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1 **Circadian Regulation of the *GLYCINE-RICH RNA-BINDING PROTEIN* Gene by the Master**
2 **Clock Protein CIRCADIAN CLOCK-ASSOCIATED 1 Is Important for Plant Innate**
3 **Immunity**

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19 **Running title:** Circadian regulation of *GRP7*
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Highlight

This research establishes a direct transcriptional control by the master clock regulatory protein CCA1 on a downstream target gene *GRP7* for its circadian expression and function in pathogen defense.

Abstract

Recent studies have demonstrated the importance of temporal regulation of pathogen defense by the circadian clock. However, our understanding of the molecular basis underlying this role of the circadian clock is still in its infancy. We report here the mechanism by which the Arabidopsis master clock protein CCA1 regulates an output target gene *GRP7* for its circadian expression and function in pathogen defense. Our data firmly establish that CCA1 physically associates with the *GRP7* promoter via the predicted CCA1 binding motif, evening element (EE). Site-directed mutagenesis study showed that while individual EE motifs differentially contribute to robust circadian expression of *GRP7*, abolishing all four EE motifs in the proximal *GRP7* promoter disrupts rhythmicity of *GRP7* expression and results in misalignment of defense signaling mediated by *GRP7* and altered pathogen responses. This study provides a mechanistic link of the circadian regulation of an output gene to its biological function in pathogen defense, underscoring the importance of temporal control of plant innate immunity.

Keywords and abbreviations

Keywords: Circadian clock, pathogen defense, defense signaling, salicylic acid, jasmonic acid, rhythm, transcription translation feedback loop, evening element.

Abbreviations:

TTFLs: interlocking transcription-translation feedback loops

EE: evening element

CBS: CCA1 binding site

CCA1: CIRCADIAN CLOCK-ASSOCIATED 1

GRP7: GLYCINE-RICH RNA-BINDING PROTEIN

LHY: LATE ELONGATED HYPOCOTYL

TOC1: TIMING OF CAB EXPRESSION

PRR1: PSEUDO-RESPONSE REGULATOR 1

LUX: LUX ARRHYTHMO

ELF3: EARLY FLOWERING 3

SA: salicylic acid

JA: jasmonic acid

ROS: reactive oxygen species

ChIP: chromatin immunoprecipitation

ChIP-seq: ChIP-coupled with high throughput sequencing

EMSA: electrophoretic mobility shift assays

LD: 12 h light/12 h dark cycle

LL: continuous light

Col-0: Columbia-0

67 Ws: Wassilewskijia
68 LUC: luciferase
69 Dpi: day post infection
70 RI: rhythmicity index
71 LOF: loss of function
72 GOF: gain of function

Introduction

Organisms in all three domains of life have evolved the ability to integrate time cues with their development and responses to environmental assaults. A precisely tuned circadian clock is critical for growth and development of many organisms and it also confers fitness and competitive advantages under stress conditions (Greenham and McClung, 2015; Kinmonth-Schultz *et al.*, 2013). Emerging evidence has established circadian regulation of plant innate immunity (Karapetyan and Dong, 2018; Lu *et al.*, 2017). However, our understanding of the molecular basis underlying this role of the circadian clock is still rudimentary.

Although the molecular components of the plant circadian clock largely differ from those of other organisms, the basic principle of clock function remains the same; that is the core clock components form interlocking transcription-translation feedback loops (TTFLs) to calibrate the circadian clock and to keep timing in a precise, self-sustaining manner (Greenham and McClung, 2015; Hsu and Harmer, 2014; Nohales and Kay, 2016). *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) is a master clock regulator involved in multiple clock TTFLs. One such clock TTFL involves the morning gene *LATE ELONGATED HYPOCOTYL* (*LHY*), which is homologous to *CCA1*, and the evening gene *TIMING OF CAB EXPRESSION 1* (*TOC1*; also known as *PSEUDO-RESPONSE REGULATOR 1* (*PRR1*)). Both *CCA1* and *LHY* proteins repress transcription by binding to the *TOC1* promoter and in their own promoters via some well-defined motifs, e.g., evening element (EE) and *CCA1* binding site (CBS) (Kamioka *et al.*, 2016; Nagel *et al.*, 2015). In turn, *TOC1*, together with its interactor *CCA1* HIKING EXPEDITION, represses *CCA1* and *LHY* expression. *CCA1* also forms additional TTFLs with other core clock genes, such as the morning genes *PRR9* and *PRR7*, the afternoon gene *PRR5*, and the evening genes *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING 3* (*ELF3*), and *ELF4*. *CCA1* represses expression of these genes by binding to their promoters and these *CCA1* targets further regulate each other's expression and that of *CCA1* through numerous intricate TTFLs.

The circadian clock regulates a myriad of biological processes, including defense against pathogens. Disrupting the normal function of a number of core clock genes compromised plant responses to a broad-spectrum of pathogens, including bacteria, oomycete, and fungi, in addition to insects (Lu *et al.*, 2017). The role of the circadian clock in plant defense is also manifested in daily oscillatory expression of numerous defense genes and accumulation of signaling molecules, such as salicylic acid (SA), jasmonic acid (JA), and reactive oxygen species (ROS) (Goodspeed *et al.*, 2012; Lai *et al.*, 2012; Zheng *et al.*, 2015; Zhou *et al.*, 2015). In the presence of pathogens, the host activates acute defense responses, including reprogramming of expression of defense-related genes and drastic increases in SA and other defense compounds. Although no longer rhythmic, some acute defense responses were shown to be gated by the circadian clock (Goodspeed *et al.*, 2012; Zhang *et al.*, 2019; Zheng *et al.*, 2015). Both SA and JA signaling are important for circadian regulation of plant defense against pathogens of varying styles (Ingle *et al.*, 2015; Li *et al.*, 2018).

Individual core clock genes likely play differential roles in affecting output pathways, including pathogen defense. Consistent with this idea, chromatin immunoprecipitation (ChIP) coupled with high throughput sequencing (ChIP-seq) experiments showed that some Arabidopsis core clock components, including the *CCA1*, *TOC1*, and the *PRR* proteins, had distinct as well as overlapping occupancies in target gene promoters (Huang *et al.*, 2012; Kamioka *et al.*, 2016; Liu *et al.*, 2013;

Liu *et al.*, 2016; Nagel *et al.*, 2015; Nakamichi *et al.*, 2012). How core clock proteins regulate expression of the target genes and what the biological consequences of such regulations remain to be fully elucidated.

The master clock regulator CCA1 is predicted to bind to the conserved EE and CBS motifs in some target gene promoters (Kamioka *et al.*, 2016; Nagel *et al.*, 2015). For the EE motif alone, there are over 5,000 predicted sites within the 1,000 bp upstream region of gene promoters in the Arabidopsis genome, suggesting that CCA1 can regulate a large number of genes for their expression and subsequently function in many biological processes (Nagel *et al.*, 2015). A large-scale ChIP-seq study further supports this idea by revealing more than 3,600 genes whose promoters were occupied by CCA1 (Nagel *et al.*, 2015). Interestingly, this study showed that CCA1 targets detected by ChIP-seq did not entirely overlap with those harboring the predicted EE and/or CBS motifs. Only about 12% predicted EE motifs in the gene promoters were associated with CCA1 occupancy under the conditions tested (Nagel *et al.*, 2015). In addition, about 30% of high-confidence CCA1 binding targets that contain perfectly matched EE motif in the gene promoters did not cycle in diurnal conditions. CCA1 was further shown to bind to gene promoters without the known EE and CBS motifs. Thus, these observations suggest that additional evidence should be provided to validate CCA1 transcriptional targets obtained from bioinformatic predictions and ChIPseq studies under specific conditions. Furthermore, it is important to elucidate how CCA1 regulates circadian expression and function of its target genes, an area of research of scarce knowledge.

We previously reported a defense role of CCA1, which is through the potential downstream target gene *GLYCINE-RICH RNA-BINDING PROTEIN* (*GRP7*; At2g21660), also known as *COLD AND CIRCADIAN REGULATED 2*, encoding an RNA binding protein (Fu *et al.*, 2007; Staiger *et al.*, 2003; Zhang *et al.*, 2013). *GRP7* is a well-characterized defense gene important for resistance against bacterial and fungal pathogens (Fu *et al.*, 2007; Kim *et al.*, 2008; Nicaise *et al.*, 2013; Zhang *et al.*, 2013). Expression of *GRP7* displays robust circadian rhythm with an evening peak (Fu *et al.*, 2007; Heintzen *et al.*, 1997; Kim *et al.*, 2008; Nicaise *et al.*, 2013; Zhang *et al.*, 2013). Because loss of function in *GRP7* does not affect clock activity (Green *et al.*, 2002; Staiger *et al.*, 2003), *GRP7* is generally not considered as a part of the core clock TTFLs; rather it is considered as a part of a slave oscillator in the clock output pathway (Heintzen *et al.*, 1997; Staiger *et al.*, 2003). Because of its robust circadian expression, the *GRP7* promoter driving expression of the luciferase gene has been widely used as a reporter to assess the clock activity. *GRP7* was predicted to be a CCA1 target gene because its promoter is enriched with motifs recognized by CCA1 and it was among over 3,600 target genes of CCA1 identified in a ChIP-seq study (Nagel *et al.*, 2014; Zhang *et al.*, 2013). Further evidence is needed to firmly establish that *GRP7* is a direct transcriptional target of CCA1. More importantly, how CCA1 binding to the *GRP7* promoter and its regulation of *GRP7* circadian expression affect the gene function in pathogen defense remains to be addressed.

To better understand how the circadian clock regulates output pathways, we investigated the molecular mechanisms by which CCA1 affects circadian expression of *GRP7* and illuminated the importance of this regulation in one clock output, pathogen defense. Via electrophoretic mobility shift assays (EMSA) and ChIP experiments, we provided strong evidence to support a direct association of CCA1 with the *GRP7* promoter at the predicted EE motifs. We generated luciferase

reporter lines under the control of the wildtype *GRP7* promoter or *GRP7* promoter mutant variants. Luciferase analysis indicated that each of the four EE motifs in the proximal *GRP7* promoter contributed differentially to robust circadian expression of *GRP7*. Abolishing all four EE motifs disrupted rhythmic *GRP7* expression and resulted in misaligned defense signaling involving JA and SA, rendering the *GRP7* misexpressing plants with altered responses to both biotrophic pathogen *Pseudomonas syringae* and necrotrophic pathogen *Botrytis cinerea*. Together, our data underscore a critical role of the circadian clock in plant defense and provide a mechanistic view of circadian control of pathogen defense.

Materials and methods

Plant material and growth conditions

Most genotypes used in this paper are in the *Arabidopsis thaliana* (L.) accession Columbia-0 (Col-0) background unless otherwise indicated. Plants were grown in growth chambers with 60% humidity, 22°C, 12 h light/12 h dark cycle (LD), and 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. The *CCA1:GFP-CCA1* seed was a kind gift from Steve Kay at University of Southern California (Pruneda-Paz *et al.*, 2009). *CCA1:GFP-CCA1* is in Wassilewskijia (Ws) background. The single mutants, *cca1-1*, *grp7-1*, and *jar1-1*, and the plant overexpressing *CCA1* (*CCA1ox*) were described previously (Zhang *et al.*, 2013). The triple mutant *cca1-1lhy-20-toc1-101* was generated by genetic cross and the homozygous mutants were confirmed in the F₂ generation with PCR markers corresponding to each mutation. Additional transgenic plants were generated in this study (For details, see below Construction of *GRP7* variants and plant transformation). Primers used for detecting the mutations and transgenes are listed in Supplementary Table S1.

Construction of *GRP7* variants and plant transformation

In order to amplify the *GRP7* gene fragment, PCR was performed using Col-0 genomic DNA as the template and primers *grp7*sense and *grp7*antisense. The genomic fragment is 2.9 kb, including 1350 bp upstream of the translation start site and 392 bp downstream of the 3' untranslated region. It was first cloned into the pMiniT plasmid (NEB) and then moved to the pGreenII plasmid, using the fragment digested with Xba I (5' end) and Hind III (3' end). A stop codon was inserted at the 3' end of the *GRP7* gene to prevent read-through expression of the downstream lacZalpha fragment on the vector. This was done by digesting *pGreenII-GRP7* with Hind III and Xho I and ligating the fragment to a double-stranded oligonucleotide containing two primers, Stop Duplex Top and Stop Duplex Bottom. This construct was designated as *GRP7wt*.

To make *GRP7* promoter variants that had EE motif deletions and were fused transcriptionally to the reporter luciferase gene (*LUC*), we first built a vector called *pGRI21* by inserting the *LUC* gene in front of the CAMV 35S terminator, using Gibson Assembly (Gibson *et al.*, 2009). The *LUC* gene was amplified from the pGL3basic (Promega) with the primers NotI_{Fluc} and XbaI_{Fluc}. Second, we conducted site directed mutagenesis on the *GRP7wt* construct in order to introduce 8-bp deletion at each position containing an EE motif in the *GRP7* promoter, -123, -270, -294, or -315 bp upstream the translation start (ATG) of the gene. Finally, we moved the promoter variants to the *pGRI21* vector. We designated these *GRP7* promoter variants as the follows: *pGRP7wt* with no EE motif deletion and $\Delta 123EE$ with the EE motif deletion at -123 bp, $\Delta 270EE$ at -270 bp,

$\Delta 294EE$ at -294 bp, and $\Delta 315EE$ at -315 bp. For example, to make the $\Delta 123EE$ construct, *GRP7_{wt}* was digested with *Xba* I and *EcoR* V and Gibson Assembly was used to combine the resulting fragment with two overlapping fragments, fragment 1 generated by pGreenXba I and *grp7*-50R and fragment 2 by *grp7*-50F and *grp7*-EcoRV. The *GRP7* promoter variant was amplified with primers GRP7sense and GRP7PstI and subsequently inserted into the *pGR121* vector at the Not I and Pst I sites. The promoter region was fully sequenced to ensure that the EE motif was deleted, and no extra mutations were introduced. The $\Delta 270EE$, $\Delta 294EE$, and $\Delta 315EE$ reporters were similarly constructed and confirmed as $\Delta 123EE$, using primers specific for EE motif deletions at -270, -294, and -315 bp sites, respectively.

To make the Δ quad construct containing all four EE motif mutations, the $\Delta 123EE$ construct was digested with the enzymes KasI and EcoR1 and then combined via Gibson assembly with a G block (IDT), which had the sequence of the *GRP7* promoter between the KasI and EcoR1 sites except for EE deletions at the -270, -294, and -315 bp positions. This Δ quad promoter fragment was used to fuse with the LUC gene to generate the Δ quad reporter or to replace the promoter region of *GRP7_{wt}* to generate a genomic construct with four EE motif deleted in the gene promoter, *GRP7 Δ quad*.

To make the *pGRP7* promoter deletion constructs, we used two primer pairs, one has GRP7Pro_Not1_500F and GRP7Pro_Pst1_R and the other has GRP7Pro_Not1_1000F and GRP7Pro_Pst1_R, to generate the 482 bp and 998 bp fragments of the *GRP7* promoter, respectively. The fragments were cloned to the binary *pGR121* by replacing the CAMV 35S promoter, using the Not I and Pst I sites.

Constructs containing the promoter variants (*pGRP7_{wt}*, Δ quad, $\Delta 123EE$, $\Delta 270EE$, $\Delta 294EE$, $\Delta 315EE$, *pGRP7*-482, and *pGRP7*-998) fused the *LUC* gene reporter were transferred to Col-0. Constructs containing the *GRP7_{wt}* or *GRP7 Δ quad* genomic fragment were transferred to the *grp7-1* mutant. The *pGRP7_{wt}* reporters was also transferred to the arrhythmic mutant *cca1-1lhy-20toc1-101*. *Agrobacterium*-mediated floral dipping method was used to generate independent transformants for each construct followed by selection for homozygous plants at the T₂ and later generations, using herbicide selection conferred by the binary vector. The transgenic plants were further confirmed by PCR using primers specific to each transgene, BASTA resistance conferred by the vector, and/or sequencing. Primers used for generating the constructs and detecting the transgenes are listed in Supplementary Table S1.

Pathogen infection

Pseudomonas syringae pv. *maculicola* ES4326 strain DG3 (*PmaDG3*) was used for bacterial infection. LD-entrained 25-d-old plants were moved to the continuous light (LL) condition 1 d before the infection. Plants were infected by evenly spraying freshly cultured *PmaDG3* at 1×10^7 cfu/ml concentration. At 3 d post infection (dpi), leaf discs of 7 mm in diameter from the infected leaves were excised using a core borer and ground in 10 mM MgSO₄. The surface area of each leaf disc is 38 mm. Serial dilutions of the ground mixture were made and plated on LB plates containing Kanamycin. *Botrytis cinerea* strain BO5-10 was a kind gift from Tesfaye Mengiste at Purdue University. *Botrytis* culture preparation, infection, and plant disease symptom scoring were previously described (Zhang *et al.*, 2013).

Electrophoretic mobility shift assays

DNA fragments (probes 1-3) from the *GRP7* promoter were generated by PCR amplification and purified using a PCR purification kit (Qiagen). Each probe (60 ng) was end-labeled with γ -[^{32}P]-dATP, using T_4 polynucleotide kinase (Thermo Scientific) at 37°C for 30 min. Labeled probes were purified and used for CCA1-GST binding assays (Wang *et al.*, 2011b). The construct containing *CCA1-GST/pGEX-3X* was a kind gift from Steve Kay at University of South California. CCA1-GST recombinant protein was induced and purified as previously described (Wang *et al.*, 2014). Each binding reaction included 2 μL 5X EMSA buffer [125 mM HEPES-KOH (pH 7.5), 12.5 mM DTT, 5 mM PMSF, 250 mM KCl], 2 μL 50% glycerol, 1 μg poly-dIdC, 20-80 ng CCA1-GST recombinant protein, 1 μL labeled probe, in a total volume of 10 μL . For a competition assay, cold DNA fragments were added at the indicated concentrations to the binding reaction. The EMSA reactions were resolved on a 6% non-denaturing polyacrylamide gel to separate free probes from DNA-protein complexes. The gels were dried and exposed to X-ray film for 2–4 days.

Chromatin immunoprecipitation (ChIP) assays

Fourteen-day-old seedlings of *CCA1:GFP-CCA1* and its ecotype control *Ws* ecotype (Pruneda-Paz *et al.*, 2009) were harvested at ZT1, immediately cross-linked under vacuum by immersion into 30 mL of 1% formaldehyde for 20 min followed by reaction quenching with 2 ml 2M glycine for 5 min. The seedlings were rinsed with sterile water, ground in liquid nitrogen to a fine powder, and extracted for DNA and protein complexes. The chromatin immunoprecipitation reaction was performed with anti-GFP antibodies (IP) (Abcam, product code 290) or whole rabbit IgG (mock) (Jackson ImmunoResearch cat 011-000-003) coated onto Dynabeads Protein G (Invitrogen cat 10004D). The immunocomplexes were washed and eluted from the beads followed by Proteinase K digestion and reverse crosslinking overnight at 65°C. Chromatin DNA was purified using a Qiagen QIAquick PCR purification Kit and used in real-time qPCR with primers for specific amplicons (Supplementary Table S1), using IP, mock, or input tubes for each pair of primers. Fold enrichment for each amplicon in plants expressing *CCA1:GFP-CCA1* or in *Ws* was normalized with the input DNA and the internal controls (fragments from the *UBQ10* (AT4G05320) gene and the *ACTIN2* (AT3G18780) gene, respectively) and was calculated using the equation $2^{(\text{Ct input}-\text{Ct IP})/2^{(\text{Ct input}-\text{Ct mock})}}$.

Luciferase assay

Seedlings expressing the *LUC* reporter were grown on agar plates containing 1/2 MS media with 1% sucrose in LD and at 22°C for 4 d. Seedlings were transferred to 96-well plates with one seedling per well for 1 d in LD (180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) followed by 1 d in LL (90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density). The plants were then measured for luminescence at 1 h intervals for five days in LL, using an Omega Luminescence Reader (BMG LABTECH, Inc.). The amplitude, period, and phase were calculated with the R package MetaCycle and rhythmicity index was calculated with the R package Wavelet Methods for Time Series Analysis.

RNA extraction and quantitative RT-PCR

The 4th to 7th leaves of 25-d-old plants were collected at the indicated times and under specific light regimes. For *P. syringae*-induced gene expression, the infection was conducted at ZT1 in LD or 25 h in LL and leaves were harvested at the indicated times post infection. Total RNA was extracted and reverse transcribed to produce cDNA, which was used in quantitative PCR with Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific) and gene-specific primers (Supplementary Table S1). Relative quantification of gene expression was normalized using *PDF2* (AT1G13320) and *ACTIN2* (AT3G18780) as internal controls (Czechowski *et al.*, 2005; Wang *et al.*, 2011a).

SA measurement

LD-entrained 25 d old plants were transferred to LL for 1 d and harvested for SA extraction followed by measurement using a high-performance liquid chromatography (HPLC) system (Shimadzu LC-20AT) as previously described (Wang *et al.*, 2011a).

Seedling growth assay with MJ treatment

Surface sterilized seeds were grown on ½MS plates containing 1% sucrose for 4 d. The seedlings were transferred to a 24-well tissue culture plate containing 0, 1, or 10 µM MJ. Seedlings were imaged for morphology and measured for root length 4 d post treatment.

Statistical analyses

Statistical analyses were performed using Prism 8 (GraphPad Software, LLC.). Details of experimental design, the number of replicates, and data presentation were indicated in the relevant Figure legends and method sections.

Results

GRP7 is a direct transcriptional target of CCA1

The *GRP7* promoter has four predicted EE motifs within -350 bp to the translation start site (ATG) and one CBS motif at -1369 bp of the gene promoter (Fig. 1A). To firmly establish if *GRP7* is a direct transcriptional target of CCA1, we first conducted electrophoretic mobility shift assays (EMSA) to assess the binding of CCA1 to the EE motifs proximal to the *GRP7* promoter (Pruneda-Paz *et al.*, 2009). We found that CCA1-GST recombinant protein bound to the EE motif-containing probes, probe 1 and probe 2 (Fig. 1B, C). Probe 3, which is about 3 kb upstream of the ATG site and does not contain a recognizable CCA1 binding motif, was not bound by CCA1-GST. The binding of CCA1-GST to probe 1 and probe 2 was specific because this binding was competed off by each corresponding cold probe (Fig. 1C, D). The negative control probe, probe 3, did not reduce CCA1 binding to probe 1, lending additional support for the binding specificity of CCA1 to probe 1 (Fig. 1D).

To further test CCA1 binding to the *GRP7* promoter *in planta*, we performed ChIP experiments, using a functional *GFP-CCA1* driven by the native *CCA1* promoter (*GFP-CCA1*) that complemented a *cca1* mutant (Pruneda-Paz *et al.*, 2009). Consistent with the EMSA results, we

found that CCA1 bound to two regions that contain the EE motif (*GRP7a* and *GRP7b*) but not to the control regions (*GRP7c*, *GRP7d*, the *UBQ10* and *ACTIN 2* genes) (Fig. 1A, E). Together, these data clearly establish that *GRP7* is a direct transcriptional target of CCA1.

Consistent with previous reports (Heintzen *et al.*, 1997; Wang and Tobin, 1998), our qRT-PCR data showed that transcripts of both *CCA1* and *GRP7* oscillated in a diurnal manner with distinct phases, *CCA1* was expressed in the morning while *GRP7* was expressed in the afternoon (Supplementary Fig. S1). Infection with the virulent bacterial strain *PmaDG3* suppressed expression of both genes while inducing the defense marker gene *PR1* (Supplementary Fig. S1). To test how CCA1 affects *GRP7* transcription, we quantified expression of *GRP7* in *CCA1* misexpressing plants, using qRT-PCR. We found that in the absence of *PmaDG3*, *CCA1* overexpression (*CCA1ox*) plants had no rhythmic expression of *GRP7* but higher transcript abundance overall, compared with Col-0 (Fig. 1F, left panel). Upon *PmaDG3* infection, expression of *GRP7* was suppressed in these plants and was still higher in *CCA1ox* than in Col-0 and *cca1-1* (Fig. 1F, right panel). These results support a positive correlation of *CCA1* and *GRP7* at the transcriptional level.

Robust circadian expression of *GRP7* requires EE motifs in the *GRP7* promoter

To test how CCA1 binding to the *GRP7* promoter affects circadian expression of *GRP7*, we first constructed a luciferase (*LUC*) reporter under the control of the wildtype *GRP7* promoter (1350 bp upstream of ATG), *pGRP7wt*. The construct was transferred to Col-0 and homozygous transgenic lines were obtained and tested for clock activity, using luminescence assays. As expected, *pGRP7wt* lines showed robust rhythmic luminescence (Fig. 2A). Then we used site-directed mutagenesis to delete four individual EE motifs in the *GRP7* promoter and fused the promoter variants to the luciferase gene. The resulting reporter lines are $\Delta 123EE$, $\Delta 270EE$, $\Delta 294EE$, and $\Delta 315EE$; each with an EE deletion at -123 bp, -270 bp, -294 bp, and -315 bp relative to the translation start site of the *GRP7* gene, respectively. Fig. 2 shows that all individual EE deletion mutants ($\Delta 123EE$, $\Delta 270EE$, $\Delta 294EE$, and $\Delta 315EE$) had the same period as the wildtype reporter (*pGRP7wt*). Compared with *pGRP7wt*, reduced amplitude was observed with the $\Delta 123EE$, $\Delta 294EE$, and $\Delta 315EE$ reporters (Fig. 2A, B, D, E). The $\Delta 123EE$ reporter, which had an EE deletion closest to the ATG site among the four *GRP7* promoter variants, also showed a leading phase (Fig. 2B). Interestingly, the $\Delta 270EE$ reporter behaved similarly to the wildtype reporter, suggesting a minimum contribution of the motif at the -270 bp position to *GRP7* circadian expression (Fig. 2C). Together, these data revealed that individual EE motifs in the *GRP7* promoter differentially affect circadian expression of the gene.

To see how these four EE motifs contribute together to *GRP7* expression, we deleted all four motifs ($\Delta quad$) while maintaining the rest of the promoter sequence the same as the *pGRP7wt* reporter. Compared with *pGRP7wt*, the $\Delta quad$ plants lost the robust temporal pattern (Fig. 3A). Thus, this result supports the importance of the EE motifs in maintaining circadian expression of *GRP7*.

We further analyzed the rhythmicity index (RI), a quantitative measurement of the rhythmic regularity (Leise *et al.*, 2013). To gauge the RI range for rhythmicity properties, we first analyzed the control plants. The *pGRP7wt* reporter in Col-0 showed rhythmic waveforms with regular peaks and troughs in luminescence assays (Fig. 3A). The RI values of the *pGRP7wt* plants were generally

above 0.7 (Fig. 3B), indicating the robustness of circadian expression. We generated additional control plants by transforming the arrhythmic mutant *cca1-1lhy-20toc1-101* with *pGRP7wt*. The *pGRP7wt* reporter lost rhythmicity in *cca1-1lhy-20toc1-101* plants and the RI values of these plants were about 0.2 (Fig. 3C, D). Compared with these two types of control plants, the RI values of three selected independent Δ *quad* transformants in Col-0 were between 0.7 and 0.2 (Fig. 3B). Thus, these results suggest that the Δ *quad* reporter expressed weaker but not fully abolished rhythmicity in the Col-0 background.

To test whether this residual rhythmicity of the Δ *quad* reporter is due to unidentified *cis*-elements upstream of the EE motifs we tested, we made two promoter deletion fragments fused with the *LUC* reporter, *pGRP7-998* and *pGRP7-482*, carrying a 998 bp and 482 bp promoter fragment, respectively. Compared with *pGRP7wt*, these two promoter deletions did not affect clock parameters, including the amplitude, period, phase, and rhythmicity index (Supplementary Fig. S2). Thus, we concluded that the -482 bp-ATG fragment had a major contribution to circadian expression of *GRP7*. The effect of additional *cis*-element(s) in the 1350 bp promoter region might only be manifested when all four EE elements are abolished.

Circadian expression of *GRP7* is critical for pathogen defense

Our data clearly establish a direct transcriptional control of *GRP7* circadian expression by the master clock regulator CCA1. Because *GRP7* is an important defense gene, we set out to elucidate the biological significance of this regulation in pathogen defense. To this end, we constructed two *GRP7* genomic constructs, *GRP7wt* and *GRP7 Δ quad*, both carrying the 1350 bp promoter fragment, the full genomic DNA region, and 392 bp downstream of the 3' untranslated region. *GRP7wt* has the wildtype promoter while *GRP7 Δ quad* has the same promoter with four EE motifs deleted. The constructs were transferred to the *grp7-1* mutant (SALK_039556) to obtain independent transformants.

Compared with Col-0, *grp7-1* was more susceptible to spray-infection of the bacterial pathogen *PmaDG3* and more resistant to the necrotrophic pathogen *Botrytis cinerea* (Figs. 4 and S3). These results are consistent with previous reports on pathogen responses of the *grp7-1* mutant (Fu *et al.*, 2007; Lee *et al.*, 2012). When being infected with *PmaDG3* or *Botrytis*, *GRP7wt* plants behaved like Col-0, showing a complementation of *grp7-1* for its enhanced susceptibility to *PmaDG3* and enhanced resistance to *Botrytis* (Supplementary Fig. S3A, B, C, D). The *grp7-1* mutant harbors a T-DNA insertion right before the first exon and did not accumulate *GRP7* transcripts (Supplementary Fig. S3E), suggesting that it is a null mutant. Consistent with the rescuing phenotypes, the *GRP7wt* transgene displayed robust expression rhythms in the *grp7-1* background, similar to the expression pattern of the endogenous gene in Col-0 (Supplementary Fig. S3E).

Interestingly, we observed two types of *GRP7 Δ quad* plants that showed opposite defense phenotypes (Fig. 4). *GRP7 Δ quad* lines 1 and 2 behaved similarly to the *grp7-1* mutant, showing enhanced *PmaDG3* susceptibility while *GRP7 Δ quad* lines 3 and 4 were more resistant to *PmaDG3*. These experiments were performed at both subjective morning and subjective evening and similar results were obtained (Figs. 4 and S4). With *Botrytis* infection in the subjective morning, *GRP7 Δ quad* lines 1 and 2 and the *grp7-1* mutant were more resistant while *GRP7 Δ quad* lines 3 and 4 were more susceptible. We further quantified *GRP7* expression in these plants. All these

GRP7 Δ quad plants lost robust rhythmicity of *GRP7* expression observed in Col-0 (Fig. 4E). The transcript level of the transgene in lines 1 and 2 was slightly higher than that in *grp7-1*. Plants from lines 3 and 4 plants accumulated a medium level of the transgene transcript, which was lower than the highest peak observed in Col-0. These results indicate that the loss of a temporal regulation of *GRP7* expression activates *GRP7* function at times of day when the endogenous gene is not expressed, leading to altered responses to pathogens of varying lifestyles. In addition, a threshold level of *GRP7* transcripts might be necessary to endow *GRP7* function.

The opposite defense phenotypes against *PmaDG3* and *Botrytis* in *GRP7* misexpressing plants prompted us to further examine which downstream signaling pathways are affected by *GRP7*. SA and JA are two defense signaling molecules that differentially activate plant defense against pathogens of varying lifestyles; SA activates resistance against biotrophs, such as *PmaDG3*, and JA is required for defense against necrotrophs, such as *Botrytis* (Campos *et al.*, 2014; Glazebrook, 2005). SA and JA are also known to antagonize each other's function under some conditions. We set out to first examine JA signaling with these *GRP7* misexpressing plants. We found that *grp7-1* was hypersensitive to root inhibition induced by methyl jasmonate (MJ), a JA agonist (Fig. 5). The *GRP7**wt* plants showed wildtype response to MJ treatment while *GRP7 Δ quad* lines 1 and 2 behaved similarly to *grp7-1*, *GRP7 Δ quad* lines 3 and 4 plants were less sensitive to MJ than *grp7-1* but similar as WT. We then quantified via qRT-PCR expression of the JA marker gene *PDF1;2* in selected *GRP7* misexpressing plants. We found that compared with Col-0, expression of *PDF1;2* was higher in *grp7-1* and *GRP7 Δ quad* line 1 at LL9 and LL13 in a day, respectively, compared with Col-0 while the *GRP7 Δ quad* lines 3 had a similar expression of *PDF1;2* as WT (Supplementary Fig. S5). Expression of the SA marker gene *PR1* remained similarly low in *GRP7* misexpression and Col-0 plants. This result supports that *GRP7* plays a role in JA signaling.

To further test whether SA contributes to *grp7-1*-conferred defense phenotype, we quantified the total SA level in a time course post *PmaDG3* infection. Our data show that *PmaDG3* infection induced similar levels of SA accumulation in *grp7-1* and Col-0 (Supplementary Fig. S6A) and slightly increased *PR1* expression in *grp7-1* than in Col-0 at 24 hpi (Supplementary Fig. S6B). This result suggests that *GRP7* has a minor contribution to the SA pathway.

Discussion

While it is well recognized that disrupting the circadian clock predisposes plants to pathogen infection, detailed mechanisms underlying circadian regulation of host defense have not been well understood. To deepen our understanding of the molecular basis by which core clock genes regulate pathogen defense, we investigated in-depth in this study how CCA1 controls circadian expression of a target gene, *GRP7*, and how this regulation impacts host-pathogen interactions. Our data highlight the importance of temporal control of defense signaling and pathogen response in the host.

Using CCA1 and its downstream target gene *GRP7* as a model, we provided data to illustrate the molecular mechanism by which CCA1 regulates *GRP7* circadian expression. The proximal *GRP7* promoter region (-350 bp relative to ATG) has four EE motifs, which are well defined CCA1 binding sites in clock-controlled genes. In addition, the *GRP7* promoter was among 3,600 gene promoters associated with CCA1 in a large-scale ChIPseq study (Carre and Kay, 1995; Harmer *et*

487 *al.*, 2000; Nagel *et al.*, 2015; Wang *et al.*, 1997). Through EMSA and ChIP experiments, we
488 demonstrated a physical association of CCA1 with the EE motifs in the *GRP7* promoter (Fig. 1),
489 firmly establishing that *GRP7* is a direct transcriptional target of CCA1.

490
491 Expression of *GRP7* is known to have a robust circadian pattern with an afternoon peak (Zhang *et*
492 *al.*, 2013). Studies have demonstrated the importance of the EE motif in rhythmicity of clock
493 regulated genes, including *GRP7* (Harmer and Kay, 2005; Michael and McClung, 2002; Mikkelsen
494 and Thomashow, 2009). Specifically, Harmer *et al* reported that both *GRP7* fragments, -523 bp-
495 ATG (containing four EE motifs) and -203 bp-ATG (containing only one EE motif) conferred a
496 similar rate of rhythmicity in transgenic seedlings carrying the corresponding construct (Harmer
497 *et al.*, 2000). Abolishing the EE motifs, either causing mutation in the single EE motif of the 203
498 bp promoter or through sequence deletion to make a 158 bp-fragment of the *GRP7* promoter
499 (containing no EE motif), drastically reduced the rate of rhythmic seedlings. This study
500 demonstrated the importance of the EE motif in general clock rhythmicity. However, it is unclear
501 how individual EE motifs in the proximal *GRP7* promoter contribute to *GRP7* rhythmicity,
502 especially quantitatively affecting clock parameters, including the period, amplitude, and phase.
503 Neither is it clear whether the DNA sequence upstream of the four EE motifs proximal to the *GRP7*
504 promoter affects circadian expression of this gene. We provided experimental data in this report
505 to answer these questions. We first constructed the LUC reporter lines driven by the wildtype
506 *GRP7* promoter (1350 bp; *pGRP7wt*). As expected, this construct conferred strong rhythmicity in
507 Col-0 (Fig. 2). Building on this construct, we further generated LUC reporter lines under the
508 control of *GRP7* promoter variants, either through promoter deletion or site-directed mutagenesis.

509
510 Our site-directed mutagenesis indicated that of the four EE motifs in the *GRP7* proximal promoter
511 region, the EE motif at -270 bp appeared to be dispensable for *GRP7* circadian expression (Fig.
512 2). Individual mutations in other three EE motifs at -123 bp, -294 bp, or -315 bp (relative to the
513 translation start site) did not change the period of the LUC reporter but significantly reduced the
514 amplitude, suggesting that these three EE motifs contribute additively to the level of *GRP7*
515 expression. In addition, the EE motif at -123 bp, also affected the phase of the LUC reporter. While
516 indicating that the EE motifs in the *GRP7* promoter had redundant influence on *GRP7* rhythmic
517 expression, these results also underscore the importance of the promoter context adjacent to these
518 EE motifs to render specific influence on circadian regulation of gene expression. It is possible
519 that in the presence of CCA1, a repressive factor associates with the EE motif at -123 bp site early
520 in the day to delay the onset of *GRP7* transcription. In the absence of CCA1, the lack of such a
521 repressor results in a leading phase of *GRP7* expression. Similar promoter-context-dependent
522 phase change was reported before. For instance, it is well known that EE motifs in different clock-
523 regulated genes are associated with diverse expression phases. Even for the same *TOC1* gene
524 promoter, a transcriptional fusion with the LUC report showed a 6 h lagging phase, compared with
525 a translational fusion reporter (Alabadi *et al.*, 2001; Michael and McClung, 2002).

526
527 While our data showed a differential contribution of the four EE motifs to circadian regulation of
528 *GRP7* expression, deleting all four EE motifs ($\Delta quad$) abolished robust rhythm of the *GRP7*
529 transcript, underscoring the importance of CCA1 physical binding to the EE motifs for *GRP7*
530 expression. Interestingly, the $\Delta quad$ reporter still exhibits a weak rhythm (Fig. 3). Such a weak
531 rhythm was also observed when the reporter was transiently expressed in tobacco (Data not

shown). It is possible that the 1350 bp *GRP7* promoter fragment has additional *cis* element(s) contributing to the weak rhythm observed in the Δ *quad* reporter.

Our promoter deletion experiments further showed that compared with the 1350 bp *GRP7* promoter fragment, both promoter deletions, -998 bp-ATG and -482 bp-ATG, had similar period, amplitude, phase, and rhythm index, (Supplementary Fig. S2). While they indicate that the proximal *GRP7* promoter region (-482 bp to ATG), which is enriched with four EE motifs, is mainly responsible for circadian regulation of *GRP7* expression, these data also suggest that the contribution by additional *cis*-element(s) in the 1350 bp promoter region might be better manifested in the absence of the four EE motifs residing in the -482 bp to ATG region. We also recognize that there are cyclic modifications at the chromosomal level. For instance, T-DNA insertions without known clock regulatory elements in the Arabidopsis genome resulted in over 20% transformants showing rhythmic expression of the transgene (Harmer *et al.*, 2000; Michael and McClung, 2003). The residual rhythm that we observed with the Δ *quad* reporter could also partly reflect the basal level of gene expression affected by diurnal chromatin remodeling in Col-0. Such diurnal chromatin remodeling is likely abolished when the circadian clock is disrupted in the *cca1-lhy-20-toc1-101* mutant (Fig. 3).

How does physical association of CCA1 to the *GRP7* promoter affect circadian expression of the gene? We showed that the lack of individual EE motifs reduced the amplitude of *GRP7* and deleting all four EE motifs abolished robust *GRP7* rhythmicity (Figs. 2 and 3). We further observed a positive correlation between the transcript levels of *CCA1* and *GRP7* (Figs. 1f and S1). This positive correlation was also reported previously (Green and Tobin, 1999; Mizoguchi *et al.*, 2002). In addition, co-suppression of *CCA1* and *GRP7* were observed under stress conditions, including activation of JA signaling and pathogen infection (Figs. 1f and S1; (Attaran *et al.*, 2014; Gao *et al.*, 2020; Zhang *et al.*, 2019)). Although CCA1 is generally considered as a transcriptional repressor within clock TTFLs, some prior studies and our data reported here suggest a transcriptional activator role of CCA1 for *GRP7* regulation. Similar to CCA1, increasing evidence shows that in addition to their known roles in transcriptional repression, core clock genes can act as transcriptional activators, e.g. LUX (Zhang *et al.*, 2019) and REVEILLE8 (RVE8) (Hsu *et al.*, 2013), to promote expression of other core clock genes and/or output genes. It is possible that this positive role of CCA1 in transcription regulation could be due to CCA1 repression of a repressor of *GRP7*, leading to a positive regulation of *GRP7* expression.

Our data further showed that circadian regulation is critical for *GRP7* function in pathogen defense. While the *GRP7* gene driven by the wildtype *GRP7* promoter (*GRP7*_{wt}) rescued the *grp7-1* mutant in pathogen resistance, abolishing circadian expression of *GRP7* by using the mutant promoter with all four EE motifs deleted (*GRP7* Δ *quad*) resulted in hyperactivation of the *GRP7* function during times of day when the endogenous gene is normally not expressed (Fig. 4). Such a gain of function (GOF) of *GRP7* likely requires a threshold expression of the transgene because two of the *GRP7* Δ *quad* lines (lines 1 and 2) with a low level of *GRP7* transcripts behaved like *grp7-1* in pathogen resistance.

GRP7 encodes an RNA binding protein and plays a role in RNA alternative splicing (Staiger *et al.*, 2003). Disrupted temporal expression of *GRP7* is unlikely to affect the biochemical function of the *GRP7* protein. However, when the protein is constitutively present during a day, it may

activate or suppress some target RNA substrates at times of day when they otherwise should not be activated or suppressed, resulting in the changes in defense signaling and ultimately pathogen responses. One pathway that might be affected by *GRP7* in this manner is JA signaling, which is known to be critical for plant defense against necrotrophic pathogens, such as *Botrytis*, while suppressing defense against the biotrophic pathogens, such as *P. syringae*. In support of the JA regulatory role of *GRP7*, loss of function (LOF) in *GRP7* (*grp7-1* and *GRP7Δquad* lines 1 and 2) conferred hypersensitivity to JA signal activation in MJ-induced root inhibition assay, *Botrytis* resistance, and *P. syringae* susceptibility (Figs. 4, 5, S3, and (Fu *et al.*, 2007; Lee *et al.*, 2012)). We further observed a higher expression of the JA marker gene *PDF1;2* in *grp7-1* and *GRP7Δquad* lines 2 at earlier times of a day, compared with Col-0 (Supplementary Fig. S5). Consistent with these data, *GRP7 GOF* in *GRP7Δquad* lines 3 and 4 plants or in plants overexpressing *GRP7* (driven by the CAMV 35s promoter (*GRP7OX*)) led to opposite defense responses to *Botrytis* and *P. syringae*, compared with the *GRP7 LOF* plants (this research and (Lee *et al.*, 2012)). *GRP7OX* also resulted in lower *PDF1;2* expression than wildtype control (Streitner *et al.*, 2010). Interestingly, *GRP7OX* further led to higher accumulation of SA and expression of *PR1* (Hackmann *et al.*, 2014). However, we did not observe a major change in SA levels and *PR1* expression between Col-0 and *grp7-1* upon *P. syringae* infection (Supplementary Fig. S6). Nevertheless, our previous data showed that *P. syringae*-susceptible mutants with a strong impact on the SA pathway usually compromised SA accumulation and/or *PR1* expression during early infection time points (before 24 hpi) under our experimental conditions (Lee *et al.*, 2008; Lu *et al.*, 2003; Song *et al.*, 2004; Zhang *et al.*, 2019). The importance of early SA accumulation and *PR1* expression in a resistance response was further supported in time-serial experiments with different *P. syringae* strains that differentially activate effector triggered immunity, effector triggered susceptibility, and PAMP triggered immunity (Hamdoun *et al.*, 2013). Taken together, we believe that our data suggest that *GRP7* has a minor contribution to the SA pathway. It is possible that the effect of *GRP7* on SA signaling could be better manifested when this gene is hyperactivated, e.g. in the *GRP7OX* plants.

Our data clearly demonstrate that circadian expression of *GRP7* and its regulation by *CCA1* are critical for the function of *GRP7* in pathogen defense. Abolishing the binding of *CCA1* to *GRP7* promoter disrupts *GRP7* expression rhythm and renders *GRP7 GOF* during times of day when the endogenous gene is inactive. Such *GRP7 GOF* likely impacts defense signaling and results in altered responses to pathogen challenges, involving JA and SA signaling. Thus, *GRP7* is a molecular link that connects the master core clock gene *CCA1* to pathogen defense. Our detailed mechanistic dissection of *CCA1* regulation of *GRP7* should shed lights on how *CCA1* influences other transcriptional targets for their expression and function in clock output pathways. However, *GRP7* does not explain all output events influenced by *CCA1*; for instance, *cca1-1* flowers early but *grp7-1* is late flowering (Steffen *et al.*, 2019; Streitner *et al.*, 2008). Such discrepancies can be readily explained that *GRP7* is only one of many transcriptional targets of *CCA1*. Another reason could be that there are additional transcriptional mechanisms to control *GRP7* circadian expression besides *CCA1*. For instance, the *CCA1* homologs, *LHY* and *RVE8*, are also known to bind to the EE motif in gene promoters (Alabadi *et al.*, 2001; Hsu *et al.*, 2013). Additional non-EE related motifs in *GRP7* may also account for circadian regulation of *GRP7*. Taken together, it is important to elucidate mechanisms of action of core clock proteins in order to better understand how the circadian clock sustains precise self-regulation and influences a myriad of output events, expanding our knowledge of the clock gene regulatory networks.

Supplemental data

The following supplementary data are available at JXB online.

Fig. S1. Expression of *GRP7* and *CCA1* is suppressed by *P. syringae* infection.

Fig. S2. *GRP7* promoter deletion analysis.

Fig. S3. Complementation of *grp7-1* by *GRP7wt*.

Fig. S4. Bacterial growth with *PmaDG3* infection at subjective evening.

Fig. S5. Expression of *PDF1;2* and *PR1* in *GRP7* misexpressing plants.

Fig. S6. SA accumulation and *PR1* expression in *grp7-1* and Col-0.

Table S1. Primers used in this report.

Acknowledgements

We thank the members in the Lu laboratory for their assistance in this work. We thank Drs. C. Robertson McClung, Stacey Harmer, Jian Hua, and Barbara Kunkle for helpful discussions. We thank Dr. Tanya Leise at Amherst College for sharing with the R code for rhythmicity index analysis.

Author contributions

MG performed pathogen infection, qRT-PCR, clock assays, and root assays; CZ did EMSA and CHIP experiments; MG, WA, OK, JW, and LW cloned *GRP7* constructs and transformed plants with the constructs; JA did part of qRT-PCR experiments and assisted clock assays and plant transformation. AH assisted plant transformation and transgenic plant selection. HL designed experiments, conducted genetic crosses, and wrote the manuscript with input from all coauthors.

Conflicts of interest

The authors declare no conflicts of interest.

Funding

This work was supported by a grant from National Science Foundation (NSF 1456140) to HL.

Data availability

The data supporting the findings of this study are available within the paper and within its supplemental material published online.

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Figure legends

Fig. 1. *GRP7* is a direct transcriptional target of CCA1. (A) A scheme to show positions of the EE motifs (asterisks), probes used for EMSA (top), and amplicons for ChIP experiments (bottom). (B) EMSA assays with probe 1 or 3. (C) EMSA assays with probe 2. (D) Competition for the probe 1 position with cold probe 1 or 3. The probes were end-labeled with γ - ^{32}P and incubated with purified recombinant CCA1-GST protein at the indicated amount in (B) and (C), and 40 ng in (D). CCA1-GST protein was not included in the reaction shown in the first lane of each panel. Unlabeled (cold) fragments (probe 1, 2, or 3) at the indicated fold more than isotope-labeled probes 1 or 2 were used in the competition assays in (C) and (D). The reactions were resolved on 6% native PAGE gels followed by gel drying and exposure to X-ray film. (E) ChIP experiments. Anti-GFP antibodies were used for IP and mock treatment was used as a control. *GRP7* amplicons were quantified after normalization with the internal controls (*UBQ10* and *ACTIN2*). The Wassilewskijia (Ws) ecotype was used as a control. (F) *GRP7* expression by qRT-PCR. LD-entrained 25-d old plants were transferred to LL. *PmaDG3* infection was conducted at 25 h after the onset of LL and samples were collected at the indicated times for RNA extraction. Data are presented as mean ($n=3$) \pm SD. Experiments in (B)-(E) were repeated three times and in (F) two times and similar results were obtained.

Fig. 2. The EE motifs contribute differentially to circadian expression of *GRP7*. Luminescence traces, amplitude, period, and phase change were shown for the LUC reporter driven by *GRP7* promoter variants. (A) *pGRP7wt*. (B) $\Delta 123EE$. (C) $\Delta 270EE$. (D) $\Delta 294EE$. (E) $\Delta 315EE$. Three homozygous independent transformants were used for each construct except for *pGRP7wt*, which had four lines. Seedlings were grown in LD for four days and were transferred to 96-well plates containing 1/2 MS agar medium with 0.5% sucrose and 0.25 mM D-luciferin for 1 d in LD followed by transferring to LL. LUC activity was recorded at 1-h intervals for five days and analyzed for amplitude, period, and phase with the R package MetaCycle. The bars at x-axis of the graph showing luminescence traces on the left of each panel indicate the entraining light-dark cycle, with open bars indicating subjective day and the gray bars indicating subjective night. RLU: Relative luminescence units. The color indicates genotypes, black for *pGRP7wt* line 1, magenta for line 1, blue for line 2, and gray for line 3 of transgenic plants carrying each construct in (B) to (E). Data represent mean \pm SEM ($n=12$). Statistical analysis was performed by One-way ANOVA post-hoc Tukey's HSD test. Different letters indicate significant difference among the samples ($P<0.05$). These experiments were repeated three times with similar results.

Fig. 3. Robust circadian expression of *GRP7* requires all four EE motifs. (A) Luminescence traces of $\Delta quad$ in Col-0. (B) Rhythmicity index of $\Delta quad$ in Col-0. (C) Luminescence traces of *pGRP7wt* in *cca1-1lhy-20toc1-101 (clt)*. (D) Rhythmicity index of *pGRP7wt* in *cca1-1lhy-20toc1-101*. *pGRP7wt* line 1 that expressed *GRP7:LUC* in Col-0 was used as a control (shown in Fig. 2A). The bars at x-axis of the graph showing luminescence traces of each panel indicate the light-dark cycle, with open bars indicating subjective day and the gray bars indicating subjective night. RLU: Relative luminescence units. Data represent mean \pm SEM ($n=12$). Statistical analysis was performed by One-way ANOVA post-hoc Tukey's HSD test. Different letters indicate significant difference among the samples ($P<0.05$). These experiments were repeated three times with similar results.

Fig. 4. Circadian expression is critical to maintain the normal function of *GRP7*. (A) Images of leaves spray-infected with *PmaDG3* (1×10^7 cfu/ml). (B) Bacterial growth (n=6). (C) Images of plants spray-infected with *Botrytis* (2×10^5 spores/ml). (D) Disease rating of plants infected with *Botrytis*. The rating scale was as the follows: 1 = no lesion or small, rare lesions; 2 = lesions on 10% to 30% of a leaf; 3 = lesions on 30% to 50% of a leaf; 4 = lesions on 50% to 70% of a leaf; 5 = lesions on over 70% of a leaf. (E) Quantification of *GRP7* expression by qRT-PCR (n=3). For (A) – (D), 25-day old plants grown in LD were transferred to LL for 25 h before being spray-infected with pathogens in the subjective morning hour 1 (LL25). Plants were then kept in LL for 3 days followed by assessment of disease symptoms and pathogen growth. For (E), LD-entrained 25-d old plants were transferred to LL for 25 h and then collected for leaf samples at the indicated subjective hour of a day in LL. Data represent mean \pm SEM. Statistical analysis was performed with One-way ANOVA with post-hoc Tukey's HSD test. Significant difference was indicated by different letters among the genotypes in (B) and between Col-0 and other genotypes in the same disease scale category in (D) ($P < 0.05$). These experiments were repeated two times with similar results.

Fig. 5. Root inhibition assay with methyl jasmonate treatment. (A) MJ response of the *GRP7^{wt}* plants. (B) MJ response of the *GRP7 Δ quad* plants. 4-d old seedlings were treated with MJ or water as a mock control for 4 d followed by imaging and root length measurement. The fold difference of seedling root length was calculated as the ratio of seedling root length with water treatment divided by seedling root length with MJ treatment. *jar1-1* is a MJ insensitive mutant and was used as a negative control. Data represent mean \pm SEM (n=6). Statistical analysis was performed with One-way ANOVA with post-hoc Tukey's HSD test. Different letters indicate significant difference among the plants treated with the same MJ concentration ($P < 0.05$). These experiments were repeated three times with similar results.