Alternative splicing of circadian clock genes correlates with temperature in field-grown sugarcane

Running title: Rhythms of alternative splicing in sugarcane correlate with temperature

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Abstract

Alternative Splicing (AS) is a mechanism that generates different mature transcripts from precursor mRNAs (pre-mRNAs) of the same gene. In plants, a wide range of physiological and metabolic events are related to AS, as well as fast responses to changes in temperature. AS is present in around 60% of intron-containing genes in Arabidopsis, 46% in rice and 38% in maize and it is widespread among the circadian clock genes. Little is known about how AS influences the circadian clock of C4 plants, like commercial sugarcane, a C4 crop with a complex hybrid genome. This work aims to test if the daily dynamics of AS forms of circadian clock genes are regulated by environmental factors, such as temperature, in the field. A systematic search for AS in five sugarcane clock genes, *ScLHY*, *ScPRR37*, *ScPRR73, ScPRR95* and *ScTOC1* using different organs of sugarcane sampled during winter, with 4 months old plants, and during summer, with 9 months old plants, revealed temperature- and organ-dependent expression of at least one alternatively spliced isoform in all genes. Expression of AS isoforms varied according to the season. Our results suggest that AS events in circadian clock genes are correlated with temperature.

Keywords: alternative splicing, circadian clock, diel rhythms, field experiment, gene expression, sugarcane

1. Introduction

During gene expression in a eukaryotic cell, pre-mRNAs undergo splicing to remove introns and join exons in a mature transcript, generating an open reading frame (ORF) for protein synthesis. Splicing is largely co-transcriptional in yeast, *Drosophila melanogaster*, mammals and *Arabidopsis thaliana* (L.) Heynth. (Beyer and Osheim, 1988; Oesterreich et al., 2016; Saldi et al., 2016; Godoy Herz et al., 2019; Jabre et al., 2019). Alternative splicing (AS) is a mechanism that generates different RNAm transcripts from a single gene. As a result of AS, the mature mRNAs represent another level of gene expression regulation at the post-transcriptional level by, for example, insertion of premature termination codons (PTC), which can target some AS isoforms to degradation by the nonsense-mediated mRNA decay (NMD) pathway (Filichkin and Mockler, 2012; Kalyna et al., 2012; Marquez et al., 2012). Alternatively, those transcripts carrying PTCs could produce truncated polypeptides missing functional domains and motifs that can compete with the corresponding functional protein (Mastrangelo et al., 2012; Reddy et al., 2013; Seo et al., 2011). In addition, AS can increase protein by producing mRNAs from the same gene that encode protein variants with different diversity in function, localization, and stability (Chaudhary et al., 2019; Syed et al., 2012). AS is a ubiquitous process observed from *Drosophila* to humans and plants (Filichkin et al., 2010; Graveley, 2005; Kornblihtt et al., 2013; Marquez et al., 2012). In plants, a wide range of physiological and metabolic events and responses are related to AS. This mechanism is so widespread that it was reported in more than 60% of intron-containing genes in Arabidopsis, 46% in rice and 38% in maize (Chamala et al., 2015; Filichkin et al., 2015c; Marquez et al., 2012; Min et al., 2015; Staiger and Brown, 2013a; Thatcher et al., 2014; Zhang et al., 2010) . There is evidence of organ and tissue-specific alternative transcript forms and even alternative transcript isoforms in different subcellular locations

65 (Kriechbaumer et al., 2012; Nagashima et al., 2011; Remy et al., 2013; Vaneechoutte et
66 al., 2017). AS impacts development, from the early gametic cell specification to the seed
67 maturation (Fouquet et al., 2011; Liu et al., 2009; Moll et al., 2008; Sugliani et al., 2010;
68 Szakonyi and Duque, 2018; Zhang et al., 2016) and even flowering time and floral
69 development (Rosloski et al., 2013; Severing et al., 2012; Zhang et al., 2011). Both biotic
70 and abiotic stress responses are also closely related to AS (Shang et al., 2017a; Staiger
71 and Brown, 2013a; Wang et al., 2018). Plants under stress conditions change their AS
72 patterns dramatically (Calixto et al., 2018, 2019; Ding et al., 2014; Filichkin et al., 2015a;
73 Palusa et al., 2007; Staiger and Brown, 2013a). Also, many circadian clock genes generate
74 alternative transcript forms with PTCs under different environmental conditions (Calixto
75 et al., 2016; Filichkin et al., 2010, 2015b; James et al., 2012c, 2012a; Jones et al., 2012).
76 The presence of alternative transcripts in the circadian clock genes is highly conserved
77 among different plant species, such as Arabidopsis, *Populus alba* L., *Brachypodium*
78 *distachyon* (L.) P. Beauv., and rice (*Oryza sativa* L.) – all C3 plants (Filichkin and
79 Mockler, 2012). Little is known about how AS influences the circadian clock of C4 plants.

80 The circadian clock is a 24 h endogenous timekeeper mechanism that anticipates the
81 Earth's day/night and seasonal cycles (Hsu and Harmer, 2014; McClung, 2019; Millar,
82 2016). Like AS, the circadian clock is associated with growth, photosynthesis, and
83 biomass in plants, so these two regulatory mechanisms may act together, or even regulate
84 each other (Dodd et al., 2005; Harmer, 2009; Lai et al., 2012; Lu et al., 2005; Staiger and
85 Brown, 2013a; Syed et al., 2012). The circadian clock consists of multiple interlocked
86 transcription-translation feedback loops connected with input pathways that feed the
87 circadian clock function with environmental cues, such as light and temperature, and with
88 output pathways that are responsible for coordinating several major metabolic and
89 physiological processes (Haydon et al., 2013; Hsu and Harmer, 2014; Pokhilko et al.,

90 2012). In Arabidopsis, the main loop consists in three different components: *CIRCADIAN*
91 *CLOCK ASSOCIATED* 1 (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), expressed
92 around dawn and *TIMING OF CHLOROPHYLL A/B BINDING PROTEIN 1* (*TOC1*),
93 expressed around dusk (Alabadí et al., 2001). Closely associated with this loop are the
94 *PSEUDO-RESPONSE REGULATORS 7*, *3* and *9* (*PRR7*, *PRR3*, *PRR9*) (Locke et al.,
95 2005; Nakamichi et al., 2010; Para et al., 2007; Zeilinger et al., 2006). The components
96 of the central loop and the associated PRRs are conserved among other plant species,
97 including crops like rice, maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and
98 sugarcane (*Saccharum* hybrid) (Calixto et al., 2015; Hotta et al., 2013; Khan et al., 2010;
99 Murakami et al., 2007). The sugarcane circadian clock, although sharing conserved
100 components with other plants, may have a broader influence over sugarcane physiology,
101 with 32% of sugarcane transcripts showing rhythms under circadian conditions (Hotta et
102 al., 2013).

103 Sugarcane is a C4 grass that stores large amounts of sucrose in its stems, which can reach
104 as much as 700 mM or 50% of the culm dry weight (Moore, 1995). Its genome is
105 exceptionally complex, showing aneuploidy and a massive autopolyploidy that can range
106 from six to fourteen copies of each chromosome (Garcia et al., 2013). The genome size
107 of commercial modern sugarcane is estimated to be around 10 Gb (Chan et al., 2018; de
108 Setta et al., 2014). Because modern sugarcane cultivars are interspecific hybrids
109 progenies from *Saccharum officinarum* L. and *Saccharum spontaneum* L., about 80% of
110 sugarcane chromosomes comes from *S. officinarum*, 10% comes from *S. spontaneum* and
111 10% are recombinants of these two species (Cuadrado et al., 2004; D'Hont, 2005; D'Hont
112 et al., 1996). Sugarcane is a valuable commodity, responsible for 80% of sugar and 40%
113 of ethanol worldwide (FAOSTAT, 2015). The remaining biomass from sugarcane can
114 also be used for bioenergy production: the bagasse can be either burned to generate

115 electricity or have its cell wall hydrolyzed to yield simple sugars, which can be fermented
116 to produce second-generation biofuel (Amorim et al., 2011).

117 Although a great deal of data has been generated about the plant circadian clock,
118 sugarcane, and AS, the majority of these studies have been performed under highly
119 controlled experimental conditions. Such conditions are essential for reproducibility and,
120 for the circadian clock, a constant environment is one way to demonstrate the inner
121 mechanism generating self-sustained rhythms, as well as rhythmic responses. However,
122 those conditions are far from the environment that crops face in nature, with fluctuations
123 and complex interactions between abiotic and biotic variables (Annunziata et al., 2017,
124 2018; Shalit-Kaneh et al., 2018). In order to better understand the relationship between
125 the circadian clock and AS and how this relationship impacts on crops, it is essential to
126 expand experiments to field conditions. Indeed, essential in-field studies using
127 Arabidopsis (Annunziata et al., 2018; Richards et al., 2012) and rice (Izawa et al., 2011a;
128 Nagano et al., 2012; Sato et al., 2011) show that the complex natural cyclic environment
129 has a broader impact on rhythmic gene expression. So far, no studies have approached
130 the AS profile on circadian clock genes under such conditions.

131 In this study, we examined whether the daily dynamics of AS forms of circadian clock
132 genes are regulated by environmental factors in the field. We used sugarcane organs
133 extracted from field-grown plants when individuals were 4-months-old, during the
134 Brazilian winter, and 9-months-old, during the Brazilian summer. We investigated the
135 AS profile of sugarcane circadian clock genes in this fluctuating natural environment.
136 Data shows that there is at least one alternatively spliced form for each of the five
137 circadian clock genes analyzed. During winter, when temperatures are lower, alternative
138 transcripts are more highly expressed than in summer, with higher temperatures, which

139 suggests that AS might be related to the fluctuating environmental temperature in the
140 field. The different organs also showed different levels of AS and leaf has most of the
141 diversity in AS events. Collectively, our data suggest temperature correlates with AS in
142 the circadian clock of sugarcane plants grown in a natural environment, possibly as a
143 mechanism t of dynamic adjustment of the circadian clock.

144

145 **2. Material and Methods**

146 **2.1. Field Conditions and Plant Harvesting**

147 The sugarcane field where the experiment was conducted was located at the Federal
148 University of São Carlos, campus Araras, in São Paulo state, Brazil (22°21′25″ S,
149 47°23′3″ W, at an altitude of 611 m). The soil of the site was classified as a Typic
150 Eutroferric Red Latosol. Sugarcane tillers from the commercial variety SP80-3280
151 (*Saccharum* hybrid) were planted in soil in April/2012. Field design had 8 plots (**Figure**
152 **S1A**). Each plot had 4 rows containing 20 tillers each. Only sugarcane plants from both
153 central lines were used in order to avoid border effects. Sugarcane individuals were
154 randomly picked from two plots in order to avoid variability of both the local environment
155 and individual plants. Data on environmental conditions was acquired from a local
156 weather station (**Figure S1B - C**). Leaves +1 (L1), a source organ and the first fully
157 photosynthetically active leaf in sugarcane, were sampled from the selected individual
158 plants during two different seasons, and therefore different developmental stages. In the
159 first harvest, 4-months-old plants were sampled in August/2012, during winter; in the
160 second harvest, 9-months-old plants were sampled in January/2013, during summer. In
161 winter, dawn was at 6:30, and dusk was at 18:00 (11.5 h day/12.5 h night). In summer,
162 dawn was at 5:45, and dusk was at 19:00 (13.25 h day/10.75 h night). To compare the

163 rhythms of samples harvested in different seasons, the time of harvesting were normalized
164 to a photoperiod of 12 h day/ 12 h night using the following equations: for times during
165 the day: $ZT = 12*T*P_d^{-1}$, where ZT is the normalized time, T is the time from dawn (in
166 hours), and P_d is the length of the day (in hours); for times during the night: $ZT = 12 +$
167 $12*(T - P_d)*P_n^{-1}$, where ZT is the normalized time, T is the time from dawn (in hours), P_d
168 is the length of the day (in hours), and P_n is the length of the night (in hours). Because the
169 9-month-old plants had their culms fully developed, internodes 1 and 2 (I1) and internode
170 5 (I5) were also sampled. Both internodes are sink tissues with different profiles:
171 internodes 1 and 2 mostly undergo intense cell division and elongation, whereas internode
172 5 undergoes sucrose storage. For every time point, 9 individuals were randomly selected
173 in the assigned plots and harvested from the culm up. After that, those 9 individuals were
174 separated into three pools of three individuals, each pool formed a biological replicate
175 and then their leaves +1 were extracted. For all harvests, plants were sampled every 2 h
176 for 26 h, starting 2 h before dawn. In total, the time course consisted of 14 time points in
177 each harvest/season. After every time point sampling, a process that took less than 30
178 mins on average, tissue was immediately frozen in liquid nitrogen.

179

180 **2.2. RNA Extraction**

181 Sugarcane leaves previously frozen in liquid nitrogen were pulverized using dry ice and
182 a grinder. Then, 100 mg of this ground tissue was used for total RNA extractions using
183 Trizol (Life Technologies, Carlsbad, CA, USA), followed by treatment with DNase I
184 (Life Technologies, Carlsbad, CA, USA) and cleaned with RNeasy Plant Mini Kit
185 (QIAGEN, Valencia, CA, USA). The quality and quantity of each RNA sample were

186 checked using an Agilent RNA 6000 Nano Kit Bioanalyzer (Agilent Technologies, Palo
187 Alto, CA, USA). All RNA samples were stored at -80ºC.

188

189 **2.3. cDNA Synthesis**

190 cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR
191 (Life Technologies, Carlsbad, CA, USA) starting from 5 µg of total RNA. For all
192 reactions, both Oligo(dT) and Random Hexamers primers were used. All cDNA samples
193 were stored at -20ºC.

194

195 **2.4. PCR Reactions**

196 Primers used in PCR reactions were designed using the software PrimerQuest Tool (IDT)
197 (http://www.idtdna.com/primerquest/home/index). Each pair of primers was gene-
198 specific and amplified fragments ranging from 242 bp to 805 bp (**Table S1**). All PCR
199 reactions were carried out using Go Taq DNA Polymerase (Promega, Madison, WI, USA)
200 and following the manufacturer's protocol. Briefly, each 20-µL PCR reaction contained 2
201 µL of template, 10 µM of each primer, 4 µL of 5x Green Go Taq Buffer, 0.15 µL of Go
202 Taq DNA Polymerase, 2 mM of dNTPs. PCR conditions were: an initial step at 94ºC for
203 2 mins, followed by 20 – 30 cycles of 94ºC for 15 s, 50ºC for 15 s, 72ºC for 30 s, followed
204 by a final extension of 72ºC for 5 min. PCR reactions using primers amplifying control
205 genes *ScGAPDH* and *ScPP2AA2* were performed for all cDNA samples. Reactions
206 containing negative control using RNA as template and positive control using genomic
207 DNA as template were carried out. All PCR-amplified fragments were analyzed by taking
208 10 µL of reaction and run on an electrophoresis gel of 1.5% agarose (Life Technologies,

209 Carlsbad, CA, USA) and 1x TBE (50 mM Tris-HCl pH 8, 50 mM Boric Acid, 1mM
210 EDTA).

211

212 **2.5. High-Resolution RT-PCR**

213 High-Resolution RT-PCR (HR RT-PCR) reactions were performed based on Simpson
214 et al. (2008) and Simpson et al. (2019). For all reactions, the forward primer was labeled
215 with 6-carboxyfluorescein (FAM). Reactions consisted of a final volume of 20 µL
216 which had 2 µL of cDNA, 10 µM of each primer, 2 µL of 10x PCR Reaction Buffer
217 with MgCl2 (Roche Life Science, Indianapolis, IN, USA), 0.15 µL Taq DNA
218 Polymerase (Roche Life Science, Indianapolis, IN, USA), and 2 mM of dNTPs. The
219 PCR detailed program was: an initial step at 94ºC for 2 min, followed by 22 – 26 cycles
220 of 94 ºC for 15 s, 50ºC for 15 s, 70ºC for 30 s, followed by a final extension of 70ºC for
221 5 mins. Once PCR reactions were complete, 1 µL of each reaction was added to a mix
222 containing 9 µL of Hi-Di Formamide (Applied Biosystems, Life Technologies,
223 Carlsbad, CA, USA) and 0.5 µL of GeneScan 500 LIZ Size Standard (Applied
224 Biosystems, Carlsbad, CA, USA). The RT-PCR products were separated on an ABI
225 3730 Automatic DNA Sequencer (Applied Biosystems, Life Technologies, Carlsbad,
226 CA, USA). The results were analyzed using GeneMapper fragment analysis software
227 (Applied Biosystems, Carlsbad, CA, USA). LOESS (locally estimated scatterplot
228 smoothing) regression was used to detect trends in the data. The maximum value of the
229 LOESS curve between ZT0 and ZT22 was considered the peak of the rhythm. The code
230 to fully reproduce our analysis is available on GitHub
231 (https://github.com/LabHotta/AlternativeSplicing) and archived on Zenodo
232 (http://doi.org/10.5281/zenodo.3509232).

233

234 **2.6. Cloning and Sequencing**

235 In order to identify alternatively spliced forms, as well as differentially expressed alleles,
236 RT-PCR fragments were cloned and sequenced. For this, PCR fragments were purified
237 using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA). Each purified
238 fragment was cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) following
239 the manufacturer's protocol. Briefly, each reaction contained 3 µL of purified PCR
240 product, 5 µL of Rapid Ligation Buffer, T4 DNA Ligase, 1 µL of pGEM-T Easy Vector
241 (50 ng) and 1 µL T4 DNA Ligase (3 Weiss units/µL). Ligation reactions were incubated
242 overnight at 4ºC. 2 µL of each ligation reactions were used for heat-shock transformation
243 of 50 µL of JM109 High-Efficiency Competent Cells (Promega, Madison, WI, USA),
244 following manufacturer's instructions. Transformed cells were plated on
245 LB/ampicillin/IPTG/X-gal media and incubated overnight at 37ºC. Random colonies
246 were selected to use in plasmid extraction using QIAprep Miniprep Kit (QIAGEN,
247 Valencia, CA, USA). Positive plasmids were confirmed by PCR reactions, digestions
248 using restriction enzymes *PstI* and *Nco1* (Promega, Madison, WI, USA) and Sanger
249 sequencing. In order to identify alternative splicing events and single-nucleotide
250 polymorphisms, results were compared to sugarcane genomic sequences, and sugarcane
251 transcripts from Sugarcane Assembled Sequences (SAS) from SUCEST (http://sucest-
252 fun.org/) using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). *In silico*
253 translation was carried out using ExPaSy translation tool
254 (http://web.expasy.org/translate/).

255

256 **3. Results**

257 **3.1. Identification of Alternatively Spliced Forms of Sugarcane Circadian Clock**
258 **Genes**

259 In Arabidopsis, alternative splicing (AS) events of the circadian clock genes are well
260 known (James et al., 2012b). In order to investigate AS of sugarcane circadian clock
261 genes, it is important to first determine and annotate their genomic sequences/structures.
262 We first described the gene structure of previously identified homologs *ScLHY*, *ScPRR73*,
263 *ScPRR95* and *ScTOC1* (Hotta et al., 2013). These homologs were identified by Hotta et
264 al. (2013) using BLAST searches of Arabidopsis and rice circadian clock coding DNA
265 sequence (CDS), on the sugarcane Expressed Sequence Tags (ESTs) collection database
266 at SUCEST (http://sucest-fun.org/). This study identified only CDS sequences of
267 sugarcane circadian clock genes but not their genomic sequences due to the relatively few
268 genomic sequences publicly available at that time for the sugarcane cultivar of interest,
269 plus a high percentage of incomplete genomic sequences (de Setta et al., 2014; Vicentini
270 et al., 2012). Here we used the SUCEST sequences (Hotta et al., 2013) to search for their
271 genomic sequences in an unpublished sugarcane genomic database (Souza et al.,
272 unpublished) We identified genomic contigs for *ScLHY* (previously identified as
273 *ScCCA1*), *ScTOC1*, *ScPRR73* (previously identified as *ScPRR3*) and *ScPRR95*
274 (previously identified as *ScPRR59*) (**Table S2**). A new homolog, *ScPRR37*, was identified
275 here for the first time in sugarcane (**Table S2, Figure 1**). The sugarcane genes were
276 annotated by comparison with genomic sequences of circadian clock homologs from
277 barley and sorghum (Calixto et al., 2015). The exon/intron structures are shown in **Figure**
278 **1**; introns contained the canonical GT..AG dinucleotides at the exon-intron boundaries.
279 The putative CDS of each gene analyzed was translated *in silico* in order to confirm intact
280 open reading frames (ORFs).

281 To identify AS transcripts, RT-PCR was performed on cDNA synthesized from L1 RNA,
282 collected at three different time points, which corresponded to the highest gene expression
283 for each clock gene, based on the findings from Hotta et al. (2013). RT-PCR used pairs
284 of primers to generate overlapping amplicons to cover the full length of the transcripts
285 for each sugarcane circadian clock genes, *ScLHY*, *ScPRR37*, *ScPRR73*, *ScPRR95,* and
286 *ScTOC1*. RT-PCR products were cloned and sequenced, which confirmed the annotation
287 and identified AS events. Confirmation of different transcript isoforms was carried out
288 by using two different approaches combined: RT-PCR product size and fragment cloning
289 followed by sequencing.

290

291 **Table 1 - AS events found in the sugarcane circadian clock genes analyzed**

Gene	AS event*	Effect on transcript
ScLHY	Intron 1 retained (I1R)	Inserts a PTC on the transcript
	Intron 5 retained (I5R)	Creates a PTC on Exon 6
ScPRR37	Alternative exon 2 (Ex2a)	Inserts PTCs in the transcript
	Exon 3 skipped (E3S)	Inserts PTCs on Exon 4
	Intron 3 retained (I3R)	Creates PTC on Exon 3
	Intron 6 retained (I6R)	Inserts a PTC on the transcript
	Intron 7 retained (I7R)	Creates PTCs on Exon 8
	Alternative 3' splice site in exon 4	Protein stays in frame, removes 30 nucleotides from the PRR domain
ScPRR73	Intron 2 retained (I2R)	Inserts a PTC on the transcript
	Alternative 5’ splice site in exon 4 and alternative 3’ splice site in exon 5	Protein stays in frame, removes 245 nucleotides from transcript
	Intron 6 retained (I6R)	Inserts a PTC on the transcript
ScPRR95	Alternative 3' splice site in exon 5	Inserts a PTC on Exon 5
	Intron 7 retained (I7R)	Inserts a PTC on the transcript
ScTOC1	Intron 1 retained (I1R) combined with Exons 2 and 3 skipped (E23S)	Inserts a PTC on the transcript
	Exon 3 skipped (E3S)	Inserts a PTC on the transcript

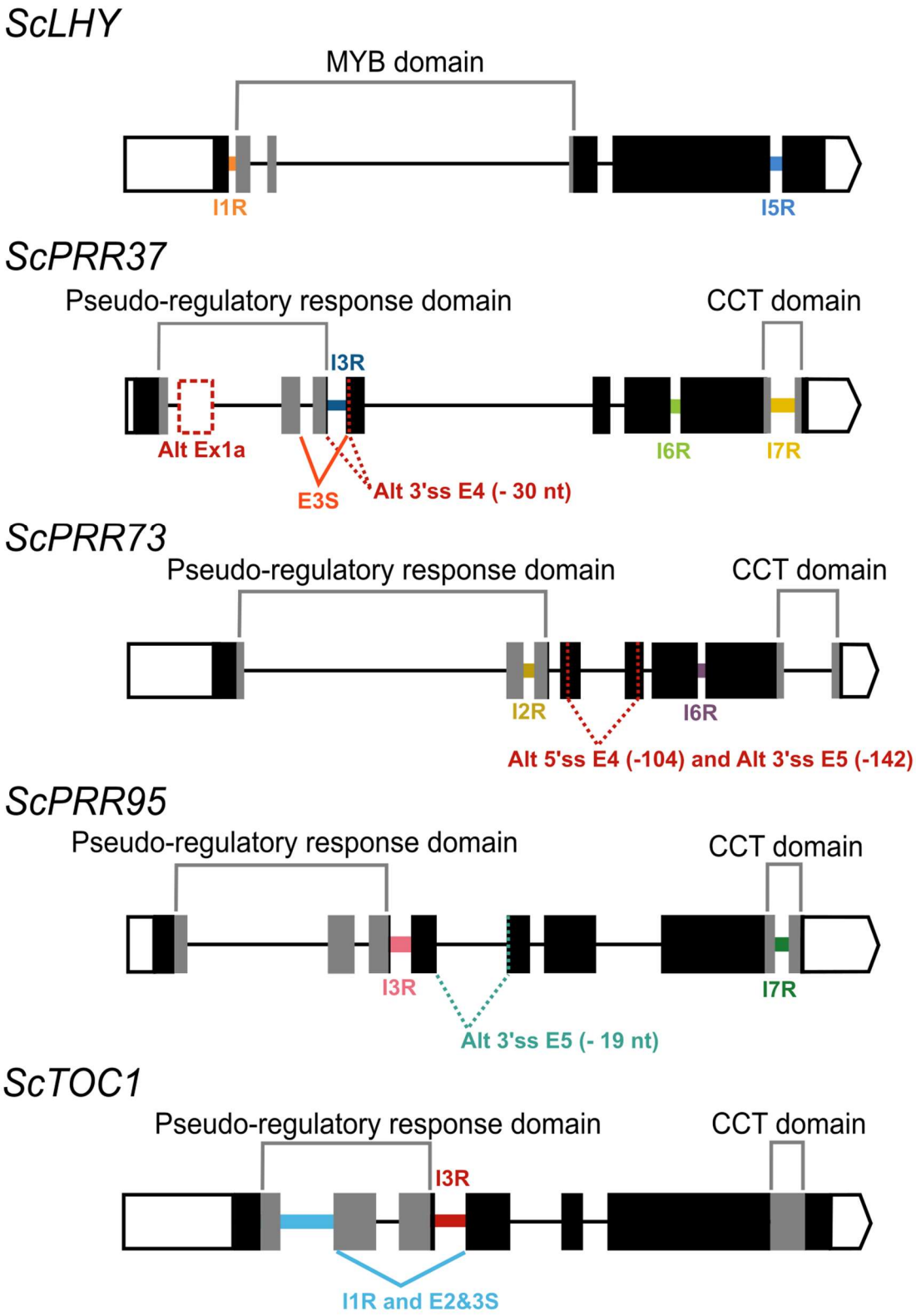
*All AS events were confirmed by Sanger sequencing.

292

293 We found evidence of AS events in all five genes analyzed (**Table 1**). Intron retention
294 (IR) was the most frequent AS event identified, resulting in the inclusion of premature
295 termination codons (PTC) in all cases (**Figure 1**). All five genes had at least two IR
296 events, and *ScTOC1* had retention of the first intron (I1R) combined with the skipping of
297 exons 2 and 3 (E23S). *ScLHY* had retention of introns 1 (I1R) and 5 (I5R). *ScPRR37* had
298 retention of introns 3 (I3R), 6 (I6R) and 7 (I7R). *ScPRR73* had retention of introns 2 (I2R)
299 and 6 (I6R). *ScPRR95* had two introns retained: intron 3 (I3R) and intron 7 (I7R) (**Figure**
300 **1**). All the other introns in the genes were efficiently spliced. Exon skipping (ES) was
301 detected in *ScPRR37* (E3S) and *ScTOC1*, the latter having two exons skipped at once
302 (E23S), combined with I1R (**Figure 1**). Alternative 3' splice sites (Alt 3' ss) were found
303 in both *ScPRR37* and *ScPRR95* in exon 4 (E4) and exon 5 (E5), respectively (**Figure 1**).
304 In *ScPRR73*, we found an AS event between exons 4 and 5 involving an alternative 5'
305 splice site (Alt 5'ss) in exon 4 (E4) being spliced to an Alt3'ss in exon 5. This AS event
306 removes 104 nt and 142 nt from exons 4 and 5, respectively (total of 246 nt), but remains
307 in frame and potentially produces a protein missing 82 amino acids compared to the wild-
308 type protein.

309 It is possible that some of the alternative sequences we found are not AS events but
310 different haplotypes since sugarcane is highly polyploid and aneuploid. To exclude this
311 possibility, we obtained the sequences for circadian clock genes from four different
312 sugarcane sequencing projects (Garsmeur et al., 2018; Riaño-Pachón and Mattiello, 2017;
313 Souza et al., unpublished; Zhang et al., 2018). We found 11 sequences for the 5' portion
314 of the *ScLHY*: 4 from *S. spontaneum* (Zhang et al., 2018), 6 from the commercial
315 *Saccharum* hybrid SP80-3280 (Riaño-Pachón and Mattiello, 2017; Souza et al.,
316 unpublished) and 1 from the commercial *Saccharum* hybrid R570 (Garsmeur et al., 2018).
317 All *ScLHY* sequences had the complete intron 1 (**Figure S2**). Similarly, all 16 sequences

318 that had the end portion of *ScLHY* contained the complete intron 5. (**Figure S2**). All other
319 detected AS events had 7 to 15 sequences supporting the conclusion that these alternative
320 sequences are not the result of different sugarcane haplotypes (**Figures S3-S6**).



321

322 **Figure 1 – Alternative splicing events identified in the sugarcane circadian clock**
323 **genes.** Alternative splicing (AS) events are shown in the gene structure of *ScLHY*,
324 *ScPRR37*, *ScPRR73*, *ScPRR95,* and *ScTOC1*. White boxes show 5' and 3' UTR regions;
325 black boxes show exons; black lines represent introns. The main protein domains are in
326 grey. AS events are shown in colored solid lines, colored dotted lines or in dotted white
327 boxes and the splicing events are illustrated below each event. Different colors were
328 chosen to mark the AS events investigated later, whereas events that were not investigated
329 further are in dark red. IR = intron retention; ES = exon skipping; Alt 5' ss = alternative
330 5' splice sites; Alt 3' ss = alternative 3' splice sites; Alt Ex = alternative exon.

331

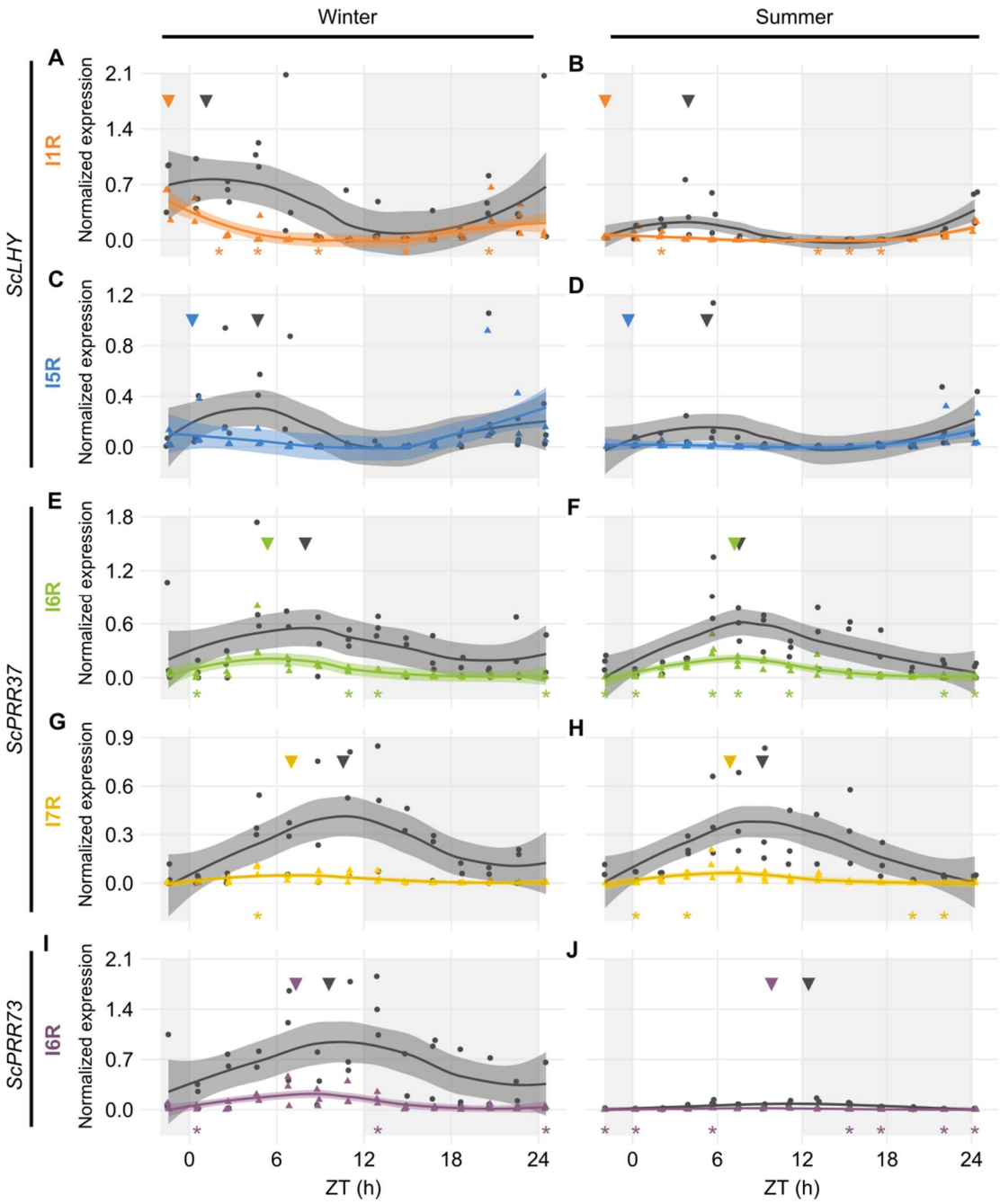
332 **3.2. Expressed AS forms in different seasons in sugarcane leaves**

333 We used HR RT-PCR (Simpson et al., 2019) to examine the daily dynamics of the
334 expressed isoforms of the sugarcane circadian clock genes in two different seasons, winter
335 and summer, using field-grown plants that were 4 and 9 months old, respectively. Briefly,
336 the HR RT-PCR system uses fluorescently labelled primers to amplify across an AS
337 event, followed by fragment analysis in an automatic DNA sequencer that quantifies the
338 relative levels of RT-PCR products and thereby splicing ratios that reflect different splice
339 site choices. The primers used for the HR RT-PCR assays had the same sequence of those
340 used to amplify each gene region on RT-PCR experiments (**Table S1**). Leaf +1 (L1),
341 internode 1 and 2 (I1), and internode5 (I5) samples, harvested every 2 h during 26 h,
342 starting from 2 h before dawn were used. As reference genes to normalize data, we used
343 *ScGAPDH* and *ScPP2AA2* (Iskandar et al., 2004; James et al., 2012a). As the experiments
344 were done in different seasons, we have normalized the time of sampling to fit in a 12 h
345 day/ 12 h night photoperiod such that ZT00 is set to dawn, and ZT12 is set to dusk.

346 From the five sugarcane circadian clock genes analyzed, HR RT-PCR experiments
347 detected high levels of AS in *ScLHY*, *ScPRR37,* and *ScPRR73* (**Figure 2**), but not in
348 *ScPRR95* and *ScTOC1*, in L1 (**Figure S7**). In general, the AS isoform peaked earlier than
349 the FS form in 8 of the 9 conditions assayed (**Figure 2A-I**), apart from *ScLHY,* where the
350 FS forms peaked at the same normalized time in winter and summer samples.

351 *ScLHY* had confirmed events of intron 1 and 5 retention (I1R and I5R) in both harvests
352 (**Figure 2A-D**). The peak of expression of the *ScLHY* alternative isoforms did not match
353 the peak of expression of the fully spliced functional (FS) isoform. *ScLHY* I1R peaked
354 close to dawn in winter and summer samples, while the FS for this region peaked one
355 hour after dawn in winter plants (ZT01) and five hours after dawn (ZT05) in summer
356 plants (**Figure 2A-B**). *ScLHY* I5R peaked at dawn for plants in winter samples, while it
357 was not considered expressed for plants in summer samples (**Figure 2C-D**). The FS form
358 for this region peaked between ZT04-05 for plants in both winter and summer samples
359 (**Figure 2**).

360 *ScPRR37* I6R had a peak at ZT05 in winter samples, but at ZT07 in summer samples
361 (**Figure 2E and F**). The corresponding FS isoform had a peak at ZT08 in both conditions.
362 In turn, *ScPRR37* I7R had a peak at ZT07 in both winter and summer plants, but the
363 corresponding FS isoform had a peak at ZT090 and ZT11 in winter and summer samples,
364 respectively (**Figure 2G and H**). *ScPRR37* I3R and E3S were not detectable using HR
365 RT-PCR (**Figure S7A-B**). Both the *ScPRR73* I6R and its FS isoform only had high levels
366 in winter samples, with a peak at ZT07 (**Figure 2I-J**). The corresponding FS isoform had
367 a peak at ZT10.



368

369 **Figure 2 – Diel expression profile of fully spliced and alternative transcript isoforms**
370 **in different seasons.** Biological replicates (circles and triangles) and their LOESS curve
371 (continuous lines ± SE) of fully spliced (FS, black) and alternative transcript forms (AS,
372 colored) for the winter samples (4-month-old plants, left) the summer samples (9-month-
373 old plants, right). **(A,B)** *ScLHY* gene expression shows levels of I1R (orange) and **(C,D)**
374 I5R events (blue). **(E,F)** *ScPRR37* gene expression shows levels of I6R (green), and

375 (**G,H**) I6R (yellow); (**I,J**) *ScPRR73* gene expression shows levels of I2R (purple).
376 Inverted triangles show the time of the maximum value of the LOESS curve. The light-
377 gray boxes represent the night period. Statistical significance was analyzed by paired
378 Student's t-test, * $p < 0.05$.

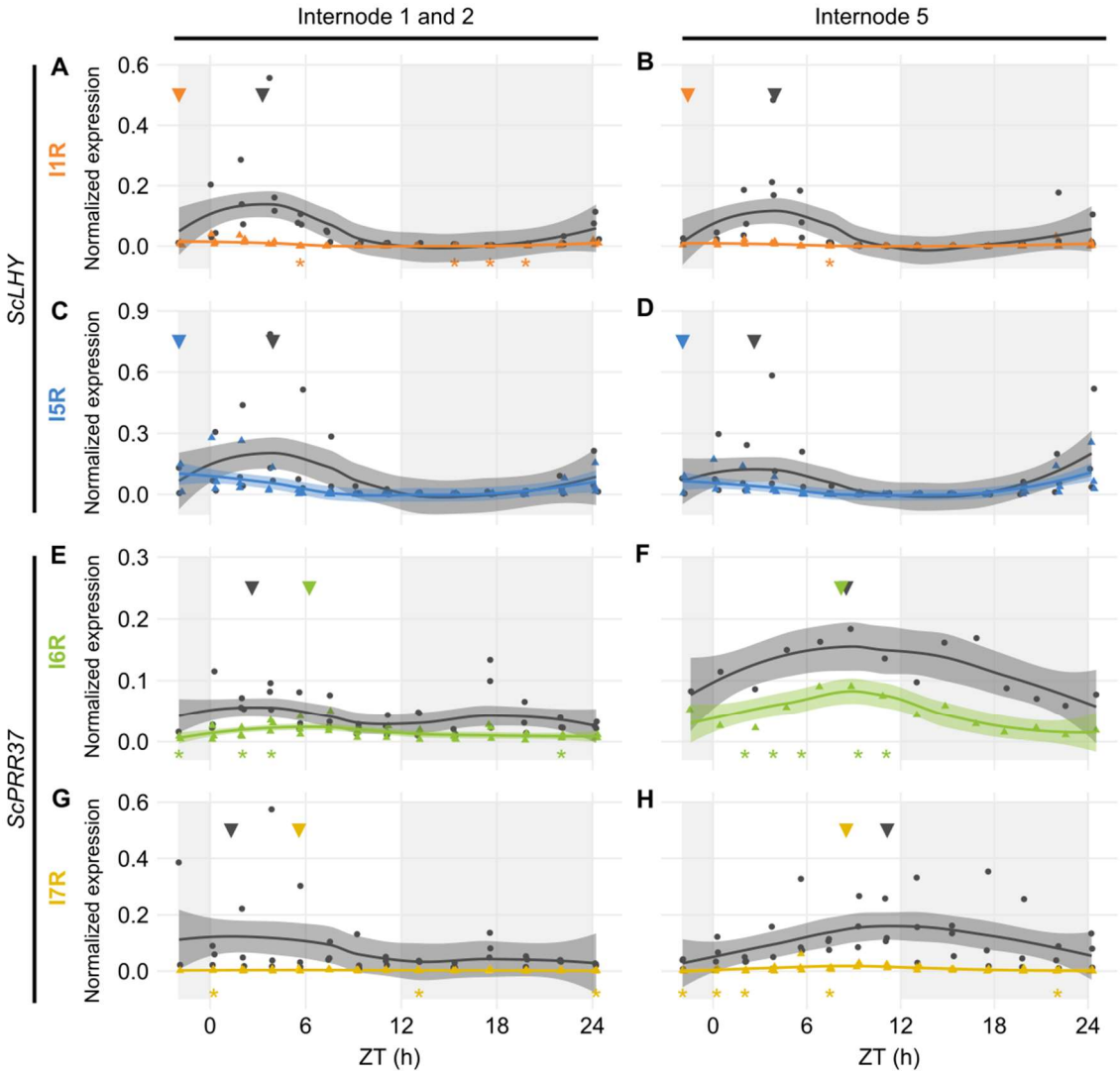
379

380 **3.3. Expressed AS forms in the different source-sink sugarcane organs**

381 We extended the investigation to two other sugarcane organs, internodes 1 and 2 (I1) and
382 internode 5 (I5). These internodes have different physiology: I1 has a high cellular and
383 metabolic activity, whereas I5 is the first internode to actively accumulate sucrose. Only
384 the plants harvested in the summer (9-month-old) had developed internodes that could be
385 harvested. We only measured AS forms in genes *ScLHY* and *ScPRR37*, which were the
386 homologs featuring the highest AS transcript expression in L1 (**Figure 2**). The main
387 difference in expression levels between organs was observed in *ScPRR37* I6R, that had
388 significantly higher levels in leaves during the day compared to the internodes (One-way
389 ANOVA with post-hoc Tukey HSD test, * $p < 0.05$, **Figure S8**).

390 In both internodes, *ScLHY* showed detectable levels of both AS events observed in L1.
391 *ScLHY* I1R-containing transcript levels were very low with a peak 2 h before dawn
392 (ZT22), with the FS isoform peaking between ZT03-04 (**Figure 3A-B**). *ScLHY* I5R was
393 also identified in both internodes, at higher levels than *ScLHY* I1R. The AS isoform
394 peaked at ZT22, while the FS form peaked between ZT03-04 (**Figure 3C-D**). During the
395 end of the night, *ScLHY* I1R levels were significantly higher in the leaves compared to
396 the internodes (One-way ANOVA with post-hoc Tukey HSD test, * $p < 0.05$, Figure S8).

397 The *ScPRR37* homolog featured variable levels of FS and AS isoforms when compared
398 to both internodes. In I1, *ScPRR37* I6R and its FS isoform did not have a clear rhythm,
399 with the I6R showing low levels of expression, and the FS isoform showing two peaks.
400 In I5, *ScPRR37* I6R and its FS isoform had higher levels and a peak between ZT08-09
401 (**Figure 3E-F**). In both internodes, the I7R isoform was expressed at very low levels, but
402 the FS isoform for that transcript region peaked at ZT01 in I1 and ZT11 in I5 (**Figure**
403 **3G-H**).



404

405 **Figure 3 – Diel expression profile of fully spliced and alternative spliced transcript**
406 **isoforms in internodes.** Biological replicates (circles and triangles) and their LOESS

curve (continuous lines ± SE) of fully spliced (FS, black) and alternative spliced (AS, colored) transcript forms of sugarcane circadian clock genes during the summer harvest in internode 1 and 2 (left) and internode 5 (right). **(A, B)** *ScLHY* gene expression shows levels of I1R (orange) and **(C, D)** I5R (blue); **(E, F)** *ScPRR37* gene expression shows levels of I6R (green) and **(G, H)** I7R (yellow). Inverted triangles show the time of the maximum value of the LOESS curve. The light-gray boxes represent the night period. Statistical significance was analyzed by paired Student's t-test, * $p < 0.05$.

3.4. Alternative splicing events are dependent on the time of the day and temperature

After the identification of rhythms in FS and AS transcripts, we tried to identify rhythms in their relative levels by examining the log of the splicing ratio of the AS to the FS transcripts (log(AS/FS)) from the HR RT-PCR data. *ScLHY*, *ScPRR37,* and *ScPRR73* showed evidence of splicing rhythms (**Figure 4**), but only *ScLHY* had more than a 10-fold difference between the maximum and the minimum log(AS/FS) (77-fold) (**Figure 4A**). The AS:FS ratios of all the time-point samples and organs were grouped, as they showed similar rhythmic patterns. In general, all AS events of a gene showed a similar rhythmic pattern. The only exception was E3S in *ScPRR37* (**Figure 4D**), that had a different phase from the other AS events found in *ScPRR37* (**Figure 4B**). The AS events observed in *ScLHY* and E3S in *ScPRR37* had a peak at the end of the night, between ZT20 and ZT24, and a trough between ZT06 and ZT08. In contrast, *ScPRR73* AS events and the remaining *ScPRR37* AS events had a peak between ZT05 and ZT06, and a trough between ZT16 and ZT18.

430 The rhythmic changes in the log(AS/FS) values could be explained by changes in the
431 expression of putative regulatory genes, such as splicing factors or spliceosomal protein
432 genes. In a previous work, we have identified 6,705 rhythmic transcripts in L1, 3,755 in
433 I1 and 3,242 in I5 in field-grown sugarcane (Dantas et al., 2019). Fourteen spliceosome-
434 related transcripts in the oligo array were expressed in all three organs (**Table S2**): 9
435 transcripts were rhythmic only in L1, one was rhythmic in L1 and I1, one was rhythmic
436 in L1 and I5, and one was rhythmic in I1 and I5 (**Table S3**). In L1, 9 of the 11 rhythmic
437 transcripts peaked between ZT09 and ZT13, with a trough between ZT03 and ZT5
438 (**Figure 4E**). In the internodes, most of the transcripts peaked at ZT00 (**Figure 4F**).

439 To test if the temperature was a factor in the regulation of AS, we correlated temperature
440 information with log(AS/FS) values. Only *ScLHY* AS events showed a significant
441 negative correlation (**Figure 5**). The negative correlation was found in both *ScLHY* AS
442 events, at both harvests/seasons, and all organs. This suggests that the AS regulation of
443 *ScLHY* genes are temperature-dependent.

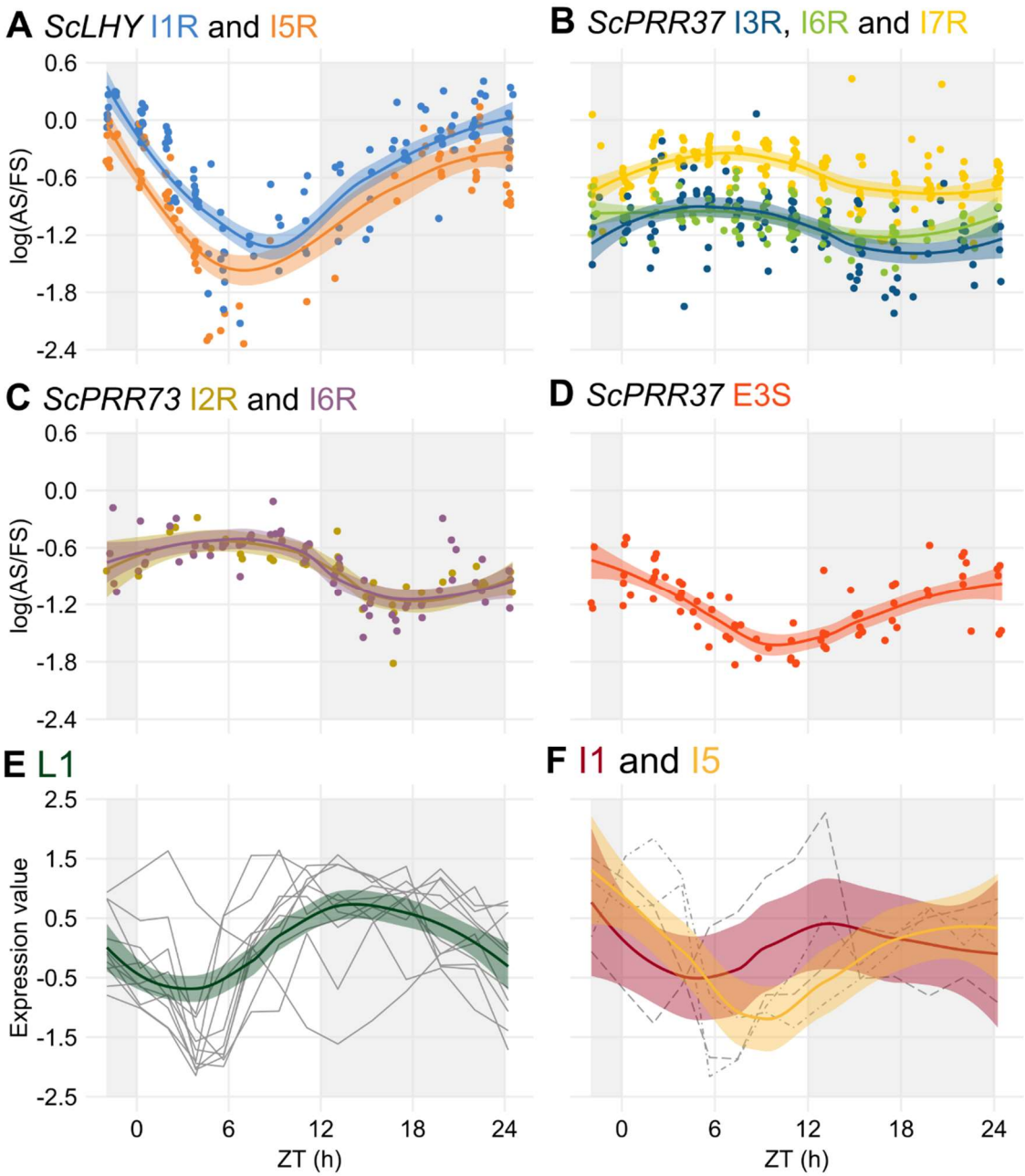
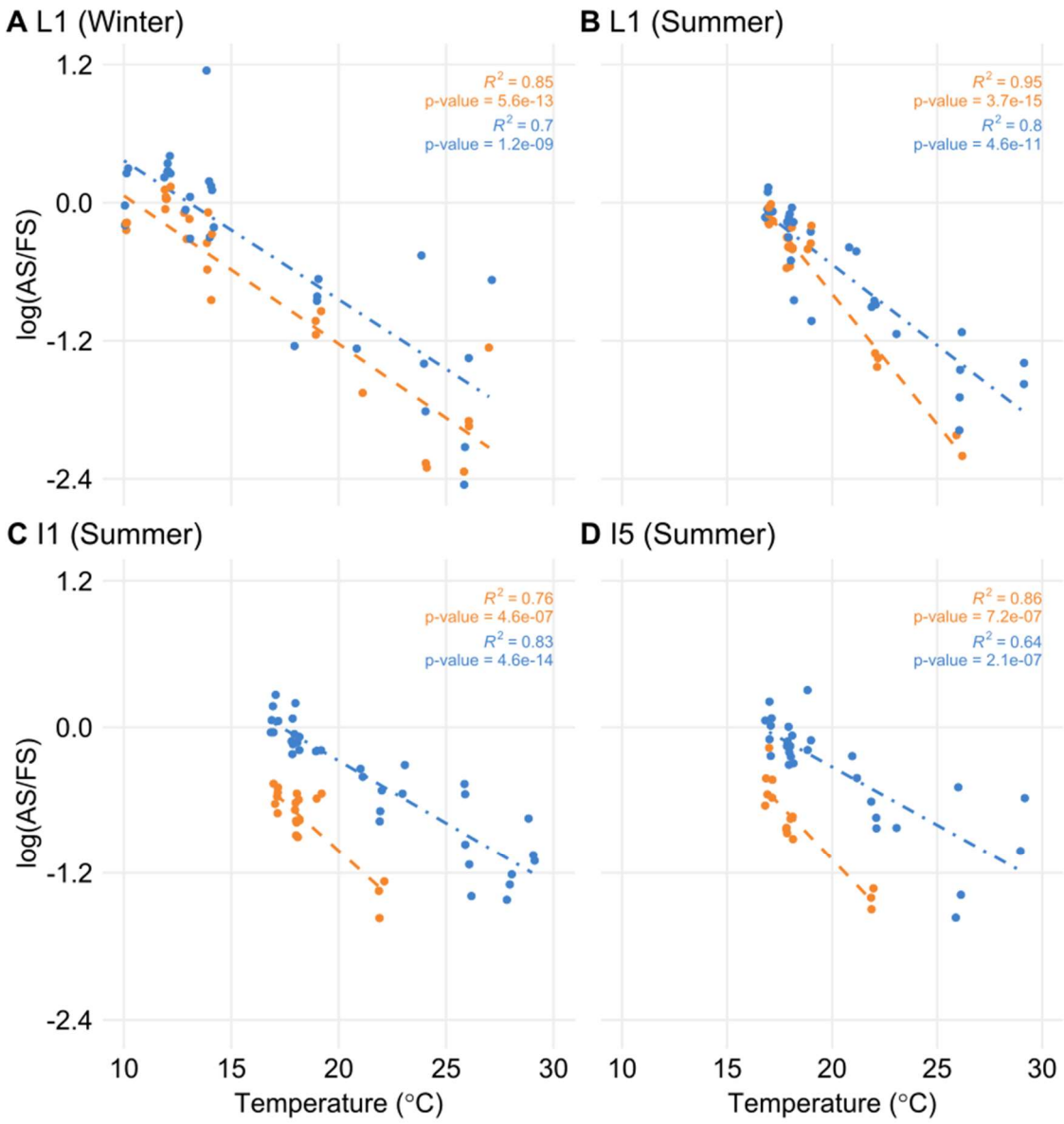


Figure 4 – Alternative splicing is rhythmic in sugarcane in field conditions. (**A-D**) The logarithm of the ratio of the expression levels of an AS isoform to its FS isoform, annotated as the log(AS/FS), was plotted against the normalized time of the day (ZT). **(A)** *ScLHY* I1R (orange) and I5R (blue); *ScPRR37* I3R (dark blue), **(B)** I6R (green) and I7R (light yellow); **(C)** *ScPRR73* I2R (gold) and I6R (purple); and **(D)** *ScPRR37* E3S (red). (**E-F**) Normalized expression levels of rhythmic splicing-related transcripts taken from oligo array data (Dantas et al., 2019) in (**E**) leaves +1 (L1,

452 green), and internodes 1 and 2 (I1, red) and (**F**) internode 5 (I5, yellow). Individual
453 expression profiles were drawn in gray. LOESS regression was used to draw the
454 trends in the data in all panels (continuous line ± SE). The light-gray boxes represent
455 the night period.



456

457 **Figure 5 – The proportion of alternative spliced and fully spliced forms has a**
458 **negative correlation with ambient temperature.** The logarithm of the ratio of the
459 expression levels of AS and FS isoforms, annotated as the log(AS/FS), was plotted
460 against the ambient temperature for *ScLHY* I1R (orange) and I5R (blue). Regression

461 lines were added for each group of ratios. R^2 and *P-value* were calculated for each
462 regression. Negative correlations were significant in leaf +1 (L1) in the **(A)** winter
463 (4-month-old plants), and **(B)** summer, **(C)** internode 1 and 2 (I1)**,** and **(D)** internode
464 5 (I5), both in the summer (9-month-old plants).

465 **4. Discussion**

466 In this paper, pioneer information on AS events in the sugarcane circadian clock core
467 genes *ScLHY*, *ScPRR37*, *ScPRR73*, *ScPRR95*, and *ScTOC1* in field-grown sugarcane
468 plants are presented (**Figure 1**). As for Arabidopsis and barley in previous studies, AS is
469 widespread among circadian clock genes (Calixto et al., 2016; Filichkin et al., 2010;
470 Filichkin and Mockler, 2012; James et al., 2012a). The circadian clock homolog
471 *CCA1/LHY* in rice also displays the conserved I1R AS event, suggesting conserved
472 patterns in AS events between the two species (Filichkin et al., 2015b). In barley, AS
473 events were described for the homologs *HvLHY*, *HvPRR37*, *HvPRR73,* and *HvGI*. There
474 are conserved AS isoforms expressed in both Arabidopsis and barley for *HvLHY* and
475 *HvPRR37* (Calixto et al., 2016). The conservation of expression of AS forms across
476 different plant species highlights the role that AS plays in gene expression of circadian
477 clock genes. Our data in sugarcane identified at least one AS event in each of the clock
478 gene homologs analyzed. Given the ploidy of sugarcane, it was necessary to demonstrate
479 that the alternative transcript sequences did not originate from haplotypes but were *bona*
480 *fide* AS events. By examination of existing sequenced genomic data, we conclude that
481 they are AS events. The most frequent AS event in the sugarcane circadian clock genes
482 was intron retention (IR), which was detected in all five genes. The presence of transcripts
483 containing retained introns might also be an indication of partially spliced transcripts but
484 the other introns in these genes were efficiently removed. The presence of retained introns

485 in transcript isoforms can lead to post-transcriptional regulation of gene expression
486 (Reddy et al., 2013). Since retained introns usually insert premature termination codons
487 (PTCs) in the transcript, they could be substrates for degradation via the nonsense-
488 mediated decay (NMD) pathway. However, in plants, transcripts with detained introns
489 appear to avoid NMD since their abundance is unaffected in NMD mutants (Kalyna et
490 al., 2012; Marquez et al., 2012). Furthermore, such transcripts have been shown to remain
491 in the nucleus and thus avoid the NMD machinery (Göhring et al., 2014). Nuclear intron
492 detention is now recognized as an important post-transcriptional regulatory mechanism
493 (Jacob and Smith, 2017). Intron retention transcripts with PTCs can also potentially give
494 rise to C-terminally truncated and dysfunctional proteins (Mastrangelo et al., 2012; Reddy
495 et al., 2013; Seo et al., 2011). Other AS events in sugarcane circadian clock genes that
496 were in frame and did not insert a PTC in the transcript were the alternative 5' splice site
497 in ScPRR37 exon 3 (Alt 5'ss E3) and the *ScPRR73* alternative splice site between exons
498 4 and 5: Alt 5'ss E4 (-104) and Alt 3' ss E5 (-142) (**Table S2**). In both cases, there is the
499 removal of nucleotides from the coding sequence. *ScPRR37* Alt 5'ss E3 removes 30
500 nucleotides (10 amino acids) from the PRR domain. The resulting sequence is likely to
501 be translated into a defective protein. In *ScPRR73*, the combination of Alt 5'ss E4 (-104)
502 and Alt 3' ss E5 (-142) removes 246 nucleotides (82 amino acids) from the coding
503 sequence but still leaves an ORF. However, it is possible that the loss of sequence could
504 affect the normal function of the *ScPRR73* CTT domain. Therefore, the AS events
505 identified in the sugarcane core circadian clock genes either produce transcripts that are
506 likely to be kept in the nucleus and degraded or that are translated into incomplete proteins
507 that are likely to be functionally defective. Thus, AS has an important role in regulating
508 expression and production of core clock proteins. This might have a direct impact on the
509 clock-dependent plant metabolism and physiology.

510 The levels of alternative transcript isoforms can vary under stress conditions (Calixto et
511 al., 2018; Shang et al., 2017b; Staiger and Brown, 2013b), at specific developmental
512 stages (Szakonyi and Duque, 2018) or in different cell tissues (Shen et al., 2014; Thatcher
513 et al., 2014). In previous work using microarrays, we found that rhythmic expression at
514 the gene level was very organ-specific in sugarcane (Dantas et al., 2019). Our data shows
515 differences in the AS in leaf and internodes at the transcript level. In L1, circadian clock
516 transcripts undergo AS at higher relative levels than in I1 and I5 at the end of the night
517 (**Figure 2 and Figure 3**). In addition, the splicing ratios, log(AS/FS), had rhythms in
518 *ScLHY*, *ScPRR37*, and *ScPRR73*. In *ScPRR37*, the intron retention events peaked in the
519 middle of the day, while the exon skipping event peaked at the end of the night, suggesting
520 that the temporal regulation of these two types of AS events are independent of each other
521 (**Figures 4B and 4D**). Although their expression profiles differ, the consequences of
522 *ScPRR37* I3R and E3S events are likely to be similar. I3R introduces PTCs after exon 3
523 and E3S removes exon 3 (part of the PRR domain). Arabidopsis *PRR7* also has two
524 mutually exclusive AS events in a similar region of the gene: retention of intron 3 (I3R)
525 and skipping of exon 4 (E4S), both of which give nonproductive mRNAs (James et al.,
526 2012a). The switch from intron retention (mainly during the day) to exon skipping
527 (mainly during the night) in *ScPRR37* may reflect rhythmic changes in specific splicing
528 factors. The splicing ratio rhythms of *ScLHY* and *ScPRR73* showed the same pattern,
529 irrespective of the sampling season or organ (**Figure 4A-C**). This means that these
530 splicing ratios are not organ-specific and are environmentally and circadian clock-
531 regulated in order to have the same distribution during the day and the night regardless of
532 their duration. To try and relate the rhythmic changes in splicing ratios of clock genes to
533 the expression of splicing factor or spliceosomal protein genes, we examined the
534 expression of the splicing-related genes that were rhythmic in L1 in our previous

535 microarray study (**Figure 4E**) (Dantas et al., 2019). The gene-level expression of the
536 majority of these genes peak around dusk (between ZT11 and ZT13), which does not
537 coincide with the peaks in splicing ratios observed in circadian clock genes. The list of
538 splicing-related genes in the microarray analysis was not extensive (**Table S1**) and many
539 splicing factors are alternatively spliced to regulate the level of productive, protein-coding
540 transcripts (Reddy et al., 2013; Staiger and Brown, 2013a). Transcript level RNA-seq will
541 be required to more accurately measure protein-coding transcripts of splicing factors to
542 identify candidate regulators of AS of the core clock genes analyzed here.

543 Another interesting observation is the noticeable variation in the AS transcripts across the
544 different organs analyzed (**Figure 3**). The differences in transcript expression between
545 source and sink tissues might reflect their metabolical differences. While L1 is a fully
546 photosynthetically active leaf in sugarcane, therefore undergoing photosynthesis, both
547 internodes sink in the assimilated carbon for different purposes: cell division and
548 elongation in I1 and sucrose storage in I5. In *Arabidopsis*, sucrose has been shown to
549 decrease *PRR7* levels, which decrease *ScLHY* levels as a consequence (Frank et al., 2018;
550 Haydon et al., 2013). Thus, differences in *ScPRR37* I6R and *ScLHY* I1R levels (Figure 3
551 and S8) could be due to the sucrose that is stored in the internodes. In turn, these
552 differences in the levels of circadian clock genes might affect circadian clock outputs. We
553 have found that transcriptional rhythms are very organ-specific in sugarcane (Dantas et
554 al., 2019).

555 In Arabidopsis, the circadian clock is associated with photosynthesis (Dodd et al., 2009),
556 cell division (Fung-Uceda et al., 2018), and sugar accumulation (Graf et al., 2010; Graf
557 and Smith, 2011; Ko et al., 2016). Taken together, these data suggest that the circadian
558 clock might regulate the different sugarcane organs in distinctive ways. It is already

559 known that metabolites can feedback to regulate the circadian clock, as data show in
560 Arabidopsis that photosynthetic sugars regulate clock functioning (Haydon et al., 2013).
561 Considering that in Arabidopsis different tissues are enriched with different levels of
562 circadian clock transcripts (Endo et al., 2014), a similar phenomenon might occur in
563 sugarcane and explain the different organ transcript expressions and profiles, as well as
564 helping to keep each organ different metabolic and physiologic profiles.

565 We found that the splicing ratios from *ScLHY* AS transcripts are negatively correlated
566 with temperature in all organs. Previous studies on the presence of AS in circadian clock
567 genes in Arabidopsis and barley revealed that, under controlled conditions, the AS status
568 of circadian clock genes is regulated by temperature, especially *LHY* and its paralog
569 *CCA1* (Calixto et al., 2016, 2018; Filichkin et al., 2015a; James et al., 2012a and b; Kwon
570 et al., 2014; Marshall et al., 2016; Park et al., 2012; Seo et al., 2012). Lower temperatures
571 led to increased abundance of alternative non-productive forms of *LHY*, *PRR7* and *PRR5*
572 in Arabidopsis (James et al., 2012a). This could affect the expression of fully spliced
573 transcripts of the circadian clock genes, promoting a functional modulation in the
574 circadian clock central oscillator, which might be reflected by altered temporal control of
575 the clock outputs. In Arabidopsis, cold temperatures reduced the amplitude of
576 *CCA1*/*LHY*, as well as disrupted the circadian clock function (Bieniawska et al., 2008).
577 Considering the natural environment context, where plants like sugarcane face
578 fluctuations in temperature on a daily and yearly basis, the continuous temperature-
579 regulation of AS of the circadian clock network could have a more profound impact on
580 metabolism and, ultimately, on crop yield.

581 The data in our work shows that from winter to summer, as the temperature increases
582 (**Figure S1B**), the expression of alternative forms of the circadian clock genes decreases,

583 noticeably for *ScLHY* (**Figure 2A-D**). In Arabidopsis, there is evidence linking the
584 circadian clock with sugar accumulation as starch through *CCA1* and *LHY* (Miller et al.,
585 2012; Ng et al., 2014; Ni et al., 2009) and in field-grown maize, a C4 plant like sugarcane,
586 two *CCA1* homologs are associated to photosynthesis and, therefore, sugar accumulation
587 (Ko et al., 2016). All this evidence allows us to speculate that there might be differences
588 in the sugar accumulation by the field-grown sugarcane from winter to summer, but a
589 metabolomic analysis focused on sucrose and hexoses content would be necessary to
590 bring evidence to support such speculation.

591 Recent studies featuring experiments conducted in field conditions in Arabidopsis, rice
592 and tomato highlight the differences in gene expression, circadian regulation and plant
593 metabolism compared to experiments conducted inside growth chambers (Annunziata et
594 al., 2017, 2018; Higashi et al., 2016; Izawa et al., 2011b; Shalit-Kaneh et al., 2018).
595 Because AS has an impact on the regulation of gene expression, which impacts circadian
596 regulation and plant metabolism, it is important to start investigating the dynamic
597 adjustment of AS in response to a fluctuating environment. RNA-seq data from time-
598 series experiments revealed that the AS status of Arabidopsis transcriptome is widely
599 responsive to changes in temperature (Calixto et al., 2018). By progressively lowering
600 temperature, rapid changes in the spliced forms of transcripts were detected, which
601 suggests that AS might also act to regulate low-temperature responses and how plants
602 tolerate such stress (Calixto et al., 2018). The Arabidopsis circadian clock also acts in
603 regulating plant abiotic stress tolerance (Grundy et al., 2015). This also suggests that both
604 AS and the circadian clock might act in synergy to help plants to cope with temperature
605 changes in both the short and long term. In the field, this regulation might be even more
606 important, due to the unexpected fluctuations in light, temperature, and humidity in which
607 plants are exposed.

608 Our data show that AS occurs in sugarcane circadian clock genes and that the different
609 transcript isoforms show a dynamic expression profile in sugarcane grown under field
610 conditions. Furthermore, *ScLHY* AS regulation correlates with temperature in sugarcane
611 circadian clock genes. Thus, the changes in expression of alternative isoforms of *ScLHY*
612 transcripts observed across winter and summer might illustrate the combined effect of
613 both the circadian clock and AS regulation and AS in *ScLHY* might be a key mechanism
614 that allows the continuous dynamic adjustment of the circadian clock by temperature in
615 sugarcane. It is important to start further studies on the impact of the seasonal variation
616 on the AS isoforms of the circadian clock gene expression and, ultimately, sugarcane
617 metabolism and yield.

618

619 **5. Author Contributions**

620 CTH and JWSB designed this research. LLBD and CTH harvested the biological material
621 and carried out BLAST analyses. LLBD processed all samples and carried out cloning,
622 HR RT-PCR and SNP analyzes. LLBD, CPGC, JWSB, and CTH participated in the
623 interpretation of genomic annotation and HR RT-PCR data. MMD contributed with
624 cloning. MSC contributed with the plants and space for the field experiment. LLBD and
625 CTH drafted the manuscript. All authors participated in its correction and have read and
626 approved the final manuscript. CTH and JWSB acquired the funding.

627

628 **6. Conflict of Interest Statement**

629 The authors declare that the research was conducted in the absence of any commercial or
630 financial relationships that could be construed as a potential conflict of interest.

7. Acknowledgments

The present study was supported by the São Paulo Research Foundation (FAPESP) [grant nos. 11/00818-8 and 15/06260-0; BIOEN Program], and by the Serrapilheira Institute (grant no. Serra-1708-16001). L.L.B.D. was supported by FAPESP scholarships [grants 11/08897-4 and 15/10220-3]. C.P.G.C and J.W.S.B. were supported by funding from the Biotechnology and Biological Sciences Research Council (BBSRC) [BB/K006568/1 and BB/N022807/1] and the Scottish Government Rural and Environment Science and Analytical Services division (RESAS) [to J.W.S.B.].

8. Supplementary Material

The Supplementary Material for this article can be found online at:

(future link here)

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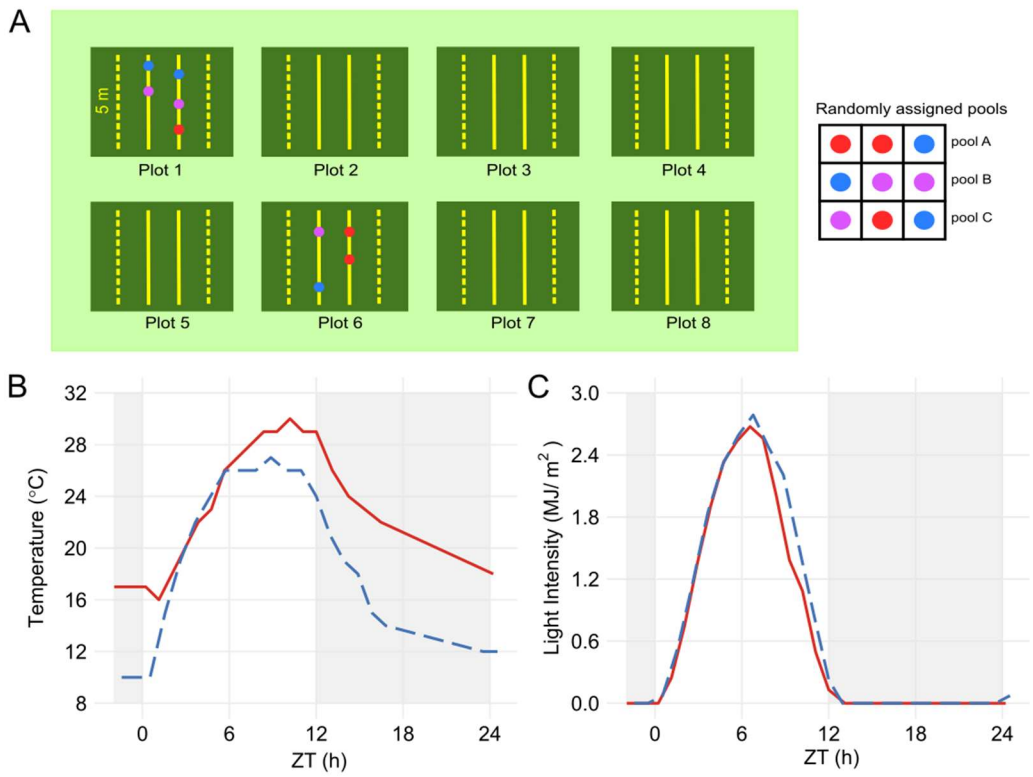
998 **Supplemental Figures**

999 **Supplemental Figure 1**



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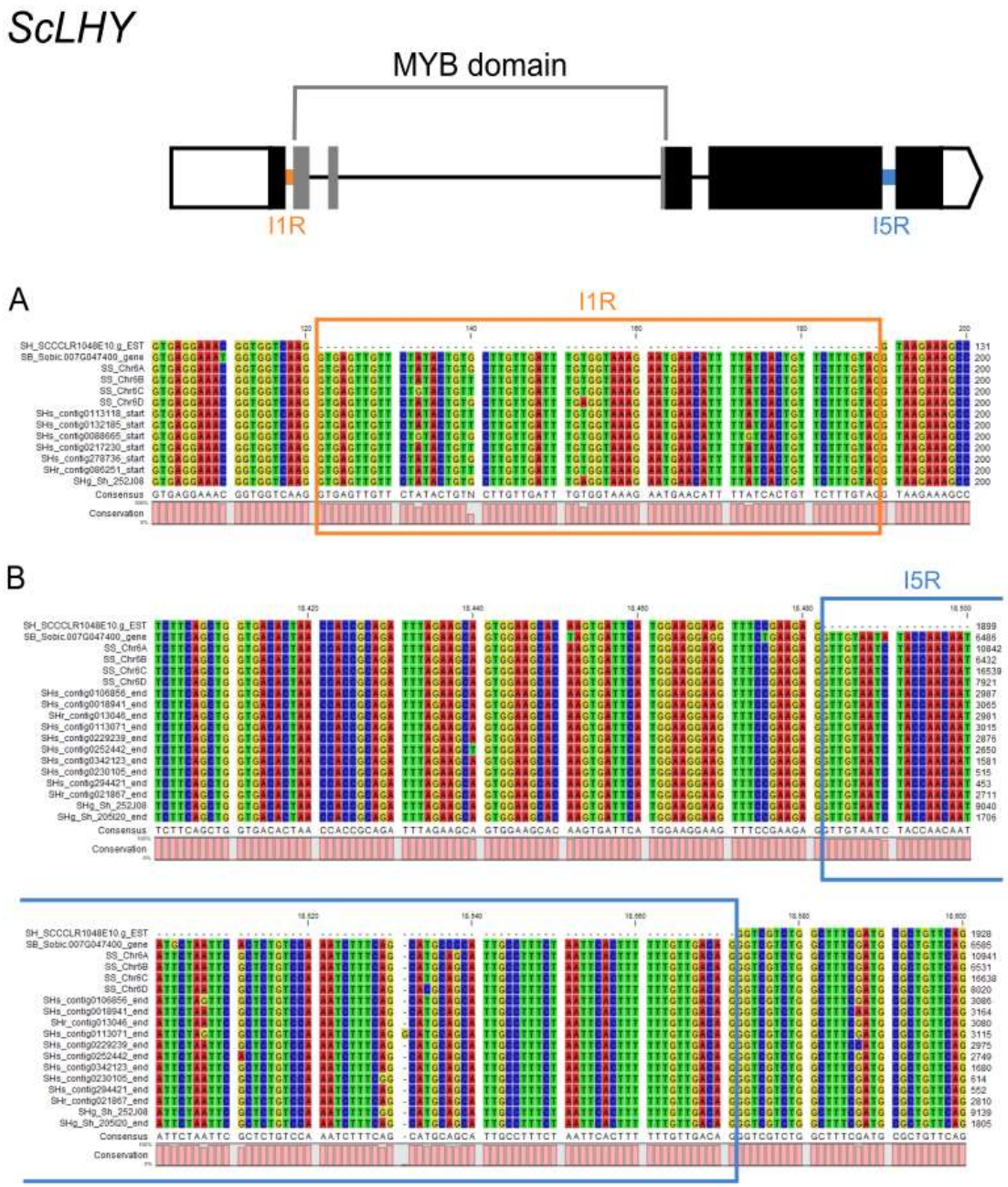
1001 **Supplemental Figure 1 – Field design and environmental conditions. (A)** The
1002 experimental field consisted of 8 plots, that were surrounded by two lines of sugarcane.
1003 There were 4 lines per plot, each line 5 m long and filled with 20 tillers of commercial
1004 sugarcane (variety SP80-3280). The border lines (dashed yellow lines) were not used in
1005 our experiment in order to avoid border effects. Plants from the two central lines of two
1006 plots were randomly picked and randomly assigned to three pools. Temperature **(B)** and
1007 light intensity **(C)** during the summer sampling (red continuous line) and the winter
1008 sampling (dashed blue line) were obtained from a nearby weather station. The time points
1009 were normalized for a 12h photoperiod. The light-gray boxes represent the night period.

Supplemental Figure 2



Supplemental Figure 2 – Alignment of two different regions of ScLHY from different genomic sequences. Sequences from different sugarcane genome projects that were identified as *ScLHY* were aligned in order to compare the regions containing the I1R and I5R AS events in different haplotypes. The regions containing (**A**) the intron 1 (orange) and (**B**) intron 5 (blue) were highlighted to show that all the sequenced

1017 haplotypes have the same gene structure. Sequences are from *Sorghum bicolor* (SB)
1018 genome (McCormick et al., 2018), *S. spontaneum* (SS) (Zhang et al., 2018), *Saccharum*
1019 hybrid (SP80-3280): SHs (Souza et al., unpublished), SHr (Matiello et al., 2017), and
1020 *Saccharum* hybrid (R570) (SHg) (Garsmeur et al., 2018).

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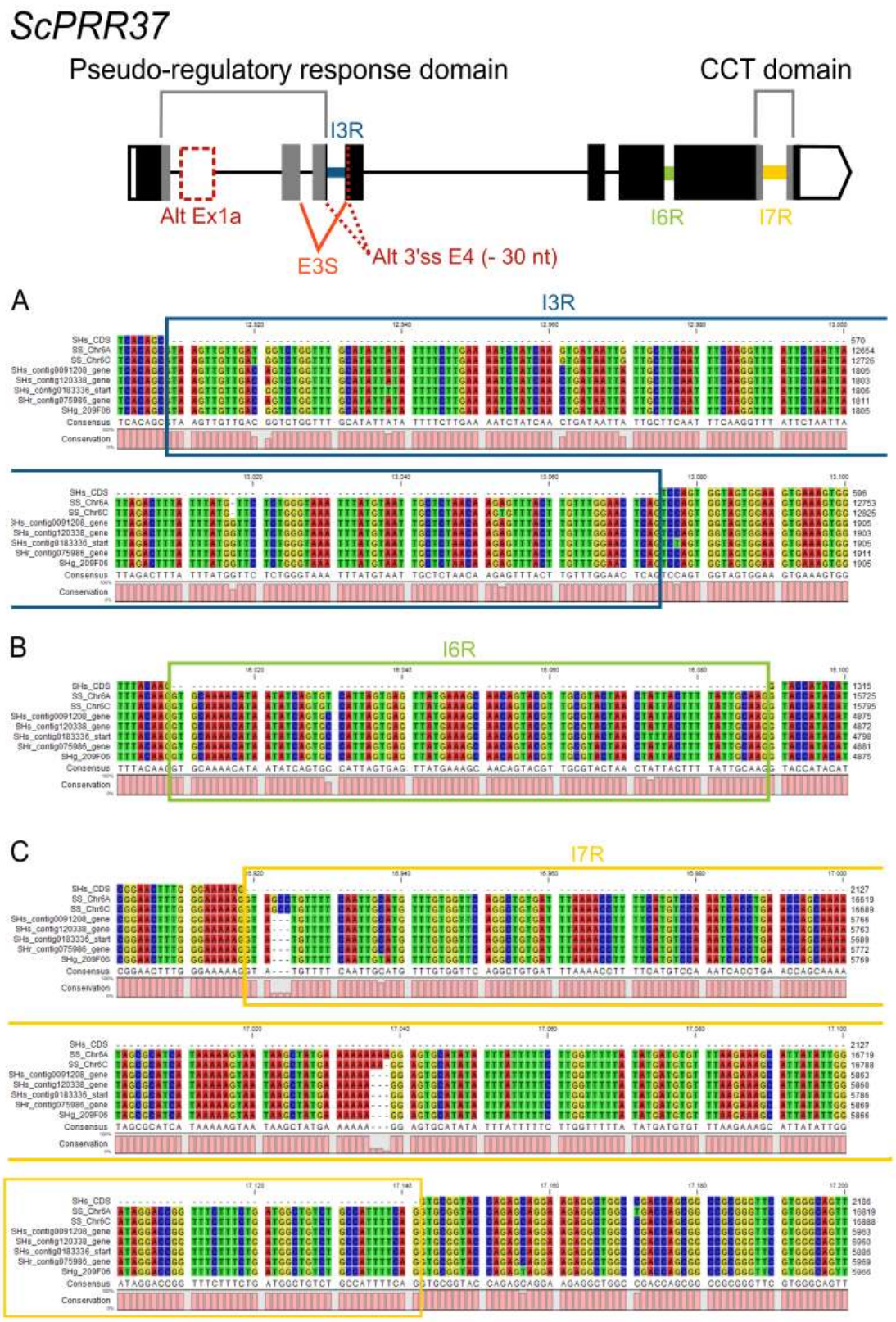
1022 **Supplemental Figure 3**



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1024 **Supplemental Figure 3 – Alignment of three different regions of *ScPRR37* from**
1025 **different genomic sequences.** Sequences from different sugarcane genome projects that
1026 were identified as *ScPRR37* were aligned in order to compare the regions containing the
1027 I3R, I6R, and I7R AS events in different haplotypes. The regions containing (**A**) the intron
1028 3 (dark blue), (**B**) intron 6 (green) and (**C**) intron 7 (yellow) were highlighted to show that
1029 all the sequenced haplotypes have the same gene structure. Sequences are from *S.*
1030 *spontaneum* (SS) (Zhang et al., 2018), *Saccharum* hybrid (SP80-3280): SHs (Souza et al.,
1031 unpublished), SHr (Matiello et al., 2017), and *Saccharum* hybrid (R570) (SHg)
1032 (Garsmeur et al., 2018).

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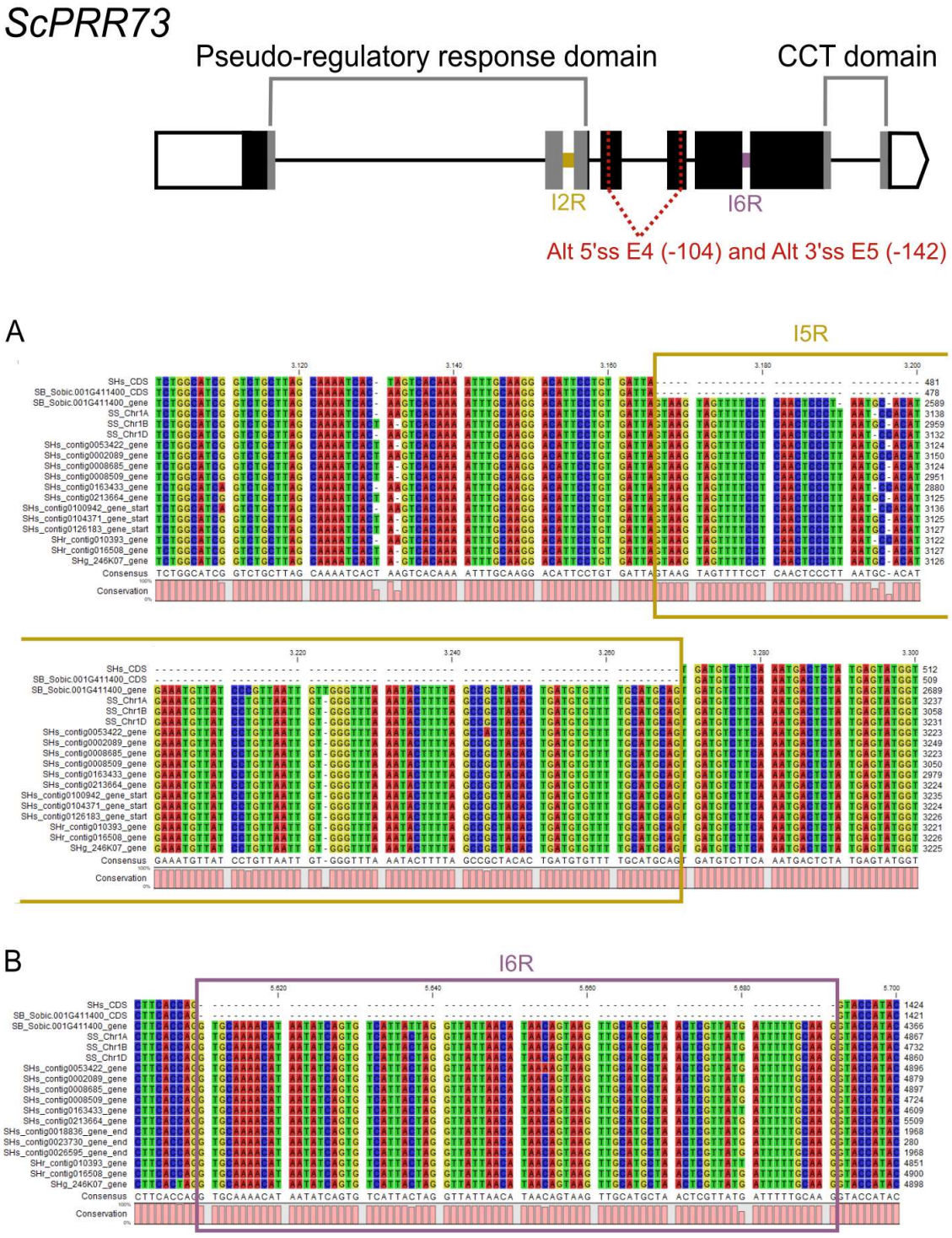
Supplemental Figure 4



Supplemental Figure 4 – Alignment of two different regions of *ScPRR73* from different genomic sequences. Sequences from different sugarcane genome projects that were identified as *ScPRR73* were aligned in order to compare the regions containing the

1040 I2R and I6R AS events in different haplotypes. The regions containing (**A**) the intron 2
1041 (gold) and (**B**) intron 6 (purple) were highlighted to show that all the sequenced
1042 haplotypes have the same gene structure. SS sequences are from *S. spontaneum* (Zhang
1043 et al., 2018). SHs are from the *Saccharum* hybrid (SP80-3280) sequenced by Souza et al.
1044 (unpublished), and SHr were sequenced by Matiello et al. (2017). SHg are from the
1045 *Saccharum* hybrid (R570) sequenced by Garsmeur et al. (2018).

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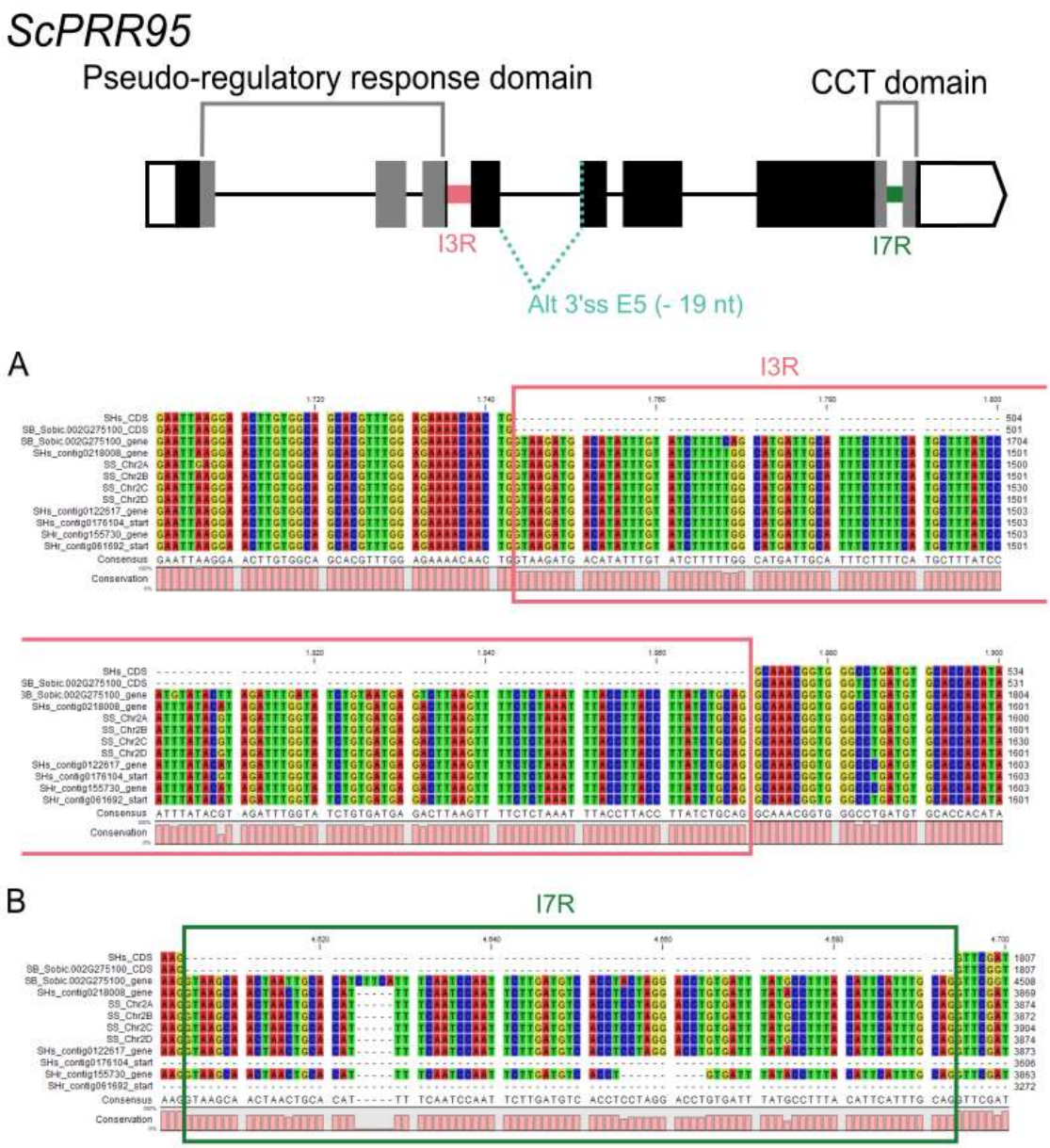
1048 **Supplemental Figure 5**



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1050 **Supplemental Figure 5 – Alignment of two different regions of *ScPRR95* from**
1051 **different genomic sequences.** Sequences from different sugarcane genome projects that
1052 were identified as *ScPRR95* were aligned in order to compare the regions containing the
1053 I3R and I7R AS events in different haplotypes. The regions containing (**A**) the intron 3
1054 (pink) and (**B**) intron 7 (dark green) were highlighted to show that all the sequenced
1055 haplotypes have the same gene structure. SS sequences are from *S. spontaneum* (Zhang

1056 et al., 2018). SHs are from the *Saccharum* hybrid (SP80-3280) sequenced by Souza et al.
1057 (unpublished), and SHr were sequenced by Matiello et al. (2017). SHg are from the
1058 *Saccharum* hybrid (R570) sequenced by Garsmeur et al. (2018).

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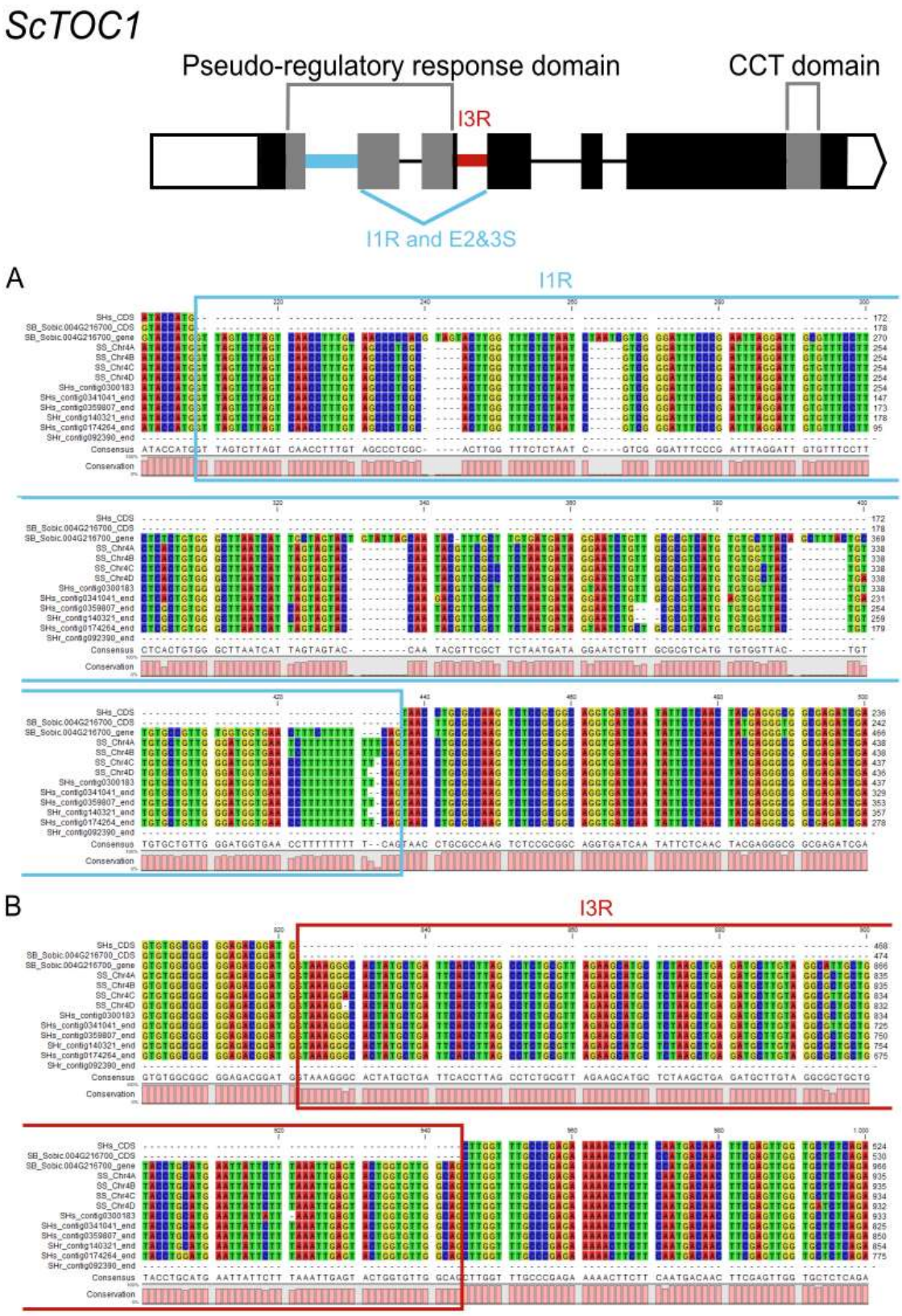
1061 **Supplemental Figure 6**



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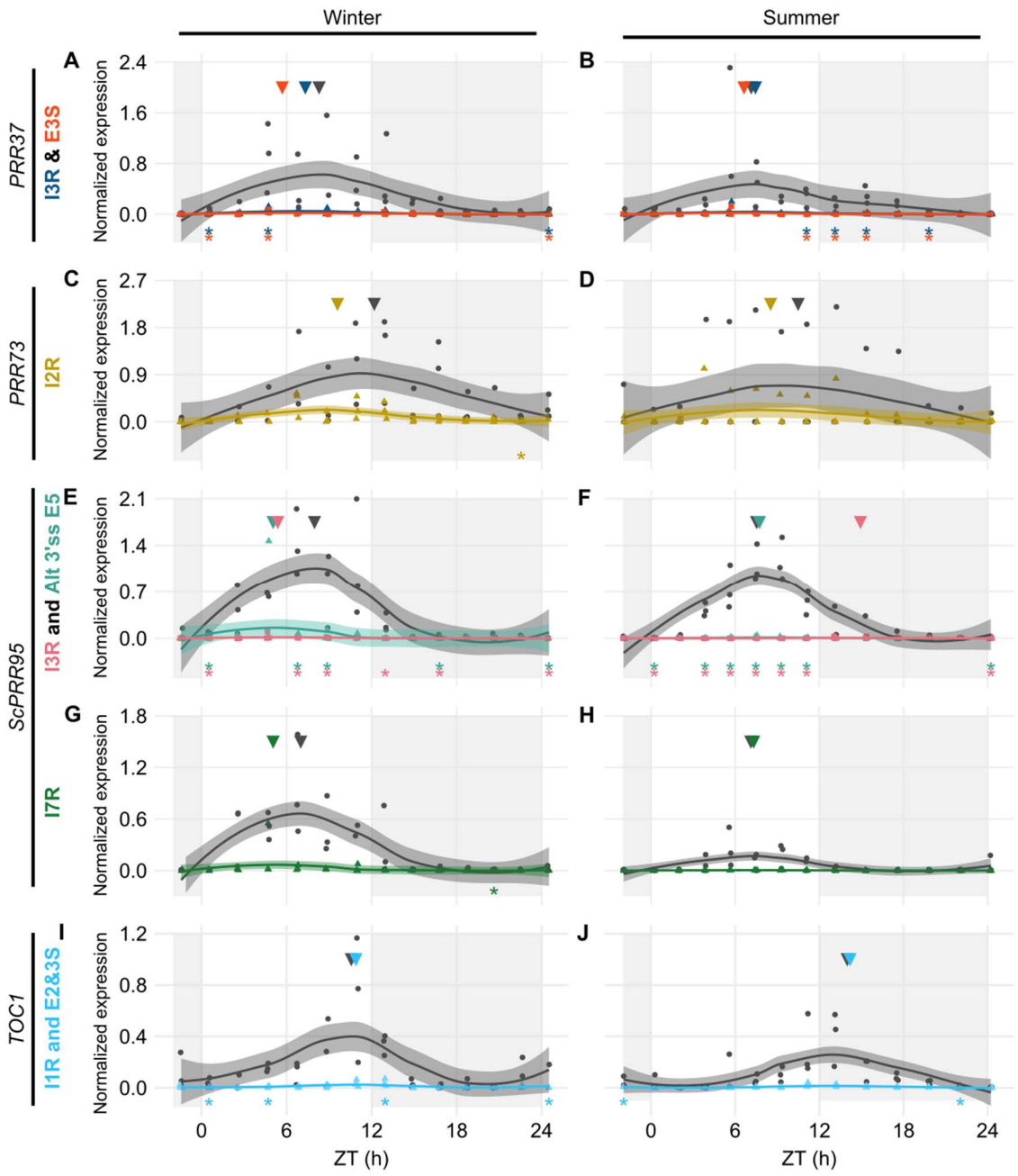
1063 **Supplemental Figure 6 – Alignment of two different regions of *ScTOC1* from**
1064 **different genomic sequences.** Sequences from different sugarcane genome projects that
1065 were identified as *ScTOC1* were aligned in order to compare the regions containing the
1066 I1R and I3R AS events in different haplotypes. The regions containing (**A**) the intron 1
1067 (light blue) and (**B**) intron 3 (red) were highlighted to show that all the sequenced
1068 haplotypes have the same gene structure. SS sequences are from *S. spontaneum* (Zhang
1069 et al., 2018). SHs are from the *Saccharum* hybrid (SP80-3280) sequenced by Souza et
1070 al. (unpublished), and SHr were sequenced by Matiello et al. (2017). SHg are from the
1071 *Saccharum* hybrid (R570) sequenced by Garsmeur et al. (2018).
1072

1073 **Supplemental Figure 7**



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1075 **Supplemental Figure 7 – Diel expression profile of fully spliced and alternative**
1076 **transcript isoforms in different seasons.** Biological replicates (circles and triangles) and
1077 their LOESS curve (continuous lines ± SE) of fully spliced (FS, black) and alternative
1078 transcript forms (AS, color) for the winter samples (4-months old plants, left) the summer
1079 samples (9-months old plants, right). These AS forms, though detected by HR RT-PCR,

1080 were not highly expressed. *ScPRR37* gene expression shows levels of I3R (orange) and
1081 E3S (dark blue) (**A-B**); *ScPRR73* gene expression shows levels of I2R (gold) (**C-D**);
1082 *ScPRR95* gene expression shows levels of I3R (pink) and Alt 5'ss E5 (teal) (**E-F**), and
1083 I7R (dark green) (**G-H**). *ScTOC1* gene expression shows levels of I1R/E23S (cyan) (**I-**
1084 **J**). Inverted triangles show the time of the maximum value of the LOESS curve. The
1085 light-gray boxes represent the night period. Statistical significance was analyzed by
1086 paired Student's t-test, * $p < 0.05$ (C-D, G-J) or by One-way ANOVA with post-hoc
1087 Tukey HSD test, * $p < 0.05$ (A-B, E-F), each color corresponding to a test of an AS form
1088 against the FS form.

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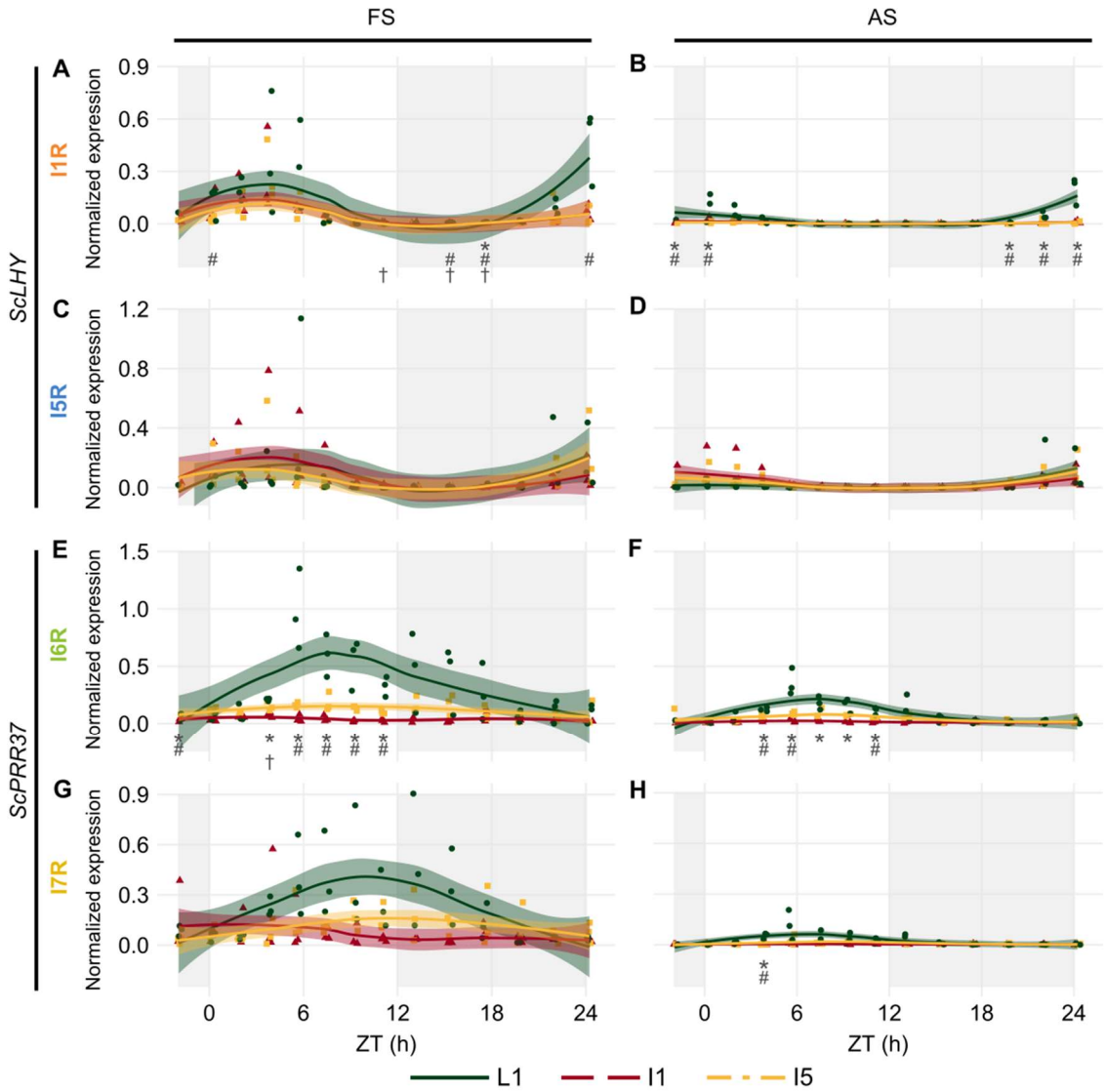
1091 **Supplemental Figure 8**



1092

1093 **Supplemental Figure 8 – Diel expression profile of fully spliced and alternative**
1094 **spliced transcript isoforms in leaves and internodes.** Biological replicates (circles and
1095 triangles) and their LOESS curve (continuous lines ± SE) of fully spliced (left column)
1096 and alternative spliced (right column) transcript forms of sugarcane circadian clock genes
1097 during the summer harvest in leaves (green), internode 1 and 2 (red) and internode 5
1098 (yellow). **(A, B)** *ScLHY* gene expression shows levels of I1R and **(C, D)** I5R; **(E, F)**
1099 *ScPRR37* gene expression shows levels of I6R and **(G, H)** I7R. The light-gray boxes
1100 represent the night period. Statistical significance was analyzed by One-way ANOVA

with post-hoc Tukey HSD test, * $p < 0.05$ when comparing L1 against I1, # $p < 0.05$ when comparing L1 against I5, and † $p < 0.05$ when comparing I1 against I5.

1104 **Table S1 – List of sugarcane gene-specific primers and their sequences.** Primers in
1105 bold indicate forward (FW) sequences, as well as the primers bearing the 6-FAM tag
1106 used in the HR RT-PCR assays. The other sequences indicate reverse (RV) sequences.
1107 Normalizer primers are highlighted in green.

Gene	Sequence (5' → 3')
ScGAPDH	**CACGGCCACTGGAAGCA**
	TCCTCAGGGTTCCTGATGCC
ScPP2AA2	**GCGCCTTAACTCCATCAGAA**
	GCTCTACACCTCCAACATAAGG
ScLHY	**CTGTTGCACATTTCCCTTCTG**
	TTTGAGCGTGACTTCTGATCT
	ACCTACCACAGGAGCTGATA
	GATAAATGCTTGTGGCGTTCTT
ScTOC1	**ATTCTTCTCTGCGACGGCGA**
	TAGGCCTATCGTCTGTCTCGT
*Sc*PRR37	**GGAATGACAATCTGAGGAGCAG**
	AGTTCATTCTTACGTATAGGCTTGG
	TGTGGAGATGGTGCCAATAC
	GCAACATCACCCTGTCCTTAT
	GAGCAACTCAGGCAGTAACA
	GCATAGCTTGCTTTACGAACTG
ScPRR73	**GAGAGGTTCCTACCTGTGAAGA**
	CTGTGTCTGGATGCCACTTT
	GTGACGAGTATGAGAACGACAG
	GAGTTCCTAGGAGCACCTATTTC
	CAAGATGCCCGATGGAAATG
	GACGTTACTCTCTCCCTGTTAG
	GGGTGTCTCAGTTGGTCATAAT
	TGAACCTTGCGCTGCTT
*Sc*PRR95	**AGTGGACTTCCTTGTGAAACC**
	GAACCAGCCACTTCATCTCTAC
	ATTCTTCGGGAATCAGGAGAAC
	CAGTTTCTGCTTCTGTTGTTGTAG

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1110 **TableS2 – SAS number, GeneID, and ProbeID of genes associated with the**
1111 **Spliceosome in the Sugarcane array (Dantas et al., 2019).**

Gene	SAS	Gene ID	Probe ID
ABH1	SCCCCL4004G07.g	TC136430	GS1_SS_06377_20908
CDC5	SCJFRZ2005G12.g	TC116361	GS1_SS_18244_14104
LSM8	SCCCAM2004E11.g	TC126680	GS1_SS_22616_12065
LSM36B	SCCCLR2002D01.g	TC124904	GS1_SS_10307_02774
	SCEZAM1081G05.g	TC132981	GS1_SS_24478_05144
Prp3	SCEPAM1022H06.g	TC123686	GS1_SS_04144_21793
RCF1	SCVPAM1059D07.g	TC133378	GS1_SS_16968_10290
SC35	SCSFSB1101H12.g	TC113063	GS1_SS_22263_08222
SmD2	SCACLR2014D10.g	TC117969	GS1_SS_07124_11310
SmF	SCEPLB1043F04.g	TC140865	GS1_SS_02829_12937
SR34	SCCCLR2C03A06.g	TC114613	GS1_SS_12661_19184
STA1	SCUTSB1075G02.g	TC130590	GS1_SS_17461_08595

1112

1113 **Table S3 – Spliceosome-related transcripts showing rhythmicity in field conditions**
1114 **according to Dantas et al. (2019).** Sugarcane Assembled Sequences (SAS) were taken
1115 from the SUCEST-FUN database (http://sucest-fun.org).

Organ	Gene	Sugarcane Assembled Sequences (SAS)	Arabidopsis homologs	Phase (ZT)
Internodes 1 and 2	*Prp3*	SCEPAM1022H06.g	At1g28060	0
	LSM8	SCCCAM2004E11.g	At1g65700	11
Internode 5	*ABH1*	SCCCCL4004G07.g	At2g13540	0
	Prp3	SCEPAM1022H06.g	At1g28060	0
Leaf +1	*RCF1*	SCVPAM1059D07.g	At1g20920	2
	LSM8	SCCCAM2004E11.g	At1g65700	9
	SmD2	SCACLR2014D10.g	At2g47640	9
	CDC5	SCJFRZ2005G12.g	At1g09770	11
	LSM36B	SCCCLR2002D01.g	At3g59810	11
		SCEZAM1081G05.g	At5g27720	11
	SR34	SCCCLR2C03A06.g	At1g02840	11
	ABH1	SCCCCL4004G07.g	At2g13540	13
	SC35	SCSFSB1101H12.g	At5g64200	13
	SmF	SCEPLB1043F04.g	At4g30220	13
	STA1	SCUTSB1075G02.g	At4g03430	22

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1117