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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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- 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

33 Introduction

34

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

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

56 The plant circadian clock drives the rhythmic expression of many genes involved in abiotic
57 stress responses. About 40% of cold-responsive genes and 50% of heat and drought-responsive
58 genes exhibit circadian rhythmicity in *Arabidopsis* (Bieniawska *et al.*, 2008; Covington *et al.*, 2008;
59 Mizuno & Yamashino, 2008). Rhythmic expression of abiotic stress-responsive genes was also
60 reported in soybean and barley (Habte *et al.*, 2014; Marcolino-Gomes *et al.*, 2014). The clock also
61 ensures that plants respond to environmental stress signal in a manner that is appropriate for the
62 time of the day (a phenomenon known as “gating”). For example, maximal drought-induced
63 changes in gene expression are observed at dusk (Wilkins *et al.*, 2010; Kielbowicz-Matuk *et al.*,
64 2014), and drought or heat treatments given at different times of the day can also result in
65 differential expression of distinct sets of genes (Wilkins *et al.*, 2010; Rienth *et al.*, 2014).

66 The circadian clock also controls the production of the stress-response hormone, abscisic
67 acid (ABA), suggesting that the clock may act to potentiate responses to heat, drought and
68 osmotic stress during the day by controlling the production of this phytohormone (Lee *et al.*, ,
69 Burschka, 1983 #4483; McAdam *et al.*, 2011), The expression of multiple ABA biosynthetic
70 enzymes oscillate in *Arabidopsis*, tomato, maize and sugarcane suggesting rhythmic control at
71 the level of ABA biosynthesis (Thompson *et al.*, 2000; Covington *et al.*, 2008; Michael *et al.*, 2008;
72 Fukushima *et al.*, 2009; Khan *et al.*, 2010; Hotta *et al.*, 2013, Mizuno, 2008 #4477). Multiple
73 components of ABA signaling pathways as well as many ABA responsive transcripts exhibit
74 circadian regulation (Michael *et al.*, 2008; Mizuno & Yamashino, 2008; Seung *et al.*, 2012; Liu *et*
75 *al.*, 2013). ABA also feeds back onto the clock mechanism to influence its function (Hanano *et al.*,
76 2006).

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

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

100 Multiple ABA receptors have been identified (Guo *et al.*, 2011), but downstream signal
101 transduction pathways have only been elucidated for one family of such proteins, known as
102 pyrabactin resistance (PYR)-like (PYL) or regulatory component of ABA receptor (RCAR) (Park
103 *et al.*, 2009). Binding of ABA to PYL/RCAR receptors results in inactivation of the co-receptor, a
104 protein phosphatase 2C (PP2C) and to the activation of a specific group of kinases termed SNF1-
105 related kinases 2 or SNRK2 (Ma, Yue *et al.*, 2009; Park *et al.*, 2009). SNRK2 kinases
106 phosphorylate ABA-responsive transcription factors, which bind ABA-responsive elements
107 (ABREs) in the promoters of ABA-responsive genes to regulate their expression (Fujii *et al.*,
108 2009).

109 Previous work suggested possible mechanisms for the regulation of ABA responses by
110 the central oscillator. The rhythmic production of ABA was proposed to be controlled by the PRR5,
111 7, and 9 proteins, because analysis of a triple mutant (*prr5,7,9*) revealed increased ABA levels
112 (Fukushima *et al.*, 2009). On the other hand, TOC1 was proposed to suppress ABA signaling by
113 inhibiting expression of the ABA-binding protein ABAR (also known as CHLH or GUN5).
114 Consistent with this hypothesis, *TOC1*-overexpressing plants had widely open stomata
115 throughout diel cycles and exhibited increased sensitivity to drought, whereas plants with reduced
116 expression of *TOC1* had the opposite phenotype (Legnaioli *et al.*, 2009). However, the function
117 of ABAR in ABA signalling remains controversial (Hubbard *et al.*, 2010), and the observed effects
118 of TOC1 on ABA responses may be indirect. One potential mechanism would be through
119 regulation of LHY and CCA1 expression, as these proteins are known to potentiate ABA-mediated
120 responses to low temperatures in the morning (Mikkelsen & Thomashow, 2009; Dong *et al.*,
121 2011).

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

131

132 **Materials and Methods:**

133

134 **Plant material and growth conditions**

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

141

142 **Chromatin immunoprecipitation (ChIP)**

143 Tissue cross-linking and chromatin extraction was carried out as described by Gendrel *et al.*
144 (2002). For each immunoprecipitation, 250 μl of chromatin was added to 2 mls of ChIP dilution
145 buffer (167 mM NaCl, 16.7 mM Tris-HCl pH8, 1.2 mM EDTA, Triton X-100, 1 mM PMSF and
146 protease inhibitors) and pre-cleared with protein A Dynabeads (Invitrogen). Samples were
147 incubated overnight at 4°C with anti-LHY antibody (1:200) (Kim *et al.*, 2003). The immuno-
148 complexes were isolated by incubation with protein A Dynabeads for 2 h at 4°C. The beads were
149 washed as described (Haring *et al.*, 2007) with the addition of three extra high salt buffer washes.

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

156

157 **Deep sequencing of ChIP samples**

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

164

165 **Analysis of ChIP-seq data**

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

168 were retained. Results of the alignment are summarized in Table S1. LHY binding regions were
169 then identified as genomic regions that showed over-representation of reads in the wild-type ChIP
170 sample as compared to the input DNA sample (in ChIP-seq 1), or to the *lhy-21* mutant ChIP
171 sample (in ChIP-seq 2). Peak analysis was carried out using the MACS2 software version
172 2.0.10.20120913 (Zhang *et al.*, 2008) following the recommended procedure for analysing ChIP-
173 seq data for transcription factor binding. The parameter determining the number of duplicates
174 retained was set to auto (*-keep-dup*), the q value threshold was set to 0.01 (*-q*), the genome size
175 set to dm (*-g*) and the size of the window for the initial genome scan was set to 200 (*-bw*). Binding
176 regions were assigned to closest gene facing away from them.

177

178 **Motif analyses**

179 200 bp sequences were retrieved on either side of the center of each binding region, and short
180 sequence motifs that were over-represented within these sequences relative to the whole genome
181 were identified using the DREME software in discriminative mode (Bailey, 2011). Control
182 sequences were composed of 1000 random 400 bp regions from each chromosome. Promoters
183 were scanned for matches to sequence motifs using FIMO (Grant *et al.*, 2011) and motif matches
184 to transcription factor binding sites were identified using TOMTOM (Gupta *et al.*, 2007), based on
185 the *Arabidopsis* PBM and DAP motif databases (Franco-Zorrilla *et al.*, 2014; O'Malley *et al.*,
186 2016).

187

188 **Ethanol induction of *ALCpro::LHY* expression**

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

192

193 **Gene expression analyses**

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

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

205

206 **Gene Ontology (GO)-term analyses**

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

210

211 **Germination experiments**

212 Seeds for these experiments were produced from plants that were grown and harvested
213 simultaneously. Seeds were plated onto MS-agar plates containing varying concentrations of ABA
214 or sorbitol, and stratified for 3 days in constant darkness at 4°C. Plates were then transferred to
215 22°C and constant light (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$) conditions and germination was scored daily based on
216 radical emergence.

217

218 **Plant growth experiments in the presence of ABA**

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

224

225 **ABA quantification by mass spectrometry**

226 *Arabidopsis* seeds were sown onto soil in 24 well plastic trays. Following stratification at 4°C for
227 3 nights in darkness, plants were grown under 16L 8D cycles (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light), 70%
228 (RH) at 22°C. All trays were initially watered every 3 days by soaking in water troughs until the
229 topsoil appeared damp. After 14 days, drought condition trays were no longer watered. After a
230 further 10 days, rosette samples were harvested and flash frozen. Samples were homogenised
231 by adding two chilled 3 mm glass beads (Lenz) to each sample before loading into an MM300
232 Tissue Lyser (Retsch) and shaking for 1 minute at 30Hz. 400 μl of extraction buffer, (10% MeOH
233 and 1% acetic acid (v/v), Fisher Scientific OptimaTMLC/MS grade components, containing the
234 labelled ABA standard Abscisic acid-d6 (Chiron)) was added to 10 mg of tissue. Samples were
235 placed on ice for 30 minutes then centrifuged at 10,000 x g at 4°C for 10 minutes. The supernatant

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

248

249 **Results and discussion**

250 **Genome-wide identification of LHY binding regions**

251 We used chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) to
252 identify genome-wide binding regions for the LHY transcription factor. ChIP was carried out using
253 a polyclonal antibody to the full-length LHY protein, which gave highly significant enrichment for
254 the known binding target *TOC1* from wild-type extracts, as compared to *lhy-21* mutant extracts
255 (Fig. 1a). Samples for sequencing were harvested from plants that were grown under 12L 12D
256 cycles for 10 days then transferred to constant light. Tissue was collected 26 hours after the last
257 dark to light transition, corresponding to the peak of LHY protein accumulation (Kim *et al.*, 2003;
258 Adams *et al.*, 2015) and maximum ChIP enrichment for *TOC1*, *ELF4* and *PRR7* promoter
259 sequences (Fig. 1b, arrow). Two experiments were carried out. The first (ChIP-seq 1) comparing
260 wild-type (Col) ChIP samples to wild-type input DNA. The second (*ChIP-seq 2*) comparing wild-
261 type and knock-out mutant (*lhy-21*) samples (Fig. 1c). ChIP-seq 2 effectively controlled for
262 potential cross-reactivity of the antibody with LHY-related proteins, but reduced the sensitivity of
263 detection for a number of known LHY targets, due to residual peaks identified at these locations
264 (as illustrated for *ELF3* in Fig. 1c, and for other clock-related loci in Fig. S1). For example, *FKF1*,
265 *CBF1* and *TOC1* sequences were ranked first, second and third in ChIP-seq 1 based on their q-
266 values for over-representation relative to the control sample, but were ranked 1823, 2998 and
267 4128 in ChIP-seq 2. Nevertheless, we reasoned that sequences that were identified in both
268 experiments would identify high confidence binding targets for LHY.

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

284

285 **Characterisation of LHY binding sites**

286 As expected for a transcription factor, 72% of confirmed LHY binding regions were located within
287 500 bp of the transcriptional start site (TSS) of a gene (Fig. 2a). Of those, 90% were located
288 upstream of the TSS and 10% in the 5'-untranslated region of the gene.

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

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

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

313

314 **Comparison with CCA1 binding targets**

315 LHY and CCA1 are almost identical within their DNA-binding domains and are thought to have
316 largely redundant roles as part of the circadian oscillator (Carré & Kim, 2002; Mizoguchi *et al.*,
317 2002). Comparisons between the set of 722 confirmed LHY targets and the 1306 and 439 high
318 confidence CCA1 binding loci identified by Nagel *et al.* (2015) and Kamioka *et al.* (2016) identified
319 400 and 193 genes in common, respectively (Fig. S2a). 150 genes were common to all 3 datasets.
320 This confirmed that LHY and CCA1 have overlapping sets of binding targets, but also suggested
321 potential differences in specificity. Consistent with this hypothesis, analyses of LHY- and CCA1-
322 specific target promoters identified different over-represented motifs (Fig. S2b). While the EE
323 motif was highly over-represented in both set of promoters, the ABRE motif was only over-
324 represented in LHY-specific target promoters. 177 matches to the ABRE were identified based
325 on $p < 0.0001$ within a test set of 315 LHY-specific targets, but only 68 were identified within the
326 same number of CCA1-specific promoters (Fig. S2d). The most closely related over-represented
327 motif within CCA1-specific targets was A(C/T)ACGT. Comparison with known transcription factor
328 binding motifs identified matches to two NAC transcription factor binding sites, ATAF1 and NAC55
329 (Franco-Zorrilla *et al.*, 2014; O'Malley *et al.*, 2016). These results suggest that LHY may have a
330 specific role to regulate ABA responses through interaction with ABA-responsive transcription
331 factors.

332

333 **Confirmation of regulatory interactions**

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

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

348

349 **Functional characterisation of LHY binding targets**

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

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

371 2002; Lopez-Molina *et al.*, 2002) and the negative regulator of *ABI5* function, *AFP3* (Lopez-Molina
372 *et al.*, 2003). Further elements of ABA signaling pathways and several enzymes involved in the
373 ABA biosynthesis pathways were identified in only one ChIP-seq experiment. Several of these
374 genomic targets were confirmed in ChIP-PCR experiments and in-vitro genomic DNA pull-down
375 experiments (Fig. S3 and S4), including the protein phosphatases *ABI1* and *ABI2*, which act to
376 repress the pathway in the absence of ABA (Leung *et al.*; Gosti *et al.*). We therefore investigated
377 the effect of LHY on expression of these binding targets, as well as on ABA accumulation and
378 downstream responses.

379 380 **LHY inhibits ABA biosynthesis**

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

391 392 **Mis-expression of LHY results in altered responses to exogenous ABA**

393 The expression of multiple components of ABA signal transduction pathways was altered
394 following *AlcPro::LHY* induction (Fig. 5). Expression of the negative regulators of ABA responses,
395 *ABI1* and *ABI2* was reduced relative to control plants within two hours of ethanol treatment,
396 suggesting that LHY might act to promote ABA responses by relieving the inhibition of the of ABA
397 signaling pathway. However, this hypothesis was contradicted by the repression of a number of
398 positive regulators of ABA responses, including the *SNRK2.2* kinase and the ABA responsive
399 transcription factors *ABF1*, *ATA1B*, *ATHB5* and *ATHB6*, and the induction of a negative regulator,
400 *AFP3*.

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

405 2015). Expression of both genes was induced 4 h after spraying plants with 10 μ M ABA (Fig. 6a).
406 This induction was suppressed in *lhy-11* plants and enhanced in the subjective night in *LHY-ox*
407 plants, indicating that LHY acts to promote these ABA responses. Responses to osmotic stress,
408 which induce the production of endogenous ABA, were consistent with these findings. *LHY-ox*
409 plants exhibited elevated expression of ABA-responsive genes *RD29A*, *LT130*, *LATE*
410 *EMBRYOGENESIS ABUNDANT (LEA)* and *ABA-RESPONSIVE PROTEIN (ABR)* in the
411 presence of 100 mM sorbitol (Fig. S5), suggesting that LHY also acts under physiologically-
412 relevant conditions to potentiate this ABA-dependent stress response. As none of these genes
413 was identified as a genomic target for LHY in ChIP-seq experiments, and *RD29A* expression was
414 slightly inhibited, rather than induced, in response to induction of the *ALCpro::LHY* transgene (Fig.
415 S6), sensitization of these genes to exogenous ABA and to sorbitol is likely to result from
416 enhanced signaling through the core ABA response pathway. LHY inhibits the expression of the
417 *ABI1* and *ABI2* protein phosphatases, which function as regulatory subunits of the ABA receptors
418 (PYR/PYLs) and repress downstream responses in the absence of ABA. We propose that
419 repression of *ABI1* and *ABI2* transcription by LHY ensures high amplitude induction of *RD29A*
420 and *LT130* transcription, by lowering the threshold for activation of the signaling pathway by ABA.

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

431 The observation that *LHY* potentiates the effect of ABA on *RD29A* and *LT130* expression
432 but antagonizes its effect on germination may reflect the different stages of development at which
433 these experiments were carried out. *LHY* may affect ABA responses differently in seeds as
434 compared to 7-day old seedlings. However, *LHY*-overexpression also attenuated the inhibitory
435 effect of ABA on growth in 10-day old plants (Fig. 6c). Similar results were obtained when plants
436 were exposed to salt or to drought conditions, which induce the production of endogenous ABA
437 (Fig. S8 and S9). While the smaller surface area of *LHY-ox* rosettes may contribute to their

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

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

451
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459 **Author contributions.** SA and JG performed experimental research; SRV, NPD and IAC carried
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462 **Accession numbers:**

463 ChIP-seq 1 and ChIP-seq 2 datasets were deposited on the Gene Expression Omnibus database
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774

775 **Fig. legends**

776 **Fig. 1. Genome-wide identification of LHY binding sites in *Arabidopsis thaliana*.** (a) Quality
777 assessment of ChIP samples used for sequencing in ChIP-seq 2. Enrichment for a known target
778 sequence of LHY (*TOC1*) was determined by quantitative PCR and compared to a control locus
779 (*ACTIN*). Data are means and standard deviations at least from 5 independent experimental
780 replicates for wild-type (Ws) and *lhy-21* mutant samples, respectively. (b) ChIP-PCR analysis of
781 wild-type samples harvested at different times of the day. Plants were grown under 12L12D cycles
782 then transferred to constant light at time zero. White and hatched bars above the chart indicate
783 subjective days and nights, respectively. Enrichment for *TOC1*, *ELF4*, *PRR7* promoter and *ACTIN*
784 3'UTR sequences was determined relative to input DNA samples (c) Comparison of results from
785 both ChIP-seq experiments at the *ELF3* locus (ATG29530). Note that the q-values reported here
786 are distinct from those reported in Table 1 and Table S2, because they indicate local
787 overrepresentation rather than overrepresentation over the binding region as a whole. Reads
788 mapped to the forward strand are shown in red, those to the reverse strand in blue. ChIP-seq 2
789 results for other clock-related loci are shown in Fig. S1. (d) Comparison of binding targets
790 identified in ChIP-seq 1 and ChIP-seq 2, based on q-value thresholds of 10^{-10} and 10^{-20} ,
791 respectively.

792
793 **Fig. 2: Characterisation of LHY binding sites in *Arabidopsis*.** (a) Position of 1000 highest
794 ranking peaks in ChIP-seq 2 relative to transcriptional start sites. (b) Histogram showing the
795 proportion of rhythmic LHY binding targets that peak at different phases of the circadian cycle as
796 compared to the genome-wide set of rhythmically expressed genes. Data for confirmed LHY
797 binding targets were retrieved from the Diurnal database (Mockler *et al.*, 2007), using the constant
798 light, LL23 dataset (Edwards *et al.*, 2006) and a correlation coefficient cut-off of 0.8. White and
799 hatched bars above the chart indicate subjective days and nights, respectively, and * and &
800 indicate p values for over- and under-representation, respectively relative to the genome-wide set
801 of rhythmic genes, determined using a hypergeometric test (* and &, $p < 0.05$; ** and &&, $p < 0.01$;
802 *** and &&& $p < 0.001$). (c) Motifs identified from the 1000 highest ranking peaks in ChIP-seq 2.
803 Sequences are shown as positional weight matrices (PWMs) where the height of each letter
804 represents the probability of having the corresponding base at that position.

805

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

818
819 **Fig. 4. LHY regulates ABA accumulation. (a)** *NCED3* transcript levels in wild-type, *lhy-11* and
820 *LHY-ox* seedlings (grey, white and black bars, respectively). *Arabidopsis* plants were grown for 7
821 days under 12L12D cycles on MS-agar plates before transfer to constant light. Tissue was
822 harvested either 3 or 15 hours after dawn. Transcript levels were determined by quantitative RT-
823 PCR and expressed relative to ACTIN. **(b)** Overexpression of *LHY* results in reduced ABA levels
824 under drought conditions. *lhy-11*, *LHY-ox* and wild-type seedlings were grown in a randomised
825 configuration on soil and entrained to 16L8D cycles. Plants received water every third day for the
826 first 14 days, then watering was withheld entirely from the drought set for the next 10 days.
827 Rosette samples were then harvested at 3-hour intervals across a 24-hour period for ABA
828 quantification. Data represents the mean from technical triplicates for a pooled sample of 2
829 biological replicates. White and black bars above the chart indicate days and nights, respectively.
830 Error bars indicate standard errors. * and + indicate p-values from t-tests comparing *LHY-ox* and
831 *lhy-11* to the wild type, respectively (* and +, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

832
833 **Fig. 5. Induction of LHY expression from the *ALCpro::LHY* transgene results in altered**
834 **expression of multiple components of ABA signaling pathways.** Wild-type *Arabidopsis*
835 plants carrying the *ALCpro::LHY* transgene were grown under 12L12D light-dark cycles then
836 transferred to constant light at the start of the experiment. Expression of *ALCpro::LHY* was
837 induced using 6% ethanol (v/v). Different sets of plants were treated at 4-hour intervals over the
838 duration of one circadian cycle, and tissue was harvested 2 hours later. mRNA levels were
839 determined either using Nanostring technology and normalized relative to *UBC12* (a,d,j) or by

840 quantitative PCR and normalized to *ACTIN* (b,c,e,f,g,h). Times indicate when the tissue was
841 harvested. Data from *ALCpro::LHY* plants (filled bars) were compared to data from wild-type
842 plants (white bars). Data shown in panels a, d, j are means and standard deviations from two
843 independent biological replicates. Data shown in other panels are mean and standard errors of
844 technical triplicates for a single experiment. * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as
845 determined by t-tests.

846

847 **Fig. 6. Mis-expression of *LHY* results in altered responses to ABA. (a)** Induction of *RD29A*
848 and *LT130* expression by ABA in wild-type, *lhy-11* and *LHY-ox* plants (grey, white and black bars,
849 respectively). *Arabidopsis* plants were grown under light-dark cycles for 7 days then transferred
850 to constant light at time zero. At each time point a set of plants was sprayed with 25 μM ABA or
851 vehicle (methanol) and tissue was harvested after 3 hours for RNA extraction. Times indicate
852 when the tissue was harvested. Transcript levels were determined by quantitative PCR and were
853 calculated relative to *ACTIN*. Data represents the mean of technical triplicates for a single
854 experiment, with error bars showing standard errors. Results were consistent across 3
855 independent experiments. **(b)** Germination of wild-type, *lhy-11* and *LHY-ox* seeds (grey, white
856 and black symbols, respectively) in the presence of 2 μM ABA. Data represents the mean
857 percentage of germination from 3 independent progenies from individual plants and error bars
858 indicate standard deviations. * and + indicate p-values from t-tests comparing *LHY-ox* and *lhy-11*
859 to the wild type, respectively (* and +, $p < 0.05$; **and ++, $p < 0.01$; ***and +++, $p < 0.001$). **(c)** Effect
860 of exogenous ABA on seedling growth. Seedlings were grown under 12L 12D cycles on MS-agar
861 plants. At the time indicated by the vertical dashed line, plants were transferred to fresh plates
862 with or without ABA (10 μM). Aerial photographs were taken daily for rosette size measurements.
863 Data represents the means from 192 plants across 2 independent experiments, and error bars
864 indicate standard deviations. Asterisks indicate p-values from t-tests comparing the experimental
865 treatment to the control condition at each time point (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

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

Gene name	Gene ID	-log ₁₀ (q-values)*	
		ChIP-seq 1	ChIP-seq 2
<i>LHY</i>	AT1G01060	29	56
<i>CCA1</i>	AT2G46830	61	14
<i>PRR9</i>	AT2G46790	93	81
<i>PRR7</i>	AT5G02810	99	35
<i>PRR5</i>	AT5G24470	78	64
<i>PRR1/TOC1</i>	AT5G61380	182	15
<i>LUX/PCL1</i>	AT3G46640	N/A	67
<i>BOA/NOX</i>	AT5G59570	127	85
<i>ELF3</i>	AT2G25930	63	91
<i>ELF4</i>	AT2G40080	107	31
<i>GI</i>	AT1G22770	162	47
<i>RVE6</i>	AT5G52660	N/A	30
<i>LNK1</i>	AT5G64170	N/A	13
<i>LNK2</i>	AT3G54500	27	24
<i>CHE/TCP21</i>	AT5G08330	77	135
<i>LWD2</i>	AT3G26640	N/A	41
<i>FKF1</i>	AT1G68050	260	38
<i>CDF1</i>	AT5G62430	22	102
<i>CDF2</i>	AT5G39660	N/A	128
<i>CKB4</i>	AT5G52660	N/A	30
<i>JMJD5</i>	AT5G52660	N/A	30

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

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

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

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

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

Supplementary information

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

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

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

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

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

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

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

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

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

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

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

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

Table S4. Phase distribution of confirmed LHY binding targets.

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

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

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