Circadian control of ABA biosynthesis and signaling pathways revealed by genome-wide analysis of LHY binding targets

Authors: [1,5]Sally Adams, [1,4,5]Jack Grundy, [2,3]Siren R. Veflingstad, [2]Nigel P. Dyer, [4]Matthew A. Hannah, [2]Sascha Ott and [1,*]Isabelle A. Carré.

Affiliations:
[1] School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK
[2] Systems Biology Centre, University of Warwick, Coventry CV4 7AL, UK
[3] Department of Statistics, University of Warwick, Coventry CV4 7AL, UK
[4]Bayer CropScience N.V., Technologiepark 38, 9052 Ghent, Belgium
[5] These authors contributed equally to this work.

***Corresponding author:**
Dr Isabelle Carre
School of Life Sciences
University of Warwick
Coventry CV4 7AL
Phone: +44 2476 523 544
isabelle.carre@warwick.ac.uk

Twitter account: @ia_carre

LHY controls the rhythmic production of ABA and circadian changes in ABA responsiveness in *Arabidopsis*.

ORCID information:
Dr Sascha Ott: https://orcid.org/0000-0002-5411-8114
Dr Isabelle Carre: https://orcid.org/0000-0002-0548-737
Dr Matthew Hannah: https://orcid.org/0000-0002-4889-490X
Dr Nigel Dyer: https://orcid.org/0000-0001-6158-0510

Word counts:
Total (Introduction, Materials and Methods, Results, Discussion, Acknowledgements, Author contributions and Accession numbers): 5440
Abstract: 192
Introduction: 1318
Material and Methods: 1343
Results and discussion: 2624
Acknowledgements: 80

6 Figures
3 tables

9 Supplementary Figures
7 Supplementary tables

Abstract

- The LATE ELONGATED HYPOCOTYL (LHY) transcription factor functions as part of the oscillatory mechanism of the Arabidopsis circadian clock. This paper reports the genome-wide analysis of its binding targets and reveals a role in the control of abscisic acid (ABA) biosynthesis and downstream responses.
- LHY directly repressed expression of NCED enzymes, which catalyse the rate-limiting step of ABA biosynthesis. This suggested a mechanism for the circadian control of ABA accumulation in wild-type plants. Consistent with this hypothesis, ABA accumulated rhythmically in wild-type plants, peaking in the evening. LHY-overexpressing plants had reduced levels of ABA under drought stress, whereas loss of function mutants exhibited an altered rhythm of ABA accumulation.
- LHY also bound the promoter of multiple components of ABA signalling pathways, suggesting that it may also act to regulate responses downstream of the hormone. LHY promoted expression of ABA-responsive genes responsible for increased tolerance to drought and osmotic stress but alleviated the inhibitory effect of ABA on seed germination and plant growth.
- This study reveals a complex interaction between the circadian clock and ABA pathways, which is likely to make an important contribution to plant performance under drought and osmotic stress conditions.

Plain language summary:

Plant, like animals possess a circadian clock which allows them to adapt their physiology predictable changes in environment conditions linked to the day-night cycle. We show here that the circadian clock contributes to drought and osmotic stress tolerance by controlling the production and plant's ability to respond to a key stress response hormone, abscisic acid.

Keywords: Abscisic acid/ *Arabidopsis* / circadian clock / Chromatin immunoprecipitation / abiotic stress

Introduction

Drought represents a major threat to food security, and salinity imposes limitations on the land that can be used for agriculture, hence there is considerable interest in developing crops with improved resilience to these environmental stresses. Recent evidence suggests that the plant circadian clock contributes to drought and osmotic stress tolerance, and that optimization of its function represents a potential strategy for crop improvement (Grundy *et al.*, 2015). Thus, plants with abnormal function of the central oscillator exhibit altered tolerance to drought, osmotic stress, salinity and cold temperatures (Kant *et al.*; Nakamichi *et al.*, 2012; Kim *et al.*, 2013; Sanchez-Villarreal *et al.*, 2013; Kolmos *et al.*, 2014; Fornara *et al.*, 2015; Miyazaki *et al.*, 2015).

The mechanism by which the plant circadian clock contributes to abiotic stress tolerance is not well understood. However, the expression of multiple oscillator components is altered in response to heat or cold (Pruneda-Paz *et al.*; Gould *et al.*, 2006; Legnaioli *et al.*, 2009; Filichkin *et al.*, 2010; James *et al.*, 2012; Chow *et al.*, 2014; Kolmos *et al.*, 2014; Nagel *et al.*, 2014; Box *et al.*, 2015), and changes in the amplitude of circadian rhythms in response to cold temperatures lead to the altered expression of thousands of genes (Bieniawska *et al.*, 2008). This results in altered growth patterns and may be important for vegetative yield at high temperatures (Box *et al.*, 2015, Kusakina *et al.*, 2014). The circadian oscillator was proposed to act as a master regulator of plant growth, development and physiology, integrating the effects of multiple environmental signals to influence the overall phenotype of the organism (Sanchez & Kay, 2016). However, the most immediate contribution of the plant circadian clock is to allow the plant to anticipate predictable changes in environmental stress conditions, due to the daily rotation of the earth.

The plant circadian clock drives the rhythmic expression of many genes involved in abiotic stress responses. About 40% of cold-responsive genes and 50% of heat and drought-responsive genes exhibit circadian rhythmicity in *Arabidopsis* (Bieniawska *et al.*, 2008; Covington *et al.*, 2008; Mizuno & Yamashino, 2008). Rhythmic expression of abiotic stress-responsive genes was also reported in soybean and barley (Habte *et al.*, 2014; Marcolino-Gomes *et al.*, 2014). The clock also ensures that plants respond to environmental stress signal in a manner that is appropriate for the time of the day (a phenomenon known as “gating”). For example, maximal drought-induced changes in gene expression are observed at dusk (Wilkins *et al.*, 2010; Kiełbowicz-Matuk *et al.*, 2014), and drought or heat treatments given at different times of the day can also result in differential expression of distinct sets of genes (Wilkins *et al.*, 2010; Rienth *et al.*, 2014).

The circadian clock also controls the production of the stress-response hormone, abscisic acid (ABA), suggesting that the clock may act to potentiate responses to heat, drought and osmotic stress during the day by controlling the production of this phytohormone (Lee *et al.*, , Burschka, 1983 #4483; McAdam *et al.*, 2011), The expression of multiple ABA biosynthetic enzymes oscillate in *Arabidopsis*, tomato, maize and sugarcane suggesting rhythmic control at the level of ABA biosynthesis (Thompson *et al.*, 2000; Covington *et al.*, 2008; Michael *et al.*, 2008; Fukushima *et al.*, 2009; Khan *et al.*, 2010; Hotta *et al.*, 2013, Mizuno, 2008 #4477). Multiple components of ABA signaling pathways as well as many ABA responsive transcripts exhibit circadian regulation (Michael *et al.*, 2008; Mizuno & Yamashino, 2008; Seung *et al.*, 2012; Liu *et al.*, 2013). ABA also feeds back onto the clock mechanism to influence its function (Hanano *et al.*, 2006).

The mechanism by which the circadian oscillator drives rhythmic changes in ABA levels and influences plants' sensitivity to the hormone remains to be fully elucidated. The oscillatory mechanism of the clock is based on a transcriptional-translational feedback loop composed of three inhibitory steps (Pokhilko *et al.*, 2012; Carré & Veflingstad, 2013). The LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED-1 (CCA1) transcription factors are expressed in the early morning (Genoud *et al.*, 1998; Wang & Tobin, 1998) and bind to Evening Element (EE) motifs (AAAATATCT) in the promoters of *PSEUDO-RESPONSE REGULATORS (PRR) 9, 7, 5* and of *PRR1*, also known as *TIMING OF CAB2 EXPRESSION1*, or *TOC1* (Harmer *et al.*, 2000; Matsushika *et al.*, 2000; Strayer *et al.*, 2000; Alabadi *et al.*, 2001; Adams *et al.*, 2015). As LHY and CCA1 protein levels decline in the afternoon, the PRR proteins are expressed in successive waves and act to repress *LHY* and *CCA1* transcription until the following morning (Nakamichi *et al.*, 2012). This repression is lifted late at night through the action of an Evening Complex composed of EARLY FLOWERING 3 and 4 (ELF3 and ELF4) and LUX ARRHYTHMO (LUX, also known as PHYTOCLOCK 1 or PCL1) (Helfer *et al.*, 2011; Nusinow *et al.*, 2011). This allows expression of *LHY* and *CCA1* transcripts to rise at dawn and the cycle to start again.

ABA is synthetized from β-carotene. The early steps of its biosynthesis, leading to the production of Xanthoxin, take place the chloroplast. Later steps leading to the production of abscisic aldehyde and ABA take place in the cytoplasm. The rate-limiting step for ABA biosynthesis is thought to be the conversion of ABA precursors 9-cis- Violaxanthin or 9-cis-Neoxanthin to Xanthoxin, which is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes (Thompson *et al.*, 2007). NCED3 is the most highly expressed NCED enzyme in root and stem tissues. It is highly induced under drought conditions and plays a major role in ABA production in response to water deficit (Iuchi *et al.*; Tan *et al.*, 2003; Ruggiero *et al.*, 2004).

Multiple ABA receptors have been identified (Guo *et al.*, 2011), but downstream signal transduction pathways have only been elucidated for one family of such proteins, known as pyrabactin resistance (PYR)-like (PYL) or regulatory component of ABA receptor (RCAR) (Park *et al.*, 2009). Binding of ABA to PYL/RCAR receptors results in inactivation of the co-receptor, a protein phosphatase 2C (PP2C) and to the activation of a specific group of kinases termed SNF1-related kinases 2 or SNRK2 (Ma, Yue *et al.*, 2009; Park *et al.*, 2009). SNRK2 kinases phosphorylate ABA-responsive transcription factors, which bind ABA-responsive elements (ABREs) in the promoters of ABA-responsive genes to regulate their expression (Fujii *et al.*, 2009).

Previous work suggested possible mechanisms for the regulation of ABA responses by the central oscillator. The rhythmic production of ABA was proposed to be controlled by the PRR5, 7, and 9 proteins, because analysis of a triple mutant (*prr5,7,9*) revealed increased ABA levels (Fukushima *et al.*, 2009). On the other hand, TOC1 was proposed to suppress ABA signaling by inhibiting expression of the ABA-binding protein ABAR (also known as CHLH or GUN5). Consistent with this hypothesis, *TOC1*-overexpressing plants had widely open stomata throughout diel cycles and exhibited increased sensitivity to drought, whereas plants with reduced expression of *TOC1* had the opposite phenotype (Legnaioli *et al.*, 2009). However, the function of ABAR in ABA signalling remains controversial (Hubbard *et al.*, 2010), and the observed effects of TOC1 on ABA responses may be indirect. One potential mechanism would be through regulation of LHY and CCA1 expression, as these proteins are known to potentiate ABA-mediated responses to low temperatures in the morning (Mikkelsen & Thomashow, 2009; Dong *et al.*, 2011).

Physiological responses downstream of the clock are primarily controlled at the level of transcription (Adams & Carré, 2011). Genome-wide analyses of binding sites for TOC1/PRR1, PRR5, PRR7 and CCA1 previously suggested a role for these proteins in the regulation of abiotic stress responses (Huang *et al.*, 2012; Nakamichi *et al.*, 2012; Liu *et al.*, 2013; Nagel *et al.*, 2015; Kamioka *et al.*, 2016). We now report the genome-wide analysis of LHY binding sites, and show that it directly controls expression of genes associated with ABA biosynthesis and the rhythmic accumulation of this hormone. Furthermore, LHY regulates the expression of ABA signaling components and downstream response genes to potentiate some ABA responses while inhibiting others.

Materials and Methods:

Plant material and growth conditions

The *LHY-ox* line (L*er* ecotype), which overexpresses the LHY protein, the loss of function mutants *lhy-11* and *lhy-21* (Ler and Ws ecotypes, respectively) and the transgenic line carrying the *ALCpro::LHY* construct were described previously (Schaffer *et al.*, 1998; Mizoguchi *et al.*, 2002; Hall *et al.*, 2003; Knowles *et al.*, 2008). Seeds were sown on MS-agar plates in the absence of sucrose and stratified in the dark for 3 days at 4°C, then grown under 12-h photoperiods at 22°C under 100 µmol m^{-2} s^{-1} white light unless otherwise stated.

Chromatin immunoprecipitation (ChIP)

Tissue cross-linking and chromatin extraction was carried out as described by Gendrel *et al.* (2002). For each immunoprecipitation, 250 µl of chromatin was added to 2 mls of ChIP dilution buffer (167 mM NaCl, 16.7 mM Tris-HCl pH8, 1.2 mM EDTA, Triton X-100, 1 mM PMSF and protease inhibitors) and pre-cleared with protein A Dynabeads (Invitrogen). Samples were incubated overnight at 4°C with anti-LHY antibody (1:200) (Kim *et al.*, 2003). The immuno-complexes were isolated by incubation with protein A Dynabeads for 2 h at 4°C. The beads were washed as described (Haring *et al.*, 2007) with the addition of three extra high salt buffer washes.

DNA to be analysed by quantitative PCR was eluted from protein A beads in the presence of 10% Chelex according to Nelson *et al.* (2006). For sequencing purposes, protein A beads were resuspended in 100 µl of TE and treated with RNase A at 37°C for 20 minutes. SDS was added to a final concentration of 0.5% and the samples digested with proteinase K for 2 h at 50°C. 8 µl of 5 M NaCl was added and the samples were incubated overnight at 65°C in order to reverse cross-links. The DNA was then purified using the MinElute PCR purification kit (Qiagen).

Deep sequencing of ChIP samples

Library preparation and sequencing was conducted at the University of Utah's Bioinformatic Core facility. For ChIP-seq1 35 bp single read were obtained using an Illumina GA II sequencer. For ChIp-seq 2, 50 bp single reads were obtained using an Illumina HiSeq 2000 sequencer. The libraries were prepared using the Illumina TruSeq DNA sample prep kit according to the instructions of the manufacturer. At least 4 independent ChIP samples were pooled for the generation of each library.

Analysis of ChIP-seq data

Sequence reads were aligned to the *Arabidopsis* genome (TAIR 9 version) using Bowtie (Langmead *et al.*, 2009). Default settings were used, except that only uniquely mapped reads

were retained. Results of the alignment are summarized in Table S1. LHY binding regions were then identified as genomic regions that showed over-representation of reads in the wild-type ChIP sample as compared to the input DNA sample (in ChIP-seq 1*),* or to the *lhy-21* mutant ChIP sample (in ChIP-seq 2). Peak analysis was carried out using the MACS2 software version 2.0.10.20120913 (Zhang *et al.*, 2008) following the recommended procedure for analysing ChIP-seq data for transcription factor binding. The parameter determining the number of duplicates retained was set to auto (-*keep-dup*), the q value threshold was set to 0.01 (-q), the genome size set to dm (-g) and the size of the window for the initial genome scan was set to 200 (-*bw*). Binding regions were assigned to closest gene facing away from them.

Motif analyses

200 bp sequences were retrieved on either side of the center of each binding region, and short sequence motifs that were over-represented within these sequences relative to the whole genome were identified using the DREME software in discriminative mode (Bailey, 2011). Control sequences were composed of 1000 random 400 bp regions from each chromosome. Promoters were scanned for matches to sequence motifs using FIMO (Grant *et al.*, 2011) and motif matches to transcription factor binding sites were identified using TOMTOM (Gupta *et al.*, 2007), based on the *Arabidopsis* PBM and DAP motif databases (Franco-Zorrilla *et al.*, 2014; O'Malley *et al.*, 2016).

Ethanol induction of *ALCpro::LHY* expression

Seedlings were grown on MS-agar plates for 2 weeks under 12 h-photoperiods before transfer to continuous light (LL). At the time of induction, 5 ml of ethanol (6% v/v) was added directly to the roots of the plants to induce expression of the transgene.

Gene expression analyses

Total RNA was extracted from seedlings using the Plant RNeasy kit (Qiagen) and contaminating genomic DNA removed by treatment with DNaseI (SIGMA). First-strand cDNA synthesis was carried out using Revert-aid H-Minus M-MuMLV Reverse transcriptase (Fermentas) and primed using random DNA hexamers. Quantitative PCR was conducted using a Stratagene MX3005P detection system (Agilent Technology) and SYBR Green Jumpstart Reagent (SIGMA). Expression levels were calculated relative to the constitutively expressed gene *ACT2* (At3g18780). Alternatively, RNA samples were sent for digital gene expression analysis using a Nanostring nCounter System (Geiss *et al.*, 2008) at the University Health Network Microarray

Centre in Toronto and analysed using the probe set described as part of Supplementary Table S5. Transcript expression levels were normalized relative to the constitutively expressed gene *UBC21* (AT5G25760).

Gene Ontology (GO)-term analyses

The Biomap output of the Virtual Plant software (Katari *et al.*, 2010) was used to identify functional categories that were statistically over-represented within the set of LHY regulatory targets as compared to the whole genome.

Germination experiments

Seeds for these experiments were produced from plants that were grown and harvested simultaneously. Seeds were plated onto MS-agar plates containing varying concentrations of ABA or sorbitol, and stratified for 3 days in constant darkness at 4°C. Plates were then transferred to 22°C and constant light (50 µmol m^{-2} s^{-1}) conditions and germination was scored daily based on radical emergence.

Plant growth experiments in the presence of ABA

Arabidopsis seeds were sown onto nylon membranes (Sefar) on MS medium, stratified for 3 nights at 4◦C and grown under 12L 12D at 22◦C. After 10 days, the nylon membranes containing the seedlings were then transferred to new plates containing varying concentrations of ABA. Plants were photographed at 7 and 10 days, then daily for the remaining 8 days of the experiment. Rosette area was then analysed using the rosettR software (Tome *et al.*, 2017).

ABA quantification by mass spectrometry

Arabidopsis seeds were sown onto soil in 24 well plastic trays. Following stratification at 4◦C for 3 nights in darkness, plants were grown under 16L 8D cycles (100 µmol m^{-2} s^{-1} white light), 70% (RH) at 22◦C. All trays were initially watered every 3 days by soaking in water troughs until the topsoil appeared damp. After 14 days, drought condition trays were no longer watered. After a further 10 days, rosette samples were harvested and flash frozen. Samples were homogenised by adding two chilled 3 mm glass beads (Lenz) to each sample before loading into an MM300 Tissue Lyser (Retsch) and shaking for 1 minute at 30Hz. 400 µl of extraction buffer, (10% MeOH and 1% acetic acid (v/v), Fisher Scientific OptimaTMLC/MS grade components, containing the labelled ABA standard Abscisic acid-d6 (Chiron)) was added to 10 mg of tissue. Samples were placed on ice for 30 minutes then centrifuged at 10,000 x g at 4◦C for 10 minutes. The supernatant

was removed and placed in a new microfuge tube. The pellet was extracted again using 400 µl of extraction buffer without labelled standard. After centrifugation, the supernatant was removed and combined with the previous supernatant which resulted in a total volume of 800 µl. Extraction blanks (no plant tissue) and solvent blanks (no plant tissue or labelled standard) were also created as controls. 15 µl of each sample was then loaded onto a Xevo TQ-S UPLC-MS/MS system (Waters) and analysed by HPLC-electrospray ionisation/MS-MS. Chromatographic separation was performed using a C18 100 mm x 2.0 mm column (Acquity), at 35◦C. Machine optimisation, collision energies, solvent gradients and other operation details were performed as described in Forcat *et al.* (2008). Samples were analysed in technical triplicate with a solvent blank run between each sample to prevent carry-over of compounds. Extraction blanks were run systematically throughout the sample list to ensure there was no contamination between samples. Data was acquired and analysed using the MassLynx suite (Waters).

Results and discussion

Genome-wide identification of LHY binding regions

We used chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) to identify genome-wide binding regions for the LHY transcription factor. ChIP was carried out using a polyclonal antibody to the full-length LHY protein, which gave highly significant enrichment for the known binding target *TOC1* from wild-type extracts, as compared to *lhy-21* mutant extracts (Fig. 1a). Samples for sequencing were harvested from plants that were grown under 12L 12D cycles for 10 days then transferred to constant light. Tissue was collected 26 hours after the last dark to light transition, corresponding to the peak of LHY protein accumulation (Kim *et al.*, 2003; Adams *et al.*, 2015) and maximum ChIP enrichment for *TOC1*, *ELF4* and *PRR7* promoter sequences (Fig. 1b, arrow). Two experiments were carried out. The first (ChIP-seq 1) comparing wild-type (Col) ChIP samples to wild-type input DNA. The second *(ChIP-seq 2) c*omparing wild-type and knock-out mutant (*lhy-21)* samples (Fig. 1c). ChIP-seq 2 effectively controlled for potential cross-reactivity of the antibody with LHY-related proteins, but reduced the sensitivity of detection for a number of known LHY targets, due to residual peaks identified at these locations (as illustrated for *ELF3* in Fig. 1c, and for other clock-related loci in Fig. S1). For example, *FKF1*, *CBF1* and *TOC1* sequences were ranked first, second and third in ChIP-seq 1 based on their q-values for over-representation relative to the control sample, but were ranked 1823, 2998 and 4128 in ChIP-seq 2. Nevertheless, we reasoned that sequences that were identified in both experiments would identify high confidence binding targets for LHY.

A summary of the read alignment and peak detection process is provided in Table S1, and a full list of LHY binding sites identified in both experiments based on false-discovery rate-corrected p vales (q-values) less that 0.01 is provided in Table S2. In order to identify putative regulatory targets for LHY, each of these binding sites was annotated according to the closest downstream gene. Alternatively, when located in a genic region, it was allocated to that gene. Sets of high confidence LHY binding targets were then defined based on conservative q-value thresholds of 10^{-10} for ChIP-seq 1, and 10^{-20} for ChIP-seq 2, corresponding to strong peaks of read enrichment. 722 loci were identified in both sets and are thereafter designated as "confirmed targets" (Fig. 1d; Table S2). This included many established LHY binding targets, such as the core clock components *ELF3*, *ELF4*, *PRR5*, *PRR7*, *PRR9* and LHY itself (Adams *et al.*, 2015). However, these criteria excluded the known binding targets *TOC1*, *LUX* or *CCA1*, because *TOC1* and *CCA1* were associated with relatively high q values in *ChIP-seq 2* (10^{-15} and 10^{-14}, respectively), and because *LUX* was not identified as a binding target in *ChIP-seq 1* (Table 1). This suggests that many of the genes identified in either in ChIP-seq 1 or in *ChIP-seq 2* with less significant q-values are also binding targets for LHY.

Characterisation of LHY binding sites

As expected for a transcription factor, 72% of confirmed LHY binding regions were located within 500 bp of the transcriptional start site (TSS) of a gene (Fig. 2a). Of those, 90% were located upstream of the TSS and 10% in the 5'-untranslated region of the gene.

In order to investigate the circadian expression pattern of LHY binding targets, data were retrieved from the Diurnal database (Mockler *et al.*, 2007) based on experiments carried out in constant light conditions (Edwards *et al.*, 2006). Consistent with the rhythmic binding of the LHY protein to its target loci (Fig. 1b), 53 % of high confidence LHY binding targets were found to exhibit rhythmic expression patterns in constant light, as compared to 23% genome-wide (Table S3). Genes that peaked in the evening (from 8 till 14 h after subjective dawn) were over-represented, and genes expressed at other times of the day were under-represented relative to the genome-wide set of rhythmically expressed genes (Fig. 2b, Table S4). As previously described for CCA1 (Nagel *et al.*, 2015), a large fraction (46%) of confirmed LHY binding targets did not exhibit rhythmic expression in constant light, suggesting that the clock may also act via LHY to regulate non-rhythmic processes.

A *de novo* search for short sequence motifs that were significantly over-represented within LHY-binding regions identified the Evening Element (EE: AAATATCT or AGATATTT) as the most highly represented motif (Fig. 1c). The EE, previously shown to bind LHY and the related

transcription factor CCA1 in gel-shift assays, was only found in 383 out of 1000 top-ranking binding regions examined, suggesting that LHY may also be recruited to target promoters through interactions with other transcription factors, as previously demonstrated at the *LHY* and *CCA1* promoters (Adams *et al.*, 2015). Additional motifs within LHY binding regions included the sequences AAAG, which may bind the cycling DOF factors CDF1, 2 and 3 to modulate the timing of rhythmic gene expression (Imaizumi *et al.*, 2005); TGGGCC which is a binding site for TCP transcription factors and may also mediate the effect of rhythmic transcription factors such as TCP21/CHE (Pruneda-Paz *et al.*, 2009); and C/GACGTGG, which functions as an Abscisic Acid Regulated Element (ABRE) and may act to regulate their level of expression in response to ABA (Hattori *et al.*, 2002).

Comparison with CCA1 binding targets

LHY and CCA1 are almost identical within their DNA-binding domains and are thought to have largely redundant roles as part of the circadian oscillator (Carré & Kim, 2002; Mizoguchi *et al.*, 2002). Comparisons between the set of 722 confirmed LHY targets and the 1306 and 439 high confidence CCA1 binding loci identified by Nagel *et al.* (2015) and Kamioka *et al.* (2016) identified 400 and 193 genes in common, respectively (Fig. S2a). 150 genes were common to all 3 datasets. This confirmed that LHY and CCA1 have overlapping sets of binding targets, but also suggested potential differences in specificity. Consistent with this hypothesis, analyses of LHY- and CCA1-specific target promoters identified different over-represented motifs (Fig. S2b). While the EE motif was highly over-represented in both set of promoters, the ABRE motif was only over-represented in LHY-specific target promoters. 177 matches to the ABRE were identified based on $p<0.0001$ within a test set of 315 LHY-specific targets, but only 68 were identified within the same number of CCA1-specific promoters (Fig. S2d). The most closely related over-represented motif within CCA1-specific targets was A(C/T)ACGT. Comparison with known transcription factor binding motifs identified matches to two NAC transcription factor binding sites, ATAF1 and NAC55 (Franco-Zorrilla *et al.*, 2014; O'Malley *et al.*, 2016). These results suggest that LHY may have a specific role to regulate ABA responses through interaction with ABA-responsive transcription factors.

Confirmation of regulatory interactions

In order to test whether the binding interactions identified were good evidence for regulatory interactions, we analysed changes in expression levels of 98 loci, 2 h after induction of an ethanol-responsive *LHY* transgene *(ALCpro::LHY)* (Knowles *et al.*, 2008). Transcripts to be monitored

were selected to include LHY targets with a wide range of ChIP-seq q values (10^{-260} to 10^{-4} in ChIP-seq 1; 10^{-125} to 10^{-6} in ChIP-seq 2) and rhythmic expression patterns (arrhythmic genes, and rhythmic genes with phases ranging from 0-23), as well as control, non-target loci. As we expected that responses to LHY-induction might be time of day-dependent, the experiment was repeated at 4-hour intervals over the duration of the circadian cycle. Results are summarized in Table 2 and the full dataset is available as Table S5. 72% of confirmed regulatory targets (50 out 69) were repressed in response to *ALCpro::LHY* induction, showing that LHY functions primarily as an inhibitor of transcription. 15 out of 18 genes that were only identified in ChIP-seq 2 were also repressed, suggesting that these may also be functional regulatory targets. For many genes, the effect of LHY induction was only observed at specific times of the day, indicating that their regulation by LHY was gated.

Functional characterisation of LHY binding targets

In order to get clues to the range of processes that may regulated by LHY, a gene ontology (GO)-term over-representation analysis was carried out based on confirmed binding targets (Table 3 and Table S6). This revealed binding of LHY to genes associated with circadian rhythms and photoperiodic responses, listed in Table 1. Genomic targets also included components of light response pathways, such as the blue light photoreceptors *CRYPTOCHROME 2* and *PHOTOTROPIN* and the light-responsive transcription factor *PHYTOCHROME-INTERACTING 4* (*PIF4*) (Ahmad *et al.*, 1998; Christie *et al.*, 1999; Huq & Quail, 2002). In addition, LHY was found upstream of many genes associated with responses to biotic and abiotic stress. This included the transcriptional regulators *COLD-BINDING FACTOR* (*CBF) 1,2, 3*, *4* and *COLD-REGULATED* (*COR) 27*, which play key roles in responses to low temperatures (Gilmour *et al.*, 1998; Mikkelsen & Thomashow, 2009), and *DEHYDRATION RESPONSIVE ELEMENT BINDING* (*DREB*) *2A*, *B* and *C* which mediate responses to drought and salinity (Liu *et al.*, 1998), and *JAZ* proteins, which function as negative regulators of jasmonic acid responses and regulate responses to drought and extremes of temperatures (Chini *et al.*, 2007; Zhao *et al.*, 2016).

Genes involved in ABA responses were highly over-represented in the dataset, suggesting another mechanism by which LHY might regulate environmental stress responses (Fig. 3, Table S7). Confirmed LHY targets included two regulatory subunits of ABA receptors, *PYL7/RCAR2* and *PYL8/RCAR3* (Ma, Y. *et al.*, 2009; Park *et al.*, 2009), five protein phosphatase co-receptors, *PP2C/HAI2*, *PP2CG1*, *PP2CA, ABI1 and ABI2* (Park *et al.*, 2009; Antoni *et al.*, 2012), the downstream protein kinases, *SNRK2.2* and *SNRK2.3* (Boudsocq *et al.*, 2004), the ABA-responsive transcription factors *ABI3*, *ABI5* and *ATHB6* (Giraudat *et al.*, 1992; Himmelbach *et al.*,

2002; Lopez-Molina *et al.*, 2002) and the negative regulator of *ABI5* function, *AFP3* (Lopez-Molina *et al.*, 2003). Further elements of ABA signaling pathways and several enzymes involved in the ABA biosynthesis pathways were identified in only one ChIP-seq experiment. Several of these genomic targets were confirmed in ChIP-PCR experiments and in-vitro genomic DNA pull-down experiments (Fig. S3 and S4), including the protein phosphatases *ABI1* and *ABI2*, which act to repress the pathway in the absence of ABA (Leung *et al.*; Gosti *et al.*). We therefore investigated the effect of LHY on expression of these binding targets, as well as on ABA accumulation and downstream responses.

LHY inhibits ABA biosynthesis

Expression of *NCED 3* was strongly repressed in LHY-overexpressing plants (*LHY-ox*, Fig. 4a), suggesting that LHY may negatively regulate ABA accumulation. This was confirmed by testing the effect of overexpression and loss of function of LHY on ABA levels under drought. In wild-type plants, ABA accumulation was rhythmic under drought conditions, and peaked in the evening approximately 12 hours after subjective dawn (Fig. 4b). The phase of this rhythm was advanced in the loss of function mutant *lhy-11*, as expected for an oscillation that is under the circadian control in *Arabidopsis* (Mizoguchi *et al.*, 2002). On the other hand, ABA levels were markedly reduced and arrhythmic in *LHY-ox* plants. These results suggest a model for the circadian control of ABA accumulation under drought conditions, in which inhibition of *NCED* gene expression by LHY results in reduced accumulation of ABA in the morning.

Mis-expression of LHY results in altered responses to exogenous ABA

The expression of multiple components of ABA signal transduction pathways was altered following *AlcPro::LHY* induction (Fig. 5). Expression of the negative regulators of ABA responses, *ABI1* and *ABI2* was reduced relative to control plants within two hours of ethanol treatment, suggesting that LHY might act to promote ABA responses by relieving the inhibition of the of ABA signaling pathway. However, this hypothesis was contradicted by the repression of a number of positive regulators of ABA responses, including the *SNRK2.2* kinase and the ABA responsive transcription factors *ABF1*, *ATAIB, ATHB5* and *ATHB6*, and the induction of a negative regulator, *AFP3*.

To investigate the net effect of LHY on ABA-mediated abiotic stress responses, we therefore tested the effect of LHY overexpression or loss of function on the well-characterised ABA responsive genes, *DESSICATION RESPONSIVE PROTEIN 29A* (*RD29A*) and *LOW-TEMPERATURE INDUCED -30* (*LTI30*) (Yamaguchi-Shinozaki & Shinozaki, 1994; Shi *et al.*,

2015). Expression of both genes was induced 4 h after spraying plants with 10 μM ABA (Fig. 6a). This induction was suppressed in *lhy-11* plants and enhanced in the subjective night in *LHY-ox* plants, indicating that LHY acts to promote these ABA responses. Responses to osmotic stress, which induce the production of endogenous ABA, were consistent with these findings. *LHY-ox* plants exhibited elevated expression of ABA-responsive genes *RD29A*, *LTI30*, *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) and *ABA-RESPONSIVE PROTEIN* (*ABR*) in the presence of 100 mM sorbitol (Fig. S5), suggesting that LHY also acts under physiologically-relevant conditions to potentiate this ABA-dependent stress response. As none of these genes was identified as a genomic target for LHY in ChIP-seq experiments, and *RD29A* expression was slightly inhibited, rather than induced, in response to induction of the *ALCpro::LHY* transgene (Fig. S6), sensitization of these genes to exogenous ABA and to sorbitol is likely to result from enhanced signaling through the core ABA response pathway. LHY inhibits the expression of the *ABI1* and *ABI2* protein phosphatases, which function as regulatory subunits of the ABA receptors (PYR/PYLs) and repress downstream responses in the absence of ABA. We propose that repression of *ABI1* and *ABI2* transcription by LHY ensures high amplitude induction of *RD29A* and *LTI30* transcription, by lowering the threshold for activation of the signaling pathway by ABA.

We also tested the effect of exogenous ABA on germination and seedling growth. Wild-type seeds plated on media containing ABA exhibited delayed germination. While LHY overexpression or loss of function did not affect germination under control conditions, in the presence of ABA the germination delay was less pronounced with *LHY-ox* seed, whereas *lhy-11* seed completely failed to germinate (Fig. 6b). Hypersensitivity to osmotic and salt-inhibition of germination was previously reported for the *lhy-12* and *lhy/cca1* double mutant (Kant *et al.*, 2008). Consistent with this observation, we found that germination of the *lhy-11* mutant was impaired under osmotic stress, whereas *LHY*-overexpression resulted in improved seed germination (Fig. S7). Altogether, these results suggest that *LHY* may act to mitigate the inhibitory effect of ABA on seed germination.

The observation that *LHY* potentiates the effect of ABA on *RD29A* and *LTI30* expression but antagonizes its effect on germination may reflect the different stages of development at which these experiments were carried out. *LHY* may affect ABA responses differently in seeds as compared to 7-day old seedlings. However, *LHY*-overexpression also attenuated the inhibitory effect of ABA on growth in 10-day old plants (Fig. 6c). Similar results were obtained when plants were exposed to salt or to drought conditions, which induce the production of endogenous ABA (Fig. S8 and S9). While the smaller surface area of *LHY-ox* rosettes may contribute to their

superior performance under conditions due to reduced water loss, this does not explain their ability to maintain growth on agar plates containing ABA.

In conclusion, these data suggest that the LHY transcription factor plays a complex role in the modulation of ABA biosynthesis and ABA responses. LHY drives the rhythmic accumulation of ABA, ensuring peak accumulation of the phytohormone at dusk when water deficit is most severe in leaves (Caldeira *et al.*, 2014). This may have an anticipatory function, enabling plants to activate drought-tolerance processes at the time when they are predictably needed. LHY also acts to potentiate responses to ABA in the morning, which may ensure high amplitude responses to unexpectedly hot or dry conditions in the day-time. LHY also regulates expression of ABA-responsive genes in a direct manner, and this may explain the suppression of specific ABA responses such as germination and growth inhibition. This work reveals an intricate coupling between the circadian clock and ABA pathways, which is likely to make an important contribution to plant performance under drought and osmotic stress conditions.

Acknowledgments. The research was funded by BBSRC grant BB/F022832/1 and BBSRC studentship ALFBB0011JGS to JG. The *ALCpro::LHY* transgenic line was a generous gift from Elaine Tobin. The generation and sequencing of ChIP-Seq libraries were carried out at the University of Utah Bioinformatic Core Facility. Nanostring analyses were carried out by the University Health Network Microarray Centre in Toronto. We thank Jonathan Moore for help with the initial stages of ChIP-Seq data analyses. ABA measurements were carried out by the Proteomics facility at the University of Warwick.

Author contributions. SA and JG performed experimental research; SRV, NPD and IAC carried out bioinformatic analyses; IAC, SO and MAH designed and directed the research; IAC wrote the paper with assistance from SA, SRV, JG, NPD and SO.

Accession numbers:

ChIP-seq 1 and ChIP-seq 2 datasets were deposited on the Gene Expression Omnibus database under the accession numbers GSE103785 and GSE52175, respectively.

References

Adams S, Carré IA. 2011. Downstream of the plant circadian clock: output pathways for the control of physiology and development. *Essays Biochem.* **49**: 53-69.

Adams S, Manfield I, Stockley P, Carré IA. 2015. Revised morning loops of the *Arabidopsis* circadian clock based on analyses of direct regulatory interactions. *PLoS ONE* **10**(12): e0143943.

Ahmad M, Jarillo JA, Smirnova O, Cashmore AR. 1998. Cryptochrome blue-light photoreceptors of *Arabidopsis* implicated in phototropism. *Nature* **392**(6677): 720-723.

Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Mas P, Kay SA. 2001. Reciprocal regulation between *TOC1* and *LHY*/*CCA1* within the *Arabidopsis* circadian clock. *Science* **293**(5531): 880-883.

Antoni R, Gonzalez-Guzman M, Rodriguez L, Rodrigues A, Pizzio GA, Rodriguez PL. 2012. Selective inhibition of clade A phosphatases type 2C by PYR/PYL/RCAR abscisic acid receptors. *Plant Physiol.*: 1532-2548.

Bailey TL. 2011. DREME: motif discovery in transcription factor ChIP-seq data. *Bioinformatics* **27**: 1653–1659.

Bieniawska Z, Espinoza C, Schlereth A, Sulpice R, Hincha DK, Hannah MA. 2008. Disruption of the *Arabidopsis* circadian clock is responsible for extensive variation in the cold-responsive transcriptome. *Plant Physiology* **147**(1): 263-279.

Boudsocq M, Barbier-Brygoo H, Laurière C. 2004. Identification of Nine Sucrose Nonfermenting 1-related Protein Kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **279**(40): 41758-41766.

Box Mathew S, Huang BE, Domijan M, Jaeger Katja E, Khattak Asif K, Yoo Seong J, Sedivy Emma L, Jones DM, Hearn Timothy J, Webb Alex AR, et al. 2015. *ELF3* Controls Thermoresponsive Growth in *Arabidopsis*. *Current Biology* **25**(2): 194-199.

Caldeira CF, Jeanguenin L, Chaumont F, Tardieu F. 2014. Circadian rhythms of hydraulic conductance and growth are enhanced by drought and improve plant performance. *Nature communications* **5**. Article number: 5365.

Carré IA, Veflingstad SR. 2013. Emerging design principles in the *Arabidopsis* circadian clock. *Seminars in Cell & Developmental Biology* **24**(5): 393-398.

Carré IA, Kim J-Y. 2002. MYB transcription factors in the *Arabidopsis* circadian clock. *Journal of Experimental Botany* **53**(374): 1551-1557.

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR et al. 2007. The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**: 666–671.

Chow BY, Sanchez SE, Breton G, Pruneda-Paz JL, Krogan NT, Kay SA. 2014. Transcriptional regulation of *LUX* by *CBF1* mediates cold input to the circadian clock in *Arabidopsis*. *Current Biology* **24**(13): 1518-1524.

Christie JM, Salomon M, Nozue K, Wada M, Briggs WR. 1999. LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (NPH1): binding sites for the chromophore flavin mononucleotide. *Proc Natl Acad Sci U S A* **96**(15): 8779-8783.

Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL. 2008. Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biology* **9**(8): R130.

Dong MA, Farré EM, Thomashow MF. 2011. *CIRCADIAN CLOCK-ASSOCIATED 1* and *LATE ELONGATED HYPOCOTYL* regulate expression of the *C-REPEAT BINDING FACTOR* (*CBF*) pathway in Arabidopsis. *Proceedings of the National Academy of Sciences* **108**(17): 7241-7246.

Edwards K, Anderson P, Hall A, Salathia N, Locke J, Lynn J, Straume M, Smith J, Millar A. 2006. *FLOWERING LOCUS C* mediates natural variation in the high-temperature response of the *Arabidopsis* circadian clock. *Plant Cell* **18**: 639-650.

Filichkin SA, Priest HD, Givan SA, Shen R, Bryant DW, Fox SE, Wong W-K, Mockler TC. 2010. Genome-wide mapping of alternative splicing in *Arabidopsis thaliana*. *Genome Research* **20**(1): 45-58.

Forcat S, Bennett MH, Mansfield JW, Grant MR. 2008. A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in plants following biotic and abiotic stress. *Plant Methods* **4**: 16.

Fornara F, de Montaigu A, Sánchez-Villarreal A, Takahashi Y, Ver Loren van Themaat E, Huettel B, Davis SJ, Coupland G. 2015. The GI–CDF module of *Arabidopsis* affects freezing tolerance and growth as well as flowering. *The Plant Journal* **81**(5): 695-706.

Franco-Zorrilla JM, López-Vidriero I, Carrasco JL, Godoy M, Vera P, Solano R. 2014. DNA-binding specificities of plant transcription factors and their potential to define target genes. *Proceedings of the National Academy of Sciences* **111**(6): 2367-2372.

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park S-Y, Cutler SR, Sheen J, Rodriguez PL, Zhu J-K. 2009. In vitro Reconstitution of an ABA Signaling Pathway. *Nature* **462**: 660-664.

Fukushima A, Kusano M, Nakamichi N, Kobayashi M, Hayashi N, al. e. 2009. Impact of clock-associated *Arabidopsis* pseudo-response regulators in metabolic coordination. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 7251-7256.

Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, et al. 2008. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotech* **26**(3): 317-325.

Gendrel A-V, Lippman Z, Yordan C, Colot V, Martienssem RA. 2002. Dependence on heterochromatic Histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*. *Science* **297**: 1871-1873.

Genoud T, Millar AJ, Nishizawa N, Kay SA, Schafer E, Nagatani A, Chua NH. 1998. An *Arabidopsis* mutant hypersensitive to red and far-red light signals. *Plant Cell* **10**(6): 889-904.

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF. 1998. Low temperature regulation of the *Arabidopsis CBF* family of *AP2* transcriptional activators as an early step in cold-induced *COR* gene expression. *The Plant Journal* **16**(4): 433-442.

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM. 1992. Isolation of the *Arabidopsis ABI3* Gene by Positional Cloning. *Plant Cell* **4**(10): 1251-1261.

Gosti F, Beaudoin N, Serizet C, Webb AA, Vartanian N, Giraudat J. 1999. *ABI1* protein phosphatase 2C is a negative regulator of abscisic acid signaling. *Plant Cell* **11**: 1897-1910.

Gould P, Locke J, Larue C, Southern M, Davis S, Hanano S, Moyle R, Milich R, Putterill J, Millar A, et al. 2006. The molecular basis of temperature compensation in the *Arabidopsis* circadian clock. *Plant Cell* **18**: 1177-1187.

Grant CE, Bailey TL, Noble WS. 2011. FIMO: scanning for occurrences of a given motif. *Bioinformatics* **27**: 1017–1018.

Grundy J, Stoker C, Carre IA. 2015. Circadian regulation of abiotic stress tolerance in plants. *Frontiers in plant science* **6**: 648.

Guo J, Yang X, Weston DJ, Chen J-G. 2011. Abscisic acid receptors: past, present and future. *J Integr Plant Biol* **53**: 469-479.

Gupta S, Stamatoyannopoulos JA, Bailey TL, Noble WS. 2007. Quantifying similarity between motifs. *Genome Biology* **8**: R24.

Habte E, Müller LM, Shtaya M, Davis SJ, von Korff M. 2014. Osmotic stress at the barley root affects expression of circadian clock genes in the shoot. *Plant, Cell & Environment* **37**(6): 1321-1327.

Hall A, Bastow RM, Davis SJ, Hanano S, McWatters HG, Hibberd V, Doyle MR, Sung S, Halliday KJ, Amasino RM, et al. 2003. The *TIME FOR COFFEE* gene maintains the amplitude and timing of *Arabidopsis* circadian clocks. *The Plant Cell* **15**(11): 2719-2729.

Hanano S, Domagalska MA, Nagy F, Davis SJ. 2006. Multiple phytohormones influence distinct parameters of the plant circadian clock. *Genes Cells* **11**(12): 1381-1392.

Haring M, Offermann S, Danker T, Horst I, Peterhansel C, Stam M. 2007. Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. *Plant Methods* **3**(1): 11.

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA. 2000. Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* **290**(5499): 2110-2113.

Hattori T, Totsuka M, Hobo T, Kagaya Y, Yamamoto-Toyoda A. 2002. Experimentally determined sequence requirement of ACGT-containing abscisic acid response element. *Plant and Cell Physiology* **43**(1): 136-140.

Helfer A, Nusinow DA, Chow BY, Gehrke AR, Bulyk ML, Kay SA. 2011. *LUX ARRHYTHMO* encodes a nighttime repressor of circadian gene expression in the *Arabidopsis* core clock. *Current Biology* **21**(2): 126-133.

Himmelbach A, Hoffmann T, Leube M, Höhener B, Grill E. 2002. Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in *Arabidopsis*. *The EMBO journal* **21**(12): 3029-3038.

Hotta CT, Nishiyama MY, Souza GM. 2013. Circadian rhythms of sense and antisense transcription in sugarcane, a highly polyploid crop. *PLoS ONE* **8**(8): e71847.

Huang W, Pérez-García P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P. 2012. Mapping the core of the *Arabidopsis* circadian clock defines the network structure of the oscillator. *Science* **336**(6077): 75-79.

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI. 2010. Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes & Development* **24**(16): 1695-1708.

Huq E, Quail PH. 2002. PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in *Arabidopsis*. *The EMBO journal* **21**(10): 2441-2450.

Imaizumi T, Schultz TF, Harmon FG, Ho LA, Kay SA. 2005. FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in *Arabidopsis*. *Science* **309**(5732): 293-297.

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K. 2002. Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. **27**(4):325-33.

James AB, Syed NH, Bordage S, Marshall J, Nimmo GA, Jenkins GI, Herzyk P, Brown JWS, Nimmo HG. 2012. Alternative splicing mediates responses of the *Arabidopsis* circadian clock to temperature changes. *Plant Cell* **24**(3): 961-981.

Kamioka M, Takao S, Suzuki T, Taki K, Higashiyama T, Kinoshita T, Nakamichi N. 2016. Direct repression of evening genes by CIRCADIAN CLOCK-ASSOCIATED1 in the *Arabidopsis* circadian clock. *The Plant Cell* **28**(3): 696-711.

Kant P, Gordon M, Kant S, Zolla G, Davydov O, Heimer YM, Chalifa-Caspi V, Shaked R, Barak S. 2008. Functional-genomics-based identification of genes that regulate *Arabidopsis* responses to multiple abiotic stresses. *Plant, Cell & Environment* **31**(6): 697-714.

Katari MS, Nowicki SD, Aceituno FF, Nero D, Kelfer J, Thompson LP, Cabello JM, Davidson RS, Goldberg AP, Shasha DE, et al. 2010. VirtualPlant: A Software Platform to Support Systems Biology Research. *Plant Physiology* **152**(2): 500-515.

Khan S, Rowe SC, Harmon FG. 2010. Coordination of the maize transcriptome by a conserved circadian clock. *BMC plant biology* **10**: 126.

Kiełbowicz-Matuk A, Rey P, Rorat T. 2014. Interplay between circadian rhythm, time of the day and osmotic stress constraints in the regulation of the expression of a *Solanum Double B-box* gene. *Annals of Botany* **113**(5): 831-842.

Kim JY, Song HR, Taylor BL, Carre IA. 2003. Light-regulated translation mediates gated induction of the *Arabidopsis* clock protein LHY. *The EMBO journal* **22**(4): 935-944.

Kim W-Y, Ali Z, Park HJ, Park SJ, Cha J-Y, Perez-Hormaeche J, Quintero FJ, Shin G, Kim MR, Qiang Z, et al. 2013. Release of SOS2 kinase from sequestration with GIGANTEA determines salt tolerance in *Arabidopsis*. *Nature communications* **4**: 1352.

Knowles SM, Lu SX, Tobin EM. 2008. Testing time: can ethanol-induced pulses of proposed oscillator components phase shift rhythms in *Arabidopsis*? *J Biol Rhythms* **23**: 463-471.

Kolmos E, Chow BY, Pruneda-Paz JL, Kay SA. 2014. HsfB2b-mediated repression of *PRR7* directs abiotic stress responses of the circadian clock. *Proceedings of the National Academy of Sciences* **111**(45): 16172-16177.

Kusakina J, Gould PD, Hall A. 2014. A fast circadian clock at high temperatures is a conserved feature across *Arabidopsis* accessions and likely to be important for vegetative yield. *Plant, Cell & Environment* **37**(2): 327-340.

Langmead B, Trapnell C, Pop M, Salzberg S. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10**(3): R25.

Lee KH, Piao HL, Kim H-Y, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee I-J, Hwang I. 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell* **126**(6): 1109-1120.

Legnaioli T, Cuevas J, Mas P. 2009. *TOC1* functions as a molecular switch connecting the circadian clock with plant responses to drought. *Embo J* **28**: 3745-3757.

Leung J, Merlot S, Giraudat J. 1997. The *Arabidopsis ABSCISIC ACID-INSENSITIVE2* (*ABI2*) and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *The Plant Cell* **9**: 759-771.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *The Plant Cell* **10**: 1391-1406.

Liu T, Carlsson J, Takeuchi T, Newton L, Farré EM. 2013. Direct regulation of abiotic responses by the *Arabidopsis* circadian clock component *PRR7*. *The Plant Journal* **76**(1): 101-114.

Lopez-Molina L, Mongrand S, Kinoshita N, Chua N-H. 2003. AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. *Genes & Development* **17**(3): 410-418.

Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT, Chua N-H. 2002. ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *The Plant Journal* **32**(3): 317-328.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E. 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**: 1064-1068.

Marcolino-Gomes J, Rodrigues FA, Fuganti-Pagliarini R, Bendix C, Nakayama TJ, Celaya B, Molinari HBC, de Oliveira MCN, Harmon FG, Nepomuceno A. 2014. Diurnal oscillations of soybean circadian clock and drought responsive genes. *PLoS ONE* **9**(1): e86402.

Matsushika A, Makino S, Kojima M, Mizuno T. 2000. Circadian waves of expression of the APRR1/TOC1 family of pseudo-response regulators in *Arabidopsis thaliana*: insight into the plant circadian clock. *Plant and Cell Physiology* **41**(9): 1002-1012.

McAdam SAM, Brodribb TJ, Ross JJ, Jordan GJ. 2011. Augmentation of abscisic acid (ABA) levels by drought does not induce short-term stomatal sensitivity to CO2 in two divergent conifer species. *Journal of Experimental Botany* **62**(1): 195-203.

Michael TP, Breton G, Hazen SP, Priest H, Mockler TC, Kay SA, Chory J. 2008. A morning-specific phytohormone gene expression program underlying rhythmic plant growth. *PLOS Biology* **6**(9): e225.

Mikkelsen M, D. , Thomashow M, F. . 2009. A role for circadian evening elements in cold-regulated gene expression in *Arabidopsis*. *The Plant Journal* **60**(2): 328-339.

Miyazaki Y, Abe H, Takase T, Kobayashi M, Kiyosue T. 2015. Overexpression of *LOV KELCH PROTEIN 2* confers dehydration tolerance and is associated with enhanced expression of dehydration-inducible genes in *Arabidopsis thaliana*. *Plant cell reports* **34**(5): 843-852.

Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carre IA, Coupland G. 2002. LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. *Developmental Cell* **2**(5): 629-641.

Mizuno T, 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 and Cell Physiology* **49**(3): 481-487.

Mockler TC, Michael TP, Priest HD, Shen R, Sullivan CM, Givan SA, McEntee C, Kay SA, Chory J. 2007. The Diurnal project: diurnal and circadian expression profiling, model-based pattern matching, and promoter analysis. *Cold Spring Harbor Symposia on Quantitative Biology: Clocks and Rhythms* **72**: 353-363.

Nagel DH, Doherty CJ, Pruneda-Paz JL, Schmitz RJ, Ecker JR, Kay SA. 2015. Genome-wide identification of CCA1 targets uncovers an expanded clock network in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **112**: E4802-4810.

Nagel DH, Pruneda-Paz JL, Kay SA. 2014. *FBH1* affects warm temperature responses in the *Arabidopsis* circadian clock. *Proceedings of the National Academy of Sciences* **111**(40): 14595-14600.

Nakamichi N, Kiba T, Kamioka M, Suzuki T, Yamashino T, Higashiyama T, Sakakibara H, Mizuno T. 2012. Transcriptional repressor PRR5 directly regulates clock-output pathways. *Proc. Nat. Acad.Sci. USA* **109**: 17123-17128.

Nelson JD, Denisenko O, Bomsztyk K. 2006. Protocol for the fast chromatin immunoprecipitation (ChIP) method. *Nat. Protocols* **1**(1): 179-185.

Nusinow DA, Helfer A, Hamilton EE, King JJ, Imaizumi T, Schultz TF, Farre EM, Kay SA. 2011. The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. *Nature* **475**(7356): 398-402.

O'Malley RC, Huang S-SC, Song L, Lewsey MG, Bartlett A, Nery JR, Galli M, Gallavotti A, Ecker JR. 2016. Cistrome and Epicistrome Features Shape the Regulatory DNA Landscape. *Cell* **165**: 1280-1292.

Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow T-FF, et al. 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068-1071.

Pokhilko A, Fernández AP, Edwards KD, Southern MM, Halliday KJ, Millar AJ. 2012. The clock gene circuit in *Arabidopsis* includes a repressilator with additional feedback loops. *Molecular Systems Biology* **8**(1): 574. doi:510.1038/msb.2012.1036.

Pruneda-Paz Jose L, Breton G, Nagel Dawn H, Kang SE, Bonaldi K, Doherty Colleen J, Ravelo S, Galli M, Ecker Joseph R, Kay Steve A. A genome-scale resource for the functional characterization of *Arabidopsis* transcription factors. *Cell Reports* **8**(2): 622-632.

Pruneda-Paz JL, Breton G, Para A, Kay SA. 2009. A functional genomics approach reveals CHE as a component of the *Arabidopsis* circadian clock. *Science* **323**(5920): 1481-1485.

Rienth M, Torregrosa L, Luchaire N, Chatbanyong R, Lecourieux D, Kelly MT, Romieu C. 2014. Day and night heat stress trigger different transcriptomic responses in green and ripening grapevine (*vitis vinifera*) fruit. *BMC plant biology* DOI: 10.1186/1471-2229-14-108

Ruggiero B, Koiwa H, Manabe Y, Quist TM, Inan G, Saccardo F, Joly RJ, Hasegawa PM, Bressan RA, Maggio A. 2004. Uncoupling the effects of abscisic acid on plant growth and water relations. Analysis of *sto1/nced3*, an abscisic acid-deficient but salt stress-tolerant mutant in *Arabidopsis*. *Plant Physiology* **136**(2): 3134-3147.

Sanchez SE, Kay SA. 2016. The Plant Circadian Clock: From a Simple Timekeeper to a Complex Developmental Manager. *Cold Spring Harb Perspect Biol* **8**: a027748.

Sanchez-Villarreal A, Shin J, Bujdoso N, Obata T, Neumann U, Du S-X, Ding Z, Davis AM, Shindo T, Schmelzer E, et al. 2013. *TIME FOR COFFEE* is an essential component in the maintenance of metabolic homeostasis in *Arabidopsis thaliana*. *The Plant Journal* **76**(2): 188-200.

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carré IA, Coupland G. 1998. The *late elongated hypocotyl* mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* **93**: 1219-1229.

Seung D, Risopatron JPM, Jones BJ, Marc J. 2012. Circadian clock-dependent gating in ABA signalling networks. *Protoplasma* **249**(3): 445-457.

Shi H, Chen Y, Qian Y, Chan Z. 2015. *Low Temperature-Induced 30* (*LTI30*) positively regulates drought stress resistance in *Arabidopsis*: effect on abscisic acid sensitivity and hydrogen peroxide accumulation. *Frontiers in plant science*

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Mas P, Panda S, Kreps JA, Kay SA. 2000. Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. *Science* **289**: 768-771.

Tan B-C, Joseph LM, Deng W-T, Liu L, Li Q-B, Cline K, McCarty DR. 2003. Molecular characterization of the *Arabidopsis 9-cis epoxycarotenoid dioxygenase* gene family. *The Plant Journal* **35**(1): 44-56.

Thompson AJ, Jackson AC, Parker RA, Morpeth DR, Burbidge A, Taylor IB. 2000. Abscisic acid biosynthesis in tomato: regulation of *zeaxanthin epoxidase* and *9-cis-epoxycarotenoid dioxygenase* mRNAs by light/dark cycles, water stress and abscisic acid. *Plant Molecular Biology* **42**(6): 833-845.

Thompson AJ, Mulholland BJ, Jackson AC, McKee JMT, Hilton HW, Symonds RC, Sonneveld T, Burbidge A, Stevenson P, Taylor IB. 2007. Regulation and manipulation of ABA biosynthesis in roots. *Plant, Cell & Environment* **30**(1): 67-78.

Tome FA-O, Jansseune K, Saey B, Grundy J, Vandenbroucke K, Hannah MA, Redestig H. 2017. rosettR: protocol and software for seedling area and growth analysis. *Plant Methods* **13**: 13.

Wang Z-Y, Tobin EM. 1998. Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED (CCA1)* gene disrupts circadian rhythms and suppresses its own expression. *Cell* **93**: 1207-1217.

Wilkins O, Bräutigam K, Campbell MM. 2010. Time of day shapes *Arabidopsis* drought transcriptomes. *The Plant journal : for cell and molecular biology* **63**(5): 715-727.

Yamaguchi-Shinozaki K, Shinozaki K. 1994. A novel cis-acting element in an *Arabidopsis* gene involved in responsiveness to drought, low temperature, or high salt stress. . *The Plant Cell* **6**: 251-264.

Zhang Y, Liu T, Meyer C, Eeckhoute J, Johnson D, Bernstein B, Nusbaum C, Myers R, Brown M, Li W, et al. 2008. Model-based Analysis of ChIP-Seq (MACS). *Genome Biology* **9**(9): R137.

Zhao G, Song Y, Wang C, Butt HI, Wang Q, Zhang C, Yang Z, Liu Z, Chen E, Zhang X, et al. 2016. Genome-wide identification and functional analysis of the TIFY gene family in response to drought in cotton. *Molecular Genetics and Genomics* **291**(6): 2173-2187.

Fig. legends

Fig. 1. Genome-wide identification of LHY binding sites in *Arabidopsis thaliana*. (a). Quality assessment of ChIP samples used for sequencing in ChIP-seq 2. Enrichment for a known target sequence of LHY (*TOC1*) was determined by quantitative PCR and compared to a control locus (*ACTIN*). Data are means and standard deviations at least from 5 independent experimental replicates for wild-type (Ws) and *lhy-21* mutant samples, respectively. **(b)** ChIP-PCR analysis of wild-type samples harvested at different times of the day. Plants were grown under 12L12D cycles then transferred to constant light at time zero. White and hatched bars above the chart indicate subjective days and nights, respectively. Enrichment for *TOC1*, *ELF4*, *PRR7* promoter and *ACTIN* 3'UTR sequences was determined relative to input DNA samples **(c)** Comparison of results from both ChIP-seq experiments at the *ELF3* locus (ATG29530). Note that the q-values reported here are distinct from those reported in Table 1 and Table S2, because they indicate local overrepresentation rather than overrepresentation over the binding region as a whole. Reads mapped to the forward strand are shown in red, those to the reverse strand in blue. ChIP-seq 2 results for other clock-related loci are shown in Fig. S1. **(d)** Comparison of binding targets identified in ChIP-seq 1 and ChIP-seq 2, based on q-value thresholds of 10^{-10} and 10^{-20}, respectively.

Fig. 2: Characterisation of LHY binding sites in *Arabidopsis*. (a) Position of 1000 highest ranking peaks in ChIP-seq 2 relative to transcriptional start sites. **(b)** Histogram showing the proportion of rhythmic LHY binding targets that peak at different phases of the circadian cycle as compared to the genome-wide set of rhythmically expressed genes. Data for confirmed LHY binding targets were retrieved from the Diurnal database (Mockler *et al.*, 2007), using the constant light, LL23 dataset (Edwards *et al.*, 2006) and a correlation coefficient cut-off of 0.8. White and hatched bars above the chart indicate subjective days and nights, respectively, and * and & indicate p values for over- and under-representation, respectively relative to the genome-wide set of rhythmic genes, determined using a hypergeometric test (* and &, $p<0.05$; ** and &&, $p<0.01$; *** and &&& $p<0.001$). **(c)** Motifs identified from the 1000 highest ranking peaks in ChIP-seq 2. Sequences are shown as positional weight matrices (PWMs) where the height of each letter represents the probability of having the corresponding base at that position.

Fig. 3. Binding of LHY to components of ABA biosynthesis and signalling pathways. The diagram illustrates the mechanism underlying transcriptional responses to ABA in *Arabidopsis*. Pointed and blunt arrows indicate activatory and inhibitory interactions, respectively. Expression of ABA-responsive genes is driven by a number of ABA-responsive transcription factors, which are activated by phosphorylation by SNRK2 kinases. In the absence of ABA the pathway is repressed through the action of protein phosphatases (PP2As family) which inactivate SNRK2s by dephosphorylation. ABA binding to its receptors (the PYL/RCAR family) results in inhibition of PP2As, and activation of SNRK kinases and of downstream transcription factors. The genes listed at each step of the pathway indicate components that were identified as binding targets for LHY. Normal fonts indicate binding targets identified in a single ChIP-seq experiment and bold fonts indicate binding confirmed either by ChIP-seq or by ChIP-PCR. Corresponding data are provided in Table S7, Figs. S3 and S4

Fig. 4. LHY regulates ABA accumulation. (a) *NCED3* transcript levels in wild-type, *lhy-11* and *LHY-ox* seedlings (grey, white and black bars, respectively). *Arabidopsis* plants were grown for 7 days under 12L12D cycles on MS-agar plates before transfer to constant light. Tissue was harvested either 3 or 15 hours after dawn. Transcript levels were determined by quantitative RT-PCR and expressed relative to ACTIN. **(b)** Overexpression of *LHY* results in reduced ABA levels under drought conditions. *lhy-11*, *LHY-ox* and wild-type seedlings were grown in a randomised configuration on soil and entrained to 16L8D cycles. Plants received water every third day for the first 14 days, then watering was withheld entirely from the drought set for the next 10 days. Rosette samples were then harvested at 3-hour intervals across a 24-hour period for ABA quantification. Data represents the mean from technical triplicates for a pooled sample of 2 biological replicates. White and black bars above the chart indicate days and nights, respectively. Error bars indicate standard errors. * and + indicate p-values from t-tests comparing *LHY-ox* and *lhy-11* to the wild type, respectively (* and +, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Fig. 5. Induction of *LHY* expression from the *ALCpro::LHY* transgene results in altered expression of multiple components of ABA signaling pathways. Wild-type *Arabidopsis* plants carrying the *ALCpro*::*LHY* transgene were grown under 12L12D light-dark cycles then transferred to constant light at the start of the experiment. Expression of *ALCpro*::*LHY* was induced using 6% ethanol (v/v). Different sets of plants were treated at 4-hour intervals over the duration of one circadian cycle, and tissue was harvested 2 hours later. mRNA levels were determined either using Nanostring technology and normalized relative to *UBC12* (a,d,j) or by

quantitative PCR and normalized to *ACTIN* (b,c,e,f,g,h). Times indicate when the tissue was harvested. Data from *ALCpro*::*LHY* plants (filled bars) were compared to data from wild-type plants (white bars). Data shown in panels a, d, j are means and standard deviations from two independent biological replicates. Data shown in other panels are mean and standard errors of technical triplicates for a single experiment. * indicates p <0.05, ** p<0.01 and *** p<0.**001** as determined by t-tests.

Fig. 6. Mis-expression of *LHY* results in altered responses to ABA. (a) Induction of *RD29A* and *LTI30* expression by ABA in wild-type, *lhy-11* and *LHY-ox* plants (grey, white and black bars, respectively). *Arabidopsis* plants were grown under light-dark cycles for 7 days then transferred to constant light at time zero. At each time point a set of plants was sprayed with 25 µM ABA or vehicle (methanol) and tissue was harvested after 3 hours for RNA extraction. Times indicate when the tissue was harvested. Transcript levels were determined by quantitative PCR and were calculated relative to *ACTIN*. Data represents the mean of technical triplicates for a single experiment, with error bars showing standard errors. Results were consistent across 3 independent experiments. **(b)** Germination of wild-type, *lhy-11* and *LHY-ox* seeds (grey, white and black symbols, respectively) in the presence of 2 µM ABA. Data represents the mean percentage of germination from 3 independent progenies from individual plants and error bars indicate standard deviations. * and + indicate p-values from t-tests comparing *LHY-ox* and *lhy-11* to the wild type, respectively (* and +, p<0.05; **and ++, p<0.01; ***and +++, p<0.001). **(c)** Effect of exogenous ABA on seedling growth. Seedlings were grown under 12L 12D cycles on MS-agar plants. At the time indicated by the vertical dashed line, plants were transferred to fresh plates with or without ABA (10 µM). Aerial photographs were taken daily for rosette size measurements. Data represents the means from 192 plants across 2 independent experiments, and error bars indicate standard deviations. Asterisks indicate p-values from t-tests comparing the experimental treatment to the control condition at each time point (* p<0.05; ** p<0.01; *** p<0.001).

Table 1. Binding of LHY to the promoters of circadian clock-associated genes in *Arabidopsis*.

Gene name	Gene ID	-log10(q-values)* ChIP-seq 1	ChIP-seq 2
LHY	AT1G01060	29	56
CCA1	AT2G46830	61	14
PRR9	AT2G46790	93	81
PRR7	AT5G02810	99	35
PRR5	AT5G24470	78	64
PRR1/TOC1	AT5G61380	182	15
LUX/PCL1	AT3G46640	N/A	67
BOA/NOX	AT5G59570	127	85
ELF3	AT2G25930	63	91
ELF4	AT2G40080	107	31
GI	AT1G22770	162	47
RVE6	AT5G52660	N/A	30
LNK1	AT5G64170	N/A	13
LNK2	AT3G54500	27	24
CHE/TCP21	AT5G08330	77	135
LWD2	AT3G26640	N/A	41
FKF1	AT1G68050	260	38
CDF1	AT5G62430	22	102
CDF2	AT5G39660	N/A	128
CKB4	AT5G52660	N/A	30
JMJD5	AT5G52660	N/A	30

*when multiple peaks were present upstream of a gene, q values given correspond to the most significant.

Table 2. Regulatory function of LHY binding interactions in *Arabidopsis*. The functionality of LHY binding interactions was tested by assaying changes in expression of LHY binding targets upon induction of the *ALCpro::LHY* transgene. “Unconfirmed targets” indicates genes that were identified in only one of the two ChIP-seq experiments. “Induced” or “repressed” indicate increases or decreases in expression levels detected at one or more time points. “Other” indicates increased expression at some time points and decreased at others.

Numbers	**no effect**	**negative**	**positive**	**other**	**total**
confirmed LHY targets	8	50	6	5	69
unconfirmed LHY targets	3	15	0	1	19
non LHY-targets	2	2	5	1	10

Table 3. GO-term over-representation analysis of high confidence LHY binding targets in *Arabidopsis*.

	Number of genes	Observed Frequency	Number of genes	Expected Frequency	p-value
Responses to light					
response to light stimulus	48	8.20%	450	2.30%	7.15E-11
response to red light	10	1.70%	54	0.30%	0.000385
response to UV	9	1.50%	66	0.30%	0.00533
response to blue light	8	1.40%	52	0.30%	0.00545
response to far red light	7	1.20%	42	0.20%	0.00798
circadian rhythm	13	2.20%	48	0.20%	1.01E-06
Biotic and abiotic stress responses					
response to cold	34	5.80%	264	1.30%	1.21E-09
heat acclimation	5	0.90%	14	0.10%	0.00328
response to water deprivation	25	4.30%	196	1%	5.52E-07
response to osmotic stress	30	5.10%	413	2.10%	0.000425
response to salt stress	28	4.80%	387	2%	0.000804
response to wounding	14	2.40%	137	0.70%	0.00264
response to biotic stimulus	36	6.20%	582	3%	0.00134
response to fungus	19	3.20%	159	0.80%	3.32E-05
Hormone responses					
response to abscisic acid stimulus	26	4.40%	317	1.60%	0.000241
response to jasmonic acid stimulus	17	2.90%	152	0.80%	0.000241
response to gibberellin stimulus	14	2.40%	112	0.60%	0.000447
response to ethylene stimulus	15	2.60%	130	0.70%	0.000522
response to auxin stimulus	21	3.60%	250	1.30%	0.00104
regulation of post-embryonic development	15	2.60%	174	0.90%	0.00682

Supplementary information

Fig. S1. Graphical representation of LHY ChIP-Seq data at the promoters of clock-associated genes.

Fig. S2. Comparison between LHY and CCA1 binding targets.

Fig. S3. ChIP-PCR confirmation of LHY binding to the promoters of the *ABI1*, *ABI2*, *ABI5*, *AFP3*, *ATHB6* and *SnRK2.2* genes .

Fig. S4. In vitro confirmation of LHY binding to the *ABI1*, *ABF3* and *SNRK2.2* promoters.

Fig. S5. Effect of *LHY* overexpression and loss of function on expression of ABA-responsive genes under osmotic stress conditions.

Fig. S6. Effect of ethanol-induction of the *ALCpro::LHY* transgene on expression of *RD29A*.

Fig. S7. Effect of overexpression and loss of function of *LHY* on seed germination under osmotic stress.

Fig. S8. Effect of *LHY* overexpression and loss of function on plant growth under severe drought.

Fig. S9. Effect of *LHY* overexpression and loss of function on plant growth under mild drought and salinity.

Table S1. Summary of the ChIP-seq alignment process.

Table S2. LHY binding targets identified by ChIP-seq.

Table S3. Rhythmicity of high confidence LHY binding targets in constant light.

Table S4. Phase distribution of confirmed LHY binding targets.

Table S5. Gene expression changes in response to ethanol induction of the *ALCpro::LHY* transgene.

Table S6. GO-term analysis of LHY binding targets.

Table S7. Binding of LHY and CCA1 to elements of ABA biosynthesis and signalling pathways.