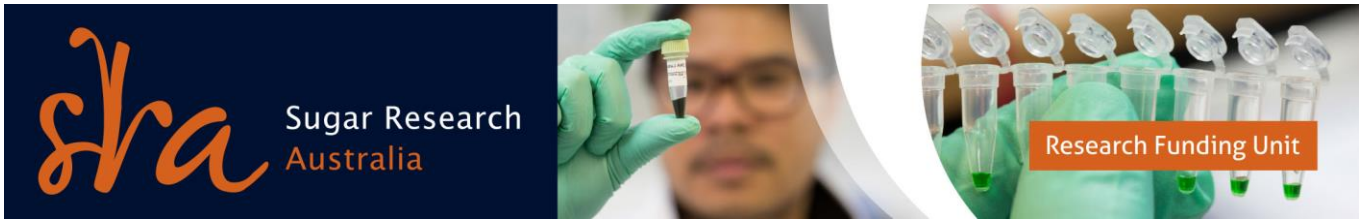

Final Report

Faster flowering – new opportunities for genetic improvement: final report 2012/024

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Research organisations: CSIRO

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Key Focus Area (KFA): Optimally-adapted varieties, plant breeding and release

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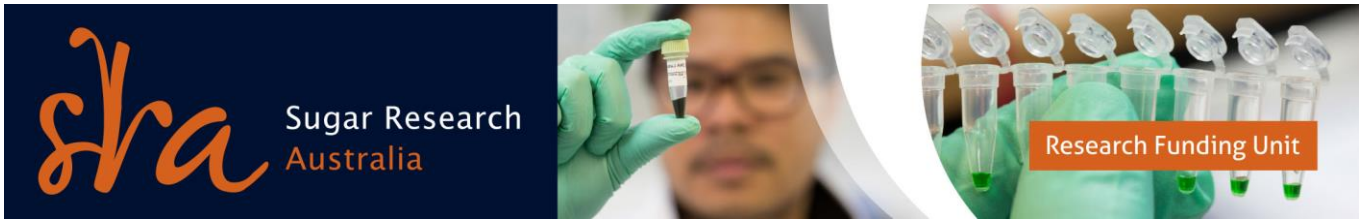

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PART A

Section 1: Executive Summary

In sugarcane, flowering is essential to breeding but is not a pre-requisite for commercial production. Consequently, our knowledge of the processes that control flowering lags behind other crops where the seed is the commercial product. Recently, sugarcane breeders have successfully used photoperiod treatments to harmonise flowering times in parents for crossing, however genetic improvement is still hampered by the inconsistent flowering of clones and by the long cycling time. Important gains could result from technology to induce flowering 'on demand' without the current environmental and infrastructure constraints. The ability to induce flowering in younger plants would significantly reduce the generation-to-generation cycle time and therefore speed up: (i) recurrent selection and introgression breeding; (ii) transfer of GM events from a single cultivar into a range of backgrounds; and (iii) production of inbred lines for genetic studies. These tools would speed up variety improvement and allow the development of new genetic resources. The aim of this project was to develop knowledge and test methods to induce flowering more frequently.

We demonstrated that the photoperiod treatment developed by the breeding program could be replicated in a controlled environment facility, resulting in the production of flowers. By moving separate sets of mature, receptive plants through the facility in turn, 3-4 experimental cycles of induction could be achieved in a year. Samples of leaves collected from plants exposed to the photoperiod treatment provided the material to identify and characterise the genes that are likely to be involved in the control of flowering in sugarcane. Sugarcane homologues of 29 genes were identified by their sequence homology with known genes in sorghum and maize. These genes could be classified into three groups according to their timing of activity in the developmental pathway: (i) genes involved in circadian rhythm and sensing of daylength; (ii) genes involved in transmission of the signal to the meristem and the determination of meristem fate; (iii) genes involved in development of structures in the inflorescence. For a selected set of key indicator genes, we demonstrated that expression responds to daylight, and that the photoperiod treatment induces changes to their expression patterns that coincide with the change from vegetative to reproductive growth. We also showed that there were variations in expression of the flowering genes in a plant variety that does not flower consistently in the field.

Potential methods for inducing early flowering were assessed and two strategies were tested. By analysis of gene expression patterns, we demonstrated that smut whips, which can occur in a very young plant, do not share a common developmental pathway with inflorescence production. Although there is a superficial resemblance and both structures are associated with repression of genes controlling vegetative growth, this pathway did not identify a mechanism to overcome the maturity requirement for flowering. Chemical

induction of flowering was then tested by the application of 19 hormone analogues and bio-active compounds to growing plants. We developed a method for topical application of the chemicals which successfully induced changes to plant growth and could be used with extremely small quantities of chemicals. The treatments were applied over a period of 7-9 weeks and plants were harvested 1-4 weeks after the last treatment. Plants were analysed for morphological features including leaf blade length/area/weight, leaf sheath length, internode length/diameter, overall stalk length/weight and anatomy of the apical meristem. Although none of the treatments resulted in the production of a flower, some of the features of reproductive growth were induced, in particular, a diagnostic increase in the ratio of leaf sheath to leaf blade length.

The major output of the project is new knowledge to underpin the control of flowering in sugarcane. Results have been communicated to sugarcane researchers through papers presented at ASSCT and ISSCT conferences and discussions at those meetings. We plan to publish results on gene expression and chemical induction as papers in international journals so that results can be used by other researchers. Throughout the project, communication was maintained with Dr Felicity Atkin at SRA Meringa Research Station to ensure that the experimental conditions were relevant to the conditions used in the breeding program. Dr Atkin also provided advice on selection of plant varieties and on analysis of plant features that indicate a positive response to photoperiod induction. These discussions helped to identify other opportunities for research to improve the efficiency of crossing, for example, by managing pollen fertility. Further work will be required to achieve control of the vital flowering process in sugarcane.

Section 2: Background

The morphology of the sugarcane plant is typical for a species of the Poaceae family. The stalk is generated by an apical meristem which gives rise to a repeating series of phytomers, consisting of internodes with attached blade-shaped leaves. When flowering is induced, the morphology changes dramatically, as the meristem changes from indeterminate growth to a determinate growth pattern. During the development of the reproductive meristem within the furled immature leaves, the stalk elongates and the leaf blades become shorter. The inflorescence emerges from the sheath of the final leaf (flag leaf) as a branched panicle (Clements 1975, Rae et al. 2014).

Sugarcane breeding for variety improvement relies on crosses between clones that are flowering at the same time. However, achieving synchronous flowering between desired parents is not always possible, resulting in opportunistic rather than planned crosses. Although flowering is essential to breeding, it is not a pre-requisite for commercial production of sugarcane. Consequently, our knowledge of the processes that control flowering

lags behind other crops where the seed is the commercial product. Research over many years has clarified some of the requirements for induction of flowering in sugarcane.

Flowering is influenced by genotype and by a number of environmental factors including photoperiod, temperature, moisture, age and nutrition. Photoperiod is a key factor in many species, and the inductive signal may be short days or long days or a combination. It has been shown that progressive reduction in daylength (or more correctly, progressive increase in night length) is essential for initiation of flowering in sugarcane. Berding and colleagues have tested the effects of various starting daylengths (Berding et al. 2010), various rates of decreasing daylength from the same starting daylength (Berding et al. 2007) and a discontinuous pattern starting with a 60 sec day^{-1} reduction in daylength until panicle development and then various decreasing rates of daylength (Berding et al. 2010). The influence of temperature has also been tested, with an optimum daily range between 18 and 31˚C (Coleman 1963; Pereira *et al.* 1983, Shanmugavadivu and Gururaja Rao 2009) and adequate water (Paliatseas and Chilton 1956) and nutrients (Brunkhorst 2001; Brunkhorst 2003) were shown to be essential requirements. Even when conditions are optimal, the induction and development time vary between sugarcane clones, with a range of 6.5 – 14 weeks from initiation to emergence observed (MacColl 1977). It has also been reported that flowering time is linked to latitude of origin for some clones, suggesting that there is genetic variation in the responses to induction (MacColl 1977; Moore and Nuss 1987). Following the initial conversion of the meristem to reproductive growth, sugarcane requires a continuing signal of decreasing daylengths in order to produce a mature flower. If the signal is not sustained, the meristem can revert to vegetative growth at various stages in the process, resulting in incomplete flowers or hybrid structures (Moore and Nuss 1987).

Various strategies have been used to speed up or delay the emergence of a flower (Moore and Botha 2014). Sugarcane breeders in Australia and other countries have successfully used photoperiod facilities (PF), in which environmental variables are controlled, to maximize and synchronize flowering in parental clones for the crossing program.

Recently the genetic control of the transition to reproductive growth has been investigated in model species and the knowledge is now being used to control processes in crop plants such as heading in cereals. The genes involved in floral induction have been well characterised in model plants such as Arabidopsis and rice, and homologues have been identified in major crop species such as wheat and

sorghum (Greenup et al. 2009; Izawa et al. 2003; Koornneef et al. 1998; Vicentini et al. 2012). It has been shown that these pathways are highly conserved between species and therefore it is likely that knowledge from sorghum could inform sugarcane, since these species are closely related. Several of the genes involved in floral induction in sugarcane have now been identified (Dornelas and Rodriguez 2001; Dornelas and Rodriguez 2006). There is also evidence that flowering in some species can be induced by bio-active chemicals such as hormone analogues that overcome the need for environmental and physiological triggers.

There would be many potential benefits from understanding and controlling flowering time in sugarcane. Genetic improvement is still hampered by the inconsistent flowering of clones and by the long cycling time. Important gains could result from technology to induce flowering 'on demand' without the current environmental and infrastructure constraints. The ability to induce flowering in younger plants would significantly reduce the generation-to-generation cycle time and therefore speed up: (i) recurrent selection and introgression breeding; (ii) transfer of GM events from a single cultivar into a range of backgrounds; and (iii) production of inbred lines for genetic studies. These tools would speed up variety improvement and allow the development of new genetic resources.

In addition, a better knowledge of the genetics behind flowering could lead to strategies for reducing flowering in field-grown plants. Although there have been conflicting opinions on the impact of flowering on yield, it is now clear that flowering can limit plant growth and therefore yield when there is a long gap between flowering and harvesting (Moore and Berding 2014). This situation often occurs in northern Australian growing regions, where heavy flowering is experienced (Berding and Hurney 2005; Jackson et al. 2012). In order to maximise yields, a number of strategies are currently used to limit field flowering, including selection of reluctant flowering genotypes. During early stage selection trials in the Northern and Herbert breeding programs, clones with >75% flowering attract a penalty of minus one unit of CCS (Jackson et al. 2012). Although there has been research into delaying flowering in order to synchronise crosses, there has been very little research on reducing overall flowering. However, studies in model systems show that this is possible, for example, the genetic manipulation of specific genes in rice resulted in delayed or no flowering (Kobayashi et al. 2012).

This project aimed to address the need for better control of flowering in sugarcane, particularly focussing on novel methods to induce flowering more frequently. Initially, the signalling pathway that controls flowering in sugarcane was characterised in plants subjected to photoperiod treatment in the CSIRO controlled environment facility. Recent discoveries on control of flowering in related grass crops provided a framework for this approach in sugarcane. Diagnostic tools for the response to the flowering signal were developed and tested in varieties that respond differently. Potential chemical inducers of flowering were then screened, making use of small molecule chemical libraries which are now commercially available. The results are described in detail in the following section.

Section 3: Outputs and Achievement of Project Objectives

Project objectives, methodology, results and discussion

Objectives 1 and 2. Define the signalling pathway for floral induction in sugarcane and test the hypothesis that genetic diversity in the response to induction is related to changes in the signalling pathway.

Background

The genes that regulate flower initiation and development were first described in the model plant Arabidopsis, which flowers in response to long days. Subsequently it has been shown that the central pathway of flowering genes is highly conserved, even between plants which are only distantly related and which are induced by a different set of environmental conditions.

In Arabidopsis grown in 16 h days, the clock-associated gene *GI* activates expression of the *CO* gene which in turn activates expression of the *FT* gene (Fig. 1) (Alvarez-Buylla et al. 2010; Koorneef et al. 1998). The *FT* gene has recently been confirmed as the source of the long-sought florigen, a factor which moves from the leaf to the shoot apex to initiate conversion of the vegetative meristem to the reproductive phase. Homologues of these genes have been identified in long day (LD) grass species including wheat, where an additional requirement for vernalisation also interacts with the central flowering genes.

In species that flower in response to short days (SD plants) the central pathway is also conserved but the genes respond differently to environmental cues. Rice is a facultative short day (SD) plant which flowers quickly in response to short days (< 10 h light), but will eventually flower even if kept under long days. The orthologous genes in the central regulatory module of rice are designated *OsGI*, *Hd1* and *Hd3a* respectively (Fig. 1). Rice also contains a second florigen gene, *RFT1*, which is regulated differently to *FT* and an additional inducer, *Ehd1*, which is not found in Arabidopsis (Tsuji et al. 2011). In the tropical SD species, sorghum, homologues of *GI*/*OsGI*, *CO*/*Hd1*, *FT*/*Hd3a* and *Ehd1*have also been identified (Fig. 1) (Murphy et al. 2011).

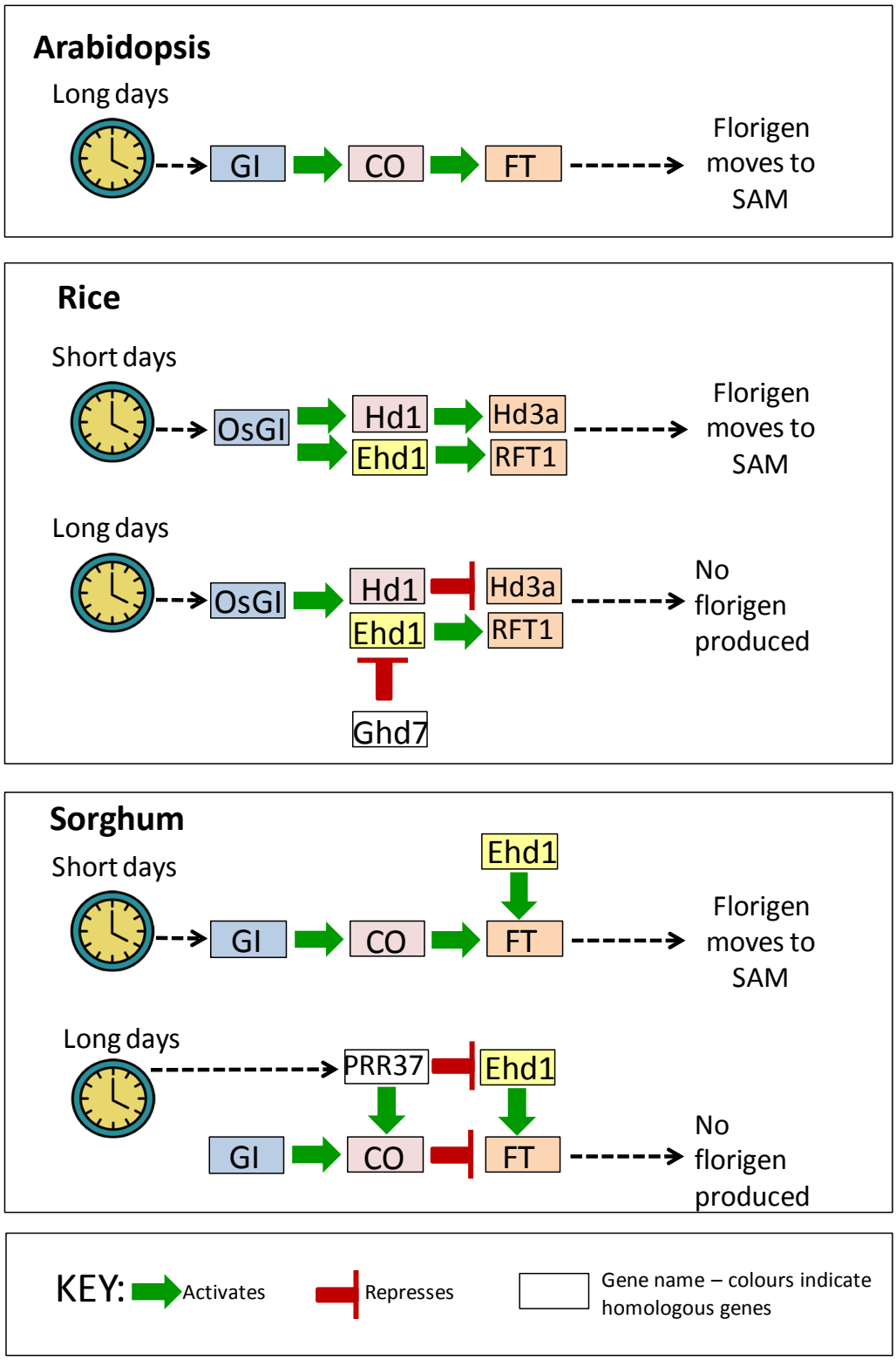


Figure 1. Genes involved in the early signalling pathway for flowering in Arabidopsis, rice and sorghum. The central pathway is highly conserved between species. Additional genes in rice and sorghum regulate the response to daylength. The interactions shown take place in the leaves and culminate in the production of a mobile signal ("florigen") which moves to the shoot apex.

Both rice and sorghum have mechanisms to suppress the expression of flowering genes in LD conditions. In both species, the *CO* equivalent becomes a repressor of the florigen genes under LD conditions. Additional genes operate through different pathways in the two species to regulate the LD expression of the florigen genes (Fig. 1). In rice, the expression of the florigen inducer, *Ehd1* is held in check by the *Ghd7* gene under long day conditions (Tsuji et al 2011). In sorghum, the unrelated *PRR37* gene represses the florigen gene directly and also indirectly by repressing the sorghum *Ehd1* homologue (Murphy et al. 2011).

Sugarcane behaves in many ways as a SD plant, requiring an induction period of decreasing daylength, although the photoperiod requirements for flower development are more complex than in other SD grasses, possibly due to the more complex genome and hybrid structure of cultivars. Based on the similarities between sugarcane and sorghum, homologues of *GI*, *CO*, *FT*, *Ehd1* and *PRR37* would be expected to occur in sugarcane.

Upon reaching the shoot apical meristem, the florigen triggers the expression of a series of genes that control flower development. The signalling pathway has been best described in Arabidopsis, although studies have also focussed on rice and maize. Grass inflorescences have a more complex structure than Arabidopsis, requiring multiple meristem types to initiate, in turn, branches, spikelets and florets (Thompson and Hake 2009).

In Arabidopsis, the arrival of the FT florigen at the meristem activates the SOC1 transcription factor which acts as an integrator of multiple flowering signals including daylength. Upregulation of SOC1 is one of the earliest events in inflorescence development (Lee and Lee 2010). SOC1 activates the floral meristem identity gene LFY (LEAFY) via AGL24 (Moyroud et al. 2010). A second floral meristem identity gene, AP1 (APETALA 1) is induced directly by FT. The two meristem identity genes, LFY and AP1 prevent the reversion of the meristem to the vegetative state and activate the floral organ identity genes which control the production of sepals, petals, stamens and carpels. These organ identity genes include transcription factors belonging to the MADs-box and AP2 gene families.

Many of the genes identified in the Arabidopsis floral development pathway are also found in grasses although some have evolved slightly different roles (Thompson and Hake 2009). OsSOC1 from rice is expressed in vegetative tissues and is also induced during floral initiation. A maize homologue, ZmMADS1, is expressed in spikelet primordia during floral development (Lee and Lee 2010). Homologues of LFY from rice and maize, known as RFL and ZFL respectively, are expressed in the developing inflorescence and control branching, so that rfl mutants show reduced branches (Moyroud et al. 2010). Transcription factors with homology to floral organ identity genes were identified amongst sugarcane EST sequences by Dornelas and Rodriguez (2001) but no functional analysis was performed.

In this study, putative sugarcane homologues of genes that are involved in flowering were identified. A representative set of these genes were then tested for two key indicators of a role in the flowering pathway in sugarcane: (i) expression in response to daylight; (ii) expression in response to decreasing daylength over a period of four weeks. Genes involved in controlling flowering would be expected to show changes in activity

under these conditions. Some of this information has been published in the proceedings of the 2013 ISSCT Congress and reprinted in Sugar Tech. This publication is included as Appendix 1:

Glassop, D, Rae, AL and Bonnett GD. (2013) Sugarcane flowering genes and pathways in relation to vegetative regression. Proceedings of the International Society of Sugar Cane Technologists Congress, CD.

Glassop, D, Rae, AL and Bonnett GD. (2013) Sugarcane flowering genes and pathways in relation to vegetative regression. Sugar Tech 16, 235–240.

Methodology

Identification of gene homologues

Sugarcane genes were identified by sequence homology to genes that are known to be involved in flowering in other plant species. The best matches amongst the sugarcane sequences were identified by using sorghum sequences which in turn were selected by homology with rice and maize sequences. Primers were designed according to conserved sequences and used in PCR reactions with genomic DNA and cDNA as the template. Genomic and cDNA clones were obtained for a number of these genes and sequencing of these clones confirmed that the correct sequences had been amplified (Table 1).

Gene expression analysis by real time quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from plant tissue by using the QIAGEN Plant RNeasy mini kit. cDNA was synthesised with the QIAGEN cDNA synthesis kit and real-time PCR was performed with the Applied Biosystems ViiA™ 7 Real Time PCR Instrument using Syber Green. Primers for RT-qPCR were designed using conserved sequences across the alleles that had been identified as described above (Table 1). In cases where no PCR product could be amplified, primers were designed according to EST sequences from public databases; this approach was only used for the genes with putative roles in later floret development that were required for the smut experiments. For each RT-qPCR reaction, 30-35 ng cDNA was used together with 1 µL mixed primers (2.4-3.0 µM primers for gene of interest and 0.3-3.0 µM reference gene primers (25s rRNA, ADF and GAPDH); also including 10 µM ITS primers in plants infected with the smut fungus) and 6 µL Syber Green (Life Technologies Corp.). The thermocycler conditions were standard on the ViiA7 Applied Sciences instrument; an initial 50°C for 2 min, 95°C for 10 min then 40 cycles of 95°C for 15 s and 60°C for 60 s. Results were normalised to the expression of the reference gene. PCR primer efficiencies were tested by construction of a standard curve and the PCR products were cloned and sequenced to confirm that correct sequences were amplified.

Replication of photoperiod induction in the controlled environment facility

In the sugarcane breeding programs at SRA and other organisations, photoperiod treatments have been used successfully to induce flowering in varieties that may not flower consistently in the field. Plants are exposed to natural elements during the day and brought into a photoperiod house in the evenings with controlled

temperature and light. However under these conditions, flowering can only be achieved once per year, thus restricting the experiments required to analyse flowering genes. To overcome this limitation, we have conducted experiments in a Controlled Environment Facility (CEF) to ensure that floral induction and flower production can be achieved.

Experiments over many years have defined the optimum temperatures, starting daylength and rate of decrease in daylength for the photoperiod treatment houses. Mimicking this methodology, replicate sett-grown plants were grown in pots for 4-5 months before applying a treatment involving the reduction of daylength in the controlled environment chamber. From a starting daylength of 12 h 55 min, the daylength was progressively reduced by 1 min day^{-1} for 104 days until flowers were mature. During this period, the daylight temperature was 29°C and humidity 60% while the night-time temperature was 24 °C and the humidity 90%. The average light intensity in the chamber was 550 µmol m^{-2} s^{-1}. During the treatment period, the external morphology of the plants was monitored and samples were taken at weekly or fortnightly intervals to examine the structure of the apical meristem. Initially the treatment was tested on variety Q174 (a cultivar known to flower reliably at Meringa) and then subsequently tested and applied to other varieties.

Collection of leaf samples

For analysis of gene expression over a 24 hour cycle of daylight and darkness, leaf samples were collected from plants of variety Q174. Replicate plants were grown from setts for 4-5 months then maintained in the CEF at a daylength of 12 h 55 min, with other conditions as above. Samples were collected at 3 h intervals through a 24 h day-night cycle. Four replicate plants were harvested at each timepoint. RNA was extracted from the spindle leaf (the unfurled roll of immature leaves), the youngest fully mature leaf (TVD, top visible dewlap) and a mature leaf from each plant. The expression of the following genes was measured by RT-PCR: ccLHY, GI, PRR1, FT-A, FT-C, LFY, SOC1 and TFL.

For analysis of gene expression during the early induction period, samples were collected from plants of variety Q208 and Q183 over the first four weeks of photoperiod treatment. Variety Q208 is known to flower frequently in the field and consistently responds to treatment in the photoperiod facility, while Q183 flowers extremely infrequently and does not respond reliably to the photoperiod treatment. Replicate plants were grown from setts for 4-5 months in the CEF at a daylength of 12 h 55 min and then treated with progressive reduction of daylength, as described above. Plants were harvested at three time points (10 am, 1 pm and 4 pm) at weekly intervals. Samples of spindle leaf, the youngest fully mature leaf (TVD) and a mature leaf from each plant were collected at each sampling point. The expression of the following genes was measured by RT-PCR: ccLHY, GI, PRR1, CO, FT-A, LFY and TFL.

Results and Discussion

Homologues of flowering genes were identified in sugarcane

Analysis of the sugarcane sequences available on GenBank allowed the identification of putative homologues of many of the genes involved in flower initiation and development. Table 1 lists the names of the genes, the corresponding sorghum sequences and the homologous sugarcane cDNA or EST sequences that were identified by searching GenBank. The table also includes the sequences that successfully amplified the correct sequences for use in measuring relative gene expression by RT-qPCR.

The genes have been listed in 3 classes, according to likely role in the signal pathway:

(i) Genes involved in maintenance of circadian rhythm and perception of daylength ("clock" genes).

(ii) Genes involved in transmission of a signal to the apical meristem and determination of meristem fate ("signalling" genes).

(iii) Genes involved in development of floral structures.

Published results suggest that some genes are likely to retain similar roles in sugarcane while other may evolve new roles. In the next section of the study, the likely involvement of the sugarcane genes in the flowering process was assessed.

Table 1. Genes associated with flowering that were analysed in sugarcane by this study. Genes were identified either by homology to sorghum genes, or from sugarcane sequences published by Hotta et al. 2013. Primers were then designed according to the sugarcane sequences that were accessed from public databases. Cloning and sequencing of PCR products was carried out to confirm that the correct genes were being amplified. Genes highlighted in green are involved in circadian rhythm and sensing of daylength. Genes highlighted in blue are involved in transmission of the signal to the meristem and the determination of meristem fate. Genes highlighted in purple are involved in development of floral structures. Reference genes for qPCR are shown without highlighting. Gene sequences were used in a range of experiments in this study: D, diurnal expression; P, expression during photoperiod induction; S, expression during smut infection.

Gene	Sorghum homologue	Homologous sugarcane clones identified in databases	Forward primer	Reverse primer	qPCR products cloned and confirmed by sequencing	Applications
CCA1 (Circadian Clock Associated 1)	Sb07g003870	SCACLV1023E11, TC134518, TC119270, TC125303, TC123186	CCA1 qPCR for4 ATGAGAAGGTGAAGCAAGCCT	CCA1 qPCR rev4 - TGCTTCTAAATCTGCGGTGGT	Yes. Aligns with cDNA.	D, P
Sc **CCA1**		Hotta et al. 2013	ScCCA1-F - CCACCACGGCCTAAAAGAAA	ScCCA1-R - TGGTTTTGTTGACTTGTCATTTGG	Yes. Aligns with cDNA.	P
ccLHY (Late Elongated Hypocotyl)	Sb06g026500	SCEZSB1093G09, CA096635, CA096025, CA195413	ccLHY qPCR F - TCACCAGCTTCCTCGGTTTA	ccLHY qPCR R-2 - ATCGTTGGAAGTGGTGGC	Yes. Aligns with genomic clones.	D, P
GI (Gigantea)	Sb03g003650	SCELB2070C07 TC131885 TC119369 TC118140 TC154473 TC118369 TC123067 TC116381 TC148895 CA159698 CA174160 TC150700 TC149241 CA263552 CA211907 CF571168 TC124982	So GI qPCR F - ACATGCCGAAGGAGTTGAAG	So GI qPCR R - GTGCAGTGGCATCGATAGTG	Yes. Aligns with TC123067	D, P
Ma1 (Sorghum	Sb06g057900		Ma1 qPCR for1 - TCAAAAGGT	Ma1 qPCR rev1 - TGCGTCTG	Yes. Aligns with Sb06g05790	

Maturity Locus 1)			G CTGTTGACTT	AA TGCCACTTT C	0 - PRR37 gene	
PRR1 (Pseudoresponse regulator 1)			PRR1 qfor GAGCCTGAC CAGAGAGAC AAGAT	PRR1 qrev CATATGCT AGAAATGC AGACGAC	Yes. Aligns with SbPRR1 cDNA and TC124158.	D, P
PRR3 (Pseudo response regulator 3)		Hotta et al. 2013	ScPRR3-F - GACCCAGTT TTCCAACCCA AT	ScPRR3-R - CCCTCCGT GCTACTGT CCAA	Yes. Aligns with Sobic.005G0 44400.1 CDS	
PRR7 (Pseudo response regulator 7)		Hotta et al. 2013	ScPRR7-F - CAAGTAATT CACCCCAAA TCAGAGATA	ScPRR7-R - TCCCATAG ATTCATCTT TATTCTCCT TAT	Yes. Aligns with sorghum sequence. Sobic.001G4 11400	
PRR5/9 (Pseudo response regulator 5/9)		Hotta et al. 2013	ScPRR59-F - CACCGATGG CATCCCTATT C	ScPRR59-R - TCTTGCCAC ATGGATGT TTTG	Yes. Aligns with sorghum sequence Sobic.002G2 75100.1 CDS.	
PRR37 (Pseudo response regulator 37)	JF801178 - 90	TC121913, TC124755, TC116034, TC112873, CA239707, TC117691	PRR37 qPCR for1 - CTATCTGGC ATCGGTCTG CT	PRR37 qPCR rev1 - TGCTCCCTT CGACAAAC ACT	Yes. Aligns with Sb01g41140 0 - PRR37	
TOC1a/PRR1 (Timing of CAB expression 1 / Pseudo response regulator 1)	Sb04g02619 0	SCCCSB1002 H04, TC132008, TC124158, TC153786, CA065665, CA167119, CF576433	ScTOC1a-F - TGCCCCAAC CCCACTACTC	ScTOC1a-R - CTTCGCTG GAGCAGGA TATTTT	Yes. Aligns with CA167119	D, P
AGL20 (Agamous Like 20)	Sb01g03057 0	SCSBAM1088 B05	So SOC for A - CCGCTACAG GACGTACAC AA	SOC1 qRT rev1 - TCTAAATTT T CACCCAAA AA CTTTC	Yes. Aligns with cDNA.	S
CO (Constans), also called **Hd1** (Heading date 1)	Sb10g01005 0	CA233930, CA244633	CO Hd1 qRT for CAGCGTGGT GTACTGCCA C	CO Hd1 qRT rev1 GCGACGCA ACGCGGTT GGC	Yes. Aligns with genomic clones.	P
EHD1 (Early heading date 1)	Sb01g01998 0		Sb EHD qPCR F1 TTTGACATCC	Sb EHD qPCR R1 GGAAATCG	Yes. Aligns with	

			CCACCGTGAT	GAAGCCCCACTG	Sb01g019980.	
FT-A (Flowering Locus T, allele A)	Sb03g001700	TC123488, CA284643, CA297966 (partial cDNA clones)	FT qRT for - GACATGCGCACCTTCTACAC	FT qRT rev2 - CGAGCTGTTGGAAGAGCAGA	Yes. Aligns with cDNA.	D, P, S
FT-C (Flowering Locus T, allele C)	Sb04g008320	CA147028 - cDNA clone	qRT for1, So FTC qRT for1 (note: will only work on cDNA as it crosses an intron.) GGCCTATACCCTAGTTATG	qRT rev1, So FTC qRT rev1 (will only work on cDNA as it crosses an intron.) TCTCGGCCAAAGCTATCAT	Yes. Aligns with cDNA.	D, P, S
Id10 (Indeterminate 10)	Sb01g021480	TC116683	Id10 F2 - aggcgcattccaaggtct	Id10 R2 - agccctgtgcgtgatgaag		S
Kn1 (Knotted 1)	Sb01g009480	TC118803	Kn1 qF1 - gatggagttcatgcgaaggg	Kn1 qR1 - accttcttgatcctcctcag		S
LFY (Leafy)	Sb06g027340	AY789622, CA223974	LFY qRT for1 CGCGACGCGGTGATG	LFY qRT rev1 GCCTCAGCTCCTTCTTCCTC	Yes. Aligns with genomic DNA.	D, P, S
SOC1 (Suppressor of Constans1)	Sb01g049020	TC135043	So SOC1 qfor GCATCAGGGGAAGAAAGACA	SoSOC1 qrev GGCTGCTTCTTGCACTTTTC	Yes. Aligns with TC135043.	D
TF1 (Terminal Flower 1)	Sb08g003210	CF571229	So pTF1 qF114 - gggtgattggagaagttc	So pTF1 qR 275 - TGAAGAAAGA CCGCAAGT		D, P, S
TSFT (Twin Sister of Flowering Locus T)	Sb08g008180		TSFT So qFor1 GGCGTTATACACACTGGTGA	TSFT qrev1 - GCACCAGCTCCTTCTGGTAT	Yes. Aligns with genomic clones.	S
AP1 (Apetala1) OsMADS14 variant	Sb01g007790	TC134443	So pAP1 qfor - TGCAGGAGGAGAACAAGGTT	So pAP1 qrev - TGGTCTGCTGTTGAGTTTGG	Yes. Aligns with cDNA clones.	S
AP1 (Apetala1) OsMADS15 variant	Sb02g001090	SCQGLR1085G190, SCQSFL3034B09, SCBFFL5069	So pMADS15 qfor - TCATGATGAGGCAGGATCAG	So pMADS15 qrev - CAGCCACC	Yes. Aligns with Sb cDNA	

		A04 – cDNA clone EST; SCMCFL5012 B02 - cDNA clone		TCTTCACCT CTC		
Lg2 (Liguleless 2)	Sb03g040530	CA259385	So Lg2 qF2 - ttgccaactaca ctgctctc	So Lg2 qR2 - tctggtgcaac gtctgct		S
PI (Pistillata) also called MADSTF	Sb09g020770	TC119595, CA161140	PI qRT for1 - TGTGGGATG A GAAGCACAA G	PI qRT rev1 - CCTTTCAG AT GCCTGAGC TG		S
Ra3 (Ramosa 3)	Sb02g039820	CA065391	So Ra3 qF 138 - gacaaggttttc gagttcg	So Ra3 qR 315 - ccagagaatc gtgcacct		S
SVP (Short vegetative phase) also called **AGL24** (Agamous like 24)	Sb01g044810	EST sequences	SoSVPqfor TCTCAGCTGC AGTCACAT	SVP qREV A1 TTAAGGCT GGTTTGAG CA	Yes. Aligns with SVP genomic clone.	S
Ts2 (Tasselseed 2)	Sb01g038050	TC123484	So Ts qF1 - aagcgccgatct agagac	So Ts qR1 - caccttcccttc caaccgct		S
ADF (Actin depolymerizing factor			ADF F1 - ctactactgtgga tttgtacgccatt atag	ADF R1 - ggacctttttta cacagcaaca aac	Yes. Aligns with TC124458	D, P
GAPDH (Glyceraldehyde-6-phosphate dehydrogenase) (Iskandar et al. 2004)			GAPDHF - CACGGCCAC TGGAAGCA	GAPDH - TCCTCAGG GTTCCTGA TGCC.	Yes. Aligns with CA254672.	D, P
25s rRNA (Iskandar et al. 2004)			25s rRNA 1F - ATAACCGCA TCAGGTCTCC AAG	25s rRNA 1R - CCTCAGAG CCAATCCTT TTCC	Yes. Aligns with Saccharum hybrid cultivar R570 clone BAC 039D18.	D, P, S
ITS (Internal transcribed spacer from *Sporisorium scitamineum*)			CTTGGTCATT TAGAGGAAG TAA	GATCCGCC AGCTCTTTC GTAAT		S

(Gardes and Bruns 1993)						

Photoperiod treatment was successfully replicated in the CEF

Approximately 74 days after commencing the daylength reduction, changes to the plant morphology were observed; specifically an increased distance between the dewlaps of the two youngest leaves and a reduced blade length of the youngest leaf. These changes indicated the elongation of the spike bearing the flower (known as "bolting") and the emergence of the flag leaf. The plants were maintained in the controlled environment chamber until flowers emerged. Figure 2 shows flowering stalks of Q174 in the controlled environment chamber.

Figure 2. Panicles produced on plants of variety Q174 that were exposed the reduced photoperiod treatment in the controlled environment facility.

There was no relationship between internode number at the time of induction and flowering, although all of the plants tested were mature. Of the 33 plants in the treatment, 31 plants produced a flower. The two plants that did not flower were dissected to examine the state of development of the apical meristem. Conversion of the vegetative meristem to the floral meristem had occurred in both plants but the floral structure had died at about 10 – 15 cm in length; the reason for death is unknown.

Examination of the meristem from plants harvested at 1 week intervals during the photoperiod treatment showed diagnostic changes to the meristem (Fig. 3). After two weeks of treatment, the meristem retained the domed shape typical of the vegetative state. After three weeks, a slight elongation of the meristem could be observed when compared with the original shape. This observation agrees with published reports suggesting that conversion of the meristem from the vegetative state to the reproductive state requires approximately 20 inductive daylengths. The elongated shape, which is characteristic of the reproductive meristem, became more pronounced over the next few weeks. The inflorescence was visible as a small projection from the apex of the meristem after 6 weeks of treatment. After 8 weeks, a branched floral structure was visible, increasing in size by 10 weeks.

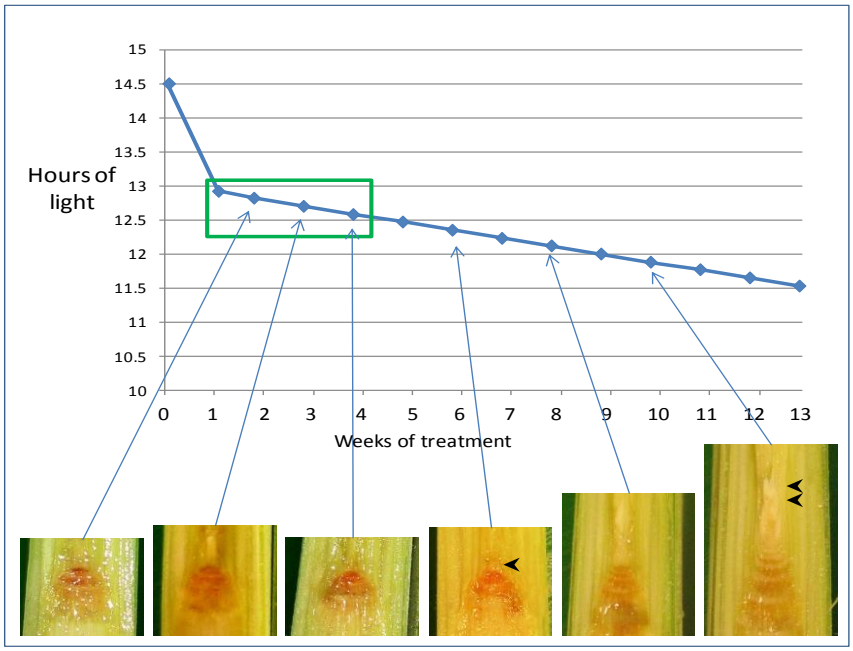


Figure 3. Photoperiod treatment and meristem conversion in sugarcane grown in a controlled environment chamber. The graph shows the length of the daylight period during the photoperiod treatment for induction of flowering. Daylength was reduced by 1 min per day for 13 weeks. The green box shows the period of sample collection for analysis of early gene expression responses. Longitudinal sections through the meristem region after 2, 3, 4, 6, 8 and 10 weeks of treatment show that the meristem changes from a domed shape to an elongated shape during the treatment. The floral meristem is visible after 6 weeks (arrowhead) and a branched floral initial is visible after 8 weeks, increasing in size at 10 weeks (double arrowhead).

These initial experiments demonstrated that the CEF was suitable for floral induction experiments and the ability to conduct experiments as required. Subsequently, the photoperiod treatment was applied to groups of plants which were harvested at different time points in the process.

Gene expression responds to daylight

The analysis of gene expression focussed on the daylength perception ("clock") and signalling genes in the first two stages of development. These genes are most likely to show early responses to changes in daylength that influence the development of the apical meristem.

The expression of genes over a 24 h day/night cycle are shown in Figures 4 and 5. Most of the genes tested showed significant changes in expression throughout a diurnal cycle. GI increased rapidly when the light was switched on and expression remained at a high and consistent level until the light was switched off. Expression of GI through the dark period remained low (Fig. 4B). Contrasting patterns were found for the other two clock genes. The expression of ccLHY remained low during the light period but increased during the dark period, reaching a maximum at 3 am (Fig. 4A). Expression of PRR1 increased throughout the daylight period then fell rapidly when the lights were switched off, so that the maximum expression occurred at the end of the day (Fig. 4C). These patterns of expression are consistent with results from other plant species, where the timing and ratio of expression of these genes has been shown to be important in sensing changes in daylength. In

Arabidopsis, the transcript abundance of five PRR genes peak sequentially between sunrise and nightfall, with PRR1 the last to peak near the end of the day. It has been shown that the fall in expression of ccLHY when the lights are switched off is related to the increase in the transcriptional repressor gene, ccLHY at this time (Fujiwara et al. 2008).

For ccLHY, GI and PRR1, comparisons were made between the expression in the spindle leaves, the youngest fully expanded leaves and the mature leaves. Minor differences in expression between these three tissues were detected. There was a trend for lower expression in the mature leaf compared to the other two tissues, but this was not tested statistically. There is evidence from physiological studies that the spindle leaf has the major role in perception of daylength and signaling in sugarcane. Previously published studies have tested the impact on flowering of removing various types of leaf from sugarcane stalks. Removal of the spindle leaf was shown to delay flowering (Moore and Berding, 2014; Shanmugavadivu and Gururaja Rao 2010), presumably by reducing the amount of signal reaching the apical meristem. In dicot plants, the signaling molecules are produced by mature leaves rather than immature leaves. There are few published studies on the relative roles of different leaf types in signaling in other grass species, but Sasani et al. (2009) showed that the FT gene was more highly expressed in younger leaves in barley, consistent with our findings in sugarcane.

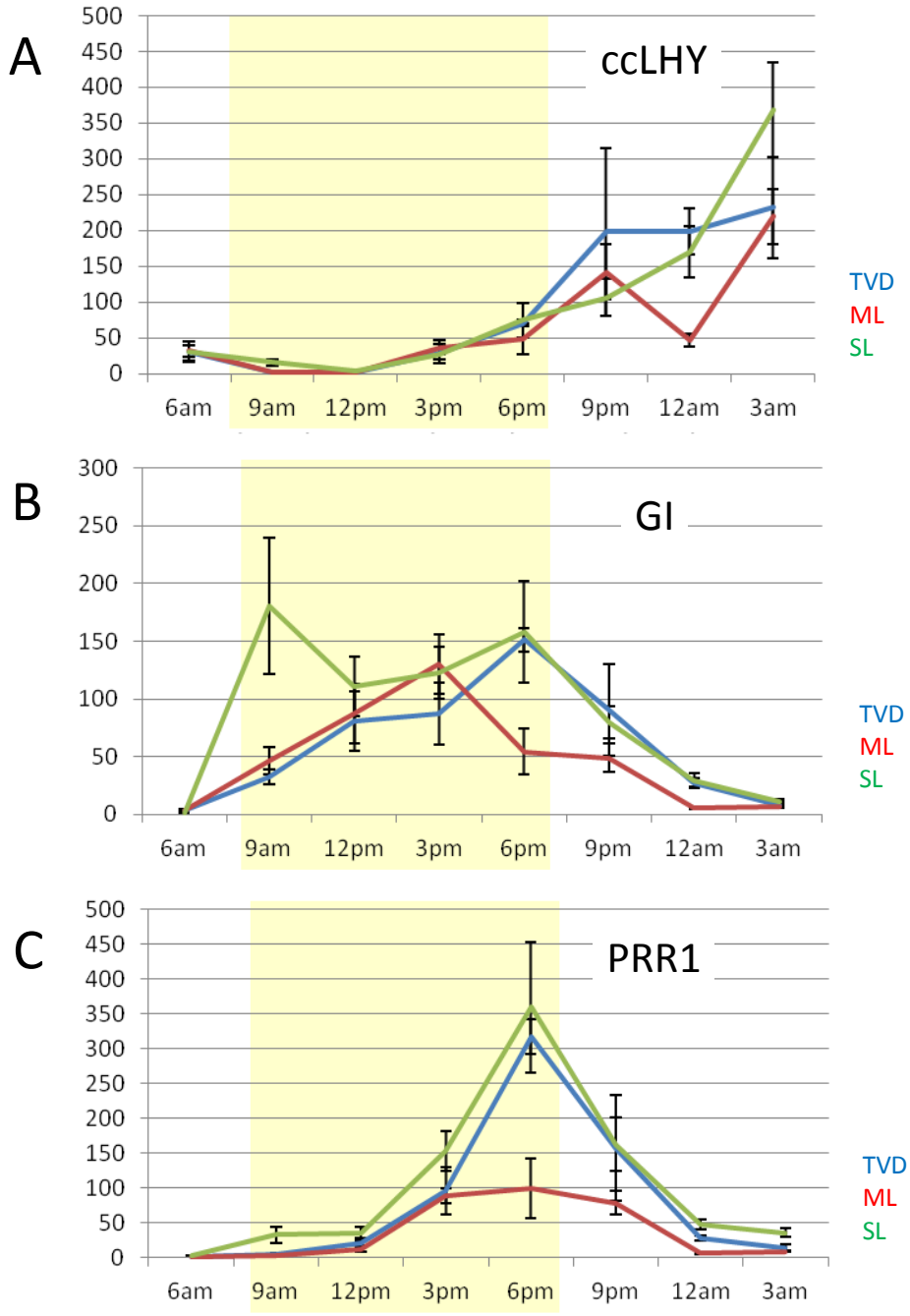


Figure 4. Expression of genes involved in maintenance of circadian rhythm and perception of daylength ("clock genes") during a 24 h diurnal cycle in the controlled environment facility. At each time point, four replicate plants of variety Q174 were harvested and samples were taken from the spindle leaf (SL), youngest mature leaf (TVD, top visible dewlap) and mature leaf (ML, equivalent to TVD-2). The genes measured by RT-qPCR were ccLHY (A),GI (B) and PRR1 (C). Results are shown as the mean and standard error relative to the expression of the reference gene encoding 25s rRNA. The light period 8 am to 7 pm is indicated by yellow shading.

The results showed that the expression of the signalling genes also changed throughout a diurnal cycle (Fig. 5). *FT-A,* and *SOC1* both showed sharp increases in expression immediately after the lights were switched on and expression then fell again (Fig. 5A, C). The peak of *FT-C* expression occurred slightly later in the morning,

although this gene was much more weakly expressed than FT-A (Fig. 5A). In contrast, the expression of LFY was bimodal, with the higher expression period lasting from late afternoon until early morning, falling again before dawn to remain low for most of the day (Fig. 5B). TFL1 was the least responsive to daylight, although appeared to have reduced expression just before dawn (Fig. 5D).

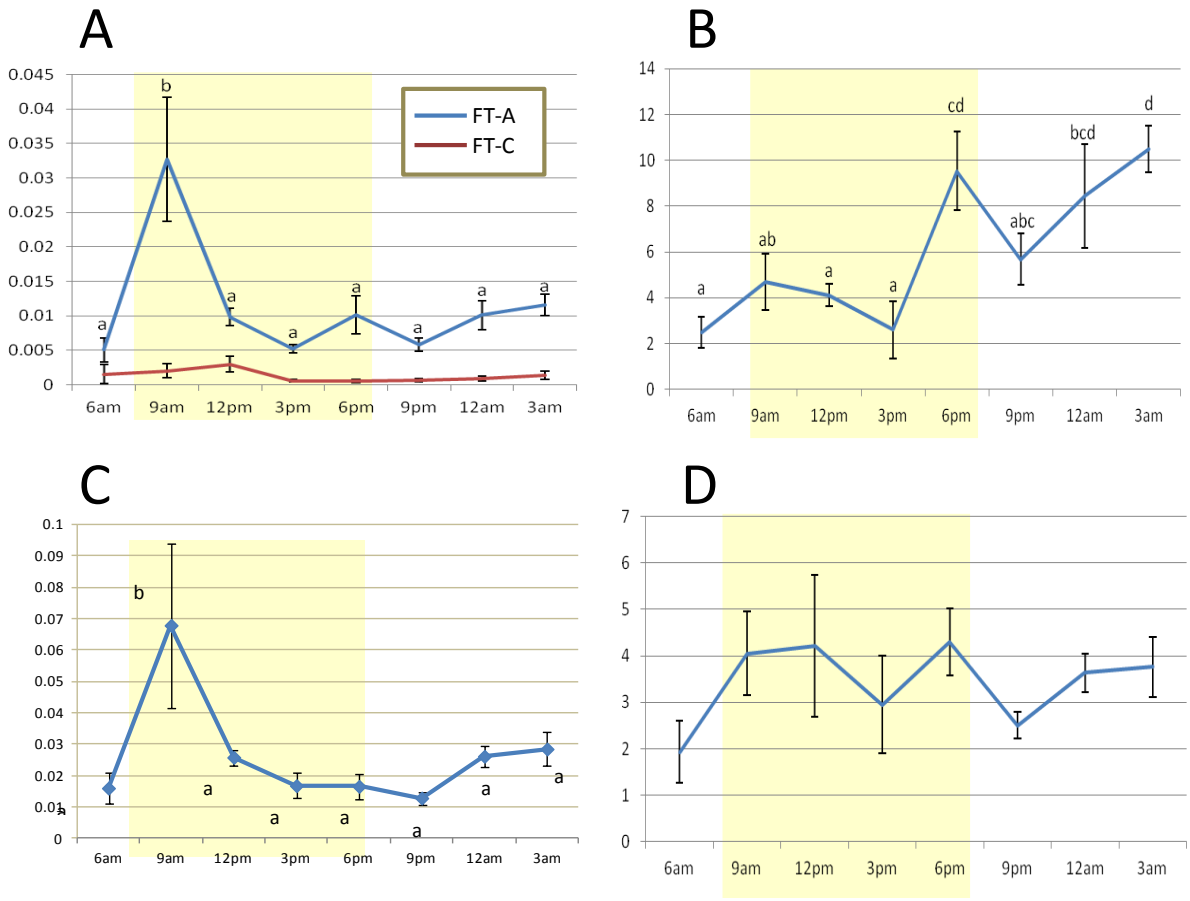


Figure 5. Diurnal expression of genes involved in transmission of the signal to the meristem and the determination of meristem fate. Expression was measured by real time qPCR using samples of spindle leaf from variety Q174 harvested over a 24h period in the controlled environment facility. The genes measured were FT-A and FT-C (A), LFY (B), SOC1 (C) and TFL1 (D). Results are shown as the mean and standard error from four replicate plants harvested at each time point, relative to the expression of the reference gene encoding 25s rRNA. Points marked with the same letter are not significantly different at P = 0.01. The light period 8 am to 7 pm is indicated by yellow shading.

Gene expression responds to changes to photoperiod treatment

After establishing that the selected genes responded to light, the effect of progressive reduction in daylength on gene expression was tested. Replicate plants of variety Q208 and Q183 were grown in the CEF and treated with progressive reduction of daylength, as described above. Samples of leaf were collected at weekly intervals

at three time points during the day: 10 am (4 h after the start of the light period), 1 pm (7 h of light) and 4 pm (10 h of light).

The results confirmed the hypothesis that the pattern of diurnal expression of the genes would change over the induction period. Results are shown in Figure 6 for the “clock” genes ccLHY, GI and PRR1 in Figure 7 for the “signaling” genes CO, FT-A, LFY and TFL.

The clock genes all showed reduced expression over the daylight period as the photoperiod treatment proceeded. Analysis of ccLHY expression at the start of the photoperiod induction treatment showed that the highest expression occurred at the 4 pm sampling time (Fig. 6A). However, the intensity of expression at 4 pm decreased markedly over the 4 week induction period and no change was observed in expression at other time points. This suggests that the amplitude of expression of ccLHY through the diurnal cycle was flattened. Analysis of GI expression at the start of the photoperiod induction treatment showed that the highest expression occurred at 1 pm and 4 pm (Fig. 6C). As the daylength decreased progressively, the intensity of the peak expression also decreased, so that the gene was found to be expressed at a uniformly low level throughout the day after 4 weeks of induction. Expression of PRR1 showed a similar reduction in amplitude of the highest expression at 4 pm (Fig. 6E). This suggests that either the level of expression overall was reduced or the peak of expression had shifted to a later time point. Because the sampling timepoints in this experiment did not provide information about expression during the dark period, we were not able to test whether the peak expression had shifted to a later time.

Figure 6:

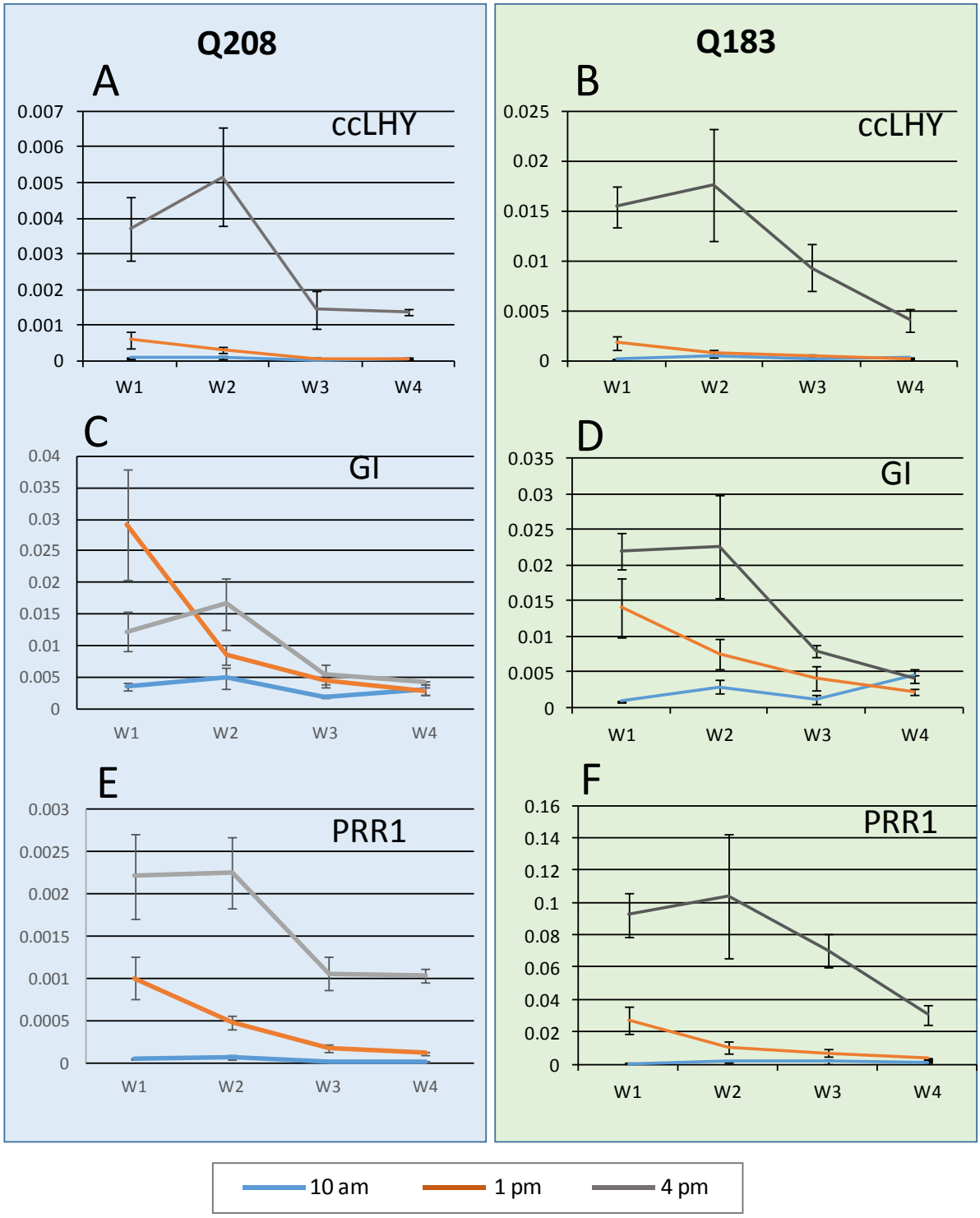
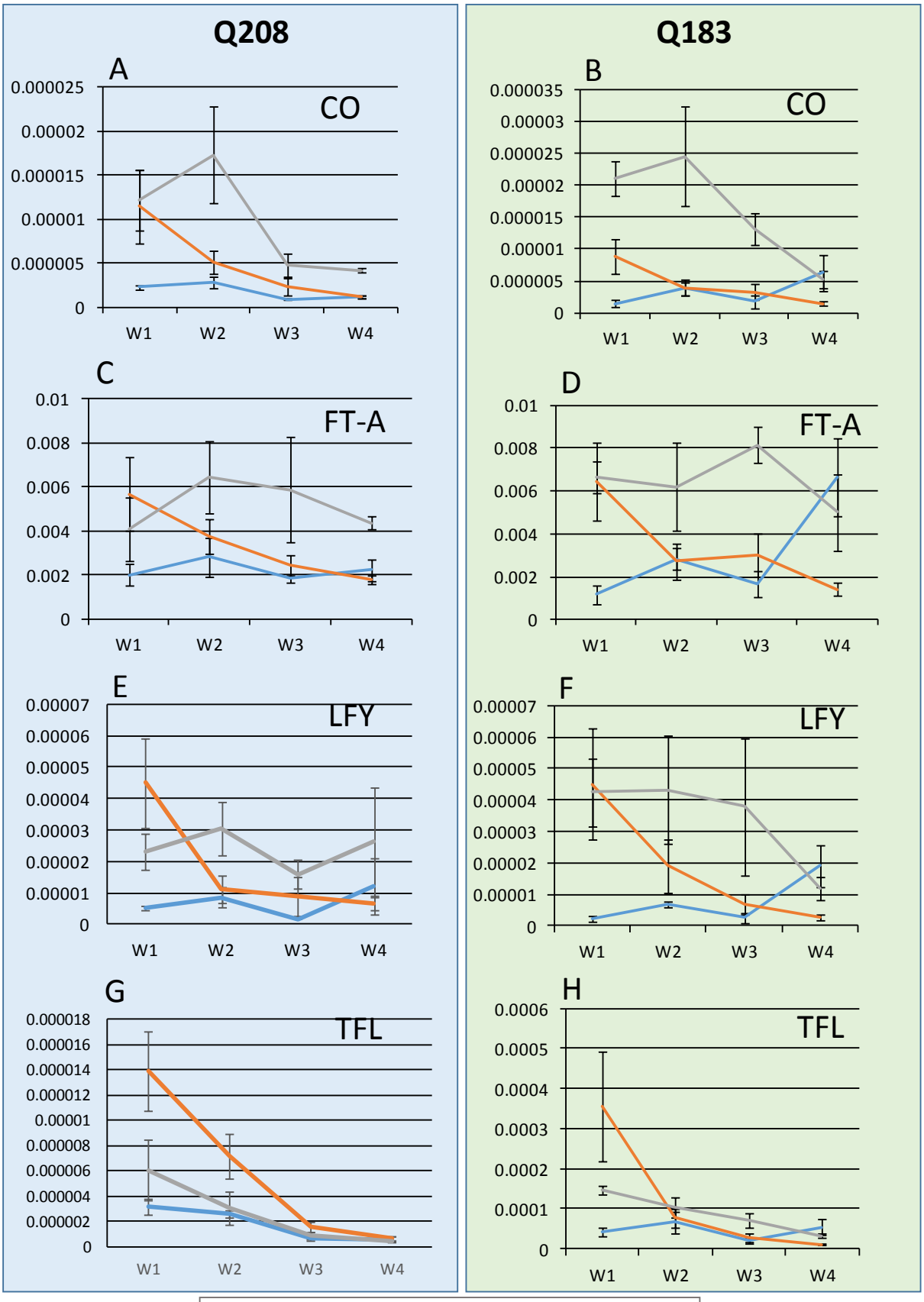


Figure 6. Expression of genes involved in maintenance of circadian rhythm and perception of daylength ("clock genes") in the spindle leaf of sugarcane varieties Q208 (left hand panel) and Q183 (right hand panel). Samples of spindle leaf were collected over a four week period of photoperiod induction consisting of progressive reduction in daylength in the controlled environment facility. Samples were collected at three time points during the day: 10 am (4 h after the start of the light period), 1 pm (7 h of light) and 4 pm (10 h of light). The genes measured by RT-qPCR were ccLHY (A, B), GI (C, D) and PRR1 (E, F). Results are shown as the mean and

standard error from four replicate plants harvested at each time point, relative to the expression of the reference gene encoding ADF.

Figure 7. Expression of genes involved in transmission of the signal to the meristem and the determination of meristem fate in the spindle leaf of sugarcane varieties Q208 (left hand panel) and Q183 (right hand panel). Samples of spindle leaf were collected over a four week period of photoperiod induction consisting of progressive reduction in daylength in the controlled environment facility. Samples were collected at three time points during the day: 10 am (4 h after the start of the light period), 1 pm (7 h of light) and 4 pm (10 h of light). The genes measured by RT-qPCR were CO (A, B), FT-A (C, D), LFY (E, F) and TFL (G, H). Results are shown as the mean and standard error from four replicate plants harvested at each time point, relative to the expression of the reference gene encoding ADF.

Figure 7:



Over the four week induction period, the expression of the "signaling" genes either fell or remained relatively consistent. Analysis

of CO and FT-A expression showed that in Week 1, expression was low at 10 am and highest at 1 pm and 4 pm (Fig. 7A, C). The expression at 4 pm increased in Week 2 but then fell over the remaining weeks. A similar trend was observed for expression of LFY (Fig. 7E). The expression of TFL1 fell at all timepoints over the four week period, resulting in extremely low expression (Fig. 7G).

Although the fall in expression of TFL1 is consistent with expectations (Neuffer et al. 1997), published results suggest that CO, FT-A and LFY should increase in expression in response to the photoperiod signal. Small increases in expression of these genes were observed in the first two weeks and in combination, these changes may be sufficient to trigger the transition from vegetative to reproductive growth. Although it has been suggested that signaling in sugarcane occurs over an extended period, our results suggest that the critical period for signaling occurs within the first week or two of a change in daylength. Weekly sampling may not have been frequent enough to detect the peak expression of each gene.

In summary, the patterns of expression during the induction phase and during a diurnal cycle are good evidence that the selected genes are involved in perception of daylength. However, the results from the diurnal experiment highlight the importance of measuring expression at a particular and consistent time of the day. For the analysis of gene expression over the 4 week early induction period, critical changes may not have been captured because they occurred within the first two weeks of a change in daylength. By combining the present results with gene functions known from other species, a model can be proposed for the pathway of genes acting in sugarcane to induce flowering (Fig. 8).

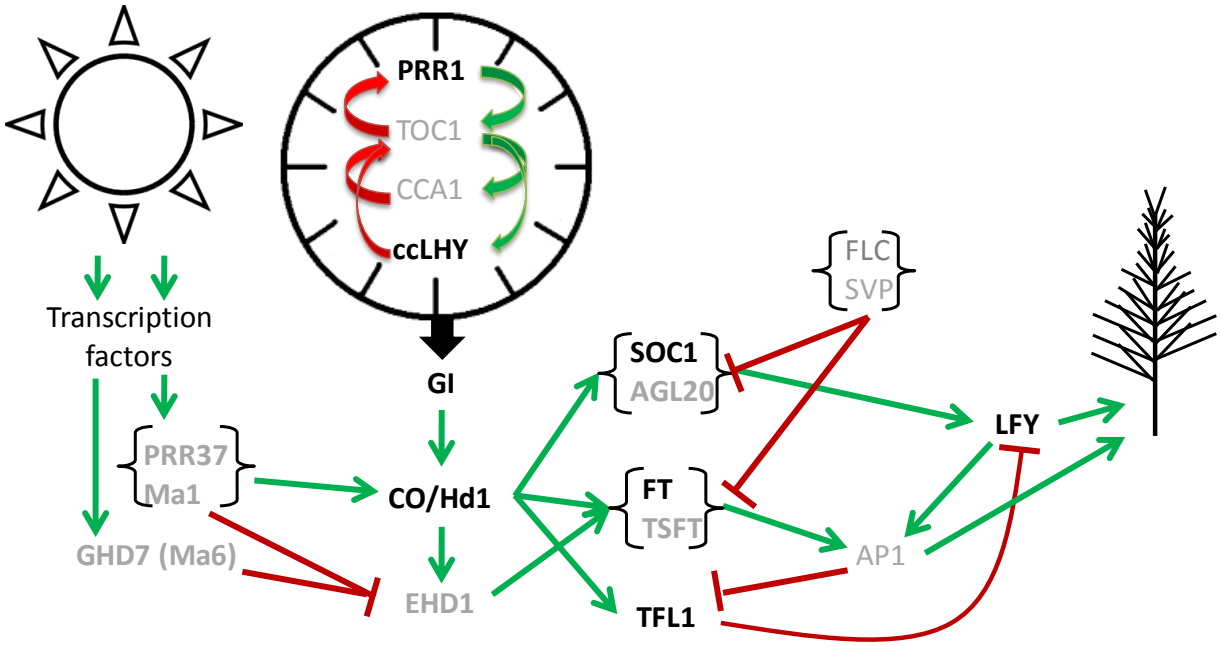


Figure 8. Proposed roles of flowering pathway genes in sugarcane. Transcript abundance for the genes in black has been measured with RT-qPCR. Genes in the circle (clock face) are associated with the circadian rhythm and daylength perception and influence GI and CO expression. Additional genes that respond directly to sunlight also influence the expression of CO and FT through intermediaries. Positive regulation is indicated by green arrows and repression is indicated by red lines.

Differences in gene expression patterns were detected in a sugarcane variety that does not respond to photoperiod induction

The analysis of gene expression was carried out using plants from variety Q208, which is known to flower frequently in the field and consistently responds to treatment in the photoperiod facility, and also variety Q183, which flowers extremely infrequently and does not respond reliably to treatment in the photoperiod facility at Meringa. Any differences in the way that these two varieties respond to the photoperiod induction treatment may contribute to an explanation of the different developmental outcomes. The results are shown in Figure 6 for the "clock" genes ccLHY, GI and PRR1 in Figure 7 for the "signaling" genes CO, FT-A, LFY and TFL, alongside the results for variety Q208 which have been discussed above. A limitation of this experiment is that plants were not maintained in the CEF until maturity to confirm that Q183 did not respond to the CEF photoperiod treatment. Although we have assumed that Q183 would behave the same way as in the field, the results should be interpreted with caution until the behavior of Q183 in the CEF is confirmed experimentally.

When the expression patterns obtained from the two varieties were compared, differences in some genes were observed. For the expression of GI (Fig. 6C, D), a peak was observed at 1 pm in Q208 after a week of photoperiod treatment, but in Q183, the peak expression occurred later in the day at 4 pm. Since GI has a role in integrating the signals from the "clock" genes, non-synchronous cycling of expression could disrupt the transmission of the photoperiod signal to the next step of the pathway. For example, this may underlie the differences in the expression of the FT-A gene in variety Q183, which varied significantly from the pattern observed in Q208. By the end of the 4 week treatment period, the diurnal pattern of expression of FT-A in Q183 had reversed, showing lowest expression in the middle of the day. The TFL1 gene is a repressor of the transition from vegetative to reproductive development and flowering cannot proceed unless the level of TFL1 expression falls. This fall was observed consistently in variety Q208. Although the expression of TFL1 also fell in variety Q183, the level of expression was at least an order of magnitude higher than the expression in Q208 and therefore the level of TFL1 in Q183 may not have fallen low enough to permit flowering to proceed.

There is evidence for differences in the expression of flowering pathway genes between a variety that flowers readily and a variety that is reluctant to flower. In order to confirm that these differences are causative, a larger set of varieties would need to be tested. As the flowering pathway is complex, it is likely that a number of different mechanisms could lead to a failure to flower.

Objective 3. Test physiological and chemical methods to induce flowering for application in immature plants or non-flowering varieties

Background

Review of the published literature identified the following potential methods for manipulating flowering:

(i) Photoperiod modification. Currently, the most reliable method for ensuring flowering is the use of a photoperiod facility to control daylength and temperature. Experiments over many years have defined the optimum starting daylength and rate of decrease.

(ii) Control of juvenile period. Studies have shown that the sugarcane plant must reach a certain stage of maturity before flowering can be initiated, either by natural photoperiod or artificial induction. During the juvenile period, the plant is unable to respond to photoperiods which would normally be inductive. In growing regions where flowering occurs early in the crop cycle and limits further increases in yield, later planting is recommended as a means of preventing flowering, since the plants are then too young to respond when the normal inductive period occurs at the end of summer. Reducing or removing the juvenile period for sugarcane is a possible method for inducing earlier flowering through earlier response to photoperiod induction.

Genetic variation in the length of the juvenile period has been observed amongst sugarcane varieties and particularly amongst different accessions of *Saccharum spontaneum*. It is likely that *S. spontaneum* varieties that

are adapted to growing regions in higher latitudes or altitudes have evolved to respond to environmental cues within a shorter growing season, including reduced juvenile period and different daylength requirements (Moore and Berding, 2014).There is the potential for selection of genes or alleles of genes that alter the length of the juvenile period. This method has been used in sorghum to adapt cultivars for growth in a variety of latitudes (Rooney and Aydin 1999).

Although many plants show a similar unresponsive juvenile phase, the genetic mechanisms that control this are only beginning to be defined (Lee and Lee 2010 and references therein). In Arabidopsis, the transition from juvenile to adult phase is controlled by the SPL family of transcription factors. The SPL genes are silenced by a microRNA which is highly expressed in young plants but progressively decreases with plant age. Altering the balance between the expression of these two genes affects the length of the juvenile phase and thus flowering time. There is evidence that flowering is regulated through the influence of SPL on the floral development integrator gene, SOC1. Recently, a maize mutation known as *Corngrass1* was identified as overexpression of a microRNA that silences the SPL genes, producing a plant that remains in the juvenile state and is slow to flower (Chuck et al. 2011). It is possible that overexpression of SPL may allow earlier flowering.

(iii) Leaf removal. Experiments have shown that removing leaves can delay or promote flowering, depending on the location of the leaves relative to the apex and the timing of removal. As the major signal that triggers flower initiation is generated in the leaves, these treatments probably block signalling to the apical meristem. Removal of the immature (spindle) leaves can delay flowering, presumably by removing the leaves which, when mature, would produce the inductive florigen that moves to the apex to initiate the change in the meristem (Moore and Berding, 2014). Removal of old leaves has been shown to promote flowering (Brett and Harding 1974). These studies suggest that mechanisms to alter the delivery of leaf-derived signal compounds could be used to control flowering.

(iv) Plant hormones. Hormones and hormone analogues are potential methods to induce early flowering. For example, gibberellins have been implicated in the development of flowers in many species by controlling the rate of developmental transitions. It is likely that gibberellins interact with the genes that initiate conversion of the meristem. Experiments have shown that gibberellin induces the expression of the LFY gene, partly by inducing the SOC1 gene (Lee and Lee 2010).

Gibberellins were first identified from rice infected with the fungus, *Gibberella* (now *Fusarium*), which caused the plants to grow tall and spindly. The morphological changes induced in sugarcane by infection with the smut fungus may also reflect the action of hormones or hormone-like chemicals produced by the fungus. Although there is some debate about whether the smut whip (sorus) is a modified floral structure (Sivanesan and Waller 1986), there is good evidence that infection alters the developmental patterning of the meristem, even in juvenile plants. An attractive feature of this hypothesis is that a sorus can be produced by very young plants, while photoperiod induction of flowering is only effective in more mature plants. Therefore, an understanding of the changes induced by smut infection may offer a way to alter the meristem structure earlier in development.

(v) Ectopic gene expression. Expression of the genes that initiate flower development has been used experimentally to promote flowering in GM plants from other species. For example, constitutive expression of the FT (florigen) gene triggers flowering in wheat even in the absence of the normal requirement for vernalisation (Kim et al. 2009). In model dicot species such as Arabidopsis and pea, leaves that have been induced to express FT can induce flowering when grafted onto a second non-induced plant. These experiments were critical in defining the mobile signal produced by the FT gene to stimulate changes at the apical meristem.

In this study, the application of hormones and hormone analogues was tested for induction of flowering. Following hormone induction, the plants were analysed for the morphological features consistent with the transition to reproductive growth. An experiment was also conducted to test whether the production of the smut whip shares a developmental pathway with the development of an inflorescence. Plants inoculated with the smut fungus were tested for expression of genes in the flowering pathway. The results of the smut inoculation study were published in the Proceedings of the 2014 ASSCT Annual Conference which is included as Appendix 2:

Glassop D, Bonnett G, Croft B, Bhuiyan S, Aitken K and Rae A. (2014) Flowering-related genes are not involved in the development of the smut whip. Proceedings of the Australian Society of Sugar Cane Technologists 36, CD.

Methodology

Smut inoculation

One-eyed setts of varieties Q174, Q117, Q183 and Q208 were inoculated with smut spores (*Sporisorium scitamineum* Ehrenb. ex Link) by the injection method as used by Karen Aitken (CSIRO) and Shamsul Bhuiyan (SRA) for smut resistance screening experiments in project CPI026. Un-inoculated control setts were also included. Staining of inoculated setts with Trypan Blue was used to detect fungal hyphae in inoculated plants. Setts were maintained in a humid environment for 30 days in vermiculite before harvesting the young plants. Previous experiments have shown that a sorus can develop in these seedlings in as little as 60 days and therefore 30 days was chosen as a time point to examine changes to the gene expression patterns that precede changes to structure. The samples were freeze-dried and stored for later extraction of RNA. Infection was confirmed by a diagnostic PCR assay as described by Magarey et al. (2008). Any plants that tested positive in the PCR assay were assumed to be infected, regardless of whether they had been deliberately inoculated. The expression of 14 flowering-related genes in these plants was assessed by RT-PCR using the methods described above and calculated relative to the expression of the 'housekeeping' gene 25s rRNA. The results were analysed by ANOVA and principal component analysis. For this study, the "clock" genes were not investigated. The genes selected included key genes involved in the transition of the meristem from vegetative to reproductive (TF1, Id10, Kn1, LFY, FT-A, FT-C, TSFT, AGL20 and SOC1) and genes involved in the formation of rachis and floret structures at

later stages of inflorescence development (AP1, SVP, Lg2, PI, Ra3 and Ts2). Genes and primer sequences are listed in Table 1.

Chemicals applied to sugarcane plants

Published results suggest that gibberellins and gibberellin-like molecules are able to control plant maturation and induce flowering in many species. Over 100 gibberellins have been identified and at least 13 have been tested for their activity in flowering in various plant species. Previously, most gibberellins were not available commercially, which limited the range of compounds that could be tested. However, a commercial supplier of synthetic gibberellins has recently been identified (OlChemIm Ltd in the Czech Republic).

In addition to the gibberellins, a small number of plant hormones were also selected based on their published roles in floral induction. Salicylic acid has been shown to decrease the number of days to flower emergence compared to un-treated controls for gladiolus (Sajjad et al. 2014). Mung bean seed weight and quality were increased in plants sprayed with salicylic acid under normal and saline conditions (Lofti and Ghassemi-Golezani 2015). Photo-insensitive *Lemna* (duckweed) can be induced to flower with the application of salicylic acid (Khurana et al. 2014). The application of abscisic acid assisted with sunflower flowering while under drought stress (Hussain et al. 2014). Putrescine application was able to overcome malformation in mango flowers, allowing the development of the fruit (Singh et al. 2014). Despite high temperatures, putrescine treated cotton plants were able to produce flowers compared to untreated plants (Bibi et al. 2010). Exogenous application of putrescine or spermidine improved grain yield of a salt-sensitive rice cultivar (Ndayiragije and Lutts 2007). Application of spermine to pistachio trees resulted in improved yields (Khezri et al. 2010).

The chemicals tested are shown in Table 2. Gibberellins were purchased from OlChemin Ltd. and remaining chemicals from Sigma-Aldrich (Australia).

Table 2. Plant hormones and hormone analogues tested in this study for induction of flowering in sugarcane. The chemicals were tested in a series of experiments indicated by: Inj, injection method; Top1, trial of topical application method; Top2, final set of topical applications.

Chemical name	**Abbreviation**	**Use in experiments**
Gibberellic acid 1 (CAS: 545-97-1)	GA1	Inj, Top2
Gibberellic acid 3 (CAS: 77-06-5)	GA3	Inj, Top1, Top2
Gibberellic acid 4 (CAS: 468-44-0)	GA4	Inj, Top2
Gibberellic acid 5 (CAS: 561-56-8)	GA5	Inj, Top1, Top2
Gibberellic acid 7 (CAS: 510-75-8)	GA7	Inj, Top2
Gibberellic acid 4 + 7 (70:30 mixture)	GA4+7	Inj
Gibberellic acid 9 (CAS: 427-77-0)	GA9	Inj, Top2
Gibberellic acid 9 methyl ester (CAS: 2112-08-5)	GA9ME	Top2

Gibberellic acid 12 aldehyde (CAS: 19436-07-8)	GA12A	Inj, Top2
Gibberellic acid 13	GA13	Inj
Gibberellic acid 19	GA19	Inj
Gibberellic acid 20 (CAS: 19143-87-4)	GA20	Inj, Top2
Gibberellic acid 24	GA24	Inj
Gibberellic acid 44	GA44	Inj
Salicylic acid (CAS: 69-72-7)	SA	Top2
Abscisic acid (CAS: 21293-29-8)	ABA	Top2
Putrescine (CAS: 110-60-1)		Top2
Spermidine (CAS: 124-20-9)		Top2
Spermine (CAS: 71-44-3)		Top2

Hormone application to sugarcane plants in the CEF: (i) Injection method

Two methods for application of chemicals were tested: an injection method and a topical application.

The injection method was initially tested using a dye solution. Aliquots (100 μL) of a water-soluble red food-colouring dye were injected by syringe into the stalks of replicate plants in approximately the region of the apical meristem. Following injection, the plants were allowed to grow normally in the glasshouse and samples were harvested after 24 h or after 4 days. Plants were bisected longitudinally and examined for the location of the dye. The dye staining confirmed that the region of the meristem had been correctly identified. After 24 h, the red dye had moved both upwards and downwards from the point of injection so that it was visible in both the immature leaves and the subtending internode. The pattern of staining was similar in the plants harvested 4 days after injection. The trial showed that the injection method was suitable for application of the test compounds.

For the first experiment, replicate plants of varieties Q208 and Q183 (three plants of each variety per chemical) were grown to 4.5 months age in long-day (non-inductive) conditions in the controlled environment facility. These varieties were chosen because Q208 is a frequent-flowering type while Q183 is a reluctant flowering type which appears to respond less readily to photoperiod induction. Plants were injected with aliquots (100 μL of 1μg/μL solution) of each chemical in the region of the apical meristem. The injections continued three times per week for five weeks while plants were maintained in long day conditions in the controlled environment facility. Untreated control plants were grown in the same conditions. Following the period of injections, the plants were maintained for eight weeks before harvesting and assessment by analysis of the growth habit. Selected plants were dissected to examine the internal meristem structure.

Hormone application to sugarcane plants in the CEF: (ii) Topical application method

Due to concerns that the injection method may adversely affect the meristem development, the method for delivery of the chemicals to the plant apex was changed to a topical application. The applications were also continued over a longer period.

Treatments were initiated on plants that were 20-26 weeks old. Gibberellic acids 3 and 5 were selected for the first experiment using topical application. Gibberellins were prepared in 95% ethanol and 0.1% Tween 20 at 10 µg μL^{-1}. The gibberellic acids were applied as a 10 µL (100 µg) drop to the inside of the outer leaf of the furled spindle as close to the meristem region as possible. Treatments were applied either weekly or twice per week with three controls consisting of no treatment, or 95% ethanol: 0.1% Tween 20 applied weekly or twice per week. Treatments were applied at approximately 4 h prior to the lights being turned off. Treatments were applied for 7-9 weeks and plants were harvested 1-4 weeks after the last treatment.

Topical application was used in two series of experiment (Table 2). In the first series, only gibberellins 3 and 5 were tested on a single sugarcane variety (Q208) and weekly application was compared to twice-weekly application. In the second series of experiments, a much larger set of chemicals (14) was used in twice-weekly applications to two sugarcane varieties, Q208 and Q183.

Harvesting and analysis of plants

The plants that had been treated with chemicals by injection were analysed for changes to the growth habit. During the normal transition to reproductive growth, the meristem undergoes a recognizable sequence of changes from:

(i) vegetative meristem producing bilaterally arranged leaves and nodal buds with subsequent elongation of the internodes

(ii) inflorescence meristem with spirally arranged cell division foci accompanied by cessation of internode elongation

(iii) floral meristem with multiple branches

(iv) complex floral meristem with secondary and tertiary branches

(v) spikelet and floret differentiation

If the inductive pressure is removed during this process, growth of the meristem can “revert” from floral to vegetative. The reversion phenotypes indicate the stage of development that had been reached by the inflorescence meristem before reversion occurred (Moore and Nuss 1987). For example, reversion after Stage (ii) above typically results in a section of sugarcane stalk with leaves arranged in a spiral and a zigzag node arrangement, often appearing as “a short section of small misshapen internodes midway in an otherwise normal stalk” (Alexander 1973). If reversion occurs after Stage (iii) above, the multiple meristems covering the apex

produce multiple spindle leaves, giving rise to the structure known as a "witch's broom". When analyzing the plants treated with the gibberellin analogues, we made use of these characteristic phenotypes as indicators that inflorescence development had been induced.

Plants treated by topical application were analysed for morphological features. Measurements were collected from leaves and internodes that had received the treatment. Measurements included leaf blade length, leaf sheath length, leaf blade area, leaf blade weight (fresh and dry weight), internode length and internode diameter. Overall stalk length and weight was also recorded. Plants were also dissected to allow examination of the meristem for floral structures that may have occurred as a result of treatment.

Statistical analysis was completed using ANOVA in Genstat.

Results and Discussion

The smut whip (sorus) does not share a developmental pathway with inflorescence development

The expression of 14 genes was assessed in smut infected and non-infected control seedlings and a range of relative expression levels was detected. The gene expression was assessed for individual varieties and analysis of variance showed no significant difference between the infected and control plants for varieties Q117, Q183 and Q208. When the results were analysed with PCA, segregation between the infected and non-infected plants was only observed in variety Q174 (Refer to Appendix 2). In Q174, the expression of six genes (*Kn1, Ra3, Ts2, FT-C, LFY* and *SVP*) was significantly increased in infected plants compared to control plants. The increased expression of *FT-C* and *LFY* in infected plants is consistent with a transition away from vegetative growth of the meristem; these genes are a requirement for cessation of vegetative growth and commencement of reproductive growth (Adrian *et al.,* 2009). However the expression of Kn1 and SVP was not consistent with this transition. In maize, these genes are involved in maintaining the meristem in a vegetative state (Koltai and Bird, 2000) and would therefore be expected to decrease in infected plants. The remaining two genes, *Ra3* and *Ts2*, are involved in the control of branching in maize tassels (Sheridan, 1988; McSteen *et al.,* 2000).

In summary, while there is a superficial resemblance between the sorus and an inflorescence, the results suggest that there is no common developmental pathway beyond the cessation of the vegetative growth pattern which occurs in both processes.

Hormone application by injection influenced growth patterns but results were not consistent

Several phenotypes of internode and node structure were found. In some cases there was no change to the structure compared to controls, so that plants continued to produce bilaterally arranged leaves and buds separated by elongated internodes. In some plants, the stalk arrangement changed from bilateral to spiral or zig-zag nodes and in one case, the production of a witch's broom was observed. Although these changes were

promising, the patterns were not consistent between replicate plants treated with the same chemical. In addition, it was clear that application of the chemical by injection had caused some physical damage to the meristematic region resulting in truncated leaves and in some cases, death of the apex. Following this trial, the method of application of the chemicals was changed so that chemical solutions were applied to the surface of the spindle leaf bundle as close to the meristem as possible.

Topical hormone application successfully influenced growth patterns and induced some changes that are characteristic of reproductive growth

Topical application experiment 1 (GA3 and GA5 only).

The application of GA3 and GA5 to Q208 plants caused significant changes to the plant morphology (Fig. 9):

(i) Stalk length and weight. There was a significant increase in stalk length in the treated plants compared to the controls. The application of GA twice weekly resulted in slightly longer stalks than the weekly application (Fig. 9A). Stalk weight (Fig. 9B) did not differ between treatments. The increased stalk length is consistent with previously published research which reported increases from as much as twice the controls (Anonymous 1956; Marth *et al.* 1956; Bates 1957; Coleman 1958; Villareal and Santos 1958; Coleman 1959; Coleman *et al.* 1960; Bull 1964; Moore 1980; Moore and Ginoza 1980).

(ii) Internode length and diameter. Internode length was significantly increased and applications twice per week resulted in significantly longer internodes than once per week applications, (Fig. 9C, D). This result is consistent with increased internode length reported by Moore and Buren (1978) and in other plants (Botha *et al.* 2013). There was no difference between GA_3 and GA_5 (Fig. 9C). While internode length increased, the internode diameter significantly decreased, resulting in long thin internodes (Fig.9D). Thinner internodes were also reported by Moore and Buren (1978).

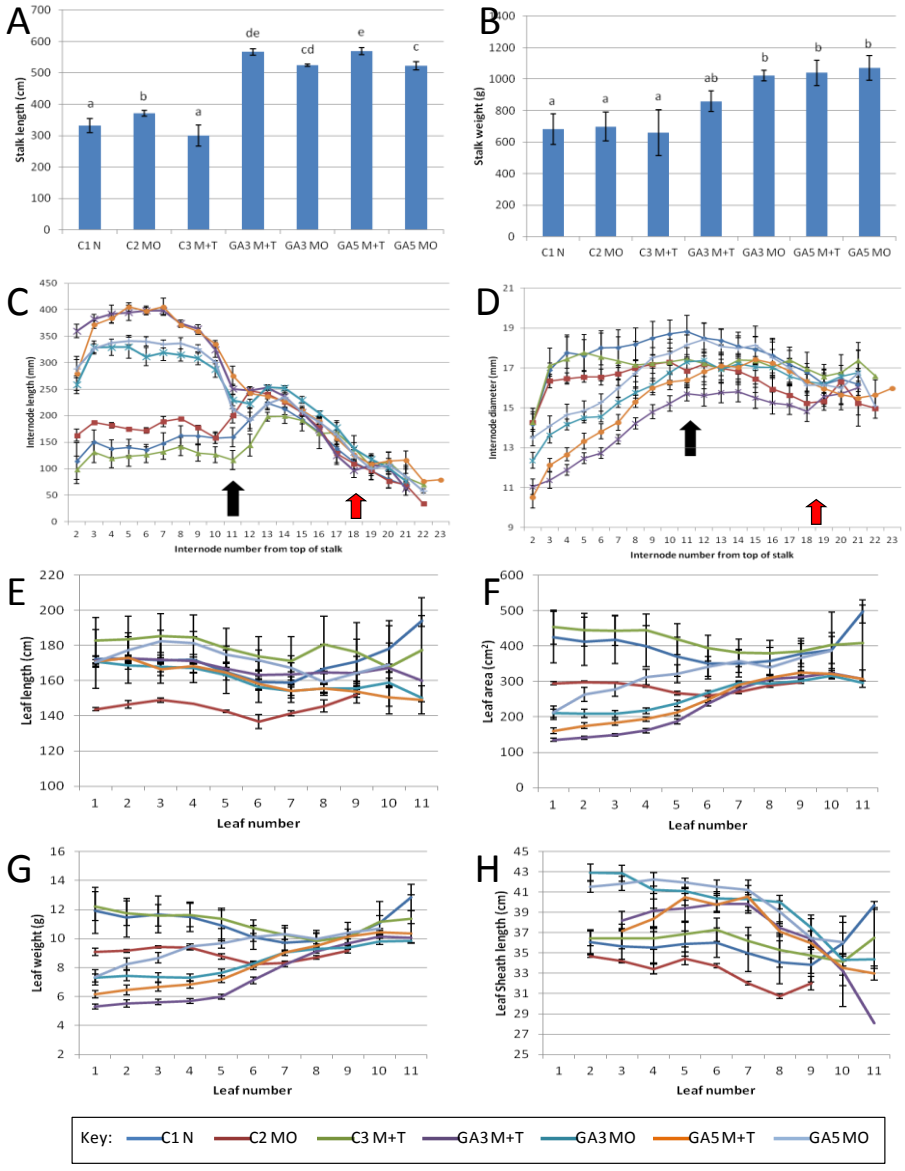
(iii) Leaf blade and sheath. While there were significant differences for leaf blade length and surface area, the gibberellic acid treatments did not separate from the controls and therefore the difference was unrelated to GA application. (Fig. 9E). The leaf blade area and blade weight of the top 5 leaves from those plants that had GA applied twice per week was significantly greater than those that received only a weekly application (Fig. 9F). Leaves that had been laid down prior to the initiation of treatments did not show any effect from the addition of GA, with changes only occurring in leaves initiated after GA was being perceived at the meristem. This is consistent with Bates' (1957) observation that already mature internodes are not affected by GA.

The length of the leaf sheath increased significantly with GA application and also with twice weekly application compared to once weekly (Fig. 9H). There was no significant difference between GA_3 and GA_5.

Figure 9. Morphology of Q208 plants treated with GA3 and GA5: (A) Stalk length, (B) stalk weight, (C) internode length, (D) internode diameter, (E) leaf blade length, (F) leaf blade area, (G) leaf blade weight and (H) leaf sheath length. The black arrows in A and B indicate the last fully expanded internode at the time of the first application of the treatments. The red arrow indicates the last fully expanded internode when the plants were moved from the glasshouse to the CEF. The leaves were numbered so that leaf 1 was the youngest leaf with a visible dewlap and was classified as being attached to internode 1.

Treatments and controls: C1 – no treatment, C2 MO – ethanol/tween 20 solution applied once per week, C3 M+T – ethanol/tween 20 solution applied twice per week, GA3 M+T – GA_3 applied twice per week, GA3 MO – GA_3 applied once per week, GA5 M+T – GA_5 applied twice per week and GA5 MO – GA_5 applied once per week. Controls N = 4, GA treated N = 6. The bars represent standard errors. Treatments with the same letter are not significantly different ($P<0.05$).

Figure 9:



(iv) Ratios of organs. The ratio of internode length to leaf sheath length was different between the control and treated plants. Figure 10 shows a comparison of internode length versus sheath length, with trendlines for the control and treated groups of plants. In the control lines, the trend was that the sheath was longer than the internode that it was attached to. In contrast, in the GA treated lines, the ratio of sheath length to internode length decreased, although both internode length and sheath length increased as a result of the GA treatment.

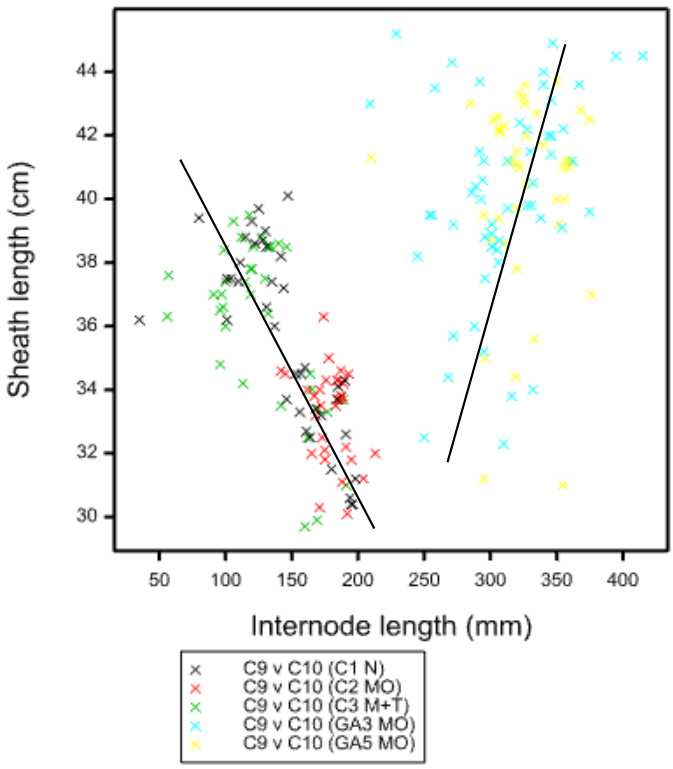


Figure 10. Relationship between leaf sheath length and internode length. Markers represent each leaf, numbered from the stalk apex down as indicated in Figure 9 showing individual replicates. Lines indicate trends in the controls (black, red and green) compared to the GA treatments (blue and yellow). C1 – no treatment, C2 MO – ethanol/tween 20 solution applied once per week, C3 M+T – ethanol/tween 20 solution applied twice per week, GA3 MO – GA_3 applied once per week, and GA5 MO – GA_5 applied once per week.

It was also interesting to note that the ratio of leaf sheath length to leaf blade length changed in the treated plants. In control plants, the ratio of leaf sheath length to leaf blade length was relatively constant. In the plants treated with GA, the leaf sheath length increased but there was no corresponding increase in the leaf blade length. This increase in sheath to blade ratio is a diagnostic feature of the change from vegetative to reproductive growth. As shown in Figure 11, leaves produced after the perception of the photoperiod induction signal typically show progressive increases in the ratio of sheath to blade lengths. The results suggest that GA treatment was able to induce some of the features of reproductive growth in the meristem.

(v) Meristem structure. At the time of harvest the meristem was dissected to determine whether the application of gibberellic acid had resulted in a transition of the meristem away from vegetative growth to floral production. No altered meristems were observed.

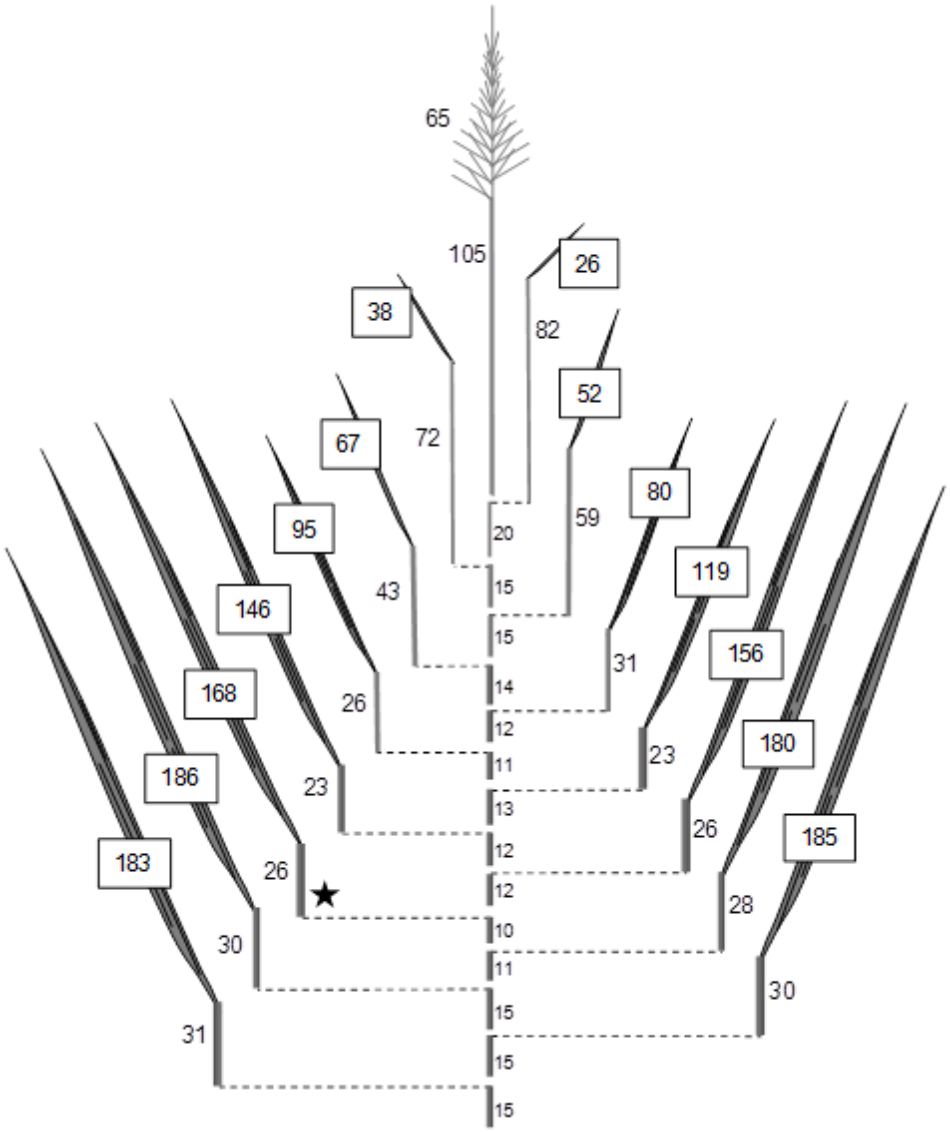


Figure 11. Diagram of a flowering culm showing the relative sizes of internode, leaf sheath and leaf blade, redrawn from Clements (1975). The numbers beside each organ represent the length (cm) derived from an average of ten measured culms. The leaf with the top visible dewlap (TVD) at the initiation of floral development is marked with a star. Following induction, internodes become longer, causing "bolting" of the culm. Leaf sheaths also become longer, while the blades are progressively reduced, culminating in the flag leaf. (Rae et al. 2014)

While the application of GA_3 or GA_5 did not result in production of a flower, some of the features of reproductive growth were induced. The application of GA resulted in a phenotype of increased stalk height and weight, lengthened but thinner internodes, increased leaf sheath length and reduced leaf area and weight; all consistent with published literature. A key result was the increase in leaf sheath to leaf blade ratio, characteristic of reproductive growth. The results showed that the topical method of chemical application resulted in the successful uptake of the chemical. Based on these results, a larger set of chemicals was tested on plant varieties Q208 and Q183.

Topical application experiment 2 (14 chemicals)

The application of 14 hormones produced results which were consistent with the trial set using GA3 and GA5 only, although differences were observed between the two cultivars in their responses to the chemicals.

(i) Stalk length and weight. In Q183, increased stalk height was observed in response to 13 chemicals (all except putrescine) while only seven of these chemicals also caused an increase in stalk height in Q208 plants (Table 3). Increased stalk weights were observed for a smaller number of treatments (Table 3).

Table 3. Changes to stalk length and stalk weight in sugarcane plants of varieties Q208 and Q183 following application of hormones. Treatments were assessed as causing an increase (Inc), decrease (Dec) or no significant difference (NSD) when compared to controls at two significance levels, P<0.05 (*) and P<0.01 (**). Three replicate stalks were analysed for each treatment.

Chemical	Stalk length		Stalk weight	
	Q183	Q208	Q183	Q208
GA_1	Inc *	Inc *	Inc **	NSD
GA_3	Inc **	Inc **	Inc *	Inc *
GA_4	Inc **	Inc *	Inc *	NSD
GA_5	Inc **	Inc **	Inc **	NSD
GA_7	Inc **	Inc *	Inc **	NSD
GA_9	Inc **	Inc *	Inc **	Inc *
GA_9me	Inc *	NSD	NSD	NSD
GA_{12}a	Inc **	NSD	NSD	NSD
GA_{20}	Inc **	Inc *	NSD	NSD
Salicylic acid	Inc *	NSD	Inc *	NSD
Abscisic acid	Inc *	NSD	Inc *	NSD
Putrescine	NSD	NSD	NSD	NSD
Spermidine	Inc **	NSD	NSD	NSD
Spermine	Inc *	NSD	NSD	NSD

(ii) Internode length and diameter. The analysis focused on the youngest 11 internodes for each plant, since the more mature internodes had already formed before treatment commenced. Seven of the treatments resulted in increased internode length for both cultivars and an additional three treatments similarly affected Q208 only. The internode diameter was significantly increased for all treatments in Q183 when compared to the control plants, whereas only two treatments had a similar effect on Q208 (Table 4). The increased internode diameter was an unexpected result and conflicts with the observations reported by Moore and Buren (1978).

Table 4. Changes to internode length and internode diameter in sugarcane plants of varieties Q208 and Q183 following application of hormones. Treatments were assessed as causing an increase (Inc), decrease (Dec) or

no significant difference (NSD) when compared to controls at two significance levels, P<0.05 (*) and P<0.01 (**). Three replicate stalks were analysed for each treatment.

Chemical	Internode length		Internode diameter	
	Q183	Q208	Q183	Q208
GA_1	Inc **	Inc **	Inc **	NSD
GA_3	Inc **	Inc **	Inc **	NSD
GA_4	Inc **	Inc **	Inc **	NSD
GA_5	Inc **	Inc **	Inc **	NSD
GA_7	Inc **	Inc **	Inc **	NSD
GA_9	Inc **	Inc **	Inc **	Inc **
GA_9me	NSD	NSD	Inc **	NSD
GA_{12}a	NSD	NSD	Inc **	Inc *
GA_{20}	Inc **	Inc **	Inc **	NSD
Salicylic acid	NSD	Inc **	Inc **	NSD
Abscisic acid	NSD	NSD	Inc **	NSD
Putrescine	NSD	NSD	Inc **	NSD
Spermidine	NSD	Inc *	Inc **	NSD
Spermine	NSD	Inc **	Inc **	NSD

(iii) Leaf blade and sheath. Leaf sheath length was increased in both cultivars by seven of the treatments and a further five treatments caused increases only in Q208 (Table 5). Reduced leaf blade length was observed following five treatments in Q183 plants, but unexpectedly, 11 of the treatments caused increases in leaf blade length in Q208 plants. Both increases and decreases to leaf area were observed. The fresh weight of the leaves was generally increased in those treatments where leaf length and/or area were increased.

Table 5. Changes to leaf sheath length, leaf blade area, leaf area and leaf fresh mass (FM) in sugarcane plants of varieties Q208 and Q183 following application of hormones. Treatments were assessed as causing an increase (Inc), decrease (Dec) or no significant difference (NSD) when compared to controls at two significance levels, P<0.05 (*) and P<0.01 (**). Three replicate stalks were analysed for each treatment except where indicated by #.

Chemical	Sheath length		Leaf length		Leaf area		Leaf FM	
	Q183	Q208	Q183	Q208	Q183	Q208	Q183	Q208
GA_1	Inc **	Inc **	NSD	Inc **	NSD	Dec **	NSD	NSD
GA_3	Inc **	Inc **	NSD	Inc **	NSD	Dec **	NSD	Dec *
GA_4	Inc **	Inc **	Dec *	NSD	Dec *	Dec **	Dec *	Dec **

GA_5	Inc **	Inc **	NSD	Inc **	NSD	NSD	Inc *	NSD
GA_7	Inc **	Inc **	Dec **	Inc **	NSD	NSD	NSD	NSD
GA_9	Inc **	Inc **	Dec **	Inc **	NSD	NSD	NSD	Inc *
GA_9me	NSD	NSD	Dec *	NSD	Inc *	NSD	Inc **	NSD
GA_{12}a	NSD	NSD	NSD	NSD	Inc **	NSD	Inc **	Inc **
GA_{20}	Inc **	Inc **	NSD	Inc **	NSD	NSD	NSD	NSD
Salicylic acid	NSD	Inc **	Dec *	Inc **	Inc **	Inc *	Inc **	Inc **
Abscisic acid	NSD	Inc **	NSD	Inc **	Inc **	Inc *	Inc **	Inc **
Putrescine	NSD #	Inc **	NSD #	Inc **	NSD #	NSD	NSD #	Inc **
Spermidine	NSD	Inc **	NSD	Inc **	Inc **	Inc **	Inc **	Inc **
Spermine	NSD	Inc **	NSD	Inc **	Inc **	NSD	Inc **	Inc **

(iv) Ratios of organs. In the previous experiments, the ratio of leaf sheath length to leaf blade length changed in the treated plants, consistent with the transition towards reproductive growth. These changes to organ ratios were not consistently found with the larger set of chemicals. Increasing sheath length and decreasing leaf blade length were observed in Q183[A] with the addition of GA4, GA7 and GA9.

(v) Meristem structure. No altered meristems were observed.

Objective 4. Develop a plan to use new knowledge/technology in variety improvement

The project did not produce outputs which can be directly used in the breeding program, however an important benefit of conducting the project has been improved communication between biotechnology researchers and the sugarcane breeding scientists. These discussions have lead to the identification of other research needs and should lead to strong and productive collaborations in the future.

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Section 4: Outputs and Outcomes

Key knowledge outputs:

- The photoperiod treatment for induction of flowering can be replicated in a controlled environment chamber, allowing more frequent experimentation compared to existing facilities.
- The genes that are likely to be involved in control of flowering in sugarcane have been identified
- The smut whip is unlikely to be a modified floral structure
- A method has been developed for application of small quantities of bio-active chemicals to the growing point of sugarcane
- Treatment with hormones can induce some of the characteristic features of reproductive growth

Other outputs:

- Better communication between biotechnology researchers and scientists in the sugarcane breeding program and the pathology program
- Increased researcher skills

Scientific paper outputs:

- Paper presented at ISSCT 2013 Congress and also published in Sugar Tech (Appendix 1)
- Paper presented at ASSCT 2014 Conference (Appendix 2)
- Poster and short talk presented at ISSCT Breeding and Molecular Biology combined workshop 2015

The outcomes of this work will be improved ability to target research through use of the knowledge and skills gained and continued collaboration between researchers. Ultimately this research will underpin new methods for controlling flowering, leading to better sugarcane varieties through achieving optimal crosses between parents.

Section 5: Intellectual Property (IP) and Confidentiality

The project has not developed protectable IP. The most effective way to achieve the outcomes is to publish the results so that they can be used widely by other researchers.

Section 6: Industry Communication and Adoption of Outputs

The key findings of the project so far have been communicated to the audiences of the 2014 ASSCT Conference and the 2013 and 2015 ISSCT Conferences.

The conference presentations generated questions and follow-up discussions with other researchers who were interested in using the results and the techniques in their own programs. There was interest in using the knowledge of genes that control flowering to design new strategies to restrict flowering in GM plants.

The work has not been communicated widely to the public. As the project outputs are likely to be useful to other researchers, the results will be published in international science journals.

Section 7: Environmental Impact

There was no environmental impact from conducting the project. The flowering induction experiments were conducted in a contained research facility. The smut experiments were conducted at the SRA Woodford Research Station.

Section 8: Recommendations and Future Industry Needs

This study generated new methods and a significant amount of new knowledge. Although some of these outputs have been published, presented and discussed at meetings of the sugar industry and research community, the remaining data should be disseminated as widely as possible to other researchers so that it can be used to underpin new research.

R1. Finalise the publication of results from this project in international scientific journals.

The knowledge base developed in the project can be applied to benefit other purposes. For example, methods have now been defined for testing growth parameters in a controlled environment facility and testing small quantities of growth-regulating chemicals. In the future, these methods may be helpful in analysing the effects of chemical application or the changes in GM sugarcane varieties.

R2. Call upon the expertise developed in the project to assist with similar analyses of sugarcane growth and development.

The project has identified other potential research that could assist with the control of flowering in sugarcane. For example, it is likely that allelic variants of some of the genes identified can control the timing of production of flowers. The current program of introgression of new *Saccharum spontaneum* genotypes has the potential to bring in new alleles that can shorten or lengthen the juvenile (non-receptive) period in hybrid varieties. The knowledge from the current project could add value to the introgression work.

R3. Investigate introgression lines for flowering time behavior to identify new genetic methods to synchronise or delay flowering.

The knowledge of genes involved in flowering could be leveraged to increase sugarcane yields through a GM strategy to reduce or delay flowering. There is anecdotal evidence that a similar strategy was pursued in Brazil but was stopped for political reasons. International biotechnology companies are actively seeking and using GM control strategies in sugarcane and other crops. By altering the expression of key flowering genes in GM varieties, continued vegetative growth of the stalks would allow increases in stalk weight, TCH and sugar yield in production systems. For breeding, incorporation of genetic control elements could permit flowering to be restored.

R4. Investigate the potential for a GM strategy to increase yields by delaying flowering

Section 9: Publications

Glassop, D, Rae, AL and Bonnett GD. (2013) Sugarcane flowering genes and pathways in relation to vegetative regression. Proceedings of the International Society of Sugar Cane Technologists Congress, CD.

Glassop, D, Rae, AL and Bonnett GD. (2013) Sugarcane flowering genes and pathways in relation to vegetative regression. Sugar Tech 16, 235–240. doi:10.1007/s12355-013-0284-z

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