CARLA PRISCILA COELHO

MOLECULAR REGULATORY MECHANISM OF FLORAL TRANSITION BY FT/TFL1 ORTHOLOGS AND THE AUTONOMOUSLY EXPRESSED ScID1 MONOCOT-SPECIFIC TRANSCRIPTION FACTOR IN SUGARCANE

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, área de Concentração em Biologia Molecular e Celular, para a obtenção do título de Doutor.

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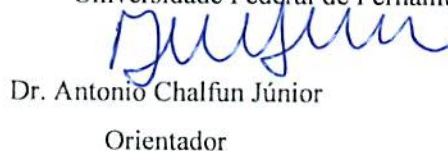

Dr. Antonio Chalfun Júnior

Orientador

LAVRAS – MG

2013

A meus pais, pelo apoio e dedicação incessante. Por serem exemplos e inspiração diante de todos os obstáculos da vida

DEDICO

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"Change is not something that we should fear. Rather, it is something that we should welcome. For without change, nothing in this world would ever grow or blossom, and no one in this world would ever move forward to become the person they're meant to be."

Author Unknown

ABSTRACT

PEBP members, *FT* and *TFL1*, play an essential role as mobile signals controlling the change from vegetative to reproductive growth. Recent discoveries on PEBP family members in plants have suggested a more complex function beyond flowering induction in diverse plant species. *ID1* is a monocot-specific zinc finger transcription factor that is predominantly expressed in immature leaves of maize, regardless of the day-length. Comparative studies among available genomes such as Arabidopsis, rice and maize are an important resource for the identification and characterization of orthologs in sugarcane, a complex polyploid plant that has not been completely sequenced yet. Flowering in sugarcane is an important factor that negatively affects cane yield and loss of sugar/ethanol production from this important perennial bioenergy species. Aiming at understanding the genetic control of the floral induction in sugarcane, a search for putative flowering time genes at the SUCEST database was performed and putative *FT*, *TFL1* and *ID1* orthologs (*ScFT1* and *ScFT2*, *ScTFL1* and *ScID1*, respectively) were isolated. Quantitative and semi-quantitative expression analyses demonstrate that these genes share the same expression patterns as their putative orthologs. The closely related C4 model plant *Setaria viridis* was transformed with the flowering time gene *SiID1* to test for function. Identification of several PEBP members in sugarcane EST database and characterization of three *FT/TFL1* orthologs show its involvement not only in flowering time but also affecting plant reproductive architecture in Arabidopsis. According to the expression analysis, *ScID1* may be involved in flowering time independently of photoperiod in sugarcane, because the expression pattern was constant in different ZT times in both short- and long-day conditions. *Setaria ID1* (*SiID1*) homolog was introduced to *Setaria* calli to check for functions. For the first time, flowering time candidates have been characterized in sugarcane. Altogether, the results suggest that the candidates may be valuable target for breeding programs, aiming at generating of low florigenic varieties and higher biomass production

Keywords: Floral induction. Florigen. Monocots. *Saccharum spp.* Bioenergy.

RESUMO

Os membros da família PEBP, *FT* e *TFL1*, atuam como sinais móveis no controle da mudança do crescimento vegetativo para o reprodutivo. Descobertas recentes envolvendo os membros da família PEBP em plantas sugerem uma função mais complexa além da indução do florescimento em diversas espécies vegetais. *ID1* é um fator de transcrição do tipo "zinc-finger" específico de monocotiledôneas, que é predomimantemente expresso em folhas imaturas de milho, independente do fotoperíodo. Estudos comparativos entre genomas disponíveis como o de Arabidopsis, milho e arroz, são importantes fontes para a identificação e caracterização de ortólogos em cana-de-açúcar, uma planta poliploide complexa, cujo genoma não foi completamente sequenciado ainda. O florescimento em cana-de-açúcar é um fator importante que afeta negativamente a produção de açúcar e etanol nessa importante espécie bioenergética. Buscando-se entender o controle genético da indução floral de cana-de-açúcar, uma busca por genes de indução floral no banco de dados do SUCEST foi realizado e possíveis ortólogos *FT*, *TFL1* e *ID1* (*ScFT1* e *ScFT2*, *ScTFL1* e *ScID1*, respectivamente) foram isolados. Análises de expressão quantitativa e semiquantitativa demonstraram que esses genes compartilham do mesmo padrão de expressão que seus ortólogos. A planta modelo do tipo C4, *Setaria viridis*, foi transformada com o gene de indução floral *SiID1* para testar sua função. Identificação de diversos membros da família PEBP no banco de dados de cana-de-açúcar e a caracterização de três ortólogos *FT/TFL1* indicam seu envolvimento, não somente na transição floral, mas também na arquitetura reprodutiva de Arabidopsis. De acordo com análise de expressão, *ScID1* pode estar envolvido na indução floral de cana-de-açúcar independentemente do fotoperíodo, uma vez que seu padrão de expressão foi constante em diferentes tempos ZT em condições de dias curtos e dias longos. O homólogo *Setaria ID1* (*SiID1*) foi introduzido em calos de *Setaria* calli para verificar suas possíveis funções. Pela primeira vez, genes candidatos de indução floral foram caracterizados em cana-de-açúcar. Conjuntamente, os resultados sugerem que os candidados podem ser alvos importantes para programas de melhoramento, objetivando-se a geração de variedades menos florigênicas e maior produção de biomassa.

Palavras-chave: Transição floral. Florígeno. Mocotiledôneas. *Saccharum spp.* Bioenergia.

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FIRST PART

1 INTRODUCTION

1. 1 To Flower or not to flower: that is the question

Floral induction is an important event in which higher plants undergo several changes from vegetative growth to produce reproductive structures and to ensure the establishment offspring. The timing of flowering is a crucial aspect of development as it guarantees that seeds will survive under unfavorable external and/or internal conditions. Flowering plants respond differently to specific environments to which they were introduced, including day length, temperature, humidity and light intensity. Being at the right place and at the right time of development ensures the production of seeds through floral transition, in which different pathways interconnect floral meristem identity genes, flowering time genes and inflorescence meristem identity genes to produce flowers at an appropriate time. Genes involved in flowering time and organ identity are associated with several developmental processes, in addition to floral transition. This phenomenon indicates the existence of redundant gene functions in floral transition and other regulatory processes. However, it is necessary to better understand how the specification of organ types is linked to several developmental processes.

2 LITERATURE REVIEW

2.1 Floral transition regulatory mechanisms

Plants undergo different development phases. The switch from vegetative to reproductive development depends on endogenous and environmental signaling cascades which is marked by differentiation of the shoot apical meristem (SAM), where cells undergo changes in identity and function (BERNIER; PERILLEUX, 2005; FLETCHER, 2002). The SAM is a dome shaped structure, site for the organogenesis, and it is formed by different cell types. Several changes occur in SAM cells, such as the increase in the number of plasmodesmata, which enhance the distribution of molecules to the SAM which will change its identity. Cell division is observed hours after the floral evocation, indicating that the SAM is preparing the coordination of changes to mediate floral transition (BERNIER; PERILLEUX, 2005; COLASANTI; SUNDARESAN, 2000). Altered patterns of cell division are accompanied by changes in cell morphology in the SAM under floral transition, leading to the establishment of the inflorescence zonation and floral structures initiation. In terms of physiological changes, there is an increase in cell respiration, RNA levels and protein synthesis, which is related to a reprogramming of SAM transcriptome. Therefore, the SAM is a key structure of developmental processes such as floral transition. The main functions of the SAM is to maintain a group of undifferentiated cells and to initiate the formation of lateral organs such as leaves and flowers (COLASANTI; SUNDARESAN, 2000). Mechanisms underlying floral induction have been extensively studied in Arabidopsis, which allowed a large amount of information to be accumulated. The flowering process is triggered by a number of environmental and endogenous signals which converge to a specific signal that will lead to reproductive development and floral morphogenesis. Six signaling pathways are well described in Arabidopsis: photoperiodic, autonomous, vernalization, ambient temperature, gibberellin and

age-dependent control (FORNARA; MONTAIGU; COUPLAND, 2010). Many plants depend on day length as the main environmental cue to signal for floral transition in changing seasons. This pathway is highly conserved among plants and it has been highlighted that the function of the *FLOWERING LOCUS T (FT)*, is a key component that transmit the signals from the leaves to the SAM, through companion cells of the phloem (GIAKOUNTIS; COUPLAND, 2008). FT and TWIN SISTER OF FT (TSF) are expressed under long-day conditions in Arabidopsis to promote flowering. FT is transcriptionally activated by the zinc-finger CONSTANS (CO) protein. CO is encoded by the *CO* gene precisely under long-day conditions, and its post-transcriptional regulation occurs toward the end of the light period, meaning that FT activation follows the external coincidence model to trigger floral transition (SUAREZ-LOPEZ et al., 2001; VALVERDE et al., 2004). Under short-day conditions, the rhythm *CO* expression is altered by the repressor DAY NEUTRAL FLOWERING (DNF) and is not sufficient to induce *FT* expression (MORRIS et al., 2010). It has been shown that the release of FT protein from companion cells is sufficient for floral induction in Arabidopsis and that *FT* mRNA is not required in cells in which the protein is active, indicating, therefore, that FT mRNA does not act as the transmissible signal (CORBESIER et al., 2007; JAEGER; WIGGE, 2007; MATHIEU et al., 2007). However, new evidences have reported that *FT* mRNA also moves through the phloem and contributes to the florigen signaling in floral transition (LI et al., 2011; LU et al., 2012). These cascades are responsible to the down-regulation of floral repressors and up-regulation of floral integrators.

Floral activation and repression share a common mechanism, in which FT forms a protein complex with FLOWERING LOCUS D (FD) and induces *APETALA1* (*AP1*) and *LEAFY* (*LFY*) expression under long-day inductive conditions. By

contrast, floral repression is triggered by the expression of *TERMINAL FLOWER 1* (*TFL1*), which acts in a negative feedback loop to repress *AP1*, of which mechanism is dependent upon a protein complex formed between TFL1, 14-3-3 and FD (AHN et al., 2006; JAEGER et al., 2013). *TFL1* acts as a transcriptional repressor and its function is to counterbalance the floral activation of *FT* (HANANO; GOTO, 2011; JAEGER et al., 2013).

Although many genes have been identified as flower-specific genes, few are known to specify vegetative development. It is also important to identify floral repressor genes, since an alteration in the activity of this type of flowering time gene may lead to a new shoot architecture in which flower organ development is ceased and leaves are developed (SUNG et al., 2003).

2.2 Floral organ determination - the conserved ABCE model

The MADS domain are a class of DNA-binding transcription factors encoded by the MADS-box genes, expressed in the four whorls where they specify organ identity (PELAZ et al., 2000; SMACZNIAK et al., 2012). More than a hundred members of the MADS family have been previously identified in Arabidopsis (PARENICOVA et al., 2003). The MADS-box genes were classified as an ABC model in which each of the genes would act individually or in pairs to determine organ fate. MADS-box heterodimers formation with floral induction proteins indicate that expression of flowering proteins may prevent activity of flower induction proteins in the flower by regulatory feedback loops (FOLTER et al., 2005). For instance, AP1 not only specifies organ identity but also plays a role in the transition from inflorescence to floral meristem identity (FERRANDIZ et al., 2000).

Floral organs are specified by floral-meristem identity homeotic genes that activate a class of genes which act in combination in order to confer organ identity in each whorl. A-class genes are responsible for specification of the outer whorl, the sepals. This class is consisted of *APETALA1* and *APETALA2* expression in this specific domain. B-class along with A-class genes determine petals by expressing *APETALA 3 (AP3)* and *PISTILLATA (PI)*, B-class along with C-class determine stamen formation by a synergic expression of B-class genes and *AGAMOUS (AG)*, and finally C-class would determine carpel identity by the expression of AG by itself (HONMA; GOTO, 2001; PELAZ et al., 2000; SAEDLER et al., 2001; SMACZNIAK et al., 2012; THEISSEN, 2001). However, the simplicity of the model was not enough to understand the entirety of floral formation, as some genes could not be classified so easily. FBP2, for instance, was isolated from Petunia, and the expression pattern of FBP2 seemed to belong to the three floral meristem inner whorls (ANGENENT et al., 1992). Later on, *SEPALLATA (SEP)* genes were characterized as *FBP2* in Arabidopsis, with the activity of B- and C-class genes depending on the expression of these genes. An E-class consisting of *SEP* genes were, then, characterized, and a "floral quartet model" was established based on the interactions of these four classes of genes to determine floral fate (DITTA et al., 2004; FERRARIO et al., 2003; PELAZ et al., 2000; THEISSEN, 2001). Five major floral homeotic MADS-domain proteins (*AP1*, AP3, PI, AG, and SEP3) interact in floral tissues as proposed in the floral quartet model, therefore, B-class proteins AP3 and PI would form complexes with A-class AP1 and E-class SEP in order to specify petals, and with C-class AG proteins in order to specify stamens. Class C along with E-class would determine carpel organs in the fourth whorl. The discovery

of four SEP genes (*SEP1-4*) suggests that all four SEP genes are required for the development of sepals (DITTA et al., 2004).

Targeted proteomics approaches established a MADS-domain protein interactome which strongly supports a mechanistic link between MADS-domain proteins and chromatin remodeling factors. Members of other transcription factor families were identified as interaction partners of floral MADS-domain proteins, suggesting various specific combinatorial modes of action (FOLTER et al., 2005; SMACZNIAK et al., 2012).

MADS box protein complexes are a highly evolutionarily conserved facet of flower development. Results involving homolog ABCE genes in grasses suggest that floral organ development occurs in a similar manner as in dicots (FERRARIO et al., 2003; LIU et al., 2013; MUNSTER et al., 2001; THEISSEN; MELZER, 2007; ZAHN et al., 2005). Furthermore, a group of MADS-interactors, related to chromatin remodeling factors, such as BELL1, was identified (BRAMBILLA et al., 2007; SMACZNIAK et al., 2012). For instance, chromatin-associated proteins were found to interact with AP1 in immune-precipitation assays, which could indicate a possible role in epigenetic regulation of target genes during the transition from inflorescence to floral meristem identity (SMACZNIAK et al., 2012). Therefore, epigenetics is another mechanism underlying inflorescence architecture differentiation in plants.

2.3 Floral transition epigenetic regulation

Chromatin modifying systems are important components in developmental processes such as the onset of the reproductive program. The chromatin-remodeling factors' repression mechanism is largely conserved among

eukaryotes. In animals, the formation of protein complexes alters DNA access to the transcriptional machinery; similarly, it is expected that plants present a conserved mechanism (LAURIA; ROSSI, 2011; PINEIRO et al., 2003; SIMON; TAMKUN, 2002).

In order to maintain similar sized flowers and the same number of floral organs, the timing to induce floral transition is strictly regulated, not only by transcriptional factors but also at a chromatin modification level (HE; AMASINO, 2005). Epigenetic silencing is an event by which a product of genes works as a multimeric complex in order to repress flowering by inhibiting the activity of a specific floral promoter, such as FT, or by global repression of the entire flowering program (SUNG et al., 2003).

Several floral repressors/activators were reported to be targets of chromatin remodeling processes. For instance, vernalization is an event by which cold leads to epigenetic silencing of floral repressors (HEO; SUNG, 2011). A cis-regulatory region of FLC in non-vernalized plants mediates histone deacetylation of FLC chromatin, consequently upregulating *FLC* expression and causing late flowering phenotypes in Arabidopsis (Figure 1). By contrast, after vernalization, FLC is silenced by chromatin modification (HE; AMASINO, 2005).

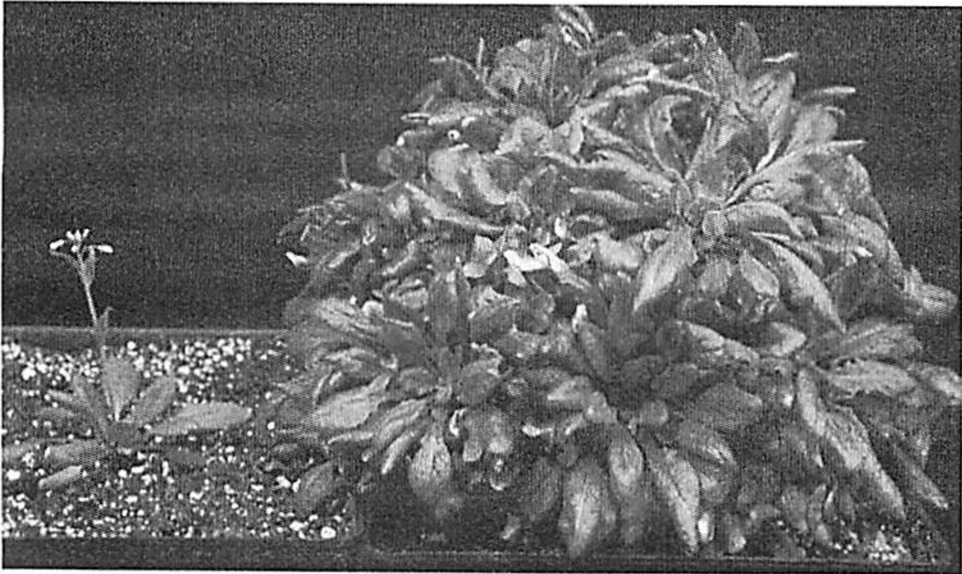

Figure 1 - Phenotypes generated by FLC manipulation. On the left, a *FLC* plant was transformed with a wild-type *FLC* transgene, in which the transgene is repressed by *FLOWERING LOCUS D* (*FLD*), *FVE* and autonomous regulators; on the right, a 295 bp cis-regulatory region of FLC is deleted, preventing deacetylation of FLC chromatin and inducing FLC expression (HE; AMASINO, 2005).

Similarly, a negative feedback loop exists between AGAMOUS (AG), which induces the differentiation of floral organs, and WUSCHEL (WUS), responsible for the maintenance of floral stem cells. Also, a regulatory mechanism involves the expression of an intermediate gene, *KNUCKLES* (*KNU*), which possess a binding site for AG in its promoter. However, a gap of two days exists between the binding of AG and the actual KNU expression, which is a consequence of epigenetic histone modifications, such as a Polycomb Repressing Complex (PRC)-mediated H2K27me3 marks, which is removed after two days of AG activity (ITO; SUN, 2009; SUN et al., 2009).

CURLY LEAF (CLF) is a plant homolog of a SET domain Polycomb Repressive Complex2 (PRC2) which directly targets *FT*, *SEP3* and *FLC* by *CLF* H2K27m3 chromatin enrichment. Interestingly, it has been suggested that, although *CLF* mutation leads to early flowering plants. This phenotype may represent a balance between opposing activities of floral induction and floral repression genes, such as *FT* and *FLC* (LOPEZ-VERNAZA et al., 2012).
Another SET domain protein, EARLY FLOWERING IN SHORT DAYS (EFS), is characterized by H3K4 methyltransferases, which is enrolled in order to methylate target genes (SOPPE; BENTSINK; KOORNNEEF, 1999). A family of plant proteins, represented by the EARLY BOLTING IN SHORT DAYS (EBS), function as putative chromatin-interacting factors. EBS acts by repressing FT activity under short-day conditions, being part of a transcriptional repressor complex involved in changes of chromatin structures (PINEIRO et al., 2003).
Altogether, transcriptional and post-transcriptional regulatory mechanisms such as epigenetic systems, suggest that phase transition is triggered by a dynamic cross-talk between multi-level controlled process.

2.4 Flowering induction and floral formation in grasses

Floral induction is a conserved process among higher plants, but specific responses evolved in plants from diverse environmental conditions, such as day-length. Plants cultivated in temperate climates respond differently compared to those grown in tropical regions (GREENUP et al., 2009). Cereals such as barley and wheat are long-day plants and present FT expression under such conditions. On the other hand, rice *FT* ortholog, *Hd3a*, is induced under short day

conditions, mechanism controlled by a gate system in which a pair of floral regulators, *Ehd1 (Early heading date 1)* and *Ghd7* (*Grain number, plant height and heading date)*, play central role (ITOH et al., 2010). There are distinct mechanisms controlling Hd3a/RFT1 in monocot rice, and this is due to the emergence of *Ghd7*, a florigen repressor under long days (ITOH et al., 2010; XUE et al., 2008). Under short days, *Ehd1* is activated by blue light and both Oryza *sativa INDETERMINATE1 (OsID1)* and *Oryza sativa MADS51 (OsMADS51)* which leads to the induction of *Hd3a* expression (GREENUP et al., 2009; ITOH et al., 2010; KOMIYA et al., 2008) (Figure 2). Alternatively, under long-day conditions, rice FT-homolog, *RFT1*, is solely induced, and plants present late flowering phenotypes (KOMIYA; YOKOI; SHIMAMOTO, 2009).

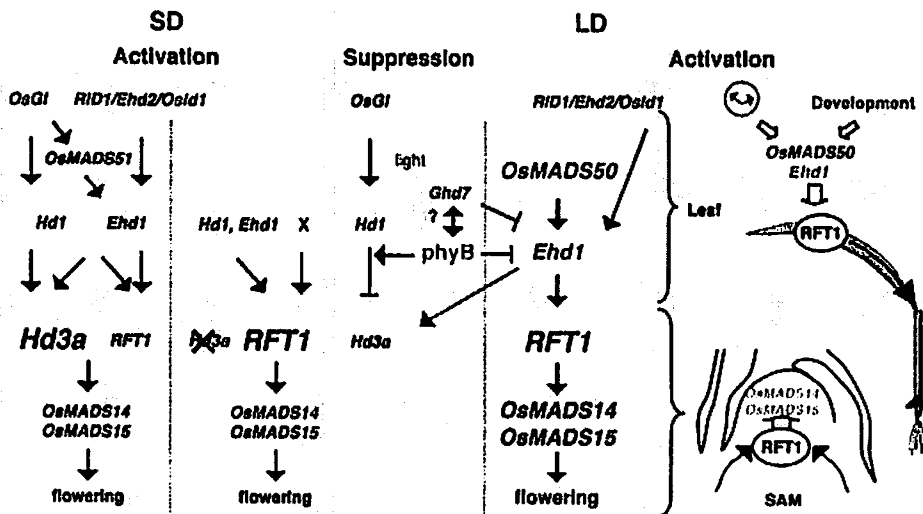

Figure 2 – Molecular pathway of rice floral induction (KOMIYA; YOKOI; SHIMAMOTO, 2009).

In maize, a day neutral plant, transition to flowering is dependent upon the expression of the *INDETERMINATE1* (*ID1*) zinc-finger transcription factor, a product of an autonomously regulated gene. The IDD-family member of transcription factors, ID1, is involved in phase transition not only in maize but in related species such as rice (AGARWAL et al., 2007; COLASANTI et al., 2006; ENGLBRECHT; SCHOOF; BÖHM, 2004).

The gene product of *ID1* binds to a specific sequence of DNA, suggesting its role in expression of other genes (KOZAKI; HAKE; COLASANTI, 2004). *ID1* plants are incapable of flowering at the correct time and show late flowering phenotype compared to the wild-type. *OsID1/RID1/EHD2* is an *ID1* ortholog in rice which plays a role in controling *Hd3a/RFT1* (MATSUBARA et al., 2008; PARK et al., 2008; WU et al., 2008). Finally, ID1-*like* proteins isolated from rice and sorghum suggest that this protein is conserved between monocot plants and that it plays a crucial role in the floral induction of both autonomously and day-length controlled plants (COLASANTI; YUAN; SUNDARESAN, 1998; CONEVA et al., 2012). *ID1* is expressed specifically to maize young leaves (KOZAKI; HAKE; COLASANTI, 2004) and its expression is connected to *ZEA MAYS CENTRORADIALIS8* (*ZCN8*) induction (LAZAKIS; CONEVA; COLASANTI, 2011; MENG; MUSZYNSKI; DANILEVSKAYA, 2011). *ZCN8* is a floral regulator ortholog of *FLOWERING LOCUS T* (*FT*) and it is suggested that its activity is involved in external signal transduction to the apical meristem. Similarly to rice, sugarcane is a monocot plant in which floral induction occurs by day length shortening , cool temperatures at night and high temperatures during the day, and high humidity (ARALDI et al., 2010). However, little is known about the molecular regulatory mechanisms involved in floral transition in *Saccharum* spp. species. Recently, several flowering time candidates were

identified by *in silico* analysis at the Sugarcane Expressed Sequence Tag (SUCEST) database, however gene function is yet to be dissected in this species (COELHO et al., 2013).

2.5 *FLOWERING LOCUS T:* beyond flowering time

Dynamic interaction processes connect different external and internal signals to regulate *FLOWERING LOCUS T* (*FT*) transcription in order to induce flowering (Figure 3). It has also been suggested that interaction among floral integrators such as FT and floral homeotic genes exists in the SAM. For instance, AP1 is a direct target of the FT-FD complex, and both *AP1* and *LFY* upregulate each other in a positive feedback loop in order to maintain floral meristem identity. Also, FT-FD in association with STM (SHOOT MERISTEMLESS) and BELL-like proteins PNY PNF (PENNYWISE POUND-FOOLISH) induces AP1 (POSE; YANT; SCHMID, 2012). To deepen the complexity, floral transition is not the only developmental process in which FT plays a role.

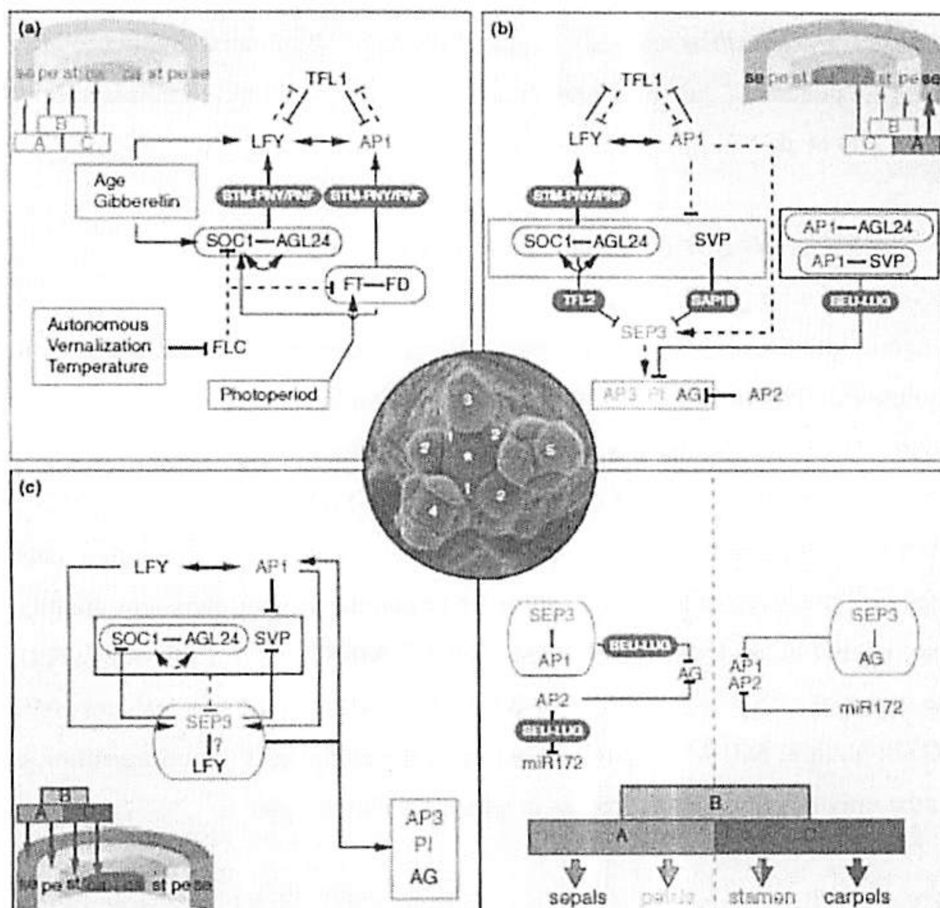

Figure 2 - Genetic networks controlling flower development (POSE; YANT; SCHMID, 2012).

Strikingly, diverse functions of FT and its interacting transcription factors have been reported. Transcription factor target maps allowed the identification of single transcription factor involved in multiple developmental processes, which permitted to link processes which at a first sight were not connected to each other (POSE; YANT; SCHMID, 2012). The discovery that FT works in alternative processes beyond flowering time is an unexpected and important

milestone. For instance, in tomato, a photoperiod-insensitive plant, the FT homolog, SFT (SINGLE FLOWER TRUSS) regulates growth and termination, flower morphology and alters shoot architecture, characterized by fewer leaflets per compound leaf, shorter internodes and thinner stems (LIFSCHITZ et al., 2006).

FT/TFL1-like genes identified in gymnosperms, *FTL1* and *FTL2*, prevented flowering when ectopically expressed in Arabidopsis, and FTL2 promoted growth cessation in spruce. Collectively, these data indicate that the native function of FT-like genes in gymnosperms is not flowering time induction (GYLLENSTRAND et al., 2007; KARLGREN et al., 2011; KLINTENAS et al., 2012).

Tobacco and sugar beet FT-like genes play antagonist roles in floral initiation, which suggests functional divergence in several FT-like genes from the PEBP family (HARIG et al., 2012; PIN et al., 2010). Also, a FT homolog in potato, StSP6A, is involved in tuberization, while its paralog, StSP3D, is involved in floral transition (NAVARRO et al., 2011). Interestingly, a new role in stomata opening has been reported for FT, which was found to be autonomously expressed in guard cells and an activator of H^+-ATPases (KINOSHITA et al., 2011).

To understand the diverse function of FT-like genes, sequence analyses allow the identification of at least three sites in FT- and TFL1-like genes which are essential to its function as a floral activator: site 85, segment B, and several positions at segment C (AHN et al., 2006; KLINTENAS et al., 2012; PIN et al., 2010).

Altogether, these data indicate that floral transition may be one of the pleiotropic effects controlled by FT orthologs among diverse multifaceted functions in plant

development, which may contribute to plant diversification and evolution (PIN; NILSSON, 2012).

2.6 Floral transition and its implication in bioenergy crop production

Flowering transition is closely associated to production yield in crop species. The increasing population and limited oil availability has led to large investments in the production of bioethanol from bioenergy crops such as sugarcane, switchgrass, Miscanthus, maize and sorghum. Therefore, a better physiological understanding regarding developmental processes, such as phase transition, is important to the development of more productive crops to attend the production of renewable energy (REGALADO, 2010).

Sugarcane is a bioenergy crop which presents C4 carbon metabolism and is the main source of sugar production in the world (D'HONT et al., 2008). Brazil is the main sugarcane producing country, mostly in the north-northeast and South-Center regions (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2013).

As an example of how environmental factors affect flowering time, the North-Northeast region of Brazil is located at the tropics and, therefore, production is affected by floral inductive conditions, such as shorter day-length and inductive temperature (ARALDI et al., 2010). Transition to reproductive growth in sugarcane is an undesirable event since it leads to the translocation of sugar from the accumulation in the stalks to the developing inflorescence, leading to a decrease in sucrose yield per plant and, subsequently, a lower production of ethanol (BUTTERFIELD; D'HONT; BERDING, 2001). Therefore, it is important to develop strategies to avoid/delay floral transition in this species.

In sorghum, for instance, functional modification of the *PSEUDORESPONSE REGULATOR PROTEIN 37* (*PRR37*) gene, involved in floral transition, has led to an increase in biomass productivity for biofuel production (MURPHY et al., 2011). Similarly, silencing of a FT ortholog in tobacco plants led to an extended vegetative phase and higher biomass production (HARIG et al., 2012). Collectively, as floral initiation is intimately connected to biomass production and, as more recently reported, involved in other important developmental processes, it is of main interest to dissect flowering time genes in bioenergy crops aiming at generating more productive plants in a food and fuel demanding scenario.

3 Hypotheses

Little is known about the molecular regulatory mechanisms of floral transition in sugarcane. This work aims, for the first time, at identifying sugarcane putative flowering time candidates and dissecting the function of key orthologs, such as FT/TFL1 and ID1, involved in phase transition. Thus, three main hypotheses were raised:

1 - The photoperiodic pathway of floral transition is mostly conserved between sugarcane and related species.

2 - ScFT and ScTFL1 orthologs play antagonist roles in sugarcane floral induction.

3 - ScID1 ortholog induces flowering through the autonomous pathway in sugarcane.

CONCLUSIONS AND FUTURE WORK

1 CONCLUSIONS

The results observed here indicate that floral transition is, at least in part, conserved in sugarcane when compared to related species. It was also possible to identify one ortholog, ScID1, which is possibly involved in the autonomous pathway of floral transition, one TFL1 ortholog (ScTFL1) and at least five FT-like genes (ScFT1, ScFT2, ScFT3, ScFT4 and ScFT5) in sugarcane. The candidates ScFT1 and ScFT2 were characterized by its overexpression in Arabidopsis plants and both of them presented late flowering phenotypes.

It is noteworthy that, for the first time, flowering time genes were characterized in *Saccharum* spp. and the identification of more than one FT ortholog support recent findings that several FT may be involved, not only as floral activators, but also as floral repressors, depending on the structure of specific regions of the FT protein. Strikingly, pleiotropic effects of sugarcane FT orthologs overexpression in Arabidopsis suggest its involvement in other developmental processes such as meristem-associated functions. Altogether, this work supports the hypothesis of diverse FT roles in different species.

At this point, it was not possible to identify the FT-promoting signal for floral transition. However, sequence analysis suggest that one of the three additional candidates, identified at the SUCEST database, is the sugarcane florigen, because of the presence of a conserved region at segment B, which is believed to be required for phase transition.

2 FUTURE WORK

Future work will be needed to confirm the function of the additional FT candidates in floral promotion; analysis of *oscar* seeds development to verify phenotype consistency; establishment of *Setaria viridis* transformation protocol to be used as a model plant for C4/monocot-specific genes, such as ScID1. It will be interesting to verify whether a similar protein complex is formed between the characterized ScFT/ScTFL1 and a sugarcane FD ortholog by protein-protein interaction essays, such as yeast two-hybrid (Y2H) and Bimolecular Fluorescence Complementation (BiFC). Ultimately, sugarcane plants will be transformed with these genes in order to confirm their function on floral transition and its possible relationship with sucrose accumulation..

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SECOND PART

PAPER 1

A proposed model for the flowering signaling pathway of sugarcane under photoperiodic control

The following chapter is entitled "**A proposed model for the flowering signaling pathway of sugarcane under photoperiodic control**" and contains data from the published paper:

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ABSTRACT

Molecular analysis of floral induction in *Arabidopsis* has identified several flowering time genes related to four response networks defined by the autonomous, gibberellin, photoperiod and vernalization pathways. Although grass flowering processes include ancestral functions shared by both monocots and dicots, they have developed their own mechanisms to transmit floral induction signals. Despite its high production capacity and its important role in biofuel production, almost no information is available about the flowering process in sugarcane. We searched the Sugarcane Expressed Sequence Tags database to look for elements of the flowering signaling pathway under photoperiodic control. Sequences showing significant similarity to flowering time genes of other species were clustered, annotated and analyzed for conserved domains. Multiple alignments comparing the sequences found in the sugarcane database and those from other species were performed and their phylogenetic relationship assessed using MEGA 4.0 software. Electronic Northerns were run with Cluster and TreeView programs, allowing us to identify putative members of the photoperiod controlled flowering pathway of sugarcane.

Keywords: Floral induction; Photoperiodism; Saccharum spp.; Flowering time genes; SUCEST; Biomass yield

1 INTRODUCTION

Transition from vegetative to reproductive growth is an important event in the development of higher plants. The change to a reproductive program is manifested in vegetative tissues and is regulated by both environmental and

endogenous factors. The shoot apical meristem (SAM) is a population of undifferentiated cells that produce leaves and branches during vegetative growth. Under environmental and endogenous responses, SAM undergoes an identity change and produces floral primordia. Molecular analysis of floral induction has been extensively developed in *Arabidopsis* (Simpson and Dean, 2002). These studies have identified numerous flowering time genes that act in four response networks: autonomous, gibberellin (Baurle and Dean, 2006), photoperiod and vernalization (Mouradov et al., 2002; Parcy, 2005).

The light-dependent flowering pathway can be controlled by two mechanisms; light quality and day length (Bernier and Periellux, 2005). These environmental factors lead to a cascade of responses that directly affect expression of the *PHYB (PHYTOCHROME B)*, *CRY2 (CRYPTOCHROME 2)* and *PHYA (PHYTOCHROME A)* genes. A circadian clock-controlled mechanism integrates the inductive long-day photoperiod signals, which leads to the expression of *GIGANTEA* (*GI*), followed by the activation of *CONSTANS* (*CO*) expression and finally the induction of the *FLOWERING LOCUS T* gene (*FT*). The *FT* gene product acts as a leaf-synthesized florigen that migrates through the phloem to the SAM to cause flowering.

Although grass flowering processes utilize some functions also found in dicots, they also have developed their own mechanisms to produce floral induction signals (Colasanti and Coneva, 2009). Rice plants possess some genes that are absent in dicot plants, such as *GHD7* (*Grain number, Plant Height* and *Heading Date7*) and *EHD1* (*EARLY HEADING DATE1*) (Greenup et al., 2009). These genes, which integrate information about short-day (SD)-induced flowering, act independently of the *GI-CO-FT* pathway.

Sugarcane, a monocot plant, is the main source of sugar production, representing almost two thirds of the world production. A better understanding of the flowering process in sugarcane, a SD plant, is important because, among other factors, it is related to crop yield. The transition to reproductive growth leads to translocation of some of the sugar to the developing inflorescence, thus diverting the stored sugar away from stalks and, consequently, decreasing crop sugar yield.

Studies involving the flowering process in this crop plant will contribute to future insights into sugarcane genetics, physiological processes related to sucrose content/translocation and the use of biotechnology approaches to increase sugar production. However, research related to the characterization of sugarcane flowering time genes is scarce.

The Sugarcane EST project (Figueiredo et al., 2001), SUCEST, encompasses a collection of 240,000 ESTs generated from 26 cDNA libraries constructed from different organs and/or tissues at different developmental stages, including apical meristem, flowers, leaf roll, seeds, internode, stem bark, etiolated leaves, lateral buds and stem bark (Vettore et al., 2001).

In this study, we employed *in silico* analyses to search the SUCEST database for putative orthologs of flowering time genes that are involved in the photoperiod-controlled floral inductive signaling pathway in sugarcane.

2 MATERIAL AND METHODS

2.1 Database searches and alignments

Homologs of functionally characterized genes involved in the flowering signaling pathway under photoperiodic control (*GI*→*CO*→*FT*) were identified by data mining in the SUCEST database (http://compbio.dfci.harvard.edu/tgi/plant.html) using plant gene (BLASTn) and protein (tBLASTn) sequences as bait. Sequences with significant similarity (e-value>10^{-4}) were selected and submitted to clustering by the CAP3 program (Huang and Madan, 1999), forming the EST contigs and singlets.

The *Saccharum officinarum* EST-contigs and EST-singlets obtained were manually annotated, and data validation was performed by local tBLASTx and tBLASTn searches of the retrieved sequences against the GenBank database. Selected sequences were then used as bait in another search against the SUCEST database, aiming at uncovering additional reads, as well as to remount incomplete clusters. This process was repeated until no more new significant reads were found. ORFs (open reading frames) of validated sequences were obtained through the ORFinder tool, from NCBI (http://www.ncbi.nlm.nih.gov), and their protein sequences were generated through the translation tool found in the ExPASY (http://www.expasy.ch) protein database. The protein sequence alignments were performed by the ClustalW program (Thompson et al., 1994), using default parameters.

2.2 Phylogenetic analysis

The putative orthology of the deduced amino acid sequences of sugarcane transcripts, compared to homologs from other species, was assessed by phylogenetic trees formed by MEGA software, version 4.0 (Tamura et al., 2007), with the neighbor-joining comparison model (Saitou and Nei, 1987), *p*-

distance method and pair-wise suppression. Bootstrap values from 1000 replicates were used to assess the robustness of the trees.

2.3 *In silico* gene expression analysis

In silico qualitative gene expression profiling was performed using virtual Northern blot analyses. For each EST-contig and EST-singlet, frequencies of reads that form each EST contig and EST singlet in the libraries in which they were expressed was calculated. This procedure required that the data be previously normalized to give a more accurate idea of the degree of expression of the sequences in each treatment and plant organ when all libraries were considered in this work.

Normalization consisted of multiplying each read by the quotient between the number of reads from the library where it was expressed and the sum of reads of all libraries where expression was found. The results were plotted in a matrix and gene expression patterns among ESTs and libraries were obtained by hierarchical clustering, performed by the Cluster v.3 program (Eisen et al., 1999). Graphic outputs were generated by TreeView v.1.6 software (Eisen et al., 1999) and presented in a color scale from black to red, where red indicated higher expression levels. Undetectable expression was noted in gray.

3 RESULTS

The main components of the flowering pathway under photoperiodic control were compiled, and their sequences were used to search sugarcane EST-contigs. Taken together, the results of the phylogenetic analyses, electronic Northern and blastp searches allowed for the identification of candidates for several genes that may be involved in the sugarcane flowering pathway under photoperiodic control.

Analyses at the SUCEST database revealed 16 reads related to the *GI* gene, clustered into 7 contigs and 2 singlets. As shown in the phylogenetic tree (Figure 1a) and in TABLE 1, GiC1 and GiC5 peptide candidates showed high similarity with maize *GIGANTEA* (*ZmGI*), with amino acid identity ranging from 90% to 97%. It was possible to divide the phylogenetic tree into 2 subgroups, with one group corresponding to neutral day plants, which included GiC5 and another group corresponding to long- and short-day plants, including the GiC1. The electronic Northern showed that these contigs are expressed in six (GiC1) and twelve (GiC5) different libraries, in no tissue specific manner, including tissues where the *GI* typically acts, such as apical meristem surrounding immature and mature leaves and in the inflorescence. Blastp analyses revealed high identity of GiC1 (90%) and GiC5 (97%) with a maize *GI* ortholog.

Twenty-three reads related to *CO* were found and clustered into 1 contig and 8 singlets. The motif analyses showed that all sequences contained conserved domains for the family of *CO*-like genes, and they could be categorized into the three subgroups of *CO* family genes (Wenkel et al., 2006; Griffiths et al., 2003). The results for *CO* gene similarity (Figure 1b) indicated that sugarcane

possesses some candidates for each subtype of the *CO*-like superfamily. This result was confirmed by blastp analyses, since the candidates found could be related to *COL1*, *COL5*, *COL6* and *COL10*. Additionally, contig CoC1 was considered a candidate for the *CO* gene because it showed a high level of similarity with maize HD1 protein (81% identity). Phylogenetic analyses showed that this singlet was grouped with *CO* orthologs of related species, such as rice and maize, and the electronic Northern suggested that its expression was specific to inflorescence tissue.

Candidates for putative *EHD1* and *GHD7* orthologs were clustered into 5 contigs and 4 singlets derived from 20 reads, and 4 contigs and 2 singlets derived from 11 reads, respectively. It was possible to identify some candidates for *EHD1* and *GHD7*, which are monocot-specific genes, in the SUCEST database. According to phylogenetic analyses and the electronic Northern, *Ehd1C4* is closely related to the rice *EHD1* gene (*OsEHD1*) and is detected in leaf libraries, such as mature leaf tissues (Figure 1c). After comparing conserved domains, it was possible to identify the CCT domain of the *Ghd7* gene. The candidate Ghd7C4 (Figure 1d) showed very high abundance in the mature leaf tissue library. Through blastp analyses, Ghd7C4 was found to correlate with the barley *COL7* gene, with an identity of 67%.

It was possible to detect 20 reads for the *FT* gene in searches, clustered into 3 contigs and 5 singlets, all of them containing the PEBP conserved domain and with high similarity to the maize *ZCN* superfamily (Danilevskaya et al., 2008). Blastp results revealed two candidates for *TFL1*-like genes in the SUCEST database; FtC3 and FtS2. These candidates showed high similarity to a rice putative *TERMINAL FLOWER1* (*TLF1*) (83% and 79%, respectively), and an electronic Northern showed that these sequences are expressed in the leaves

(FtS2) and lateral bud (FtC3) tissues. Results of the phylogenetic analyses suggested that the FtS1 and FtS5 EST-contigs were the most related to *ZCN26* (Figure 1e), and the electronic northern showed expression in root-shoot transition libraries. Phylogenetic results suggested that FtC2 and FtC1 are candidates of the *FT*-like I group orthologs, *ZCN14-ZCN15* and *ZCN19-ZCN25*, respectively. FtS2, FtC3, FtS1 and FtS5 could be categorized as candidates for the *FT*-like II group, related to *ZCN8* and *ZCN26*. A candidate for a *MOTHER of FT* (*MFT*)-like subfamily gene, *ZCN11*, was also found, i.e., the FtS4 EST-contig.

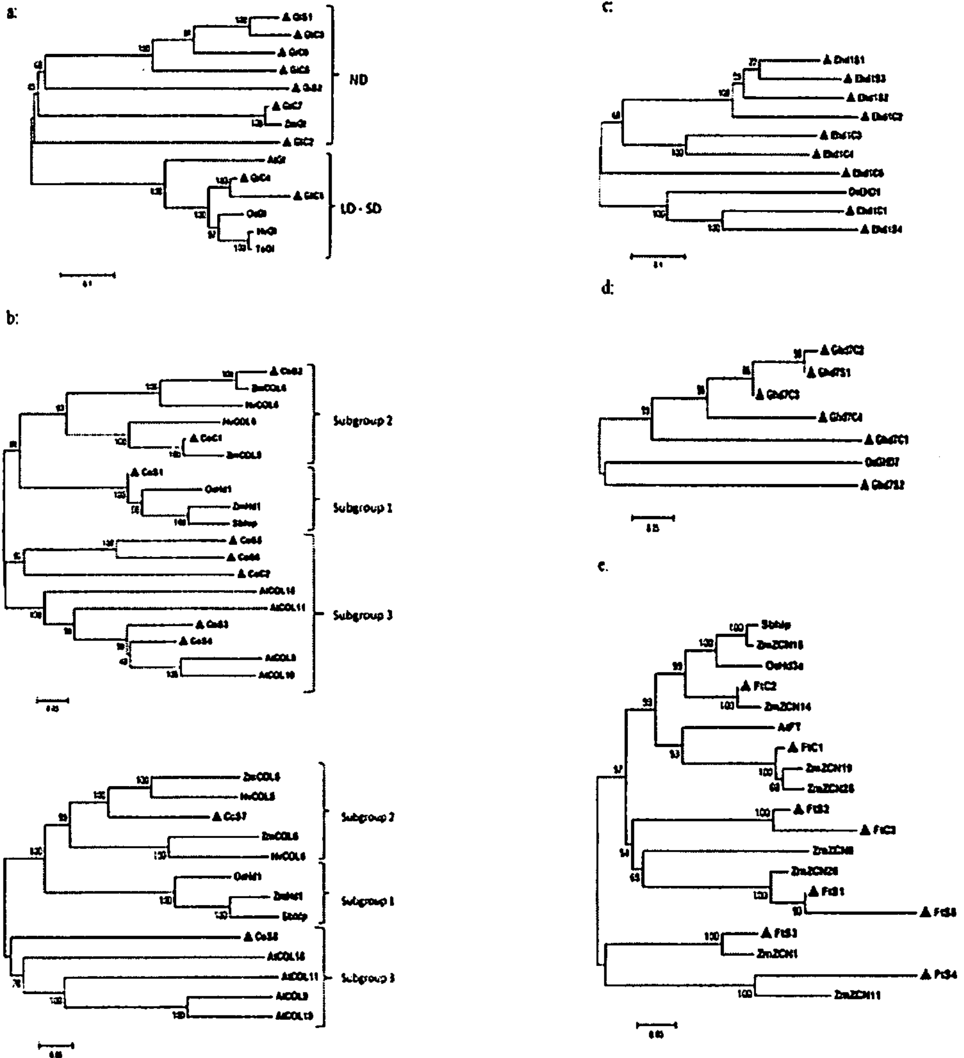

Figure 1. Phylogenetic analyses involving the sugarcane putative genes of the flowering pathway under photoperiodic control (triangles) and homolog sequences obtained from the NCBI database related to **A.** *GI*; **B.** *CO*; **C.** *EHD1*; **D.** *GDH7*; **E.** *FT*. Neighbor-joining trees were built for sugarcane-deduced amino acids and protein sequences from other species aligned with ClustalW2. Bootstrap values from 1000 replications were used to assess the robustness of the trees. Genetic distances are shown at the given scales. The protein sequences from other species and their respective accession numbers are as follows: **A.** *Zea mays* [ZmGI (ABZ81992.1)], *Arabidopsis*

thaliana [AtGI (AAF00092.1)], *Oryza sativa* [OsGI (BAD68052.1)], *Hordeum vulgare* [HvGI (ACM49849.1)], *Triticum aestivum* [TaGI (AAL08497.2)]. **B.** *Arabidopsis thaliana* [AtCOL9 (NP_187422.1), AtCOL10 (NP_199636.1), AtCOL11 (NP_193260.2), AtCOL15 (NP_174126.1)], *Zea mays* [ZmCOL5 (NP_001147679.1), ZmCOL6 (NP _001148229.1), ZmHd1 (ABW82153.1)], *Hordeum vulgare* [HvCOL5 (AAL99264.1), HvCOL6 (AAL99267.1)], *Oryza sativa* [OsHd1 (BAI59731.1)], *Sorghum bicolor* [Sbhip (XP_002436860.1)]. **C.** *Oryza sativa* [OsEHD1 (ABF95340.1)]. **D.** *Oryza sativa* [OsGHD7 (ACA14489.1)]. **E.** *Zea mays* [ZmZCN1 (ABW96224.1), ZmZCN8 (ABX11010.1), ZmZCN11 (NP_001106264.1), ZmZCN14 (NP_001106251.1), ZmZCN15 (ABW96237.1), ZmZCN19 (NP_001106256.1), ZmZCN25 (NP_001106257.1), ZmZCN26 (ABW96244.1)], *Oryza sativa* [OsHd3a (BAF15064.1)], *Arabidopsis thaliana* [AtFT (AAF03936.1)], *Sorghum bicolor* [Sbhip (XP_002436509.1)]

Table 1. Comparison of the sugarcane ESTs related to the flowering pathway under photoperiodic control found in the SUCEST database and their best hit of Blastp analysis at NCBI database.

Category	Contig/aa	Blastp	E-value	Identity	Positives
GI	GiC1/514	DAA06172.1| TPA_inf: gigantea 1A [Zea mays]. 1162aa	0.0	424/469 (90%)	437/469 (93%)
	GiC2/332	DAA06172.1| TPA_inf: gigantea 1A [Zea mays]. 1162aa	0.0	311/330 (94%)	315/330 (95%)
	GiC3/193	BAD68053.1| putative gigantea [Oryza sativa Japonica Group]. 261aa	$4e^{-92}$	143/186 (77%)	156/186 (84%)
	GiC4/289	CAB56058.1| gigantea homologue [Oryza sativa]. 975aa	$9e^{-175}$	252/284 (89%)	261/284 (92%)
	GiC5/271	DAA06172.1| TPA_inf: gigantea 1A [Zea mays]. 1162aa	$6e^{-180}$	261/269 (97%)	266/269 (99%)
	GiC6/184	DAA06172.1| TPA_inf: gigantea 1A [Zea mays]. 1162aa	$2e^{-88}$	140/161 (87%)	151/161 (94%)
	GiC7/317	DAA06172.1| TPA_inf: gigantea 1A [Zea mays]. 1162aa	0.0	297/309 (96%)	304/309 (98%)
	GiS1/117	AF469490_1 gigantea-like protein [Triticum aestivum].190aa	$1e^{-42}$	70/83(84%)	75/83 (90%)
	GiS2/87	ABR26000.1| gigantea protein [Oryza sativa Indica Group]. 169aa	$3e^{-27}$	56/87 (64%)	64/87 (74%)
CO	CoC1/127	AAM74069.1|AF490474_1 CONSTANS-like protein [Hordeum vulgare subsp. vulgare]. 323aa	$1e^{-56}$	93/119 (78%)	102/119 (86%)
	CoC2/206	NP_195607.2| B-box type zinc finger-containing protein [Arabidopsis thaliana]. 183aa	$3e^{-73}$	116/207 (56%)	145/207 (70%)
	CoS1/75	ABW82153.1| Hd1 [Zea mays]. 398aa	$2e^{-35}$	63/78 (81%)	68/78 (87%)
	CoS2/279	NP_001148229.1| CONSTANS-like protein CO6 [Zea mays]. 364aa	$2e^{-142}$	242/267 (91%)	245/267 (92%)
	CoS3/116	NP_199636.1| zinc finger protein CONSTANS-LIKE 10 [Arabidopsis thaliana]. 373aa	$3e^{-54}$	83/114 (73%)	97/114 (85%)

	CoS4/91	NP_199636.1| zinc finger protein CONSTANS-LIKE 10 [Arabidopsis thaliana]. 373aa	$7e^{-38}$	65/90 (72%)	72/90 (80%)
	CoS5/163	ACC85612.1| zinc finger protein [Phyllostachys edulis]. 256aa	$5e^{-65}$	110/158 (70%)	118/158 (75%)
	CoS6/159	ACF35275.1| B-box zinc finger protein [Bambusa oldhamii]. 256aa	$9e^{-64}$	106/145 (73%)	113/145 (78%)
	CoS7/152	NP_001147679.1| CONSTANS-like protein CO5 [Zea mays]	$5e^{-48}$	91/135 (67%)	100/135 (74%)
	CoS8/136	AAZ86536.1| truncated COL1 [Lolium perenne]. 168aa	$1e^{-66}$	96/135 (71%)	108/135 (80%)
FT	FtC1/109	ABW96241.1| ZCN19 [Zea mays]. 175aa	$2e^{-73}$	104/109 (95%)	104/109 (95%)
	FtC2/115	ABW96236.1| ZCN14 [Zea mays]. 236aa	$6e^{-79}$	113/115 (98%)	114/115 (99%)
	FtC3/85	BAD73176.1| putative terminal flower1 [Oryza sativa Japonica Group]. 180aa	$2e^{-35}$	57/69 (83%)	61/69 (88%)
	FtS1/175	ABW96244.1| ZCN26 [Zea mays]. 187aa	$3e^{-119}$	163/173 (94%)	169/173 (98%)
	FtS2/179	BAD73176.1| putative terminal flower1 [Oryza sativa Japonica Group]. 180aa	$3e^{-95}$	136/272 (79%)	148/172 (86%)
	FtS3/173	ABW96224.1| ZCN1 [Zea mays]. 173aa	$3e^{-113}$	158/173 (91%)	163/173 (94%)
	FtS4/128	ABW96234.1| ZCN11 [Zea mays]. 180aa	$7e^{-48}$	81/93 (87%)	86/93 (92%)
	FtS5/78	ABX11026.1| ZCN26 [Zea mays]. 187aa	$3e^{-24}$	59/65 (91%)	62/65 (95%)
EHD1	Ehd1C1/215	ADX60172.1| ARR-B transcription factor [Zea mays]. 631aa	2^{e-128}	201/209 (96%)	206/209 (99%)
	Ehd1C2/130	ADX60157.1| ARR-B transcription factor [Zea mays]. 631aa	$9e^{-41}$	70/111 (63%)	84/111 (76%)
	Ehd1C3/252	ABF95340.1| Two-component response regulator-like PRR73, [Oryza sativa Japonica Group]. 473aa	$1e^{-118}$	189/237 (80%)	201/237 (85%)
	Ehd1C4/170	NP_001151536.1| two-component response regulator-like PRR95 [Zea mays]. 630aa	$2e^{-111}$	161/165(95%)	165/169 (98%)

	Ehd1C5/548	ADO51647.1| PRR59 [Zea mays]. 695aa	0.0	454/547(83%)	481/547 (88%)
	Ehd1S1/125	ADX60172.1| ARR-B transcription factor [Zea mays]. 631aa	$4e^{-59}$	92/109 (84%)	98/109 (90%)
	Ehd1S2/146	NP_001104864.1| response regulator 10 [Zea mays]. 686aa	$2e^{-92}$	130/139 (94%)	136/139 (98%)
	Ehd1S3/153	ADX60172.1| ARR-B transcription factor [Zea mays]. 631aa	$5e^{-89}$	132/146 (90%)	139/146 (95%)
	Ehd1S4/184	ADX60172.1| ARR-B transcription factor [Zea mays]. 631aa	$2e^{-38}$	85/144 (59%)	102/144 (71%)
GHD7	Ghd7C1/152	AF490474_1 CONSTANS-like protein [Hordeum vulgare subsp. vulgare]. 323aa	$5e^{-48}$	117/153 (76%)	120/153 (78%)
	Ghd7C2/75	NP_001148229.1| CONSTANS-like protein CO6 [Zea mays]. 364aa	$3e^{-49}$	67/71 (94%)	67/71 (94%)
	Ghd7C3/73	AAL99266.1| CONSTANS-like protein CO6 [Hordeum vulgare subsp. vulgare]. 357aa	$5e^{-25}$	63/75 (84%)	67/75 (89%)
	Ghd7C4/105	AAL99269.1| CONSTANS-like protein CO7 [Hordeum vulgare subsp. vulgare]. 244aa	$1e^{-25}$	69/103 (67%)	71/103 (69%)
	Ghd7S1/89	NP_001148229.1| CONSTANS-like protein CO6 [Zea mays]. 364aa	$1e^{-46}$	77/81(95%)	77/81 (95%)
	Ghd7S2/127	AAL99270.1| CONSTANS-like protein CO8 [Hordeum vulgare subsp. vulgare]. 247aa	$2e^{-16}$	44/81 (54%)	51/81 (63%)

4 DISCUSSION

Previous studies in Arabidopsis and rice have shown that, although some pathways are conserved between monocot and dicot plants, each group has developed specific mechanisms to control the flowering process.
Almost nothing is known about the flowering pathway in sugarcane, although this process is extremely important and related to crop yield. A search for five flowering time genes in sugarcane, *GIGANTEA, CONSTANS, EARLY HEADING DATE1, HEADING DATE7* and *FLOWERING LOCUS T*, which are both monocot- and dicot-specific genes, was performed in the SUCEST database.

4.1 *Gigantea*

GI is a large protein that is nuclear localized (Huq et al., 2000) and regulates flowering through the integration of circadian rhythm periods, acting upstream of the *CO* gene (Samach and Coupland, 2000). Analysis of this gene was performed since sugarcane floral induction is controlled by photoperiod and the circadian clock. The circadian clock is a pacemaker that controls rhythmic processes that occur within a period of 24 h (Hayama and Coupland, 2003). Mutation of *GI* both impairs circadian rhythms and delays the flowering process, suggesting that its functions is to couple the circadian clock to the day-length perception inducing downstream genes, including those specifically related to the floral transition, such as *CO* and *FT* (Mizoguchi et al., 2005). *GI* is highly conserved in higher plants, including monocot species such as rice (Hayama et al., 2002). A functional hierarchy of *GI-CO-FT* acting together to connect the

circadian oscillator to the flowering pathway has been established (Mizoguchi et al., 2005). The two candidates found in sugarcane contain a conserved domain present in *GI*, although the sequences found encode incomplete ORFs. The contig GiC2 was grouped with GI proteins of day-neutral plants, and the GiC1 contig was grouped with *GI* of long- and short-day plants. This information supports the idea that each candidate may act differently, depending on the environmental conditions in which sugarcane is subjected. Results from Higuchi and colleagues (2011) suggest that in *Pharbitis nil*, a SD plant with an absolute requirement for short-day photoperiods to induce flowering, a *GI* ortholog functions as a suppressor of flowering through the repression on an *FT* ortholog. Although sugarcane is a SD plant (Araldi et al., 2010), other variables interact with photoperiod signals to determine floral induction, such as low temperatures. Taken together with the electronic Northern (Figure 2a), it is possible to predict that the *GI* ortholog (GiC1) is important in the sugarcane flowering network, although its specific function (such as inducing or repressing downstream *FT-like* genes) needs to be confirmed by functional analyses.

4.2 *Constans*

The *CO* gene, downstream of *GI*, is a key regulatory protein that integrates signals from the circadian clock to control flowering (Putterill et al., 2001; Valverde, 2011). *CO* expression exhibits a circadian rhythm under continuous light, in which *CO* has a diurnal expression pattern with a peak in the night, regulated by the circadian clock (Hayama and Coupland, 2003). Rice, which is a short day plant (SD), has a *CO* ortholog (the *Hd1* gene) whose expression is repressed under LD and induced under SD floral inductive photoperiods (Izawa

et al., 2003). Since sugarcane is also a SD plant, a mechanism of flowering control via *CO* orthologous genes may be shared between these two species. All the *CO*-like candidates found contained an amino terminus B-box superfamily conserved domain, which regulates protein-protein interactions (Torok and Elkin, 2000), and/or the carboxyl terminus CCT domain. They could be grouped into specific classes of the *CO*-like gene family. Cereals possess specific classes that are absent in Arabidopsis; i.e., the group I class genes that contain a single B-box domain and the group IV class, which lacks the B-box domain and has only the CCT domain (Griffiths et al., 2003). No group IV candidate genes were found in sugarcane. Complete ORFs of possible *CO* orthologs in sugarcane could be found, such as CoS1, which has the B-box superfamily conserved domain. CoC1 and CoC2 transcripts were present in different leaf libraries (Figure 2b). Blastp analyses identified barley and Arabidopsis *CO*-like genes; phylogenetic analyses suggested that they are related to subgroups I and III, respectively. The blastp and the phylogenetic results showed a closer relationship of CoC1 to monocot *CO* orthologs, suggesting that this contig may be the candidate of *CO* in sugarcane. Functional characterization needs to be performed to verify under which conditions (SD or LD) this gene is induced and/or repressed to determine whether or not this mechanism of control is shared between sugarcane and rice.

4.3 *Early heading date1*

A putative rice *Ehd1* B-type response regulator (RR) domain was detected in all sugarcane *EHD1* candidates found at the SUCEST database. In rice, this gene acts as a floral inducer under short-day conditions by controlling the expression

of the *FT* gene, and independently of *Hd1* gene, induces expression of *FT*-like genes after SD treatment in *Hd1*-deficient strains. Rice *Hd1* is expressed only under SD conditions, but *Ehd1* is expressed in both conditions, independent of *Hd1* (Doi et al., 2004). As in rice, a candidate sugarcane ortholog of *Ehd1* may perform this function. A complete ORF of Ehd1C4 was found to possess the RR conserved domain. Additionally, this contig was detected in leaf libraries (Figure 2c), indicating that sugarcane may possess a two-component flower signaling pathway, such as in rice plants (Doi et al., 2004; Endo-Higashi and Isawa, 2011).

4.4 *Heading date7*

The *Ghd7* gene is responsible for inactivation of *Ehd1* under LD conditions in rice. Similar to *Ehd1*, the *Ghd7* gene is a monocot-specific gene, so far found only in rice, whose expression is related to crop grain number (Xue et al., 2008). GHD7 protein contains a CCT motif, which mediates protein-protein interaction and nuclear localization, as found in CO proteins. *Ghd7* candidates found in sugarcane ESTs database are very similar to rice *Ghd7* and *CO-like* genes because they share the same conserved domain. Due to this feature, blastp analyses found sequences related to *CO* genes (Table 1). However, when the analyses for *CO* genes were performed, these genes were not found, suggesting that they are *Ghd7* candidates. *Ghd7* contributes to the adaptation of rice cultivars to cold-climate regions (Xue et al., 2008), and it has been recently revealed that *Ghd7* transcription is mediated through phytochrome signaling and is gated in a photoperiod-dependent manner (Itoh et al., 2010). Electronic Northern results showed high abundance of potential *Ghd7* orthologs in sugarcane leaf tissue libraries, such as Ghd7C4, as in rice, where *Ghd7* is

strongly expressed in the blades of fully expanded leaves (Xue et al., 2008). This suggests that this candidate may have an important function in the flowering process (FIGURE 2d), although expression in leaves alone does not mean that this gene is involved in the floral induction. There is a correlation between the *Ehd1* levels and *Ghd7* induction under non-inductive LD conditions, where *Ghd7* represses transcription of *Ehd1,* thus subsequently affecting expression of *Heading date 3a* (Hd3a, a rice florigen) (Itoh et al., 2010). The existence of this pathway may be predicted in sugarcane, since candidate orthologs were found in SUCEST.

4.5 *Flowering locus T*

The *FT* gene, which encodes a phosphatidylethanolamine binding protein (PEBP)-related protein, is highly conserved in plants (Kobayashi et al., 1999). The FT protein has been found to move to the SAM, via the phloem, where it acts as a floral stimulus by activating the FD transcription factor (Corbesier et al, 2007). *FT*-related genes have been found in monocot plants such as wheat (19 genes), maize (30 genes, according to Chardon and Damerval, 2005; and 25 genes according to Danilevskaya et al., 2008) and rice (19 genes). Within the *ZCN* superfamily there are three major subfamilies: *FT*-like, *MFT*-like and *TFL1*-like (Danilevskaya et al., 2008). Candidates for all members of the *ZCN* superfamily could be found, including members of the *FT*-like I group, comprising monocot floral activators such as *Hd3a* (Danilevskaya et al., 2008). Maize possesses *TFL1*-like genes, named *ZCN1* to *ZCN6* genes, which when expressed ectopically modify the flowering time and inflorescence architecture in transgenic maize plants and maintain the indeterminacy of vegetative

meristems (Danilevskaya et al., 2010). *TLF1*, which is a meristem identity gene, is an antagonist of the *FT* gene, despite the similarity in their sequences (Kobayashi et al., 1999; Tahery et al., 2009). The candidate FtS2 is a possible sugarcane *FT* ortholog; it contains the complete PEBP superfamily domain, and transcripts have been found in mature leaf libraries (Figure 2e), as would be expected of a potential florigen-encoding gene. Despite a blastp analysis suggesting that FtS2 is highly similar to TFL1 protein, which is an antagonist of the FT protein, FtS2 is expressed in mature leaf tissues, indicating that it is more likely an FT ortholog rather than TFL1, which is expressed in inflorescence meristem tissues (Tahery et al., 2009).

4.6 *ZCN8*

Recent findings show that *ZCN8* is an *FT* ortholog gene in maize, teosinte and tropical short-day maize (Lazakis et al., 2011; Meng et al, 2011). Exposure of teosinte plants to short-day photoperiods that cause flowering is correlated with a large increase in *ZCN8* expression. A more moderate increase in ZCN8 expression has been observed in maize, a neutral-day plant, under SD conditions. Other evidence has shown that *ZCN8* is involved in photoperiod sensitivity, acting as a florigen, since ectopic expression in *Arabidopsis* rescues the *ft* mutant phenotype (Lazakis et al., 2011). Moreover, *ZCN8* silencing experiments showed late flowering of transgenic maize plants (Meng et al., 2011). No clear ortholog of *ZCN8/FT* was found in blastp searches, and the expression profile showed no transcript in leaf tissues (Figure 2e). However, through phylogenetic analyses, the FtS1 and FtS5 EST-contigs were the most related to *ZCN8*. Together, this information suggests that *ZCN*-like superfamily

gene function is conserved between the species, and that some of them may act as floral activators in sugarcane.

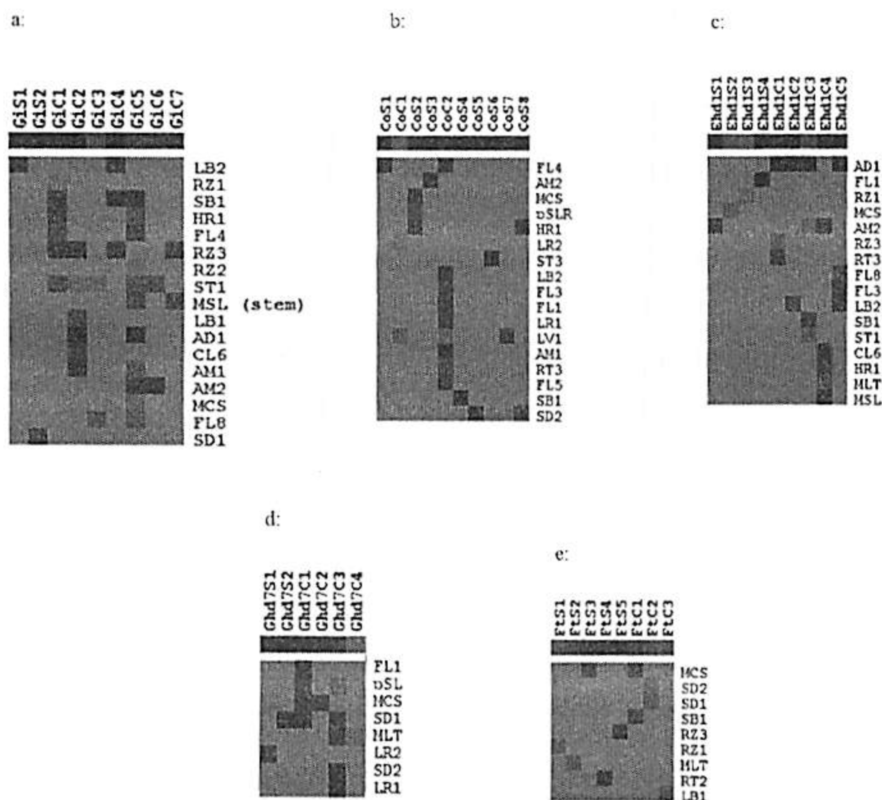

Figure 2. *In silico* expression profile of putative elements of the flowering pathway under photoperiodic control: **A.** *Gigantea*; **B.** *Constans*; **C.** *Early heading date1*; **D.** *Heading date7*; **E.** *Flowering locus T*. The normalized numbers of reads for the transcripts in each library are represented in a scale from black to red. The contigs (C) and singlets (S) are represented as columns and the sugarcane libraries as lines. Sugarcane libraries are as follows: FL4 (developed inflorescence and rachis), AM2 (apical meristem and tissues surrounding of immature plants), MCS (stem), pSRL (leaf roll including apex after floral induction), HR1 (seedling inoculated with *Herbaspirilum rubrisubal*), LR2 [(leaf roll from field-grown adult plants (small insert)], ST3 (fourth apical stalk internodes of adult plants), LB2 (lateral buds from adult plants), FL3 (base of developing inflorescence), FL1 (inflorescence at the beginning of development), LR1 [leaf roll from field-grown adult plants (large insert)], LV1 (etiolated leaves from *in*

vitro-grown seedlings), AM1 (apical meristem and tissues surrounding of mature plants), RT3 (root apex from adult plants), FL5 (developed inflorescence), SB1 (stalk bark from adult plants), SD2 (developing seeds), AD1 (seedlings inoculated with *Gluconacetobacter diazot*), FL1 [inflorescence at beginning of development (1 cm long)], RZ1 [shoot-root transition zone from young plants (large insert)], RZ3 (shoot-root transition zone from adult plants), FL8 [developing inflorescence and rachis (10 cm long)], ST1 (first apical stalk internodes of adult plants), CL6 [pool of sugarcane calli submitted to low temperature (4°C)], MLT (mature leaf tissue), MSL (sugarcane mature stem library), SD1 [developing seeds (large insert library)], RT2 [root tips (0.3 cm long) from adult plants], LB1 (lateral buds from field-grown adult plants), pSL (leaves after floral induction), RZ2 [shoot-root transition zone from young plants (small insert)].

A model for the photoperiodic mechanism of the flowering pathway (Figure 3) is proposed based on a comparative transcriptome and sequence conservation analysis. This preliminary study of the flowering time genes in sugarcane provides basic information for in-depth studies relating to the flowering process in this important crop. Further analyses of the genes identified will also provide an in-depth understanding of the photoperiodic control of this crucial metabolic process. Moreover, functional characterization will help to unravel the molecular basis of the flowering process in different varieties of sugarcane with distinct florigenic potential.

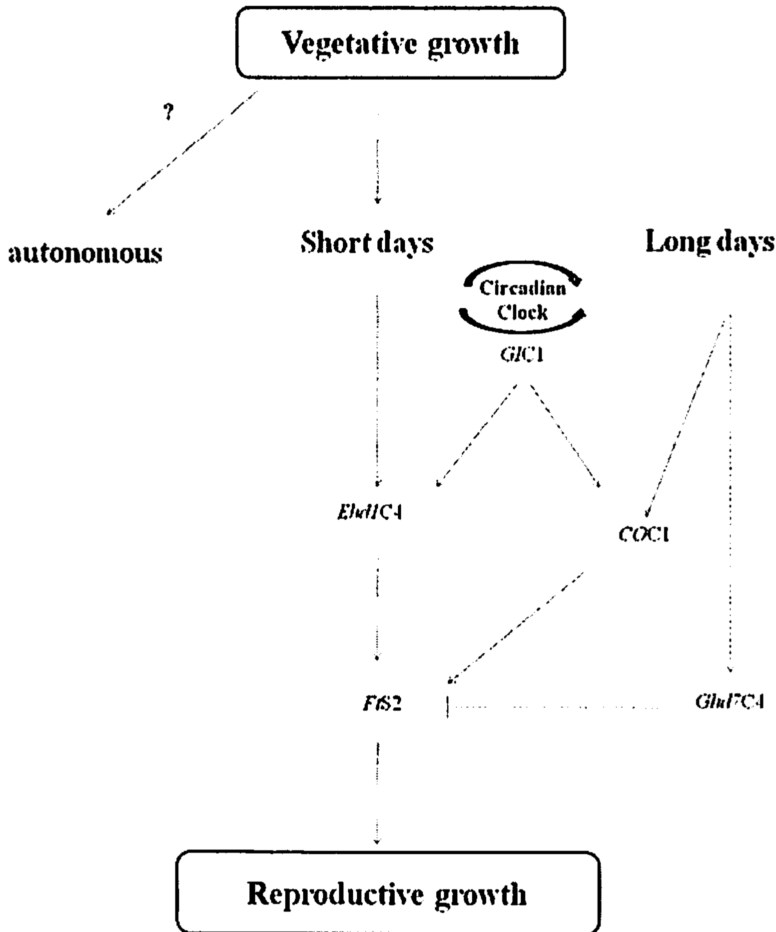

Figure 3. A hypothetical model of the gene network controlling floral induction under photoperiodic control in sugarcane. Photoperiodism and circadian clock controls the pathway by regulation of *GI*, *EHD1*, and *CO* ortholog genes. Two distinct mechanisms are assumed to be involved in the regulation of the *FT* ortholog gene, one under long-day and short-day conditions, which induces *FtS2* expression and another mechanism through the component *GHD7* ortholog, which suppresses *FtS2* expression in non-inducing conditions.

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PAPER 2

Putative sugarcane FT/TFL1 members delay flowering time and alter reproductive architecture in Arabidopsis

The following chapter entitled: "**Putative sugarcane *FT/TFL1* members delay flowering time and alter reproductive architecture in Arabidopsis.**" contains data of the paper that was submitted to the peer-reviewed journal *Frontiers in Plant Genetics and Genomics*.

ABSTRACT

Floral induction in crop species is directly related to crop yield. Although most crop species are evolutionarily distant from Arabidopsis, the flowering process is mostly conserved in higher plants. Comparative studies among available genomes such as Arabidopsis, rice and maize are an important resource for the identification and characterization of orthologs in sugarcane, a complex polyploid plant, that has not been sequenced completely yet. PEBP members, *FT* and *TFL1,* play an essential role as mobile signals controlling the transit from vegetative to reproductive growth. Recent discoveries on PEBP family members in plants have suggested a more complex function beyond flowering induction in diverse plant species, and furthermore, that FT/TFL1 seem to be partially conserved between annual and perennial plants. Perennial plants depend on the maintenance of growth through several season conditions, therefore, they need to control the increasing of age to coordinate competence to flower. Flowering in sugarcane is an important factor that negatively affects cane yield and loss of sugar/ethanol production from this important perennial bioenergy species. Here we identify several PEBP members in sugarcane EST database and show that two *FT/TFL1* orthologs in sugarcane are involved not only in flowering time but also affect plant reproductive architecture in Arabidopsis.

Keywords: *Saccharum* spp., bioenergy crop, floral induction, *FT*-like genes, orthologs, PEBP family

1 INTRODUCTION

Flowering time is a crucial and highly controlled mechanism in plants, as it impacts reproductive success and survival (Imaizumi and Kay, 2006). Plants

have developed a core signaling pathway which is responsible for the integration of day-length perception into developmental reprogramming of the cells. Signals are initiated outside of the SAM and a cascade of responses is triggered, ultimately reaching the SAM where cell changes occur, leading to the formation of floral structures instead of leaves. Six signaling pathways are described: photoperiodic, autonomous, vernalization, gibberellin, ambient temperature and age-dependent control (Fornara et al, 2010). FT/TFL1 are phosphatidylethanolamine-binding protein (PEBP) family members that are similar to mammal PEBP proteins (Ahn et al, 2006; Banfield et al, 1998). TFL1 is responsible for the maintenance of inflorescence in Arabidopsis. Although *TFL1* presents a highly conserved sequence to *FT* mRNA, it acts antagonistically by delaying floral commitment (Hanzawa et al, 2005; Ahn et al, 2006). TFL1 also binds to FD to repress downstream genes such as *AP1* and *LFY* in the central zone of the meristem (Ratcliffe et al., 1999; Hanano and Goto, 2011). Opposing function of *TFL1* and floral meristem genes reflects on specific expression to separate domains. *TFL1* is expressed in central cells of the SAM and floral meristem genes are concentrated to the peripheral cells (Mandel et al., 1992; Weigel et al., 1992; Kempin et al., 1995; Bradley et al., 1997). When floral meristem identity genes expression is reduced, flowers present shoot-like characteristics (Irish and Sussex 1990; Schultz and Haughn 1991, 1993; Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Shannon and Meeks-Wagner, 1993). Upon floral transition, *TFL1* is up-regulated to maintain the indeterminate inflorescence meristem and to counterbalance *FT* expression (Shannon and Meeks-Wagner, 1991; Bradley et al., 1997; Ratcliffe et al., 1999; Conti and Bradley, 2007; Hanano and Goto, 2011; Jaeger et al, 2013).

Several structural and biochemical aspects of the FT protein support the hypothesis that FT is the major component of the florigen complex that triggers floral evocation in the SAM (Taoka et al, 2013). FT is encoded by the *FT* gene which is expressed in phloem specific tissues under inductive long day conditions (Takada and Goto, 2003; An et al, 2004), FT requires intercellular trafficking from companion cells (Jaeger and Wigge, 2007; Mathieu, 2007) and FT homologs were found to act similarly in related species (Tamaki et al, 2007; Lifschitz et al, 2006; Lin et al, 2007; Corbesier et al, 2007; Meng et al, 2011; Lazakis et al, 2011; Kojima et al, 2002). For instance, in rice, the FT ortholog, Heading date3 (Hd3a), is a mobile signal synthesized in the leaves and capable of being transited to the SAM (Kojima et al, 2002; Tamaki et al, 2007), Zea mays CENTRORADIALIS8 (ZCN8) is a leaf-produced molecule that induces flowering when over-expressed in Arabidopsis under control of a phloem-specific promoter (Meng et al, 2011; Lazakis et al, 2011), tomato *SINGLE FLOWER TRUSS* (*SFT*) dependent graft-trasmissible elements complement developmental defects in *sft* mutants and substitute long-day conditions in Arabidopsis (Lifschitz et al, 2006), and the *Beta vulgaris FT2 (BvFT2)* is needed for normal flower initiation in sugarbeet (Pin et al, 2010).

Distinct from Arabidopsis, several plants show more than one *FT* homolog and sequence analysis suggests that a few variation in specific regions of the gene are responsible for alternative functions, such as floral repression (Ahn et al, 2006; Hanzawa et al, 2005; Harig et al, 2012; Pin et al, 2010; Blackman et al, 2010). This observation has raised the hypothesis that the FT ancestor is a floral repressor (Karlgren et al, 2011; Harig et al, 2012). Thus, despite the classic role of FT in flowering time, recent discoveries have associated it to other meristem-related mechanisms (Shalit et al, 2009; Navarro et al, 2011; Bohlenius et al,

2006), consolidating FT as a key mobile signal that is related to diverse developmental changes in plant species.

Perennial plants depend on the maintenance of growth through several seasons, balancing nutritional status, biomass accumulation and alternating between vegetative and reproductive growth over the years. Flowering time genes seem to be partially conserved between annual and perennial plants (Albani and Coupland, 2010), however perennial plants also need to control the increasing of age to coordinate competence to flower. In perennial *Arabis alpina*, sensitivity to vernalization depends on the age of the plant, condition by which a PEBP member, *AaTFL1*, sets a threshold to control age-dependent pathway to flowering (Wang et al, 2001; Bergonzi et al, 2013). In the perennial sugarcane, a qualitative short-day plant, little is known about the genetic control of floral induction. It has been observed that shortening of the days and cooler temperatures promote floral induction. This event is undesirable in commercial cultivars, because it leads to diversion of sucrose that has been accumulated in the stalks to produce reproductive structures (Berding and Hurney, 2005). Genome size and complexity is still a limitation for genetic improvement in sugarcane, hampering the understanding of molecular mechanisms that underlie physiological processes such as floral transition. The understanding of the genetic aspects of flowering time in sugarcane will provide a basis for the development of new strategies to increase agronomical traits such as increased biomass by manipulating floral promoter/repressor proteins in this important bioenergy crop. For the first time, we provide evidence that two sugarcane PEBP members alter flowering time and floral architecture in Arabidopsis.

2 Material and methods

2.1 Plant growth conditions and genotyping

Sugarcane plants were grown in the greenhouse under long-day 14 hour days at 27°C and 10 hour nights at 22°C and short-day inductive conditions of 12 hour days in the field with 20-20-20 fertilizer supplied with micronutrients added as required. Arabidopsis plants, ecotype *Columbia (Col-0)* were cultivated in Conviron growth chambers under 16hour days at 23°C and 8 hour nights at 21°C, light intensity 120μmol.m^{-2}.s^{-1}, and humidity of 60%. Arabidopsis segregating plants were genotyped for the transgene using the Sigma REDExtract-N-Amp Plant PCR Kit (Sigma Biosciences) following manufacturer's instructions, and PCR was performed using kanamycin primers - KanrF: 5'-ATACTTTCTCGGCAGGAGCA-3' and KanrR: 5'-ACAAGCCGTTTTACGTTTGG-3'.

2.2 Isolation and cloning of *FT/TFL1* homologs from sugarcane leaves

Mature and immature leaf tissues from sugarcane plants under inductive and non-inductive conditions were collected for total RNA extraction (TRIzol Reagent) and genomic DNA (as described previously (Colasanti et al, 1998) . For RNA essays, complementary cDNA was synthesized using the qScript cDNA SuperMix (Quanta Bioscences) according to the manufacturer's instructions. Sequences were amplified using specific primers designed at the UTR region of the genes: *ScTFL1F*: 5'-GTCCGATTAGCTTGCTGCAT-3';

ScTFL1R: 5'-GGCCATGCTCATAACTTTGG-3'; *ScFT1F*: 5'-ATATGGCTAATGACTCCCTGACG-3'; *ScFT1R*: 5'-CTGGACATGAGGGGTAGGTAAAT-3'. Genomic and complementary *ScTFL1/ScFT1* sequences were cloned to the CloneJET PCR Cloning Gene (Thermo Scientific) and sequenced.

2.3 Phylogenetic analysis of the ScTFL1 and ScFT1 candidates with orthologs of related species

Deduced amino acid sequences of sugarcane ScTFL1/ScFT1 compared to homologs from other species, were aligned with translated sequences for Arabidopsis *TFL1* and *FT; ZCN1, ZCN2* and *ZCN8* (maize), *RCN1 and Hd3a* (rice), tobacco *NtFT1*to *NtFT4*, sugarbeet *BvFT1* and *BvFT2*, in BioEdit 7.1.3.0 (Hall, 1999). Phylogenetic trees were constructed by MEGA software, version 4.0 (Tamura et al., 2007), with the neighbor-joining comparison model (Saitou and Nei, 1987), *p*-distance method and pair-wise deletion. Bootstrap values from 1000 replicates were used to assess the robustness of the trees (Felsenstein, 1985). Gene structure information for homologs was accessed at the Phytozome 9.1 genome database available online (www.phytozome.net).

2.4 Construction of overexpression vector and Arabidopsis transformation

Candidate genes for *TFL1* and *FT* were cloned into a Gateway entry vector, pDONR-221, using the BP recombination reaction and the subsequent products were recombined with the destination vector pK2GW7 by a LR clonase,

originating the expression vector 35S::*ScTFL1* and 35S::*ScFT1*. Gateway sites were inserted to the sequencing primers for the cloning reaction as follow; ScTFL1gatF: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGTCCGATTAGCTTGCTGCAT-3' and ScTFL1gatR: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGCCATGCTCATAACTTTGG-3' and ScFT1gatF: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGGCTAATGACTCCCTGACG-3' and ScFT1gatR: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGACATGAGGGGTAGGTAAAT-3'. *Agrobacterium tumefaciens* strain GV3101::pMP90 containing the over-expression constructs was introduced to Arabidopsis plants by floral dip (Clough and Bent, 1998). *Agrobacterium* containing *ScTFL1* and *ScFT1* over-expression constructs were introduced to the Columbia (*Col-0)* ecotype. Four independent lines of T2 plants (in *Col-0* background) were selected for phenotypic analyses of *ScTFL1* and *ScFT1* overexpression.

2.5 Scanning Electron Microscopy (SEM)

Multipe inflorescences were harvested from *ScTFL1* over-expressed plants and image was captured with a Hitachi Tabletop TM-1000 Scanning Electron Microscope. Dimension bars were added using the ImageJ software.

3 Results

3.1 Isolation of a *TFL1* homolog from *Saccharum* spp. and expression pattern in different tissues

Candidates for the *FT/TFL1* family were identified from the sugarcane EST database, SUCEST (Coelho et al, 2013; Vettore et al, 2001). Candidate FtS3, from now on referred as *ScTFL1*, was the unique assembled complete sequence identified that belongs to the TFL1-*like* subfamily, presenting a 93% and 84% amino acid identity to maize *ZCN1* and *ZCN2*, respectively; 92% to rice *RCN1* and 70% to *TFL1* (Figure 1-D). As expected, ScTFL1 sequence is more similar to rice and maize homologs, compared to Arabidopsis (Figure 1-B). TFL1 is highly conserved between species, as has been reported for several flowering time genes in different species (Taylor et al, 2010; Hecht et al, 2005; Danilevskaya et al, 2010; Mauro-Herrera et al, 2013).

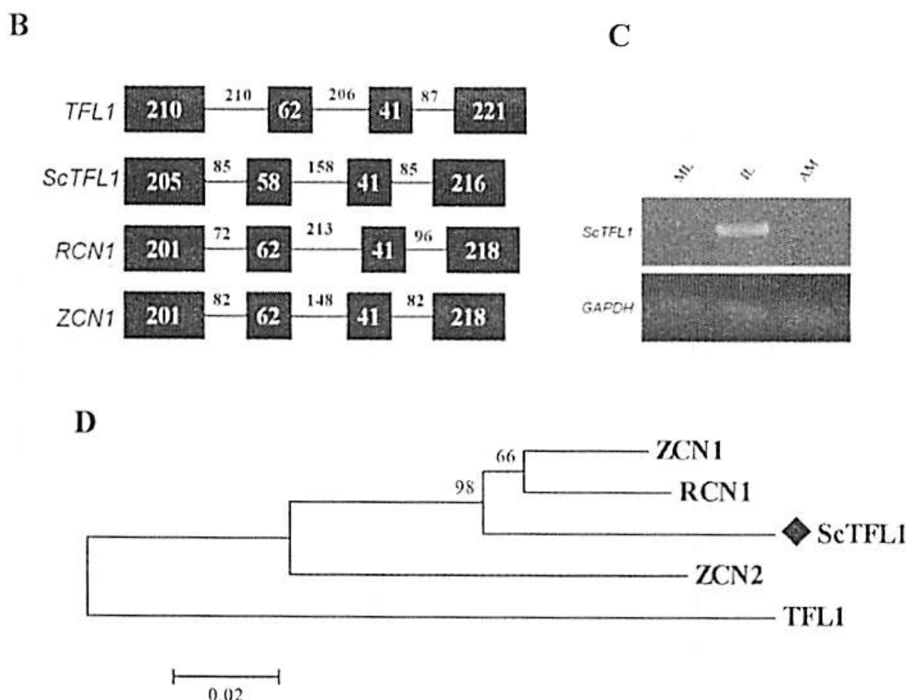


Figure 1 - Sequence conservation among TFL-like genes. A) Alignment of the ScTFL1 candidate with homologs from different species: Arabidopsis TFL1, rice RCN1; and maize ZCN1. *asterisk highlights the amino acid residue conservation in all TFL1 homologs. B) TFL1 gene structure conservation among TFL1 homologs, consisting of four exons and three introns. Boxes represent exons and lines, introns. Numbers indicate size of each exon and intron; C) Expression pattern of *ScTFL1* of different tissues by semi-quantitative PCR, sugarcane *GAPDH* endogenous control was used as control; IL: apex-surrounding immature leaves; ML: mature leaves; AM: apical meristem; D) Evolutionary relantioship of TFL1 homologs. Amino acid sequences from different species were aligned using ClustalW, the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap values from 1000 replications were used to assess the robustness of the trees. Sugarcane TFL1 candidate gene is highlighted by a diamond symbol (♦) and TFL1 homolog from related species are deposited at the Genbank database. Accession numbers: ZCN1 (ABX11003.1), ZCN2 (ABX11004.1), RCN1 (ABA95827.1) and TFL1 (AED90661.1).

Phosphatidyl ethanolamine-binding protein (PEBP) family is constituted of small proteins that was first found in mammals, responsible for the inhibition of several enzymes in mammals, such as Raf-kinase (Schoentgen et al, 1995;

Yeung et al, 1999). In plants, PEBP proteins are general regulators of signaling complexes, as shown for tomato SELF PRUNNING (SFP), a homolog of TFL1 that acts by interacting with different proteins (Pnueli et al, 2001). PEBP family consists of three subfamilies named as MFT-like, TFL1-like and FT-like (Chardon and Damerval, 2005). In sugarcane, eight candidates of the PEBP family were identified by *in silico* analysis, represented by one MFT-like, one TFL1-like and six FT-like candidates (Coelho et al, 2013). It is possible, however, that sugarcane genome contains more members. Maize is represented by six members of the *TFL1*-like subfamily, *ZCN1* to *ZCN6* (Danilevskaya et al, 2010) and in rice, four members were identified: *Oscen1* to *-4* (Nakagawa et al, 2002). The completion of the sugarcane genome sequencing project will help to identify all the PEBP members in this species.

Comparing the translated sequence of the ScTFL1 to the other homologs we observed that all of them share a histidine residue in the position 89 (H89) (Figure 1-A), which in Arabidopsis is a key position to determine TFL1 or FT activity as a floral repressor or activator, respectively (Hanzawa et al, 2005). Gene structure of ScTFL1 is similar to the related *TFL1* orthologs, consisting of three introns and four exons of similar sizes. (Figure 1-B).

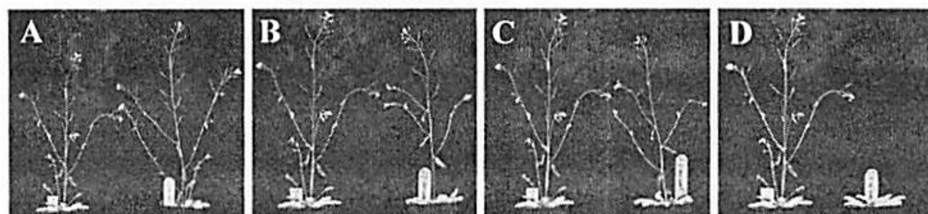


Figure 2 - Ectopic *ScTFL1* expression affects flowering in four independent lines of transgenic Arabidopsis, under long-day conditions after 43 days. (A) 35S::ScTLF1-5;

(B) 35S::ScTFL1-6; (C) 35S::ScTFL1-11; (D) 35S::ScTFL1-41. All transgenic lines (right) were compared to Col-0 wild-type plants (left).

The fourth exon, which contains the segment B, is critical for FT to function as a floral activator or TFL1 as a floral repressor (Ahn et al, 2006). This segment forms an external loop that evolves rapidly in TFL1 but not in FT, and it seems that the divergent activity in flower induction is derived from a hydrogen bond formation close to the binding pocket in TFL1 but not in FT, suggesting that this segment is crucial for the co-activation of specific yet-to-be identified FT/TFL1 interactors (Ahn et al, 2006; Taoka et al, 2011; Pin et al, 2010; Harig et al, 2012; Taoka et al, 2013). Consistent with this, FT present a tyrosine residue at position 85 (Y85) and this is a key difference for FT to function as a floral activator in Arabidopsis (Hanzawa et al, 2005), but in some species it has been reported that the FT-likes presenting the Y85 may act as a floral repressor if there is variation in the segment B (Harig et al, 2012; Pin et al, 2010).

We compared the expression pattern of *ScTFL1* at the apical meristem, mature leaves and the immature leaves surrounding the meristem of seven-month old sugarcane plants. *ScTFL1* was expressed specifically to young leaves that enfold the meristem (Figure 1-C). It has been shown before that, similar to FT, TFL1 is a mobile protein that can move from the site of transcription to peripheral areas of the meristem. In Arabidopsis, *TFL1* is expressed in young axillary meristems and is later arrested to the central core of the meristem (Conti and Bradley, 2007). *ScTFL1* expression domain suggests that this gene is active in the adjacent regions of vegetative sugarcane plants. Similarly, in the annual maize plant, *ZCN1* and *ZCN2* are expressed in both vegetative and reproductive

phases, in which *ZCN1* is detected in vascular bundles of leaf primordia and *ZCN2* in leaf axils of shoot apices (Danilevskaya et al, 2010).

3.2 Ectopic expression of *ScTFL1* altered flowering time and maintained indeterminate fate of inflorescence meristems in Arabidopsis transgenic plants

To understand the role of sugarcane TFL1 homolog in the control of floral induction, we have examined transgenic Arabidopsis plants over-expressing the *ScTFL1* driven by a constitutive promoter. Four T2 segregating independent lines (ScTFL1-5; ScTFL1-6; ScTFL1-11 and ScTFL1-41) were selected for further analysis and ten T2 plants from each line were produced. All lines flowered later than the wild-type control and the vegetative stage was extended, as an increase in the number of rosette leaves in all transgenic lines, that ranged from 15.4 to 17.7 leaves, compared to the 11.4 leaves formed in Col-0 (Table 1).

Table 1 - Flowering characteristics of four 35S::*ScTFL1* independent lines.

Plant genotype	Days to flowering	Number of leaves	Number of plants
Columbia (Col-0) background			
Col-0 wild-type	32	11.4±0.54	5
ScTFL1-5	41	15.4±1.35[a]	10
ScTFL1-6	41	14.3±1.34[a]	10
ScTFL1-11	41	15.2±1.73[a]	10
ScTFL1-41	47	17.7±1.60[a]	10

[a] Indicates that the value was statistically different from the wild-type with a $p>0.05$ by the student *t-test*

Ectopic expression of *ScTFL1* altered not only flowering time (Table 1; Figure 2D) but inflorescence structures formation that were visible in the ScTFL1-41 line (Figure 3A). Despite the late flowering phenotype, the lines ScTFL1-5, -6 and -11did not present severe effects on plant architecture (Figure 2A to C). The mild effects in these three lines could be due to segregation and an indication that ScTFL1-41 is homozygous for the transgene at this point.

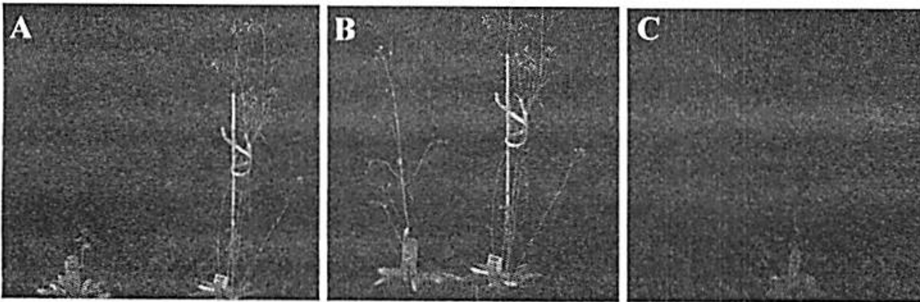


Figure 3 - Ectopic *ScTFL1* expression affects inflorescence architecture in transgenic Arabidopsis. (A) Growth of 35S::ScTLF1-41 transgenic plants (left) and Col-0 (right) under long-day conditions after 50 days; and (B) 55 days; and (C) 64 days of germination, at this point Col-0 wild-type plants have completed the life cycle. All plants are in the Col-0 background.

ScTFL1-41 plants presented extremely late flowering, highly branching phenotype (Figure 3C), shoot-*like* inflorescences, with aerial rosettes, abnormal flower formation (Figure 4) and extended life cycle (>64 days). This is also observed in Arabidopsis over-expression of *TFL1* and respective homologs in grasses, in which developmental phases are delayed with similar effects on plant architecture (Ratcliffe et al, 1998; Ratcliffe et al, 1999; Danilevskaya et al, 2010;

Nakagawa et al, 2002; Jensen et al, 2001).

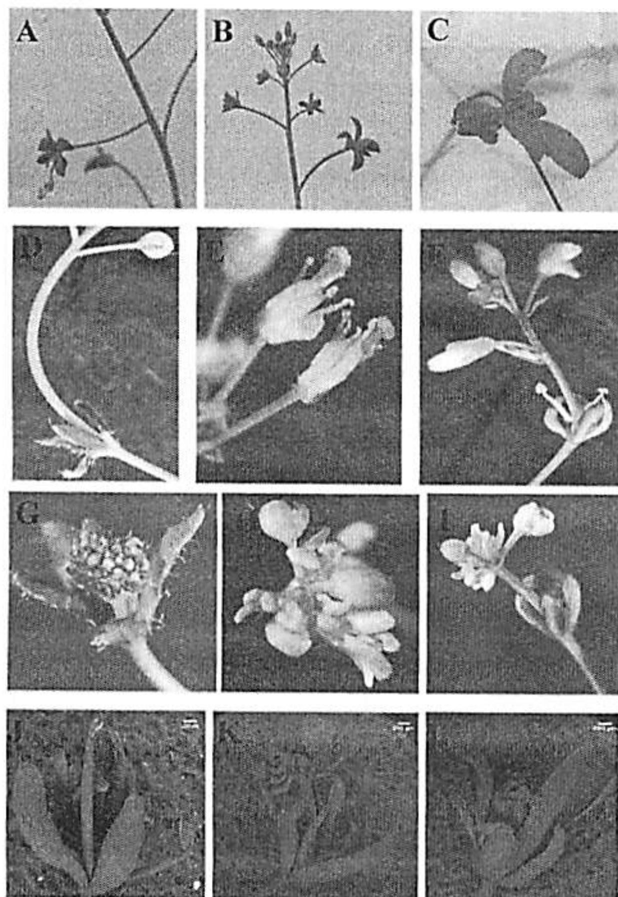


Figure 4 - Phenotypes of *ScTFL1* transgenic plants. (A-D) Examples of aerial rosettes phenotype of 35S::ScTFL1 lines. (E-I) Abnormal flower formation with emerging floral buds. Electronic microscopy showing floral buds emerging from 35S::*ScTF1* inflorescences (J-L).

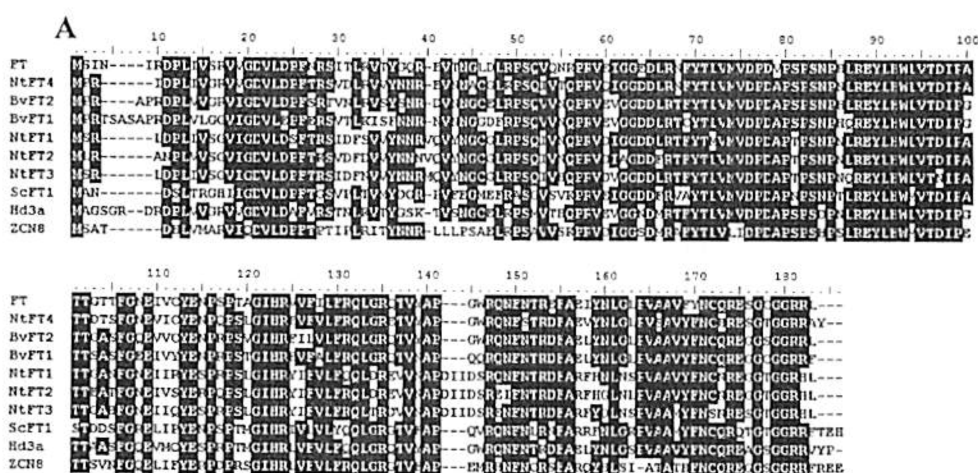
Similar aerial rosette inflorescence phenotypes observed in ScTFL1-41 plants (Figure 4-A to D) have been reported before in Arabidopsis mutants, such as *soc1-2 ful-2* double mutants (Melzer et al, 2008), and *art1* mutants (Poduska et al, 2003). Evidence of indeterminacy and/or delay of phase changes were observed by electron microscopy (Figure 4-J to L), where abnormal flowers

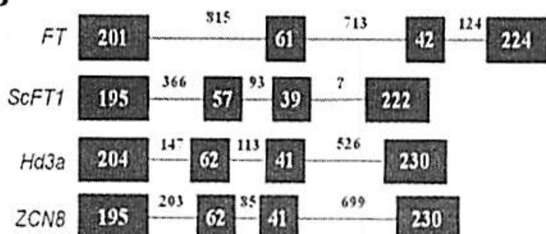
gave rise to floral buds in several flowers of ScTFL1-41 plants (Figure 4-E to I). SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and FRUITFULL (FUL) interconnect the signaling transduction from the FT to the floral meristem identity genes, such as AP1 and LFY to determine flower formation (Melzer et al, 2008). *art1* mutants phenotypes are a consequence of delay from vegetative (V) to reproductive (R) phase transitions, therefore originating a new type of metamer formation consisting of V1 → V2*→ R* → R, in which aerial rosettes are formed by the V2* stage (Poduska et al, 2003). Similarly to this, 35S::*TFL1* plants also delays reproductive transition in consequence of the prolonged vegetative phase (Ratcliffe et al, 1998; Ratcliffe et al, 1999).

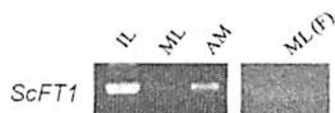
3.3 *ScFT1* is a putative FT homolog and is developmentally expressed in sugarcane tissues

In parallel to *ScTFL1* characterization, we isolated the candidate FtS1 (referred to as *ScFT1*) from mature sugarcane plants and compared the sequence and expression pattern to FT homologs. Expression of ScFT1 was not specific to mature leaves of plants under inductive flowering conditions, a common pattern of FT homologs. Interestingly, *ScFT1* was not expressed in mature leaves of young plants, but it was expressed in leaves from flowering mature plants (Figure 5-C). Like all PEBP family members, ScFT1 gene structure consists of four exons and three introns, with similar exon sizes but largely varying the number of nucleotides in the introns (Figure 5-B). We compared the translated sequence to FT homologs from different species with ScFT1 (Figure 5-A) and its sequence is more similar to candidates involved in the early flowering phenotype. ScFT1 presented 59% amino acid identity to *FT*, 59% to rice Hd3a;

57% to ZCN8 in maize; 62% to floral inductor in sugarbeet BvFT2 and 61% to the tobacco NtFT4. Sugarbeet and tobacco candidates that act antagonistically to flowering presented a lower similarity to ScFT1: NtFT1, -2, -3 were 57, 54 and 54%, respectively and the sugarbeet BvFT1, 59%.







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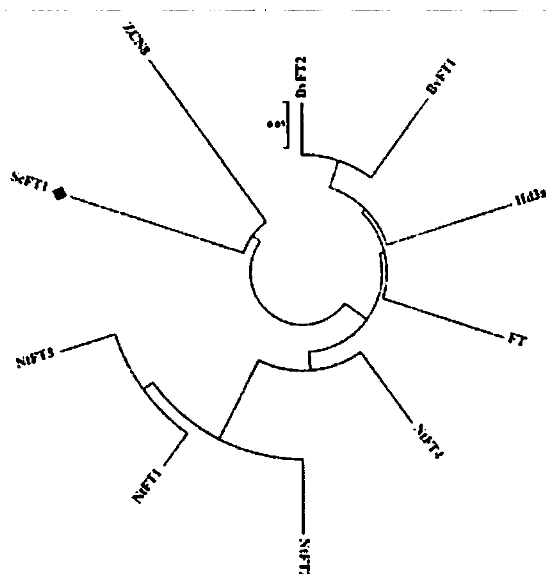


Figure 5 - Sequence conservation among FT-like genes. A) Alignment of the ScFT1 candidate with homologs from different species: Arabidopsis FT, rice Hd3a; and maize ZCN8; tobacco NtFT1-4; sugarbeet BvFT1/2. *asterisk highlights the amino acid residue conservation in all FT homologs. B) Evolutionary relantioship of TFL1 homologs. Amino acid sequences from different species were aligned using ClustalW, the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bootstrap values from 1000 replications were used to assess the robustness of the trees. Sugarcane TFL1 candidate gene is highlighted by a diamond symbol (♦) and TFL1 homolog from related species are deposited at the Genbank database. Accession numbers: BvFT1 (ADM92608.1), BvFT2 (ADM92610.1), NtFT1 (AFS17369.1), NtFT2 (AFS17370.1), NtFT3 (AFS17371.1), NtFT4 (AFS17372.1), FT (BAA77838.1), Hd3a (BAB61030.1), ZCN8 (ABX11010.1).; C) Expression pattern of *ScFT1* of different tissues by semi-quantitative PCR, IL: apex-surrounding immature leaves; ML: mature leaves; AM: apical meristem; ML(F): mature leaves of mature flowering plants.

Phylogenetic analysis of FT-like shows that FT-like floral repressors from tobacco clade together and the floral promoter NtFT4 clades with the FT-like activators, FT and Hd3a (Figure 5-D). As expected, ScFT1 clades to its closely related maize ZCN8, but not to other FT-like activators, such as FT and Hd3a.

Despite the fact that ZCN8 does not clade with FT-like floral inductors, it acts as a floral activator in maize (Lazakis et al, 2011; Meng et al, 2011). To test if sugarcane FT-like candidate is involved in flowering time, we constitutively over-expressed *ScFT1* in Arabidopsis.

3.4 Transgenic plants overexpressing *ScFT1* delayed flowering and presented abnormal siliques development

One sugarcane FT-like gene was isolated and over-expressed in Arabidopsis. Four independent lines were selected for flowering time analysis. Unexpectedly, *ScFT1* over-expression generated late flowering plants, producing an average ranging from 16.1 to 24.5 rosette leaves in four independent lines, compared to the 11.4 rosette leaves of Col-0 wild-type (Figure 6).

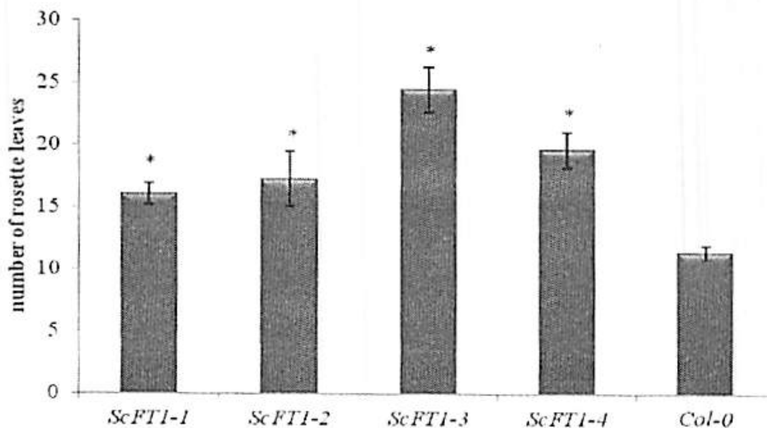


Figure 6 - Flowering time of four independent lines compared to Col-0 wild-type plants. asterisk (*) indicates that the numbers were statistically different from the wild-type with a $p>0.05$ by the student *t-test*.

The most severe phenotype in flowering time was observed in line ScFT1-3, although reproductive abnormalities were consistently observed in line ScFT1-1. *ScFT1*-1 plants presented a higher number of sterile flowers, forming abnormal shorter siliques (Figure 7-A). Most of the siliques did not form properly, leading to poor seed set, and most of the plants were sterile. In wild-type plants, open flowers self-fertilize originating seed-containing siliques. In the ScFT1 transgenic lines, open flowers seems to be unable to self-fertilize and siliques are not developed from the fertilized carpel, explaining the shorter phenotype (Figure 7-B).

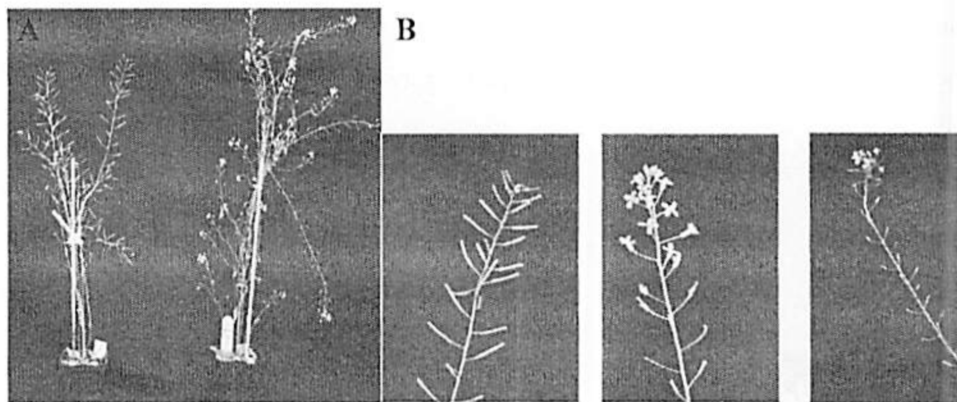
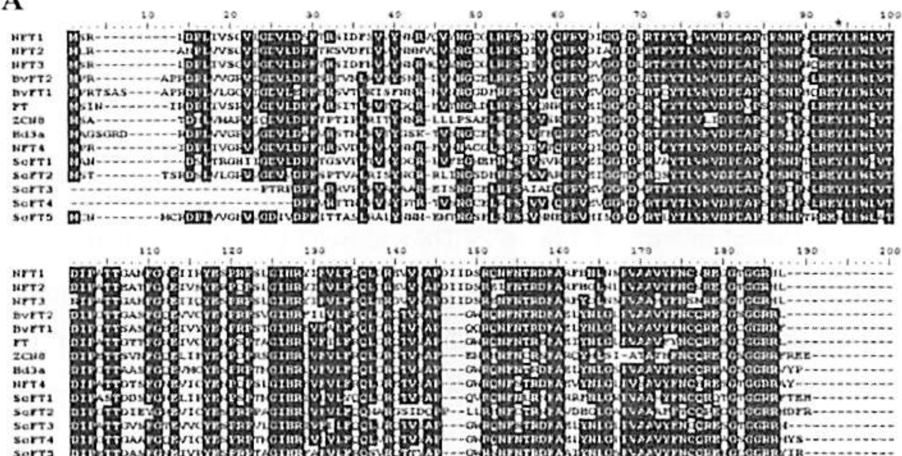


Figure 7 - Ectopic expression of ScFT1-1 affects flowering time and siliques development. (A) Comparison of development timing of ScFT1-1 (right) with Col-0 wild-type plant (left). (B) Close-up at the siliques from Col-0 (left), ScFT1-1 flowers (middle) and abnormal siliques (right).

3.5 Yet to be characterized ScFT-likes may be involved in floral induction in sugarcane

ScFT1 is not the only sugarcane FT-like candidate. Four other incomplete sequences were identified in the sugarcane EST database (SUCEST), named as ScFT2, ScFT3, ScFt4 and ScFT5.

ScFT2 is the closest related FT-like to ZCN8 and ScFT1 that was functionally analysed here. ScFT3 and ScFT4 putative homologs clade together to all floral promoter FT-like genes, Hd3a, FT and BvFT1 (Figure 8-B), indicating that we cannot rule out the hypothesis that one (or both) of them may act as a florigen in sugarcane. Functional characterization of these candidates will enlighten this hypothesis. In addition to the phylogenetic relationship between ScFT3 and ScFT4 to floral promoters, sequence analysis also indicates that these PEBP members should act as floral promoters, considering for instance, the high degree of similarity of the segment B compared to all FT floral promoting proteins (Figure 8-A). Segment B is the region where floral activators bind to and it has been reported that its conservation is the key difference among FT-like acting as floral promoters and floral repressors (Taoka et al, 2013).



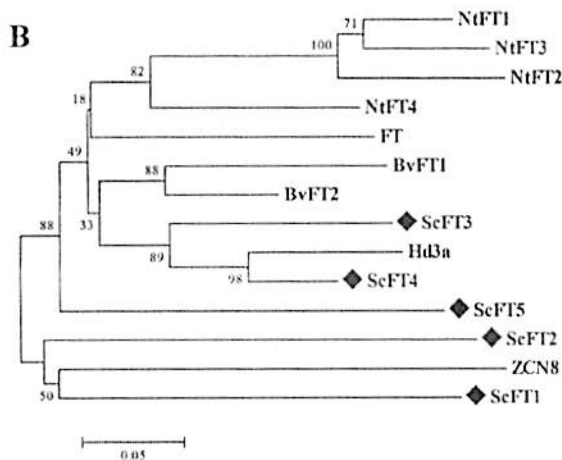


Figure 8 - Sequence analysis of ScFT candidates with other FT homologs. (A) Amino acid sequences from different species were aligned to five ScFT-like using ClustalW. Segment B is highlighted by a black line. (B) Phylogenetic tree of FT homologs. Bootstrap values from 1000 replications were used to assess the robustness of the tree. Sugarcane ScFT-like candidate genes are highlighted by a diamond symbol (♦) and FT homolog from related species are deposited at the Genbank database. Accession numbers: BvFT1 (ADM92608.1), BvFT2 (ADM92610.1), NtFT1 (AFS17369.1), NtFT2 (AFS17370.1), NtFT3 (AFS17371.1), NtFT4 (AFS17372.1), FT (BAA77838.1), Hd3a (BAB61030.1), ZCN8 (ABX11010.1).

4 DISCUSSION

4.1 ScTFL1 maintains meristem indeterminacy in sugarcane

Plant architecture is extensively and consistently altered by *ScTFL1* overexpression in Arabidopsis plants, leading to formation of aerial rosettes. Aerial rosettes consist of leaf-like structures in the apex of the inflorescences, which is an indication of floral reversion. The aerial rosette is always related to a mutation in a floral meristem identity gene, responsible for the signal transduction to *AP1* gene, integrator of all flowering pathways to determine floral organ formation. By the time *AP1* is expressed, floral determination is initiated and plants continue to flower independently of environmental signals (Hempel et al, 1997). Extended life cycle are also observed in ScTFL1 plants. This suggests that *ScTFL1* acts by extending duration of growth phases and maintenance of the inflorescence meristem in sugarcane. ScTFL1 plants present abnormal floral organ structures, which may be due to an unbalance between *TFL1* and *AP1* expression, leading to floral reversion into vegetative tissues and triggering the appearance of floral buds inside the aerial rosettes, as a consequence of indeterminate growth fate. *AP1* down-regulates *TFL1* in floral meristems and in turn, *TFL1* maintains indeterminate growth of the vegetative center (Ratcliffe et al, 1999). Although flowering prevention has not yet been observed in any single mutants, *BEL1*-like (BELL) homeobox *pennywise pound-foolish (pny pnf)* double mutants never completes floral transition (Smith et al, 2004), and *TFL1* is ectopically expressed at high levels in the vasculature of these mutants, same site for *FT* expression. This indicates that when *TFL1* is ectopically expressed, a non-flowering phenotype is observed. Late flowering is a negative agronomic trait in may crops, but not to commercial sugarcane plants;

instead, late flowering or non flowering phenotypes is a highly desirable trait that has been subject of sugarcane breeding programs (Berding and Hurney, 2005; van Heerden et al, 2010). Late flowering varieties prevent loss of sugar accumulation in the stalks derived from precocious flowering, especially in the tropics where day-length is inductive for floral transition throughout the year.

Increased axillary branching of the *ScTFL1* transgenic plants is similar to the effects of other TFL1 homologs and it has been suggested that this phenotype may be a consequence of interaction of TFL1 with hormones, since plant hormones such as auxin, cytokinin and strigolactone play a role in branching and outgrowth of plants (McSteen, 2009; Danilevskaya et al, 2010). TFL1 protein complex has already been reported and external loop seems to be the site for co-repressors/co-activators to bind and to trigger developmental responses, nevertheless these co-activators/co-repressors have not been identified yet (Taoka et al, 2013), raising the hypothesis that plant hormones could be the missing interactors linking ScTFL1 to the phenotypes observed.

Mechanisms of function of *TFL1* is less clear than *FT*, and it may vary among annual and perennial plants. It has been reported that this gene acts in an age-dependent flowering pathway in the perennial *Arabis alpina*. In this plant, *AaTFL1* is responsible for the maintenance of vegetative growth of young plants even upon inductive conditions, preventing that all the axillary meristems become determined. As the shoot ages, *AaTFL1* sets an increasing flowering threshold and the plant is capable to develop its perennial traits (Wang et al, 2011). In the perennial ryegrass, *LpTFL1* is up-regulated in the apex once the temperature and day-length increases, allowing the lateral branching and

consequently, tillering is promoted (Jensen et al, 2001). It is possible that *ScTFL1* act in a similar manner in the perennial sugarcane, justifying the expression of this gene in leaves surrounding the peripheral regions of the meristem of vegetative young sugarcane plants. It will be interesting to verify, in the future, the expression pattern of *ScTFL1* of mature plants that are flowering.

4.2 ScFT1 protein may have functions other than flowering time control in sugarcane

Evolutionary analysis of the PEBP family suggests that the FT-like and TFL1-like subfamilies arose from a common TFL1-like ancestor, and that FT-like floral promoters evolved within the angiosperm clade (Karlgren et al, 2011; Klintenas et al, 2012). Therefore, it is likely that FT-likes acting as floral repressors may still exist among angiosperms, as has been reported for tobacco (Harig et al, 2012), sugarbeet (Pin et al, 2010) and now for sugarcane.

Although we have no consistent data to completely understand the ScFT1 overexpression phenotype, we have evidence that ScFT1 may be involved in meristem activities that control flowering time and production of fertile organs. Meristem-related functions have been previously associated to FT-like proteins in diverse species (Shalit et al, 2009; Krieger et al, 2010; Bohlenius et al, 2006; Danilevskaya et al, 2011; Navarro et al, 2011).

Similar effects were observed in the *TAPETUM DETERMINANT1* (*tpd1*) mutant, that is involved in the anther development, required for the specialization of tapetal cells. In contrast, over-expression of *TPD1* activates cell division, process that involves meristem activation allowing the anther to properly form (Yang et al, 2005). *DYSFUNCTIONAL TAPETUM1* (*DYT1*) is also involved in tapetum differentiation and function, in which *dyt1* mutant

plants display siliques phenotypes similar to ScFT1 plants (Zhang et al, 2006). *BEL1* and *SHORT INTEGUMENT (SIN1)* control ovule development in Arabidopsis and *bel1* mutations cause ovule integuments into carpels, associated to ectopic expression of *AGAMOUS* (*AG*) in these tissues (Ray et al, 1994). Plant architecture of *bel1* mutant is similar to what we observed in *ScFT1* plants (Robison-Beers et al, 1992). *SIN* has been reported to be required for both flowering time and normal ovule development and also, its interaction with *TFL1* is important to normal pollen development (Ray et al, 1996).

Recently, upcoming discoveries has reported FT-like proteins acting not only as floral repressors but also in diverse developmental events, such as potato tuberization (Navarro et al, 2011), seasonal control of growth cessation in poplar trees (Bohlenius et al, 2006), termination of meristem growth and fruit yield in tomato (Shalit et al, 2009; Krieger et al, 2010), plant architecture in maize (Danilevskaya et al, 2011) and stomatal control in Arabidopsis (Kinoshita et al, 2011). All these together raise fundamental questions of whether FT-like proteins should be solely considered flowering time regulators or to be versatile mobile signals orchestrating diverse processes in plant development (Taoka et al, 2013).

FT-like genes possess critical residues required for the 14-3-3 binding and there is a variation in those that allow the (Florigen Activation Complex) FAC-like to be built and to regulate diverse developmental physiological processes (Taoka et al, 2013). All FT-like genes involved in floral promotion have a conserved region, known as segment B, located in the fourth exon, that is essential for these homologs to act as floral inductors in diverse plant species (Ahn et al, 2006; Pin et al, 2010, Harig et al, 2012). Varying FT-like segment B has been indicated to be floral repressors or involved in other developmental processes

other than floral induction, which is the case of tobacco and sugarbeet. The present work indicates that the segment B of one sugarcane FT-like, *ScFT1*, may be also a critical region for the floral promotion function in this species. Segment B of ScFT1 varied in three amino acid residues compared to FT and Hd3a floral promoters. In sugarbeet the variation of three amino acids in the segment B of two FT-like in this species are sufficient for them to act antagonistically (Pin et al, 2010). Additionally, a study developed with gymnosperms showed that no FT-like was involved in floral promotion, indicating that this function emerged during the early stages of flowering plants evolution (Klintenas et al, 2012). Comparison of several ScFT-like genes in a sugarcane EST database (SUCEST) indicates that the incomplete candidates ScFT3 and/or ScFT4 may be involved in the floral promotion of this species, considering the sequence conservation and phylogenetic relationship to FT-like homologs subfamily. Segment B of ScFT3 is identical to FT-like floral activators. Full-length of these genes need to be characterized to evaluate the effect of sequence plasticity and divergence of functions in sugarcane FT-like genes.

Conflict of Interest Statement

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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PAPER 3

ScID1 and ScFT2 homologs are involved in floral transition of *Saccharum* spp. plants

The following chapter entitled: "**ScID1 and ScFT2 homologs are involved in floral transition of *Saccharum* spp. plants**" contains data of the paper that was submitted to the peer-reviewed journal *Development*.

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ABSTRACT

Saccharum spp. is a polyploid perennial crop that flowers with shortening of the days and temperatures between 18°C and 31°C. Although floral transition is a desirable event in most crop species, this event is highly undesirable in commercial varieties of sugarcane, because it leads to the loss of sucrose accumulated in the stalks to form the reproductive organs. Physiologically, floral transition triggers a process known as "isoporização", which is consisted of water loss from the culms that become dry. ID1 is a monocot-specific putative transcription factor known to be involved in the transmission of a leaf-derived signal in the day-neutral maize independent of photoperiod. It also plays a role in the induction of the *FLOWERING LOCUS T* ortholog in maize, *ZEA MAYS CENTRORADIALIS 8.* Aiming to better understand the floral transition in this species, we have isolated putative sugarcane *ID1* and *FT* homologs, which are key genes under the control of the autonomous and photoperiodic pathways, respectively. Here we show that sugarcane has at least one ID1 ortholog (*ScID1*) that is expressed in non-photosynthetic immature leaves and regulated independently of day-length. Additionally, we report that a FT candidate in sugarcane (*ScFT2*) caused pleiotropic effects on shoot formation and late-flowering phenotypes in Arabidopsis plants. Collectively, these data provides genetic information of key genes involved in floral transition and also gives rise to the hypothesis that although sugarcane responds to short-day conditions, a putative florigen in this species may integrate signals from both photoperiod and autonomous pathways to induce flowering. Also, the identification of more than one floral repressor FT homolog supports previous studies on functional diversification of FT-like genes in diverse species.

1 INTRODUCTION

Sugarcane is an important tropical crop, used as a primary source of sugar production for hundreds of years. *Saccharum* spp. is cultivated in more than twenty million of hectares in the tropical and sub-tropical regions of the world.

Cultivation of this species has gained more attention because of the ethanol production, an important source of renewable energy (Buckeridge et al., 2012; Scortecci et al., 2012).

Saccharum has a complex genome, consisted of polyploid species. Genome of modern cultivars corresponds to hybrids of 70 to 120 chromosomes, derived from crosses of *Saccharum officinarum* and *Saccharum espontaneum* (Daniels and Roach, 1987). Most of the chromosomes were inherited from the *S. officinarum*, and 10% to 25% from *S. espontaneum* (D'Hont et al., 1998).

Conclusion of the Genome Project of EST sequencing in sugarcane (SUCEST - *Sugarcane Expressed Sequence Tag*) has led to significant advances in sugarcane genetic and molecular researches. This project allowed the generation of 26 ESTs libraries and 43.141 *clusters*, and an estimation of more than 30.000 genes (Vettore et al., 2001; Vettore et al., 2003). However, the yet-to-be sequenced genome has hampered molecular breeding by biotechnological tools (D'Hont et al., 2008).

Transition from the vegetative to reproductive growth is an important event to the development of superior plants. The change is observed in vegetative tissues, under the regulation of both environmental and endogenous factors. Plants respond to a combination of day-length, quality of light, temperature, vernalization, and endogenous status such as carbon allocation and age to guarantee optimal conditions to flower (Fornara et al., 2010; Coneva et al., 2012). Perception of environmental conditions triggers a signalling cascade that is transmitted to the shoot apical meristem (SAM). Under inductive conditions, the SAM promotes modifications in the vegetative identity meristem turning it into floral meristems, which lead to floral primordia formation. Although flowering process has been reported to be conserved among species (Battey and

Tooke, 2002; Taylor et al., 2010; Liu et al., 2013), it is important to characterize homologs in other species to identify specific mechanisms of control that may exist (Distelfeld et al., 2009; Pin et al., 2010; Pin and Nilsson, 2012; Bergonzi et al., 2013).

Although the flowering process in grasses shares some common functions with dicot plants, a few specific mechanisms of transmitting inductive signals to floral transition are specific to grasses (Colasanti and Coneva, 2009; Greenup et al., 2009). Temperate maize is a day-neutral plant, which means that floral transition is independent of day-length, and triggered by a developmentally expressed gene, *INDETERMINATE1* (*ID1*) (Colasanti et al., 1998; Coneva et al., 2012). *id1* mutants of maize are unable to flower, producing more leaves than the wild-type (Colasanti et al., 1998). Recent findings have suggested a relationship between carbohydrate allocation and autonomous flowering, indicating a role of ID1 in starch/sucrose balance (Coneva et al., 2012). The presence of this putative transcription factor in other grasses such as rice and sorghum suggests that *ID1* may define an important endogenous regulator of floral transition in monocot species (Colasanti et al., 2006; Matsubara et al., 2008; Wu et al., 2008; Coneva et al., 2012). *ID1* is exclusively expressed in immature leaves of maize (Kozaki et al., 2004), and it is related to the induction of *ZEA MAYS CENTRORADIALIS8* (*ZCN8*), an ortholog of the key florigen-encoding gene *FLOWERING LOCUS T* (*FT*), indicating that its activity may be involved in the signal transduction to the apical meristem (Lazakis et al., 2011; Meng et al., 2011).

FLOWERING LOCUS T (FT) is one of the most studied flowering time genes. The transcript product of *FT* has been characterized as the florigen, as it acts similarly in several plant species by mediating the signals from the leaves to

shoot apical meristem (SAM), where the transition to the reproductive growth is triggered (Jaeger and Wigge, 2007; Giakountis and Coupland, 2008; Notaguchi et al., 2008; Shalit et al., 2009; Harig et al., 2012; Xiang et al., 2012; Kim et al., 2013).

Here, we characterized two sugarcane flowering time candidate homologs for *FT* and *ID1* from the photoperiodic and autonomous pathway, respectively.

2 MATERIAL AND METHODS

2.1 Plant growth conditions and genotyping

Sugarcane plants, variety RB72 454, were grown in a greenhouse under either 14-hour long-day conditions at 27°C with 10-hour nights at 22°C, or 12-hour short-day inductive conditions with 20-20-20 (N-P-K) fertilizer supplemented with micronutrients added as required. Arabidopsis plants, ecotype *Columbia (Col-0), Landsberg erecta (Ler)* and *ft*-mutant, were cultivated in Conviron growth chambers under conditions of 16-hour days at 23°C with 8-hour nights at 21°C, with a light intensity of 120μmol.m^{-2}.s^{-1}, and 60% humidity. Arabidopsis segregating plants were genotyped using the Sigma REDExtract-N-Amp Plant PCR Kit (Sigma Biosciences) following manufacturer's instructions, and PCR was performed using kanamycin primers - KanrF: 5'-ATACTTTCTCGGCAGGAGCA-3' and KanrR: 5'-ACAAGCCGTTTTACGTTTGG-3'.

2.2 Isolation and cloning of *ScFT2* and *ScID1* from sugarcane leaves

Mature and immature leaf tissues from sugarcane plants under inductive and non-inductive conditions were collected for total RNA extraction (TRIzol Reagent) and genomic DNA as previously described (Colasanti et al., 1998). For RNA assays, complementary cDNA was synthesized using the qScript cDNA SuperMix (Quanta Bioscences) according to the manufacturer's instructions. Sequences were amplified using specific primers of the genes: ScID1F: 5'-ATGATGATGCTCTCTGATCTCTCGTCT-3'; ScID1R: 5'-TAAGTTGTGGCTCCAGGTC-3'; ScFT2F: 5'-CTTACACTACAAGGTCTCGAATGTC-3'; SFT2R: 5'-AAGTTGCGGGTGCAGAAGTT-3'. Genomic and complementary *ScID1* and *ScFT2* sequences were cloned to the CloneJET PCR Cloning Gene (Thermo Scientific) and sequenced.

2.3 Phylogenetic analysis of the candidates ScFT2 and ScID1 with orthologs of related species

Deduced amino acid sequence of sugarcane ScFT2 was compared to homologs from other species, by aligning them with translated sequences for Arabidopsis FT; ZCN8 (maize); Hd3a (rice); NtFT1 to NtFT4 (tobacco); and BvFT1 and BvFT2 (sugarbeet), in BioEdit 7.1.3.0 (Hall, 1999). Similarly, sugarcane ScID1 was aligned to ID1 (maize), OsID1 (rice) and SbID1 (sorghum). Phylogenetic trees were constructed by MEGA software, version 4.0 (Tamura et al., 2007), with the neighbor-joining comparison model (Saitou and Nei, 1987), *p*-distance method and pair-wise deletion. Bootstrap values from 1000 replicates were used to assess the robustness of the trees (Felsenstein, 1985). Gene structure

information for homologs was accessed at the Phytozome 9.1 genome database available online (www.phytozome.net).

2.4 Construction of overexpression vector and Arabidopsis transformation

The candidate gene for *FT* was cloned into a Gateway entry vector, pDONR-221, using the BP recombination reaction and the subsequent products were recombined with the destination vector pK2GW7 by a LR clonase, originating the expression vector 35S::*ScFT2*. It is worth to note that the sequence used to construct the overexpression vector does not include the stop codon of *ScFT2*, and therefore the protein may have an addition of eight amino acids on its C-terminal sequence. Gateway sites were inserted to the sequencing primers for the cloning reaction as follow; ScFT2gatF: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTACACTACAAGGTCTC GAATGTC-3' and ScFT2gatR: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCGGAAGTCGTGGCG-3'. *Agrobacterium tumefaciens* strain GV3101::pMP90 containing the over-expression constructs was introduced to Arabidopsis plants by floral dip (Clough and Bent, 1998). *Agrobacterium* containing *ScFT2* over-expression constructs were introduced to the Columbia *(Col-0)*, *Landsberg erecta (Ler)* and *ft*-mutant backgrounds. Four independent lines in *Col-0*, five lines in *Ler* and one line in *ft*-mutant background were selected for phenotypic analyses of *ScFT2* overexpression.

2.5 Construction of silencing vectors and *Setaria viridis* transformation

A fragment of approximately 500 base pairs of the candidate genes for *Setaria* ID1 (*SiID1*) and sugarcane *ScID1* were cloned into a Gateway entry vector, pDONR-221, using the BP recombination reaction and the subsequent products were recombined with the destination vector pIPKb007 (Himmelbach et al., 2007) by a LR clonase, originating the silencing vectors RNAi::*SiID1* and RNAi::*ScID1*. Gateway sites were inserted to the sequencing primers for the cloning reaction as follow; ScID1gatF: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAGCACTATCACCACCACA-3' and ScID1gatR: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGCTCCAGGTCTCGTTGAG-3' for *ScID1*; and SiID1RNAiF: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGAGGATTCTTGCGGCCGCGGGGACAAGTTTGTACAAAAAAGCAGGCT-3' and SiID1RNAiR: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGATCGCTGCCGCTGTTGCCGGGGGACCACTTTGTACAAGAAAGCTGGGT-3' for *SiID1*. *Agrobacterium tumefaciens* strain LBA4404 containing the RNAi::*SiID1* construct was introduced into *Setaria viridis* calli. Two days prior the transformation, the *Agrobacterium tumefaciens* strain LBA4404 was streaked onto a MG/L medium supplemented with tetracyclin (5mg/mL), rifampicin (25mg/mL) and spectinomycin (50mg/mL) and incubated at 28°C. After two days, 2 mL of liquid CIM was dispensed into the *Agrobacterium* plate and the solution was transferred to a 50 mL Falcon tube to an OD_{600} of 0.6. Acetosyringone was added to a final concentration of 200 uM and 10 uL of 10% of Synperonic per 1 mL of *Agrobacterium* suspension. 50 calli were added to 15 mL Facon tubes containing the *Agrobacterium* suspension. Calli were incubated for 5 minutes, rocking occasionally. After that, calli were poured in a petri dish

and the excess of liquid was removed and dried calli were placed on a sterile filter paper in a petri plate. Plates were incubated in the dark at 22°C for three days. After three days, the calli were transferred to CIM/timentin/hygromycin (40mg/mL) and maintained in the dark at 24°C for 16 days. In sequence, they were transferred to regeneration/timentin/hygromycin (40mg/mL) and kept under 24°C, 16-hour photoperiod, light intensity of 65 uE m^{-2} s^{-1}. The developed shoots were transferred to 1/2 MS rooting/timenting/hygromycin (40mg/mL) in Magenta boxes.

2.6 Gene expression analysis using semi-quantitative and quantitative RT-PCR

Gene and transgene expression analysis was carried out using real time RT-PCR (RT-qPCR). For the *ScID1* expression analysis, RNA was extracted as detailed above from sugarcane mature leaves, immature leaves and shoot apical meristem. For the Zeitgeber (ZT) times experiment, immature leaves were collected on ZT2, ZT6, ZT10 and ZT14 under both long days (14 hours) and short days (12 hours) conditions. Complementary DNA (cDNA) was prepared using qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. *ScFT2* primers were designed to assess differential transgene expression in the overexpression *ScFT2* lines: ScFT2qRTF: 5'-GACGCTGAGGGAGTATTTGC -3'; ScFT2qRTR: 5'-GCTGGAAGAGCACGAACAC-3' and to assess *ScID1* expression pattern in sugarcane: ScID1qPCRF: 5'-CCCTAACAATACCCTCTTCTTCC-3'; ScID1qPCRR: 5'-GGTGGTGGTGGTGATGCT-3'. A PerfeCTa SYBR Green SuperMix (Quanta Biosciences) and an Applied Biosystems 7300 Real Time

PCR instrument were used, and data was analyzed by the Pfaffl method with efficiency correction to obtain fold difference in expression (Pfaffl, 2001). Three biological replicates consisting of three technical replicates were used for sugarcane essays and three technical replicates were used for each segregating line in Arabidopsis. *Actin8* (ActinrtF: 5'-GCCGATGCTGATGACATTCA-3' and ActinrtR: 5'-CTCCAGCGAATCCAGCCTTA-3') and *ScGAPDH* (ScGAPDHF: 5'-CACGGCCACTGGAAGCA-3' and ScGAPDHR: 5'-TCCTCAGGGTTCCTGATGCC-3') were used for normalization and the calibrator was the average ΔCt for the independent line with lower expression level. Statistical significance is reported by the Student's *t-test* with $P < 0.05$. Expression of the downstream genes *AP1* (AP1rtF: 5'-GTCGATGGAGTATAACAGGC-3' and AP1rtR: 5'-AGATGGCTGATGAGAGAGC-3'); *LFY* (LFYrtF: 5'-AGCAGCAGAGACGGAGAAAG -3' and LFYrtR: 5'-GAAGGAACTCACGGCATTGT -3'); *FT* (FtrtF: 5'-CCAAGTCCTAGCAACCCTCA-3' and FTrtR: 5'-GCCACTCTCCCTCTGACAA-3') and *TFL1* (TFL1rtF: 5'-GCTCTTTCCTTCTTCTGTTTCCTCC-3' and TFL1rtR: 5'-CAGCGGTTTCTCTTTGTGCGT -3') were also investigated by semi-quantitative PCR in the two more severe 35S::*ScFT2* independent lines.

2.7 Scanning Electron Microscopy (SEM)

Multipe inflorescences were harvested from *ScFT2* over-expressed plants and image was captured with a Hitachi Tabletop TM-1000 Scanning Electron Microscope. Dimension bars were added using the ImageJ software (Abràmoff et al., 2004).

3 RESULTS

3.1 ScID1 is a putative monocot-specific transcription factor that acts in the autonomous pathway of floral induction in sugarcane

An ortholog of the autonomous regulated gene *ID1* may be involved in the onset of floral transition in sugarcane. We have isolated the gene from immature leaves of sugarcane and sequence analysis indicates that ScID1 has an identity of 84% to ID1, and the high sequence similarity may be correlated to function conservation. Alignment of ScID1 to rice and sorghum ID1 homologs also resulted in a high identity value; 54% to rice and 85% to sorghum, indicating that the function of ScID1 may be conserved in sugarcane. Analysis of the ScID1 translated sequence allowed the identification of four zinc fingers (ZF1, ZF2, ZF3 and ZF4), a nuclear localization sequence (NLS) and a C-terminal conserved domain, TRDFLG (Figure 1), that are also present in other ID1 orthologs (Colasanti et al., 2006; Matsubara et al., 2008; Wu et al., 2008).

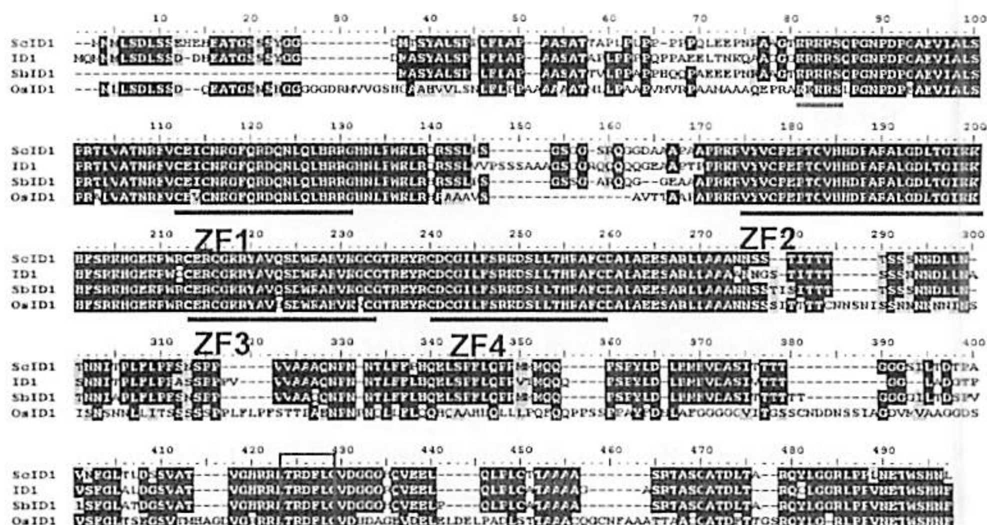
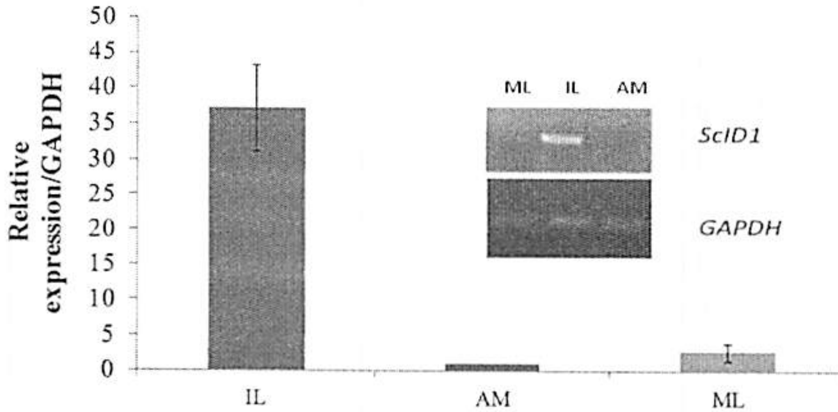


Figure 1 - Alignment of maize ID1, SbID1 (sorghum), OsID1 (rice) and ScID1 amino acid coding sequence shows a high level of identity (84%, 85% and 54%, respectively). The four conserved zinc finger domains (ZF1, ZF2, ZF3, ZF4) are indicated by a green line, the NLS region is shown by an orange bar (KRKR) and the C-terminal TRDFLG motif is boxed.

The expression pattern of *ScID1* mRNA was evaluated by quantitative and semi-quantitative PCR and the results show that the gene is expressed in immature leaves of sugarcane, similarly to the pattern observed in maize *ID1* (Figure 2A). *ID1* it is not regulated by photoperiodic cues, acting by the autonomous pathway of floral induction in maize (Wong and Colasanti, 2007). Similarly to *ID1*, it has been observed that *ScID1* is expressed in both long-day and short-day conditions with no significant alterations in different Zeitgeber (ZT) times (Figure 2B).

A



B

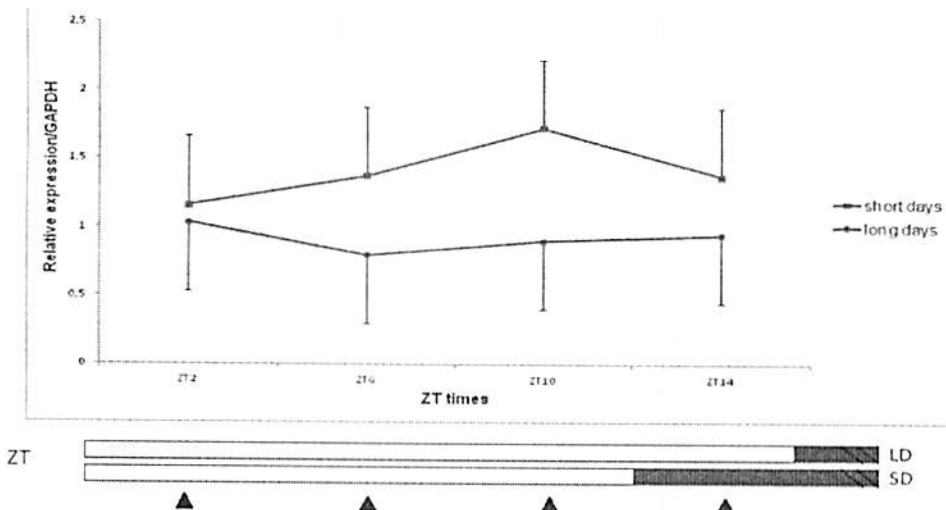


Figure 2 - Semi-quantitative and quantitative expression of the *ScID1* gene in different sugarcane tissues and day-length conditions. A) IL: immature leaves; ML: mature leaves; AM: apical meristem; B) ScID1 expression in different Zeitgeber times (ZT) under both long- and short-day conditions; ZT2: two hours after dawn; ZT6: six hours after dawn; ZT10: ten hours after dawn and ZT14: fourteen hours after dawn. Statistical significance ($P < 0.05$) between any pair of relative expression means was assessed by a *t-test*.

Altogether, the results suggest that ScID1 may be localized to the nuclei, acting as a transcription factor in the transmission of signals to trigger flowering,

independently of day-length. Future analysis will be performed to determine the sub-cellular localization of the *ScID1* product and to verify whether ScID1 plays a role in the floral transition of sugarcane.

Aiming at a better understanding of *ScID1* gene function, we started using *Setaria viridis* as a model plant, since ScID1 is a monocot-specific gene and Arabidopsis is not the appropriate model to study its function. *Setaria viridis* is a C4-monocot plant that has been used as a model for C4 photosynthesis (Brutnell et al., 2010; Li and Brutnell, 2011; Bennetzen et al., 2012). Therefore, a *Setaria italica* ID1 ortholog (*SiID1*) was isolated and used to transform *Setaria viridis*.

3.2 Transformation of *Setaria viridis*, a C4 model plant for functional analysis

The C4 *Setaria viridis* plant has been used as a model system to study C4 photosynthetic evolution (Brutnell et al., 2010; Li and Brutnell, 2011; Wang et al., 2011). We have started to transform *Setaria viridis* calli with a silencing vector containing a fragment of Setaria *ID1* ortholog (RNAi::*SiID1*). In the future, we expect to use it as a system to understand whether ScID1 regulates flowering time. A silencing vector was also constructed for the *ScID1* gene (RNAi::*ScID1*) (Figure 3). *Setaria viridis* seeds were inoculated in callus induction medium (CIM) for four weeks until calli formation was visible (Figure 3B). Most explants formed callus in the third week of cultivation and were co-cultivated in a fresh inductive medium for more two weeks.

A

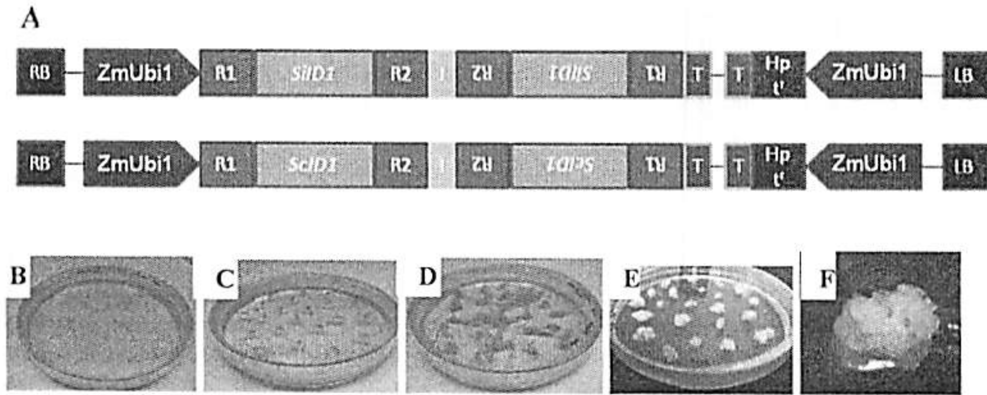


Figure 3 - Schematic representation of the destination vectors generated and regeneration of plantlets from calli from sterile seeds. (A) the Gateway RNAi vectors (pIPK007, (Himmelbach et al., 2007) used contain a fragment of both *SiID1* and *ScID1* orthologs; (B) sterile seeds inoculated on callus induction medium; (C) co-cultivated calli formed from the seeds; (D) embryogenic calli containing roots formed on subculture medium after 1 month; (E) calli after excision of roots on subculture medium; (E) example of a compact and yellow callus used for Agrobacterium transformation.

Two types of calli were observed, a non-embryogenic type that was white and watery while the embryogenic callus was compact and yellowish. Embryogenic healthy calli were selected and transformed via *Agrobacterium tumefaciens*, strain LBA4404, containing the silencing vector with a fragment of the *ID1* homolog from *Setaria viridis* (Figure 3). The bacterial culture at OD600 = 0.6 and 28 days old calli were used. Transformed calli became dark after the treatment with hygromycin, however after a few days under light conditions they started to form differentiated tissues such as leaves (Figure 4). Although the leaves seemed to be photosynthetically active after a few more days, growth of most of the shoots was arrested. A few green shoots were transferred to the rooting medium, but none have developed to a new plant.

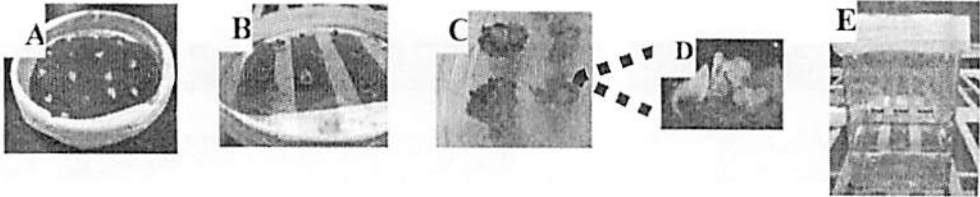


Figure 4 - Stages of Agrobacterium-mediated transformation of the SiID1 and ScID1 genes into *Setaria viridis*. (A) embryogenic calli induced from sterile seeds; (B) resistant calli cultured on selective medium for one month; (C) formation of differentiated leaves on regeneration medium after seven days; (D) close-up of the formation of green leaves from embryogenic calli; (E) differentiated shoots were excised and transferred to a rooting inducing medium.

For this reason, it was not possible to measure the transformation efficiency since no regenerated callus survived the rooting medium condition. Currently, transformation protocol optimization is being performed at Colasanti's Lab. Low transformation efficiency has been reported for *Setaria viridis* transformation, therefore it is important to improve key steps of the protocol, such as callus preparation and co-cultivation procedures.

3.3 Independent lines of 35S::*ScFT2* altered flowering time and caused pleiotropic effects in shoot architecture of Arabidopsis plants

We generated three T2 lines over-expressing *ScFT2* (candidate FtS2) in *Landsberg* background, four lines in Columbia background and one line in *ft*-mutant. Also, two T1 independent lines (referred to as *oscar1* and *oscar2*) were evaluated because they presented the most severe phenotypes. Flowering time of the four T2 lines in Col-0 background and the unique line in the *ft*-mutant was measured by numbers of rosette leaves (Table 1).

Table 1 - Flowering characteristics of 35S::*ScFT2* independent lines in *Columbia* and *ft*-mutant backgrounds.

Plant genotype	Number of rosette leaves	Number of plants
Columbia (Col-0) background		
Col-0 wild-type	11.4±0.54	5
ScFT2-9	16.5±2.94[a]	10
ScFT2-21	16.1±4.01[a]	9
ScFT2-31	18.8±1.40[a]	10
ScFT2-59	14.9±2.13[a]	10
ft-mutant background		
ft-mutant (wild-type)	23.25±3.09	4
ScFT2-1 ft	37.5±1.91[a]	4

[a] Indicates that the value was statistically different from the wild-type with a p>0.05 by the student *t-test*

Line *ScFT2-31* presented the latest flowering phenotype in Col-0 background. Plants formed 18.8 rosette leaves compared to the 11.4 leaves formed by the wild-type before bolting (Figure 5A and 5B). Main inflorescence was thicker than usual which allowed the plant to be maintained straight without any support (Figure 5C) and the primary inflorescence showed an aerial rosette phenotype (Figure 5D).

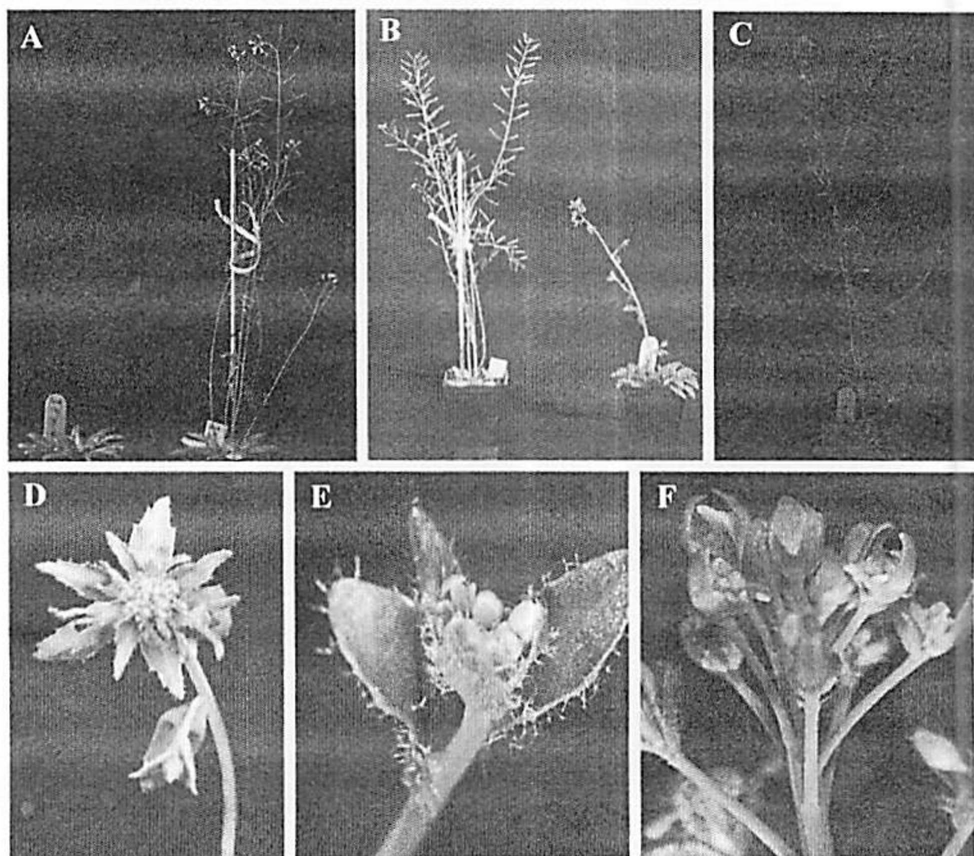


Figure 5 - *ScFT2-31* line in Col-0 background. A) Comparison between ScFT2-31 (left) with Col-0 wilt-type (right) after 35 days of germination. B) Col-0 (left) was senescing after 44 days of germination, comparing to the ScFT2-31 line (right) that was bolting. C) ScFT2-31 line after 50 days of germination. D) Close-up to the main inflorescence of ScFT2-31, showing the formation of an aerial rosette. E) Floral buds are visible in a lateral inflorescence. F) Abnormal floral buds emerge from where flowers should be forming in a ScFT2-31 plant.

At late stages, the primary and lateral inflorescences originated several buds (Figure 5E) that turned into abnormal flowers (Figure 5F). SEM shows abnormal

floral buds formation (Figure 6A and 6B) and also leaf-*like* structures, instead of sepals (Figure 6C).

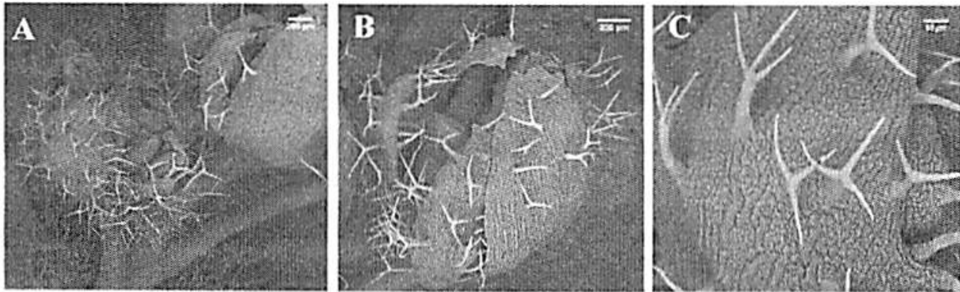


Figure 6 - Scanning electron microscopy (SEM) of inflorescence apices of line *ScFT2-31*. Bars of (A) and (B) represent 200 uM and (C), 50 uM.

Inflorescence apices formed abnormal floral organs, observed by the absence of the concentric four whorls consisted of four sepals, petals, six stamens and two fused carpels (Figure 6A and 6B). Instead, it is observed a possible flower reversion event, characterized by the formation of a mass of disorganized structures, such as a prominent carpel, absence of pistils that are surrounded by a trichome-rich leaf-like structures. Puzzle-like epidermal cells rich in branched trichomes are characteristic of leaves and not of sepals, which could indicate floral reversion (Figure 6C).

In contrast, overexpression lines in the *Landsberg* background did not result in a consistent phenotype. It was observed both early and late flowering phenotypes and several architecture defects. Line *ScFT2-7*, for instance, presented abnormal architecture, marked by absence of rosette leaves and very early flowering phenotypes (Table 2). Flowering time was not assessed for the T1 independent lines *oscar1* and *oscar2*.

Table 2 - Flowering characteristics of 35S::*ScFT2* in *Landsberg* background.

Plant genotype	Number of rosette leaves	Number of plants
Landsberg (Ler) background		
Ler wild-type	9.6±1.94	13
ScFT2-2	14.0±1.22 [a]	4
ScFT2-3	13.1±2.33 [a]	9
ScFT2-7(1)	0	1
ScFT2-7(2)	0	1
ScFT2-7(3)	18	1
ScFT2-7(4)	12	1
ScFT2-7(5)	7	1
ScFT2-7(6)	17	1
ScFT2-7(7)	8	1
ScFT2-7(8)	0	1
ScFT2-7(9)	0	1
oscar1(T1 line)	-	1
oscar2 (T1 line)	-	1

[a] Indicates that the value was statistically different from the wild-type with a $p>0.05$ by the student *t-test*

Abnormal seedlings were produced in *ScFT2-7*, characterized by absence of rosette leaves and presence of floral buds in very early stages of development (Figures 7A to 7C). Etiolated leaves were observed in *ScFT2-7(3)* (Figure 7D)

and *ScFT2-7(6)* (Figure 7E), indicating that the *ScFT2-7* over-expression line caused an abnormal meristem activity in *Landsberg*.

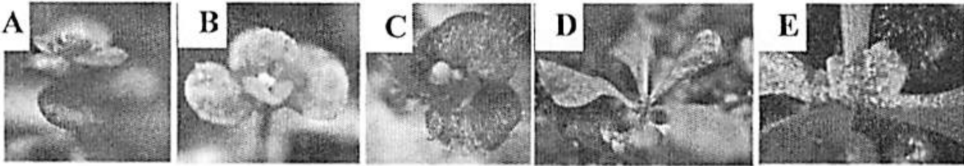


Figure 7 - T2 independent line 7 of 35S::*ScFT2* in *Ler* background showing diverse architecture phenotypes in individuals of the segregating line. A) *ScFT2-7(1)*; B) *ScFT2-7(2)*; C) *ScFT2-7(9)*; D) *ScFT2-7(3)*; E) *ScFT2-7(6)*

Later in development, individuals *ScFT2-7(3)* and *ScFT2-7(4)* presented loss of the apical dominance, leading to formation of more than one primary branch (Figure 8C and 8F). Pleiotropic effects frequently observed in *ScFT2* lines in Landsberg ecotype may be related to abnormal embryonic shoot meristem formation, which led to fusion of stems, as it is shown in Figure 8E and 8G. Phenotypes involved in floral initiation dysfunction have been associated with regulatory activities of PNY and PNF heterodimers and/or the phytohormones auxin and brassinosteroids (Azpiroz et al., 1998; Smith et al., 2004; Kanrar et al., 2006; Cheng et al., 2007; Li et al., 2010; Krizek, 2011; Yamaguchi et al., 2013).

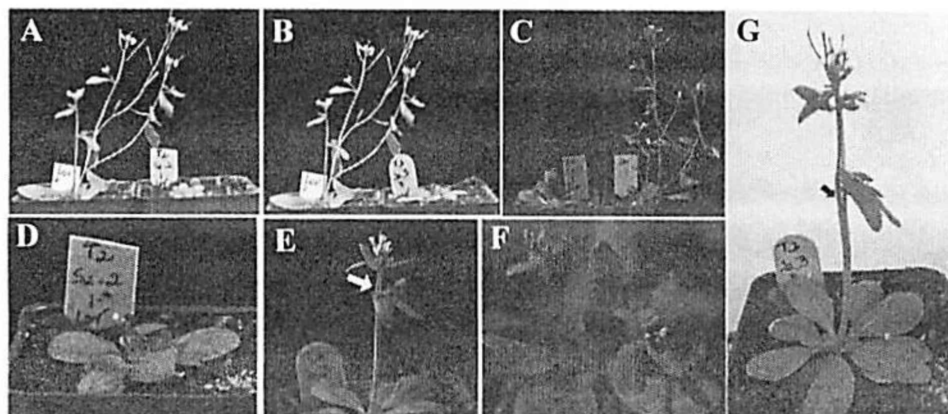


Figure 8 - Flowering time and shoot phenotypes of *ScFT2* lines in Ler background. A) Comparison between *ScFT2-2* (right) with Ler wilt-type (right) after 21 days of germination. B) *ScFT2-3* (right) and Ler (left) after 21 days of germination. C) *ScFT2-7* line (left) after 26 days of germination; Ler is in the right. D) Shoot architecture of *ScFT2-2* presenting a short main inflorescence. E) Formation of a fused stem in the ScFT2-3 line after 27 days of germination. F) Development of multiple rosette architectures in *ScFT2-7*. G) Close-up of *ScFT2-3* showing the dissociation site of the fused stems. Black and white arrows indicate the position where the fused stem dissociates.

oscar1 and *oscar2* independent lines developed for more than four months before start senescing. Plants formed an inflorescence shoot, but axillary shoot meristems could not develop into flowers, as in *wild-type* plants. The inability to form reproductive organs in *oscar* plants led to the formation of a highly complex branching system (Figure 9C and 9D). It was also observed down-turned axillary branches, which seems to be derived of dysfunction in organ polarity (Figure 9B).

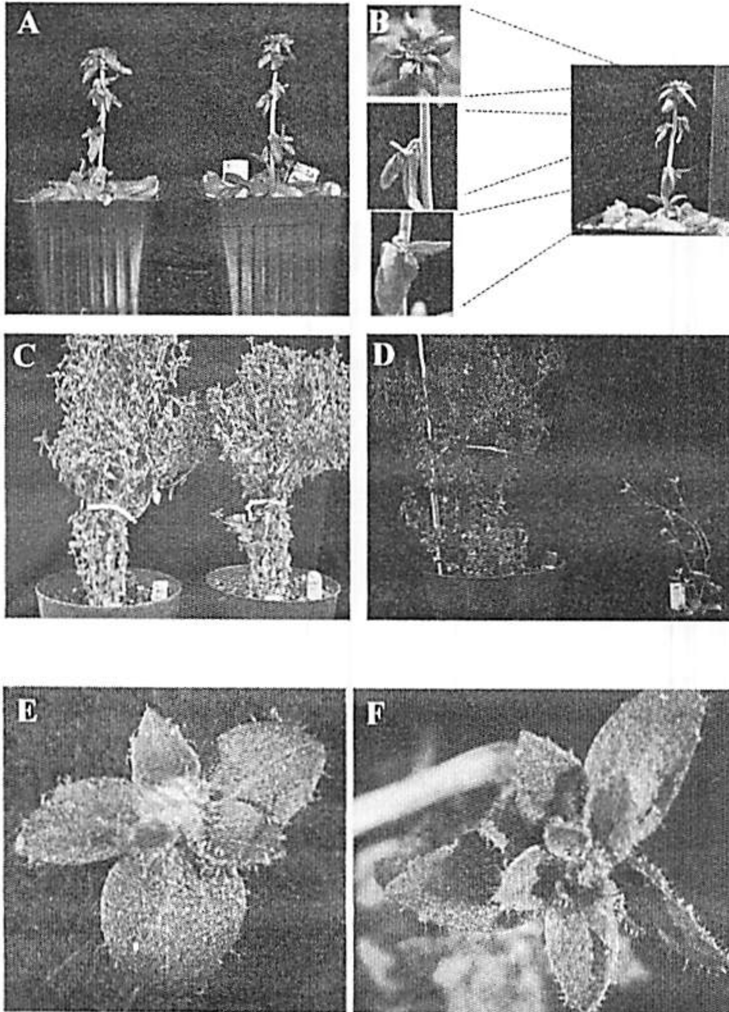


Figure 9 - *oscar1* and *oscar2* phenotypes. A) T1 independent lines of 35S::*ScFT2* in Ler background, named *oscar1* (left) and *oscar2* (right). B) *oscar2* leaf-*like* inflorescence apex and down-turned axillary branches. C) *oscar1*(left) and *oscar2* (right) 94 days after germination. Plants presented inflorescence formation but no floral structure have been formed. D) *oscar1* (left) after 124 days of germination compared to a *Ler* wild-type (right). E) and F) leaf-like inflorescence apex phenotypes of *oscar* plants.

A few fertile floral structures were observed after approximately four months of development in *oscar1* (Figure 10A and 10B); by the time the apical meristem

had already produced a large number of coflorescences; and *oscar2* never produced flowers. The strongly delayed phenotype is characterized by a highly branched and bushy appearance.

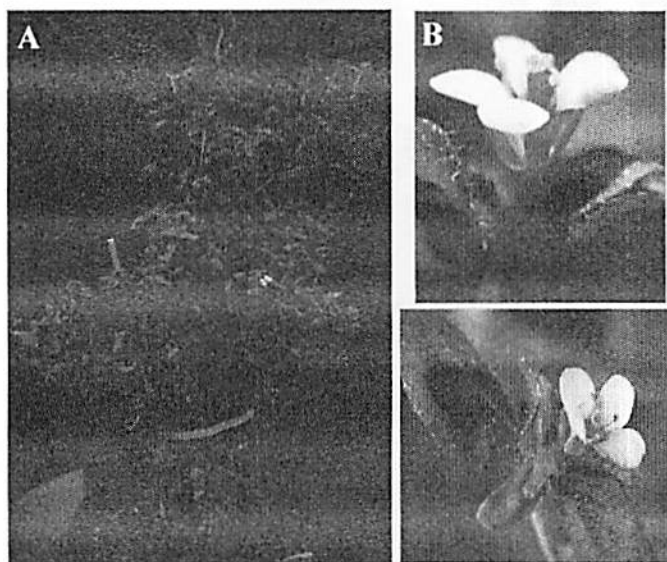
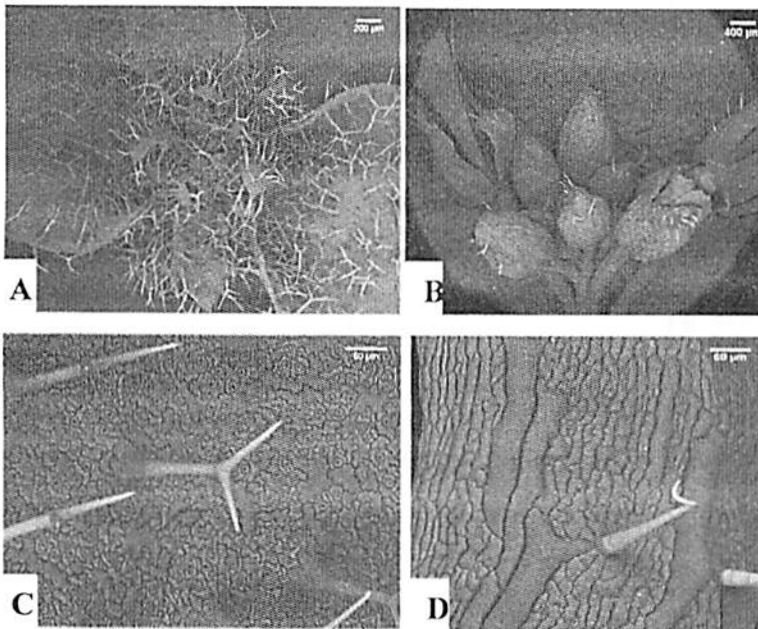


Figure 10 - *oscar1* after 104 days of germination. A) One flower emerged from one of the branches, which is highlighted in B. B) Close-up of an emerged flower in *oscar2*, only four stamens were formed instead of six.

sep1sep2sep3sep4 quadruple mutants present a similar inflorescence, in which there is no formation of any of the four whorls of the flower. Instead, only leaf-like structures are observed in this mutants (Ditta et al., 2004). To check if the inflorescence is consisted of leaf-*like* structures or whether they are formed by sepals in these plants, we analysed *oscar* apices using electron microscope. Comparing *oscar* inflorescences (Figure 11A) to *Ler* apical inflorescences (Figure 11B) it was observed that the number of trichomes was higher in the

transgenic plants. Additionally, epidermal cells are puzzle-*like* structures, rich in stomata and stelate branched trichomes, common in leaf-*like* structures of vegetative tissues (Figure 11C), compared to the elongated cells, few guard cells and unbranched trichomes of sepal tissues (Figure 11D). Interestingly, axillary inflorescences of *Ler* (Figure 11F) are similar to the apical inflorescences of *oscar* plants, presenting a high number of branched trichomes along the newly formed leaves (Figure 11E). This observation led us to believe that the apical dominance was lost in *oscar* lines, leading to a highly branched phenotype.



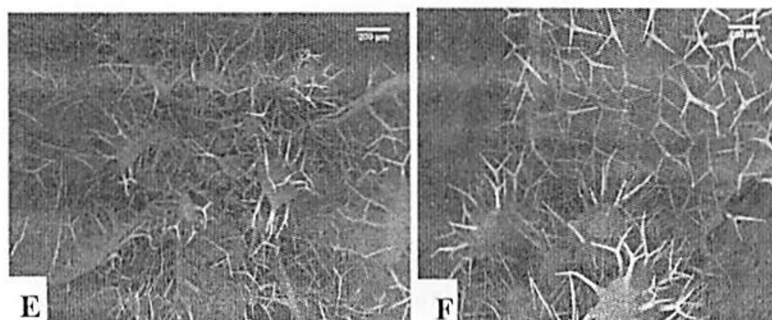


Figure 11 - Scanning electron microscopy of *oscar* inflorescences. A) Inflorescence apices of *oscar1* and *Ler* (B). (C) Adaxial side of leaf-*like* inflorescence structures and sepal-like structure of Ler (D). (E) Apical inflorescence of *oscar1* and axillary branches of Ler (F).

The 35S::*ScFT2* over-expression construct did not include the stop-codon, which resulted in the addition of eight amino acids of the vector sequence to the C-terminal region of the protein. Therefore, we cannot rule the hypothesis that the amino acids insertion in the protein sequence could have led to the effects observed here. Nonetheless, it has been proposed that a specific sequence in the fourth exon of FT members play an important role in flowering promotion (Ahn et al., 2006; Pin et al., 2010; Harig et al., 2012; Taoka et al., 2013). Segment B of floral activators-type FT, including its homologs in tobacco (NtFT4) and sugarbeet (BvFT2) are highly conserved, while this segment in delay flowering-type FT homologs is not a consensus. In accordance to that, it is more likely that the variation in the segment B of sugarcane candidate ScFT2 is the cause of the late flowering phenotypes observed. For a detailed sequence analysis of the FT homologs segment B refer to the Results section of Chapter 3.

In an attempt to explain whether the diverse effects on flowering time and shoot formation of different independent lines of *ScFT2* is a consequence of transgene

accumulation, *ScFT2* expression was assessed by real time PCR. A significant difference in expression of 35S::*ScFT2* lines was observed, which could be one of the reasons of varying phenotypes (Figure 12).

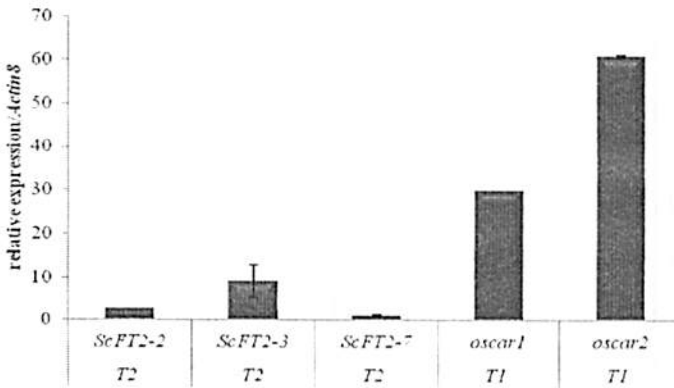


Figure 12 - Expression analysis of transgene in 35S::*ScFT2* independent lines. ScFT2-2: independent line number two; ScFT2-3: independent line number three; ScFT2-7: independent line number seven; *oscar1*: independent line number four; *oscar2*: independent line number five.

The very early flowering time phenotype of the line *ScFT2-7* may be associated to the lower transgene expression levels. In contrast, expression of *ScFT2* in *oscar* plants compared to all the others lines was up to 60 times higher, which could be an indication that the expression levels are associated to the flowering phenotype observed. Collectively, these results indicate that the severe phenotype observed in *oscar* plants, may be an indication that the FT protein complex with FD and 14-3-3 was disrupted (Taoka et al., 2011; Taoka et al., 2013).

3.4 *oscars* leaf-*like* and branching phenotypes may be a result of ectopic expression of *TFL1* and suppression of *AP1* expression

At a transcriptional level, two genes had their function disrupted in *oscar1* and *oscar2* plants (Figure 13). Endogenous FT and associated genes such as *APETALA1* (*AP1*), *TERMINAL FLOWER1* (*TFL1*) and *LEAFY* (*LFY*) were evaluated. The flowering integrator *AP1* and the floral repressor *TFL1* had an altered expression pattern compared to *LFY* expression in *oscar* plants. *LFY* expression was not altered in *oscar* inflorescence apices compared to Ler, however its expression was not enough to trigger floral formation. Consistent with the absence of flower-like structures, *AP1* mRNA could not be detected in the inflorescences of *oscar* lines. AP1 is a key floral integrator expressed in outer cells of floral meristems, that integrates all signaling pathway to determine floral promotion (Kaufmann et al., 2010). The MADS-Box genes *APETAL2* (*AP2*), *APETALA3 (AP3)*, *PISTILLATA (PI)*, *AGAMOUS* (*AG*) and *SEPALLATA (SEP1-4)* depends on the expression of *AP1* to trigger the development of the four whorls of the flower (Pelaz et al., 2000; Smaczniak et al., 2012). More interestingly, *TFL1* was not only expressed in the inflorescence apices, but ectopically expressed in cauline leaves of *oscar* plants, which may have led to a continuous negative feedback of *AP1* expression. *TFL1* down-regulates *AP1* and, in turn, *AP1* down-regulates *TFL1* after floral transition is triggered (Liljegren et al., 1999) (Figure 13).

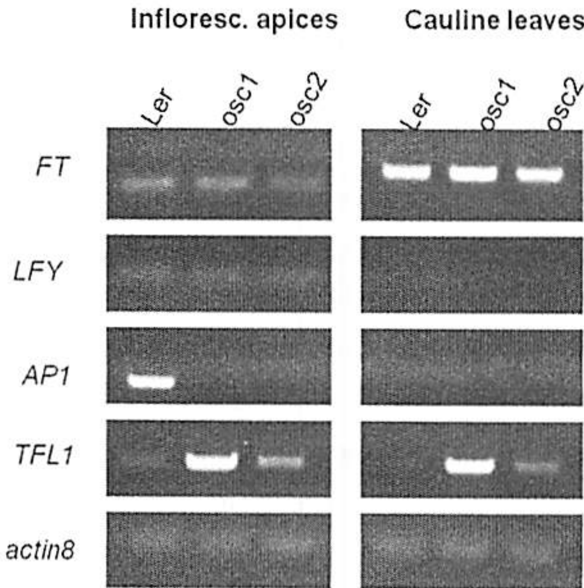


Figura 13 - Expression analysis of flowering time genes of *oscar1* and *oscar2* compared to Ler. *FT: FLOWERING LOCUST; LFY: LEAFY; AP1: APETALA1; TFL1: TERMINAL FLOWER1* and actin8 was used as an endogenous control.

This mechanism of regulation may have been disrupted and *oscar* mutants maintained the production of vegetative branches indeterminately (Figure 9C). Because FT was expressed in both *oscar1* and *oscar2*, the co-suppression hypothesis was disregarded. Also, the production of inflorescences indicate the initiation of phase transition, suggesting that FT signalling cascade from the leaves to the SAM occurs, but the transmission of the signal to the floral organ identity genes is interrupted, which explains the inability to form flowers, leading to the formation of leaf-*like* structures (Figure 9). It is possible that the protein complex formed between FT and FD/14-3-3 was disrupted because of the imbalance in the concentration of FT in the macromolecular complex (Veitia, 2010). Similar effect was also observed in a couple of protein-forming

complexes (Pineiro et al., 2003; Morris et al., 2010). Both over-expression of the floral repressors *EBS* and *DNF* and its mutants presented early flowering phenotype. To be functional, these proteins form complexes and it has been suggested that the increase of one of the molecules may cause a disruption of the complex formed by these proteins to induce flowering, preventing it to work properly (Pineiro et al., 2003; Morris et al., 2010). Similarly, two molecules of FT forms a heterohexamer complex with two 14-3-3 and FD proteins, this complex has been named as the Florigen Activation Complex (FAC) (Taoka et al, 2013). Therefore, it is possible that when *ScFT2* was over-expressed, an accumulation of FT in *oscar* lines led to an impairment of the formation of the FAC, blocking the activation of the *AP1* floral identity meristem gene and, consequently, no flower structures were developed.

3.5 Overexpression of *ScFT2* does not complement *ft*-mutant phenotype and causes pleiotropic effects in Arabidopsis

ft-mutant plants were transformed with the 35S::*ScFT2* construct (35S::ScFT2 *ft*) to check if it rescues the phenotype for flowering time. Interestingly, over-expression of *ScFT2* did not complement the *ft*-mutant phenotype, presenting a more severe delay in flowering time compared to *ft*-mutants under long-day conditions (Figure 14A and 14C). The delay in flowering time also led plants to produce more biomass, in consequence of the higher number of rosette leaves (Figure 14B and 14D). These findings support the hypothesis that *ScFT2* primarily acts as a floral repressor. Interestingly, T1 lines of these mutants

presented abnormal formation of shoot structures, similarly to the mutants in the Ler background (Figure 14E to 14H).

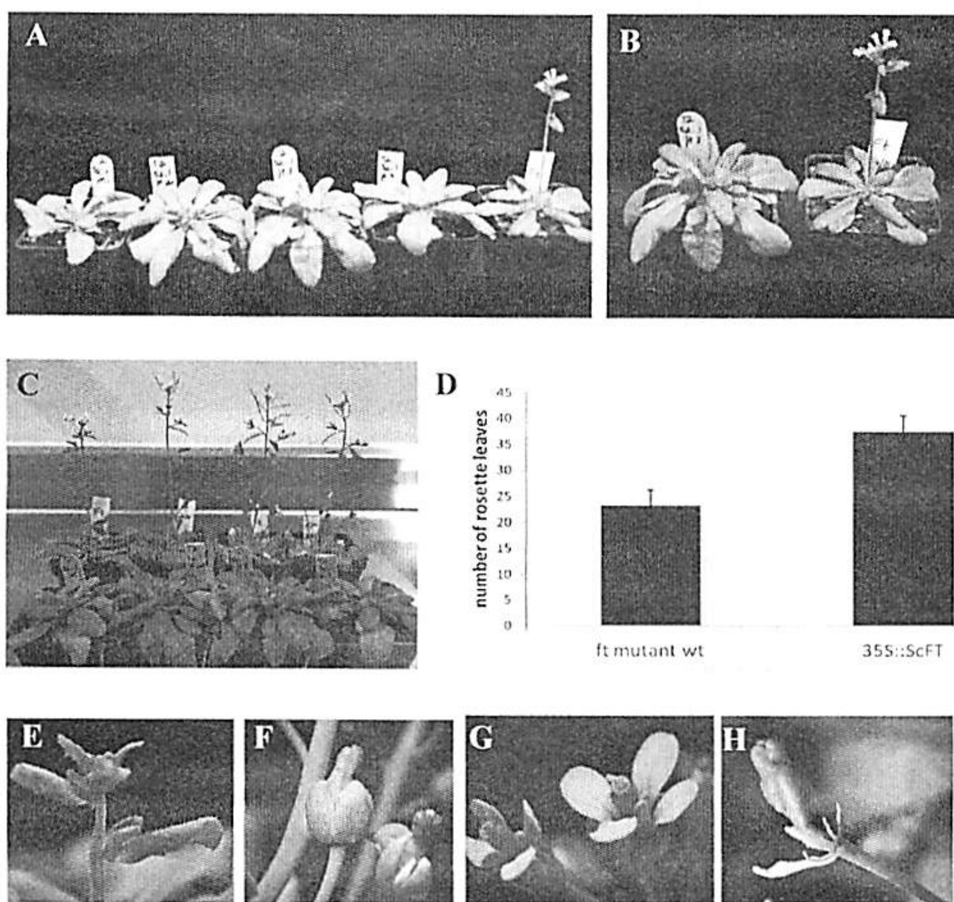


Figure 14 - Analyses of *ScFT2* overexpression in *ft*-mutant background. A) Four T2 individuals (left) compared to the ft-mutant background (right) after 29 days of germination; B) Increased biomass accumulation in the leaves of the transgenic plant (left) compared to the ft-mutant (right); C) Flowering time of *ft*-35S::ScFT2 (front line) compared to *ft*-mutant (back line) after 32 days of germination. D) Flowering time measured by number of rosette leaves. E) to H) Abnormal shoot formation observed in T1 transgenic plants.

4 DISCUSSION

4.1 ScID1 may be related to the autonomous pathway of flowering induction in sugarcane

Sugarcane responds to shortening of the days to induce flowering. In parallel, floral induction is also part of the plant's developmental program, known as the autonomous flowering (McSteen et al., 2000). ID1 is a zinc-finger transcription factor that regulates flowering in maize, independently of day-length (Colasanti et al., 1998). Differently from other flowering time genes, *ID1* is a peculiar transcript, a monocot-specific gene and expressed in immature leaves (Kozaki et al., 2004; Kozaki and Colasanti, 2006; Wong and Colasanti, 2007). Although floral identity genes are conserved among species, the control of meristem identity in the early stages of development may be less conserved (McSteen et al., 2000). Identification of an ID1 ortholog in sugarcane suggests that there is a conserved mechanism of phase transitions among monocot, as ID1 orthologs have also been identified in foxtail millet, rice and sorghum (Colasanti et al., 2006; Matsubara et al., 2008; Mauro-Herrera et al., 2013). ID1 is a developmentally expressed transcription factor that does not depend upon day/night cycles or sink to source transition of the leaves (Wong and Colasanti, 2007). Similarly to ID1 expression pattern, *ScID1* expression did not vary throughout the day under both long- and short-day conditions, indicating that ScID1 may be involved in the floral transition through the autonomous pathway in sugarcane. Additionally, *ScID1* expression is specific to immature leaves of sugarcane, which supports a possible role in signal transduction to induce downstream leaf-derived flowering time regulators.

Sugars are signaling molecules that can modulate floral transition by indicating the physiological status of plants (Roldan et al., 1999; Rolland et al., 2006). It has been demonstrated that the ratio between starch and sucrose in source leaves is altered in *id1* flowering time mutants compared to wild-type plants, favouring carbon utilization over storage (Coneva et al., 2012). Additionally, AtIDD8 regulates floral transition by modulating sugar metabolism and transport, specifically by altering levels of sucrose synthase genes (Seo et al., 2011). Also, higher sucrose content has been associated with high trehalose-6-phosphate (T6P) levels, which may represent the carbohydrate status in plants. Transgenic plants with altered levels of T6P flower extremely late under long-day inductive conditions, affecting flowering in both leaves and apex of Arabidopsis plants (Wahl et al., 2013).

Altogether, these data suggest that floral transition and sugar metabolism pathways are intimately correlated. Therefore, ScID1 may function by linking carbon status to competence to flower in sugarcane.

To further investigate the function of ScID1 in flowering time, *Setaria viridis* is being used as a model system and evaluation of altered phenotypes will enlighten our hypothesis.

4.2 *oscar1* and *oscar2* phenotypes suggest that *ScFT2* is involved in the flowering time pathway but not necessary to induce floral transition

Flowering prevention has not been observed in single mutants such as in *oscar* plants, yet several mutants develop similar effects in floral transition. *pennywise pound-foolish (pny pnf)* double mutants, for instance, never complete floral transition (Smith et al., 2004) and the double mutants *ft-1 ap1-1* and *ft-1 lfy-6*

produce cauline leaves with a spiral phyllotaxis instead of flower-like structures (RuizGarcia et al., 1997). Also, *apetala2-1 agamous-1 apetala3-1* (*ap2-1 ag3-1 ap3-1*) triple mutants and *sepallata* (*sep1 sep2 sep3 sep4)* quadruple mutants form leaf-*like* floral structures (Bowman et al., 1991; Ditta et al., 2004) (Figure 15). It has been observed that *soc1-3 ful-2* mutants acquire perennial phenotypes such as longevity by flowering delay, production of aerial rosettes and lateral branching that remained active even after silique senescence. Moreover, overexpression of *FT* in *soc1-3 ful-2* mutants provoked reversion of inflorescence meristems, forming a cushion-plant phenotype under short-day conditions (Melzer et al., 2008). This indicates that in absence of SOC1 and FUL, FT in not able to induce the transition from the vegetative to reproductive growth (Melzer et al., 2008). We show here that the over-expression of *ScFT2* in *oscar* plants causes late flowering and alters plant architecture, forming a branched shrub phenotype, caused by reversion of inflorescences to reiteration of vegetative growth.

Ectopic expression of *TFL1* in *oscar* plants supports the hypothesis that this gene may have caused *AP1*down-regulation, explaining the absence of flowers in this plants. *TFL1* becomes highly expressed in cells that remain vegetative, acting as a counterbalance to *FT* (Liljegren et al., 1999; Jaeger and Wigge, 2007; Jaeger et al., 2013). *AP1* down-regulates *TFL1* in floral meristems and maintain indeterminate growth of the vegetative center. In contrast, a strong ectopic expression of *TFL1* may lead to flowering repression (Jaeger et al., 2013).Therefore, it is conceivable that *oscar* phenotypes may be a result of the increased expression of *TFL1* in both the center and the flanks of the shoot, which triggered the *AP1* knock-out.

We also raised the hypothesis that variation in a specific region of *ScFT2* may have led to a disruption of the putative complex formed by its product with a FD and 14-3-3 homologs in sugarcane. Structure of the florigen activation complex has been determined for Hd3a complex in rice, which is a heterohexamer constituted by two Hd3a, 14-3-3 proteins and two OsFD1 molecules (Taoka et al., 2011). However, mutations in the specific binding region for 14-3-3 in Hd3a, such as the Y85, segment B and anion-binding pocket indicates that these regions are not crucial for the complex binding to occurs, and that it may be a interaction site for co-activators, transcription factors and/or other interacting molecules to activate downstream genes, such as floral meristem identity genes (Taoka et al., 2013).

Similar to FT and Hd3a, ScFT2 has the Y85 amino acid residue which is one of the factors for the candidate to be considered a floral inductor; nevertheless, our data show that the segment B is not conserved (Chapter 3) as it is in *FT* homologs that promote flowering (Ahn et al., 2006; Pin et al., 2010; Harig et al., 2012). The amino acids variation in this region could be leading to floral co-repressors to bind, instead of floral co-activators, and subsequently repressing floral structures initiation. In sugar beet, a substitution of three amino acids in the segment B of BvFT1 is enough to trigger its function as a floral antagonist (Pin et al., 2010). This may suggest that if ScFT2 is a flowering time in sugarcane, it would be unlikely that the complex have been disrupted, but that the non-consensus segment B may be the binding site for a co-repressor molecule, inactivating downstream genes, such as *AP1*.

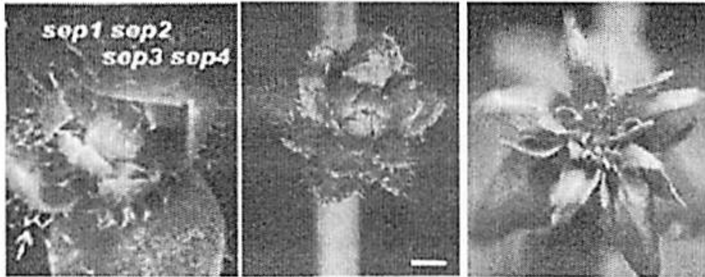


Figure 15 - Phenotypes of *sep1 sep2 sep3 sep4* (left) quadruple mutant (Ditta et al., 2004) and *ap2-1 ag-1 ap3-1* triple mutant (Bowman et al., 1991) (middle), presenting leafy-*like* inflorescence structures, similarly to *oscar* inflorescences (on the right).

It is crucial to unravel the nature of the molecules that bind to the FT/TFL1 proteins to depict the whole molecular scenario of floral transition. Up to date, there is no information whether FT can interact with transcriptional regulators to trigger plant hormone responses, however 14-3-3 proteins are known to interact with brassinosteroids, abscisic acid and gibberellins (Jaspert et al., 2011). Based on phenotypes, the results shown here suggest a possible connection between ScFT2 with hormones.

Despite the fact that the segment B is not necessary to the complex to be formed, another hypothesis to be explored is that the increased number of *ScFT2* transcripts in *oscar* lines may have led to a protein complex disruption, because of an imbalance of the components of the complex. Protein complexes are composed by a specific number of its components and if one of the factors is not balanced, the protein complex is not stabilized (Veitia, 2010).

Finally, ScFT2 is one of the FT homologs that are versatile molecules and act in regulatory processes other than flowering promotion (Hsu et al., 2006; Shalit et al., 2009; Krieger et al., 2010; Pin et al., 2010; Danilevskaya et al., 2011; Hsu et al., 2011; Kinoshita et al., 2011; Navarro et al., 2011; Pin and Nilsson, 2012).

4.3 *ScFT2* overexpression caused pleiotropic effects in Arabidopsis phase transition

Inflorescence growth is marked by internode development, flower specification and axillary branch development (Hempel and Feldman, 1994). In general, 35S::ScFT2 plants in Col-0, Ler background and 35S::ScFT2 *fl* presented pleiotropic effects in phase transition indicating abnormal shoot development, such as abnormal flower production, aerial rosettes, absence of rosette leaves and early bud development, and fusion of the pedicel to the main stem, which was accompanied with a curving of the inflorescence (Smith et al., 2004; Hibara et al., 2006; Kanrar et al., 2006; Lee et al., 2009). Collectively, phenotypes observed in ScFT2 overexpressed plants indicate that ScFT2 may be associated with the activity of transcription factors such as PNY and PNF (Kanrar et al., 2006). For instance, inflorescences of *stm-10 PNY/pny* plants frequently terminated with a central flower-like structure that lacks petals, stamens and carpels, and occurrence of miniature inflorescences and reduced internode development. Similarly, *ScFT2* plants were unable to convert inflorescence meristem into normal floral meristems, reinforcing the speculation that this flowering time gene is involved in the regulation of PNY and PNF genes controlling the inflorescence architecture in the SAM (Kanrar et al., 2006). Additional research will be addressed to assess the possible connection between ScFT2 function and the regulation of inflorescence development and floral specification events.

The inability of *ScFT2* over-expression to rescue the flowering time phenotype of the *fl*-mutant reinforces its main function as a floral repressor. Several *FT*-like have been found to exist in different plant species, performing meristem-related

activities, which suggests that the floral promotion is only one of many functions of *FT* genes (Shalit et al., 2009; Pin et al., 2010; Harig et al., 2012; Klintenas et al., 2012; Pin and Nilsson, 2012; Taoka et al., 2013).

4.4 Flowering time candidates *ScFT2* and *ScID1* as gene targets for molecular breeding in sugarcane

Sugarcane is an important crop worldwide, being one of the main sources of sugar and biofuel production. However, the lack of a sequenced genome has been hampering advances in genetics and molecular studies in this species. The identification and characterization of candidates involved in important developmental processes such as floral transition is essential to a better understanding of the physiological aspects of agronomically important traits in non-model species. Strategies such as the isolation of flowering time genes and comparison to putative orthologs in related species facilitates future research on the mechanisms underlying floral transition in important crop species. Moreover, these candidates may be possible targets for sugarcane breeding programs, used as molecular markers, to enable predicting the transition in early stages of development and, therefore, accelerate breeding process. Also, once the functional activity of ScFT2 and ScID1 in floral transition is completely unravelled it could be used as tools to delay and/or inhibit transition to reproductive habits of crops, such as sugarcane, in which biomass accumulation is highly desired.

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