

ScFT6: A Putative Candidate for Sugarcane Floral Inducer

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Abstract

One of the factors that can decrease sugarcane productivity is the flowering, because it affects the quantity and quality of feedstock, due to sucrose consumption from the stem during inflorescence emission. Photoperiodicity is the main environmental factor involved in sugarcane floral induction, which occurs by the integration of gene regulatory networks in response to environmental and endogenous stimuli. One of the genes involved in those regulatory networks is the *FLOWERING LOCUS T (FT)*, which is considered a phloem-mobile signal that stimulates floral induction in the shoot apical meristem. This work aimed to identify and characterize homologs of the *FT* gene in sugarcane, as well as to determine the putative function of these genes during floral induction. From this perspective, we have conducted *in silico* analyses of putative *FT* orthologs in sugarcane, as well as the expression levels in different photoperiodic conditions in a 24-hours-day-cycle of *ScFT6* in different plant tissues in contrasting cultivars in terms of flowering time. Three new possible *FT* orthologs were found with high similarity to *FT* homologs in other species. Among three genes identified, we highlighted *ScFT6*, which has a conserved domain and amino acids at characteristic positions for the flowering inducer phosphatidylethanolamine-binding protein gene family. Additionally, its expression occurs according to coincidental model, possibly being controlled by the circadian clock. Cultivars with distinct flowering time behavior display variable expression for the *ScFT6* gene, suggesting a possible genotypic relationship for its expression. Therefore, sugarcane has at least one putative orthologous gene in relation to *FT* that promotes floral induction.

Introduction

Sugarcane (*Saccharum* spp.) is one of the most relevant world crops due to its high production potential in both food and biofuels industrial sectors. Many countries have stood out as world sugarcane producers, with ethanol produced from it being the main source of renewable energy. The drive for this type of energy will grow stronger over time, as it is an efficient substitute for fossil fuels (1; 2; 3). However, a drawback of this commodity is the flowering process, which affects the quantity and quality of raw material produced, due to the consumption of sucrose present in the stem during inflorescence formation (4; 5).

Sugarcane flowering results in the pith formation into the stalks (the dehydration of the sugarcane stalk internal tissues), which leads to a decrease of internodes density and sucrose contents. Consequently, a high fiber relative percentage and reducing sugars become imminent. This process results in a low-quality broth in the power plant due to the high reducing sugar content and suspension fibers, which reduces the value of raw material (6). Then, there is a relationship between genotypes with higher inflorescence formation and higher pith process (4), generating considerable economic losses to the production chain.

Photoperiod is the main environmental factor affecting sugarcane floral induction. The ideal photoperiod varies from 12 to 12.5 hours when the day is shortening (Shortening-Day). This period must coincide with the time when the plants have already completed the vegetative period or juvenile phase (7). Natural

photoperiodic characteristics have been simulated in the development of research for artificial floral induction (8; 9). These researches have contributed to genetic improvement programs. However, the genetic network for flowering control of this species is still incipient, considering both different genotypic responses and its complex genome, principally by the perception of photoperiodic stimulus genes.

Usually, plants perceive photoperiodic stimulus by the leaves, which send signals that regulate the flowering transition process in the shoot apex. In *Arabidopsis thaliana*, a long-day plant, the perception between long and short days is made by the interaction between light signaling, circadian rhythms and expression of *CONSTANS (CO)* (10). The CO protein is a transcriptional regulator capable of binding to DNA sequences promoting flowering under long-day conditions in *A. thaliana*. Under these conditions, CO stimulates the expression of another transcription factor, the *FLOWERING LOCUS T (FT)*, a phloem-mobile signal that stimulates floral induction in shoot apical meristem (SAM) (11; 12).

FT is a member of the phosphatidylethanolamine binding protein (PEBP) gene family. Furthermore, *TERMINAL FLOWER1 (TFL1)*, another gene in this family, is also involved in flowering and 60% of its amino acid sequence is identical to *FT*. Whereas *FT* acts in floral induction, *TFL1* inhibits the action of *LEAFY (LFY)* and *APETALA1 (AP1)*, which are genes that participate in the floral induction, preserving SAM in its vegetative stage (13; 14; 15). As well as *FT*, *TFL1* function is also conserved among several species (15).

Long-day plants, as well as short-day plants, have their flowering process regulated by genes from the PEBP family. Rice (*Oryza sativa* L.), for instance, has a pair of *FT* orthologs known as *Heading-date3 (Hd3a)* and *RICE FLOWERING LOCUS T1 (RFT1)*, which are transcribed and translated in leaves. Their proteins are translocated through phloem to SAM, where they regulate its differentiation into floral meristem (16; 17). *Hd3a* is responsible for promoting flowering on short days, while *RFT1* is expressed under long-day conditions and, although not preventing flowering, causes its delay (18; 19; 20).

In sugarcane, two possible *FT* and *TFL* homologous genes, which belong to the PEBP protein family, have already been identified (21). *ScTFL1*, which belongs to the *TFL1*-like subfamily, causes flowering delay and increases vegetative phase length when overexpressed in *A. thaliana*. *ScFT1* has a possible involvement in meristematic activity controlling flowering time and formation of reproductive organs (21). The comparison of partial sequences of several genes similar to *ScFT* – from the EST sugarcane database (SUCEST) (22) – indicated that other candidates may be involved in floral induction (21). The identification and characterization of those genes by making use of heterologous expression analyses have provided important contributions to understanding flowering regulation. Nevertheless, elucidating the mechanisms that control flowering in sugarcane is still a missing information, despite its economic importance.

Although the discovery of genes linked to floral repression has provided a breakthrough in the face of one of the biggest productivity problems of sugarcane, until the present work, there was no evidence of genes promoting flowering, bringing new perspectives. Here we identify and characterize one novel sugarcane PEBP member and show that it is under photoperiod and circadian clock control, determining it like a

putative floral inducer gene in sugarcane. In the search to elucidate the sugarcane floral induction mechanism through a photoperiodic pathway, we show the possible functional relation among putative sequences related to *FT*, from bioinformatics, gene expression, and morphological characterization from two genotypes.

Material And Methods

In silico analysis

Sugarcane putative orthologs *FT* were identified by computational analyses in available RNA-seq libraries with nearly 48 million paired-end reads deposited at the NCBI SRA (National Center for Biotechnology Information - Sequence Read Archive) under accession numbers SRR1979656 through SRR1979669 and SRR1974519 (23). Adapter removal and quality control were performed using a Trimmomatic software version 0.36 (24), resulting in approximately 43 million paired-end reads (91%) with high quality. Transcript assembly was performed using the Trinity assembler version 2.3.2 (25). By the end of the process, 80647 possible transcripts with an N50 value of 1392 and an average length of 1065 bases were produced.

Identification of potential coding transcripts as well as their respective polypeptide chains was performed using the TransDecoder version 3.0.1 program (26). After protein prediction, *FT* sequences characterized in *Arabidopsis thaliana* and *Setaria viridis* were aligned against all predicted protein sequences using the BLAST tool version 2.4.0 (27). A new prediction step was performed from BLAST results transcripts using the ORFinder tool (<http://www.ncbi.nlm.nih.gov>) and GeneScan (28).

Multiple alignment was performed between the possible predicted sugarcane *FT* (29) and homologs of other related species using ClustalW (30). After the alignment, a phylogenetic tree was inferred using the MEGA 5.2.2 program (31) following the neighbor-joining comparison model (32) and using the Jones-Taylor-Thornton (JTT) distance matrix. The tree node reliability test was accessed via 2000 randomizations.

The protein sequences obtained were analyzed by the Conserved Domains Database (CDD) tool (33) to identify, characterize and compare them with protein domains present in *FT* sequences from species already characterized.

From the sequences obtained with *in silico* analyses, the amplification primers for the *ScFT6* were designed using the online tool OligoPerfect (Available at <http://tools.thermofisher.com/content.cfm?pageid=9716>). The designed primers quality was verified using Oligo Analyzer3.1 software (34) at the IDT (Integrated DNA Technologies) company website. Two primer pairs were designed: (Table 1) one for PCR amplification (Polymerase Chain Reaction) and the other for real-time quantitative PCR (RT-qPCR) gene expression analyses.

Plant material and growth conditions

The sugarcane seedlings from the AgMusa System™ were kindly provided by BASF. We used three cultivars: RB85-5156 and RB 85-5453 both with frequent flowering; and CTC 9003 with rare flowering. The seedlings were kept at greenhouse with shade netting of 50% spacing until transplanting. These seedlings were used for four experiments, including gene expression analysis in different tissues (1), in a 24-hour-cycle (2), in different photoperiods (3), and contrasting cultivars (4). Therefore, the experiments were conducted using pots in growth chambers for the artificial photoperiodic treatment impositions. Moreover, the greenhouse (with shade netting of 50% spacing) was used for the acclimatization, and the experimental area (outdoor) was used for the contrasting cultivars evaluation.

The seedlings were transplanted to pots (40 L for the experiment under contrasting photoperiods and 25 L for the experiment over 24-hours) with equal amounts of clay soil and sand (3:2), following fertilizations being performed in a 30-day-interval throughout the experimental periods, watered every two days to keep at the field capacity. Two shoots were kept in each pot and any other tillers were eliminated with pruning shears once a week during the experimental period. Fully expanded leaf +3 (35), immature leaf, stalk (from the internode surrounded by +3 leaf) and apical meristem were used for the gene expression analysis.

Qualitative gene expression analysis in different sugarcane tissues

Sugarcane seedlings (RB85-5156 cultivar) were kept in full sun (outdoor) and evaluated at six months of age. Samples from leaf (+3), immature leaves, stalk and apical meristem of sugarcane shoots were collected for the gene expression analysis by the semi-quantitative PCR.

Quantitative gene expression analysis over time (24-hour) in sugarcane leaves

Sugarcane seedlings (RB85-5156 cultivar), after transplanted to 25 L pots were grown by 78 days at greenhouse (with shade netting of 50% spacing). For the treatment imposition, they were transferred to the growth chambers running under short-day conditions (11 hours light, 13 hours dark) with a light flux of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ using metal vapor lamps (HQI 400W E40TUB, OSRAM), during 30 days. Plants were also kept watered every two days keeping the field capacity. After 30 days of treatment, fully expanded leaves (+3) were collected at 06, 12, 18 and 24 hours for gene expression analysis by RT-qPCR. The experiment was a completely randomized design (CRD) with four treatments (photoperiodic sample times), using three biological repetitions (three plants by the chamber) with two technical replicates.

Quantitative gene expression analysis in Shortening and Lengthening-Day conditions

The seedlings (RB 85-5156), after transplantation to 40 L pots, were grown by three months at greenhouse (with shade netting of 50% spacing), then they were transferred to growth chambers and kept in 6.2 m² controlled light (400 μmol m⁻² s⁻¹) with three metal vapor lamps (HQI 400W E40TUB[®], OSRAM) in neutral day conditions (12 hours light and 12 hours dark) for acclimatization. After twenty days of acclimatization, two treatments were imposed to the plants: one group of plants was subjected to a Shortening-Day condition (with one-minute light reduction a day) and the other to a Lengthening-Day (with an increase of one-minute light a day) according to Supplementary Figure 1. Plants were always kept watered every two days at field capacity. After 30 days of the two imposed treatments, the sampled leaves (+3) were collected and ground with liquid nitrogen for the gene expression analysis by RT-qPCR.

Growth analysis and quantitative gene expression in contrasting flowering sugarcane cultivars

For this experiment, seedlings were used from the cultivars RB 85-5453 and CTC 9003. The seedlings were transplanted to 40 L pots and kept outdoors. At 140, 160, 180, 200 and 220 days after seedling transplantation, we evaluated the stalk mean height (MSH), stalk mean diameter (MSD), +3leaf length, the width of the proximal, center and distal sections of the leaf (+3), number of fully expanded green leaves, number of young leaves, and number of dead leaves. From these values, we also estimated plant leaf area. At 140, 180 and 220 days after seedlings transplanting (March, May and June, respectively), the leaves (+3) were collected (8:00 a.m.) for further gene expression analysis.

Molecular analysis

After sampling, the tissues were ground with liquid nitrogen and stored in Deep Freezer. RNA extraction was performed following the Qiazol protocol (QIAzol[®] Lysis Reagent, Qiagen) adapted for sugarcane tissues. After RNA extraction the samples were treated with DNA-free kit (Ambion[®], Thermo Fisher Scientific) to eliminate residual DNA. The integrity of the samples was accessed by 1.2% agarose gel electrophoresis, stained with fluorescent nucleic acid dye (GelRed[®] Nucleic Acid Gel, Thermo Fisher Scientific), and visualized with a photo-imager (UVITEC Cambridge). Afterwards, the samples were quantified in a spectrophotometer (Biochrom NanoVue[®] Plus) at A260, A230 and A280 nm to determine the quantity and quality of the extracted material. Then, the samples were treated with DNA-free Ambion kit to eliminate any residual DNA from the samples. Samples with high integrity and purity were used for cDNA synthesis with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems[™]).

An assay was conducted to obtain the standard curve for the efficiency and the primer dilution. Expression of *ScFT6* was characterized in different tissues by semi-quantitative PCR making use of the

primers described in Table 1 and the cDNA obtained from each sampled tissue, being visualized through electrophoresis and by photo-imager.

The RT-qPCR was conducted in a Rotor-Gene Q Real-Time PCR (Qiagen) with the SYBR Green detection system. The cDNA volume used for each reaction was determined after quantifying primer efficiency, by using 10 μ L of reaction volume per sample with MasterMix SYBR Green[®] (Qiagen).

ScFT6 gene expression data were obtained from Rotor-Gene Series Pure Detection Software (Version 2.0.2), and relative expression was determined according to Pfaffl (36). Relative expression was performed by the comparative $\Delta\Delta$ CT (Cycle Threshold) method obtained from the reference genes GAPDH and eEF-1 α (37).

Statistical analysis of data

The experiments were conducted under split-plot system on time with two cultivars contrasting to the flowering. Data obtained from the biometric measurements were submitted to analysis of variance by the F test ($p \leq 0.05$) and, in case of the significance of the analysis of variance, the means were compared by the Tukey test ($p \leq 0.05$) or submitted to regression analysis ($p \leq 0.05$) when the interaction between cultivars and evaluation times occurred. Gene expression data was analyzed using the Rotor-Gene Series Pure Detection Software, and relative expression was calculated according to comparative $\Delta\Delta$ CT (36). The raw data of gene expression were modeled using an LMM (Linear Mixed Model) to estimate the level of significance of the interaction between treatments (38).

Results

Putative *FT* ortholog genes in sugarcane

The alignment of the already characterized *FT* sequences of *A. thaliana* and *Setaria viridis* against the predicted proteins assembled from the public RNA-seq libraries resulted in 51 possible homologous sequences. The sugarcane sequences with identity above 50% and query cover above 70% were selected. Previous studies have already identified a *TFL1* ortholog (*ScTFL1*) and five *FT*-like genes (*ScFT1*, *ScFT2*, *ScFT3*, *ScFT4* and *ScFT5*) in sugarcane (21). Here, three sequences compatible with these characteristics were identified, being named *ScFT6*, *ScTFL3* and *ScTFL4* with 174, 173 and 197 amino acids, respectively. Both mRNA and amino acid sequences were indexed with the following access codes: MN458470.1; MN458471.1; MN458472.1; respectively, at NCBI database. These three sequences were aligned with homologous sequences of *FT* gene in *A. thaliana* and different species related (Table 2).

Three possible homologs of *FT* and *TFL* genes identified in sugarcane showed high identity values in comparison with *FT* genes from other species, highlighting the *ScFT6* gene that showed the highest values. The protein sequence analysis identified PEBP conserved domain presence (Figure 1). The

presence of the PEBP domain indicates the involvement of these genes as regulators of signaling complexes in growth and development (39).

Multiple alignment analysis allowed to explore the relationships between the possible predicted sugarcane *FT*, putative *FT* previously identified (29) and homologs of other related species by ClustalW. We verified the structure and the putative functions of the obtained sequences (Figure 2). Regarding the possible *FT* genes identified in sugarcane, *ScFT6* has a tyrosine residue (Y) at position 85 that characterizes the flowering-inducing *FT* genes. The other two (*ScTFL3* and *ScTFL4*) have a histidine domain (H) that is characteristic of *TFL*-like genes considered to be floral repressors (12; 13). For sugarcane *FTs*, conserved motifs were observed between *ScFT6* relative to the previously identified *ScFT3* and *ScFT4* genes. In contrast, amino acid differences for *ScFT1* and *ScFT2* genes corroborate with possible antagonistic functions.

Phylogenetic analyses allowed the division of the putative *FTs* in sugarcane into four clades (Figure 3). The first clade represented by *AtFT* and *SvFT*, including *ScFT5* (21) and *ScFT6*. The second clade, composed by *Hd3a* homologs, grouped genes characterized in other monocotyledons with *ScFT3* and *ScFT4* previously investigated (21). The third clade is represented by *ZCN8*, including *ScFT1* and *ScFT2* (21). Finally, the fourth clade is divided into two subclades, one represented by *AtMFT*, with no sugarcane member, and the second subclade, where *ScTFL3* and *ScTFL4* were clustered, was represented by *AtTFL* and contained flowering repressor members. *ScFT6* shares a greater identity with *FT* from *A. thaliana*, an LD plant, and farther from *Hd3A* from *O. sativa*, an SD plant. Both *ScFT6* and *AtFT* share a monophyletic group, in contrast to the *TFL* sequences identified in sugarcane, as well as to the *TFL* orthologous of *Sorghum bicolor*, *Hordeum vulgare*, include *A. thaliana*.

PCR analysis of RNA isolated from different sugarcane tissue samples revealed that the *ScFT6* is transcribed in mature leaves and SAM (Figure 4), as well as its homologs in *A. thaliana*, where it can induce flowering by its expression in leaf phloem cells or SAM (40; 41). The same pattern is observed in rice, where *Hd3a* (*FT* homolog) is highly expressed in inductive photoperiodism in leaf tissues and SAM (42; 16). A more detailed analysis of the temporal pattern of expression showed that the gene is expressed according to the day-night cycle and photoperiod.

***ScFT6* gene expression over time (24-hour) in sugarcane leaves**

The expression of *ScFT6* in sugarcane, a putative *FT* homolog, follows a similar pattern of relative expression along the day (Figure 5). The results expressed in estimates of the fold-change showed that *ScFT6* is highly expressed throughout the day and has its expression significantly reduced during the night. The results showed that the expression increased from 12:00 p.m. to 6:00 p.m., decreasing until midnight. Differences between midnight and 6:00 a.m. were not detected (Supplementary Figure 2). With

this expression pattern, we can infer that the *ScFT6* gene has its expression controlled by circadian rhythms, as well as its flowering-inducing counterparts in other species (15).

***ScFT6* gene expression in Shortening-Day and Lengthening-Day conditions**

Relative expression of *ScFT6* varied as a function of day-length (Figure 6). The results of the estimates of fold-change showed a significant increase in expression of *ScFT6* under the shortening-day condition (Supplementary Figure 3). The data evidenced that *ScFT6* was about 31 times more expressed 30 days after the imposition of the photoperiod reduction. These results support the hypothesis of its function as a flowering inducer corresponding to inductive photoperiod sugarcane floral. Whereas there is strong functional evidence as to the level of identity between the *ScFT6* and their counterparts, similar responses are attributed to gene expression in photoperiodic inductive conditions (16; 43; 44).

Contrasting flowering sugarcane cultivars and *ScFT6* gene expression.

The flowering process in sugarcane occurs by induction (9; 29) and subsequent reallocation of sugar reserves present in the stalk to the floral organs (7). The enhanced vegetative development is characterized by the larger average stalk diameter and average stalk height that predominated in the cultivar RB 85-5453 compared with CTC 9003 (Figure 7). These factors are possible contributions that can lead to different frequencies flowering in these cultivars. Moreover, the cultivar RB 85-5453 displayed lower senescence and increased leaf area at the beginning of the cultivation period (Figure 8). This result can indicate discrepancies in favorable characteristics to support the transition of reproductive development. Taken together, the results suggest differences in sucrose enhanced production and storage, ensuring energy source for floral organ development.

The relative expression levels of *ScFT6* evidenced differences between RB 85-5453 and CTC 9003 cultivars (Figure 9). These results showed the effect of shorter natural photoperiods on different cultivars. In this case, the recorded photoperiod varied from 11:50 to 10:35 h of light, below the supposed critical photoperiod for sugarcane. Although the cultivar RB 85-5453 (the most responsive to the inductive photoperiod of flowering) showed higher levels of *ScFT6* expression, the fold-change estimates, based in LMM, did not indicate significant differences between cultivars along this time (Supplementary Figure 4). The expression levels of *ScFT6* accumulated throughout the day, having baseline levels during the early morning. More pronounced differences between the two contrasting cultivars may be more evident at peak times of expression, such as in the afternoon hours. Thus, the collection period in the early morning may have contributed to these results.

Discussion

The *ScFT6* gene is a putative sugarcane floral inducer

The floral transition is a critical step in the life cycle of sugarcane. The scarcity of information on floral genetic control makes it difficult to use molecular tools to minimize yield damage. Here, we first showed a homolog with high identity to the *FT* gene compatible with flowering promote function due to association with its amino acid positions. Two other homologs, compatible with the *TFL* gene, were identified with putative flowering repressor function in sugarcane, however, with lower identity values.

ScFT6 had the conserved PEBP domain, which is present in genes that act, in overall terms, as regulators of signaling complexes in growth and development (39), mainly as integrators in genetic regulatory networks, controlling the transition from vegetative to reproductive phase (11; 45; 46). These genes may represent novel regulators present in sugarcane, highlighting the *ScFT6* as a first floral promote found in such a crop. PEBP domain genes, involved in plant transition from vegetative to reproductive phase, can be classified as either floral repressors or inducers, depending on the specific amino acids present in their sequences (47). *ScFT6* had a tyrosine residue (Y) at position 85, which is characteristic of inductor *FT*, whereas *ScTFL3* and *ScTFL4* had a histidine domain (H) that is characteristic of *TFL*. These amino acid position differences imply *ScFT6* as a floral inducer and *ScTFL3* and *ScTFL4* as floral repressors.

In several plant species, histidine (H) is the most important amino acid to the PEBP family members that have a *TFL1-like* function, as well as tyrosine (Y) is key for *FT-like* function members (13). Despite the importance of residues in position 85 for the promotion of a full phenotypic expression, the protein has also to be complete. Loss of residues in other regions has shown alterations in both *TFL* and *FT* functions as well (12; 48). In some species, *FT-like* genes can repress or induce flowering. In addition to position 85 in exon 2, positions 134 and 139 in exon 4 are also important to determine either the induced or repressed phenotype expressed by those genes (49; 50).

In species that have flowering repressor *FT* homologs, most *FTs* inducers have a tyrosine (Y) at position 134 (based on alignment with *A. thaliana* *FT* protein) and another non-tyrosine residue, is found in repressive *FTs*. In sugarcane, *ScFT1* is proven to be a flowering repressor (21), and has a phenylalanine residue (F) at position 134. In addition to *ScFT1*, the *ScFT2*, *ScFT7* and *ScFT8* genes have different residues of tyrosine (Y) at position 134. Already *ScFT3*, *ScFT4*, *ScFT5* *ScFT6* have a tyrosine residue (Y) at position 134, characteristic of flowering-inducing *FT* homologues.

Regarding position 139 in the amino acid sequences of *FT* homologs, the presence of a conserved tryptophan residue (W) in this specific position most likely, can allow flowering induction. On another standpoint, *FTs* with a different amino acid in that position can inhibit flowering (49; 50). The presence of tryptophan (W) in sugarcane *FT* homologs in position 139 was observed only in *ScFT3*, *ScFT4*, *ScFT5* and *ScFT6*, thus those protein-coding genes are probably *FTs* that induce flowering.

The phylogenetic results suggest that *ScFT6* possibly acts as a flowering inducer in sugarcane and can be classified as *FT-like* due to the presence of tyrosine residue (Y) at positions 85 and 134 and tryptophan (W) at position 138 and by its clustering pattern in the phylogenetic tree. *ScFT6* appears to have diverged

from the other *FTs* in *Saccharum* spp., especially in relation to *ScFT1*, *ScFT2*, *ScFT3* and *ScFT4*, with antagonistic possible functions. In the monocots group, *FT* genes may be a more diverse group than *TFL* genes, suggest that they could have acquired functional roles in delaying flowering (51; 52), for example, *ScFT1* (21). While the *ScTFL3* and *ScTFL4* genes were suggested as possible flowering repressors and classified as *TFL*-like due to the presence of a histidine residue (H) at position 85 and their respective clustering pattern in the phylogenetic tree.

The circadian clock controls *ScFT6* gene expression

The circadian clock acts indirectly on *FT* expression (53). According to the coincidence model, there is a straight correlation between light and CONSTANS stability in a way that continuous sunlight exposition leads to its accumulation. CO is a transcription factor that, when presented in high number, promotes flowering by inducing *FT* expression in long-day plants subjected to this specific condition (54) leading ultimately to flowering induction (55; 56). This same regulatory network also inhibits flowering of short-day plants by repressing *FT* homologs when a high number of CO are present (42; 57).

By the coincidence model, it is possible to determine that the *FT* gene had its expression controlled by the circadian clock and, thus, its expression peak overlaps with the sunlight dynamics, both in long and short-day plants (55; 58). Determination of flowering time in short and long-day plants is dependent on light signaling, where the signal must coincide with sensitive phases established by endogenous circadian clock components (59; 60). *FT* and its homologs, including the possible flowering-inducing *ScFT6* gene, are controlled by such signals, and their expression is indirectly regulated by light-controlled transcription factors as well as the duration of light exposure (17; 59).

Sugarcane genes involved in the circadian clock can be compared with genes already characterized in other species and by transgenic lines. Some of these genes showed greater similarities about the patterns seen in short-day plants, grown under non-inductive photoperiods (61). Considering a 24-hour cycle, these genes have expression patterns that control the change from vegetative to reproductive growth and can be headed by *ScFT6* signaling.

ScFT6 expression is modified by photoperiod

We showed that the ability of sugarcane mature leaf to respond to shortening-day is essential for *ScFT6* expression. The effect of reducing the photoperiod on *ScFT6* transcription is suggested by the light-dependent regulation promoted, together with the regulation of the circadian clock, on the stability of a probable CO homologous protein (53; 62; 63). However, this regulation establishes the response of *ScFT6* during the shortening of the day duration, while CO is under control over long days.

The inductive photoperiod for sugarcane may have a higher connection with a regulation upstream of a possible Hd1 homolog, which is responsive to short-day conditions, as detected in *O. sativa*. In these

photoperiodic conditions, the expression of the *Hd3a* florigen is regulated positively by Hd1, suggesting that *Hd3a* is under the control of Hd1 (18). Thus, the mechanism linked to the actuation of the putative *ScFT6* florigen, under shortening-day, can be more similar to the genetic control dependent on light and the circadian clock of short-day plants.

The phenotypic result of flowering in sugarcane, for the shortening of the photoperiod, can be verified when using artificial photoperiodic growth chambers under controlled conditions (8). Under conditions of natural photoperiod (disregarding temperature and water and nutritional regime variations), flowering induction is determined shortly after the start of the day's shortening period, with complete flowering (anthesis) varying overtime throughout the year (64). This process can be certified by the results obtained with the interruption of the response, using the control management (inhibitors) of flowering in the field, which is recommended especially during this period. The synchronicity between the floral phenotype displayed in artificial and natural conditions supports the classification of sugarcane as responsive in shortening-day condition. During the shortening of the photoperiod, the increase in *ScFT6* expression indicates the possible role of this gene as florigen under shortening-day control.

Several studies have shown significant progress on the molecular mechanisms involved in the control of plant photoperiod and the circadian clock. In sugarcane, the knowledge of these mechanisms is still incipient. In parallel research, the identification and characterization of genes upstream of *ScFT* should be the next targets of this study. In that case, we expect to establish greater precision in the molecular connections of the sugarcane flowering process.

Sugarcane varieties display alternate behaviors regarding *ScFT6* expression

Different sugarcane cultivars exhibited distinct behavior regarding flowering induction and are affected in different manners by photoperiod. There are differences regarding the number of days required for flowering stimulus within the inductive photoperiod, and the required stimulus amount for the induction varies among cultivars (65). There are cultivars in which flowering induction happens, but there is no complete development of the floral organs due to suboptimal photoperiodic regimes (66) and other stressful environmental conditions. In those cases, the feedstock production is compromised leading to severe economic losses.

Although flowering is not desirable at a sugarcane field, because flowering potentially limits productivity, it is necessary for reproduction and the breeding of cultivars. In contrast, some desired crosses remain challenging due to unresponsive plant varieties. Therefore, it is likely that each cultivar's sensitivity to the photoperiod is a genetic characteristic involving *ScFT6* expression, allowing this gene to be a research tool, as a marker, for selection and also to use cultivars with better commercial characteristics.

The *ScFT6* expression levels, when evaluated throughout 24-hour-cycle, demonstrated baseline levels during the early morning. The level of transcripts detected in this period is low when comparing both

cultivars in this period. The cultivar with frequent flowering had higher transcriptional level, although not considered significant by LMM for fold-change refined comparisons. The plants were evaluated after the supposed critical photoperiod, the transcript levels were not sufficient for significant expression differences. In our results, the plants were analyzed during the extreme shortening of the day, after the critical photoperiod until mid-June. Under conditions of natural photoperiod, the plants would probably respond to the critical photoperiod from 12:00 pm to 12:30 p.m. We believe that in contrasting cultivars, the flowering induction associated to the photoperiod may be determined under a specific time and the beginning of the shortening period of the day.

When considering the control of the *ScFT6* expression pattern, studies with genetic manipulation in sugarcane may help to obtain non-responsive cultivars to floral induction by the photoperiod. It will be necessary to check for polymorphisms of *ScFT6*, in a way that is silenced its function in flowering without harm in other functions that are not linked directly to floral induction. In this sense, it will be possible to reduce or even eliminate the detrimental effect of flowering on sugarcane productivity.

The expression patterns of *ScFT6* should be assessed under different developmental stages, including in different tissues. These factors are fundamental to a better comprehension of its physiological role. Therefore, *ScFT6* gene recognition, within the flowering pathway, consists of a perspective to the resolution of one of the biggest problems that impact the sugarcane culture yield during its maturation process. Discrepancies between cultivars may be linked to complete induction for the floral transition upon reaching an *ScFT6* critical concentration possible limited by physiological characteristics.

Conclusions

Our results show that sugarcane has at least one possible *FT*-homologous gene that can induce flowering in sugarcane. This homolog is characterized as *ScFT6*, the putative florigen gene in sugarcane. The *ScFT6* gene has a conserved domain at characteristic positions of flowering inducing PEBP family genes. The flowering induction, by *ScFT6*, can be perceived under the control perspective of the photoperiod and circadian-clock, in a shortening-day condition. *ScFT6* expression occurs in sugarcane tissues in a way similar to its homologs in other species and is controlled by circadian rhythms according to the coincidence model. Analysis of the gene expression pattern supports the essential role of the mature leaves in the perception of signals that induce flowering. Moreover, corroborating the results obtained, sugarcane varieties with different flowering phenotypic can be promised to studies searching different levels *ScFT6* expression. Responsive and non-responsive cultivars to flowering can be subjected to the regulatory network control from the photoperiod by *ScFT6*

Declarations

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Tables

Table 1 Primers designed from *in silico* identification of the possible sugarcane *ScFT6* gene.

Identification	Sequence 5'→3'	Estimated Amplicon (pb)	Efficiency
<i>ScFT6</i> RT-qPCR Fw	GTGTGGAGGCAGACGATA	112	1,02
<i>ScFT6</i> RT-qPCR Rv	GCTTTGCGAAGTGGCTGG	112	1,02
<i>ScFT6</i> Fw1	CGGCTTCTTTGTTCTCTA	247	-
<i>ScFT6</i> Rv1	CTTGGTTCATTTGCTACT	247	-

Table 2 Summary of the three possible homologs of the sugarcane *FT* and *TFL* genes compared to *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa*, *Sorghum bicolor* and *Setaria italica* *FT* genes.

Name	Species	Reference	Identity (%)		
			<i>ScFT6</i>	<i>ScTFL3</i>	<i>ScTFL4</i>
<i>AtFT</i>	<i>Arabidopsis thaliana</i>	(Kardailsky et al., 1999, Kobayashi et al., 1999)	72	56	56
<i>ZCN8</i>	<i>Zea mays</i>	(Muszynski et al., 2006)	54	49	52
<i>ZCN12</i>	<i>Zea mays</i>	(Danilevskaya et al., 2008)	54	53	57
<i>Hd3a</i>	<i>Oryza sativa</i>	(Kojima et al., 2002)	68	59	62
<i>SbFT1</i>	<i>Sorghum bicolor</i>	(Wolabu et al., 2016)	68	57	62
<i>SbFT8</i>	<i>Sorghum bicolor</i>	(Wolabu et al., 2016)	55	51	55
<i>SiFT</i>	<i>Setaria italica</i>	(Bennetzen et al., 2012)	87	52	53

Figures

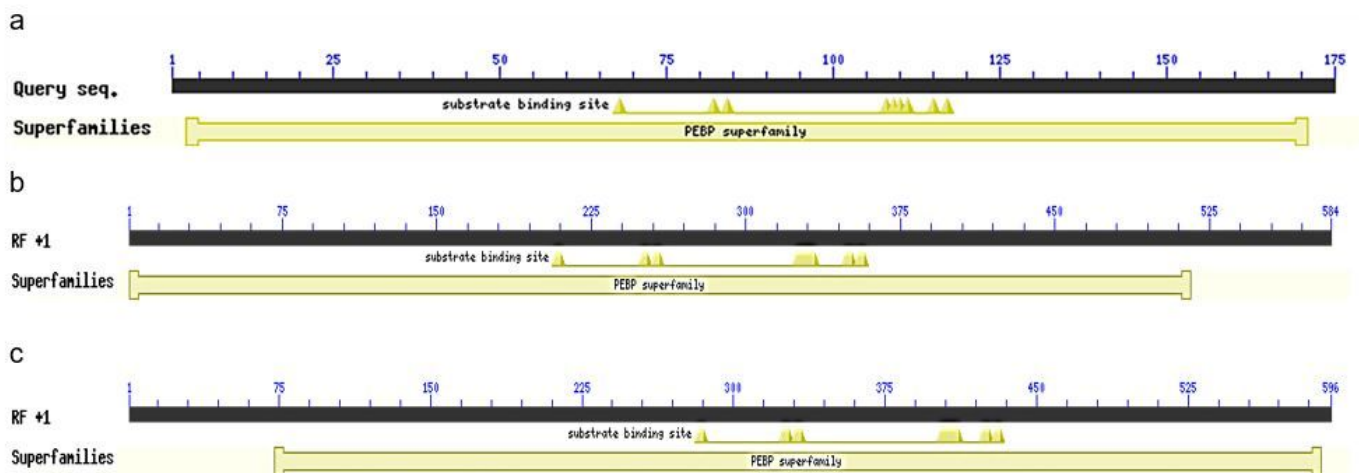


Figure 1

Conserved PEBP domain present in sugarcane *FT* and *TFL* putative orthologs: *ScFT6* (a), *ScTFL3* (b) and *ScTFL4* (c). Query seq. indicates the length of the query sequence by the number of amino acids. Small triangles indicate the amino acid involved in conserved features/sites, such as catalytic and binding sites. Data generated from .

	85		134				139	
ScFT1	H	...	F	-	A	P	Q	V
ScFT2	Y	...	D	Q	P	P	L	L
ScFT3	Y	...	Y	-	A	P	G	W
ScFT4	Y	...	Y	-	A	P	G	W
ScFT5	N	...	Y	-	A	P	G	W
ScFT6	Y	...	Y	-	A	P	G	W
ScTFL4	H	...	T	V	P	S	S	-
ScTFL3	H	...	N	P	P	S	S	-
SvFT	Y	...	Y	-	A	P	G	W
SbFT1	Y	...	Y	-	A	P	G	W
ZCN_15	Y	...	Y	-	A	P	G	W
VRN3	Y	...	Y	-	A	P	G	W
HvFT1	Y	...	Y	-	A	P	G	W
Hd3a	Y	...	Y	-	A	P	G	W
HvFT2	Y	...	Y	-	A	P	G	W
ZCN14	Y	...	Y	-	A	P	G	W
AtFT	Y	...	Y	-	A	P	G	W
AtTSF	Y	...	Y	-	A	P	G	W
SbFT6	Y	...	Y	-	A	P	G	W
SbFT11	Y	...	Y	-	A	P	G	W
SbFT5	N	...	Y	-	A	P	G	W
SbFT3	Y	...	Y	-	A	P	G	W
ZCN16	Y	...	Y	-	A	P	G	W
SbFT7	Y	...	Y	-	A	P	S	W
SbFT12	Y	...	F	A	A	P	S	R
SbFT9	Y	...	F	-	A	P	Q	V
SbFT8	Y	...	F	-	A	P	D	M
ZCN12	Y	...	F	-	A	P	D	M
ZCN8	Y	...	F	-	A	P	E	M
SbMFT1	Y	...	V	H	A	E	A	P
ZCN10	Y	...	V	H	A	E	G	P
TaMFT	Y	...	V	P	A	A	S	P
HvMFT	Y	...	V	D	A	A	S	P
AtMFT	W	...	M	V	Q	Q	P	P
ZCN11	L	...	P	P	S	L	A	P
AtTFL1	H	...	F	P	N	I	P	S
SbTFL1-2	H	...	T	V	P	S	S	-
SbTFL1-3	H	...	A	M	P	S	S	-
HvTFL1	H	...	T	V	P	S	F	-
SbTFL1-1	H	...	N	P	P	S	S	-
ZCN2	H	...	N	P	P	S	S	-
SbTFL1-4	H	...	R	P	P	S	S	-
ZCN4	H	...	R	P	P	S	S	G

Figure 2

Multiple alignment of sugarcane and other related protein sequences in a PEBP domain portion, depicting amino acid variations at position 85 of Exon 2 and positions 134 and 139 in the external loop of Exon 4.

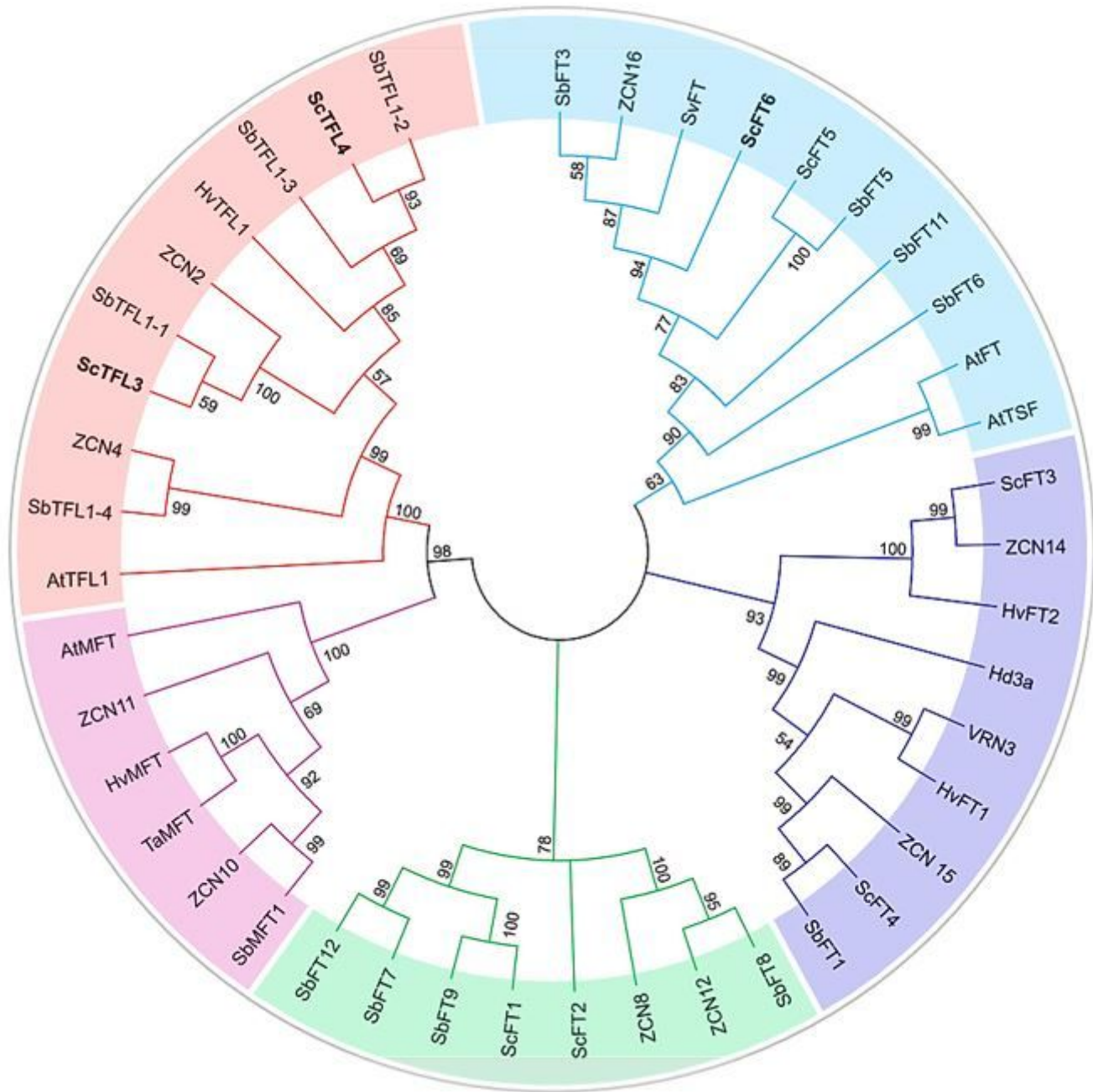


Figure 3

Phylogenetic analysis of possible FT homologs ScFT6, ScTFL3 and ScTFL4 based on the complete amino acid sequence of the proteins encoded by them. Analysis performed following the neighbor-joining comparison model (Saitou and Nei 1987) and using Jones-Taylor-Thornton (JTT) sequence distance matrix with 2000 randomizations.

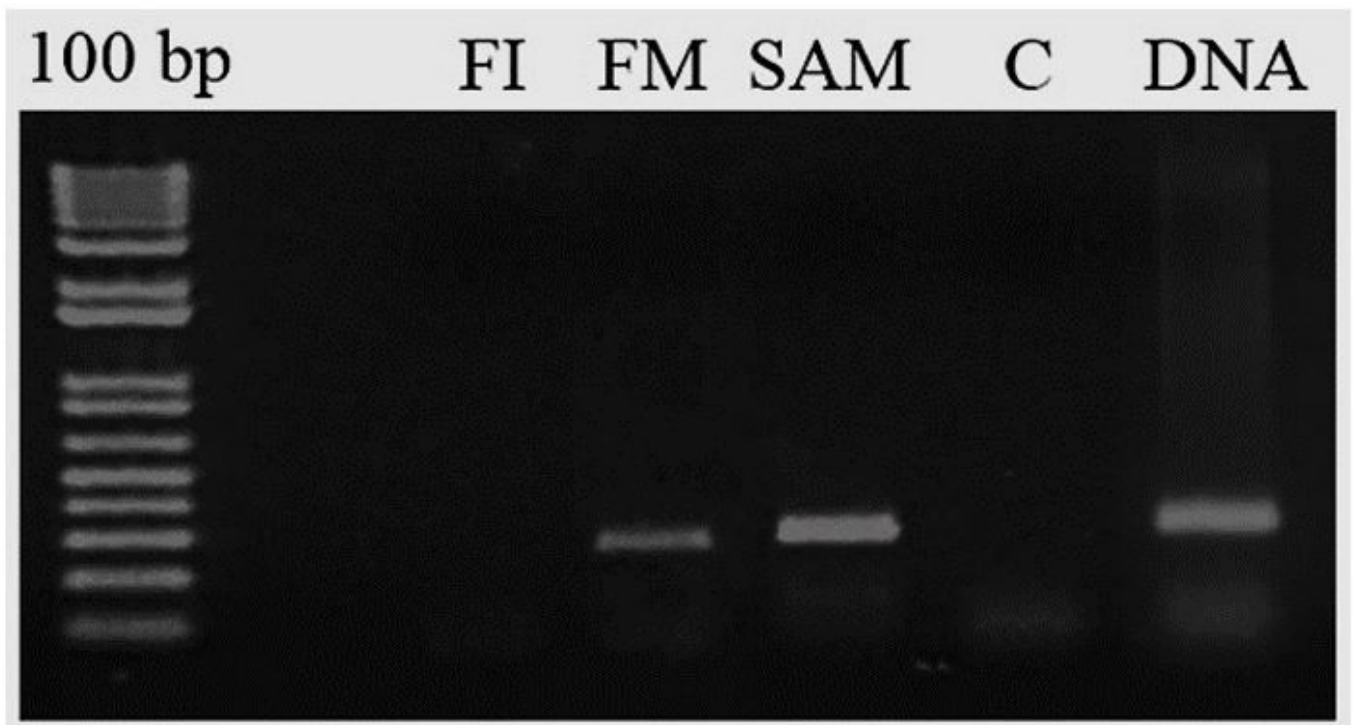


Figure 4

ScFT6 gene expression pattern in different sugarcane tissues by semi-quantitative PCR. FI: Immature Leaf; FM: Mature leaf; SAM: Shoot Apical Meristem; C: Stalk; DNA: DNA Pool - Positive Control.

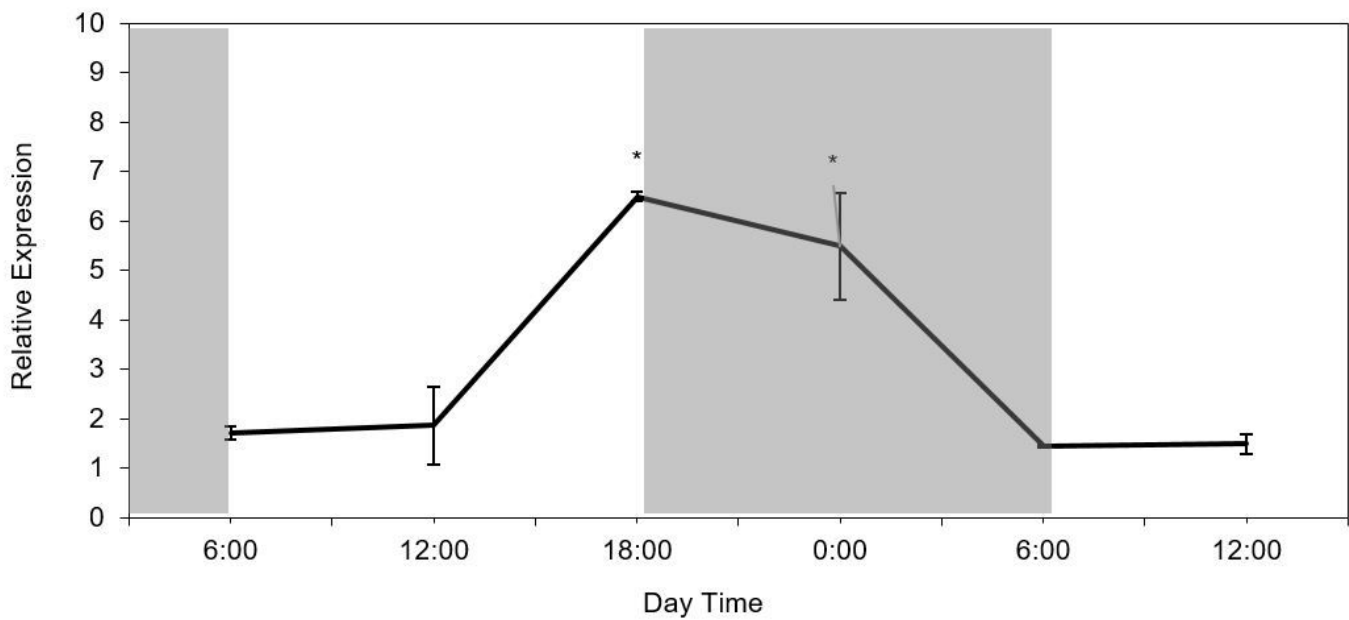


Figure 5

Gene expression profile of ScFT6 in sugarcane leaves by RT-qPCR. Data was generated over 24-hours. The expression values were obtained from three biological repetitions, and expression difference over the period of the experiment was calculated in relation to a normalizing sample (12:00 hours). Interval bars indicate the standard error. Expression values were normalized by the reference genes GAPDH and eEF1 α . * indicates a significant difference from the Linear Mixed Model analyses; significance level was determined based on the confidence intervals of the estimated fold-change values ($p \leq 0,05$).

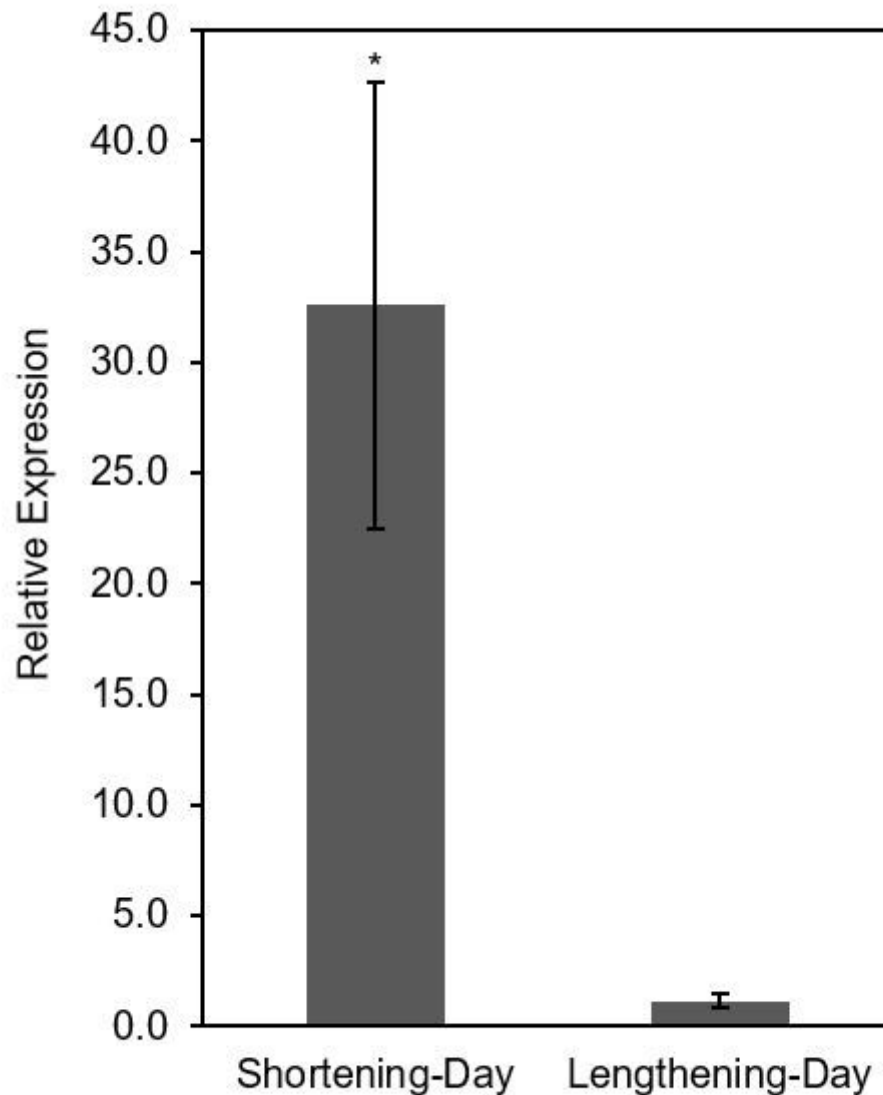


Figure 6

Gene expression profile of ScFT6 in sugarcane leaves by RT-qPCR. The bars represent the difference in expression between two photoperiodic conditions relative to a normalizing sample (day length). The expression values in each treatment were obtained from four biological repetitions and the error bars represent their standard error. Expression values were normalized by the reference genes GAPDH and

eEF1a. * indicates a significant difference from the Linear Mixed Model analyses; significance level was determined based on the confidence intervals of the estimated fold-change values ($p \leq 0,05$).

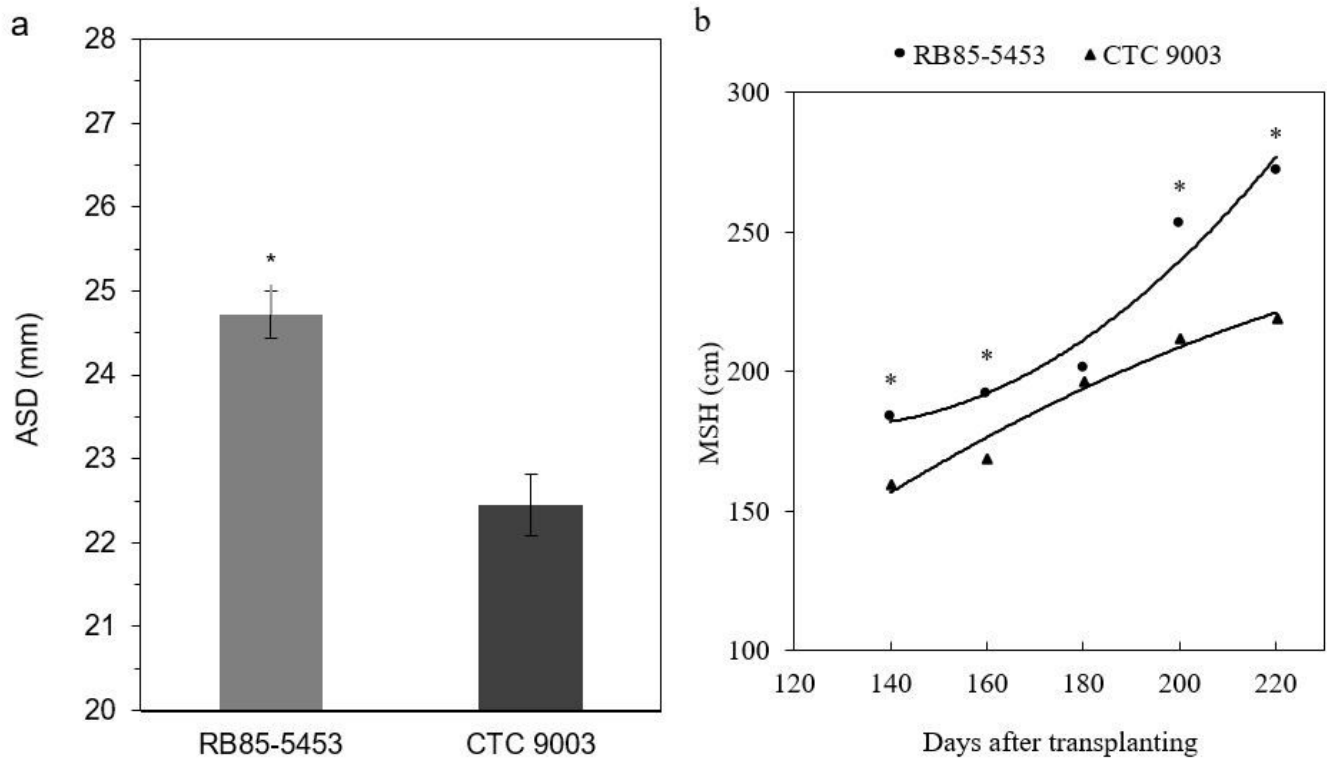


Figure 7

Average stalk diameter (a) and mean stalk height (b) of cultivars RB 85-5453 and CTC 9003. The symbol (*) indicates statistical difference between means by F test at $p \leq 0.05$. The vertical bars indicate the standard errors of the mean.

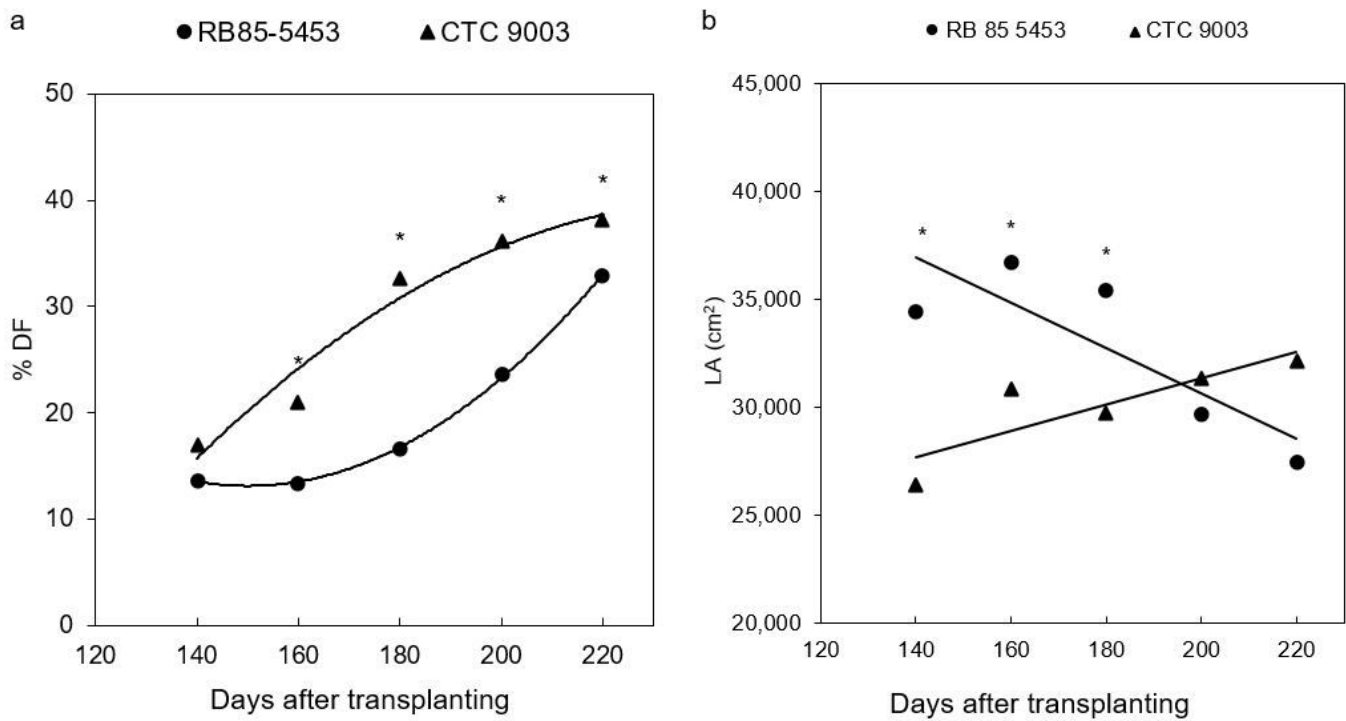


Figure 8

Percentage of dead leaves (% DF, left) and leaf area (LA, right) of cultivars RB 85-5453 and CTC 9003. The symbol (*) indicates statistical difference between means by F test at $p \leq 0.05$.

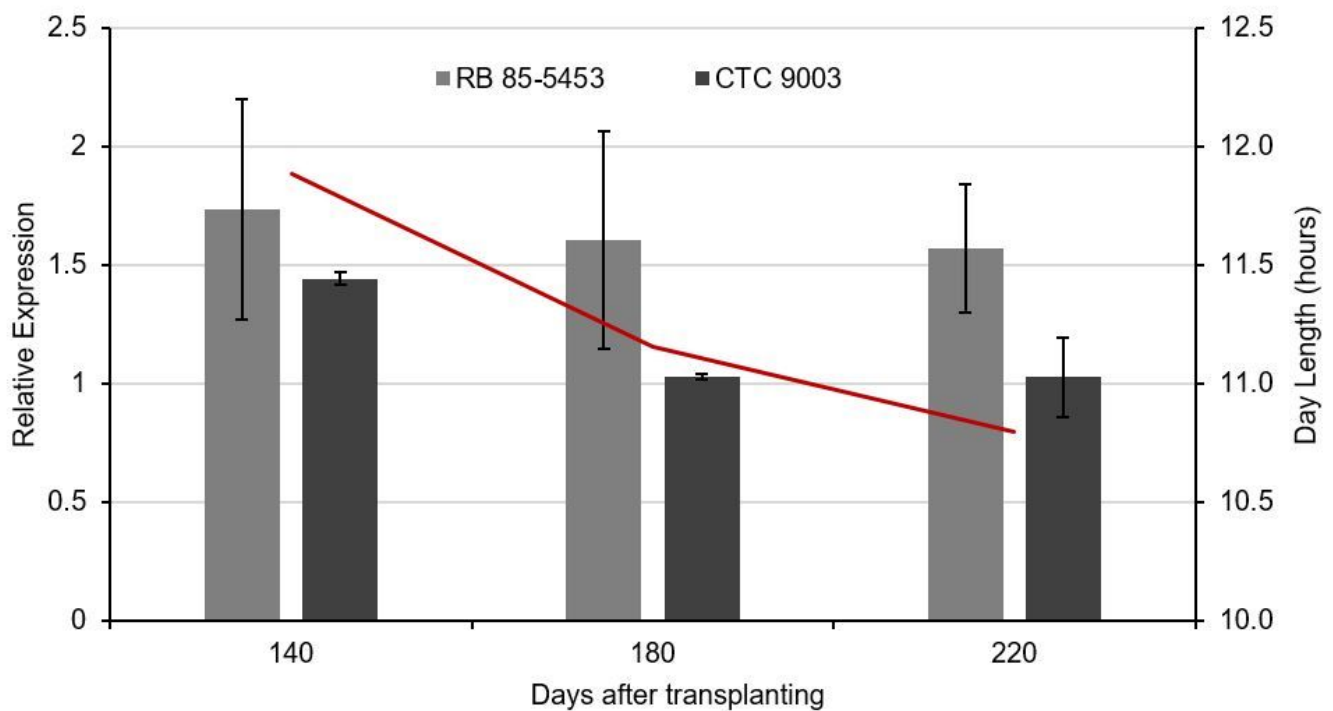


Figure 9

Relative expression profile of the ScFT6 gene in +3 leaves of two sugarcane varieties obtained by RT-qPCR. Color bars represent the difference in expression between varieties compared to a normalizing sample along the experimental period (CTC 9003 – 180 days). Each genotype expression was obtained from three biological repetitions and the bars show their standard errors. GAPDH and eEF1 α genes were used as reference genes. The line to second Y-axis indicates day length over the experimental period.

Supplementary Files

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- [SupplementaryFigure1.docx](#)
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- [SupplementaryFigure3.docx](#)
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