Alternative splicing of circadian clock genes is temperature regulated in field-grown sugarcane

Running title: Temperature-regulated rhythms of alternative splicing in sugarcane

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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 identify AS events in core circadian clock genes and the dynamic adjustment of the events as a response to temperature changes in field-grown sugarcane. 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 regulated by temperature.

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

41 **1. Introduction**

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

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

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

91 2012). In Arabidopsis, the main loop consists in three different components:
92 CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL
93 (LHY) and TIMING OF CHLOROPHYLL A/B BINDING PROTEIN 1 (TOC1)
94 (Alabadí et al., 2001). Closely associated to this loop are the PSEUDO-RESPONSE
95 REGULATORS 7, 3 and 9 (PRR7, PRR3, PRR9) (Locke et al., 2005; Nakamichi et al.,
96 2010; Para et al., 2007; Zeilinger et al., 2006). The components of the central loop and
97 the associated PRRs are conserved among other plant species, including crops like rice,
98 maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and sugarcane (*Saccharum* hybrid)
99 (Calixto et al., 2015; Hotta et al., 2013; Khan et al., 2010; Murakami et al., 2007). The
100 sugarcane circadian clock, although sharing conserved components with other plants,
101 may have a broader influence over sugarcane physiology, with 32% of sugarcane
102 transcripts showing rhythms under circadian conditions (Hotta et 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 used sugarcane organs extracted from field-grown plants when
132 individuals were 4-months-old, during the Brazilian winter and 9-months-old, during the
133 Brazilian summer. We investigated the AS profile of sugarcane circadian clock genes in
134 this fluctuating natural environment. Data shows that there is at least one alternatively
135 spliced form for each of the five circadian clock genes analyzed. During winter, when
136 temperatures are lower, alternative transcripts are more highly expressed than in summer,
137 with higher temperatures, which suggests that AS might be related to the fluctuating
138 environmental temperature in the field. The different organs also showed different levels

139 of AS and leaf has most of the diversity in AS events. Collectively, our data suggest there
140 is a strong influence of temperature over the AS in the circadian clock of sugarcane plants
141 grown in a natural environment, possibly as a mechanism regulating environmental
142 information.

143

144 ## 2. Material and Methods

145 ### 2.1. Field Conditions and Plant Harvesting

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

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

178

179 **2.2. RNA Extraction**

180 Sugarcane leaves previously frozen in liquid nitrogen were pulverized using dry ice and
181 a grinder. Then, 100 mg of this ground tissue was used for total RNA extractions using
182 Trizol (Life Technologies, Carlsbad, CA, USA), followed by treatment with DNase I
183 (Life Technologies, Carlsbad, CA, USA) and cleaned with RNeasy Plant Mini Kit
184 (QIAGEN, Valencia, CA, USA). Quality and quantity of each RNA sample were checked
185 using an Agilent RNA 6000 Nano Kit Bioanalyzer (Agilent Technologies, Palo Alto, CA,
186 USA). All RNA samples were stored at -80ºC.

187

188 **2.3. cDNA Synthesis**

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

193

194 **2.4. PCR Reactions**

195 Primers used in PCR reactions were designed using the software PrimerQuest Tool (IDT)
196 (http://www.idtdna.com/primerquest/home/index). Each pair of primers was gene-
197 specific and amplified fragments ranging from 242 bp to 805 bp (**Table S1**). All PCR
198 reactions were carried out using Go Taq DNA Polymerase (Promega, Madison, WI, USA)
199 and following the manufacturer's protocol. Briefly, each 20-µL PCR reaction contained 2
200 µL of template, 10 µM of each primer, 4 µL of 5x Green Go Taq Buffer, 0.15 µL of Go
201 Taq DNA Polymerase, 2 mM of dNTPs. PCR conditions were: an initial step at 94ºC for
202 2 mins, followed by 20 – 30 cycles of 94ºC for 15 s, 50ºC for 15 s, 72ºC for 30 s, followed
203 by a final extension of 72ºC for 5 min. PCR reactions using primers amplifying control
204 genes *ScGAPDH* and *ScPP2AA2* were performed for all cDNA samples. Reactions
205 containing negative control using RNA as template and positive control using genomic
206 DNA as template were carried out. All PCR-amplified fragments were analyzed by taking
207 10 µL of reaction and run on an electrophoresis gel of 1.5% agarose (Life Technologies,
208 Carlsbad, CA, USA) and 1x TBE (50 mM Tris-HCl pH 8, 50 mM Boric Acid, 1mM
209 EDTA).

210

211 **2.5. High-Resolution RT-PCR**

212 High-Resolution RT-PCR (HR RT-PCR) reactions were performed based on Simpson et
213 al. (2008) and Simpson et al. (2019). For all reactions, the forward primer was labeled
214 with 6-carboxyfluorescein (FAM). Reactions consisted of a final volume of 20 µL which
215 had 2 µL of cDNA, 10 µM of each primer, 2 µL of 10x PCR Reaction Buffer with $MgCl_2$
216 (Roche Life Science, Indianapolis, IN, USA), 0.15 µL Taq DNA Polymerase (Roche Life
217 Science, Indianapolis, IN, USA), and 2 mM of dNTPs. The PCR detailed program was:
218 an initial step at 94ºC for 2 min, followed by 22 – 26 cycles of 94 ºC for 15 s, 50ºC for
219 15 s, 70ºC for 30 s, followed by a final extension of 70ºC for 5 mins. Once PCR reactions
220 were complete, 1 µL of each reaction was added to a mix containing 9 µL of Hi-Di
221 Formamide (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and 0.5 µL of
222 GeneScan 500 LIZ Size Standard (Applied Biosystems, Carlsbad, CA, USA). The RT-
223 PCR products were separated on an ABI 3730 Automatic DNA Sequencer (Applied
224 Biosystems, Life Technologies, Carlsbad, CA, USA). The results were analyzed using
225 GeneMapper fragment analysis software (Applied Biosystems, Carlsbad, CA, USA).
226 LOESS (locally estimated scatterplot smoothing) regression was used to detect trends in
227 the data. The maximum value of the LOESS curve between ZT0 and ZT22 was
228 considered the peak of the rhythm.

229

230 **2.6. Cloning and Sequencing**

231 In order to identify alternatively spliced forms, as well as differentially expressed alleles,
232 RT-PCR fragments were cloned and sequenced. For this, PCR fragments were purified

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

251

252 **3. Results**

253 **3.1. Identification of Alternatively Spliced Forms of Sugarcane Circadian Clock**
254 **Genes**

255 In Arabidopsis, alternative splicing (AS) events of the circadian clock genes are well
256 known (James et al., 2012b). In order to investigate AS of sugarcane circadian clock

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

277 To identify AS transcripts, RT-PCR was performed on cDNA synthesized from L1 RNA,
278 collected at three different time points, which corresponded to the highest gene expression
279 for each clock gene, based on the findings from Hotta et al. (2013). RT-PCR used pairs
280 of primers to generate overlapping amplicons to cover the full length of the transcripts
281 for each sugarcane circadian clock genes, *ScLHY*, *ScPRR37*, *ScPRR73*, *ScPRR95,* and

282 *ScTOC1*. RT-PCR products were cloned and sequenced, which confirmed the annotation
283 and identified AS events. Confirmation of different transcript isoforms was carried out
284 by using two different approaches combined: RT-PCR product size and fragment cloning
285 followed by sequencing.

286

287 **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
	Introns 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.

288

289 We found evidence of AS events in all five genes analyzed (**Table 1**). Intron retention
290 (IR) was the most frequent AS event identified, resulting in the inclusion of premature
291 termination codons (PTC) in all cases (**Figure 1**). All five genes had at least two IR
292 events, and *ScTOC1* had retention of the first intron (I1R) combined with the skipping of
293 exons 2 and 3 (E23S). *ScLHY* had retention of introns 1 (I1R) and 5 (I5R). *ScPRR37* had
294 retention of introns 3 (I3R), 6 (I6R) and 7 (I7R). *ScPRR73* had retention of introns 2 (I2R)

295 and 6 (I6R). *ScPRR95* had two introns retained: intron 3 (I3R) and intron 7 (I7R) (**Figure**
296 **1**). All the other introns in the genes were efficiently spliced. Exon skipping (ES) was
297 detected in *ScPRR37* (E3S) and *ScTOC1*, the latter having two exons skipped at once
298 (E23S), combined with I1R (**Figure 1**). Alternative 3' splice sites (Alt 3' ss) were found
299 in both *ScPRR37* and *ScPRR95* in exon 4 (E4) and exon 5 (E5), respectively (**Figure 1**).
300 In *ScPRR73*, we found an AS event between exons 4 and 5 involving an alternative 5'
301 splice site (Alt 5'ss) in exon 4 E4 being spliced to an Alt3'ss in exon 5. This AS event
302 removes 104 nt and 142 nt from exons 4 and 5, respectively (total of 246 nt), but remains
303 in frame and potentially produces a protein missing 82 amino acids compared to the wild-
304 type protein.

305 It is possible that some of the alternative sequences we found are not AS events but
306 different haplotypes since sugarcane is highly polyploid and aneuploid. To exclude this
307 possibility, we obtained the sequences for circadian clock genes from four different
308 sugarcane sequencing projects (Garsmeur et al., 2018; Riaño-Pachón and Mattiello, 2017;
309 Souza et al., unpublished; Zhang et al., 2018). We found 11 sequences for the 5' portion
310 of the *ScLHY*: 4 from *S. spontaneum* (Zhang et al., 2018), 6 from the commercial
311 *Saccharum* hybrid SP80-3280 (Riaño-Pachón and Mattiello, 2017; Souza et al.,
312 unpublished) and 1 from the commercial *Saccharum* hybrid R570 (Garsmeur et al., 2018).
313 All *ScLHY* sequences had the complete intron 1 (**Figure S2**). Similarly, all 16 sequences
314 that had the end portion of *ScLHY* contained the complete intron 5. (**Figure S2**). All other
315 detected AS events had 7 to 15 sequences supporting the conclusion that these alternative
316 sequences are not the result of different sugarcane haplotypes (**Figures S3-S6**).

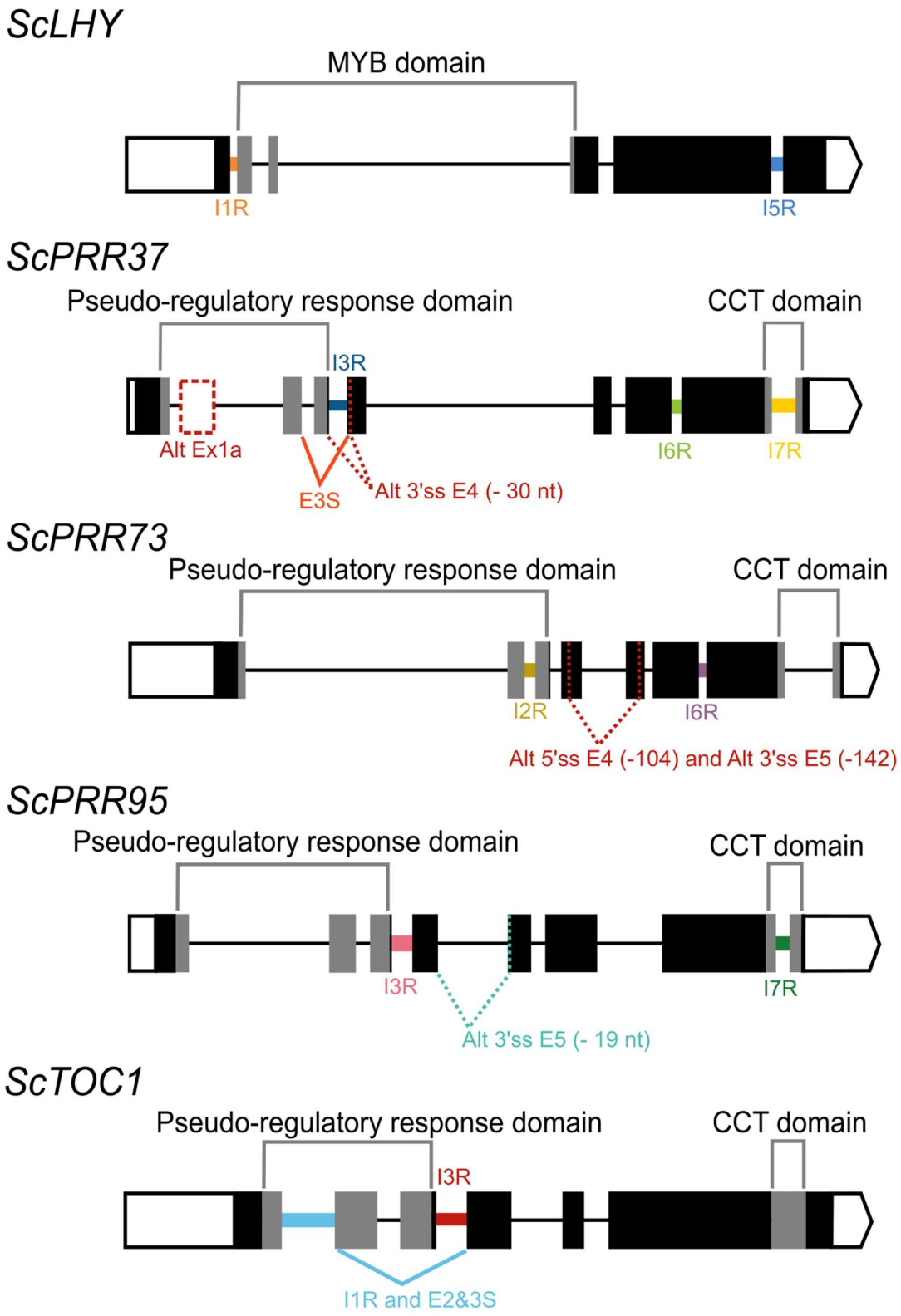


Figure 1 – Alternative splicing events identified in the sugarcane circadian clock genes. Alternative splicing (AS) events are shown in the gene structure of *ScLHY*, *ScPRR37*, *ScPRR73*, *ScPRR95,* and *ScTOC1*. White boxes show 5' and 3' UTR regions;

321 black boxes show exons; black lines represent introns. The main protein domains are in
322 grey. AS events are shown in colored solid lines, colored dotted lines or in dotted white
323 boxes and the splicing events are illustrated below each event. Different colors were
324 chosen to mark the AS events investigated later, whereas events that were not investigated
325 further are in dark red. IR = intron retention; ES = exon skipping; Alt 5' ss = alternative
326 5' splice sites; Alt 3' ss = alternative 3' splice sites; Alt Ex = alternative exon.

327

328 **3.2. Expressed AS forms in different seasons in sugarcane leaves**

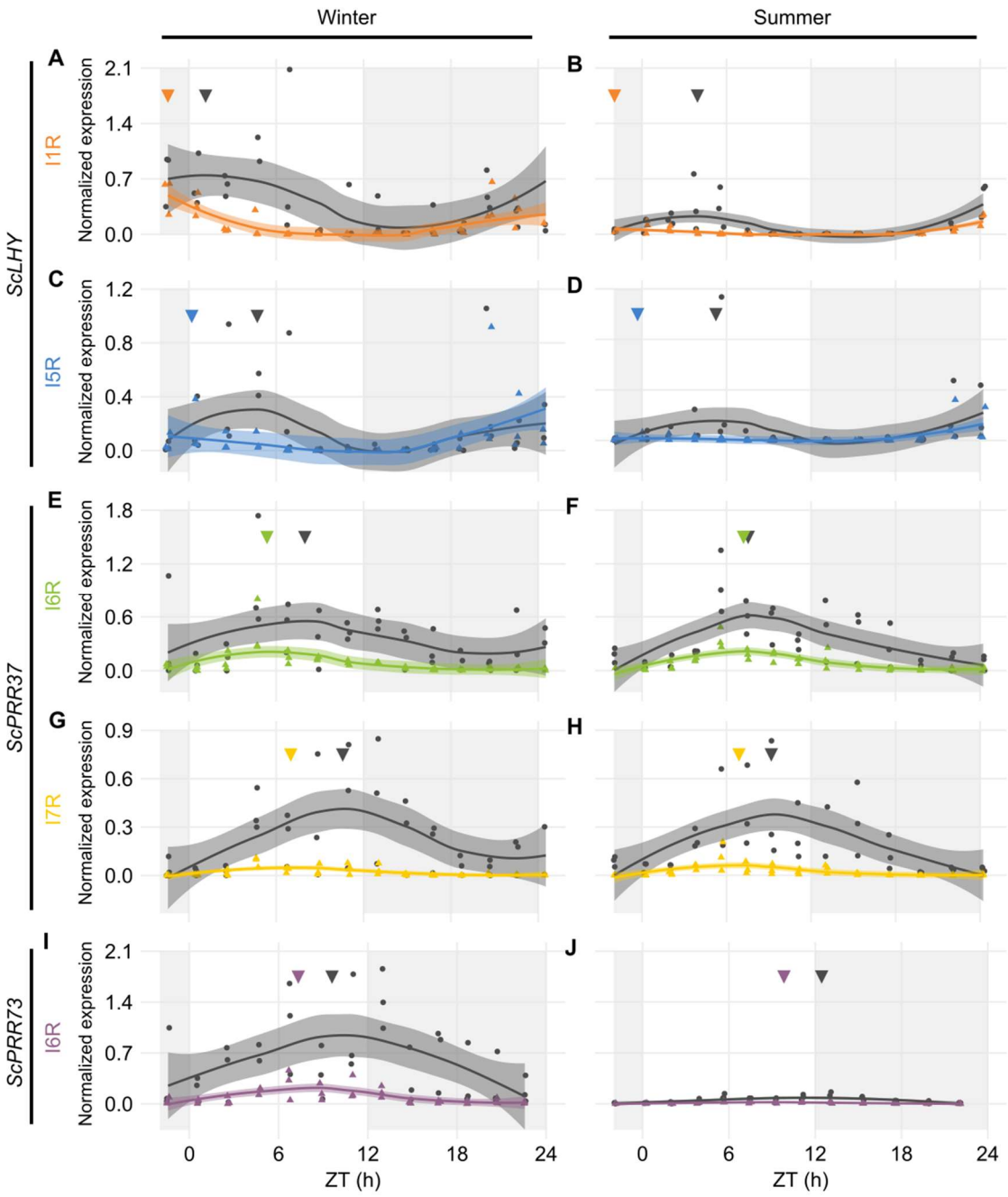
329 We used HR RT-PCR (Simpson et al., 2019) to examine the daily dynamics of the
330 expressed isoforms of the sugarcane circadian clock genes in two different seasons, winter
331 and summer, using field-grown plants that were 4 and 9 months old, respectively. Briefly,
332 the HR RT-PCR system uses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

342 From the five sugarcane circadian clock genes analyzed, HR RT-PCR experiments
343 detected high levels of AS in *ScLHY*, *ScPRR37,* and *ScPRR73* (**Figure 2**), but not in
344 *ScPRR95* and *TOC1*, in L1 (**Figure S7**). In general, the AS isoform peaked earlier than

345 the FS form in 8 of the 9 conditions assayed (**Figure 2A-I**), apart from *ScLHY,* where the
346 FS forms peaked at the same normalized time in winter and summer samples.

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

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



364

365 **Figure 2 – Diel expression profile of fully spliced and alternative transcript isoforms**
366 **in different seasons.** Biological replicates (circles and triangles) and their LOESS curve
367 (continuous lines ± SE) of fully spliced (FS, black) and alternative transcript forms (AS,
368 colored) for the winter samples (4-month-old plants, left) the summer samples (9-month-
369 old plants, right). **(A,B)** *ScLHY* gene expression shows levels of I1R (orange) and **(C,D)**
370 I5R events (blue). **(E,F)** *ScPRR37* gene expression shows levels of I6R (green), and
371 **(G,H)** I6R (yellow); **(I,J)** *ScPRR73* gene expression shows levels of I2R (purple).

372 Inverted triangles show the time of the maximum value of the LOESS curve. The light-
373 gray boxes represent the night period.

374

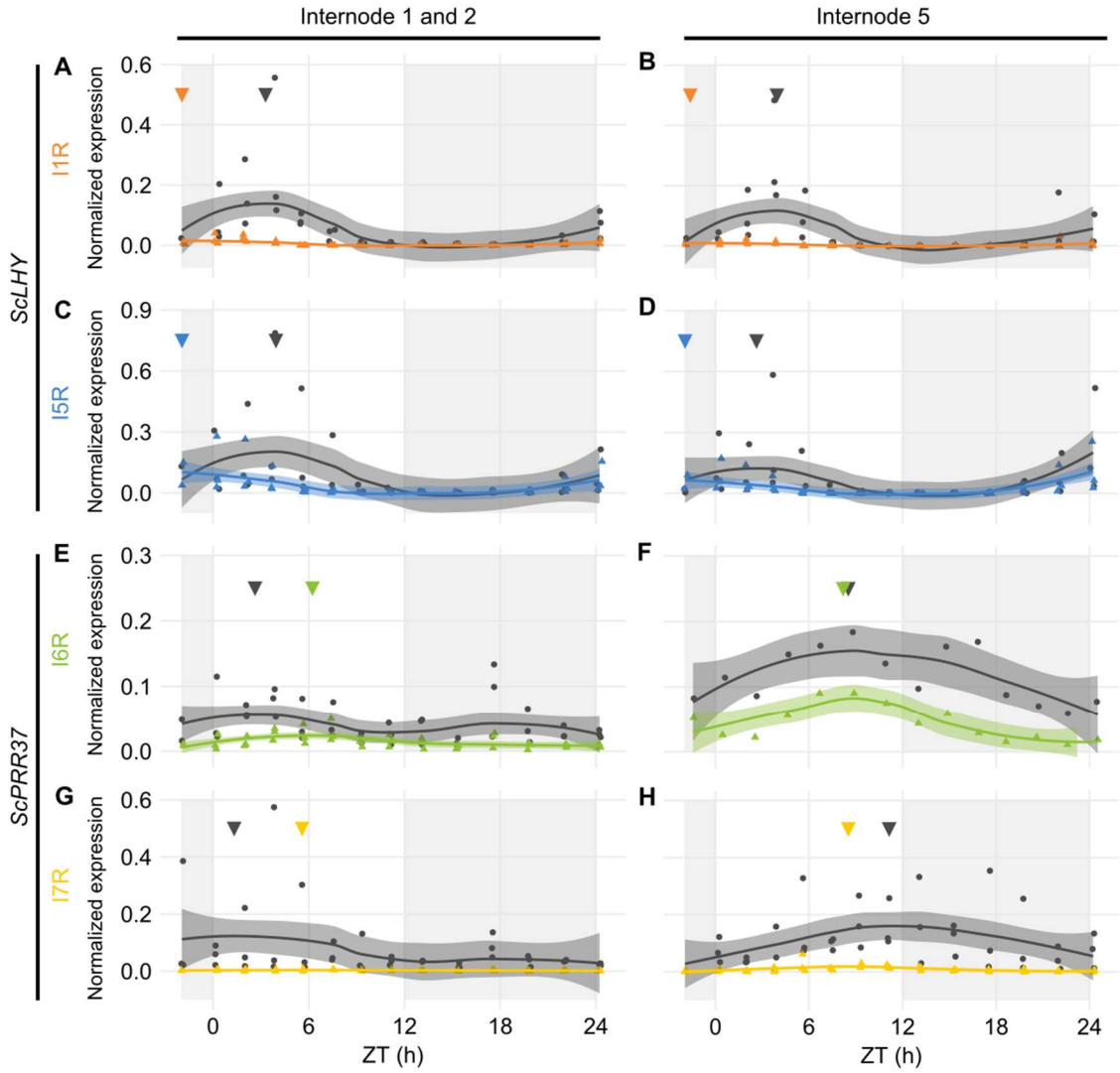
375 **3.3. Expressed AS forms in the different source-sink sugarcane organs**

376 We extended the investigation to two other sugarcane organs, internodes 1 and 2 (I1) and
377 internode 5 (I5). These internodes have different physiology: I1 has a high cellular and
378 metabolic activity, whereas I5 is the first internode to actively accumulate sucrose. Only
379 the plants harvested in the summer (9-month-old) had developed internodes that could be
380 harvested. We only measured AS forms in genes *ScLHY* and *ScPRR37*, which were the
381 homologs featuring the highest AS transcript expression in L1 (**Figure 2**). Both genes
382 had lower levels of AS in the internodes compared to the L1 (**Figure 3**).

383 In both internodes, *ScLHY* showed detectable levels of both AS events observed in L1.
384 *ScLHY* I1R-containing transcript levels were very low with a peak 2 h before dawn
385 (ZT22), with the FS isoform peaking between ZT03-04 (**Figure 3A-B**). *ScLHY* I5R was
386 also identified in both internodes, at higher levels than *ScLHY* I1R. The AS isoform
387 peaked at ZT22, while the FS form peaked between ZT03-04 (**Figure 3C-D**).

388 The *ScPRR37* homolog featured variable levels of FS and AS isoforms when compared
389 to both internodes. In I1, *ScPRR37* I6R and its FS isoform did not have a clear rhythm,
390 with the I6R showing low levels of expression, and the FS isoform showing two peaks.
391 In I5, *ScPRR37* I6R and its FS isoform had higher levels and a peak between ZT08-09
392 (**Figure 3E-F**). In both internodes, the I7R isoform was expressed at very low levels, but
393 the FS isoform for that transcript region peaked at ZT01 in I1 and ZT11 in I5 (**Figure**
394 **3G-H**).

395 In summary, there is a striking difference in the expression levels of the various clock
396 gene transcripts in internodes compared to leaves. Expression was significantly lower in
397 internodes especially for I5 and fewer AS events were detected, perhaps reflecting the
398 differences in physiology and gene expression inherent to each one of the three organs
399 analyzed.



400

401 **Figure 3 – Diel expression profile of fully spliced and alternative spliced transcript**
402 **isoforms in internodes.** Biological replicates (circles and triangles) and their LOESS
403 curve (continuous lines ± SE) of fully spliced (FS, black) and alternative spliced (AS,
404 colored) transcript forms of sugarcane circadian clock genes during the summer harvest

405 in internode 1 and 2 (left) and internode 5 (right). **(A, B)** *ScLHY* gene expression shows
406 levels of I1R (orange) and **(C, D)** I5R (blue); **(E, F)** *ScPRR37* gene expression shows
407 levels of I6R (green) and **(G, H)** I7R (yellow). Inverted triangles show the time of the
408 maximum value of the LOESS curve. The light-gray boxes represent the night period.

409

410 **3.4. Alternative splicing events are dependent on the time of the day and**
411 **temperature**

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

425 The rhythmic changes in the log(AS/FS) values could be explained by changes in the
426 expression of putative regulatory genes, such as splicing factors or spliceosomal protein
427 genes. In a previous work, we have identified 6,705 rhythmic transcripts in L1, 3,755 in
428 I1 and 3,242 in I5 in field-grown sugarcane (Dantas et al., 2019). Fourteen spliceosome-

429 related transcripts in the oligo array were expressed in all three organs (**Table S2**): 9
430 transcripts were rhythmic only in L1, one was rhythmic in L1 and I1, one was rhythmic
431 in L1 and I5, and one was rhythmic in I1 and I5 (**Table S3**). In L1, 9 of the 11 rhythmic
432 transcripts peaked between ZT09 and ZT13, with a trough between ZT03 and ZT5
433 (**Figure 4E**). In the internodes, most of the transcripts peaked at ZT00 (**Figure 4F**).

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

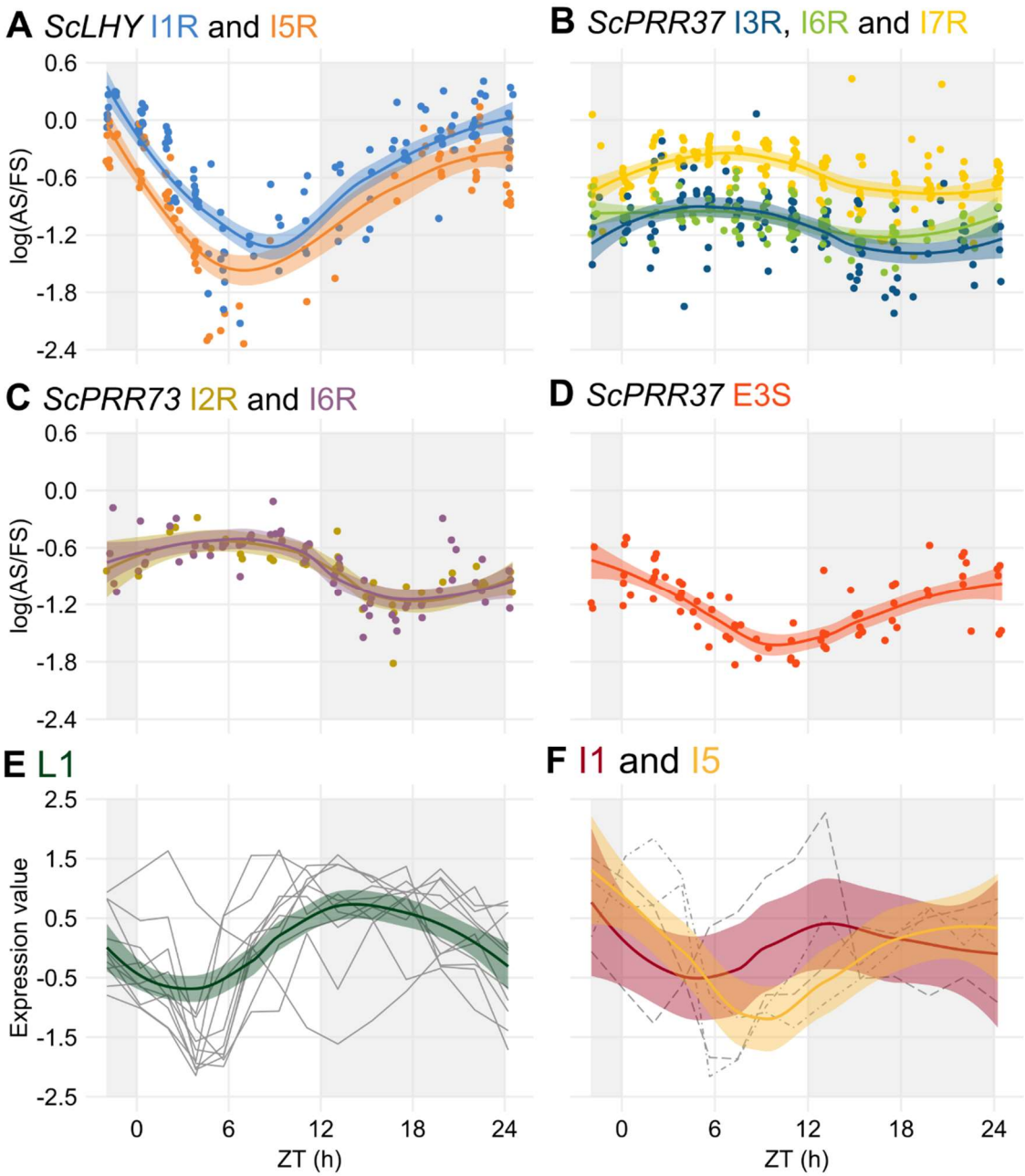
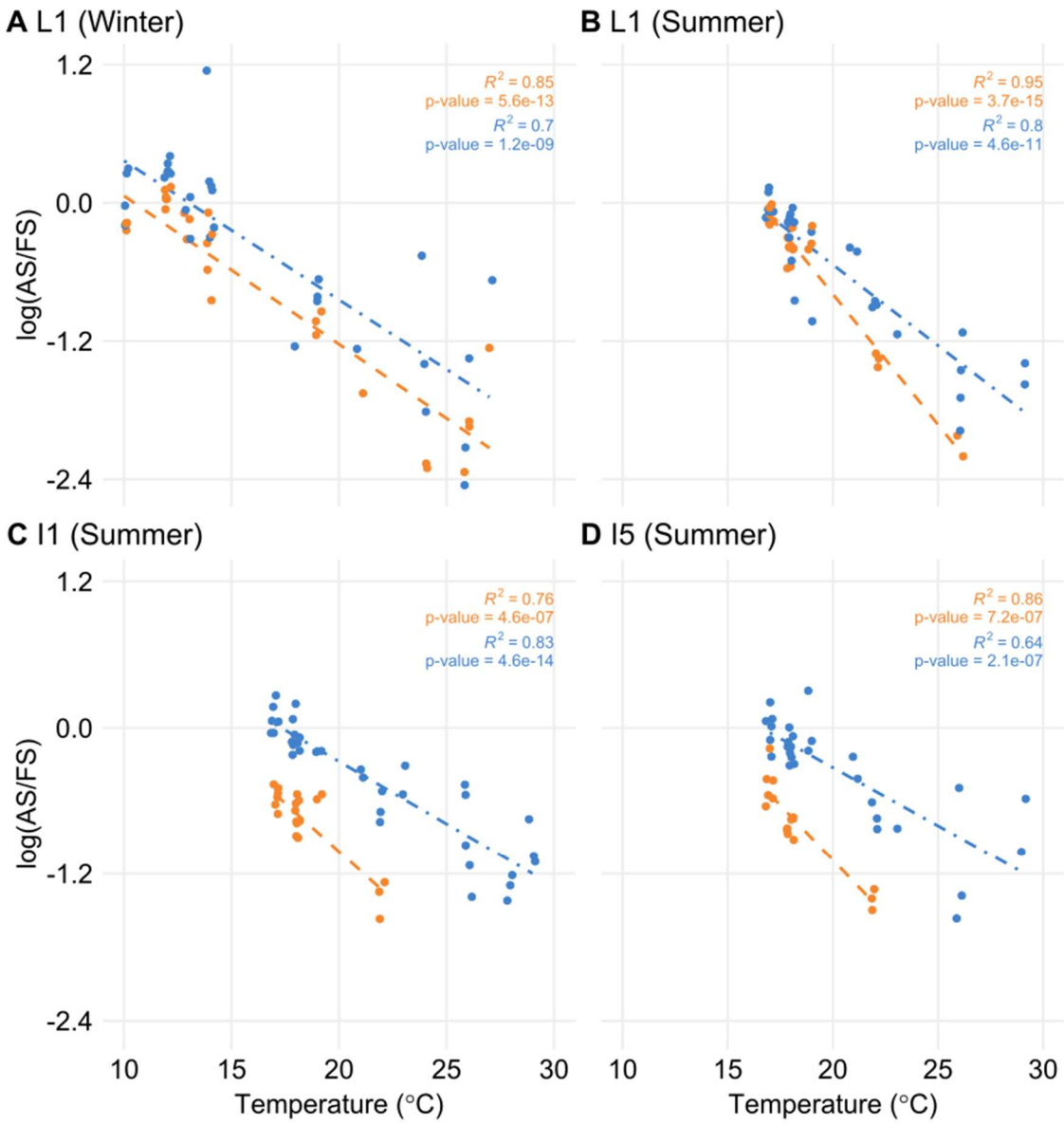


Figure 4 – Alternative splicing is rhythmic in sugarcane in field conditions. (**A-D**) The logarithm of the ratio of the expression levels of an AS isoform to its FS isoform, annotated as the log(AS/FS), was plotted against the normalized time of the day (ZT). **(A)** *ScLHY* I1R (orange) and I5R (blue); *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,

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



451

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

456 lines were added for each group of ratios. R^2 and *P-value* were calculated for each
457 regression. Negative correlations were significant in leaf +1 (L1) in the **(A)** winter
458 (4-month-old plants), and **(B)** summer, **(C)** internode 1 and 2 (I1)**,** and **(D)** internode
459 5 (I5), both in the summer (9-month-old plants).

460 **4. Discussion**

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

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

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

530 2019). The gene-level expression of the majority of these genes peak around dusk
531 (between ZT11 and ZT13), which does not coincide with the peaks in splicing ratios
532 observed in circadian clock genes. The list of splicing-related genes in the microarray
533 analysis was not extensive (**Table S1**) and many splicing factors are alternatively spliced
534 to regulate the level of productive, protein-coding transcripts (Reddy et al., 2013; Staiger
535 and Brown, 2013b). Transcript level RNA-seq will be required to more accurately
536 measure protein-coding transcripts of splicing factors to identify candidate regulators of
537 AS of the core clock genes analyzed here.

538 We found that the splicing ratios from *ScLHY* AS transcripts are negatively correlated
539 with temperature in all organs. Previous studies on the presence of AS in circadian clock
540 genes in Arabidopsis and barley revealed that, under controlled conditions, the AS status
541 of circadian clock genes is regulated by temperature, especially *LHY* and its paralog
542 *CIRCADIAN CLOCK ASSOCIATED* (*CCA1*) (Calixto et al., 2016, 2018; Filichkin et al.,
543 2015a; James et al., 2012a and b; Kwon et al., 2014; Marshall et al., 2016; Park et al.,
544 2012; Seo et al., 2012). Lower temperatures led to increased abundance of alternative
545 non-productive forms of *LHY*, *PRR7* and *PRR5* in Arabidopsis (James et al., 2012a). This
546 could affect the expression of fully spliced transcripts of the circadian clock genes,
547 promoting a functional modulation in the circadian clock central oscillator, which might
548 be reflected by altered temporal control of the clock outputs. In Arabidopsis, cold
549 temperatures reduced the amplitude of *CCA1*/*LHY*, as well as disrupted the circadian
550 clock function (Bieniawska et al., 2008). Considering the natural environment context,
551 where plants like sugarcane face fluctuations in temperature on a daily and yearly basis,
552 the continuous temperature-regulation of AS of the circadian clock network could have a
553 more profound impact on metabolism and, ultimately, crop yield.

554 Recent studies featuring experiments conducted in field conditions in Arabidopsis, rice
555 and tomato highlight the differences in gene expression, circadian regulation and plant
556 metabolism compared to experiments conducted inside growth chambers (Annunziata et
557 al., 2017, 2018; Higashi et al., 2016; Izawa et al., 2011b; Shalit-Kaneh et al., 2018).
558 Because AS has an impact on the regulation of gene expression, which impacts circadian
559 regulation and plant metabolism, it is important to start investigating the dynamic
560 adjustment of AS in response to a fluctuating environment. RNA-seq data from time-
561 series experiments revealed that the AS status of Arabidopsis transcriptome is widely
562 responsive to changes in temperature (Calixto et al., 2018). By progressively lowering
563 temperature, rapid changes in the spliced forms of transcripts were detected, which
564 suggests that AS might also act to regulate low-temperature responses and how plants
565 tolerate such stress (Calixto et al., 2018). The Arabidopsis circadian clock also acts
566 regulating plant abiotic stress tolerance (Grundy et al., 2015). This also suggests that both
567 AS and the circadian clock might act in synergy to help plants to cope with temperature
568 changes in both the short and long term. In the field, this regulation might be even more
569 important, due to the unexpected fluctuations in light, temperature, and humidity in which
570 plants are exposed.

571 Our data show That AS occurs in sugarcane circadian clock genes and that the different
572 transcript isoforms show dynamic expression profile in sugarcane grown under field
573 conditions. Furthermore, *ScLHY* AS regulation is temperature-dependent in sugarcane
574 circadian clock genes, as observed in Arabidopsis and barley. Thus, the changes in
575 expression of alternative isoforms of *ScLHY* transcripts observed across winter and
576 summer might illustrate the combined effect of both the circadian clock and AS regulation
577 and AS in *ScLHY* might be a key mechanism that allows the continuous dynamic
578 adjustment of the circadian clock by temperature in sugarcane. It is important to start

579 further studies on the impact of the seasonal variation on the AS isoforms of the circadian
580 clock gene expression and, ultimately, sugarcane metabolism and yield.

581

582 **5. Author Contributions**

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

590

591 **6. Conflict of Interest Statement**

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

594

595 **7. Acknowledgments**

596 The present study was supported by the São Paulo Research Foundation (FAPESP) [grant
597 nos. 11/00818-8 and 15/06260-0; BIOEN Program], and by the Serrapilheira Institute
598 (grant no. Serra-1708-16001). L.L.B.D. was supported by FAPESP scholarships [grants
599 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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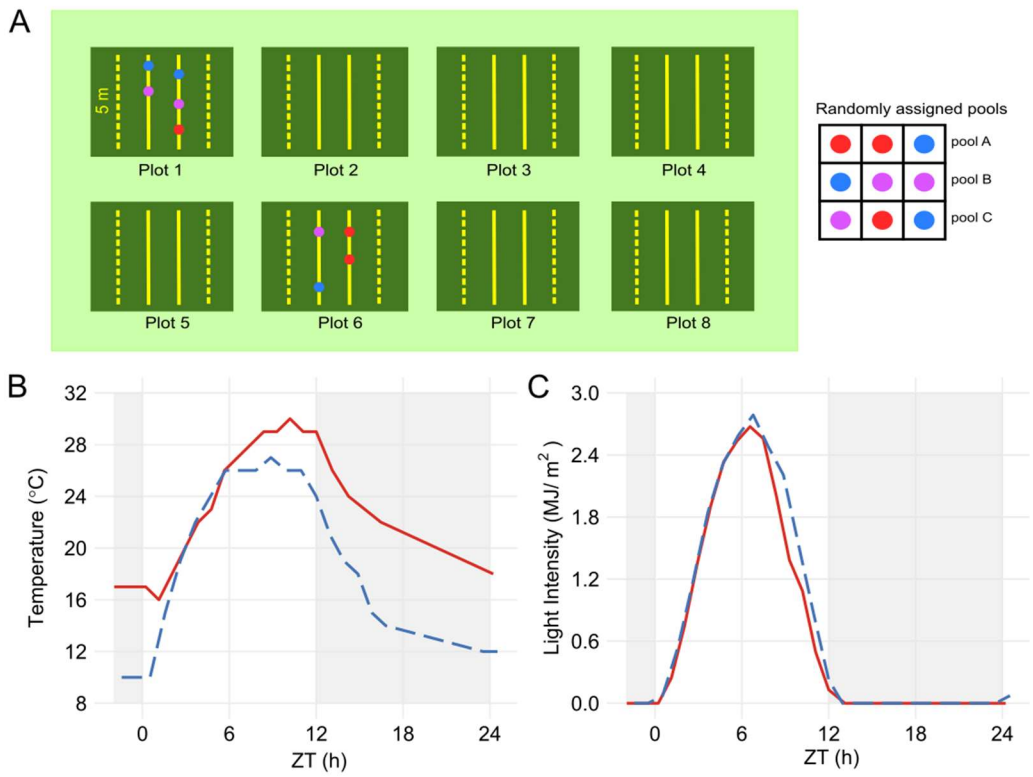
927

928 **Supplemental Figures**

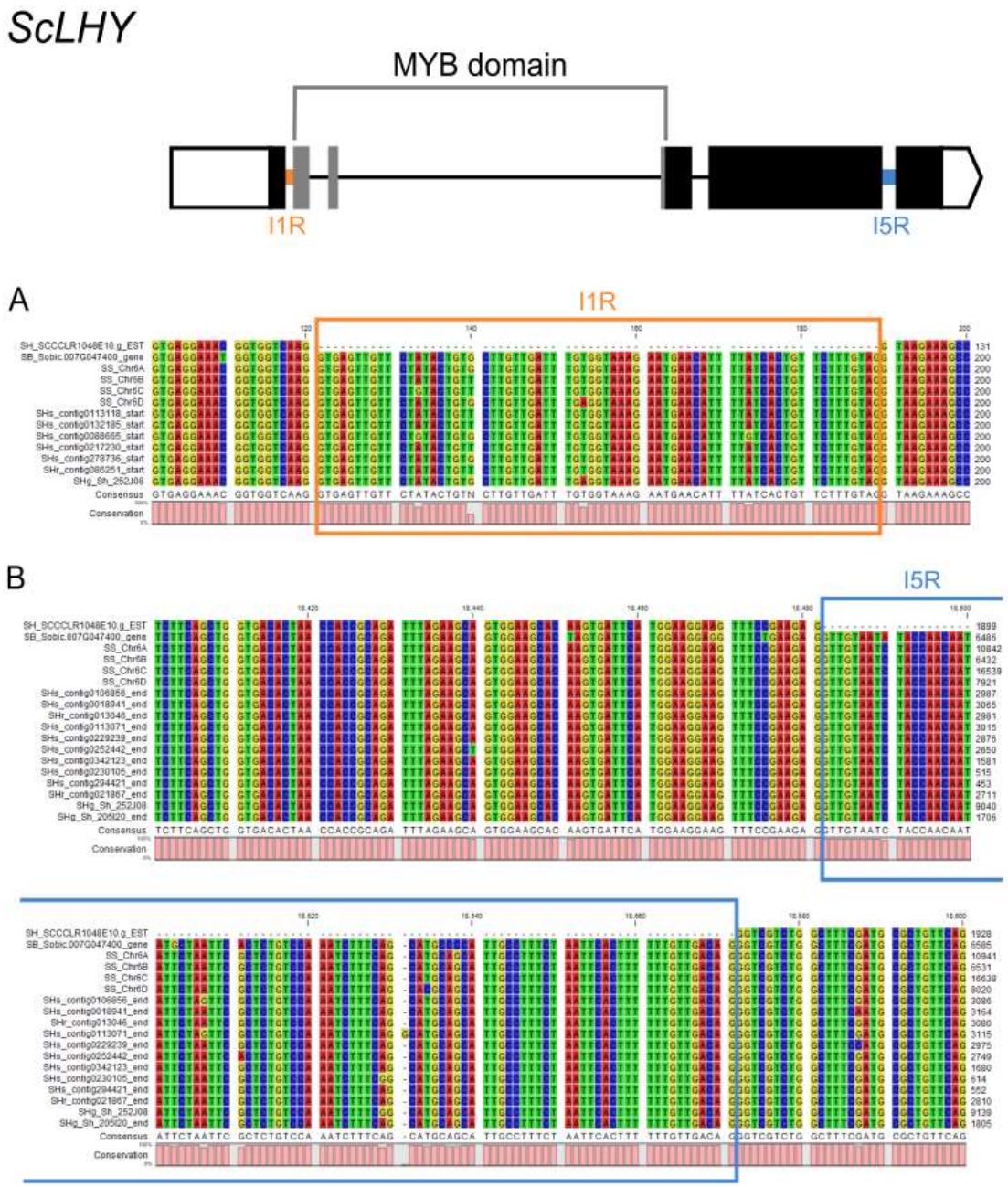
929 **Supplemental Figure 1**



930

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

940 **Supplemental Figure 2**



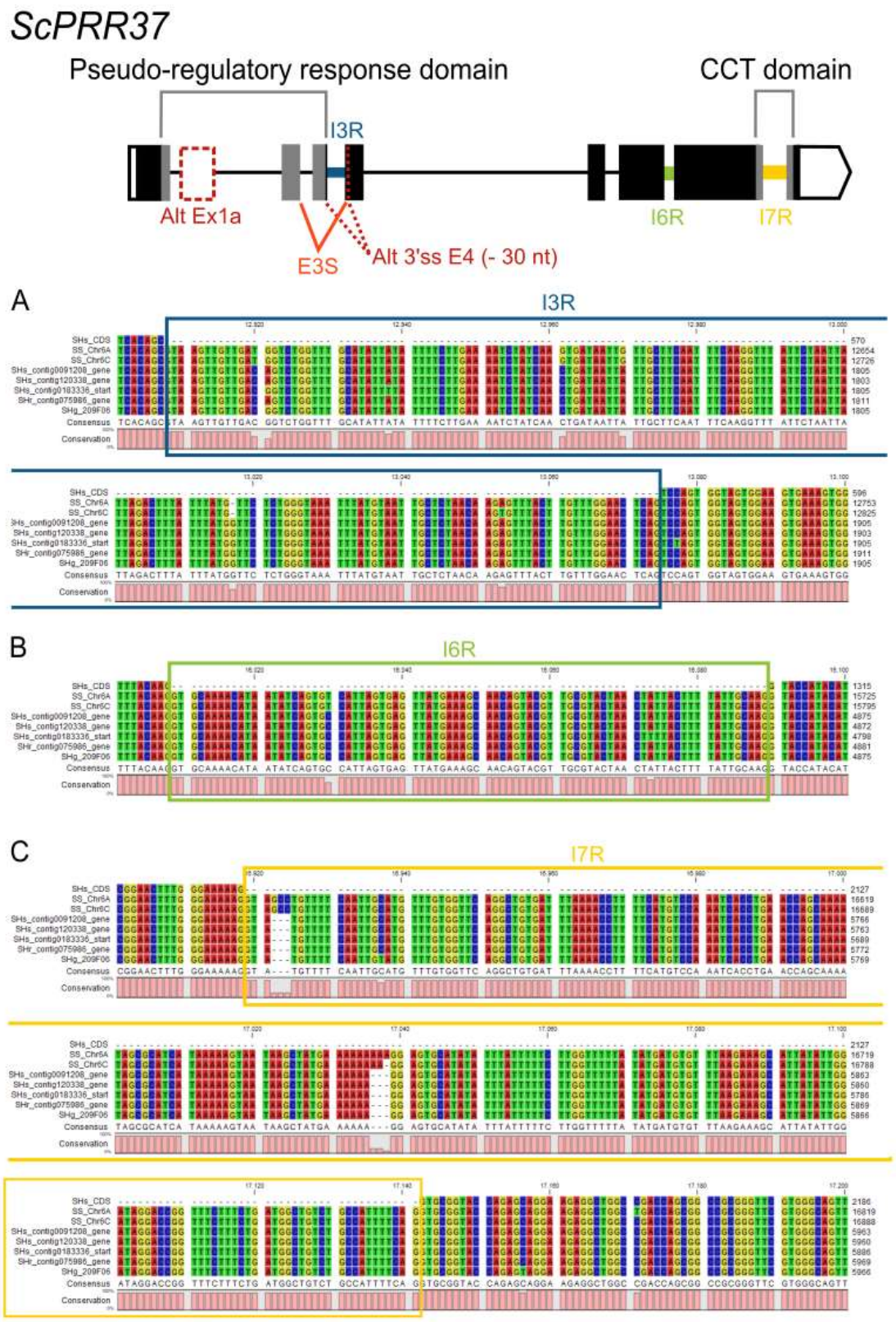
941

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

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

951

Supplemental Figure 3



954 **Supplemental Figure 3 – Alignment of three different regions of *ScPRR37* from**
955 **different genomic sequences.** Sequences from different sugarcane genome projects that
956 were identified as *ScPRR37* were aligned in order to compare the regions containing the
957 I3R, I6R, and I7R AS events in different haplotypes. The regions containing (**A**) the intron
958 3 (dark blue), (**B**) intron 6 (green) and (**C**) intron 7 (yellow) were highlighted to show that
959 all the sequenced haplotypes have the same gene structure. Sequences are from *S.*
960 *spontaneum* (SS) (Zhang et al., 2018), *Saccharum* hybrid (SP80-3280): SHs (Souza et al.,
961 unpublished), SHr (Matiello et al., 2017), and *Saccharum* hybrid (R570) (SHg)
962 (Garsmeur et al., 2018).

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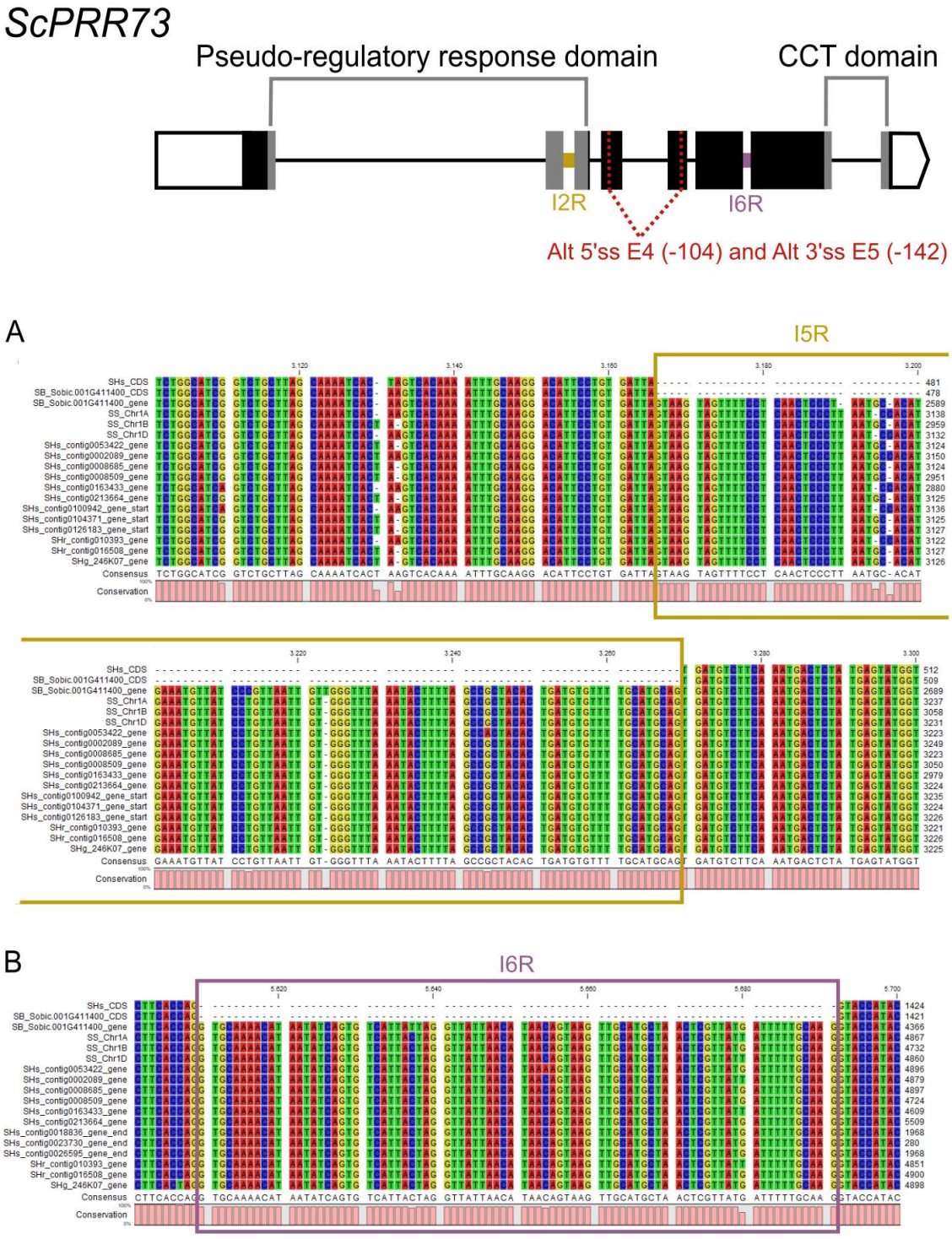
964

Supplemental Figure 4



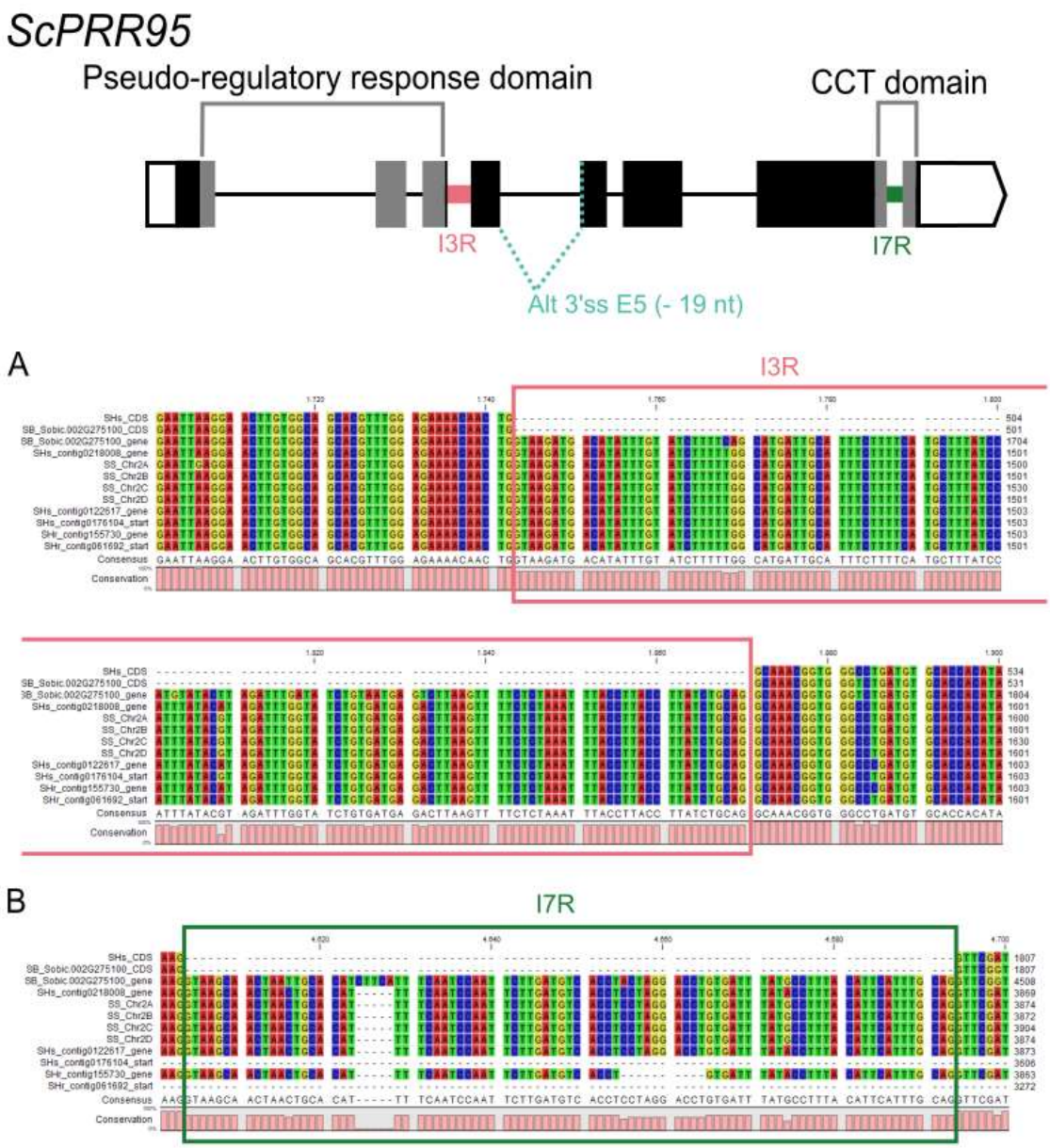
Supplemental Figure 4 – Alignment of two different regions of *ScPRR73* from different genomic sequences. Sequences from different sugarcane genome projects that were identified as *ScPRR73* were aligned in order to compare the regions containing the

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

976

977

Supplemental Figure 5



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

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

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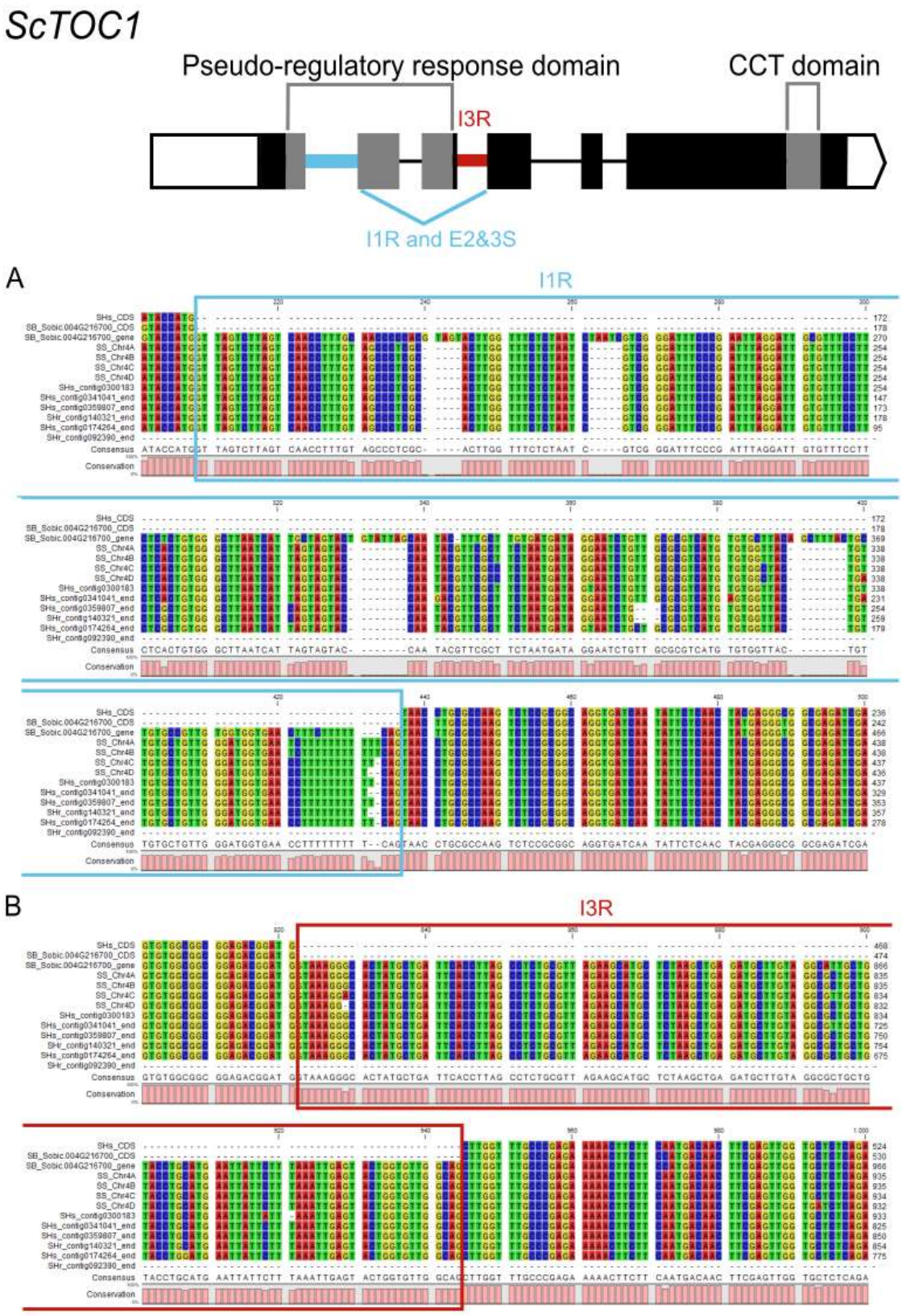
990

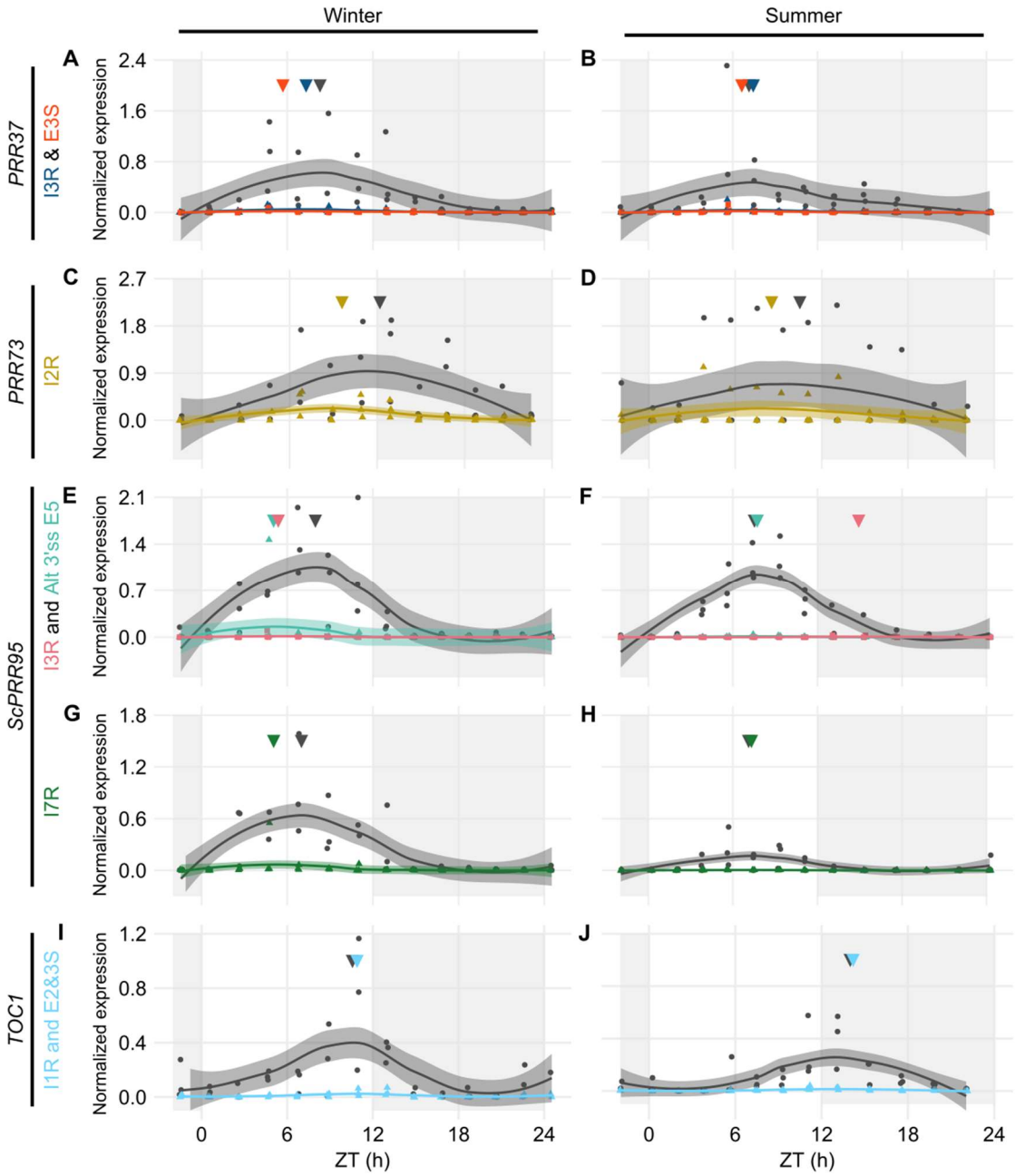
991 **Supplemental Figure 6**



992

993 **Supplemental Figure 6 – Alignment of two different regions of *ScTOC1* from**
994 **different genomic sequences.** Sequences from different sugarcane genome projects that
995 were identified as *ScTOC1* were aligned in order to compare the regions containing the
996 I1R and I3R AS events in different haplotypes. The regions containing (**A**) the intron 1
997 (light blue) and (**B**) intron 3 (red) were highlighted to show that all the sequenced
998 haplotypes have the same gene structure. SS sequences are from *S. spontaneum* (Zhang
999 et al., 2018). SHs are from the *Saccharum* hybrid (SP80-3280) sequenced by Souza et
1000 al. (unpublished), and SHr were sequenced by Matiello et al. (2017). SHg are from the
1001 *Saccharum* hybrid (R570) sequenced by Garsmeur et al. (2018).
1002

1003 **Supplemental Figure 7**



1004

1005 **Supplemental Figure 7 – Diel expression profile of fully spliced and alternative**
1006 **transcript isoforms in different seasons.** Biological replicates (circles and triangles) and
1007 their LOESS curve (continuous lines ± SE) of fully spliced (FS, black) and alternative
1008 transcript forms (AS, color) for the winter samples (4-months old plants, left) the summer
1009 samples (9-months old plants, right). These AS forms, though detected by HR RT-PCR,

1010 were not highly expressed. *ScPRR37* gene expression shows levels of I3R (orange) and
1011 E3S (dark blue) (**A-B**); *ScPRR73* gene expression shows levels of I2R (gold) (**C-D**);
1012 *ScPRR95* gene expression shows levels of I3R (pink) and Alt 5'ss E5 (teal) (**E-F**), and
1013 I7R (dark green) (**G-H**). *ScTOC1* gene expression shows levels of I1R/E23S (cyan) (**I-**
1014 **J**). Inverted triangles show the time of the maximum value of the LOESS curve. The
1015 light-gray boxes represent the night period.

1016

1017 **Table S1 – List of sugarcane gene-specific primers and their sequences.** Primers in
1018 bold indicate forward (FW) sequences, as well as the primers bearing the 6-FAM tag
1019 used in the HR RT-PCR assays. The other sequences indicate reverse (RV) sequences.
1020 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

1021

1022

1023 **TableS2 – SAS number, GeneID, and ProbeID of genes associated with the**
1024 **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

1025

1026 **Table S3 – Spliceosome-related transcripts showing rhythmicity in field conditions**
1027 **according to Dantas et al. (2019).** Sugarcane Assembled Sequences (SAS) were taken
1028 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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