1 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

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

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

Here we have measured metabolic and transcriptional rhythms in sugarcane leaves grown in the field. We show that transcript and metabolite rhythms in 4-months-old (4 mo) sugarcane have 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 Eutroferic 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, $ZT = 12 \cdot T \cdot Pd^{-1}$, 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, $ZT = 12 + 12 \cdot (T - Pd) \cdot Pn^{-1}$, 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

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

Oligoarray hybridizations

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

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

Data analysis

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

RT-qPCR analysis

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

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

Results

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

between 18.0 h and 20.0 h in 4 mo sugarcane (Fig. 1b). In contrast, *ScTOC1* peaked at 11.0 h and 11.3 h after dawn in 4 mo and 9 mo sugarcane leaves, respectively (Fig. 1b). All plants were harvested from the middle of the field to avoid margin effects.

Fig. 1. Sugarcane leaves have different *ScLHY* phases in field-grown sugarcane. (a) Leaves from sugarcane grown for 4 months old (mo) (upper photo) and 9 mo (lower photo) in the field were harvested for 26 h. Bar = 0.5 m. (b) Leaves from sugarcane grown for 4 mo and 9 mo in the field were harvested for 26 h. (A) Diel rhythms of *LATE ELONGATED HYPOCOTYL* (*ScLHY*) and *TIME OF CAB EXPRESSION 1* (*ScTOC1*) in the leaves of 4 mo (dark green) and 9 mo (light green) sugarcane leaves. When sugarcane is 4 months old (left), there is little shading between plants. In comparison, 9 mo sugarcane shade each other when the sun is at a low angle (right). Bar = 0.5 m. All biological replicates and their LOESS curve (continuous lines $\pm$ 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*).

To check if other transcriptional rhythms showed a peak change similar to *ScLHY*, we used custom Agilent oligoarrays to measure transcript accumulation in 4 mo sugarcane leaves. We

used the transcriptomic data to detect rhythms in 4 mo sugarcane leaves using WGCNA to generate co-expression modules and JTK-Cycle to identify rhythmic modules (Fig. S2). There were 8,553 expressed transcripts in 4 mo sugarcane leaves, 86% of the expressed transcripts found in 9 mo sugarcane leaves (9,891) (Fig. 2a). We considered 4,143 of the expressed transcripts were rhythmic in 4 mo sugarcane leaves (48.4%), lower than in 9 mo sugarcane leaves (68.3%, Fig. S3a). About half of the transcripts that were rhythmic in both 4 mo and 9 mo sugarcane leaves peaked earlier in the older plants (51.5%), whilst 48.2% peaked within 1 h of each other (1.65 ± 1.8 h, median $\pm$ MA, $n = 4,110$) (Fig. 2b). Thus, the phase changes can be seen in transcripts peaking during the whole 24 h cycle, and not just at the same time as *ScLHY* peaks (Fig. S3b). These phase changes were also observed using only JTK-Cycle to identify rhythmic transcripts with phase estimates using JTK_Cycle (1.56 ± 1.9 h, median $\pm$ MA, $n = 1,014$) or using LOESS (3.62 ± 0.4 h, median $\pm$ MA, $n = 1014$) (Figs S4a-d). If only transcripts with significant gene expression profile differences identified with maSigPro were used, the phases differences were 1.65 ± 1.7 h (median $\pm$ MA, $n = 1,539$) using LOESS, and 1.55 ± 2.1 h (median $\pm$ MA, $n = 527$) using JTK-Cycle (Fig. S4e).

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

(light green) or in 9 mo sugarcane leaves (dark green). The dotted lines represent the proportion of rhythmic transcripts among all annotated transcripts. **(d)** *FLOWERING LOCUS D* (*ScFLD*), **(e)** *SHORT VEGETATIVE-1* (*ScSVP-1*), **(f)** *SUPPRESSOR OF CONSTANS OVEREXPRESSION* (*ScSOC1*), and **(g)** *FLOWERING LOCUS T* (*ScFT*) rhythms measured in 4 mo and 9 mo sugarcane. All biological replicates and their LOESS curve (continuous lines $\pm$ 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.

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

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

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

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

(dark green) and 9 mo plants (light green). **(g)** When sugarcane is 4 months old (left), there is little shading between plants. In comparison, 9 mo sugarcane shade each other when the sun is at a low angle (right). All biological replicates (circles in 4 mo or triangles in 9 mo) and their LOESS curve (continuous lines $\pm$ SE) are shown. Inverted triangles show the time of the maximum value of the LOESS curve. Metabolite levels were normalized with 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 period.

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

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

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

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

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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 $\pm$ 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*).

Discussion

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

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

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

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

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

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

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

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

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

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

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

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Author Contributions

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

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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 $\pm$ 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.

Supplemental Figure 4

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

Supplemental Figure 5

Fig. S5 – Rhythms of circadian clock genes and N metabolism genes in the leaves of 4 months old and 9 months old sugarcane. Rhythms were measured using custom Agilent oligo arrays in the leaves of 4 mo and 9 mo sugarcane grown in the field. All biological replicates and their LOESS curve (continuous lines $\pm$ 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.

Supplemental Fig. 6

Fig. S6 – Rhythms of genes associated with flowering in the leaves of 4 months old and 9 months old sugarcane. Rhythms of (a) *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*ScCOP1*), (b) *CYCLING DOF FACTOR1* (*ScCDF1*), (c) *SHORT VEGETATIVE-1* (*ScSVP-2*), (d) *ScSVP-3*, (e) *APETALA1-1* (*ScAPI-1*), and (f) *ScAPI-2* were measured using custom Agilent oligo arrays in the leaves of 4 mo and 9 mo sugarcane grown in the field. All biological replicates and their LOESS curve (continuous lines $\pm$ 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.

Supplemental Figure 7

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

Supplemental Fig. 8

Fig. S8 – Metabolite rhythms that had an early phase or no phase change 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) myo-Inositol, (b) Phenylalanine and (c) Pyruvate 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 $\pm$ 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.

788 **Supplemental Fig. 9**

789

790 **Fig. S9 – Metabolite rhythms that had a phase increase smaller or equivalent to 6 h in 9**
791 **months old sugarcane compared to 4 months old sugarcane.** Leaf +1 of sugarcane grown for 4
792 months old (4 mo, dark green) and 9 mo (light green) in the field were harvested for 26 h.
793 Rhythms of (a) Valine, (b) Leucine, (c) Isoleucine, (d) Citrate, (e) Glycerate, (f) Malate, (g)
794 Aspartate, (h) Glycine, and (i) GABA in 4 mo and 9 mo sugarcane leaves. Metabolite levels were
795 normalized with Z-score. All biological replicates (circles in 4 mo and triangles in 9 mo) and their
796 LOESS curve (continuous lines $\pm$ SE) are shown. Inverted triangles show the time of the
797 maximum value of the LOESS curve. To compare the rhythms of samples harvested in different
798 seasons, the time of harvesting (ZT) was normalized to a photoperiod of 12 h day/ 12 h night. The
799 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 $\pm$ 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 11

Fig. S11 – Sugarcane leaves have different *ScPRR73* and *ScPRR37* patterns when shaded at dawn. (a-b) Diel rhythms of (a) *PSEUDO-RESPONSE REGULATOR 73* (*ScPRR73*) and (b) *ScPRR37* in the leaves of sugarcane grown on the east side (purple) and the west side (pink) of the field. (c-d) Diel rhythms of (c) *ScPRR73* and (d) *ScPRR37* in the leaves of sugarcane grown ahead of the wall (dark blue) and beyond the wall (light blue). All biological replicates and their LOESS curve (continuous lines $\pm$ 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.

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	Gene Name	SAS*	GenBank ID
Symbol			
<i>API</i>	<i>APETALA1</i>	SCQGLR1085G10.g	CA124279.1
<i>CDF1</i>	<i>CYCLING DOF FACTOR1</i>	SCMCCL6053D03.g	CA098046.1
<i>COP1</i>	<i>CONSTITUTIVE PHOTOMORPHOGENIC 1</i>	SCCCCL6003C08.g	CA096699.1
<i>ELF3</i>	<i>EARLY-FLOWERING 3</i>	SCEZLB1009F09.g	CA113166.1
<i>FLD</i>	<i>FLOWERING LOCUS D</i>	SCEQRT2090F09.g	CA138853.1
<i>FT</i>	<i>FLOWERING LOCUS T</i>	SCBFSD2035E11.g	CA278114.1
<i>GAPDH</i>	<i>GLYCERALDEHYDE-3-PHOSPHATE</i>	SCQGAM2027G09.g	CA086777.1
<i>GI</i>	<i>GIGANTEA</i>	SCJFAD1014B07.b	CA067312.1
<i>LHY</i>	<i>LATE ELONGATED HYPOCOTYL</i>	SCCCLR1048E10.g	CA167119.1
<i>PRR37</i>	<i>PSEUDO-RESPONSE REGULATOR 37</i>	Contig 0151209	-
<i>PRR59</i>	<i>PSEUDO-RESPONSE REGULATOR 59</i>	SCACLR1057G02.g	CA116370.1
<i>PRR73</i>	<i>PSEUDO-RESPONSE REGULATOR 73</i>	SCACLR1057C07.g	CA116387.1
<i>PRR95</i>	<i>PSEUDO-RESPONSE REGULATOR 95</i>	SCCCLR1077F09.g	CA120437.1
<i>RVE8</i>	<i>REVEILLE 8</i>	SCSGST1070F10.g	CA179134.1
<i>SVP-1</i>	<i>SHORT VEGETATIVE-1</i>	SCCCLR1C05A05.g	CA189804.1
<i>SVP-2</i>	<i>SHORT VEGETATIVE-2</i>	SCCCLR1072F01.g	CA119595.1
<i>SVP-3</i>	<i>SHORT VEGETATIVE-3</i>	SCCCLR2001F11.g	CA127026.1
<i>SOC1</i>	<i>SUPPRESSOR OF CONSTANS OVEREXPRESSION 1</i>	SCCCLR2C03H07.g	CA127525.1
<i>TOC1</i>	<i>TIME OF CAB EXPRESSION 1</i>	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
<i>ScGAPDH</i>	SCQGAM2027G09.g	FWD CACGGCCACTGGAAGCA RVS TCCTCAGGGTTCCTGATGCC	Papini-Terzi et al. (2005) ^a
<i>ScLHY</i>	SCCCLR1048E10.g	FWD CCACCACGGCCTAAAAGAAA RVS TGGTTTTGTTGACTTGTCATTTGG	Hotta et al. (2011) ^b
<i>ScPRR37</i>	Contig 0151209	FWD CCCTGCTATGTGGTTCCTC RVS ATTCTGCCCCTTGTTCC	
<i>ScPRR73</i>	SCACLR1057C07.g	FWD CAAGTAATTCACCCCAAATCAGAGATA RVS TCCCATAGATTCATCTTTATTCTCCTTAT	
<i>ScTOC1</i>	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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