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Research

Circadian control of abscisic acid biosynthesis and signalling pathways revealed by genome-wide analysis of LHY binding targets

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Summary

- 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 9-*cis*-epoxycarotenoid dioxygenase 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.

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.*, 2008; 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 (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; Pruneda-Paz *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 (Kusakina *et al.*, 2014; Box *et al.*, 2015). 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 (*Glycine max*) and barley (*Hordeum vulgare*) (Habte *et al.*, 2014; Marcolino-Gomes *et al.*, 2014). The clock also ensures that plants respond to environmental stress signals in a manner that is appropriate for the time of the day

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(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.*, 2006; Burschka *et al.*, 1983; McAdam *et al.*, 2011). The expression of multiple ABA biosynthetic enzymes oscillates in *Arabidopsis*, tomato (*Solanum lycopersicum*), maize (*Zea mays*) and sugarcane (*Saccharum officinarum*), 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 signalling pathways and 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 REGULATOR 9*, *7* and *5* (*PRR9*, *-7* and *-5*) and of *PRR1*, also known as *TIMING OF CAB2 EXPRESSION1* (*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 catalysed 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.*, 2002; 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 (SNRK2; Ma *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 signalling 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 LHY directly controls expression of genes associated with ABA biosynthesis and the rhythmic accumulation of this hormone. Furthermore, LHY regulates the expression of ABA signalling 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 Murashige and Skoog (MS)-agar plates in the absence of sucrose and stratified in the dark for 3 d

at 4°C, then grown under 12 h photoperiods at 22°C under 100 μmol m^{-2} s^{-1} white light unless stated otherwise.

Chromatin immunoprecipitation

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 ml of chromatin immunoprecipitation (ChIP) dilution buffer (167 mM sodium chloride, 16.7 mM tris(hydroxymethyl)aminomethane hydrochloride pH 8, 1.2 mM EDTA, Triton X-100, 1 mM phenylmethylsulfonyl fluoride 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 buffer and treated with RNase A at 37°C for 20 min. Sodium dodecyl sulfate 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 sodium chloride 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-seq 1, 35 bp single reads 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 (Illumina Cambridge Ltd, Essex, UK). At least four 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 Supporting Information Table S1. LHY binding regions were then identified as genomic regions that showed over-representation of reads in the wild-type ChIP sample compared with 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 v.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

Sequences of 200 bp were retrieved on either side of the centre 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 wk 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 DNase I (Sigma). First-strand complementary DNA synthesis was carried out using Revert-aid H-Minus M-MuMLV Reverse transcriptase (Thermo Fisher Scientific, Hemel Hempstead, UK) and primed using random DNA hexamers. Quantitative PCR was conducted using a Stratagene MX3005P detection system (Agilent Technology, Cheadle, UK) 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 Princess Margaret Genomics Centre (Toronto, Canada) and analysed using the probe set described as part of Table S5. Transcript expression levels were normalized relative to the constitutively expressed gene *UBC21* (AT5G25760).

Gene Ontology term analyses

The Biomap output of the VIRTUALPLANT software (Katari *et al.*, 2010) was used to identify functional categories that were statistically over-represented within the set of LHY regulatory targets compared with 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 d 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 three nights at 4°C and grown under 12 h : 12 h, light : dark at 22°C. After 10 d, the nylon membranes containing the seedlings were then transferred to new plates containing varying concentrations of ABA. Plants were photographed at 7 and 10 d, then daily for the remaining 8 d 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 three nights in darkness, plants were grown under 16 h : 8 h, light : dark cycles (100 μmol m^{-2} s^{-1} white light), 70% relative humidity at 22°C. All trays were initially watered every 3 d by soaking in water troughs until the topsoil appeared damp. After 14 d, drought condition trays were no longer watered. After a further 10 d, rosette samples were harvested and flash frozen. Samples were homogenized by adding two chilled 3 mm glass beads (Lenz Laborglas GmbH (Wertheim, Germany)) to each sample before loading into an MM300 Tissue Lyser (Retsch GmbH, Haan, Germany) and shaking for 1 min at 30 Hz. Then, 400 μl of extraction buffer (10% methanol and 1% acetic acid (v/v), Fisher Scientific Optima™ LC–MS-grade components (Fisher Scientific UK Ltd, Loughborough, UK), containing the labelled ABA standard ABA-d_6 (Chiron UK Ltd, Woking, UK)) was added to 10 mg of tissue. Samples were placed on ice for 30 min and then centrifuged at 10 000 ***g*** at 4°C for 10 min. 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. Then, 15 μl of each sample was loaded onto a Xevo TQ-S ultra-performance LC–MS/MS system (Waters UK, Elstree, UK) and analysed by high-performance LC–electrospray ionization-MS/MS. Chromatographic separation was performed using an Acquity C18 column (Waters UK), at 35°C. Machine optimization, 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 carryover of compounds. Extraction blanks were run systematically throughout the sample list to ensure there was no contamination between samples. Data were acquired and analysed using the MassLynx suite (Waters).

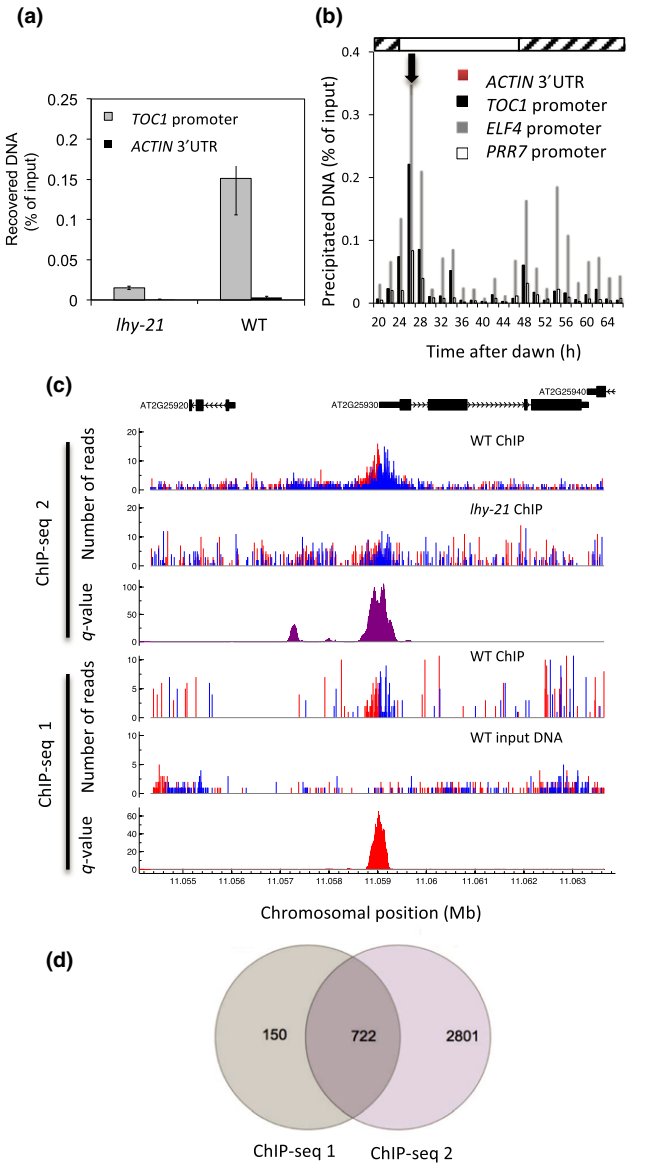


Fig. 1 Genome-wide identification of LATE ELONGATED HYPOCOTYL (LHY) binding sites in *Arabidopsis thaliana*. (a) Quality assessment of chromatin immunoprecipitation (ChIP) samples used for sequencing in ChIP-seq 2. Enrichment for a known target sequence of LHY (*TIMING OF CAB2 EXPRESSION1* (*TOC1*)) was determined by quantitative PCR and compared with a control locus (*ACTIN*). Data are means ± SD from at least five independent experimental replicates for wild-type (WT) and *lhy-21* mutant samples. (b) ChIP-PCR analysis of WT samples harvested at different times of the day. Plants were grown under 12 h : 12 h, light : dark cycles and then transferred to constant light at time zero. White and hatched bars above the chart indicate subjective days and nights respectively. Enrichment for *TOC1*, *EARLY FLOWERING* (*ELF*)*4*, *PSEUDO-RESPONSE REGULATOR 7* (*PRR7*) promoter and *ACTIN* 3′ untranslated region (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 Supporting Information Table S2, because they indicate local over-representation rather than over-representation over the binding region as a whole. Reads mapped to the forward strand are shown in red, and those to the reverse strand are 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.

Table 1 Binding of LATE ELONGATED HYPOCOTYL (LHY) to the promoters of circadian clock-associated genes in *Arabidopsis*.

		$-\log_{10}(q \text{ value})$*	
Gene name	Gene ID	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

N/A, this gene was not identified as a binding target in this experiment. *When multiple peaks were present upstream of a gene, *q* values given correspond to the most significant.

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.

Results and discussion

Genome-wide identification of LHY binding regions

We used 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, compared with *lhy-21* mutant extracts (Fig. 1a). Samples for sequencing were harvested from plants that were grown under 12 h : 12 h, light : dark cycles for 10 d and then transferred to constant light. Tissue was collected 26 h 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*

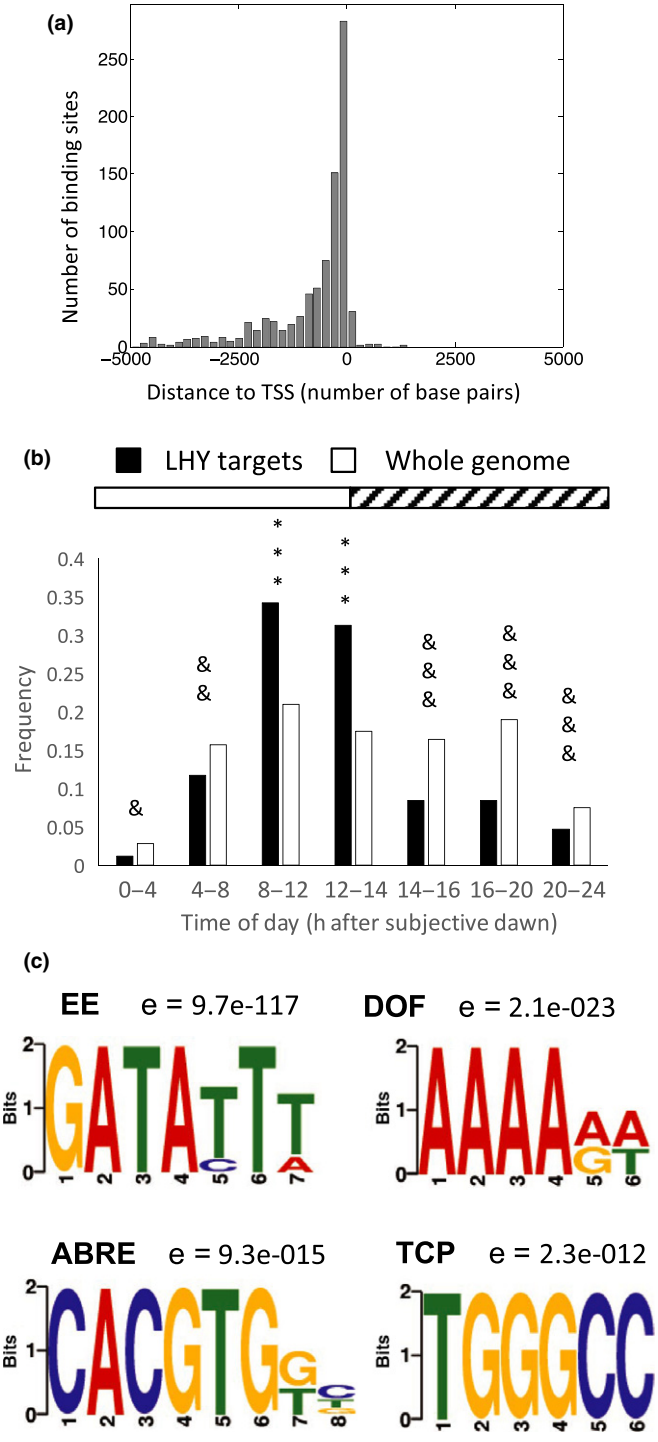


Fig. 2 Characterization of LATE ELONGATED HYPOCOTYL (LHY) binding sites in *Arabidopsis*. (a) Position of 1000 highest ranking peaks in ChIP-seq 2 relative to transcriptional start sites. TSS, transcriptional start site. (b) Histogram showing the proportion of rhythmic LHY binding targets that peak at different phases of the circadian cycle compared with 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 underrepresentation respectively relative to the genome-wide set of rhythmic genes, determined using a hypergeometric test (&, $P < 0.05$; &&, $P < 0.01$; &&&, $P < 0.001$). (c) Motifs identified from the 1000 highest ranking peaks in ChIP-seq 2. Sequences are shown as positional weight matrices, where the height of each letter represents the probability of having the corresponding base at that position. EE, evening element; ABRE, abscisic acid regulated element.

and *PRR7* promoter sequences (Fig. 1b, arrow). Two experiments were carried out. The first (ChIP-seq 1) compared wild-type (Col) ChIP samples with wild-type input DNA. The second (ChIP-seq 2) compared 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. 1(c), and for other clock-related loci in Fig. S1). For example, *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*, *COLD-BINDING FACTOR* (*CBF*)*1* 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. A total of 722 loci were identified in both sets and are hereafter 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.

Table 2 Regulatory function of LATE ELONGATED HYPOCOTYL (LHY) binding interactions in *Arabidopsis*.

	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

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 LHY targets' indicates genes that were identified in only one of the two chromatin immunoprecipitation sequencing experiments. 'Positive' and 'negative' respectively 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 3 Gene Ontology term over-representation analysis of high-confidence LATE ELONGATED HYPOCOTYL (LHY) binding targets in *Arabidopsis*.

	No. of genes	Observed frequency (%)	No. of genes	Expected frequency (%)	*P*-value
Responses to light					
Response to light stimulus	48	8.20	450	2.30	7.15×10^{-11}
Response to red light	10	1.70	54	0.30	0.000385
Response to ultraviolet light	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	1.01×10^{-6}
Biotic and abiotic stress responses					
Response to cold	34	5.80	264	1.30	1.21×10^{-9}
Heat acclimation	5	0.90	14	0.10	0.00328
Response to water deprivation	25	4.30	196	1	5.52×10^{-7}
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	3.32×10^{-5}
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

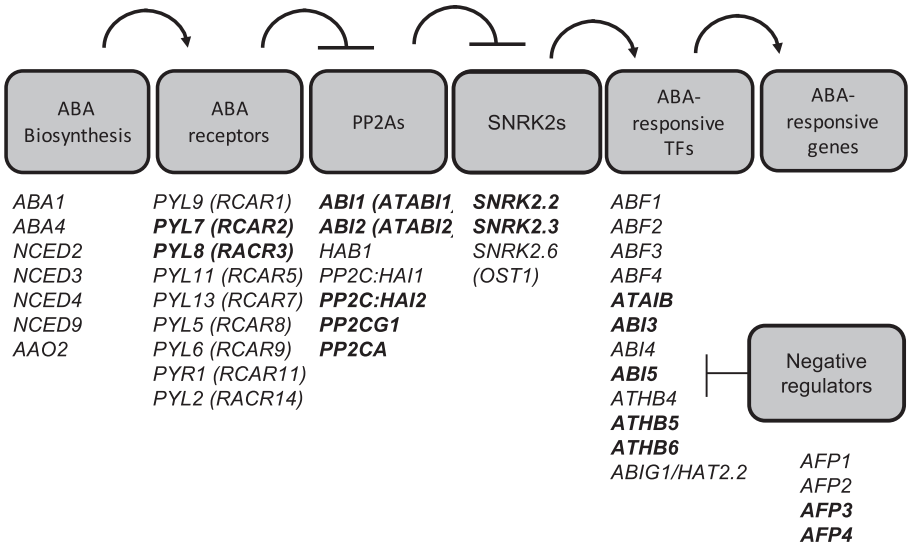


Fig. 3 Binding of LATE ELONGATED HYPOCOTYL (LHY) to components of abscisic acid (ABA) biosynthesis and signalling pathways. The diagram illustrates the mechanism underlying transcriptional responses to ABA in *Arabidopsis*. Pointed and blunt arrows indicate activatory and inhibitory interactions respectively. Expression of ABA-responsive genes is driven by a number of ABA-responsive transcription factors, which are activated by phosphorylation by SNF1-related kinases 2 (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 chromatin immunoprecipitation (ChIP) sequencing experiment, and bold fonts indicate binding confirmed either by ChIP-seq or by ChIP-PCR. Corresponding data are provided in Supporting Information Table S7 and Figs S3 and S4.

Characterization 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 of a gene (Fig. 2a). Of those, 90% were located upstream of the transcriptional start site 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, compared with 23% genome-wide (Table S3). Genes that peaked in the evening (from 8 h until 14 h after subjective dawn) were over-represented, and genes expressed at other times of the day were underrepresented 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 nonrhythmic processes.

A *de novo* search for short-sequence motifs that were significantly over-represented within LHY binding regions identified the 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 ABA 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). One hundred and fifty genes were common to all three 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). Although the EE motif was highly over-represented in both sets of promoters, the ABRE motif was only over-represented in LHY-specific target promoters. There were 177 matches to the ABRE 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.

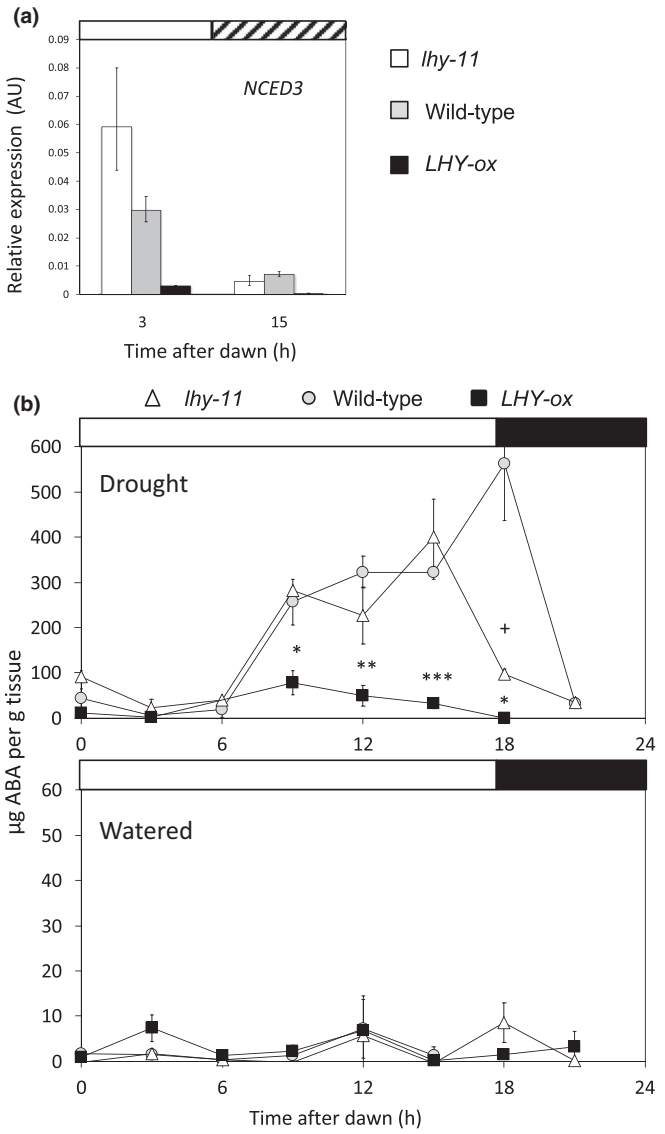


Fig. 4 LATE ELONGATED HYPOCOTYL (LHY) regulates abscisic acid (ABA) accumulation. (a) *Nine-cis-epoxycarotenoid dioxygenase 3* (*NCED3*) transcript levels in wild-type, *lhy-11* and *LHY-ox* seedlings. *Arabidopsis* plants were grown for 7 d under 12 h : 12 h, light : dark cycles on Murashige and Skoog agar plates before transfer to constant light. Tissue was harvested either 3 or 15 h after dawn. Transcript levels were determined by quantitative reverse transcription 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 randomized configuration on soil and entrained to 16 h : 8 h, light : dark cycles. Plants received water every third day for the first 14 d, then watering was withheld entirely from the drought set for the next 10 d. Rosette samples were then harvested at 3 h intervals across a 24 h period for ABA quantification. Data represent the mean from technical triplicates for a pooled sample of two biological replicates. White and black bars above the chart indicate days and nights respectively. Error bars indicate ± SE. * and + indicate *P*-values from *t*-tests comparing *LHY-ox* and *lhy-11* with the wild-type respectively (* and +, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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 to 23), as well as control, nontarget loci. As we expected that responses to LHY induction might be time-of-day dependent, the experiment was repeated at 4 h intervals over the duration of the circadian cycle. Results are summarized in Table 2, and the full dataset is available as Table S5. Of the confirmed regulatory targets, 72% (50 out 69) were repressed in response to *ALCpro::LHY* induction, showing that LHY functions primarily as an inhibitor of transcription. Fifteen 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 characterization 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 (Tables 3, 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* (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 *CBF1*, *-2*, *-3*, *-4* and *COLD-REGULATED 27*, which play key roles in responses to low temperatures (Gilmour *et al.*, 1998; Mikkelsen & Thomashow, 2009), *DEHYDRATION RESPONSIVE ELEM*

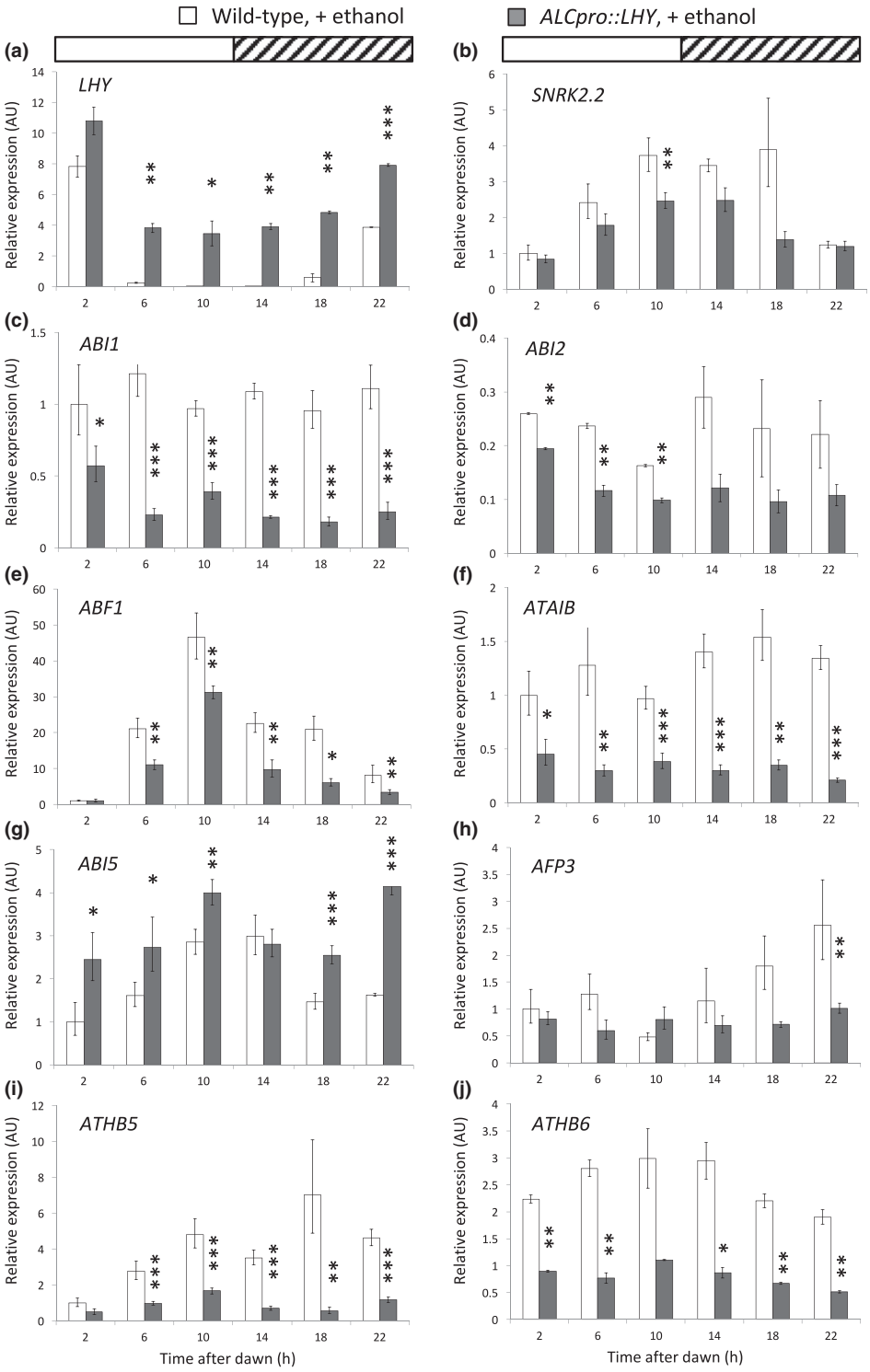
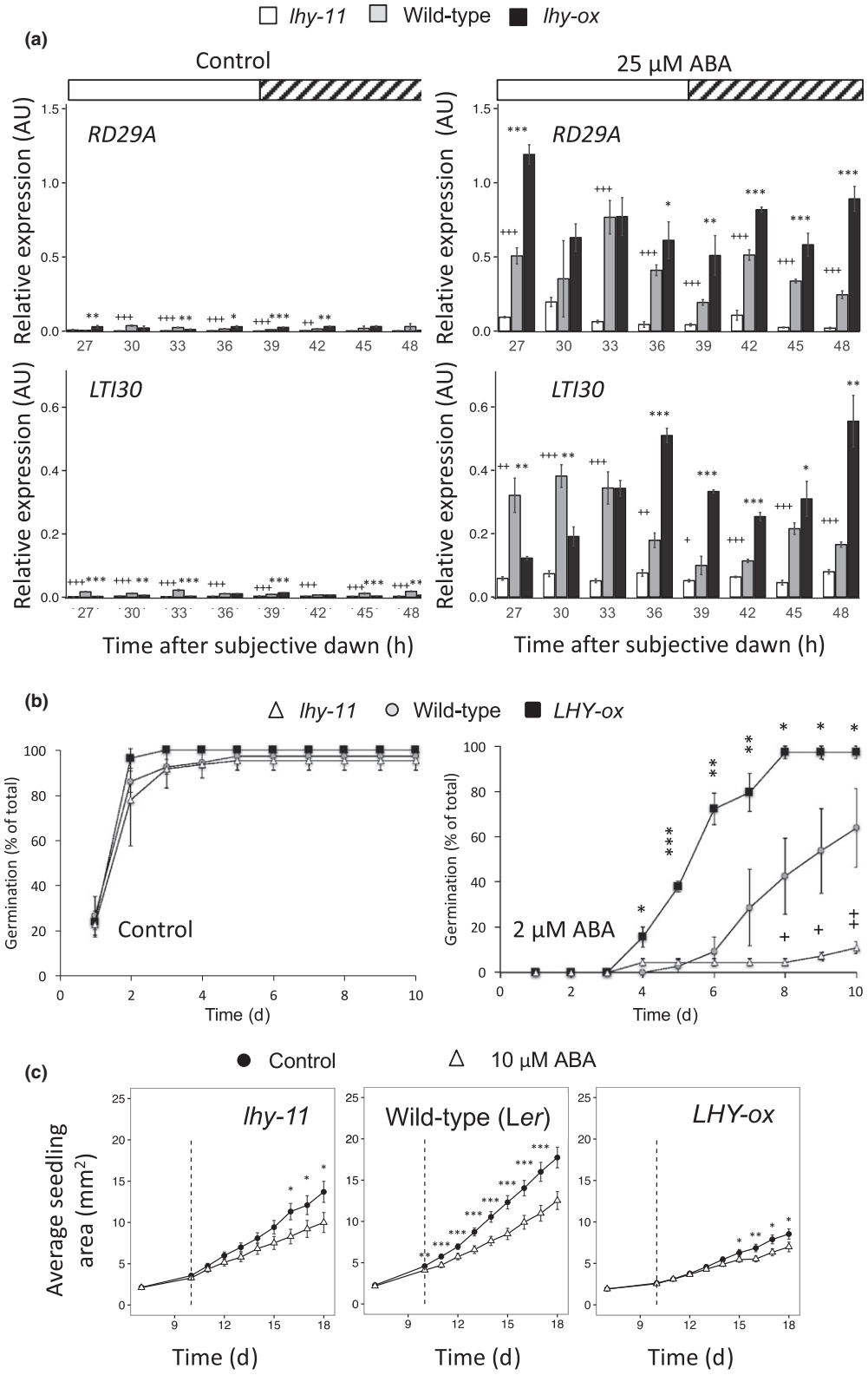


Fig. 5 Induction of *LATE ELONGATED HYPOCOTYL* (*LHY*) expression from the *ALCpro::LHY* transgene results in altered expression of multiple components of abscisic acid (ABA) signalling pathways. Wild-type *Arabidopsis* plants carrying the *ALCpro*::*LHY* transgene were grown under 12 h : 12 h, light : dark cycles and 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 h intervals over the duration of one circadian cycle, and tissue was harvested 2 h later. Messenger RNA 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–h, i). Times indicate when the tissue was harvested. Data from *ALCpro*::*LHY* plants (closed bars) were compared with data from wild-type plants (open bars). Data shown in panels (a, d, j) are means and ± SD from two independent biological replicates. Data shown in other panels are mean and ± SE of technical triplicates for a single experiment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (as determined by *t*-tests).



ENT BINDING 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 *et al.*, 2009; Park

Fig. 6 Misexpression of *LATE ELONGATED HYPOCOTYL* (*LHY*) results in altered responses to abscisic acid (ABA). (a) Induction of *DESSICATION RESPONSIVE PROTEIN 29A* (*RD29A*) and *LOW-TEMPERATURE INDUCED 30* (*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 d and 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 h for RNA extraction. Times indicate when the tissue was harvested. Transcript levels were determined by quantitative PCR and were calculated relative to *ACTIN*. Data represent the mean of technical triplicates for a single experiment, with error bars showing ± SE. Results were consistent across three independent experiments. (b) Germination of wild-type, *lhy-11* and *LHY-ox* seeds in the presence of 2 μM ABA. Data represent the mean percentage of germination from three independent progenies from individual plants, and error bars indicate ± SD. * and + indicate *P*-values from *t*-tests comparing *LHY-ox* and *lhy-11* with 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 12 h : 12 h, light : dark cycles on Murashige and Skoog 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 represent the means from 192 plants across two independent experiments, and error bars indicate ± SD. Asterisks indicate *P*-values from *t*-tests comparing the experimental treatment with the control condition at each time point (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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 signalling 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 (Figs S3, S4), including the protein phosphatases *ABI1* and *ABI2*, which act to repress the pathway in the absence of ABA (Leung *et al.*, 1997; Gosti *et al.*, 1999). 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 *c.* 12 h 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.

Misexpression 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 2 h of ethanol treatment, suggesting that LHY might act to promote ABA responses by relieving the inhibition of the ABA signalling 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-characterized 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* and *ABA-RESPONSIVE PROTEIN* 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 signalling 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 signalling 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. Whereas 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, and *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 compared with 7-d-old seedlings. However, *LHY* overexpression also attenuated the inhibitory effect of ABA on growth in 10-d-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 (Figs S8, S9). Although 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 daytime. 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. SA and JG. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

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.

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See also the Commentary on this article by Belbin & Dodd, **220**: 661–663.