Field microenvironments regulate crop diel transcript and metabolite rhythms

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Summary

- Most research in plant chronobiology was done in laboratory conditions. However, they usually fail to mimic natural conditions and their slight fluctuations, highlighting or obfuscating rhythmicity. High-density crops, such as sugarcane (*Saccharum* hybrid), generate field microenvironments with specific light and temperature due to mutual shading.
- We measured the metabolic and transcriptional rhythms in the leaves of 4-month-old (4 mo) and 9 mo sugarcane grown in the field. Most of the assayed rhythms in 9 mo sugarcane peaked >1 h later than in 4 mo sugarcane, including rhythms of the circadian clock gene, *LATE ELONGATED HYPOCOTYL* (*LHY*).
- We hypothesized that older sugarcane perceives dawn later than younger sugarcane due to self-shading. As a test, we measured *LHY* rhythms in plants on the east and the west side of a field. We also tested if a wooden wall built between lines of sugarcane changed their rhythms. The *LHY* peak was delayed in the plants in the west of the field or beyond the wall; both shaded at dawn.
- We conclude that plants in the same field may have different phases due to field microenvironments, impacting important agronomical traits, such as flowering time, stalk weight and number.

Keywords: circadian clock, crops, field experiments, microenvironments, *Saccharum* hybrid, shading, sugarcane

Introduction

Plants are sessile organisms living in constantly changing environments. Some of those changes are rhythmic due to the movements of the tilted Earth around the sun, bringing seasons, and around itself, bringing day and night. The circadian oscillator is an adaptation for life in rhythmic environments. It is an internal regulatory network that allows plants to track the time of the day by generating responses on both metabolism and physiology levels. The ability to anticipate the rhythmic changes in the environment increases plants fitness (Green *et al.*, 2002; Dodd *et al.*, 2005). Plants that cannot keep their rhythms desynchronize with the environment and assimilate less carbon (C), accumulate less biomass and have lower water use efficiency (Dodd *et al.*, 2005).

The circadian oscillator network comprises a central oscillator, input pathways and output pathways. The central oscillator generates rhythms independently of environmental cues (zeitgebers), such as light and temperature. Input pathways continuously feed the central oscillator with internal and external information, synchronizing it with environmental rhythms (Webb *et al.*, 2019). Output pathways gather temporal information from the interactions between the central oscillator and the input pathways and translate it into timely regulated metabolic and physiologic responses. The plant central oscillator includes several interlocked feedback loops based on the regulation of transcription and translation. For example, dawn is marked by an increase in transcripts of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*, not found in monocots), *LATE ELONGATED HYPOCOTYL* (*LHY*) and *REVEILLE 8* (*RVE8*) expression (Alabadí *et al.*, 2001; Rawat *et al.*, 2011; Gray *et al.*, 2017), which in turn regulate and are regulated by the expression of members of the PSEUDO RESPONSE REGULATOR (PRR) gene family during the day (Nakamichi *et al.*, 2010; Huang *et al.*, 2012). In monocots, this includes *PRR1*, *PRR37*, *PRR59*, *PRR73*, and *PRR95* (Hotta *et al.*, 2013; Calixto *et al.*, 2015; Dantas *et al.*, 2020). As LHY expression declines during the day, the expression of PRR1, usually called TIMING OF CAB

61 EXPRESSION (TOC1), increases, leading to a peak near dusk (Alabadí *et al.*, 2001). In
62 *Arabidopsis thaliana* (L.) Heynh., TOC1 degradation is regulated by an interplay between
63 ZEITLUPE (AtZTL) and GIGANTEA (AtGI) (Kim *et al.*, 2007; Cha *et al.*, 2017). In eudicots, a
64 protein complex called EVENING COMPLEX (EC) is composed of LUX ARRHTHMO (LUX),
65 EARLY FLOWERING 3 and 4 (ELF3 and ELF4) and is assembled during the night, repressing
66 many other central oscillator genes (Herrero *et al.*, 2012). The EC still needs to be confirmed in
67 monocots, even though *ELF3* is present and functional (Zhao *et al.*, 2012; Huang *et al.*, 2017).

68 Experiments done in laboratory conditions frequently fail to fully mimic natural conditions,
69 especially their rhythms (Annunziata *et al.*, 2017; Song *et al.*, 2018). For example, in rice (*Oryza*
70 *sativa* L.), mutations in homolog *OsGI* lead to a delay in flowering in short days and long days in
71 laboratory conditions. However, this phenotype was not observed in long days in the field (Izawa
72 *et al.*, 2011). In *Arabidopsis*, flowering under artificial conditions does not fully mimic flowering
73 in nature due to the light quality and spectra and the temperature gradients found in natural
74 conditions (Song *et al.*, 2018). *Arabidopsis* that was grown under artificial light also had different
75 metabolic profiles than plants grown under natural light (Annunziata *et al.*, 2017). Rhythms in the
76 transcription of plants grown in natural conditions or the field are regulated by the circadian
77 oscillator and by changes in external conditions, such as temperature, light intensity, humidity,
78 and photoperiod, or internal conditions, such as plant age and plant physiology (Nagano *et al.*,
79 2012, 2019; Matsuzaki *et al.*, 2015; Panter *et al.*, 2019; Dantas *et al.*, 2020). A better
80 understanding of plant rhythms in the field is essential to translate this knowledge to agriculture
81 (Steed *et al.*, 2021).

82 Here we have measured metabolic and transcriptional rhythms in sugarcane leaves grown in the
83 field. We show that transcript and metabolite rhythms in 4-months-old (4 mo) sugarcane have
84 earlier peaks in the day than 9 mo sugarcane, with phase shifts ranging from 2 h to 12 h. These

phase shifts are correlated with changes in the peak of expression of circadian oscillator genes. Such variation in the timing of the peaks was confirmed by experiments done on plants grown on the east and west sides of the field or on plants that were separated by a wooden wall. We conclude that external rhythms in field microenvironments regulate metabolite rhythms and transcripts associated with the sugarcane circadian oscillator.

Materials and Methods

Field conditions, plant growth, and harvesting

All three assayed sugarcane fields were located in the same area, at the Federal University of São Carlos, campus Araras, in São Paulo state, Brazil (22°21′25″ S, 47°23′3″ W; altitude of 611 m). The soil was classified as a Typic Eutroferric Red Latosol. Sugarcane from the commercial variety SP80 3280 (*Saccharum* hybrid) was used in all experiments. The environmental conditions for the three experiments were collected from a local weather station or from within the field using a handheld lux meter and a thermometer at the height of the leaves (Fig. S1). Light intensity was measured with the sensor parallel to the ground. Plants were sampled every 2 h for 26 h, starting 1.5 h before dawn. The leaves +1 of nine sugarcane individuals were harvested and separated into three pools for all experiments, each with three individuals, and each pool was considered a biological replicate. The leaves +1 are the first fully photosynthetically active leaf in sugarcane. Each sampling took less than 30 min, and the harvested tissue was immediately frozen in liquid nitrogen. In the first experiment, sugarcane was planted in April/2012 and sampled in August/2012 (4-months-old), during winter, and in January/2013 (9-months-old), during summer. In the winter harvesting, dawn was at 6:30, and dusk was 18:00 (11.5 h day/12.5 h night). In summer harvesting, dawn was at 5:45, and dusk was 19:00 (13.3 h day/10.7 h night). For the second experiment (east and west margins), plants were planted in October/2014 and harvested in

March/2015 (5 months old) (12.0 h day/12.0 h night). In the third experiment, plants (the wall experiment) were also planted in October/2014 but harvested in April/2015 (6 months old) (11.3 h day/12.7 h night). The orientation of the sugarcane was north to south. In the wall experiment, a 2 m high wall made of plywood sheets was built between the first and the second rows on the east side of the field two days before harvest day. The time of harvesting was normalized to a photoperiod of 12 h day/ 12 h night using the following equations to compare the rhythms of samples harvested in different seasons: for times during the daytime, ZT = 12*T*Pd^(-1), where ZT is the normalized time, T is the time from dawn (in hours), and Pd is the length of the day (in hours); for times during the nighttime, ZT = 12 + 12*(T - Pd)*Pn^(-1), where ZT is the normalized time, T is the time from dawn (in hours), Pd is the length of the day (in hours), and Pn is the length of the night (in hours).

Metabolome

Metabolites were extracted from all 3 biological replicates. First, 50 mg of the grounded tissue was used for MTBE: methanol: water 3:1:1 (v/v/v) extraction, as described previously (Giavalisco *et al.*, 2011). Next, the 100 µl of the organic phase was dried and derivatized as previously described (Roessner *et al.*, 2001). Finally, 1 µl of the derivatized samples were analyzed on a Combi-PAL autosampler (Agilent Technologies, St. Clara, USA) coupled to an Agilent 7890 gas chromatograph coupled to a Leco Pegasus 2 time-of-flight mass spectrometer (LECO, St. Joseph, MI, USA) in both split (1:40) and splitless modes (Weckwerth *et al.*, 2004; Ferreira *et al.*, 2018). Chromatograms were exported from Leco ChromaTOF software (version 3.25) to R-3.1.x (R Core Team, 2021). Peak detection, retention time alignment, and library matching were performed using *Target Search R-package* (Cuadros-Inostroza *et al.*, 2009). Metabolites were quantified by the peak intensity of a selective mass. Metabolites intensities were

133 normalized by dividing the fresh-weight, followed by the sum of total ion count and global outlier
134 replacement as described previously (Giavalisco *et al.*, 2011). Each metabolite value was further
135 normalized to Z-score. The principal component analysis was performed using *pcaMethods*
136 Bioconductor R package. Data ellipses were drawn for the samples harvested during the day and
137 the night (0.85 confidence level).

138

139 *Oligoarray hybridizations*

140 Oligoarrays were hybridized as described before (Hotta *et al.*, 2013; Dantas *et al.*, 2020). Briefly,
141 frozen samples were pulverized in dry, and 100 mg were used for total RNA extraction using
142 Trizol (Life Technologies, Carlsbad, USA). The RNA was treated with DNase I (Life
143 Technologies, Carlsbad, USA) and cleaned using the RNeasy Plant Mini kit (Qiagen, Hilden,
144 Germany) following the supplier's recommendations. The total RNA quality was assayed using
145 an Agilent RNA 6000 Nano Kit Bioanalyzer chip (Agilent Technologies, St. Clara, USA).
146 Labelling was done following the Low Input Quick Amp Labelling protocol of the Two-Color
147 Microarray-Based Gene Expression Analysis system (Agilent Technologies, St. Clara, USA).
148 Hybridizations were done using a custom 4×44 k oligoarray (Agilent Technologies, St. Clara,
149 USA) previously described (Lembke *et al.*, 2012; Hotta *et al.*, 2013). Two hybridizations were
150 done for each time point against an equimolar pool of all samples of each organ. Each duplicate
151 was prepared independently using dye swaps. Data were extracted using the Feature Extraction
152 software (Agilent Technologies, St. Clara, USA). Background correction was applied to each
153 dataset. A nonlinear LOWESS normalization was also applied to the datasets to minimize
154 variations due to experimental manipulation. Signals that were distinguishable from the local
155 background signal were taken as an indication that the corresponding transcript was expressed.
156 The GenBank IDs of all sugarcane genes mentioned here can be found in the Supplemental

157 Material (Table S1). The complete dataset can be found at the Gene Expression Omnibus public
158 database under the accession number GSE129543 and GSE171222.

159

160 *Data analysis*

161 For further analysis, only expressed transcripts in more than 6 of the 12 time points were
162 considered to be expressed. Identification of rhythmic transcripts was made using an algorithm
163 described in previous work (Dantas *et al.*, 2020). Analyses were done in R-3.1.x (R Core Team,
164 2021). All the time series from expressed transcripts were grouped in co-expressed modules using
165 the R package *weighted correlation network analysis* (*WGCNA*) (Langfelder & Horvath, 2008)
166 with the same parameters as before (Dantas *et al.*, 2020). Co-expression modules were considered
167 rhythmic or non-rhythmic using JTK-CYCLE (P-value of < 0.75) (Hughes *et al.*, 2010). LOESS
168 (locally estimated scatterplot smoothing) regression was used to establish the timing of the peak,
169 or the phase, of each rhythmic time series (Dantas *et al.*, 2019). As controls, we also analyzed
170 rhythms using only JTK-Cycle (P-value of < 0.05) (Hughes *et al.*, 2010) and identified only
171 transcripts with significant gene expression profile differences using the R package *maSigPro*
172 (Nueda *et al.*, 2014). Euler diagrams were done using the R package *eulerr*. Code to fully
173 reproduce our analysis is available on GitHub (https://github.com/LabHotta/Microenvironments)
174 and archived on Zenodo (http://doi.org/10.5281/zenodo.4645464).

175

176 *RT-qPCR analysis*

177 Frozen leaf samples were pulverized on dry ice using a coffee grinder (Model DCG-20, Cuisinart,
178 Stamford, USA). 100 mg of each pulverized samples were used for total RNA extractions using

179 Trizol (Life Technologies, Carlsbad, USA), according to the manufacturer's protocol. Total RNA
180 was treated with DNase I (Life Technologies, Carlsbad, USA) and cleaned using the RNeasy
181 Plant Mini Kit (Qiagen). Both RNA quality and concentration were checked using Agilent RNA
182 6000 Nano Kit Bioanalyzer chip (Agilent Technologies, St. Clara, USA). 5 µg of the purified
183 RNA was used in the reverse transcription reactions using the SuperScript III First-Strand
184 Synthesis System for RT-PCR (Life Technologies Cuisinart). The RT-qPCR reactions were done
185 using Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, USA), 10× diluted
186 cDNA, and specific primers as previously described (Hotta *et al.*, 2013). Reactions were placed in
187 96-well plates and read with the Fast 7500/7500 Real-Time PCR System (Applied Biosystems,
188 Waltham, USA). Ct determination was performed using the Fast 7500/7500 Real-Time PCR
189 System built-in software (Applied Biosystems, Waltham, USA). The $2^{-\Delta CT}$ method was used to
190 calculate relative expression, using *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*
191 (*ScGAPDH*) as a reference gene (Hotta *et al.*, 2013). All primers used can be found in the
192 Supplemental Material (Table S2).

193

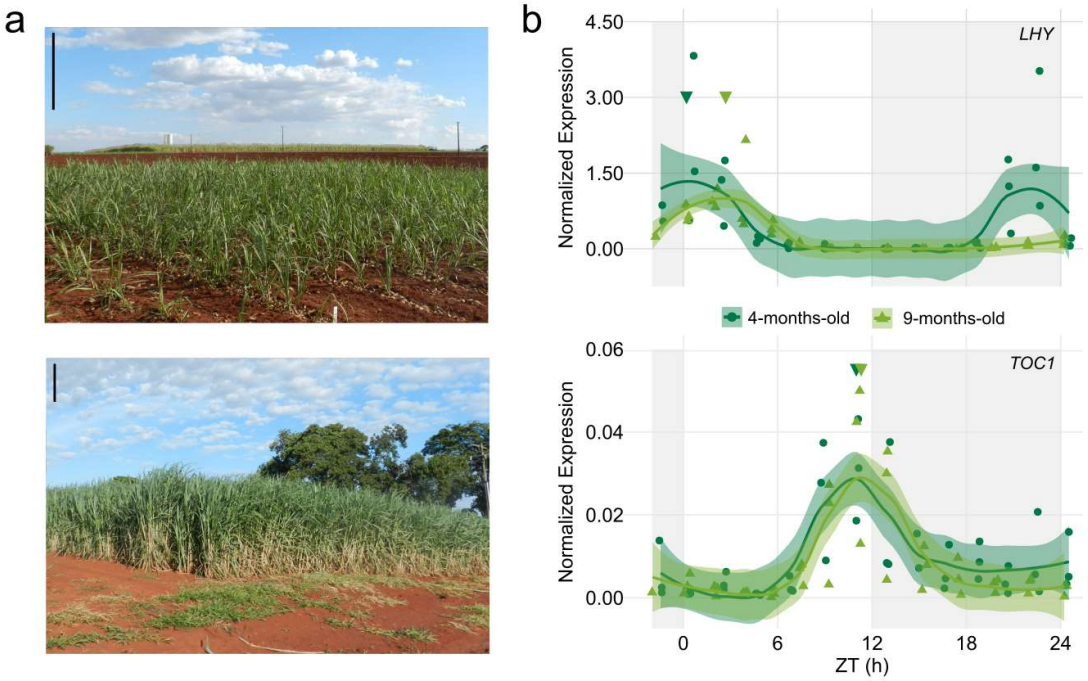
194 **Results**

195 We have measured transcriptional rhythms of leaves, internodes 1&2 and internodes 5 of 9-
196 months-old (9 mo) commercial sugarcane (*Saccharum* hybrid, cultivar SP80 3280) grown in a
197 field in previous work (Dantas *et al.*, 2020). We noticed that the circadian clock gene *LATE*
198 *ELONGATED HYPOCOTYL* (*ScLHY*) peaked hours after dawn, which is unusual, as this gene is
199 light-induced in *Arabidopsis* (Mockler *et al.*, 2007). To further investigate this disparity, we
200 measured *ScLHY* rhythms in 4 mo sugarcane leaves that had been harvested from the same field
201 (Fig. 1a). *ScLHY* peaked 0.2 h after dawn in 4 mo sugarcane leaves but peaked 2.7 h after dawn in
202 9 mo sugarcane leaves (Fig. 1b). We also observed a second *ScLHY* peak that starts increasing

203 between 18.0 h and 20.0 h in 4 mo sugarcane (Fig. 1b). In contrast, *ScTOC1* peaked at 11.0 h and

204 11.3 h after dawn in 4 mo and 9 mo sugarcane leaves, respectively (Fig. 1b). All plants were

205 harvested from the middle of the field to avoid margin effects.



206

207 **Fig. 1. Sugarcane leaves have different *ScLHY* phases in field-grown sugarcane. (a)** Leaves from
208 sugarcane grown for 4 months old (mo) (upper photo) and 9 mo (lower photo) in the field were harvested
209 for 26 h. Bar = 0.5 m. **(b)** Leaves from sugarcane grown for 4 mo and 9 mo in the field were harvested for
210 26 h. (A) Diel rhythms of *LATE ELONGATED HYPOCOTYL* (*ScLHY*) and *TIME OF CAB EXPRESSION*
211 *1* (*ScTOC1*) in the leaves of 4 mo (dark green) and 9 mo (light green) sugarcane leaves. When sugarcane is
212 4 months old (left), there is little shading between plants. In comparison, 9 mo sugarcane shade each other
213 when the sun is at a low angle (right). Bar = 0.5 m. All biological replicates and their LOESS curve
214 (continuous lines ± SE) are shown. Inverted triangles show the time of the maximum value of the LOESS
215 curve. Time series were normalized using Z-score. To compare the rhythms of samples harvested in
216 different seasons, the time of harvesting (ZT) was normalized to a photoperiod of 12 h day/ 12 h night. The
217 light-grey boxes represent the night periods. Transcript levels were measured using RT-qPCR. Relative
218 expression was determined using *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*
219 (*ScGAPDH*).

220

221 To check if other transcriptional rhythms showed a peak change similar to *ScLHY*, we used

222 custom Agilent oligoarrays to measure transcript accumulation in 4 mo sugarcane leaves. We

223 used the transcriptomic data to detect rhythms in 4 mo sugarcane leaves using WGCNA to
224 generate co-expression modules and JTK-Cycle to identify rhythmic modules (Fig. S2). There
225 were 8,553 expressed transcripts in 4 mo sugarcane leaves, 86% of the expressed transcripts
226 found in 9 mo sugarcane leaves (9,891) (Fig. 2a). We considered 4,143 of the expressed
227 transcripts were rhythmic in 4 mo sugarcane leaves (48.4%), lower than in 9 mo sugarcane leaves
228 (68.3%, Fig. S3a). About half of the transcripts that were rhythmic in both 4 mo and 9 mo
229 sugarcane leaves peaked earlier in the older plants (51.5%), whilst 48.2% peaked within 1 h of
230 each other (1.65 ± 1.8 h, median ± MA, n = 4,110) (Fig. 2b). Thus, the phase changes can be seen
231 in transcripts peaking during the whole 24 h cycle, and not just at the same time as *ScLHY* peaks
232 (Fig. S3b). These phase changes were also observed using only JTK-Cycle to identify rhythmic
233 transcripts with phase estimates using JTK_Cycle (1.56 ± 1,9 h, median ± MA, n = 1,014) or
234 using LOESS (3.62 ± 0.4 h, median ± MA, n = 1014) (Figs S4a-d). If only transcripts with
235 significant gene expression profile differences identified with maSigPro were used, the phases
236 differences were 1.65 ± 1.7 h (median ± MA, n = 1,539) using LOESS, and 1.55 ± 2.1 h (median
237 ± MA, n = 527) using JTK-Cycle (Fig. S4e).

238

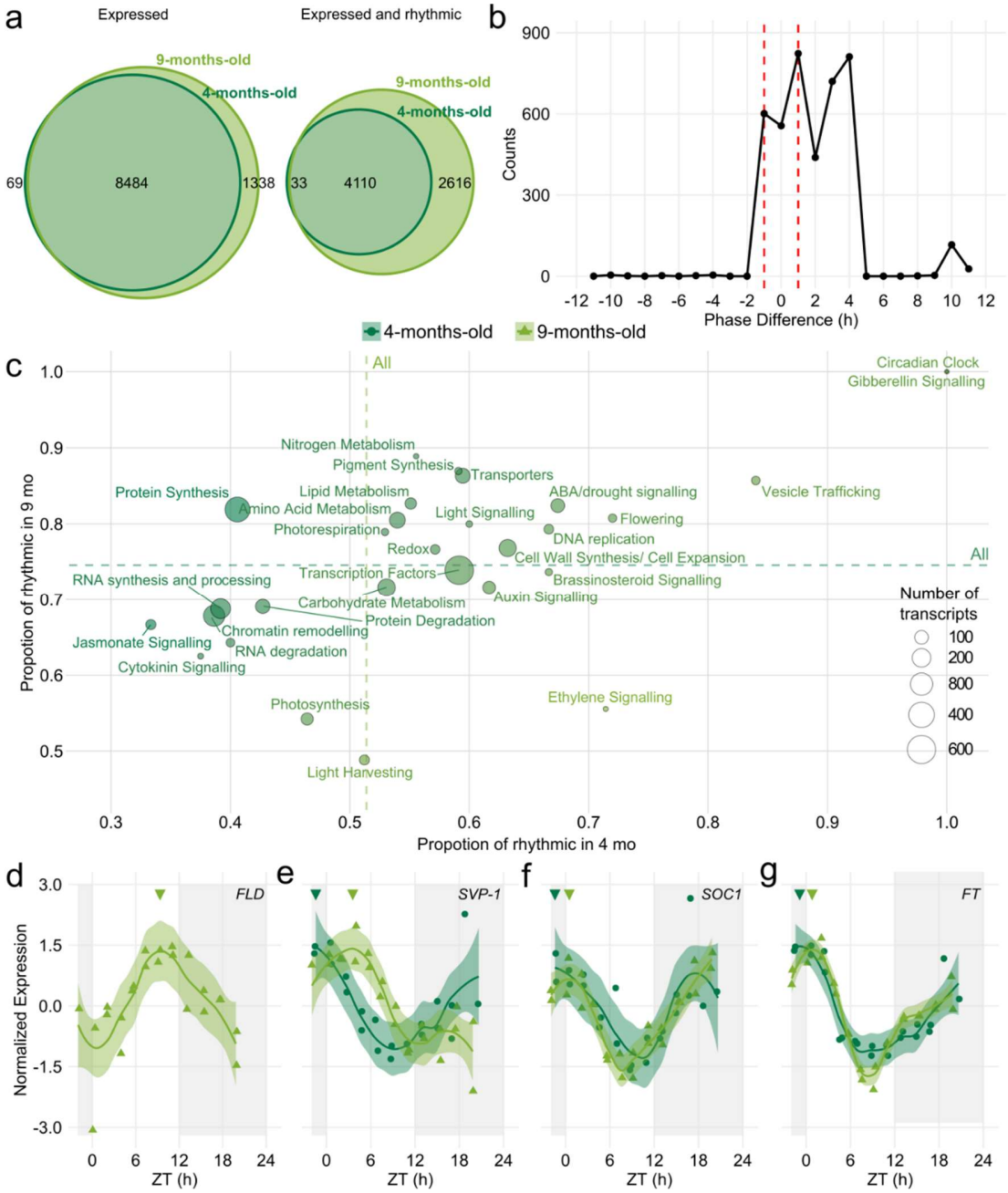


Fig. 2. Field-grown sugarcane has transcriptional rhythms with different phases. Leaves from sugarcane grown for 4 months old (4 mo) and 9 mo in the field were harvested for 26 h. **(a)** Euler diagrams of all expressed transcripts and expressed transcripts that are rhythmic in 4 mo (dark green) and 9 mo (light green) sugarcane leaves in diel conditions. **(b)** Distribution of phase differences between rhythmic transcripts from 4 mo and 9 mo sugarcane. Phase differences under ±1 h are not considered significant (red dashed line). **(c)** The proportion of rhythmic transcripts in different functional categories in 4 mo and 9 mo sugarcane leaves. The size of the circles shows the total number of expressed transcripts in each category. The colour of the circles shows if there is an increase in the proportion of rhythmic transcripts in 4 mo

248 (light green) or in 9 mo sugarcane leaves (dark green). The dotted lines represent the proportion of
249 rhythmic transcripts among all annotated transcripts. **(d)** *FLOWERING LOCUS D* (*ScFLD*), **(e)** *SHORT*
250 *VEGETATIVE-1* (*ScSVP*-1), (**f**) *SUPPRESSOR OF CONSTANS OVEREXPRESSION* (*ScSOC1*), and (**g**)
251 *FLOWERING LOCUS T* (*ScFT*) rhythms measured in 4 mo and 9 mo sugarcane. All biological replicates
252 and their LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the
253 maximum value of the LOESS curve. Time series were normalized using Z-score. To compare the rhythms
254 of samples harvested in different seasons, the time of harvesting (ZT) was normalized to a photoperiod of
255 12 h day/ 12 h night. The light-grey boxes represent the night periods.

256

257 Among the other central oscillator genes investigated, *PSEUDO RESPONSE REGULATOR 73*
258 (*ScPRR73*), *ScPRR59*, *ScGI, REVEILLE 8* (*ScRVE8*) and *EARLY FLOWERING 3* (*ScELF3*) also
259 peaked at similar times in 4 mo and 9 mo sugarcane leaves, while *ScPRR95* had an earlier peak (-
260 2.6 h) in 9 mo sugarcane leaves (Fig. S5). We also have analyzed the proportion of rhythmic
261 transcripts in different functional categories (Figs 2c and S3c). The *Circadian Clock*,
262 *ABA/drought signalling*, *Gibberellin Signalling*, *Transporters*, and *Flowering* are examples of
263 functional categories with a higher proportion of rhythmic transcripts than the average.
264 Conversely, *Chromatin Remodeling* and *RNA Synthesis and processing* are functional categories
265 with lower rhythmic transcripts than the average. Among the disproportionately rhythmic
266 categories in either 4 mo or 9 mo sugarcane leaves, *Auxin Signalling* and *Ethylene Signalling*
267 have a higher proportion of rhythmic transcripts in 4 mo sugarcane leaves. In comparison, *Protein*
268 *Synthesis* has a higher proportion of rhythmic transcripts in 9 mo sugarcane leaves (Figs 2c and
269 S3c).

270 One important pathway that could be affected by phase changes in rhythmic genes is flowering
271 signalling. Several sugarcane genes associated with flowering had their transcription patterns
272 changed. For example, *FLOWERING LOCUS D* (*ScFLD*), a histone acetylase, is only expressed
273 in 9 mo sugarcane leaves, with a peak at ZT9 (Fig. 2d). Mutation of *FLD* in Arabidopsis (He *et*
274 *al.*, 2003) and RNA interference of its homolog in rice(Shibaya *et al.*, 2016) delays flowering.
275 The flowering genes: *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*ScCOP1*) (Tanaka *et al.*,

276 2011), *CYCLING DOF FACTOR1* (*ScCDF1*)(Higgins *et al.*, 2010), *SHORT VEGETATIVE*
277 (*ScSVP-1* and *-3*) (Higgins *et al.*, 2010), *SUPPRESSOR OF CONSTANS OVEREXPRESSION 1*
278 (*ScSOC1*) (Higgins *et al.*, 2010), *FLOWERING LOCUS T* (Sc*FT*) (Abdul-Awal *et al.*, 2020), and
279 *APETALA* (*ScAP1*) (Preston & Kellogg, 2006) peak an average 2 h earlier in 4 mo sugarcane
280 leaves, except for *ScSVP-2* (Figs 2e-f and S6).

281 We also measured metabolite rhythms in 4 mo and 9 mo sugarcane leaves (Fig. 3). The metabolic
282 profiles of plants harvested during the day and the night cluster separately, except for the leaves
283 harvested 21 h after dawn (zeitgeber time 21, ZT21) in 4 mo sugarcane and leaves harvested at
284 ZT01 in 9 mo sugarcane (Fig. 3a). 31 out of 57 metabolites (54.4%) were found rhythmic in 4 mo
285 sugarcane leaves, whereas 33 out of 51 metabolites (64.7%) were found rhythmic in 9 mo
286 sugarcane leaves. Most of the 20 metabolites that were rhythmic in both plants (75%) peaked
287 later in 9 mo sugarcane (Figs 3b and S7). Thus, the phase changes observed in *ScLHY*, but not in
288 *ScTOC1*, are also widespread among transcriptional and metabolic rhythms. Only 2 metabolites,
289 Sucrose and myo-Inositol, peaked later in 4 mo sugarcane than in 9 mo sugarcane (Figs 3b and
290 S8a). As myo-Inositol has a broader peak, possibly due to a second peak, in 4 mo sugarcane
291 compared to 9 mo sugarcane, this phase-estimate might not be accurate (Fig. S8a). Three
292 metabolites peaked within a 1 h difference (Figs 3d and S8b-c), 10 metabolites peaked 1.5 h to 6
293 h later in 9 mo sugarcane (Fig. 3e and Fig. S9), and 5 metabolites peaked 8 h to 13 h later in 9 mo
294 sugarcane (Figs 3f and S10). The atypical night peak seen in *ScLHY* was also observed in
295 metabolites such as Alanine (Fig. 3e), Phenylalanine (Fig. S8b), Leucine (Fig. S9b) and Glycerate
296 (Fig. S9e).

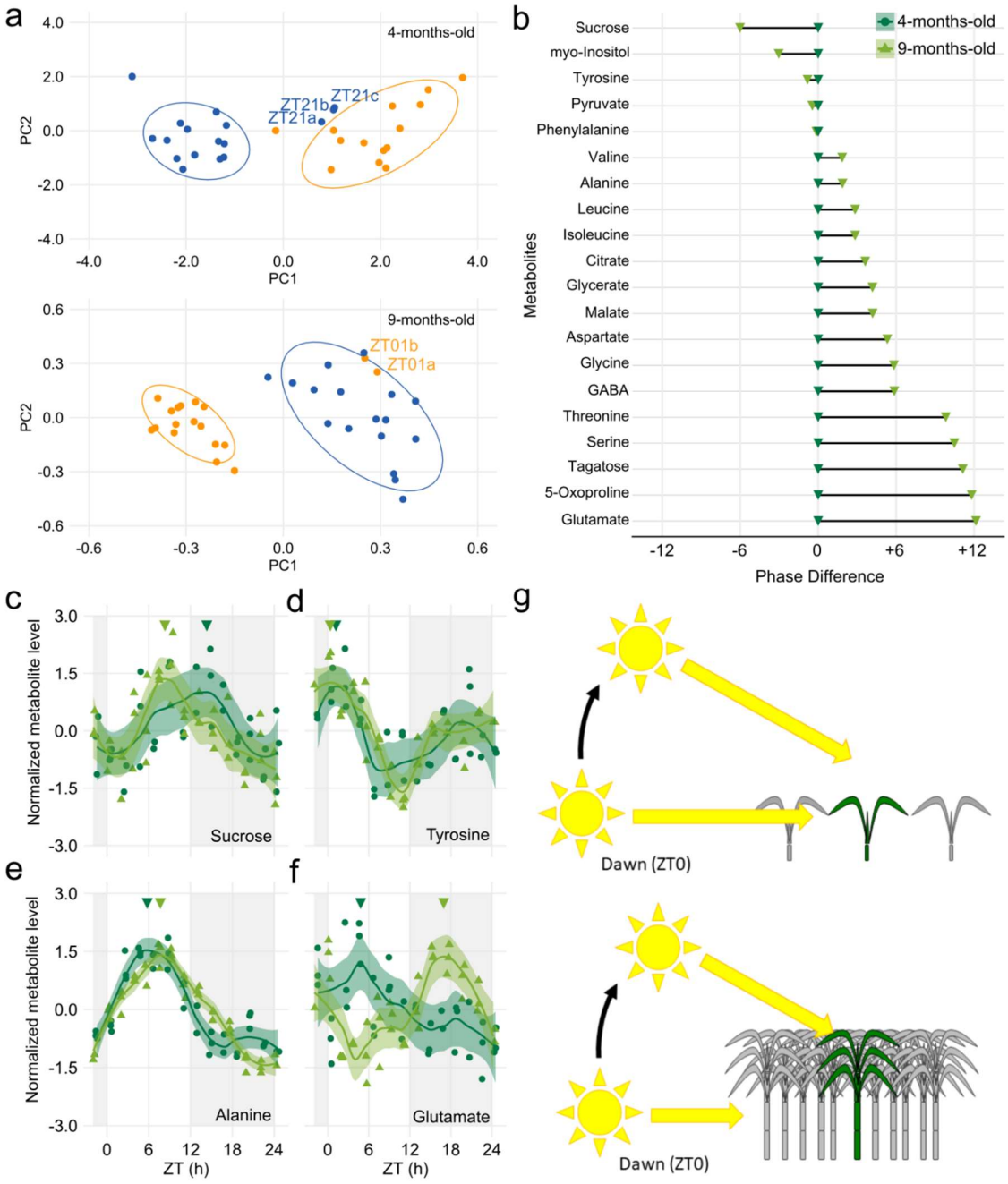


Fig. 3. Field-grown sugarcane has metabolic rhythms with different phases. (a) Principal Component Analysis (PCA) of the metabolic data from the leaves of 4 mo and 9 mo sugarcane during the day (yellow) or during the night (blue). The percentages of total variance represented by the principal component 1 (PC1) and principal component 2 (PC2) is 63.7% and 60.3%. Metabolites identified in less than 70% of the samples and samples with less than 70% of the total metabolites identified were excluded from the PCA. Data ellipses were drawn for the samples harvested during the day and the night (0.85 confidence level). **(b)** Phase difference in metabolites that were considered rhythmic in both 4 mo (dark green) and 9 mo plants (light green). **(c-f)** Rhythms of **(c)** Sucrose, **(d)** Tyrosine, **(e)** Alanine, and **(f)** Glutamate in 4 mo

306 (dark green) and 9 mo plants (light green). **(g)** When sugarcane is 4 months old (left), there is little shading
307 between plants. In comparison, 9 mo sugarcane shade each other when the sun is at a low angle (right). All
308 biological replicates (circles in 4 mo or triangles in 9 mo) and their LOESS curve (continuous lines ± SE)
309 are shown. Inverted triangles show the time of the maximum value of the LOESS curve. Metabolite levels
310 were normalized with Z-score. To compare the rhythms of samples harvested in different seasons, the time
311 of harvesting (ZT) was normalized to a photoperiod of 12 h day/ 12 h night. The light-grey boxes represent
312 the night period.

313

314 In Arabidopsis, *AtLHY* is responsive to light and temperature, marking the perception of dawn by
315 the plant circadian oscillator(Martínez-García *et al.*, 2000; Gould *et al.*, 2006). Thus, we
316 hypothesized that the observed phase changes result from the circadian oscillator of 9 mo
317 sugarcane perceiving dawn later than 4 mo sugarcane due to shading effects from neighbouring
318 plants. While young sugarcane plants interact little with their neighbours due to their short height,
319 mature sugarcane plants usually grow densely surrounded by other plants and senescent leaves
320 (Fig. 1a), shading each other, and making it difficult for low angle sunlight to reach leaves
321 uniformly in the entire field (Fig. 3g). This hypothesis also explains why the metabolic profile of
322 9 mo sugarcane leaves harvested at ZT01, before the *ScLHY* peak, was closer to the leaves
323 harvested during the night than the plants harvested during the day (Fig. 3a).

324 If our hypothesis were correct, plants that grow on the east side, which receives direct sunlight at
325 dawn, and the west side of a field would have *ScLHY* rhythms with different phases (Fig. 4a).
326 Using leaves from 6 mo plants, we estimated that *ScLHY* from sugarcane peaked 0.6 h after dawn
327 on the east side of the field and 1 h later on the west side (Fig. 4b). Light levels were also around
328 1 h late on the west side in the morning (Fig. S1c). Moreover, the *ScLHY* transcript levels
329 changed around 800 x during the day in sugarcane on the east side and 350 x in sugarcane on the
330 west side. The peak of ScLHY was 4.5 x higher in sugarcane on the east side, affecting the phase
331 and amplitude of downstream rhythms (Ni *et al.*, 2009). *ScPRR73* and *ScPRR37* on the east side
332 show one broad peak, while they have two smaller peaks on the west side (Figs S11a,b). In

333 contrast, *ScTOC1* rhythms had similar phases (Fig. 4b). We concluded that the location of a plant
334 within a field could change the perception of dawn by the circadian clock due to differences in
335 the inner field microenvironment, even if no developmental differences between plants on each
336 side of the field could be detected. In a similar experiment, grape berries (*Vitis vinifera* L.)
337 harvested from the east and the west side of a vine had different phases in sucrose rhythms
338 (Reshef *et al.*, 2019).

339 To further confirm our hypothesis, we created artificial shading by building a wooden wall
340 between the first two lines of 5 mo sugarcane on the east side of the field (Fig. 4c). The wall was
341 built two days before harvesting to avoid developmental effects and any long-term shade
342 avoidance responses caused by shading. Plants ahead the wall received direct sunlight at dawn,
343 while plants beyond the wall received direct sunlight 0.7 h later (Fig. S1e), affecting the local
344 temperature (Fig. S1f). In sugarcane growing ahead of the wall, *ScLHY* expressed in leaves
345 peaked 1.1 h after dawn, whereas *ScLHY* peaked 2.4 h after dawn in leaves beyond the wall (Fig.
346 4d). The *ScLHY* transcript levels changed 400 x during the day in sugarcane ahead of the wall and
347 950x in sugarcane beyond the wall. The peak of *ScLHY* was 1.7 x higher in sugarcane ahead of
348 the wall. The *ScPRR73* and *ScPRR37* amplitudes are lower in sugarcane beyond the wall.
349 *ScPRR37* also peaks 1.3 h later in sugarcane beyond the wall (Figs S11c,d). *ScTOC1* peaked at
350 the same time in both conditions (Fig. 4d). These last two experiments show that the phase
351 changes seen in 4 mo and 9 mo sugarcane leaves are not a consequence of seasonal or
352 developmental differences, as plants of the same age that were harvested simultaneously were
353 used. Combining the three experiments, the peaks of *ScLHY* and *ScTOC1* happened 10.4 ± 0.5 h
354 (n = 3) apart in plants not subjected to shading at dawn, and 8.7 ± 0.5 h (n = 3) apart in plants
355 shaded at dawn, which could be enough to trigger photoperiodic responses.

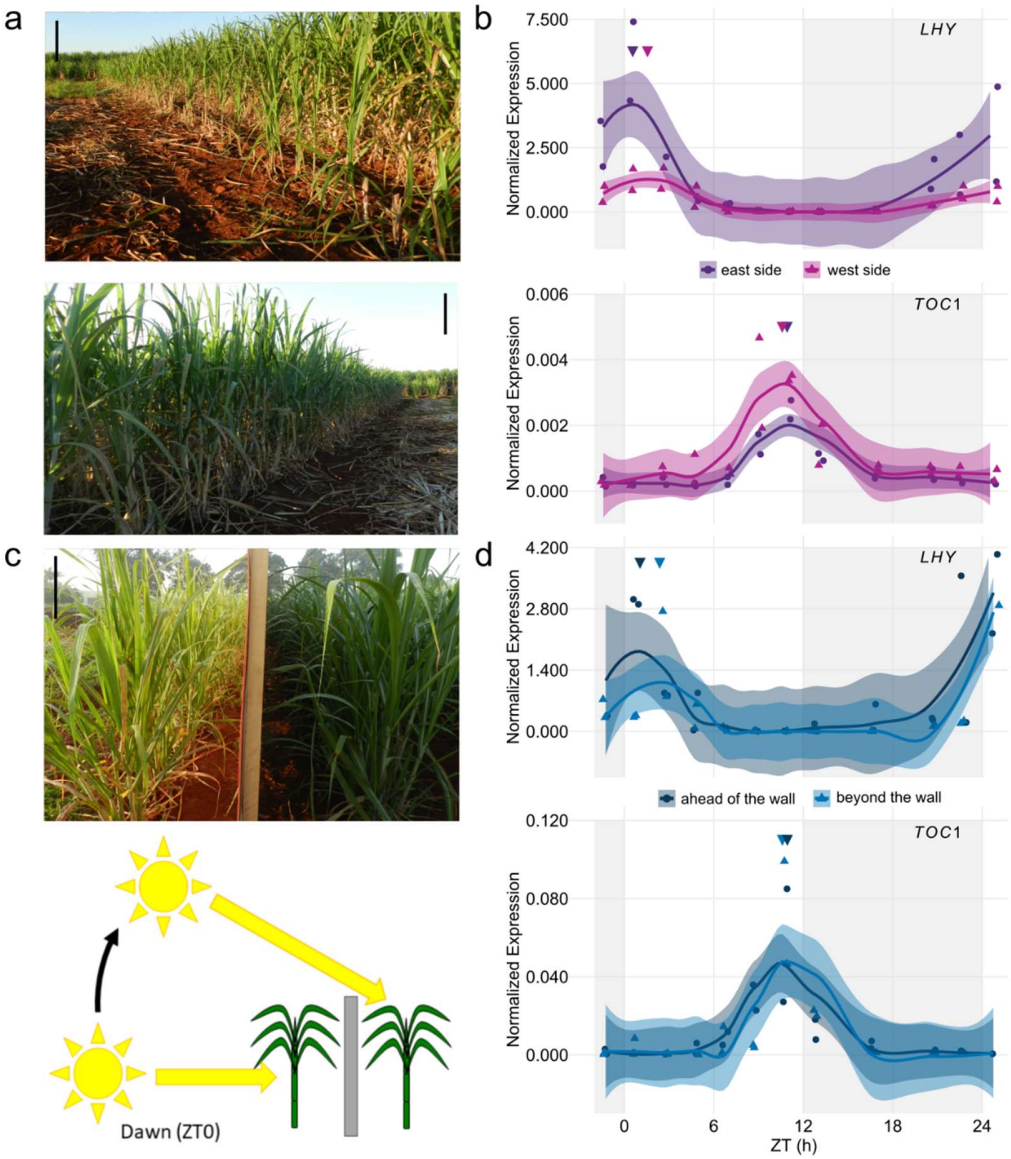


Fig. 4. Sugarcane leaves have different *ScLHY* phases when shaded at dawn. (a) Leaves from sugarcane grown on the east side (upper photo) and the west side (bottom photo) of the field were harvested for 26 h. Bar = 0.5 m. **(b)** Diel rhythms of *LATE ELONGATED HYPOCOTYL* (*ScLHY*) and *TIME OF CAB EXPRESSION 1* (*ScTOC1*) in the leaves of sugarcane grown on the east side (purple) and the west side (pink) of the field. **(c)** Leaves from sugarcane grown before a wooden wall and after the wooden wall were harvested for 26 h. The photo was taken 1 h after dawn and horizontally flipped. Bar = 0.5 m. **(d)** Diel rhythms of *ScLHY* and *ScTOC1* in sugarcane leaves grown ahead of the wall (dark blue) and beyond the wall (light blue). All biological replicates and their LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the maximum value of the LOESS curve. Time series were normalized using Z-score. To compare the rhythms of samples harvested in different seasons, the time of harvesting (ZT) was normalized to a photoperiod of 12 h day/ 12 h night. The light-grey boxes represent the night periods. Transcript levels were measured using RT-qPCR. Relative expression was determined using *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*ScGAPDH*).

370 **Discussion**

371 We found that the location of a plant within a field can change the perception of dawn by the
372 circadian clock due to differences in field microenvironments. The circadian clock regulates
373 carbon allocation, which may impact agronomic traits (Yazdanbakhsh *et al.*, 2011; Kölling *et al.*,
374 2015). Among the circadian clock genes, only *ScLHY* and *ScPRR95* had their phase changed
375 when comparing 4 mo and 9 mo sugarcane leaves, explaining why only half of the rhythmic
376 genes had phase changes. However, change in the expression of these two genes can trigger
377 changes that affect the timing of a vast number of output rhythms, as hundreds of genes are
378 regulated directly by *AtLHY* and *AtPRR95* in Arabidopsis (Harmer *et al.*, 2000; Liu *et al.*, 2016;
379 Adams *et al.*, 2018).

380 Diel rhythms could be driven by environmental rhythms, the circadian oscillator, or their synergic
381 combination. Among the external factors, temperature, light quality and quantity, photoperiod
382 and photosynthesis are known to be essential regulators of plant rhythms (Martínez-García *et al.*,
383 2000; Gould *et al.*, 2006; Frank *et al.*, 2018; Dantas *et al.*, 2019). While it is tempting to attribute
384 the phase differences to light, temperature may be an important factor driving sugarcane rhythms.
385 In *Brachypodium dystachion* (L.) P. Beauv., another grass species, changes in temperatures were
386 found to be the main environmental cue driving rhythmic behaviour (Matos *et al.*, 2014;
387 MacKinnon *et al.*, 2020). In sugarcane, temperature also regulates the rate of *ScLHY* isoform
388 formation by alternative splicing (Dantas *et al.*, 2019). Temperature rhythms were very different
389 when 4 mo sugarcane was harvested (temperature between 10°C and 27°C) from when 9 mo
390 sugarcane was harvested (temperature range between 16°C and 30°C) (Fig. S1b). This
391 temperature difference may also explain the differences in the number of rhythmic transcripts and
392 metabolites (Pyl *et al.*, 2012; Annunziata *et al.*, 2018). In Arabidopsis, differences in
393 temperatures affected the levels of *AtLHY*, *AtCCA1* and *AtPRR9* (a probable ortholog of

394 *ScPRR95*), which included a delay in peak time (Bieniawska *et al.*, 2008; Gould *et al.*, 2013;
395 Annunziata *et al.*, 2018). We should also stress that the 4 mo and 9 mo sugarcane leaves were
396 under different photoperiods (11.5 h day/12.5 h and 13.3 h day/10.7 h night, respectively), which
397 could be another factor explaining some differences among rhythms, as seen in *Arabidopsis*
398 *halleri* subsp. *gemmifera* (Matsum.) O'Kane & Al-Shehbaz in natural conditions (Nagano *et al.*,
399 2019). However, plants in the other experiments were harvested at the same time.

400 Sugarcane rhythms may also be regulated by shade avoidance responses, as the R: FR ratio could
401 be altered due to shading from other plants. Even though little is known about the regulation of
402 the circadian clock by shade-avoidance responses, changes in plant density have been correlated
403 to changes in the circadian clock genes. In *Sorghum bicolor* (L.) Moench. at high and low
404 densities, changes in transcription levels of *SbLHY* and *SbTOC1* were correlated with differences
405 in internode length (Kebrom *et al.*, 2020). As only one time point was sampled in that study, if
406 sorghum had similar phase differences as the ones we detected, they would be seen as differences
407 in expression levels, as seen when only one timepoint is considered in our experiments (Fig. S12).
408 When row spacings in sugarcane fields were changed between 1.5 m and 2.3 m, cane yields (t/ha)
409 were maintained, as the stalk numbers and weight would change in response to different plant
410 densities (Garside *et al.*, 2009; Chiluwal *et al.*, 2018).

411 Microenvironment changes due to shading also affect photosynthesis. In *Arabidopsis*,
412 photosynthetic soluble sugars have been shown to regulate the circadian oscillator (Haydon *et al.*,
413 2013; Frank *et al.*, 2018). Hence, the phase changes seen in *ScLHY* could be driven by changes in
414 photosynthesis or, specifically, by carbon assimilation into sucrose. Moreover, photosynthesis is
415 one of the factors that impact rhythms in gene expression in *A. halleri* subsp. *gemmifera* in
416 natural conditions (Nagano *et al.*, 2019). It is unclear how the circadian oscillator and metabolites
417 rhythms are interconnected in sugarcane. However, Sucrose was one of the only metabolites with

418 an earlier phase in the leaves of 9 mo sugarcane (Figs 3b,c). Indeed, Sucrose levels in the first 4 h
419 of the day were similar in 4 mo and 9 mo plants. Thus, Sucrose from photosynthesis might not be
420 the factor explaining the observed phase changes, but our analysis only covered a few
421 photosynthetic metabolites. As rhythmic differences in Sucrose were distinct from the changes
422 observed in other metabolites, it is possible that it is dependent on a different internal or external
423 factor. One possible explanation is a reduction of the photosynthesis in the afternoon due to
424 shading of sugarcane in the middle of the field, or the inhibition of photosynthesis due to Sucrose
425 accumulation in the leaves and internodes (McCormick *et al.*, 2008).

426 In 4 mo sugarcane leaves, *ScLHY* and other transcripts showed an unexpected transcriptional peak
427 in the middle of the night in 4 mo sugarcane (Figs 1b and S3a). We do not know what could have
428 triggered this peak in transcript expression, but metabolites, such as Alanine, Glycerate and
429 Phenylalanine, showed the same peak. A similar peak in the middle of the night was found in
430 *Coffea arabica* L. and attributed to moonlight effects (Breitler *et al.*, 2020). In our experiment,
431 the increase in transcriptional and metabolic levels during the night started minutes after the
432 crescent moon was set (ZT17-18). Thus, we do not think moonlight or other artificial lights with
433 similar intensity or spectrum were the factors driving these increases. However, this night peak of
434 unknown origin shows that metabolites and transcripts are interconnected and that the metabolic
435 changes brought by photosynthesis and light signalling are not the only force driving rhythms
436 during the day. For example, we have seen significant phase shifts in metabolites (> 8h) between
437 4 mo and 9 mo sugarcane. The differences, seen in Glutamate, GABA, Serine, 5-Oxoproline,
438 Tagatose and GABA (Figs 3f, S9i and S10), may be due to differences in plant age. In *Nicotiana*
439 *tabacum* (L.), a similar phase change was observed in the rhythms of Glutamate and GABA in the
440 sink and the source leaves (Masclaux-Daubresse *et al.*, 2002). This change was associated with
441 the rhythmic expression of *GLUTAMATE DEHYDROGENASE 1* (*NtGDH1*) in the source leaves

442 but no expression in sink leaves. In 4 mo sugarcane, an *ScGDH1* was arrhythmic, with expression
443 below the detection levels in many time points (Fig. S5i). In 9 mo sugarcane, *ScGDH1* was
444 rhythmic, and expression was above detection levels in all time points.

445 Flowering is a necessary process that could be affected by phase changes in rhythmic genes. As
446 the vegetative stage is harvested, flowering is usually undesired in sugarcane, as it reduces
447 sucrose yield due to decreased growth, increased side-shoot production, and pithiness (Rao,
448 1977). Sugarcane enters the reproductive phase in response to the shortening of the photoperiod
449 over 15 days, and just a 0.5 h difference in photoperiod can trigger flowering in most genotypes
450 (Midmore, 1980; Moore & Berding, 2013; Glassop *et al.*, 2014). Thus, the correct measurement
451 of dawn and dusk is essential for flowering in sugarcane. Among those genes, *ScSVP*-1, *ScSVP*-3,
452 *ScSOC1*, *ScFT*, and *ScCDF1* peak before dawn in 4 mo sugarcane leaves and after dawn in 9 mo
453 sugarcane leaves (Figs 2e-g and S6). In Arabidopsis, AtLHY and AtCCA1 reduce AtSVP protein
454 levels, a repressor of flowering (Fujiwara *et al.*, 2008). A change in the relative phase between
455 *ScLHY* and *ScSVP* may change the ScSVP protein levels, impacting flowering. We also found
456 that *ScPRR37* and *ScPRR73* show different expression patterns in plants shaded in the morning
457 that could be explained by the changes in the phase of two peaks during the day. In sorghum, the
458 second peak in *SbPRR37*, during the evening, regulates flowering (Murphy *et al.*, 2011). Thus,
459 neighbour shading could trigger flowering earlier than the actual critical photoperiod. In
460 sugarcane, the spindle, a whorl of immature leaves on the top of the plant, perceives photoperiod,
461 which could be a strategy to reduce the effects of shading from neighbour plants (Moore &
462 Berding, 2013; Glassop *et al.*, 2014; Glassop & Rae, 2019).

463 The circadian clock increases the productivity of plants when its phase matches the phase of
464 environmental rhythms (Dodd *et al.*, 2005). It is usually accepted that the rhythms of plants under
465 the same photoperiod have matching phases. However, we have evidence that field

466 microenvironmental rhythms and not astronomical rhythms regulate the plant circadian oscillator
467 of plants in natural, fluctuating conditions and transcriptional and metabolic rhythms. This
468 phenomenon can probably be observed in plants grown in natural or artificial environments, such
469 as crops, forests, and gardens, with microenvironments. Thus, rhythms in field
470 microenvironments are another factor to consider when translating knowledge from the lab to the
471 field, especially agriculture.

472

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481

482 **Author Contributions**

483 Conceptualization, LLBD, MSC, CTH; Methodology, LLBD, CTH; Software, CTH, MYN;
484 Validation, LLBD, NOL; Investigation, LLBD, NOL, CTH, CC; Resources, MSC, C. C.; Data
485 Curation, CTH, MYN; Writing – Original Draft, LLBD, CTH; Writing – Review & Editing,
486 LLBD, MSC, CC, CTH; Visualization; C. T. H.; Project Administration, CTH; Funding
487 Acquisition, CTH.

488

489 **Data availability**

490 The complete dataset can be found at the Gene Expression Omnibus public database under the
491 accession number GSE129543 and GSE171222. Code to fully reproduce our analysis is available
492 on GitHub (https://github.com/LabHotta/Microenvironments) and archived on Zenodo
493 (http://doi.org/10.5281/zenodo.4645464).

494

495 **Competing Interests**

496 The authors have no competing interests to declare.

497

498 **References**

499

500 **Abdul-Awal SM, Chen J, Xin Z, Harmon FG**. **2020**. A sorghum gigantea mutant attenuates
501 florigen gene expression and delays flowering time. *Plant Direct* **4**: e00281.

502 **Adams S, Grundy J, Veflingstad SR, Dyer NP, Hannah MA, Ott S, Carré IA**. **2018**.
503 Circadian control of abscisic acid biosynthesis and signalling pathways revealed by genome-wide
504 analysis of LHY binding targets. *New Phytologist* **220**: 893–907.

505 **Alabadí D, Oyama T, Yanovsky MJ, Harmon FG, Más P, Kay SA**. **2001**. Reciprocal
506 regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. *Science (New
507 York, N.Y.)* **293**: 880–883.

Annunziata MG, Apelt F, Carillo P, Krause U, Feil R, Koehl K, Lunn JE, Stitt M. **2018**. Response of Arabidopsis primary metabolism and circadian clock to low night temperature in a natural light environment. *Journal of Experimental Botany* **69**: 4881–4895.

Annunziata MG, Apelt F, Carillo P, Krause U, Feil R, Mengin V, Lauxmann MA, Köhl K, Nikoloski Z, Stitt M, *et al.* 2017. Getting back to nature: a reality check for experiments in controlled environments. *Journal of Experimental Botany* **68**: 4463–4477.

Bieniawska Z, Espinoza C, Schlereth A, Sulpice R, Hincha DK, Hannah MA. **2008**. Disruption of the Arabidopsis Circadian Clock Is Responsible for Extensive Variation in the Cold-Responsive Transcriptome. *Plant Physiology* **147**: 263–279.

Breitler J-C, Djerrab D, Leran S, Toniutti L, Guittin C, Severac D, Pratlong M, Dereeper A, Etienne H, Bertrand B. **2020**. Full moonlight-induced circadian clock entrainment in Coffea arabica. *BMC Plant Biology* **20**: 24.

Calixto CPG, Waugh R, Brown JWS. **2015**. Evolutionary relationships among barley and Arabidopsis core circadian clock and clock-associated genes. *Journal of Molecular Evolution* **80**: 108–119.

Cha J-Y, Kim J, Kim T-S, Zeng Q, Wang L, Lee SY, Kim W-Y, Somers DE. **2017**. GIGANTEA is a co-chaperone which facilitates maturation of ZEITLUPE in the Arabidopsis circadian clock. *Nature Communications* **8**: 3.

Chiluwal A, Singh HP, Sainju U, Khanal B, Whitehead WF, Singh BP. **2018**. Spacing Effect on Energy Cane Growth, Physiology, and Biomass Yield. *Crop Science* **58**: 1371–1384.

528 **Cuadros-Inostroza A, Caldana C, Redestig H, Kusano M, Lisec J, Peña-Cortés H,**
529 **Willmitzer L, Hannah MA**. **2009**. TargetSearch--a Bioconductor package for the efficient
530 preprocessing of GC-MS metabolite profiling data. *BMC bioinformatics* **10**: 428.

531 **Dantas LL de B, Almeida-Jesus FM, de Lima NO, Alves-Lima C, Nishiyama-Jr MY,**
532 **Carneiro MS, Souza GM, Hotta CT**. **2020**. Rhythms of Transcription in Field-Grown
533 Sugarcane Are Highly Organ Specific. *Scientific Reports* **10**: 6565.

534 **Dantas LLB, Calixto CPG, Dourado MM, Carneiro MS, Brown JWS, Hotta CT**. **2019**.
535 Alternative Splicing of Circadian Clock Genes Correlates With Temperature in Field-Grown
536 Sugarcane. *Frontiers in Plant Science* **10**.

537 **Dodd AN, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibberd JM, Millar AJ, Webb AA**.
538 **2005**. Plant circadian clocks increase photosynthesis, growth, survival, and competitive
539 advantage. *Science* **309**: 630–3.

540 **Ferreira DA, Martins MCM, Cheavegatti-Gianotto A, Carneiro MS, Amadeu RR, Aricetti**
541 **JA, Wolf LD, Hoffmann HP, de Abreu LGF, Caldana C**. **2018**. Metabolite Profiles of
542 Sugarcane Culm Reveal the Relationship Among Metabolism and Axillary Bud Outgrowth in
543 Genetically Related Sugarcane Commercial Cultivars. *Frontiers in Plant Science* **9**.

544 **Frank A, Matiolli CC, Viana AJC, Hearn TJ, Kusakina J, Belbin FE, Wells Newman D,**
545 **Yochikawa A, Cano-Ramirez DL, Chembath A, *et al.* 2018**. Circadian entrainment in
546 Arabidopsis by the sugar-responsive transcription factor bZIP63. *Current Biology* **28**: 2597-
547 2606.e6.

548 **Fujiwara S, Oda A, Yoshida R, Niinuma K, Miyata K, Tomozoe Y, Tajima T, Nakagawa M,**
549 **Hayashi K, Coupland G, *et al.* 2008**. Circadian Clock Proteins LHY and CCA1 Regulate SVP
550 Protein Accumulation to Control Flowering in Arabidopsis. *The Plant Cell* **20**: 2960–2971.

551 **Garside AL, Bell MJ, Robotham BG, Garside AL, Bell MJ, Robotham BG**. **2009**. Row
552 spacing and planting density effects on the growth and yield of sugarcane. 2. Strategies for the
553 adoption of controlled traffic. *Crop and Pasture Science* **60**: 544–554.

554 **Giavalisco P, Li Y, Matthes A, Eckhardt A, Hubberten H-M, Hesse H, Segu S, Hummel J,**
555 **Köhl K, Willmitzer L**. **2011**. Elemental formula annotation of polar and lipophilic metabolites
556 using (13) C, (15) N and (34) S isotope labelling, in combination with high-resolution mass
557 spectrometry. *The Plant Journal: For Cell and Molecular Biology* **68**: 364–376.

558 **Glassop D, Rae AL**. **2019**. Expression of sugarcane genes associated with perception of
559 photoperiod and floral induction reveals cycling over a 24-hour period. *Functional Plant Biology*
560 **46**: 314–327.

561 **Glassop D, Rae AL, Bonnett GD**. **2014**. Sugarcane Flowering Genes and Pathways in Relation
562 to Vegetative Regression. *Sugar Tech* **16**: 235–240.

563 **Gould PD, Locke JCW, Larue C, Southern MM, Davis SJ, Hanano S, Moyle R, Milich R,**
564 **Putterill J, Millar AJ, *et al.* 2006**. The Molecular Basis of Temperature Compensation in the
565 Arabidopsis Circadian Clock. *The Plant Cell* **18**: 1177–1187.

566 **Gould PD, Ugarte N, Domijan M, Costa M, Foreman J, Macgregor D, Rose K, Griffiths J,**
567 **Millar AJ, Finkenstädt B, *et al.* 2013**. Network balance via CRY signalling controls the
568 Arabidopsis circadian clock over ambient temperatures. *Molecular Systems Biology* **9**: 650.

569 **Gray JA, Shalit-Kaneh A, Chu DN, Hsu PY, Harmer SL**. **2017**. The REVEILLE Clock Genes
570 Inhibit Growth of Juvenile and Adult Plants by Control of Cell Size. *Plant Physiology* **173**: 2308–
571 2322.

572 **Green RM, Tingay S, Wang Z-Y, Tobin EM**. **2002**. Circadian rhythms confer a higher level of
573 fitness to Arabidopsis plants. *Plant Physiology* **129**: 576–584.

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

577 **Haydon MJ, Mielczarek O, Robertson FC, Hubbard KE, Webb AA**. **2013**. Photosynthetic
578 entrainment of the Arabidopsis thaliana circadian clock. *Nature* **502**: 689–92.

579 **He Y, Michaels SD, Amasino RM**. **2003**. Regulation of flowering time by histone acetylation in
580 Arabidopsis. *Science (New York, N.Y.)* **302**: 1751–1754.

581 **Herrero E, Kolmos E, Bujdoso N, Yuan Y, Wang M, Berns MC, Uhlworm H, Coupland G,**
582 **Saini R, Jaskolski M, *et al.* 2012**. EARLY FLOWERING4 recruitment of EARLY
583 FLOWERING3 in the nucleus sustains the Arabidopsis circadian clock. *The Plant Cell* **24**: 428–
584 443.

585 **Higgins JA, Bailey PC, Laurie DA**. **2010**. Comparative Genomics of Flowering Time Pathways
586 Using Brachypodium distachyon as a Model for the Temperate Grasses. *PLOS ONE* **5**: e10065.

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

589 **Huang H, Gehan MA, Huss SE, Alvarez S, Lizarraga C, Gruebbling EL, Gierer J, Naldrett**
590 **MJ, Bindbeutel RK, Evans BS, *et al.* 2017**. Cross-species complementation reveals conserved
591 functions for EARLY FLOWERING 3 between monocots and dicots. *Plant Direct* **1**: e00018.

592 **Huang W, Pérez-García P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P**.
593 **2012**. Mapping the Core of the Arabidopsis Circadian Clock Defines the Network Structure of the
594 Oscillator. *Science* **336**: 75–79.

595 **Hughes ME, Hogenesch JB, Kornacker K**. **2010**. JTK_CYCLE: an efficient nonparametric
596 algorithm for detecting rhythmic components in genome-scale data sets. *Journal of Biological*
597 *Rhythms* **25**: 372–380.

598 **Izawa T, Mihara M, Suzuki Y, Gupta M, Itoh H, Nagano AJ, Motoyama R, Sawada Y,**
599 **Yano M, Hirai MY, *et al.* 2011**. Os-GIGANTEA confers robust diurnal rhythms on the global
600 transcriptome of rice in the field. *The Plant Cell* **23**: 1741–1755.

601 **Kebrom TH, McKinley BA, Mullet JE**. **2020**. Shade signals alter the expression of circadian
602 clock genes in newly-formed bioenergy sorghum internodes. *Plant Direct* **4**: e00235.

603 **Kim W-Y, Fujiwara S, Suh S-S, Kim J, Kim Y, Han L, David K, Putterill J, Nam HG,**
604 **Somers DE**. **2007**. ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue
605 light. *Nature* **449**: 356–360.

606 **Kölling K, Thalmann M, Müller A, Jenny C, Zeeman SC**. **2015**. Carbon partitioning in
607 Arabidopsis thaliana is a dynamic process controlled by the plants metabolic status and its
608 circadian clock. *Plant, Cell & Environment* **38**: 1965–1979.

609 **Langfelder P, Horvath S**. **2008**. WGCNA: an R package for weighted correlation network
610 analysis. *BMC bioinformatics* **9**: 559.

611 **Lembke CG, Nishiyama MY, Sato PM, de Andrade RF, Souza GM**. **2012**. Identification of
612 sense and antisense transcripts regulated by drought in sugarcane. *Plant Molecular Biology* **79**:
613 461–477.

614 **Liu TL, Newton L, Liu M-J, Shiu S-H, Farré EM**. **2016**. A G-Box-Like Motif Is Necessary for
615 Transcriptional Regulation by Circadian Pseudo-Response Regulators in Arabidopsis. *Plant*
616 *Physiology* **170**: 528–539.

617 **MacKinnon KJ-M, Cole BJ, Yu C, Coomey JH, Hartwick NT, Remigereau M-S, Duffy T,**
618 **Michael TP, Kay SA, Hazen SP**. **2020**. Changes in ambient temperature are the prevailing cue
619 in determining Brachypodium distachyon diurnal gene regulation. *New Phytologist* **227**: 1709–
620 1724.

621 **Martínez-García JF, Huq E, Quail PH**. **2000**. Direct Targeting of Light Signals to a Promoter
622 Element-Bound Transcription Factor. *Science* **288**: 859–863.

623 **Masclaux-Daubresse C, Valadier M-H, Carrayol E, Reisdorf-Cren M, Hirel B**. **2002**. Diurnal
624 changes in the expression of glutamate dehydrogenase and nitrate reductase are involved in the
625 C/N balance of tobacco source leaves. *Plant, Cell & Environment* **25**: 1451–1462.

626 **Matos DA, Cole BJ, Whitney IP, MacKinnon KJ-M, Kay SA, Hazen SP**. **2014**. Daily changes
627 in temperature, not the circadian clock, regulate growth rate in Brachypodium distachyon. *PloS*
628 *One* **9**: e100072.

629 **Matsuzaki J, Kawahara Y, Izawa T**. **2015**. Punctual transcriptional regulation by the rice
630 circadian clock under fluctuating field conditions. *The Plant Cell* **27**: 633–648.

631 **McCormick AJ, Cramer MD, Watt DA**. **2008**. Regulation of photosynthesis by sugars in
632 sugarcane leaves. *Journal of Plant Physiology* **165**: 1817–1829.

633 **Midmore DJ**. **1980**. Effects of photoperiod on flowering and fertility of sugarcane (Saccharum
634 spp.). *Field Crops Research* **3**: 65–81.

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

639 **Moore PH, Berding N**. **2013**. Flowering. In: Sugarcane: Physiology, Biochemistry, and
640 Functional Biology. John Wiley & Sons, Ltd, 379–410.

641 **Murphy RL, Klein RR, Morishige DT, Brady JA, Rooney WL, Miller FR, Dugas DV, Klein**
642 **PE, Mullet JE**. **2011**. Coincident light and clock regulation of pseudoresponse regulator protein
643 37 (PRR37) controls photoperiodic flowering in sorghum. *Proceedings of the National Academy*
644 *of Sciences of the United States of America* **108**: 16469–16474.

645 **Nagano AJ, Kawagoe T, Sugisaka J, Honjo MN, Iwayama K, Kudoh H**. **2019**. Annual
646 transcriptome dynamics in natural environments reveals plant seasonal adaptation. *Nature Plants*
647 **5**: 74.

Nagano AJ, Sato Y, Mihara M, Antonio BA, Motoyama R, Itoh H, Nagamura Y, Izawa T. **2012**. Deciphering and prediction of transcriptome dynamics under fluctuating field conditions. *Cell* **151**: 1358–1369.

Nakamichi N, Kiba T, Henriques R, Mizuno T, Chua N-H, Sakakibara H. **2010**. PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. *The Plant Cell* **22**: 594–605.

Ni Z, Kim E-D, Ha M, Lackey E, Liu J, Zhang Y, Sun Q, Chen ZJ. **2009**. Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. *Nature* **457**: 327–331.

Nueda MJ, Tarazona S, Conesa A. **2014**. Next maSigPro: updating maSigPro bioconductor package for RNA-seq time series. *Bioinformatics* **30**: 2598–2602.

Panter PE, Muranaka T, Cuitun-Coronado D, Graham CA, Yochikawa A, Kudoh H, Dodd AN. **2019**. Circadian Regulation of the Plant Transcriptome Under Natural Conditions. *Frontiers in Genetics* **10**.

Preston JC, Kellogg EA. **2006**. Reconstructing the Evolutionary History of Paralogous APETALA1/FRUITFULL-Like Genes in Grasses (Poaceae). *Genetics* **174**: 421–437.

Pyl E-T, Piques M, Ivakov A, Schulze W, Ishihara H, Stitt M, Sulpice R. **2012**. Metabolism and Growth in Arabidopsis Depend on the Daytime Temperature but Are Temperature-Compensated against Cool Nights. *The Plant Cell* **24**: 2443–2469.

R Core Team. **2021**. R: A language and environment for statistical computing.

Rao PS. **1977**. Effects of Flowering on Yield and Quality of Sugarcane. *Experimental Agriculture* **13**: 381–387.

669 **Rawat R, Takahashi N, Hsu PY, Jones MA, Schwartz J, Salemi MR, Phinney BS, Harmer**
670 **SL**. **2011**. REVEILLE8 and PSEUDO-REPONSE REGULATOR5 form a negative feedback
671 loop within the Arabidopsis circadian clock. *PLoS genetics* **7**: e1001350.

672 **Reshef N, Fait A, Agam N**. **2019**. Grape berry position affects the diurnal dynamics of its
673 metabolic profile. *Plant, Cell & Environment* **42**: 1897–1912.

674 **Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie A**. **2001**.
675 Metabolic profiling allows comprehensive phenotyping of genetically or environmentally
676 modified plant systems. *The Plant Cell* **13**: 11–29.

677 **Shibaya T, Hori K, Ogiso-Tanaka E, Yamanouchi U, Shu K, Kitazawa N, Shomura A, Ando**
678 **T, Ebana K, Wu J, *et al.* 2016**. Hd18, Encoding Histone Acetylase Related to Arabidopsis
679 FLOWERING LOCUS D, is Involved in the Control of Flowering Time in Rice. *Plant & Cell*
680 *Physiology* **57**: 1828–1838.

681 **Song YH, Kubota A, Kwon MS, Covington MF, Lee N, Taagen ER, Laboy Cintrón D,**
682 **Hwang DY, Akiyama R, Hodge SK, *et al.* 2018**. Molecular basis of flowering under natural
683 long-day conditions in Arabidopsis. *Nature Plants* **4**: 824–835.

684 **Steed G, Ramirez DC, Hannah MA, Webb AAR**. **2021**. Chronoculture, harnessing the
685 circadian clock to improve crop yield and sustainability. *Science* **372**.

686 **Tanaka N, Itoh H, Sentoku N, Kojima M, Sakakibara H, Izawa T, Itoh J-I, Nagato Y**. **2011**.
687 The COP1 Ortholog PPS Regulates the Juvenile–Adult and Vegetative–Reproductive Phase
688 Changes in Rice. *The Plant Cell* **23**: 2143–2154.

Webb AAR, Seki M, Satake A, Caldana C. **2019**. Continuous dynamic adjustment of the plant circadian oscillator. *Nature Communications* **10**: 550.

Weckwerth W, Wenzel K, Fiehn O. **2004**. Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. *Proteomics* **4**: 78–83.

Yazdanbakhsh N, Sulpice R, Graf A, Stitt M, Fisahn J. **2011**. Circadian control of root elongation and C partitioning in Arabidopsis thaliana. *Plant, Cell & Environment* **34**: 877–894.

Zhao J, Huang X, Ouyang X, Chen W, Du A, Zhu L, Wang S, Deng XW, Li S. **2012**. OsELF3-1, an Ortholog of Arabidopsis EARLY FLOWERING 3, Regulates Rice Circadian Rhythm and Photoperiodic Flowering. *PLOS ONE* **7**: e43705.

Supplemental Figure 1

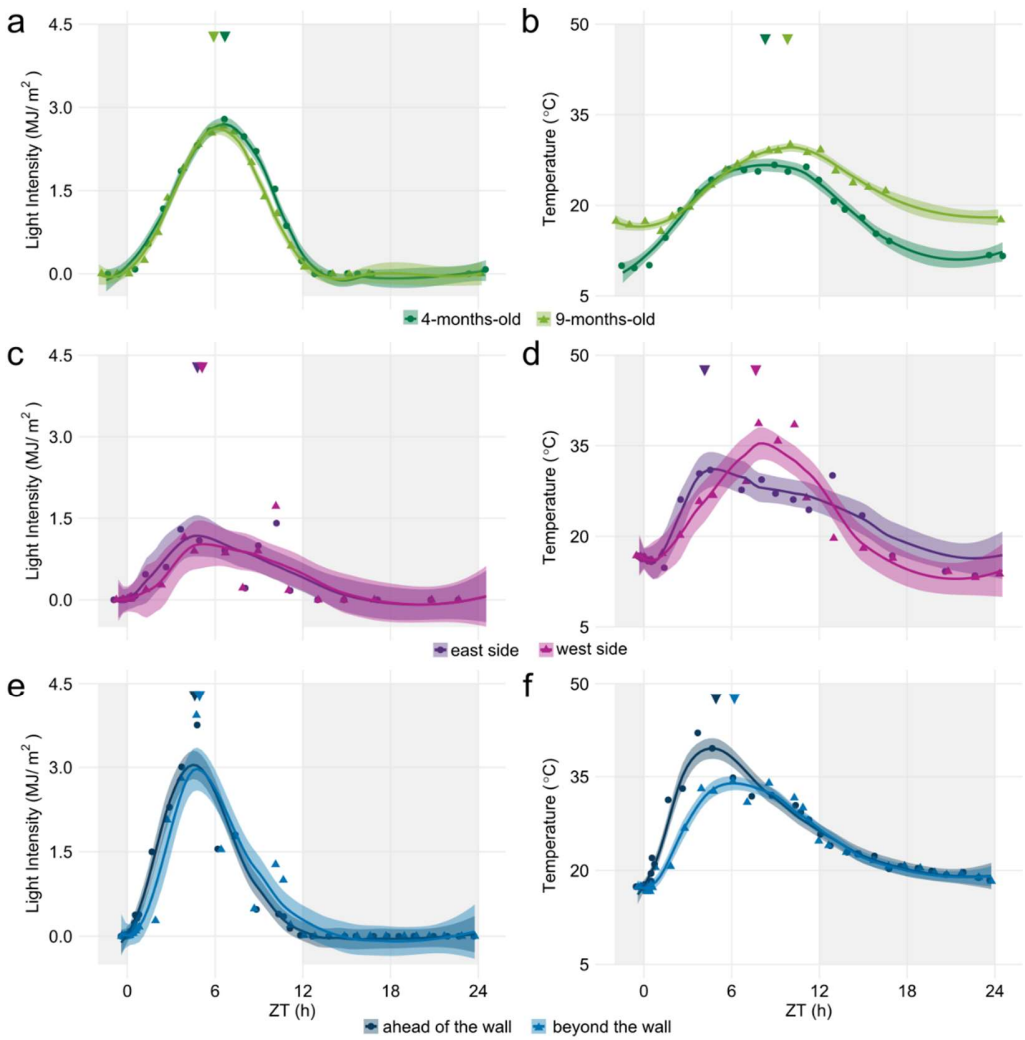
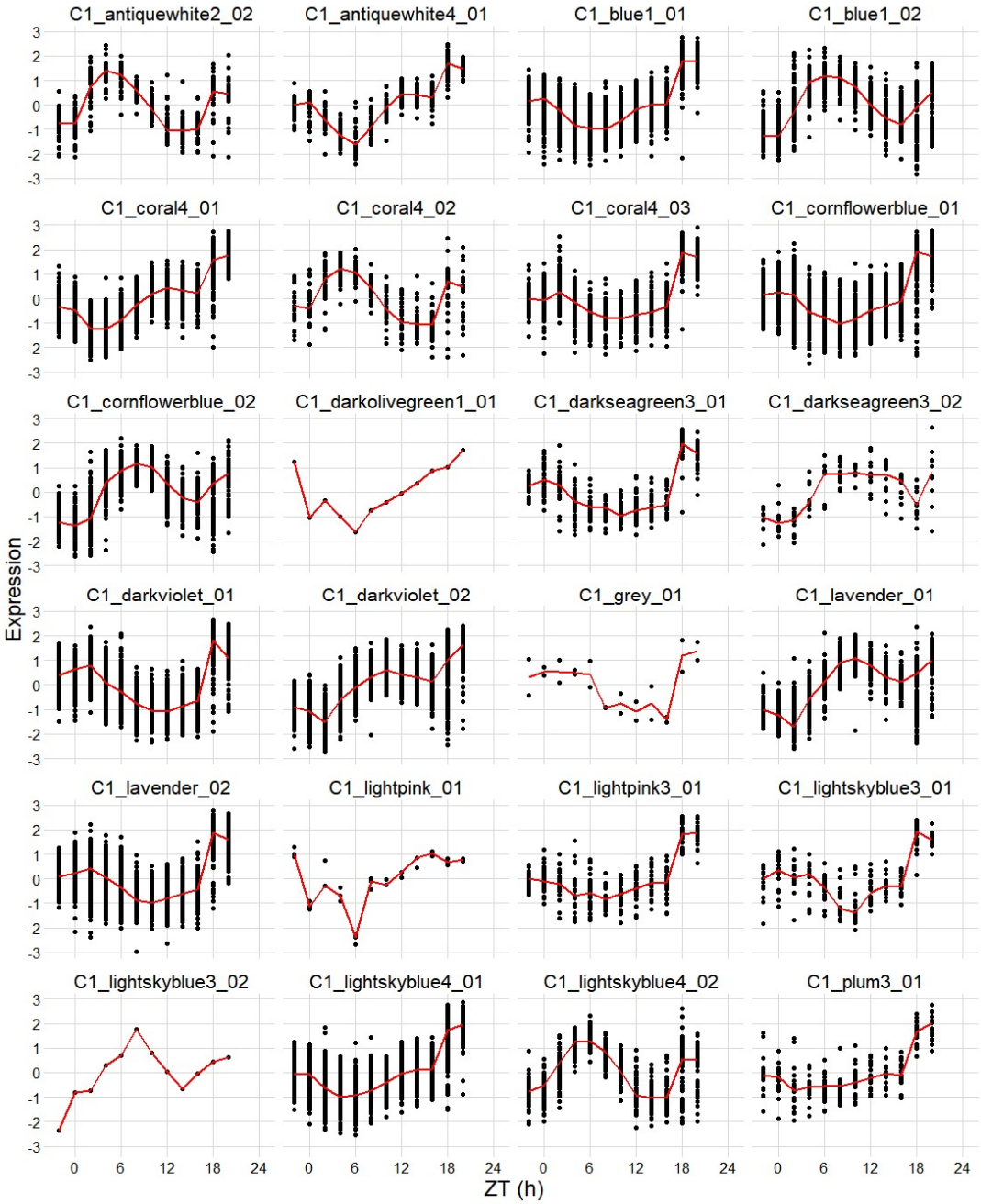


Fig. S1 – Environmental conditions in Araras (SP, Brazil). (a) Light intensity and **(b)** temperature on the day of harvesting 4 months old (4 mo) (green) and 9 mo (light green) sugarcane. Data was taken from a meteorological station nearby the sugarcane field, which underestimates microenvironmental changes. **(c)** Light intensity and **(d)** temperature on the day of harvesting 6 mo sugarcane in the east (purple) or the west side (pink) of the field. Data was taken from a handheld light meter and thermometer next to the plants inside the field. **(e)** Light intensity and **(f)** temperature on the day of harvesting 5 mo sugarcane in the east side of the field (dark blue) and sugarcane shaded by an artificial wall (light blue). Data was taken from a handheld light meter and thermometer next to the plants inside the field. All data and their LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the maximum value of the LOESS curve. To compare the rhythms of samples harvested in different seasons, the time of harvesting (ZT) was normalized to a photoperiod of 12 h day/ 12 h night. The light-grey boxes represent the night period.

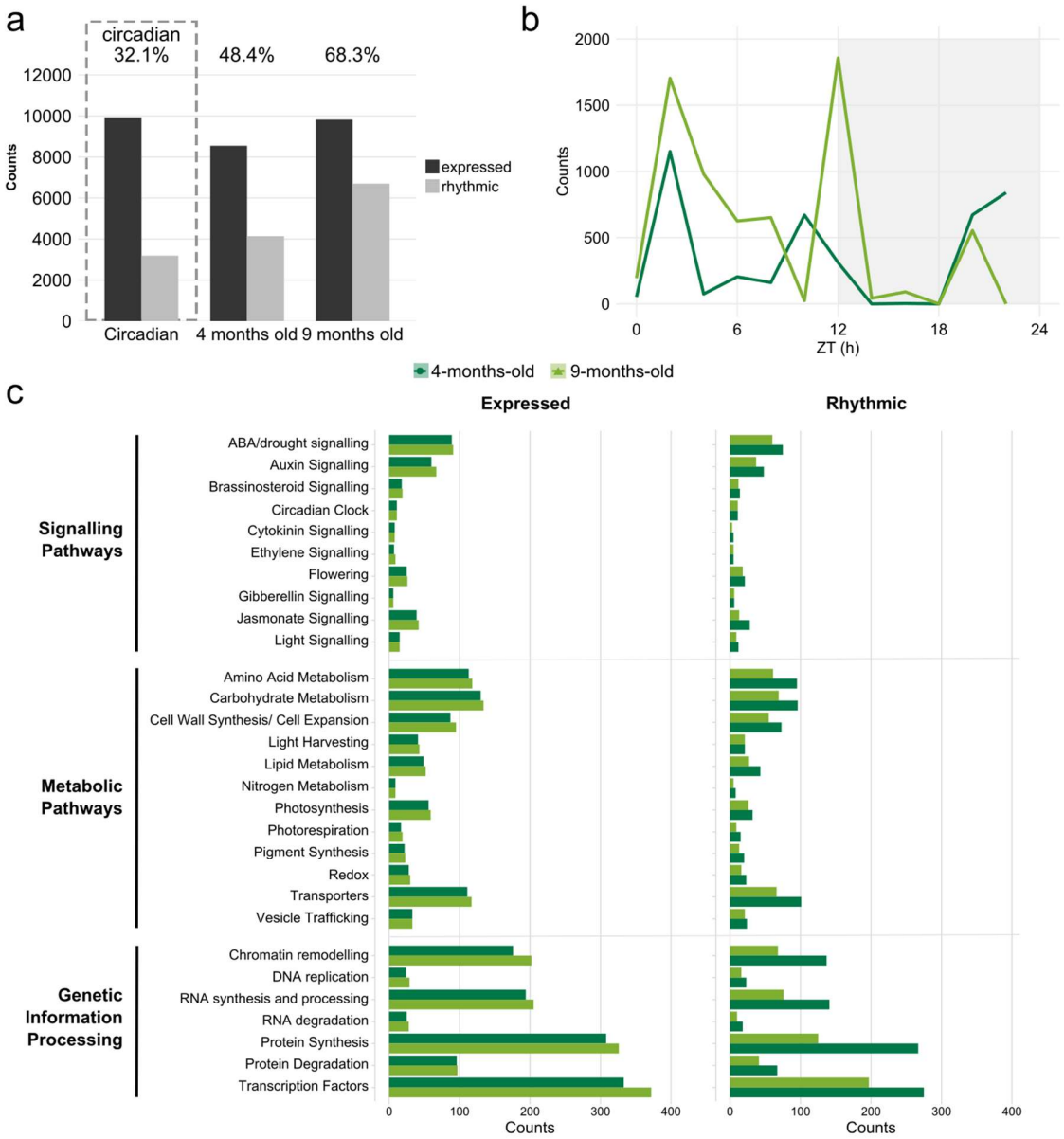
716 **Supplemental Figure 2**



717

718 **Fig. S2 – Rhythmic co-expression modules that were identified in 4-months-old sugarcane**
719 **leaves.** Weighted correlation network analysis was used to group co-expressed transcripts in
720 modules. Rhythmic modules were identified using JTK-cycle. The time course generated from
721 the median of all time points is in red.

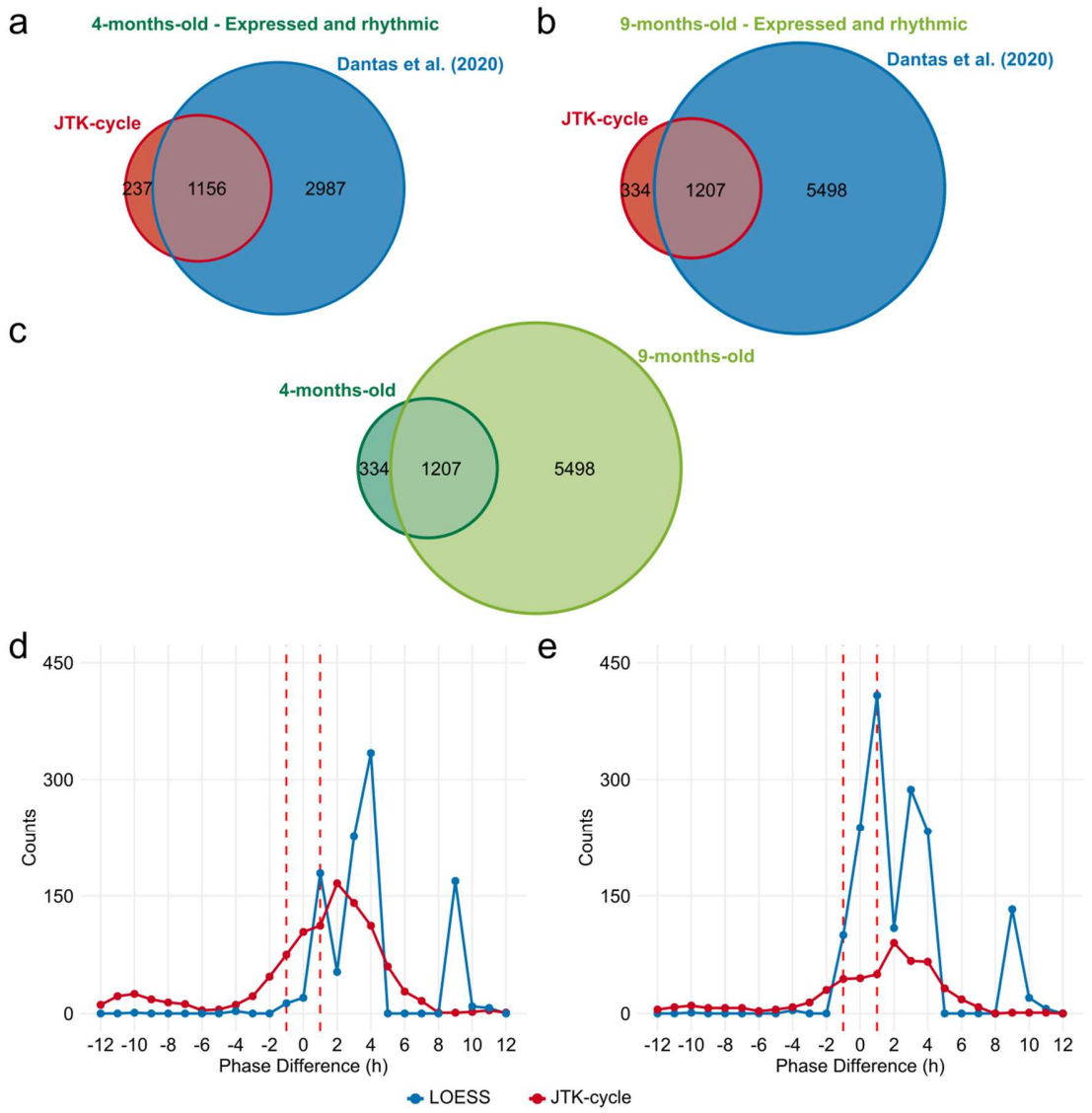
722 **Supplemental Figure 3**



723

724 **Fig. S3 –** Rhythmic co-expression modules that were identified in 4-months-old sugarcane
725 leaves. ~~9-months-old sugarcane has more rhythmic transcripts than 4-months-old~~
726 ~~sugarcane.~~ **(a)** The number of expressed and rhythmic transcripts detected in the leaves of 4-
727 months-old (4 mo) and 9 mo sugarcane in field-grown (diel) conditions and 3 mo leaves in
728 circadian conditions published in Hotta et al. (2013)(Hotta *et al.*, 2013). **(b)** Distribution of the
729 peak time of rhythmic transcripts in 4 mo and 9 mo sugarcane. **(c)** Distribution of the functional
730 categories of expressed and rhythmic transcripts in 4 mo (dark green) and 9 mo (light green)
731 sugarcane.

732 **Supplemental Figure 4**

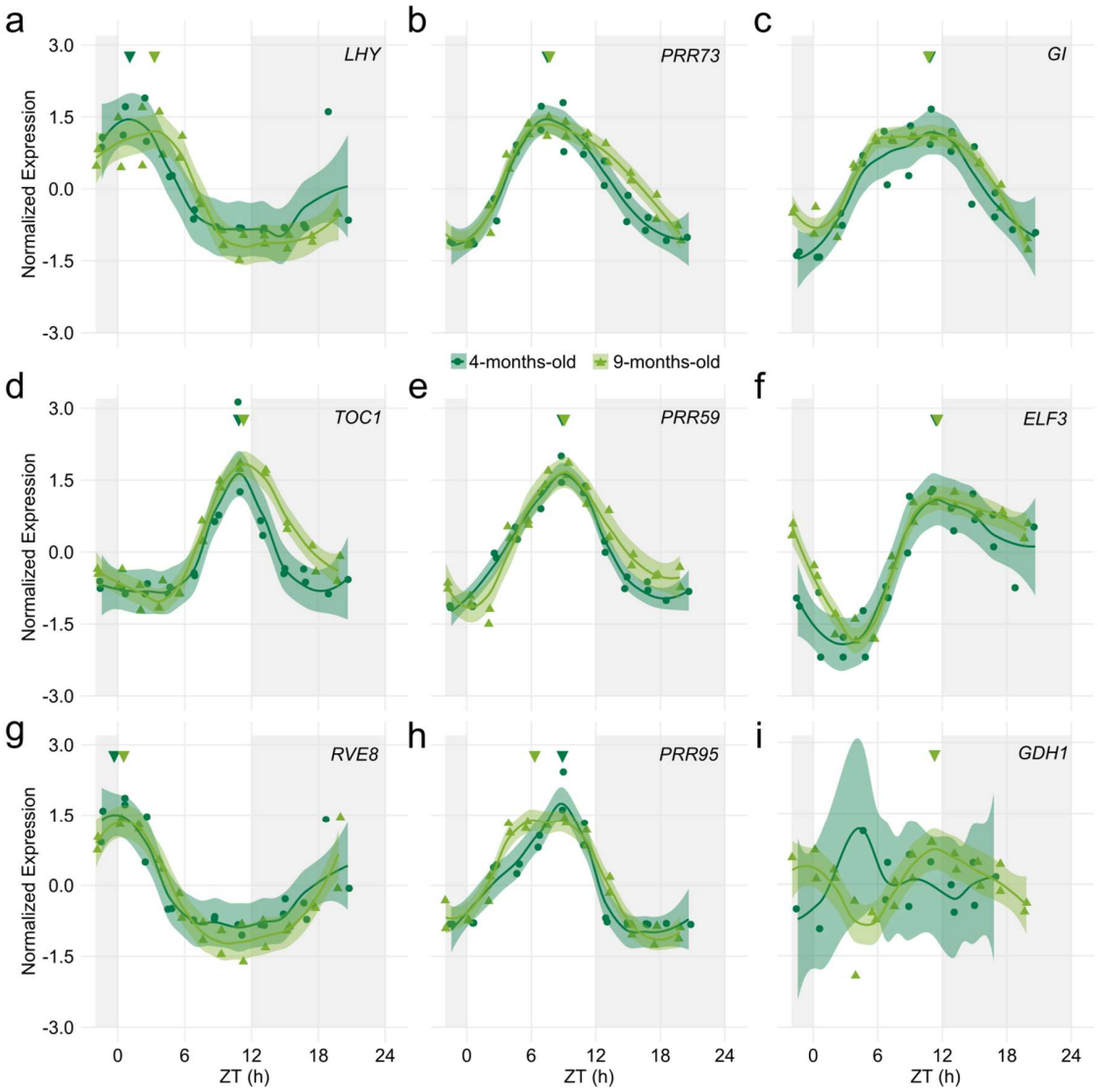


733

734 **Fig. S4 – Phase changes between rhythmic transcripts in 4-month-old and 9-month-old**
735 **sugarcane leaves. (a-b)** Rhythmic transcripts were identified using JTK-Cycle (blue) and
736 compared with the WGCNA and JTK-Cycle used in Dantas et al. (2020) transcripts (red) in **(a)** 4-
737 month-old (4 mo) and **(b)** 9 mo sugarcane leaves using Euler diagrams. **(c)** Euler diagram of the
738 rhythmic transcripts identified by JTK-Cycle in 4 mo (dark green) and 9 mo sugarcane leaves
739 (green). **(c-d)** Distribution of phase differences between rhythmic transcripts from 4 mo and 9 mo
740 sugarcane using **(d)** JTK-Cycle and **(e)** maSigPro. Phase differences were estimated using JTK-
741 Cycle (red) or LOESS (blue). Differences under ±1 h are not considered significant (red dashed
742 line).

743

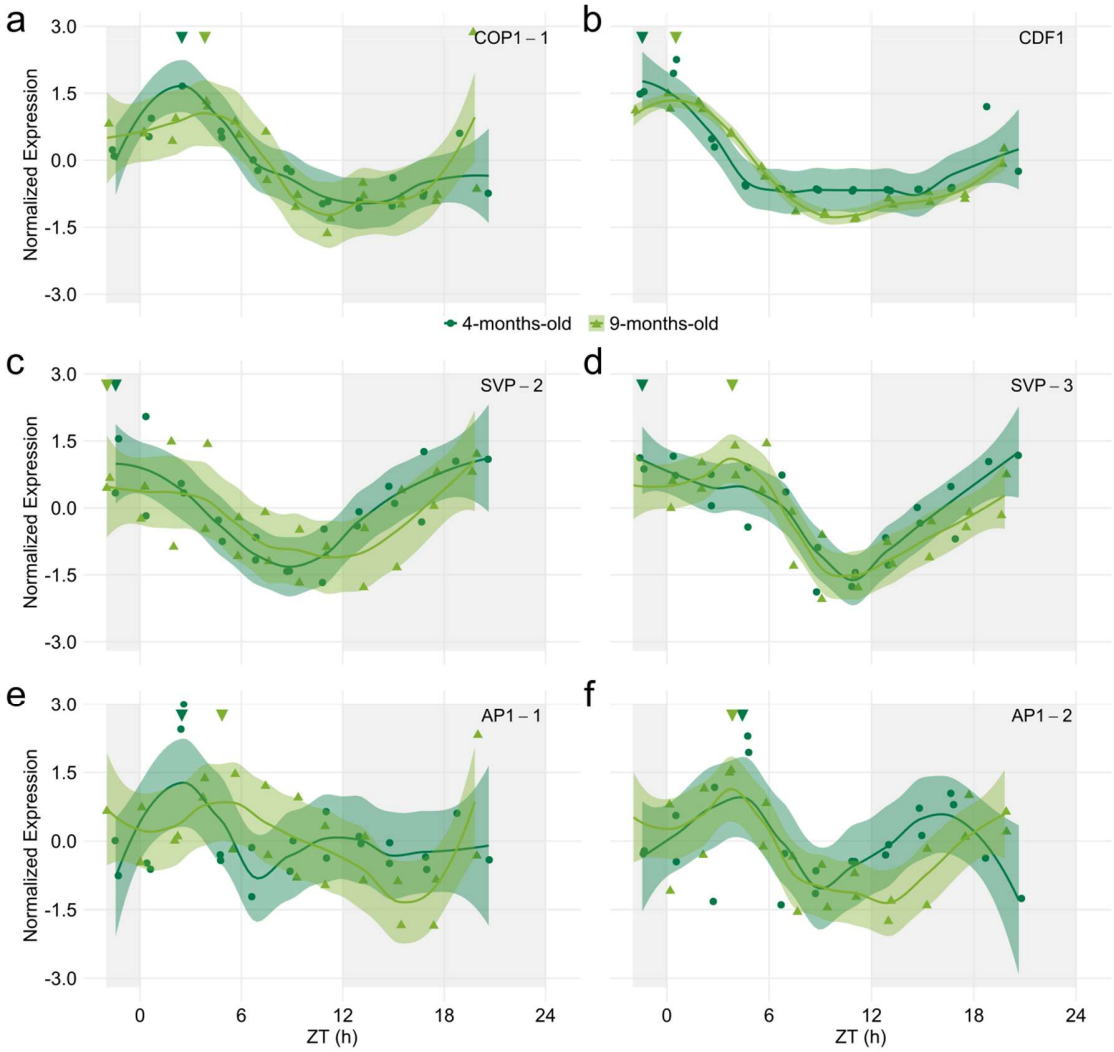
744 **Supplemental Figure 5**



745

746 **Fig. S5 – Rhythms of circadian clock genes and N metabolism genes in the leaves of 4**
747 **months old and 9 months old sugarcane.** Rhythms were measured using custom Agilent oligo
748 arrays in the leaves of 4 mo and 9 mo sugarcane grown in the field. All biological replicates and
749 their LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the
750 maximum value of the LOESS curve. Time series were normalized using Z-score. To compare
751 the rhythms of samples harvested in different seasons, the time of harvesting (ZT) was
752 normalized to a photoperiod of 12 h day/ 12 h night. The light-grey boxes represent the night
753 periods.

754 **Supplemental Fig. 6**

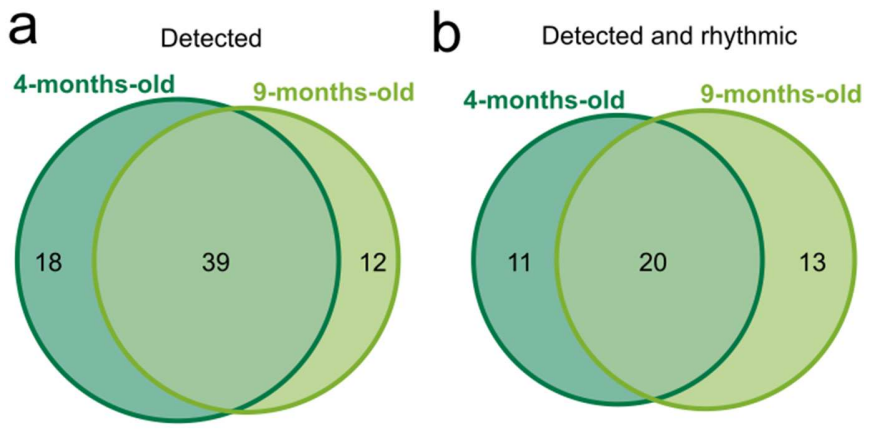


755

756 **Fig. S6 – Rhythms of genes associated with flowering in the leaves of 4 months old and 9**
757 **months old sugarcane.** Rhythms of **(a)** *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*ScCOP1*),
758 **(b)** *CYCLING DOF FACTOR1* (*ScCDF1*), **(c)** *SHORT VEGETATIVE-1* (*ScSVP*-2), **(d)** *ScSVP*-3,
759 **(e)** *APETALA1*-1 (*ScAP1*-1), and **(f)** *ScAP1*-2 were measured using custom Agilent oligo arrays
760 in the leaves of 4 mo and 9 mo sugarcane grown in the field. All biological replicates and their
761 LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the
762 maximum value of the LOESS curve. Time series were normalized using Z-score. To compare
763 the rhythms of samples harvested in different seasons, the time of harvesting (ZT) was
764 normalized to a photoperiod of 12 h day/ 12 h night. The light-grey boxes represent the night
765 periods.

766

767 **Supplemental Figure 7**



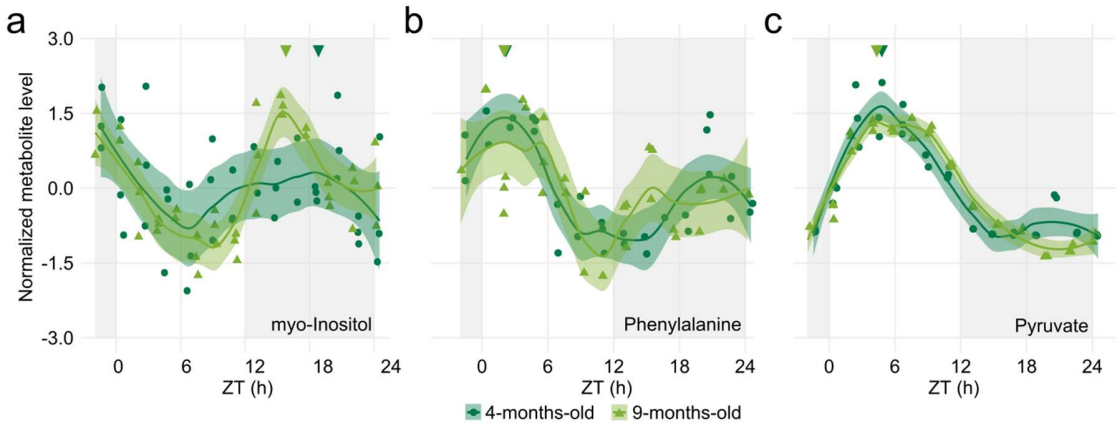
768

769 **Fig. S7 – Euler diagram of detected and rhythmic metabolites.** Euler diagrams of all **(a)**
770 detected metabolites and **(b)** rhythmic metabolites in 4 mo (dark green) and 9 mo (light green)
771 sugarcane leaves in diel conditions.

772

773

774 **Supplemental Fig. 8**



775

776 **Fig. S8 – Metabolite rhythms that had an early phase or no phase change in 9 months old**
777 **sugarcane compared to 4 months old sugarcane.** Leaf +1 of sugarcane grown for 4 months old
778 (4 mo, dark green) and 9 mo (light green) in the field were harvested for 26 h. Rhythms of **(a)**
779 myo-Inositol, **(b)** Phenylalanine and **(c)** Pyruvate in 4 mo and 9 mo Metabolite levels were
780 normalized with Z-score. All biological replicates (circles in 4 mo and triangles in 9 mo) and their
781 LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the
782 maximum value of the LOESS curve. To compare the rhythms of samples harvested in different
783 seasons, the time of harvesting (ZT) was normalized to a photoperiod of 12 h day/ 12 h night. The
784 light-grey boxes represent the night period.

785

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Supplemental Fig. 9

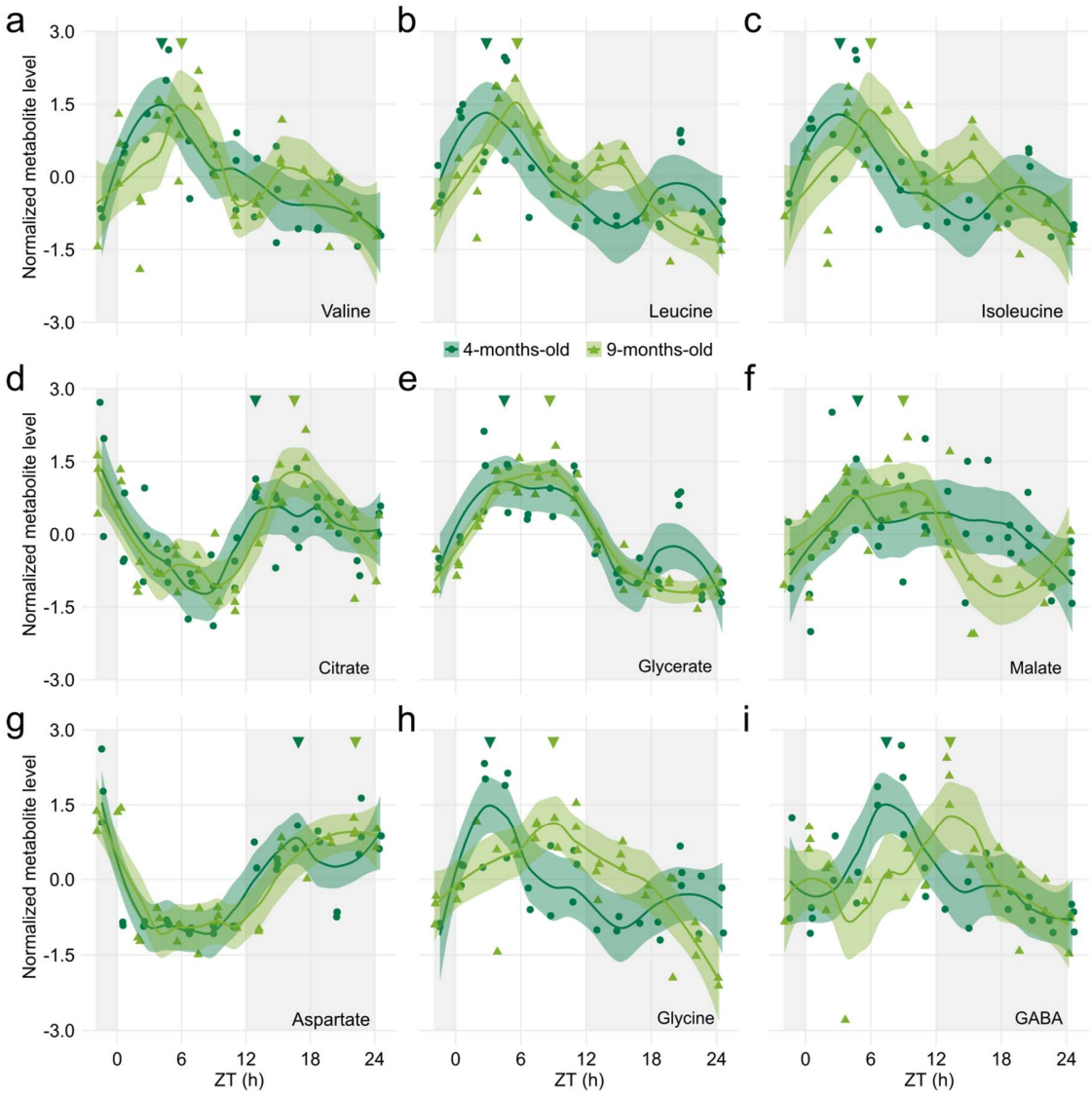


Fig. S9 – Metabolite rhythms that had a phase increase smaller or equivalent to 6 h in 9 months old sugarcane compared to 4 months old sugarcane. Leaf +1 of sugarcane grown for 4 months old (4 mo, dark green) and 9 mo (light green) in the field were harvested for 26 h. Rhythms of **(a)** Valine, **(b)** Leucine, **(c)** Isoleucine, **(d)** Citrate, **(e)** Glycerate, **(f)** Malate, **(g)** Aspartate, **(h)** Glycine, and **(i)** GABA in 4 mo and 9 mo sugarcane leaves. Metabolite levels were normalized with Z-score. All biological replicates (circles in 4 mo and triangles in 9 mo) and their LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the maximum value of the LOESS curve. To compare the rhythms of samples harvested in different seasons, the time of harvesting (ZT) was normalized to a photoperiod of 12 h day/ 12 h night. The light-grey boxes represent the night period.

Supplemental Figure 10

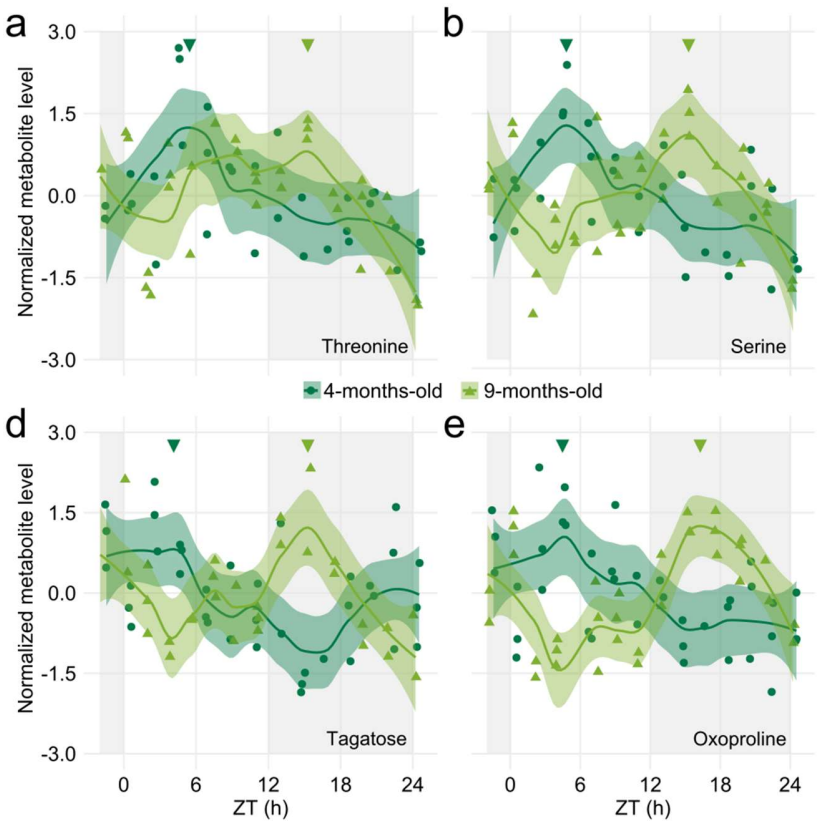
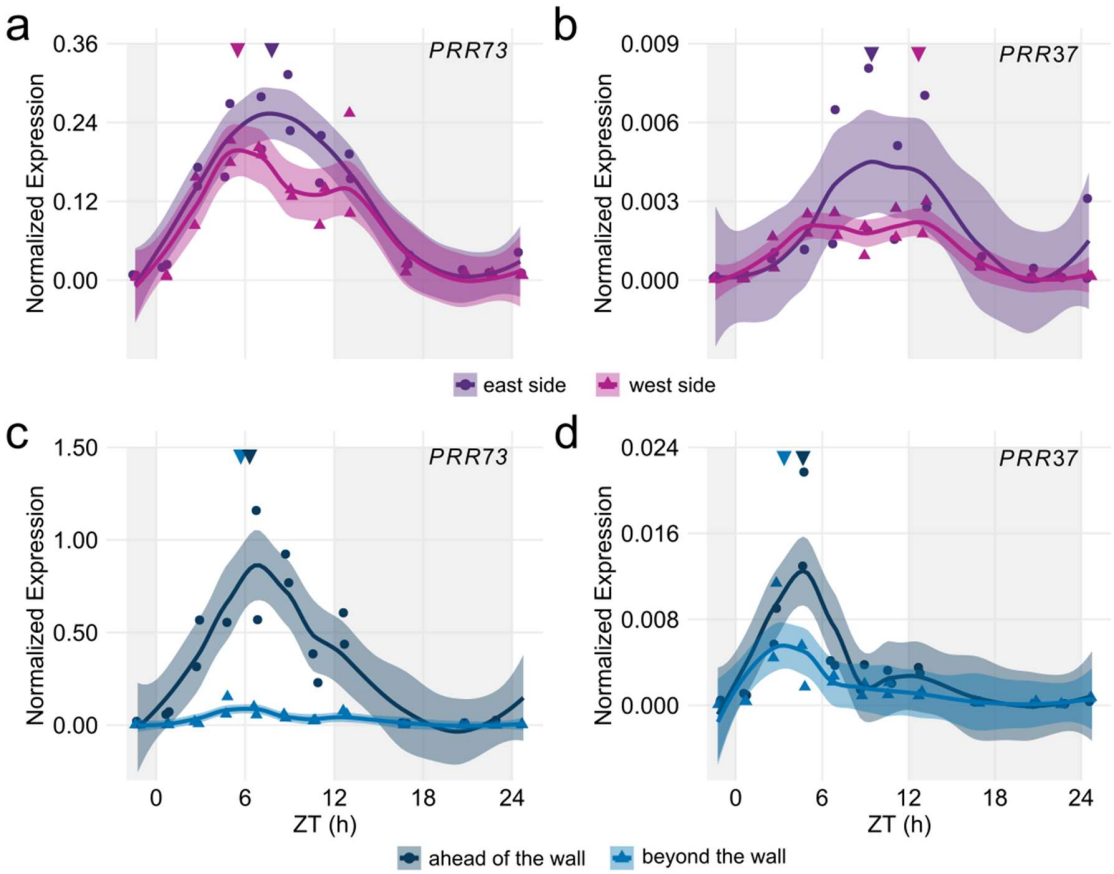


Fig. S10 – Metabolite rhythms that had a phase increase larger than 6 h in 9 months old sugarcane compared to 4 months old sugarcane. Leaf +1 of sugarcane grown for 4 months old (4 mo, dark green) and 9 mo (light green) in the field were harvested for 26 h. Rhythms of **(a)** Threonine, **(b)** Serine, **(c)** Tagatose and **(d)** Oxoproline in 4 mo and 9 mo Metabolite levels were normalized with Z-score. All biological replicates (circles in 4 mo and triangles in 9 mo) and their LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the maximum value of the LOESS curve. To compare the rhythms of samples harvested in different seasons, the time of harvesting (ZT) was normalized to a photoperiod of 12 h day/ 12 h night. The light-grey boxes represent the night period.

811 **Supplemental Figure 11**



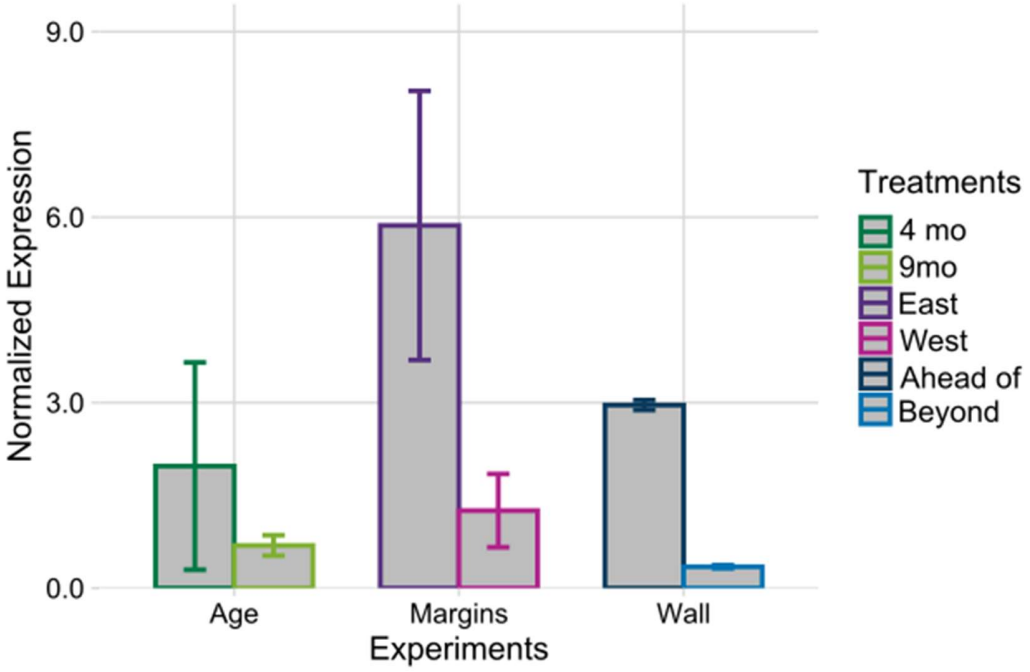
812

813 **Fig. S11 – Sugarcane leaves have different *ScPRR73* and *ScPRR37* patterns when shaded at**
814 **dawn. (a-b)** Diel rhythms of **(a)** *PSEUDO-RESPONSE REGULATOR 73* (*ScPRR73*) and **(b)**
815 *ScPRR37* in the leaves of sugarcane grown on the east side (purple) and the west side (pink) of
816 the field. **(c-d)** Diel rhythms of **(c)** *ScPRR73* and **(d)** *ScPRR37* in the leaves of sugarcane grown
817 ahead of the wall (dark blue) and beyond the wall (light blue). All biological replicates and their
818 LOESS curve (continuous lines ± SE) are shown. Inverted triangles show the time of the
819 maximum value of the LOESS curve. Time series were normalized using Z-score. To compare
820 the rhythms of samples harvested in different seasons, the time of harvesting (ZT) was
821 normalized to a photoperiod of 12 h day/ 12 h night. The light-grey boxes represent the night
822 periods. Transcript levels were measured using RT-qPCR.

823

824

825 **Supplemental Figure 12**



826

827 **Fig. S12 – Transcript levels of *ScLHY* in the first hours of the morning.** In the Age
828 experiment, 4-months-old (dark green) and 9-months-old (light leaves) sugarcane leaves were
829 used. In the Margins experiment, sugarcane leaves from the East side (purple) or the West side
830 (pink) of the sugarcane field were taken. In the Wall experiment, sugarcane leaves were taken
831 from plants ahead of the wall (dark blue) and beyond the wall (light blue) on the east side of the
832 field. Sugarcane leaves were harvested between ZT0 and ZT2 (n=3). Transcript levels were
833 measured using RT-qPCR. Relative expression was determined using *GLYCERALDEHYDE-3-*
834 *PHOSPHATE DEHYDROGENASE* (*ScGAPDH*).

835

836 **Table S1 – Sugarcane genes and GenBank IDs.**

Gene Symbol	Gene Name	SAS*	GenBank ID
AP1	*APETALA1*	SCQGLR1085G10.g	CA124279.1
CDF1	*CYCLING DOF FACTOR1*	SCMCCL6053D03.g	CA098046.1
COP1	*CONSTITUTIVE PHOTOMORPHOGENIC 1*	SCCCCL6003C08.g	CA096699.1
ELF3	*EARLY-FLOWERING 3*	SCEZLB1009F09.g	CA113166.1
FLD	*FLOWERING LOCUS D*	SCEQRT2090F09.g	CA138853.1
FT	*FLOWERING LOCUS T*	SCBFSD2035E11.g	CA278114.1
GAPDH	*GLYCERALDEHYDE-3-PHOSPHATE*	SCQGAM2027G09.g	CA086777.1
GI	*GIGANTEA*	SCJFAD1014B07.b	CA067312.1
LHY	*LATE ELONGATED HYPOCOTYL*	SCCCLR1048E10.g	CA167119.1
PRR37	*PSEUDO-RESPONSE REGULATOR 37*	Contig 0151209	-
PRR59	*PSEUDO-RESPONSE REGULATOR 59*	SCACLR1057G02.g	CA116370.1
PRR73	*PSEUDO-RESPONSE REGULATOR 73*	SCACLR1057C07.g	CA116387.1
PRR95	*PSEUDO-RESPONSE REGULATOR 95*	SCCCLR1077F09.g	CA120437.1
RVE8	*REVEILLE 8*	SCSGST1070F10.g	CA179134.1
SVP-1	*SHORT VEGETATIVE-1*	SCCCLR1C05A05.g	CA189804.1
SVP-2	*SHORT VEGETATIVE-2*	SCCCLR1072F01.g	CA119595.1
SVP-3	*SHORT VEGETATIVE-3*	SCCCLR2001F11.g	CA127026.1
SOC1	*SUPPRESSOR OF CONSTANS OVEREXPRESSION 1*	SCCCLR2C03H07.g	CA127525.1
TOC1	*TIME OF CAB EXPRESSION 1*	SCCCSB1002H04.g	CA167119.1

837 * Sugarcane Assembled Sequence.

838

839 **Table S2 – Sugarcane primers pairs used to validate oligo arrays expression levels using**
840 **RT-qPCR.**

Gene Symbol	SAS	Oligonucleotide sequence (5' -> 3')	Reference
ScGAPDH	SCQGAM2027G09.g	FWD CACGGCCACTGGAAGCA RVS TCCTCAGGGTTCCTGATGCC	Papini-Terzi et al. (2005)[a]
ScLHY	SCCCLR1048E10.g	FWD CCACCACGGCCTAAAAGAAA RVS TGGTTTTGTTGACTTGTCATTTGG	Hotta et al. (2011)b
ScPRR37	Contig 0151209	FWD CCCTGCTATGTGGTTCCTC RVS ATTCTGCCCCTTGGTTCC	
ScPRR73	SCACLR1057C07.g	FWD CAAGTAATTCACCCCAAATCAGAGATA RVS TCCCATAGATTCATCTTTATTCTCCTTAT	
ScTOC1	SCCCSB1002H04.g	FWD TTCTGCCTGAATTTGGCAAGTG RVS GGCATCGAGCACACCAATGC	Hotta et al. (2011)b

[a] Papini-Terzi et al. (2005) *DNA Research* 12:27, https://doi.org/10.1093/dnares/12.1.27

[b] Hotta et al. (2013) *PloS one* 8:e71847, https://doi.org/10.1371/journal.pone.0071847

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844