C) | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| ELF3 (D) | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| TOC1 (A) | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| TOC1 (B) | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| TOC1 (C) | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| TOC1 (D) | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| PRR5 (A) | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| PRR5 (B) | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| PRR7 (A) | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| PRR9 (A) | F ATTTGGGAATGTATTCACCTTTCA      |
|          | R ATAGCAGCCTATCTAGAATGATC       |
| PRR9 (B) | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |
| CPD      | F ACCACCCCATGGTTTCTCTAT          |
|          | R ACCACCCCATGGTTTCTCTAT          |

F, forward primer; R, reverse primer.