Expression of sugarcane genes associated with perception of photoperiod and floral induction reveals cycling over a 24-hour period

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Abstract. The genetic network resulting in the production of an inflorescence is complex, involving one or more pathways including the photoperiod, maturity, gibberellin and autonomous pathways, and induction and repression of genes along the pathways. Understanding the cyclic expression profile of genes involved with photoperiod perception and floral pathway induction in sugarcane, an intermediate–short day plant (ISD), is crucial for identifying key genes and understanding how the profile changes in response to floral induction signals under decreasing daylengths. Homologues of 21 genes, and some gene alleles, associated with photoperiod perception and the flower induction pathway were examined in sugarcane variety Q174 over a 24-h light-dark cycle. The strongest expression of these genes was seen in the immature spindle leaves and levels of expression generally decreased with increasing leaf age. Significant changes in gene expression levels during a 24-h cycle were observed for 16 of the 21 genes tested. We have now defined an important baseline for expression patterns over a 24-h cycle in non-inductive conditions in sugarcane. These results can be utilised to select the optimal time for detecting changes during floral induction, differences between varieties that are responsive/non-responsive to photoperiod induction, and to identify genes that may be manipulated to enhance or inhibit flowering.

Additional keywords: circadian cycle, diurnal cycle, flowering, photoperiod, *Saccharum*.

Introduction

The consistent increase and decrease in the level of gene expression or protein over a cycle of 24 h, known as a biological rhythm, can be controlled by endogenous (internal biological circadian clock) or exogenous (external) stimuli (Webb 2003; McWatters and Devlin 2011). Endogenous rhythms that cycle over a period of time close to 24 h are also called circadian rhythms. One of the most common exogenous rhythms is the synchronisation to the length of day and night, known as a diurnal rhythm; a key diagnostic is that these rhythms cease to persist when exposed to constant light or dark conditions (Schaffer *et al.* 2001; Webb 2003; Yeang 2015). Although many gene expression profiles correlate with the day/night 24-h cycle not all genes are directly affected by light and/or dark periods but are actually responding to fluctuations in photosynthate compounds or other internal rhythms.

Daylength is a particularly important seasonal cue that triggers the transition from vegetative to reproductive growth, resulting in the production of flowers. Floral induction is a multifaceted genetic network involving various pathways including photoperiod, gibberellins, vernalisation and autonomous signalling pathways (Mouradov *et al.* 2002; Albani and Coupland 2010; Fornara *et al.* 2010; Song *et al.* 2013).

The photoperiod pathway of floral induction is predominantly important for many species, with some plants such as wheat and *Arabidopsis* requiring a lengthening light period (long day, LD plants), whereas others, such as rice and sorghum, respond to shortening light periods (short day, SD plants), to induce flowering. The gene pathway for photoperiod response is similar between LD and SD plants; genes involved in floral induction that are highly conserved between different species have been identified for many plants including *Arabidopsis*, rice and *Brachypodium* (Blázquez 2000; Greenup *et al.* 2009; Higgins *et al.* 2010). Although as Higgins *et al.* (2010) pointed out, there are a few genes that are unique to SD or LD plants. The perception and duration of light can alter both the amplitude and timing of expression of the endogenous circadian clock genes. Changes in expression of the circadian clock genes in turn affect the expression levels or profiles of the flowering pathway genes; resulting in floral induction (Yanovsky and Kay 2003). More complex requirements for induction have also been observed in some species, including differences in the initial daylength and the duration of inductive conditions required. Sugarcane flowers when daylength decreases over a period of 15 days, otherwise it will remain or revert to its vegetative growth phase (Moore 1974; Moore and Berding 2013;

Glassop *et al.* 2014*b*). It can thus be classified as an intermediate–short day (ISD) plant but is most commonly referred to as a short day plant (Vijayasaradhy and Narasimhan 1953; Burr *et al.* 1957; Coleman 1965, 1968; Nuss and Maharaj 1992; Berding *et al.* 2007). When flowering is successfully induced, the apical meristem ceases production of vegetative nodes with attached leaves and internodes, and transitions to a determinate state. With continued inductive conditions over several weeks, a spiral pattern of lateral meristems develops, eventually giving rise to a multi-branched panicle bearing bisexual florets.

Sugarcane is unique in its ability to accumulate sucrose in the stalk to as much as 50% of the dry weight (Botha and Black 2000; Jackson 2005; Rae *et al.* 2005; Glassop *et al.* 2007; Papini-Terzi *et al.* 2009; Inman-Bamber *et al.* 2011; Dal-Bianco *et al.* 2012), but further increases in sugar content would increase sugar yield and grower profitability. Increased yield could be achieved through plant breeding but one constraint is the successful synchronised production of inflorescences required for crossing plants. Although commercial sugarcane is a hybrid with a complex polyploid and aneuploid genome (D'Hont and Glaszmann 2001), a large amount of sequence information is now available, facilitating the identification of gene homologues. Many of the flowering pathway genes have been defined in sorghum, which is the closest diploid relative to sugarcane and has a completed genome sequence. Genes along the flowering pathway can loosely be assigned to groups, including photoperiod perception, internal clock cycle and floral induction, based on their presumed role in other species. A preliminary pathway of sugarcane genes resulting in floral production can be inferred (Fig. 1). The proposed sugarcane model has been based on previously published pathways observed in *Arabidopsis*, rice, sorghum and *Brachypodium distachyon* (L.) P.Beauv., which have been tested through examination of single gene mutant plants and the production of genetically modified plants to identify genes/proteins within the pathway and their influence, through up- or downregulation, on other genes/proteins, resulting in floral induction (Blázquez 2000; Izawa *et al.* 2003; Higgins *et al.* 2010). The genes/proteins affected by the photoperiod interact with the circadian clock genes and together affect the genes associated with floral induction and the meristem floral identity genes (Fig. 1). The sequences of sugarcane homologues for genes resulting in floral induction have been elucidated from previously published work (Casu *et al.* 2007; Murphy *et al.* 2011; Hotta *et al.* 2013; Glassop *et al.* 2014*a*) or alignments with sequence databases including Sugarcane v0.1 GBrowse and the sorghum genome (Hotta *et al.* 2013; Glassop *et al.* 2014*b*; Aitken *et al.* 2016). Further analysis of temporal and spatial gene expression patterns will be needed in order to confirm these gene homologies.

In sugarcane, as in other species, the initial photoperiod signals are perceived in the leaves (Blázquez 2005) and in
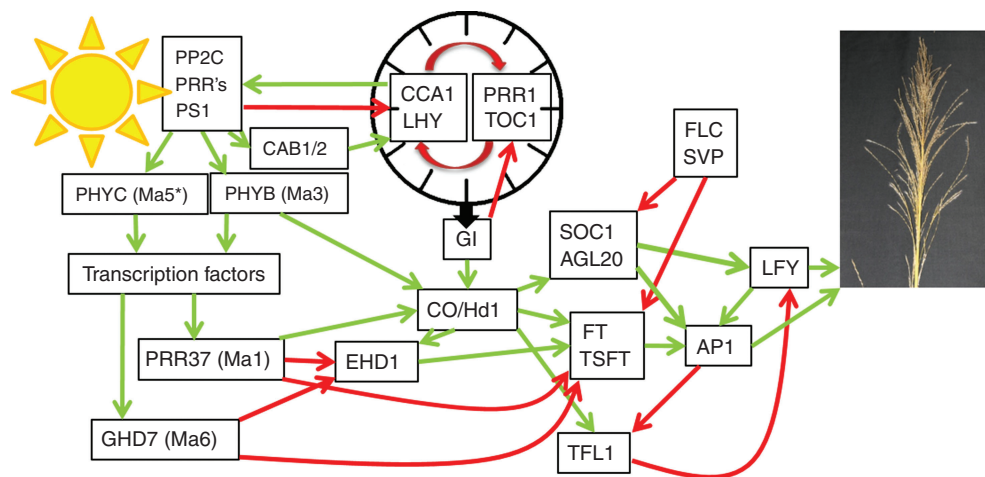

Fig. 1. Proposed genetic pathway for controlling flowering in sugarcane based on published pathways from *Arabidopsis* and cereal crops. These are pathways/interactions that are known in other species but there may be other alternative interactions in sugarcane which are not shown here. Genes associated with photoperiod perception are shown beside and below the sun image. Genes associated with internal 24-h cycle are inside the circle (clock schematic). Green and red arrows indicate positive and negative regulation, respectively, as based on published literature. Gene names: AGL20 – *AGAMOUS LIKE 20*, AP1 – *APETALA 1*, CAB1/2 – *CHLOROPHYLL a/b BINDING PROTEIN 1/2*, CCA1 – *CIRCADIAN CLOCK ASSOCIATED 1*, CO/Hd1 – *CONSTANS/HEADING DATE 1*, EHD1 – *EARLY HEADING DATE 1*, FLC – *FLOWERING LOCUS C*, FT – *FLOWERING LOCUS T*, GHD7(Ma6) – *GRAIN HEADING DATE 7/MATURITY GENE 6*, GI – *GIGANTEA*, LHY – *LATE ELONGATED HYPOCOTYL*, LFY – *LEAFY*, PHYB (Ma3) – *PHYTOCHROME B/MATURITY GENE 3*, PHYC(Ma5*) – *PHYTOCHROME C/MATURITY GENE 5*, PP2C – *PROTEIN PHOSPHATASE 2 C*, PRR's – *PSEUDO-RESPONSE REGULATOR'S*, PRR1 – *PSEUDO-RESPONSE REGULATOR 1*, PRR37(Ma1) – *PSEUDO-RESPONSE REGULATOR 37/MATURITY GENE 1*, PS1 – *PHOTYOSYSTEM 1 GENE*, SOC1 – *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*, SVP – *SHORT VEGETATIVE PHASE*, TFL1 – *TERMINAL FLOWER 1*, TOC1 – *TIME OF CHOLOPHYLL A/B BINDING PROTEIN 1*, TSFT – *TWIN SISTER FLOWERING LOCUS T*. * PHYC and Ma5 have not been confirmed as being homologous genes at this stage. (Blázquez 2000; Izawa *et al.* 2003; Higgins *et al.* 2010).

particular, in the spindle, the youngest leaves comprising a tight whorl of 6–15 immature leaves (Moore 1974). This was determined by experiments showing delayed flowering or inhibition of flowering when the spindle was removed from the plant (Panje and Raja Rao 1961; Panje *et al.* 1968; Moore and Berding 2013). Most floral induction genes are expressed in a 24-h cycle and it is the change in this expression profile that activates the transition from vegetative to reproductive growth. In order to study this vegetative to floral transition, the pattern of expression over the 24-h cycle of the genes needs to be examined in order to accurately ascertain the time of day when peaks and troughs occur and consequent changes that may occur during floral induction. In this study, the assessment of sugarcane homologues of photoperiod perception and floral induction pathway genes in leaves of varying age and in the meristem were examined in the commercial sugarcane cultivar Q174, over a 24-h cycle. This baseline knowledge of gene expression is important for identifying the genes that are likely to be involved and to determine how far the knowledge from other grasses can be translated to assist sugarcane. The results add functional information to support the roles of these genes and will underpin future work on the control of flowering in sugarcane by guiding experiments on responses to induction and modification of critical genes.

Materials and methods

Sugarcane leaf and internode labelling system

Kuijper (1915) established a numbering system that designated the youngest (top) leaf with a visible dewlap (TVD) as leaf 1 and this leaf is attached to internode 1 (Bonnett 2013). Progressing down the stalk and with increasing maturity, the leaves and internodes are consecutively numbered 2, 3 and so on. The furled immature leaves above the TVD are commonly called the spindle leaves and are attached to the meristem, defined as the portion of immature stalk above internode 1. In this study, the spindle leaves (SL) consist of all of the furled immature leaves, TVD as described above, mature leaf (ML, leaf 3) and the meristem (MS, a section of the growing tip containing immature internodes/nodes and the meristem).

Plant growth conditions

Sugarcane stalks were cut into sections that contain a single bud, also known as billets or setts. Setts of *Saccharum hybrid* Q174 were germinated in vermiculite and individual plantlets transferred to 8 L pots containing Searles Peat 80 Mix (Searles). Plants were grown in the Controlled Environment Facility (CSIRO, Queensland Bioscience Precinct, St Lucia, Qld) at 32°C and 65% temperature and humidity, respectively, during 14 h of light (500 µmol photons m^{-2} s^{-1}) and 28°C and 85% during the dark period. When plants were 9.5 months old, tissues were collected at 3-h time intervals covering a 24-h cycle. Samples included the SL, TVD, ML and MS, as defined above. At each sampling time, tissues were collected from three replicate plants. Collected samples were immediately frozen in liquid nitrogen and temporarily stored at −80°C before freeze drying.

Genes

Fifteen sugarcane genes homologous to genes associated with photoperiod perception and floral induction were identified in previously published work (Hotta *et al.* 2013; Glassop *et al.* 2014*a*); see Table S1, available as Supplementary Material to this paper. The sequences of another nine genes were identified in the functionally annotated sorghum database Phytozome (v11.0, Goodstein *et al.* 2012), and the sorghum sequences aligned against the sugarcane sequence database (Aitken *et al.* 2016); Table S1. Primers were designed to the identified sugarcane and sorghum sequences and full/partial clones were amplified from sugarcane variety Q174 genomic DNA; sequences unpublished. Genes of interest associated with photoperiod perception and floral induction pathway included *AGAMOUS LIKE 20* (*AGL20*), *APETALA 1* (*AP1*), *CHLOROPHYLL a/b BINDING PROTEIN* (*CAB2*), *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*); *FLOWERING LOCUS T – A* and *C* (*FT-A* and *FT-C*), *GRAIN HEADING DATE 7/ MATURITY GENE 6* (*GHD7/Ma6*), *GIGANTEA* (*GI*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *LEAFY* (*LFY*), *PHYTOCHROME B/ MATURITY GENE 3* (*PHYB/Ma3*), *PROTEIN PHOSPHATASE 2 C* (*PP2C*), *PSEUDO-RESPONSE REGULATOR 1/ TIME OF CHOLORPHYLL A/B BINDING PROTEIN 1* (*PRR1/ TOC1, TOC1-a, TOC1-b*), *PSEUDO-RESPONSE REGULATOR 3* (*PRR3*), *PSEUDO-RESPONSE REGULATOR 37/MATURITY GENE 1* (*PRR37/Ma1*), *PSEUDO-RESPONSE REGULATOR 7/73* (*PRR7/73*), *PSEUDO-RESPONSE REGULATOR 59* (*PRR59*), *PHOTOSYSTEM 1* (*PS1*), *SHORT VEGETATIVE PHASE* (*SVP*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *TERMINAL FLOWER 1* (*TF1*), *TWIN SISTER FLOWERING LOCUS T* (*TSFT*); see Table S1 for sugarcane accession numbers and sorghum homologues. All genes amplified in this study have been labelled after the form *Saccharum* hybrid (*Sh*), although commercial sugarcane varieties are hybrids between two species, so it is possible that some sequences are derived from *Saccharum spontaneum* and others from *Saccharum officinarum*. The housekeeping gene used to normalise the qPCR was *ACTIN DEPOLYMERISING FACTOR* (*ADF*, Casu *et al.* 2015). Primer sequences for all genes are detailed in Table S1.

Primer3 (Untergasser *et al.* 2012) was used to design primers that cross intron/exon boundaries or to produce a product that spanned an exon to exclude gDNA amplification. All previously unpublished primers were selected based on the following criteria: (i) solely amplifying cDNA, (ii) had a PCR efficiency above 95% in a standard curve qPCR, and (iii) the product was confirmed by cloning into pGEM-T Easy vector system (Promega), independent transformation events grown for plasmid extraction (QIAprep Spin Miniprep Kit, Qiagen), the sequence of the cloned DNA obtained with Sanger sequencing (Australian Genome Research Facility) and sequence analyses with CLC Main Workbench 7 (CLC bio, Qiagen) and Sequence Scanner Software (Applied Biosystems Life Technologies).

RNA extraction, cDNA synthesis and RT–PCR

Dried samples were ground to a fine powder with a ball mill (Retsch, MEP Instruments Pty Ltd) and RNA extracted following the manufacturer's instructions (Qiagen Plant

RNeasy Kit) with the introduction of a 10 min incubation at room temperature after the addition of buffer RLT/$_\beta$ME. Complementary DNA was synthesised using the Qiagen QuantiTect Reverse Transcription Kit with anchored oligo dT primers. Real-time (RT-) PCR reactions contained SYBR Green Master Mix (ThermoFisher Scientific) with ~32 ng cDNA and 2.4–3 µM primers (0.3 µM for housekeeping gene) and were run on the Applied Biosystem ViiA7 Real-Time PCR System (ThermoFisher Scientific). Further, RT-PCR analysis required technical replicates to be within 0.5 C_t value of each other and the controls without template to show no amplification for the results to be accepted (Nolan *et al*. 2006). The double delta threshold cycle (C_t) method was used to process qPCR results (Nolan *et al*. 2006).

Statistical analysis using ANOVA and Fisher's protected least significant difference was processed using GENSTAT (ver. 16.1.0.10916, VSN International Ltd). Significant differences were accepted for $P < 0.05$.

Results and discussion

The genetic control of photoperiod induced flowering has been well documented in other species like *Arabidopsis*, rice, sorghum and *Brachypodium* (Bäurle and Dean 2006; Imaizumi and Kay 2006; Colasanti and Coneva 2009; Greenup *et al*. 2009; Higgins *et al*. 2010; Murphy *et al*. 2011), illustrating the high degree of conservation within this pathway and providing an excellent starting place for elucidating the sugarcane flowering pathway. This research identified further genes associated with the sugarcane flowering pathway adding to those previously identified by Coelho *et al*. (2013), Hotta *et al*. (2013) and Glassop *et al*. (2014*a*) (as detailed in Table S1). Although the expression patterns of these genes have been assessed in various tissues and developmental stages, their role and function within the sugarcane flowering pathway still needs to be confirmed with experiments involving the production of transgenic plants or complementation of mutant lines. These tests would be necessary to cement or adjust the simplified sugarcane flowering pathway presented (Fig. 1).

Functionally annotated sorghum sequences were used to identify and clone 16 previously uncloned sugarcane homologues of photoperiod perception and flowering pathway genes. The qPCR products from each gene were sequenced to confirm that the correct gene was being amplified. Sequences identified single nucleotide polymorphisms (SNP's) that determined 1–5 variants per gene (see Fig. S1, available as Supplementary Material to this paper); however, as only a maximum of 12 clones per gene were sequenced to confirm identity and only a small section of cDNA amplified, there may be more variants than observed. Because it is a complex polyploid, sugarcane is further complicated with multiple allelic copies of each gene. Previous studies have shown that the coding regions of the homeo-alleles generally have high levels of homology, with most variation occurring in the introns and non-translated regions. Discriminating between the expression of alleles using primer sequence variants is extremely difficult and it has been necessary to use amplicon sequencing for this purpose. For example, Coelho *et al*. (2013) reported as many as 2–8 homeologues for *GI*, *TF1 L-like*, *CO*, *EHD1*, *GHD7* and

FT in a Brazilian sugarcane cultivar, with expression of different homeologues in different tissues and/or developmental stages analysed from the SUCEST database. In the present study, we took a different approach using the homology to advantage by intentionally using primers to capture cumulative allele expression. The expression profiles taken over a 24-h cycle in non-inductive conditions presented below may not represent all potential alleles, but are a good indicator of the cumulative expression in the tissues that have been identified as perceiving changes in the photoperiod and initiating the cascade of the floral induction pathway.

A further advantage of our approach was to sample separate replicate sets of plants at each point in the 24-h cycle. In previous studies, the repeat sampling from individual plants necessitated sampling over several days to eliminate individual plant variation. Our approach overcame this problem and furthermore it allowed the same tissue to be sampled at each time point (e.g. spindle) without any confounding effects due to wounding at previous sampling points.

Of the 23 sugarcane photoperiod perception and floral induction pathway genes, including gene alleles, all except five displayed significant changes in expression over the 24-h cycle within the spindle leaves (Figs 2–6). Expression over the 24 h ranged from 51 – 100% increases from minimum expression levels (Figs 2–6). Fourteen of the genes had peak expression during the light cycle and four during the dark cycle (Figs 2–6). The cycle of gene expression over 24 h has been compared with published results for sugarcane genes and to other SD plants grown under similar non-inductive photoperiods as that used to grow the sugarcane tested here where this is information is available; consequently some gene profiles have no appropriate comparison. Several expression profiles were generated using the same primers that have previously been published by Hotta *et al*. (2013) or different primers for the same gene; in some cases the expression profiles over the 24-h cycle did not match. This may highlight differences in the varieties used (RB855453, Hotta *et al*. 2013; and Q174 in this research), the different age of the plants or different growth conditions (3 month old plants grown in 12 h light (100 µmol photons m^{-2} s^{-1}) at a constant temperature of 25°C, Hotta *et al*. 2013; and 9.5 month old plants grown at 32°C during 14 h of light (500 µmol photons m^{-2} s^{-1}) and 28°C during the dark period in this research).

Variations in expression of genes that are associated with the internal clock cycle over a 24-h cycle

The endogenous clock genes regulate many genetic networks including carbon fixation, plant growth and time of flowering. Genes associated with the internal clock cycle include *CCA1*, *LHY*, *TOC1* and *PRR1* (Dong *et al*. 2012). Three sugarcane homologues of genes associated with the clock cycle were measured and all showed statistically significant variation in the level of expression over the 24 h light/dark cycle, with a greater than 65% difference in expression between the minimum and maximum values observed (Fig. 3). The expression of *ShPRR1/TOC1* increased with the onset of the light period, reaching a peak 7–10 h later, followed by a decrease to minimum expression 2 h after the onset of the dark period (Figs 2, 3*a*). Differences in expression level were
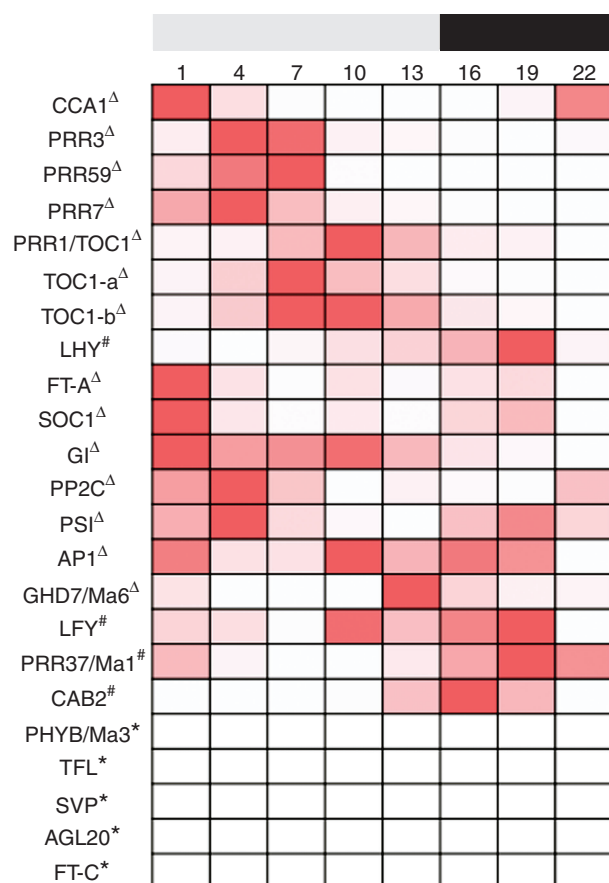
Fig. 2. Heat map of changes in gene expression over a 24-h cycle in sugarcane spindle leaves. Variation in expression over the 24-h cycle was normalised within each gene, with changes in colour from dark red to white representing the top 25% to lowest 25% levels of relative expression. The *x*-axis label at the top of the figure represents Zeitgeber time and the light grey and black bar diurnal and nocturnal period respectively. Those genes with an asterisk show no significant difference in expression over the 24-h cycle ($P < 0.05$). Genes with a triangle or hash symbol next to them indicate a major peak during the light or dark cycle, respectively. The full gene names are detailed in 'Materials and methods'. Individual gene expression graphs are supplied in Figs 3–7.

observed between different sugarcane leaf samples for *ShPRR1/TOC1*, with similar expression between the spindle and TVD leaves and minimal expression in mature leaves (Fig. 6a). However, the *ShPRR1/TOC1* expression profile was different from that reported in sugarcane variety RB855453 which had a peak at the transition from the light to the dark periods and a trough at the dark to light transition (Hotta *et al.* 2013). Therefore, the analysis was repeated using the same primers as used by Hotta *et al.* (2013), here labelled *TOC1-a* and *TOC1-b*. The expression patterns generated by these primers were very similar to the original pattern for *ShPRR1/TOC1*, with the major peak occurring 7–10 h into the light period (Fig. 3b, c) suggesting that all three pairs of primers amplify a similar set of alleles in variety Q174. It is possible that the Brazilian variety RB855453 contains different alleles of *PRR1/TOC1* which are amplified by the same primer set, but which are expressed at a different point in the 24-h cycle. The *ShPRR1/*

TOC1 profile was similar to those reported in rice and tobacco (Ogiso *et al.* 2010; Yon *et al.* 2012). Differences between rice and sorghum plants grown in SD and LD conditions were the rate of expression increasing and decreasing over the course of the light and dark periods, respectively. The profile pattern stayed the same, though there was a 2–4-fold increase in the level of expression in SD (floral inductive conditions) grown rice plants (Hori *et al.* 2013; Yang *et al.* 2014; Lee *et al.* 2016). Any similar changes in *ShPRR1/TOC1* expression levels grown under shortening daylength conditions require further investigation.

As observed for *ShPRR1/TOC1*, *ShLHY* expression was also highest in the spindle leaf, with reduced expression in the TVD and minimal expression in the mature leaf (Fig. 6b). The spindle leaf expression of *ShLHY* was at a minimum level before the onset of the light period and remained low for the first 7 h of light, then increased slowly to reach maximum expression 5 h after the onset of the dark period (Fig. 3d). This expression profile over 24 h differed from those reported in rice and tobacco, where expression started at the end of the dark period, with a sharp peak in the first part of the light period (Yon *et al.* 2012; Zhao *et al.* 2012; Hori *et al.* 2013; Lee *et al.* 2016). As the sugarcane qPCR product sequence was confirmed to be correct by alignment with known *LHY* genes, this may represent a divergent function for *LHY* in sugarcane. Expression profiles of *LHY* during floral inductive photoperiod conditions have not yet been established in sugarcane but the profile of *OsLHY* changed, with the peak occurring at the transition of the dark to the light period and expression levels were reduced by ~2-fold (Hori *et al.* 2013).

The spindle leaf expression of *CCA1* slowly increased from 4 h before the start of the light period to reach a peak at the transition from the dark to the light period followed by a sharp decrease in expression to no detectable expression 6 h after the onset of the light period (Fig. 3e). This matched the 24-h cycle expression pattern for *CCA1* seen previously in a Brazilian cultivar of sugarcane and maize (Wang *et al.* 2011; Hotta *et al.* 2013). The expression profile and levels remain the same between maize plants grown in SD and LD conditions, with the peak occurring at the transition from dark to light regardless of the length of the light period (Wang *et al.* 2011), therefore detecting changes in *ShCCA1* under photoperiod inductive conditions may not be an appropriate indicator of response and would require further investigation.

Genes associated with the clock typically have a 24-h cycle expression pattern with peaks that follow a cascade; *LHY/CCA1* → *PRR*s → *TOC1* (Staiger *et al.* 2013). This sequence of gene expression is seen in both short day plants, like rice (Murakami *et al.* 2005; Ogiso *et al.* 2010; Gao *et al.* 2014; Lee *et al.* 2016), intermediate-short day plants, like sugarcane (Hotta *et al.* 2013), and long day plants, like wheat (Murphy *et al.* 2011; Mizuno *et al.* 2016), barley (Campoli *et al.* 2012; Ejaz and von Korff 2017) and *Arabidopsis* (Pokhilko *et al.* 2012; Staiger *et al.* 2013). The peaks of the genes involved in the cascade in sugarcane may not be synchronous with the peaks observed in other species; for example, *TaPRR7* will peak in the latter half of the light period, while *ShPRR7* peaks in the middle of the light period. However it is significant that the peaks still occur in the same order: *ShLHY/CCA1* → *ShPRRs* → *ShTOC1*
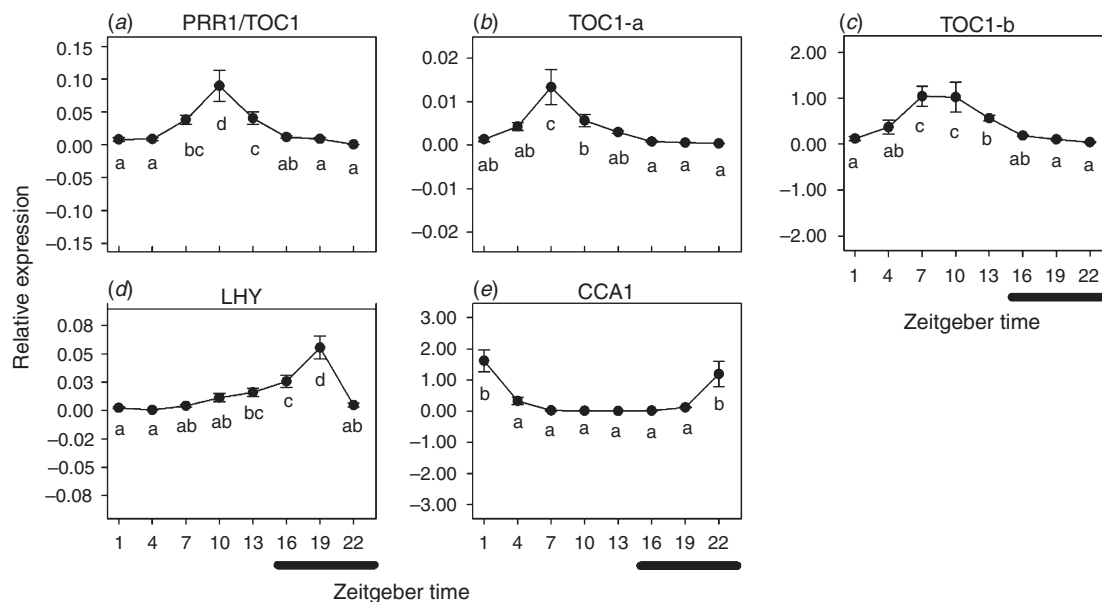
Fig. 3. Relative expression in spindle leaves of sugarcane homologues of genes associated with the internal clock cycle over a 24-h cycle. (*a*) PRR1/TOC1, (*b*) TOC1-a, (*c*) TOC1-b, (*d*) LHY, (*e*) CCA1. The *y*-axis is the expression of the gene normalised to the housekeeping gene. The line beneath the *x*-axis values indicates when the lights were off. Standard error bars are displayed (*n* = 3). Fisher's protected least significant difference test is denoted by letters, where values with the same letter are not significantly different from each other (*P* < 0.05).

(top eight genes in Fig. 2) implying that the pathway is similar but expression unique to sugarcane.

Variations in expression of genes that are associated with photoperiod perception over a 24-h cycle

The perception of light is an important signal that regulates flowering time with phytochromes discerning changes in the red/far red ratio, which in turn affects expression of several transcription factors and pseudo response regulator genes interacting with the clock cycle genes (Bolouri Moghaddam and Van den Ende 2013). Nine sugarcane homologues of genes involved in photoperiod perception were measured and all except one (*PHYB/Ma3*) showed statistically significant variation in the level of expression over the 24 h light/dark cycle, with a greater than 50% difference in expression between the minimum and maximum values observed (Fig. 4). Several different patterns of expression were observed. The expression of *ShPP2C*, *ShPRR3*, *ShPRR59* and *ShPRR7* in spindle leaves all peaked 4 h after the beginning of the light period (Fig. 4*a–d*). *ShPP2C* and *ShPRR7* expression then quickly decreased whereas *ShPRR3* and *ShPRR59* sustained maximum expression for a further 3 h before decreasing to minimum expression. *ShPRR59* and *ShPRR7* had no detectable expression during their troughs (Fig. 4*c, d*). All four genes reached their minimum expression well before the onset of the dark period and maintained low expression until the onset of light, with the exception of *ShPP2C* where expression started to increase 3 h before the beginning of the light period (Fig. 4*a*).

Similar patterns have been reported for homologues of these four genes in other species. The profile of *ShPP2C* over a 24-h

cycle (Fig. 4*a*) is similar to that observed for soybean *GmPP2C*, with a single peak starting in the latter half of the dark period and decreasing to minimal expression half way through the light period (Marcolino-Gomes *et al.* 2014). Although the *ShPRR3* pattern in cultivar Q174 differed from the expression of *PRR3* in a Brazilian cultivar of sugarcane (Hotta *et al.* 2013), the 24-h cycle pattern for *GmPRR3* and *OsPRR3* are similar to *ShPRR3* expression (Fig. 4*b*), with a single peak during the light period and minimal expression during the dark period, in non-inductive photoperiods (Filichkin *et al.* 2011; Marcolino-Gomes *et al.* 2014). Under floral inductive photoperiods the *OsPRR3* expression decreased at a slower rate with 50–80% expression at the transitions from the light to the dark period (Filichkin *et al.* 2011); expression patterns of *ShPRR3* during floral induction are yet to be examined.

The expression pattern of *PRR59* in the Brazilian sugarcane cultivar RB855453 and rice (peaking during the light period then slowly decreasing across the light/dark transition before increasing again) differed from the expression of *ShPRR59* in Q174 (Fig. 4*c*), where in Q174 the expression was already at a minimum at the same transition (Hotta *et al.* 2013). The expression profiles for *OsPRR59* in rice grown in photoperiod inductive and non-inductive conditions, both peaked at the light/dark transition, but along with the peak occurring earlier in inductive conditions, expression was only 50% of that observed in the non-induced plants.

The expression pattern of *ShPPR7* was similar in both sugarcane varieties RB855453 and Q174, with a peak during the light period and minimal expression during the dark period (Fig. 4*d*) (Hotta *et al.* 2013). When the sequences of the *ShPRR7* partial clones isolated were blasted against the NCBI nucleotide databases, they aligned with *PRR73* genes from other
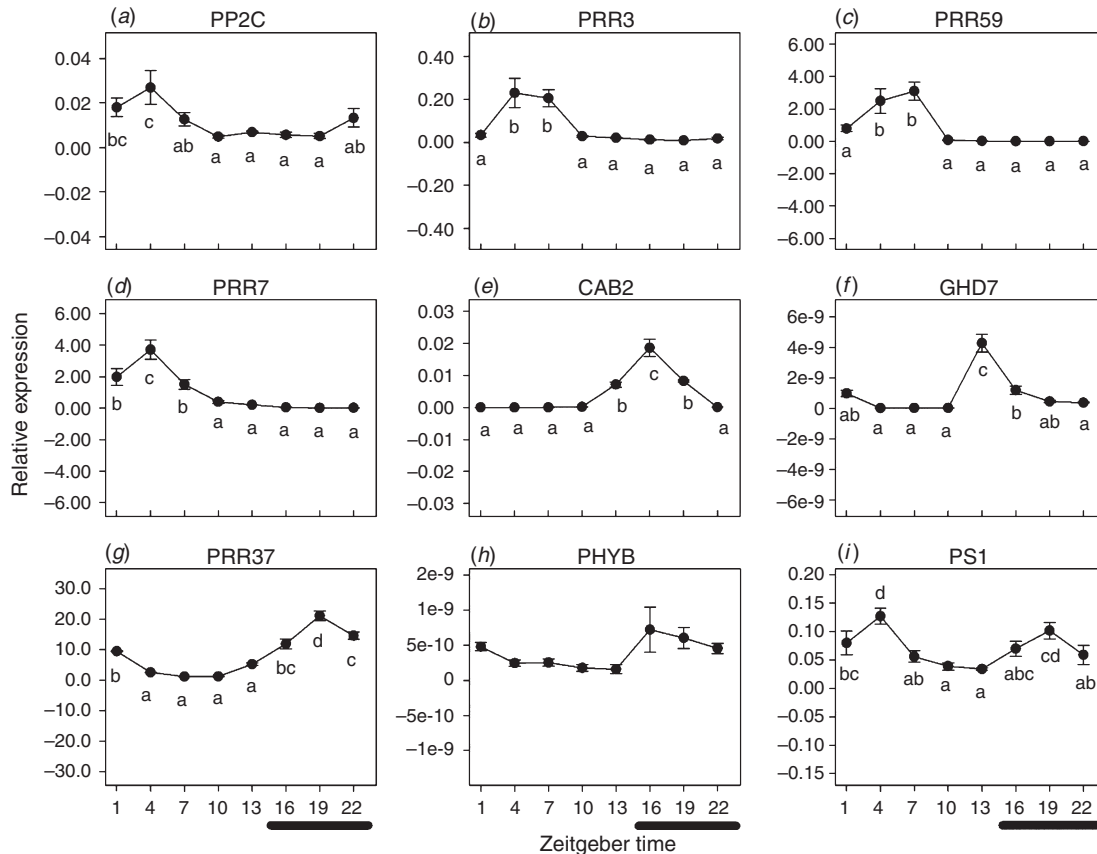
Fig. 4. Relative expression in spindle leaf of sugarcane homologues of genes associated with photoperiod perception over a 24-h cycle. (*a*) PP2C, (*b*) PRR3, (*c*) PRR59, (*d*) PRR7, (*e*) CAB2, (*f*) GHD7, (*g*) PRR37, (*h*) PHYB, (*i*) PS1. The *y*-axis is the expression of the gene normalised to the housekeeping gene. The line beneath the *x*-axis values indicates when the lights were off. Standard error bars are displayed ($n = 3$). Fisher's protected least significant difference test is denoted by letters, where values with the same letter are not significantly different from each other ($P < 0.05$). Gene expression profiles without l.s.d. letters were not significantly different over the 24-h cycle.

species; hence the *ShPRR7* 24-h cycle pattern was compared with both *PRR7* and *73* expression profiles from other species. The expression pattern of *ShPRR7* in Q174 matched the 24-h cycle profiles of rice cultivars Kasalath and Dongjin (*OsPRR73*, Murakami *et al*. 2005; Lee *et al*. 2016). The difference in the expression profile of *OsPRR73* between rice plants grown in SD or LD conditions is that there was minimal expression detected across the light/dark transition in SD plants compared with LD plants (Lee *et al*. 2016); as the rice SD profile was similar to the LD profile in sugarcane, it is difficult to infer how *ShPRR7* expression may change under floral inducing conditions and would require further testing.

In contrast to the group of genes with maximal expression in the light period, *ShCAB2*, *ShGHD7*, *ShPRR37* and *ShPHYB* expression in spindle leaves had a single sharp peak at the transition from the light to the dark period or during the dark period, then expression decreased to no detectable levels (Fig. 4*e–h*). *ShPS1* had a similar significant peak during the dark period and a second peak in the first half of the light period (Fig. 4*i*). The *ShPS1* profile was similar to the profile observed in sugarcane variety RB855453, though the expression during the dark period continued to increase and contribute to the peak during the light period (Hotta *et al*. 2013).

The expression of *ShCAB2* increased just before the light/dark transition to peak 2 h after the onset of the dark period and then decreased over the next 6 h to no detectable expression before the beginning of the light period (Fig. 4*e*); this expression profile does not match that seen in sorghum which has a single peak during the light period and no expression during the dark period, under non-inductive growth conditions (Finlayson *et al*. 1999), and there is no published data on expression under floral inductive conditions that may assist with predicting changes in *ShCAB2* expression during floral induction.

The expression patterns of *GHD7* in sorghum cv. 100M and rice, have two substantial peaks, the first during the light period and the second peak at the transition from the light to the dark period (Hori *et al*. 2013; Yang *et al*. 2014). While there was a smaller second peak for sugarcane *ShGHD7*, similar to *SbGHD7* (Fig. 4*f*), it was not significantly different from the baseline (Murphy *et al*. 2014; Yang *et al*. 2014). Within sorghum the *GHD7* gene, also known as *Ma6*, contributes to the timing of floral induction with flowering seen in plants grown in less than 10 h light but delayed if light is greater than 12 h in duration (Yang *et al*. 2014). In both sorghum and rice there is no second peak when the plants are grown under photoperiod inductive conditions; expression profiles in sugarcane during
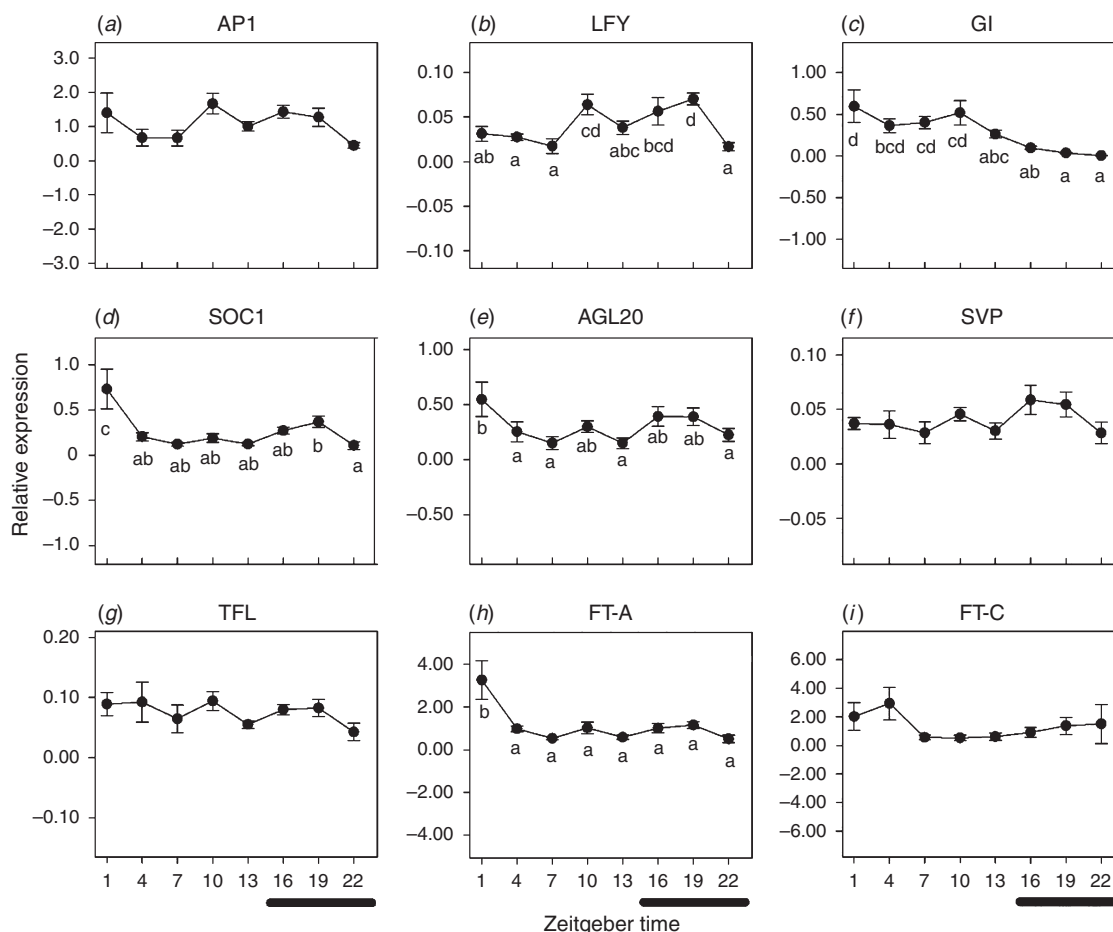
Fig. 5. Relative expression in spindle leaves of sugarcane homologues of genes associated with the floral induction pathway over a 24-h cycle. (*a*) AP1, (*b*) LFY, (*c*) GI, (*d*) SOC1, (*e*) AGL20, (*f*) SVP, (*g*) TFL, (*h*) FT-A, (*i*) FT-C. The *y*-axis is the expression of the gene normalised to the housekeeping gene. The line beneath the *x*-axis values indicates when the lights were off. Standard error bars are displayed ($n = 3$). Fisher's protected least significant difference test is denoted by letters, where values with the same letter are not significantly different from each other ($P < 0.05$). Gene expression profiles without l.s.d. letters were not significantly different over the 24-h cycle.

floral induction require further research (Hori *et al.* 2013; Yang *et al.* 2014).

While Higgins *et al.* (2010) reported that *PRR37* and *PRR73* were paralogues in *Brachypodium*, and that *PRR73* was not known to be associated with flowering, the expression profiles of *ShPRR37* were assessed separately from published *PRR73* profiles. *PRR37* expression in sorghum cv. 100M and rice, grown under long day conditions, both have one peak during the light period, with an additional peak in the dark period for sorghum (Murakami *et al.* 2005; Murphy *et al.* 2011). Under photoperiod inductive conditions the peak during the dark period is not detected in sorghum. In sugarcane the expression profile of *ShPRR37* did not match that seen in sorghum or rice as it had a single peak occurring during the dark period; to this end it is difficult to infer how *ShPRR37* expression would change during floral induction without detailed analysis over the 24 h period (Fig. 4*g*).

Unlike the other eight photoperiod perception genes, the expression of *ShPHYB* in spindle leaves did not show significant differences over the 24 h period but there was a

trend for a peak during the dark period (Fig. 4*h*). The pattern for *PHYB* in sugarcane cultivar RB855453 was different, with peak expression occurring in both the light and dark periods and lower expression at the transition from light to dark and *vice versa* (Hotta *et al.* 2013). Although the same primers were used, the different expression profiles over the 24-h cycle may be due to different growth conditions or presence of different *PHYB* alleles. In rice, grown under non-inductive conditions, there are two *OsPhyB* peaks, one in the first half of the dark period and the second at the transition from the dark to the light period (Gao *et al.* 2014). When rice is grown under floral induction conditions the expression at the beginning of the dark period increases by 50%, whereas the second peak remains at the same expression level and is not as prominent because the expression level is masked by the increasing levels of the first peak (Gao *et al.* 2014). Within sorghum the *PHYB* homologue is *Ma3*, with three alleles (Ma3, ma3 and ma3[R]) that contribute to timing of floral initiation in sorghum. The role of different alleles in floral initiation should be kept in mind when examining *PHYB*/*Ma3* in sugarcane as it is highly likely to also contain multiple alleles.
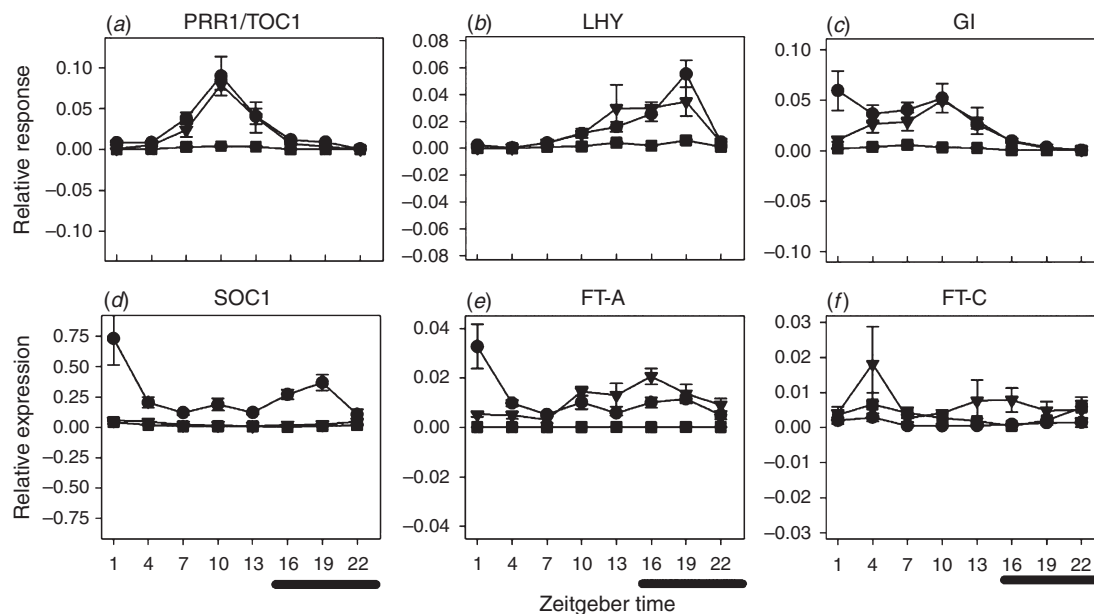
Fig. 6. Relative expression of genes in various leaf tissue. (*a*) PRR1/TOC1, (*b*) LHY, (*c*) GI, (*d*) SOC1, (*e*) FT-A, (*f*) FT-C. Expression was measured in spindle leaves (circle), leaf attached to the top visible dewlap (TVD – downward pointing triangles) and mature leaf (square). The *y*-axis is the expression of the gene normalised to the housekeeping gene. The line beneath the *x*-axis values indicates when the lights were off. Standard error bars are displayed (*n* = 3).

Fig. 7. Relative expression of sugarcane genes in meristematic tissue over a 24-h cycle. (*a*) AGL20, (*b*) SOC1, (*c*) AP1, (*d*) TSFT. The *y*-axis is the expression of the gene normalised to the housekeeping gene. The line beneath the *x*-axis values indicates whe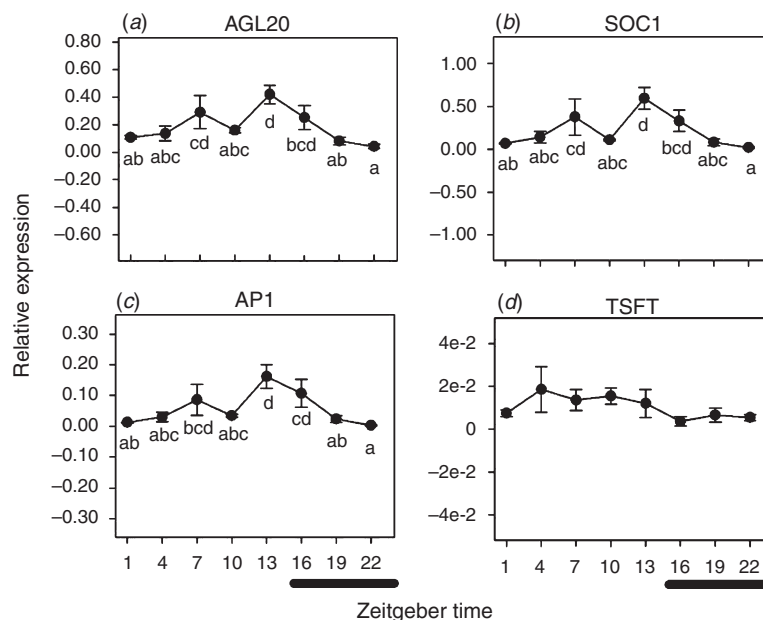n the lights were off. Standard error bars are displayed (*n* = 3). Fisher's protected least significant difference test is denoted by letters, where values with the same letter are not significantly different from each other (*P* < 0.05). Gene expression profiles without l.s.d. letters were not significantly different over the 24-h cycle.

Variations in expression of genes that are associated with the floral induction pathway over a 24-h cycle

The genes associated with the floral induction pathway can be regulated by one or more of the various flowering pathways including photoperiod, vernalisation, gibberellin and autonomous; and consequently activate *LFY* and *AP1* which are floral meristem identity genes. Nine sugarcane homologues of genes associated with floral induction were measured. Five of these genes showed statistically significant changes in expression level over the 24-h cycle, with a difference between minimum and maximum expression of greater than 50% and the remaining four genes showed no significantly different expression (Fig. 5).

ShLFY, *ShGI*, *ShSOC1*, *ShAGL20* and *ShFT-A* all showed significant changes in expression over a 24-h cycle (Fig. 5). *ShLFY* expression had a broad peak encompassing the last 4 h of the light period and the first 5 h of the dark period, with a small decrease in expression at the transition from light to dark (Fig. 5b). Within the final 2 h of the dark period, expression dropped to its minimum and remained at this level through the first 7 h of the light period (Fig. 5b). *LFY* has primarily been observed within floral meristem and floral structures; the expression and role in sugarcane leaf tissue is not known and may or may not be associated with floral transition.

The expression of *ShGI* in the spindle leaf peaked with the onset of the light period that continued for 10 h then slowly decreased with the transition to the dark period followed by minimum expression during the dark period (Fig. 5c). We note that *ShGI* expression in the TVD leaf did not have the sustained peak seen in the spindle leaf but reached a peak 10 h after the onset of the light period, and there was minimal expression in the mature leaf (Fig. 6c). The 24-h cycle expression pattern of *ShGI* in the TVD leaf is similar to the pattern seen in a Brazilian sugarcane cultivar (Hotta *et al*. 2013) and also to homologues in rice (Hori *et al*. 2013; Wang *et al*. 2013; Lee and An 2015) and soybean (Izawa *et al*. 2011; Li *et al*. 2013; Wang *et al*. 2013; Lee and An 2015). In rice and soybean, grown under floral induction conditions, the peak expression of *GI* was maintained at the transition from the light to dark period, but this is now occurring earlier in the 24-h cycle (expression has contracted with the short daylength), and in rice, expression was increased by ~25% (Filichkin *et al*. 2011; Hori *et al*. 2013; Li *et al*. 2013). Under SD conditions, expression of *LpGI* in rye grass occurred earlier in the 24-h period and expression was reduced (Gagic *et al*. 2015). Further analysis is required to determine if *ShGI* expression alters similarly to rice, soybean and rye grass during floral inductive conditions.

Despite *SOC1* and *AGL20* previously being identified as the same gene in wheat (Shitsukawa *et al*. 2007), two different sugarcane clones were obtained following blast searches with several published sequences that aligned to these genes. Both sugarcane clones aligned to different sorghum chromosomes; consequently they have been treated as separate genes but compared with published expression patterns for genes labelled both *SOC1* and *AGL20*. The expression patterns for *ShAGL20* and *ShSOC1* were similar overall (Fig. 5d, e). There was minimal expression of *ShSOC1* in the TVD and mature leaf (Fig. 6d). In the spindle leaf *ShSOC1* expression peaked sharply with the onset of the light period then quickly decreased to

minimum expression 3 h later (Fig. 5d). The presence of two peaks was reported in the soybean homologous *SOC1* gene (*GAL1*), occurring after the onset of the light period and at the end of the dark period, (Zhong *et al*. 2012). During photoperiod induction the first peak in soybean occurs at the same time, but is now at the transition from light to dark and there is no second peak (Zhong *et al*. 2012).

The expression profiles of *ShAP1*, *ShSVP* and *ShTF1* were similarly erratic over the 24-h cycle and despite 2-fold differences in expression there were no significant differences (Fig. 5a, f, g). In northern blots the rice *AP1* homologue, *OsMADS14*, appears to have consistent expression over 24 h similar to the *ShAP1* expression (Wang *et al*. 2013). In *Arabidopsis*, *TF1* expression peaked at the transition from light to dark periods under LD conditions, along with an increase in expression over that reported in SD conditions (Sanchez *et al*. 2011). Although *Arabidopsis* is a LD plants, unlike sugarcane, it is likely that changes to *ShSVP* and *ShTF1* expression would occur under inductive conditions.

The metabolite responsible for signalling changes that control and/or trigger flowering has generally been called florigen. The florigen has been identified as the product of *FT*, whereby transcripts produced in the leaves are transported to the meristem, and the translated protein affects the transition from shoot apical meristem to floral meristem in conjunction with other proteins. The role of *FT* may vary depending on the precise signalling requirements of the plant. For example, expression of *FT* is only seen in LD plants after CO protein is stabilised to achieve a threshold level, yet its homologue in rice, *Hd3a*, is expressed in SD conditions independently of *CO* level, with both pathways leading to floral induction (Greenup *et al*. 2009). Two sequences were identified in sugarcane for *FT* and these genes have been treated as alleles labelled *FT-A* and *FT-C*. The expression profiles of *ShFT-A* and *ShFT-C* were different (Fig. 5h, i), with *ShFT-C* showing no significant difference in expression over the 24-h cycle within any leaf tissue (Fig. 6f). Expression of *ShFT-A* in the spindle leaf peaked at the onset of the light period, however this peak was not observed in other leaf types and expression was minimal at all time points in the mature leaf (Figs 5h, 6e). The expression profile of *ShFT-A*, over a 24-h cycle, was similar to *FT* profiles reported in rice (known as *Hd3A* and *RFT1*; Komiya *et al*. 2008; Zhao *et al*. 2012; Wang *et al*. 2013; Lee and An 2015) and sorghum (known as *CENTRORADIALES*, *CN*; Yang *et al*. 2014). In rice the expression peak of *Hd3a* and *RFT1* was at the transition from dark to light in both photoperiod inductive and non-inductive conditions, however there was a higher level of expression under the inductive conditions (Izawa *et al*. 2002; Lee *et al*. 2016; Sun *et al*. 2016). This expression profile for *ShFT-A* is an important base line to assess changes in expression during floral induction.

Variations in gene expression in the meristem

Many of the genes involved in flowering act in specific tissues, including those genes that affect floral organ differentiation in the meristem. Despite their specific roles, cyclic expression is still observed and changes can be diagnostic of signal perception. For example, *AP1* has been associated with floral meristem

identity and changes in the 24-h cycle profile or level of expression may be seen when transitioning from vegetative to floral meristem. Of the four genes measured in the sugarcane vegetative meristem, three showed significant changes in the level of expression over the 24 h light/dark cycle, with variations between minimum and maximum expression of greater than 60% (Fig. 7). *ShAGL20*, *ShSOC1* and *ShAP1* gene expression in the meristem had similar patterns consisting of a gradual increase in expression 7 h after the start of the light period, reaching a peak at the beginning of the dark period followed by a reduction in expression to reach a minimum at the start of the light period (Fig. 7*a–c*). All three genes also displayed a sharp reduction in expression in the latter half of the light cycle (Fig. 7*a–c*). We noted that in soybean meristem the expression of *GmSOC1* and *GmSOC1-like* over 24 h showed frequent oscillation, by 20%, in non-inductive conditions, but had a peak during the dark period in inductive conditions (Na *et al*. 2013). Expression of *ShTSFT* was not significantly different over the 24-h cycle; however, there was a trend for a broad peak initiating 5 h before the onset of the dark period and persisting until 5 h before the start of the light period (Fig. 7*d*).

Conclusion

Control of flowering in sugarcane

Analysis of the gene expression patterns supports the important role of the young leaves in perception of signals that induce flowering. It has been known for some time that removal of the spindle leaves prevented floral induction in sugarcane (Coleman 1968; Julien 1971; Chu and Serapion 1972; Moore 1974; Shanmugavadivu and Rao 2010; Moore and Berding 2013; Glassop *et al*. 2014*b*), suggesting that the main source of photoperiod signals arises from the youngest leaves. In wheat, analysis of the level of *AP1* transcription in the top five leaves showed that the youngest leaf had significantly higher gene expression than seen in the older four leaves (Yan *et al*. 2003). Amongst the five sugarcane genes that were examined in multiple leaf types in the present study, the expression was generally highest in the spindle leaves, followed by the TVD leaf and lastly the mature leaf, where often there was no signal detected; the exception was *FT-C* where the differences were not significant, but there was a trend towards stronger expression in the TVD leaf (Fig. 6*f*). These results now provide a molecular explanation of the practice within the sugar industry of using partial defoliation to prevent or delay flowering.

Other factors affecting flowering, including drought and heat, are also manipulated by sugarcane growers via farming practices. For example, withholding water during the period of floral induction results in reduced flowering (Gosnell 1973). The molecular basis of these practices is not yet known but insights may be drawn from studies of signalling pathways in other species. For example, genes similar to the yeast HAP2/HAP3/HAP5 (Heme activator proteins) complex may be involved with perceiving these environmental signals and regulating flowering (Jung and Müller 2009). The various pathways that influence flowering and their integration with further environmental cues result in a complex floral

induction process which is only just starting to be analysed in sugarcane.

While flowering is not desirable on a sugarcane farm, as flowering potentially limits growth and reduces yields, it is necessary for breeding purposes. Within the sugarcane breeding programs around the world, crosses can only be made between varieties with synchronised flowering. The use of photoperiod control facilities has been successful in artificially synchronising sugarcane flowering (Berding and Moore 1996; Berding and Hogarth 2005; Berding *et al*. 2007), however some desired crosses remain unachievable due to non-responsive plant varieties. Key changes in gene expression may not be occurring in these plants in response to changing daylengths. This study identified the expression pattern of genes involved with the flowering pathway over a 24-h cycle, which provides a baseline to underpin the assessment of changes in expression when the sugarcane plant is exposed to floral inductive conditions. Knowing this baseline of gene expression will likely be very important when assessing differences between sugarcane varieties that readily flower and those that are non-responsive to floral induction. From gene expression studies in other plants under photoperiod inductive conditions, inferred expression changes in sugarcane may include reduction (*TFL1*, *SOC1*, *PRR59*, *GHD7/Ma6*, *LHY*), increases (*SOC1* – meristem, *FT-A*, *PhyB/Ma3*, *PRR1*), and altered timing of expression (*GI*, *PRR3*, *PRR7*); however, these inferences require detailed future investigation. Furthermore, the study has identified parts of the signalling pathway where sugarcane may diverge from other species. Although some parts of the pathway are likely to be conserved, some genes may function differently depending on short or long day signalling requirements of the plant. Of the sugarcane genes and gene alleles examined, 12 genes had similar expression profiles to published results, supporting the proposed flowering pathway in sugarcane and consistent with the expected results for a SD plant. However, the expression pattern of five genes did not match those reported in other SD plants also grown under non-inductive photoperiods; while this does not exclude that another allele may match the published expression profiles, it may indicate that these genes function differently in sugarcane. Understanding the control of genes in the flowering pathway in sugarcane and ascertaining any variations in gene expression patterns between responsive and non-responsive sugarcane varieties will assist with identifying key regulatory points of control that may potentially be manipulated to induce or inhibit flowering.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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