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

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

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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; Kielbowicz-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; McAdam et al., 2011). The expression of multiple ABA biosynthetic enzymes oscillates 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). 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 & Velflingstad, 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 (Ler 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 immunocomplexes 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) comparing 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 values (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 \textit{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.

\textbf{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 \textit{CRYPTOCHROME 2} and \textit{PHOTOTROPIN} and the light-responsive transcription factor \textit{PHYTOCHROME-INTERACTING 4} (\textit{PIF4}) (Ahmad \textit{et al.}, 1998; Christie \textit{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 \textit{COLD-BINDING FACTOR (CBF) 1,2, 3, 4} and \textit{COLD-REGULATED (COR) 27}, which play key roles in responses to low temperatures (Gilmour \textit{et al.}, 1998; Mikkelsen \& Thomashow, 2009), and \textit{DEHYDRATION RESPONSIVE ELEMENT BINDING (DREB) 2A, B} and \textit{C} which mediate responses to drought and salinity (Liu \textit{et al.}, 1998), and \textit{JAZ} proteins, which function as negative regulators of jasmonic acid responses and regulate responses to drought and extremes of temperatures (Chini \textit{et al.}, 2007; Zhao \textit{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, \textit{PYL7/RCAR2} and \textit{PYL8/RCAR3} (Ma, Y. \textit{et al.}, 2009; Park \textit{et al.}, 2009), five protein phosphatase co-receptors, \textit{PP2C/HAI2}, \textit{PP2CG1}, \textit{PP2CA}, \textit{ABI1} and \textit{ABI2} (Park \textit{et al.}, 2009; Antoni \textit{et al.}, 2012), the downstream protein kinases, \textit{SNRK2.2} and \textit{SNRK2.3} (Boudsocq \textit{et al.}, 2004), the ABA-responsive transcription factors \textit{ABI3}, \textit{ABI5} and \textit{ATHB6} (Giraudat \textit{et al.}, 1992; Himmelbach \textit{et al.},...
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 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,
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.

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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.

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**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*.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>ChIP-seq 1</th>
<th>ChIP-seq 2</th>
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<td>56</td>
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<td>CCA1</td>
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<td>PRR9</td>
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<td>AT5G02810</td>
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<td>PRR5</td>
<td>AT5G24470</td>
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<td>PRR1/TOC1</td>
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<td>JMJD5</td>
<td>AT5G52660</td>
<td>N/A</td>
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</table>

*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.

<table>
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<tr>
<th>Numbers</th>
<th>no effect</th>
<th>negative</th>
<th>positive</th>
<th>other</th>
<th>total</th>
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<td>confirmed LHY targets</td>
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<td>6</td>
<td>5</td>
<td>69</td>
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<tr>
<td>unconfirmed LHY targets</td>
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<td>0</td>
<td>1</td>
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<td>2</td>
<td>5</td>
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<td>10</td>
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Table 3. GO-term over-representation analysis of high confidence LHY binding targets in *Arabidopsis*.

<table>
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<tr>
<th><strong>Responses to light</strong></th>
<th>Number of genes</th>
<th>Observed Frequency</th>
<th>Number of genes</th>
<th>Expected Frequency</th>
<th>p-value</th>
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</thead>
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<td>8.20%</td>
<td>450</td>
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<td>response to red light</td>
<td>10</td>
<td>1.70%</td>
<td>54</td>
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<td>66</td>
<td>0.30%</td>
<td>0.00533</td>
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<td>response to blue light</td>
<td>8</td>
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<td>response to far red light</td>
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<td>0.00798</td>
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<tr>
<td>circadian rhythm</td>
<td>13</td>
<td>2.20%</td>
<td>48</td>
<td>0.20%</td>
<td>1.01E-06</td>
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</tbody>
</table>

**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.