MOLECULAR ANALYSIS OF *WOX9* FUNCTION IN DICOT LEAF BLADE DEVELOPMENT AND IDENTIFICATION OF SORGHUM FLORIGENS

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DEDICATION

To my:

Father: **Mr. WOLABU WATIRA ODA**

Mother: **Mrs. SHASHI KENSO BARDE**

Dedications reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

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CHAPTER I

GENERAL INTRODUCTION

This study contains two major components. The first study was conducted to characterize expression pattern and biological function of *Medicago truncatula WOX9 (MtWOX9*) in *Medicago* and *Nicotiana sylvestirs* leaf blade development in the STF/LAM1-mediated pathway. In the second project, sorghum *FLOWERING LOCUS T* (*SbFT*) genes were identified and characterized to establish a molecular roadmap for mechanistic understanding of sorghum flowering time control. In this general introduction section, I present brief introductory remarks of leaf blade development in dicots and sorghum *FT*-like genes in flowering time control.

In the plant life cycle, the shoot apical meristem (SAM) is a reservoir of undifferentiated stem cells that functions as a continuous source to produce lateral organs, including leaves, branches and flowers. The SAM has a highly organized structure, which can be subdivided, into a central zone (CZ) of pluripotent stem cells, the peripheral zone primordia that contributes to the production of lateral organs, and the rib zone (RZ) (Nakajima and Benfey, 2002). The leaf primordium starts as a bulge from a small group of pluripotent stem cells organizes itself into well-defined cell layers at the flanks of the SAM and extends laterally during primary and secondary morphogenesis. Since leaf primordia develop from a small group of undifferentiated cells on the flanks of SAM, leaves possess inherent positional relationships with the SAM (Yamaguchi et al. 2012). Thus, leaf primordium passes through well-orchestrated cell division, cell expansion, and cell differentiation patterns forming a flattened blade with three distinct axes: proximodistal (length), mediolateral (width), and dorsoventral (thickness) (Tadege 2013). Therefore, the leaf lamina (blade) is a highly organized photosynthetic structure in which cells in the upper (adaxial) and lower (abaxial) surfaces are morphologically different and are associated with different functional specializations (Tadege 2013). The adaxial

palisade mesophyll cells are specialized for solar energy capture and the abaxial spongy mesophyll cells are specialized for gas exchange. The vascular bundles in the middle mesophyll are specialized for water and nutrient translocation in which the water and minerals conducting xylem vessels are positioned adaxial to the sugar transporting phloem. Because the lamina is essentially a solar panel where energy from the sun and carbon dioxide from the atmosphere are assimilated into chemical energy (sugars), its size and design are of fundamental interest to biology from the point of view of form, function, and environmental fitness (Moon and Hake 2011; Tadege 2013). Leaves show considerable diversity in shape and size and the diversity of leaf forms can be classified into two morphological classes: simple leaves with a single unit of undivided blade whereas compound leaves consist of multiple discontinuous blade subunits, termed leaflets and both simple and compound leaves emerge from SAM (Moon and Hake 2011). Several developmental and molecular genetics studies devoted to understand the mechanisms that control leaf development have emerged in recent years in *Arabidopsis* and other model species (Efroni et al. 2010; Gonzalez and Inze 2015; Moon and Hake 2011; Nakata and Okada 2012; Tadege 2013; Tadege et al. 2011a).

The process of leaf development, from initiation to patterning, involves coordinated regulation by transcription factors, small RNAs and hormones (Kalve et al., 2014; Lin et al., 2013; Moon and Hake 2011; Tadege et al., 2011a & b; Tadege 2013; Zhang et al., 2014). Genetic analyses of model plants have identified several regulatory genes that determine the adaxial or abaxial cell fate, and these genes are required for blade outgrowth and margin development (Chitwood et al. 2007). Mutations in regulatory genes lead to abnormal leaf blade phenotypes that produces plants having narrower or smaller leaves (Kerstetter et al. 2001; McConnell et al. 2001; Siegfried et al. 1999; Waites et al. 1998; Tadege et al., 2011a; McHale 1992; Vandenbussche et al., 2009) indicating the requirement of dorsiventral establishment for flat leaf outgrowth of the lamina, as lack of either adaxial or abaxial identity in leaves results in narrow rod shaped leaves. In addition, the *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes that are plant specific transcription factors also play large role in lateral organs development as both transcriptional repressor and activators (Mukherjee et al. 2009; van der Graaff et al. 2009; Tadege et al., 2011a; Lin et al., 2013; Zhang et al., 2014).

In general, an understanding of leaf development is critical to a more general understanding of shoot development and the improved understanding of leaf development contributes directly to a more comprehensive concept of angiosperm biology (Tsukaya 2013). Furthermore, understanding leaf growth is obviously of great academic interest, but a better comprehension of the molecular basis of this process can also allow for maximizing the plant's potential for food and energy production. However, the involvement of *WOX* family genes in general and *WOX9* gene in particular and the molecular mechanism regulating the leaf blade development are not yet well understood. In this research, I investigated the role of *WOX9* in leaf blade development in *Medicago truncatula* and *Nicotiana sylvestris* and its relationship with the STF pathway. My results demonstrate that *WOX9* not only functions in leaf blade development antagonistic to the *STF* pathway, but also strongly represses flowering in tobacco, suggesting a molecular link between the mechanisms of leaf blade outgrowth and control of flowering time (see chapter II). Interestingly, this connection between leaf blade development and floral transition bridged my two projects because my second project is devoted to understanding the control of floral transition in sorghum (see chapter III).

Floral transition is a major phase change in flowering plants where developmental programs switch from vegetative growth to reproductive growth in which gametes are formed to ensure continuity to the next generation. Thus, plants coordinate the timing of their flowering with environmental changes to achieve reproductive success. Leaves also play an important role as a source of florigen that activates flowering in angiosperm. At the SAM, florigen causes changes in gene expression that lead to the subsequent generative phase known as the floral transition. This switch is regulated by multiple flowering pathways that are controlled by environmental and endogenous factors. Genetic studies in *Arabidopsis*, rice and other flowering plants defined major pathways that transduce environmental signals to integrators, predominantly *FLOWERING LOCUS T (FT)*, which induce flowering in long-day and short-day plants (Corbesier et al. 2007; Giakountis and Coupland 2008; Jaeger 2007; Lifschitz and Eshed 2006; Tamaki et al. 2007; Zeevaart 2008). *FT* homologs have been shown to play critical roles in the induction of flowering in all taxa examined under LD, SD, and day neutral conditions (Ballerini and Kramer, 2011). Several research reports indicated that the orthologs of *FT*-

like genes have been identified as floral activators in many monocot and dicot species, including wheat (*Triticum aestivum*) (Yan, Fu, et al., 2006), barley (*Hordeum vulgare*) (Faure et al., 2007), Maize *(Zea mays*) (Men et al., 2011), rice *(Oryza sativa),* (Kojima et al., 2002, Komiya et al., 2008, Komiya et al., 2009), *Medicago* (*Medicago truncatula*) (Rebecca et al., 2011), Garden pea (*Pisum sativum*) (Hecht et al., 2011), soybean (*Glycine max*) (Kong et al., 2010), tomato (*Solanum lycopersicum*) (Lifschitz et al., 2006), sugar beet (*Beta vulgaris*) (Pin et al., 2010), and sunflower (*Helianthus annuus*) (Blackman et al., 2010). The conserved FT-like protein homologues share sequence similarity at their main binding domain of phosphatidylethanolamine binding protein (PEBP) (Kardailsky et al., 1999). FT-like proteins in different species have been shown to function in a similar manner including, production in the leaf, transport to the SAM through the phloem, and interaction with FD-like proteins in the shoot apex, suggesting that this general mechanism is likely to be widely conserved across flowering plants (Hecht et al., 2011; Lifschitz et al.,2007; Lin et al., 2007).

In sorghum (*Sorghum bicolor* L. Moench), flowering time is a key agronomic trait that determines whether sorghum can be used as a grain or biomass crop. Sorghum is a typical SD plant with substantial photoperiod sensitivity. However, like maize, breeders have selected photoperiod insensitive genotypes for grain production in temperate regions. As a result, temperate sorghum can be classified as grain sorghum with attenuated photoperiod response, biomass sorghum (includes forage and energy sorghum) and sweet sorghum. Biomass and sweet sorghums require SD photoperiod for early flowering and flower very late in LDs, and were selected for increased biomass yield through longer duration of vegetative growth in temperate regions (Rooney et al., 2007; Olson et al., 2012). Currently, the manipulation of flowering time loci has been of fundamental importance to the production of high-biomass sorghum for bio-power and lignocellulosic biofuels and the timing of the floral transition is a vitally important trait in maximizing yield potential in general (Murphy et al., 2011). Despite this critical importance of flowering time for sorghum agronomy and the existence of more than 40 flowering time QTL (Mace et al., 2013), very little is known about the molecular mechanism of flowering time control in sorghum.

In this study, sorghum *FLOWERING LOCUS T* (*SbFT*) genes were identified and characterized to establish a molecular roadmap for mechanistic understanding of sorghum flowering time control. So far, 19 sorghum phosphatidylethanolamine binding protein (PEBP) family genes have been identified of which 13 are *FLOWERING LOCUS T* (*FT*)-like genes. Out of the 13 *FT*-like genes, *SbFT1, SbFT8* and *SbFT10* were identified as potential candidates for encoding florigens that activate floral transition and mediate photoperiodic responses. In addition, the result demonstrates that two of sorghum *FT* genes, *SbFT8* and *SbFT10* not only activated flowering, but also showed additional pleiotropic effects in leaf morphology, plant growth and architecture. In summary, these results together demonstrate that sorghum has at least three *FT* genes that could potentially form a florigen activation complex and mediate genotype dependent photoperiod response and flowering time variation in sorghum (see chapter III).

Major research objectives

This project has two separate major research objectives.

1. To determine the role of *MtWOX9* in *STF*-mediated leaf blade development pathway using transformation technology and biochemical analyses.

Specific objectives:

To evaluate the effect of *MtWOX9* over expression in *Medicago* and tobacco leaf blade development,

To identify the relationship of *MtWOX9* with *STF* through protein-protein interaction and its role in the *STF*-mediated leaf blade development pathway.

2. To clone and characterize sorghum *FT*-like genes to establish a molecular road map for understanding the mechanism of flowering time control in sorghum.

Specific objectives:

To clone and study the evolutionary relationship of sorghum PEBPs (*SbFT/TFL1/MFT*-like) family through their proteins and DNAs sequence alignment and phylogenetic analysis. To determine temporal and spatial expression pattern of *SbFT/TFL1/MFT*-like genes in different sorghum developmental phases and identify the possible sorghum florigen candidates and to identify, putative sorghum florigens by evaluating their interaction with SbFD and Sb14-3-3 proteins and their effect on flowering time by overexpressing in transgenic *Arabidopsis* and complementation of the *ft-1* mutant.

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CHPATER II

THE EFFECT OF ECTOPIC EXPRESSION OF *MtWOX9* IN *Medicago truncatula* AND *Nicotiana sylvestris* LEAF BLADE DEVELOPMENT.

INTRODUCTION

Leaf primordia initiation starts at the flanks of the shoot apical meristem (SAM) and extend laterally during primary and secondary morphogenesis in which growth occurs predominantly by cell division and cell expansion (Dengler and Tsukaya 2001; Donnelly et al., 1999). Since leaf primordia develop from a small group of cells on the (Chitwood et al., 2007) flanks of SAM, leaves possess inherent positional relationships with the SAM (Yamaguchi et al., 2012). After primordium initials are recruited from the shoot apical meristem, the primordium organizes itself into defined cell layers through highly regimented cell division and cell differentiation patterns, forming a flat lamina (blade) that develops along three distinct axes: proximal-distal, medial-lateral, and adaxial-abaxial. The adaxial side is the side closer to the SAM and the abaxial side is the side away from the SAM. The adaxial palisade mesophyll cells are specialized for solar energy capture and the abaxial spongy mesophyll cells are specialized for gas exchange, while the vascular bundles in the middle mesophyll are specialized for water and nutrient translocation in which the water and minerals conducting xylem vessels are positioned adaxial to the sugar transporting phloem positioned abaxial. Genetic analyses of model plants have identified several regulatory genes that determine the adaxial or abaxial cell fate, and these genes are required for blade outgrowth and margin development (Chitwood et al., 2007). Among identified adaxial and abaxial specific genes that encode transcription factors; the LOB-domain family gene *ASYMMETRIC LEAVES2 (AS2)* gene and Class III Homeodomain Leucine Zipper (*HD-ZIPIII)* family genes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*) are adaxial-specific regulators

(Iwakawa et al., 2002; McConnell et al., 2001; Waites et al., 1998), whereas *KANADI* (*KAN1, 2 and 3*), *YABBY* (*FIL, YAB2, 3 and 5*) family genes, and *AUXIN RESPONSE FACTORS* (*ARF3*/*ETT* and *ARF4*) family genes are abaxial specific regulators (Kerstetter et al., 2001; Sawa et al., 1999; Siegfried et al., 1999). Mutations in these regulatory genes lead to abnormal phenotypes of the leaf blade and mutant plants have narrower or smaller leaves than those of the wild type (Kerstetter et al., 2001; McConnell et al., 2001; Siegfried et al., 1999; Waites et al., 1998).

Although adaxial-abaxial differentiation is thought to be a prerequisite for lamina outgrowth, the presence of mutants in *Arabidopsis* that are affected only in proximal-distal or medial-lateral growth suggested that different mechanisms of cell proliferation and expansion patterns may exist to accomplish growth in length and width directions (Nakata and Okada 2012; Nakata et al., 2012; Nakata and Okada 2013; Tadege 2013; Tadege et al., 2011a). Recently, a third domain, located at the adaxial-abaxial junction of the lamina, was found to be required for cell proliferation mediated blade outgrowth (Nakata and Okada 2012; Nakata et al., 2012; Nakata and Okada 2013; Tadege 2013; Tadege et al., 2011a; Tadege et al., 2011b). The identity of this inner (middle) domain is regulated by the *WUSCHEL* related homeobox (*WOX*) family homeodomain (HD) transcription factor *STENOFOLIA (STF)* in *Medicago truncatula* (Tadege et al., 2011a; Tadege et al., 2011b) and its orthologs in other dicots, including *MAEWEST* in *Petunia x hybrida* (Vandenbussche et al., 2009), *LAM1* in *Nicotiana sylvestris* (McHale 1992; Tadege et al., 2011a), *WOX1* and *PRESSED FLOWER (PRS/WOX3)* in *Arabidopsis* (Nakata et al., 2012; Nakata and Okada 2012; Vandenbussche et al., 2009), and *LATHYROIDES* in pea (*Pisum sativum*) (Zhuang et al., 2012).

The most recent insights on blade formation have come from analysis of genes in the *WUSCHEL (WUS) RELATED HOMEOBOX (WOX)* family. The critical requirement of *WOX* genes for the development of lateral organs, including leaves and flowers, has become increasingly apparent from the identification of several mutant phenotypes in angiosperms. For example, in maize, the *narrowsheath1* and *2* (*ns1/ns2*) double mutant exhibits a leaf margin deletion phenotype (Nakata et al., 2012), the *WOX1* homologs *maewest* (*maw*) in *Petunia x hybrid* (Vandenbussche et al., 2009*), stenofolia (stf)* in *Medicago truncatula* (Tadege 2013; Tadege et al., 2011a; Tadege et al., 2011b), *lam1* in

Nicotiana sylvestris (McHale 1992*)*, and *wox1/prs* double mutant in *Arabidopsis thaliana* (Nakata et al., 2012; Vandenbussche et al., 2009*)* show narrow leaves. Similarly, in rice (*Oryza sativa*), the orthologs of *ns1* and *ns2* such as *narrow leaf2* & *3* (*nal2* and *nal3*) are shown to be involved in leaf blade outgrowth (Cho et al., 2013), suggesting that the requirement for *WOX* function in leaf blade development is conserved in both dicots and monocots. These mutants produce narrow leaf blades owing to defects in lateral cell proliferation affecting leaf width, whereas leaf length is virtually unaffected (Lin et al., 2013; Tadege et al., 2011a). Therefore, several mutation studies in different plants confirmed that *WOX1* orthologous genes are key regulators of blade outgrowth through the activation of cell proliferation in the middle domain (Nakata et al., 2012; Tadege et al., 2011a; Vandenbussche et al., 2009).

The *WUSCHEL* related homeobox (*WOX*) genes form a plant specific homeobox transcription factor superfamily in *Arabidopsis* consists of 15 members, including the founding member *WUSCHEL (WUS)* and *WOX1–WOX14* (Haecker et al., 2004; Mukherjee et al., 2009; van der Graaff et al., 2009), which are involved in the regulation of key developmental processes, including stem cell maintenance in SAM and root apical meristem (RAM), embryo apical-basal polarity patterning, and development of lateral organs. This *WOX* family has been divided into three major clades: ancient (*WOX10*, *13* and *14)* detected in as early as green algae and mosses; intermediate clade (*WOX8*, *9*, *11* and *12)* appeared in vascular plants including lycophytes; and modern clade also called the *WUS* clade (*WUS* and *WOX1–7)* found only in seed plants (van der Graaff et al., 2009). The members of the modern clade (*WUS* clade) including *WUS* and *WOX1*-*WOX7* contain intact *WUS*-box elements, whereas intermediate and ancient members show variation at this position (Lin et al., 2013; van der Graaff et al., 2009). Characterization of the *WUS*-box not only uncovered the mechanism for such conservation of functions, but also revealed the extent of functional conservation existing among *WOX* genes (Lin et al., 2013). For instance, *STF* acts as a transcriptional repressor primarily through its *WUS*-box in organizing cell proliferation during leaf blade morphogenesis, as well as during petal and carpel development (Lin et al., 2013; Tadege 2013; Tadege et al., 2011b). Although the intermediate and ancient clade *WOX* genes lack the *WUS*-box and act as transcriptional activators in transient expression assays, they can gain competence for

lam1 complementation when fused with the *WUS* box or *SRDX* repressor domain (Lin et al., 2013). Therefore, there is a possibility that ancient and intermediate clade *WOX* genes may directly or indirectly be involved in leaf blade development as repressor or activator. On the other hand, *WOX9/STIMPY* of *Arabidopsis* acts as a strong transcriptional activator and enhances the *lam1* phenotype with additional proximal-distal defects which led to a speculation that meristematic activity and lateral organ development in higher plants requires a balance between the activation and repression functions of *WOX* genes (Lin et al., 2013).

Recently, Tadege et al., (2011) reported the requirement of a *WOX1/MAW*-like gene, *STENOFOLIA* (*STF*), for blade outgrowth and leaf vascular patterning in *Medicago truncatula.* The *STF* gene is expressed at the adaxial-abaxial junction of the leaf margin throughout leaf growth and disappears in the mature fully expanded leaf suggesting that *STF* function is an ongoing requirement until blade expansion ceases (Tadege et al., 2011a). *STF* modulates blade outgrowth by promoting cell proliferation at the adaxial–abaxial junction of leaf primordium (Tadege et al., 2011a). Lin et al., (2013) also demonstrated that transcriptional repressive activity conferred by the *WUS*-box of the *STF* protein underlies this mechanism. The *WUS*-box is essential to the function of *STF* in both leaf blade outgrowth and floral organ development (Lin et al., 2013; Niu et al., 2015; Tadege et al., 2011a) as *STF* acts as transcriptional repressor primarily through its *WUS*-box in organizing cell proliferation during leaf blade morphogenesis. In fact, the *WUS* gene itself can substitute for *STF* function if expressed in the correct domain, as demonstrated by complementation of the *lam1* and *stf* mutants in *N. sylvestris* and *M. truncatula*, respectively (Lin et al., 2013). Both the blade and flower phenotypes of *lam1* can be complemented with *WUS* expressed under the *STF* promoter suggesting a conserved mechanism in stem cell maintenance and lateral organ development (Lin et al., 2013; Tadege 2013; Tadege et al., 2011a; Tadege et al., 2011b; Zhang et al., 2014). The *stf* and *lam1* mutants are severely affected only in medial-lateral (width) growth of the leaf blade and flower petal where both cell division and cell expansion are compromised. In *stf* leaf primordium, leaf founders are recruited at the right position and blade tissue is formed in both the terminal leaflet and the two lateral leaflets, but expansion of the lamina and further differentiation of tissues is arrested leaving the width of *stf* leaves less

than a third of the wild type (Lin et al., 2013; Tadege et al., 2011a). Therefore, *STF* is critical for lamina outgrowth and leaf vascular patterning in simple leaf (*N. sylvestris*) and compound leaf (*M. truncatula*) species.

On the other hand, *WOX9 or STIMPY* (*STIP*) was first identified in an activation-tagging screen through a dominant gain-of-function allele, *stip*-D (Wu et al., 2005) in *Arabidopsis.* And this study revealed that *WOX9 (STIP)* is expressed in developing embryos, proliferating tissues, in the vegetative shoot and leaf primordia in floral meristems, in emerging floral organs, in the inflorescence meristem, in the epidermal layer of the placenta and growing septum, and in the upper portion of the root meristematic zone (Haecker et al., 2004; Wu et al., 2007; Wu et al., 2005) indicating that *WOX9* is required for several developmental programs such as early embryo development, promotion of the growth of vegetative SAM by maintaining the cell division in shoot and root apex. In *Arabidopsis* gain-of function (*stip*-D) plants develop wavy leaf margins and an increased number of axillary shoots, resulting in a mildly bushy phenotype due to mis-regulated cell division in leaves (Wu et al., 2005). In addition, flower and ovule development are also affected in *stip*-D plants, leading to reduced fertility (Wu et al., 2005). On the other hand, loss of *WOX9*/*STIMPY* activity leads to a reduction in embryonic growth, failure in axillary SAM and leaf-primordia initiation and growth, and failure in primary root growth and lateral root initiation indicating that *WOX9*/*STIMPY* functions as a general positive regulator of undifferentiated growth in *Arabidopsis*. In addition, complete loss of *WOX9*/*STIMPY* function results in early embryonic arrest (Wu et al., 2007) due to the *stip* embryos show cell cycle arrest; suggesting that *WOX9*/*STIMPY* has a broader role in maintaining growth in the vegetative shoot apex, which may be the cause of the complete growth arrest of most *stip* seedlings. Lateral organs such as leaves or secondary shoots are never formed after the vegetative SAM fails in *stip* mutants (Wu et al., 2005), suggesting the vital role of *WOX9*/*STIMPY* in maintaining vegetative growth. Further investigation in *stip* mutant cell differentiation and cell-division revealed that *WOX9*/*STIMPY* maintains the ability of cell to divide in the vegetative SAM and leaf primordia. Generally, phenotypic characterization of *stip* mutant showed that growth arrests soon after germination in more than 80% of the seedlings, resulting in early seedling lethality and remaining plants are delayed in

development in comparison to the wild type, and about half of them die during later vegetative development (Wu et al., 2007; Wu et al., 2005).

As indicated above, *STF* acts mainly as a transcriptional repressor, and the evolutionarily conserved *WUS* box, which is specific to the modern/*WUS* clade *WOX* genes, partially contributes to this repressive activity (Lin et al., 2013). Since *WOX9* acts as an activator and enhances the *lam1* mutant phenotype, it will be important to investigate the function of *WOX9* in relation to lamina outgrowth in tobacco to understand how the distinct functions have evolved. The effect of *WOX9* that shares common aspect with cytokinin that upregulates *CycD3* expression to promote undifferentiated cell growth, and regulates organ initiation in shoot apex (Giulini et al., 2004; Riou-Khamlichi et al., 1999) should be investigated to understand the effect of ectopic expression defects in transgenic plant leaves. Furthermore, the complementation of *stip* mutant in *Arabidopsis* by exogenous application of sucrose indicates the functional role of *WOX9* in stimulating the cell cycle (Wu et al., 2005). In previous studies, the constitutive expression of *evergreen* (*EVG*) and *WOX9* in *Petunia* and *Arabidopsis*, respectively, causes similar defects, suggesting that the proteins are functionally very similar and the diversification of *EVG* and *WOX9* might rely on alteration in their expression pattern as reviewed in (Costanzo E et al., 2014). For example, *EVG* and *sister of evergreen* (*SOE)* in *Petunia* represent the duplication (two *WOX9*-like genes) of an ancestral gene that acquires a new function in development (Rebocho et al., 2008). However, Wu et al., (2005) reported that the functional redundancy of *WOX9* paralog is not a simple case among closely related genes as it depends on the interaction partners to have different effects in plant developmental process. It has been also mentioned that may be a general feature that of the *WOX9* family that functional redundancy is not solely determined by overall protein sequence similarity (Wu et al., 2007). More recently, *WOX9* has been shown to promote the establishment of cotyledon boundary along with the expression of *WOX2, CUC2* and *CUC3* (Lie C et al., 2012).

Among intermediate clade, *WOX9* function in leaf primordia has not been established, and it remains to be shown whether *STF* and *WOX9* antagonistically regulate the same target or set of targets during leaf blade morphogenesis (Lin et al., 2013). Based

on these findings I came to the hypothesis that *MtWOX9* acts as an activator and probably functions antagonistic to *STF* in STF-mediated leaf lamina outgrowth

In this study, spatial and temporal expression of *MtWOX9*-1 and *MtWOX9-2* in *Medicago* young leaf, shoot apex, stem, pod, and flower at different developmental stages have shown and similar pattern was also observed using GUS reporter that suggest the biological function of *WOX9* genes in plant development in general and leaf lamina development in particular. Interestingly, the highest expression of *MtWOX9-1* in *stf* mutant suggests that *STF* may have regulation effect on *MtWOX9*. Overexpression of *MtWOX9-1* in *Medicago* induced remarkable leaf blade defect with downward curling and ectopic expression of *MtWOX9-1* in *N. sylvestris* reveals strong effects in leaf blade development, as it is sufficient to modify the leaf features with the generation of unique leaf blade defects. In addition, ectopic expression of *MtWOX9-1* in *stf* (*Medicago*) and *lam1* (tobacco) dramatically worsen leaf phenotype compared with the original *stf* and *lam1* mutant by forming a more compacted radial blade with added proximal-distal defects. The higher expression of *MtWOX9-1* was correlated with the higher degree of leaf lamina twists and worsens leaf size and shape. Furthermore, detail phenotype analysis of severe phenotype of 35S::*MtWOX9-1* in tobacco wild type revealed not only significant leaf defects and arrested growth but also showed significant late flowering time in tobacco due to low expression of tobacco *FLOWERING LOCUS T1* (*NsFT1)* which was reversed by ectopic expression of the *NsFT1* gene. It is also interesting to note that ectopic expression of *NsFT1* in transgenic line as well as *lam1* complemented flowering time and also fully rescued the twisted and distorted tobacco leaf blades affected by ectopic expression of *35S::MtWOX9-1/WT* in severe transgenic lines indicating the functional interaction of *NsFT1* with *MtWOX9-1* in leaf blade out growth. Taken, together this study suggest the existence of functional interconnection among *WOX9, STF/LMA1* and *FT* genes in leaf blade development and flowering time control in tobacco plant. Recently, several research findings have reported that *FT*-like genes (florigens) not only play universal roles as floral integrator and flowering activator but also involved in pleiotropic functions as general growth hormone; affecting the various physiological process in monocot and dicot plants for different developmental programs (Böhlenius et al., 2006; Guo et al., 2015; Hiraoka et al., 2013; Krieger et al., 2010; Lee et

al., 2013; Li et al., 2015; McGarry and Ayre 2012; Melzer et al., 2008; Navarro et al., 2011; Niwa et al., 2013; Shalit et al., 2009; Smith et al., 2011). For instance, *FT*-like genes regulate stomatal guard cells opening, modulate lateral shoot outgrowth, involved in multiple steps of axillary bud development in *Arabidopsis* (Hiraoka et al., 2013; Kinoshita et al., 2011; Niwa et al., 2013; Smith et al., 2011). Furthermore, ectopic overexpression of *FT* in cotton promotes determinate growth habit in all aerial organs (McGarry and Ayre 2012). S*INGLE FLOWER TRUSS* (*SFT*) regulates reiterative growth and termination of shoots, influences leaf maturation, compound leaf architecture, stem growth, and abscission zone formation (Shalit et al., 2009). As reported in recent study of ectopic expression *G. hirsutum (GhFT1)* in wild type (WT) tobacco, *GhFT1* showed pleiotropic functions such as lateral shoots outgrow at the base, axillary buds at rosette axil, altering leaves morphology (Guo et al., 2015; Li et al., 2015).

Research goals and objectives

The goal of this research was to find out the role of *MtWOX9* in STF-mediated leaf blade development pathway using genetic transformation and biochemical analyses.

Specific objectives:

To analyze and determine the spatial and temporal expression pattern of *MtWOX9*-like genes in *Medicago* at different developmental stages.

To determine the effect of *MtWOX9* over expression in *Medicago* and tobacco leaf blade development,

To identify the nature of MtWOX9 and STF interaction and its role in the STF-mediated leaf blade development pathway

MATERIALS AND METHODS

Plant materials and growth conditions

Medicago truncatula var R108 and *stf* mutant (NF0120 & NF075) and *Nicotiana sylvestris* (tobacco) wild type and *lam1* mutant were grown in one gallon pots in greenhouse under long day (LD) conditions with 16/8 hours light/dark cycle at 27-30°C temperature, and in growth room under long day conditions of 16/8 hours light /dark cycle at 23-25°C temperature, 70-80% relative humidity, and 150 µmol.m2 light intensity.

Plant tissue sample collection

Medicago plant tissue samples (root, cotyledon, singlet, trifoliate, young leaf and old leaf at vegetative stage, shoot apex, flower and young pods) were collected throughout the developmental stage of the plant (from seedling to pod setting stages) of wild type. In addition, young leaf and shoot apex samples were collected from *stf* mutant and WT. Leaf samples of tobacco wild type, transgenic and *lam1* mutant were collected at appropriate time of specific experiment for different genes expression patterns analyses. Collected samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until processing.

RNA extraction and gene expression patterns analyses

Total RNA was isolated using TRIzol Reagent (Invitrogen) for cDNA synthesis. Reverse transcription (RT) was performed using RNA treated with DNase I (Invitrogen), an oligo (dT) primer and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instruction. Expression patterns analyses were performed using semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) and quantitative real time PCR (qRT-PCR) with specific forward and reverse primers. *Medicago* and tobacco actin primers were used as positive controls. All specific forward and reverse primers used for gene cloning and expression analysis in this study were given in Supplemental Table 1.

Gene isolation, cloning and transformation

MtWOX9-1 gene was isolated from the *Medicago* genome by BLAST search using *Arabidopsis WOX9* sequence. Full-length of *MtWOX9-1* coding sequence was amplified by RT–PCR using total RNA extracted from leaf samples. To generate transgenic plants of *Medicago*, the cDNA was sub-cloned into the pDONR207 entry vector (Invitrogen) by BP clonase reaction. The final construct was produced by an LR clonase reaction between each of the entry vectors and pMDC32 of destination vector. The resulting plasmid was transformed into *Agrobacterium tumefaciens* strain *AGL1* and *GV2260* by freeze-heat shock method and used to transform into *Medicago* wild type and *stf* mutant by agro-mediated-transformation (AMT). *Medicago* AMT performed by explant wounding and co-cultivation. Then the inoculated explant leaf disks transferred to callus inducing medium with appropriate antibiotics which followed by regeneration and rooting using SH3a, SH9 and 1/2SH9 media, respectively (Chabaud et al., 2007). Tobacco (*N. sylevesris*) wild type and *lam1* mutant plants were transformed by *35S::MtWOX9-1, SFT::GUS* and *35S::NsFT1* genes, using *Agarobacterium tumefaciens*, *GV2260* strain by leaf disks inoculation, co-cultivation and regeneration tissue culture method (Horsch et al., 1986). Sufficient homozygous transgenic lines carrying *35S::MtWOX9-1, MtWOX9-1-::GUS, STF::GUS,* and *35S::NsFT1* were generated for each transgenic background. Transgenic lines were genotyped by DNA extraction and performing RT-PCR for gene constructs insertion. Then, transgenic lines and non-transformed controls were transferred to soil and grown under LD conditions and phenotypic and molecular analyses carried out accordingly.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of MtWOX9-1 and 2 proteins of for *Medicago* and homologs from other related species were performed using BioEdit software and the ClustalW program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Neighbor-joining phylogenic tree was constructed using MEGA6.0 default settings with 1000 bootstrap replications (http://www. megasoftware.net/).

Yeast-two-hybrid assays

Yeast two-hybrid analysis was performed using the ProQuest Two-Hybrid system (Invitrogen) following the manufacturer's instructions. The full-length and different domain combination truncation protein of MtWOX9 cloned in pGBKT7-GW as bait, while the full-length and different truncation proteins of STF and partners proteins were cloned in pGADT7-GW as prey, and sets of constructs were co-transformed into Y2H Gold yeast strain (Clontech). Yeast transformants were selected on synthetic minimal double dropout medium deficient in *SD/-Leu/-Trp*. Protein interactions were assessed on triple dropout medium (TDO) deficient in *SD/-His/-Trp/-Leu* and quadruple dropout stringent medium (QDO) deficient in *SD/-His/-Trp/-Leu/-Ade*.

Bimolecular florescence complementation (BiFC) analysis and confocal microscopy

BiFC assays were conducted according to Lu et al., (2010). Briefly, *MtWOX9* and different domain truncation were cloned into pEARLEYGATE201-YN, while *STF* was cloned into pEARLEYGATE202-YC, by LR reaction. Each construct was introduced into *Agrobacterium* stain *GV2260* by freeze-heat shock method. Pairs of combinations were co-infiltrated into four weeks old *N. benthamiana* leaves. P19 was used to inhibit transgenic silencing. Yellow florescent protein (YFP) signal was observed after 48 to 60hrs of infiltration using a TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems).

RESULTS

Multiple sequence alignment and phylogenetic analysis of MtWOX9-like proteins with homologs from other related species

To study *WOX9* orthologs in *Medicago truncatula,* BLAST search of the *Medicago* genome database have been done using *WOX9* gene sequences of *Arabidopsis* and related species. On the basis of sequence homology to *Arabidopsis*, two genes were identified in *Medicago* plant and designated as *MtWOX9-1* and *MtWOX9-2* (Fig. 1a). Both *Medicago WOX9*-like genes show limited homology to each other with 45% amino acid identity. To understand the evolutionary relationship; phylogenetic analysis was performed including two *Medicago MtWOX9*-like genes, five other related species such as *NsWOX9* (*Nicotiana sylvestris), PcWOX9* (*Phaseolus coccineus*), *GmWOX9* (*Glycine max*), *VvWOX9* (*Vitis vinifera*), *PhWOX9* (*SISTER OF EVERGREEN* (*Petunia x hybrid*), *CsWOX9* (*Cucumis sativus*) and *SlWOX9* (*COMPOUND INFLORESCENCE* (*Solanum lycopersicum*) and 15 *Arabidopsis WOX* genes. All *WOX9* genes from different species were clustered together (fig. 1b). As indicated, MtWOX9-1 is closer to *Arabidopsis* WOX9 proteins with 86% aa identify, followed by PcWOX9 with 71% aa identity whereas MtWOX9-2 shows lower aa identity to tested species with 41-42% aa identity, except for *Arabidopsis* WOX9 of 64% aa identity (Fig. 1b).

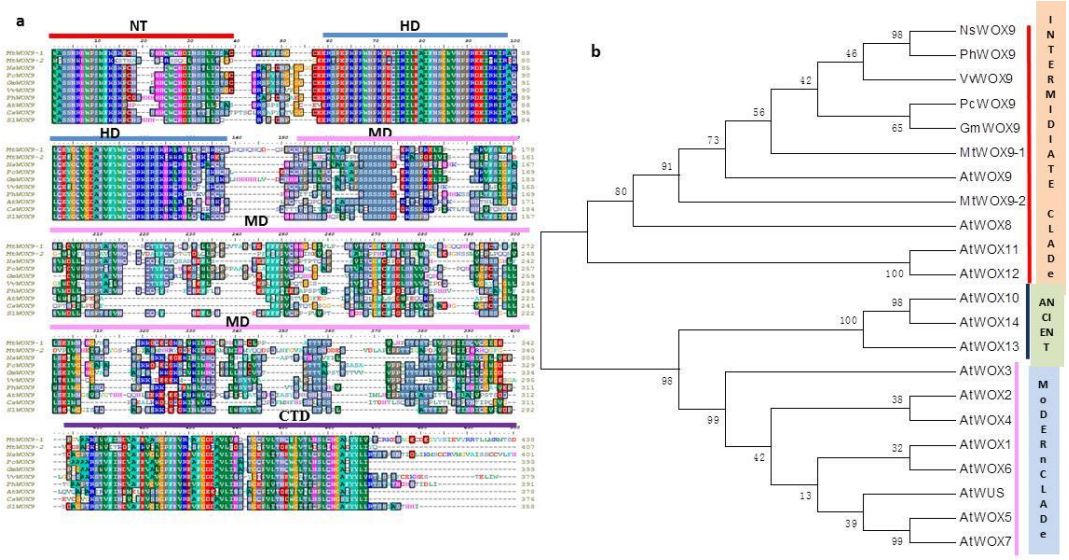

Fig. 1 Sequence comparison of *Medicago* WOX9 (MtWOX9) and related species proteins. (a) Section of multiple protein alignment of the complete protein sequences of

two MtWOX9 proteins with related AtWOX9 (*Arabidopsis thaliana*), NsWOX9 (*Nicotiana sylvestris*), PcWOX9 (*Phaseolus coccineus*), GmWOX9 (*Glycine max*), VvWOX9 (*Vitis vinifera*), PhWOX9 (SISTER OF EVERGREEN (*Petunia x hybrid*), CsWOX9 (*Cucumis sativus*) and SlWOX9 (COMPOUND INFLORESCENCE (*Solanum lycopersicum*). N-terminal domain (NTD), homeodomain (HD), middle domain (MD) and C-terminal domain (CTD) are marked by red, blue, purple and brown, respectively by horizontal thick lines. (b) Phylogenetic analysis of orthologues of the *MtWOX9*-like genes based on the amino acid sequence of the full length proteins, fifteen *Arabidopsis thaliana WOX* genes and other proteins of related species were retrieved via the NCBI server (http://www.ncbi.nlm.nih.gov/). *WOX9* genes clustered together as shown at each node/boxes. Numbers on major branches indicate bootstrap percentages for 1000 replicate analyses.

Expression of *MtWOX9* genes in different *Medicago* plant organs

To assess the biological function of *MtWOX9* genes in *Medicago truncatula* leaf and other lateral organs, transcript analysis was performed at different developmental stages of the plant of R108 variety wild type (WT). Tissue samples were collected at three developmental phases of the following organs: root, cotyledon, single leaf and trifoliate leaf (seedling phase of 10 days old); shoot apex, young leaf, old leaf and stem (vegetative phase of 4-weeks old); flower and pod at reproductive phase. *MtWOX9-1 & 2* gene expression analyses were made by semi-quantitative and quantitative real time PCR (qRT-PCR). Transcript accumulation of *MtWOX9-1* and *MtWOX9-2* were detected in young leaf, shoot apex, stem, pod and flower organs at moderate to strong expression levels (Fig. 2a-c). Although, *MtWOX9-1* and *MtWOX9-2* have similar expression pattern in leaf, stem, shoot apex, flower and pod; *MtWOX9-1* was highly expressed in flower, pod and root. In addition *MtWOX9-1* moderately expressed in leaves, shoot apex and stem as compared to *MtWOX9-2* (Fig. 2b&c) whereas, *MtWOX9-2* was highly expressed in stem and flower (Fig. 2b&c). Both genes commonly expressed in flower, stem and pod. This result revealed that *MtWOX9-1& 2* genes expression at different stages of the plant, across tested organs suggest that both genes may have specific and redundant function in various lateral organs of *Medicago* plant.

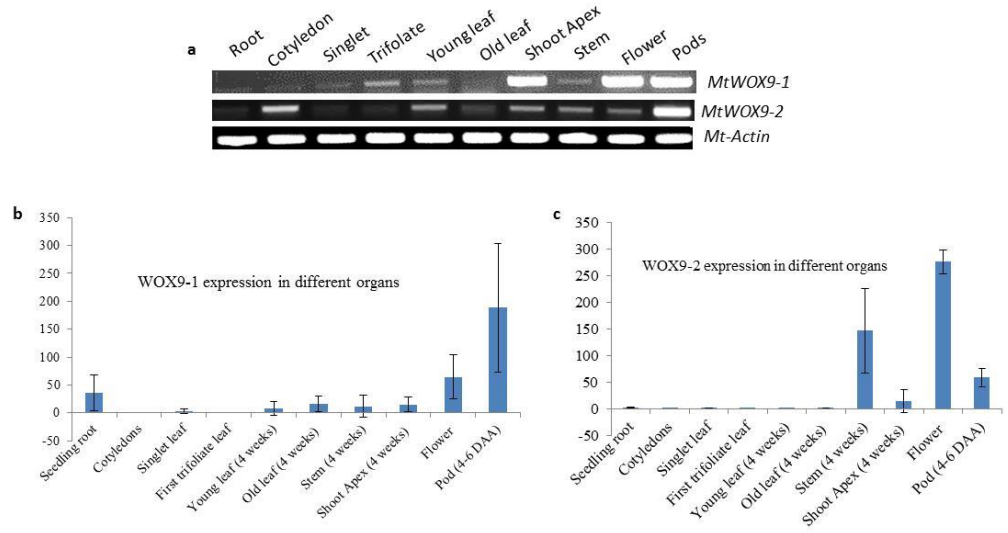

Fig. 2 Temporal and spatial expression pattern analyses of *Medicago WOX9*-like genes. (a) Expression pattern analysis of *MtWOX9-1 &2* like genes in indifferent tissues of *Medicago* R108 wild type (WT) at different growth stages (seedling to pod setting) by semi-quantitative RT PCR. (b&c) *MtWOX9-1* and *WOX9-2 e*xpression pattern analysis of by qRT-PCR under LD conditions. Expression levels were determined by semi-quantitative & quantitative RT-PCR using Sb-Actin as loading and internal control.

Furthermore, 3kb of *MtWOX9-1* gene promoter region was cloned and fused with *GUS* and transformed into *Medicago* wild type (R108) by agro-mediated-transformation (AMT) for *GUS* expression analysis for additional confirmation of the pervious *MtWOX9-1* gene expression pattern analysis. *MtWOX9-1* promoter with a β-glucuronidase (*GUS*) reporter showed strong signal in cotyledon, young leaf, stem, shoot apex, flowers and young pod & immature seed (Fig. 3a-h). However, as the leaf and seed grows older, the expression level becomes progressively weaker and confined to veins and helium, respectively. Taken together, the expression pattern and reporter *GUS* expression analysis showed similar expression patterns of *MtWOX9-1* in different organs suggesting that the genes may have biological function in leaf, stem, shoot-apex, flower, pod and immature seed which requires further molecular and genetic investigation.

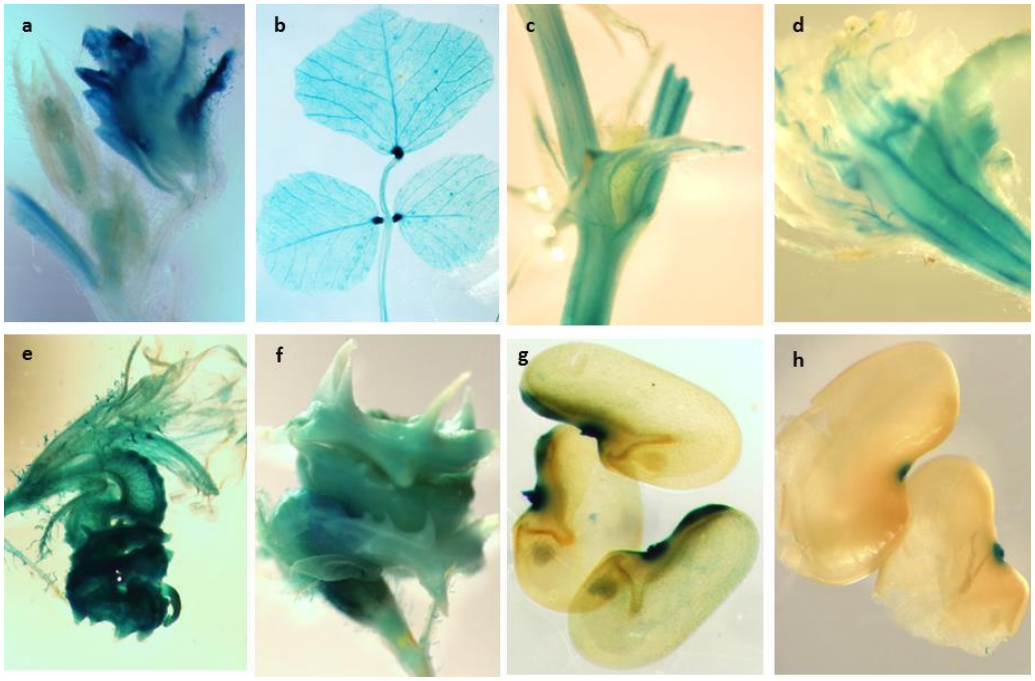

Fig. 3. *ProMtWOX9-1-GUS* staining expression pattern analysis. (a) *pMtWOX9::GUS* staining signal in the young leaf and shoot apex; (b) *pMtWOX9::GUS* staining signal in fully expanded leaf (old leaf); (c) *pMtWOX9::GUS* staining signal in stem; (d) *pMtWOX9::GUS* staining signal in flower; (e) *pMtWOX9::GUS* staining signal in the young pod; (f) *pMtWOX9::GUS* staining signal in the old pod (g&h) *pMtWOX9::GUS* staining signal in immature seed and matured seed, respectively. Strong *GUS* signals were detected in all tested organs but confined to seed helium site in matured seed.

` To study whether *MtWOX9-1* expression was affected by the presence or absence of *STF* and to insight any regulatory relationship in leaf blade development; expression pattern of *WOX9-1* was studied in young leaf and shoot apex of 4-weeks old plants of *stf* mutant (NF075 and NF0120) and wild type (R108). Interestingly, highest *MtWOX9-1* transcript accumulation was detected in both *stf* mutant alleles as compared to wild type (Fig 4) suggesting that *STF* may repress the expression of *MtWOX9-1* in leaf blade outgrowth in WT as compared the mutant.

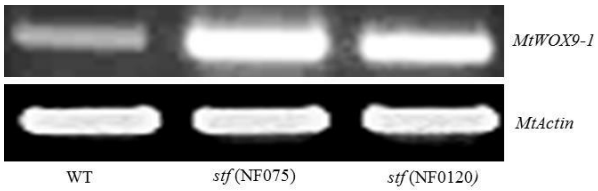

Fig. 4. Expression pattern of *MtWOX9-1*-like gene in WT vs *stf* mutant alleles (NF075 & NF0120) from plants at 4weeks old under LD condition. Expression levels were determined by semi-quantitative & quantitative RT-PCR using Sb-Actin as loading control.

Effect of *MtWOX9-1* overexpression in *Medicago truncatula* leaf blade development

To further, determine the biological function of *Medicago truncatula WOX9-1* in leaf blade development, *MtWOX9-1* gene was transformed into wild type (R108) and *stf* mutant plant driven by the 35S promoter. Transformation was confirmed by performing RT-PCR where twelve and eight independent transgenic lines in the wild type and *stf* mutant background were generated, respectively. Transgenic lines were transferred to soil and grown under LD conditions for phenotype and molecular analyses. All transgenic lines with wild type background leaves showed downward curling with triangular shape (Fig.5b). There is also prominent deep leaf margin serration and diminished venation pattern in transgenic lines (Fig. 5b) as compared to wild type (Fig. 5a). Moreover, the fertility of transgenic lines was reduced with poor pod and seed setting potential. Interestingly, ectopic expression of *MtWOX9-1* in *stf* mutant worsens leaf morphology in particular and arrests plant growth in general (Fig. 5d-i). In addition, the medial-lateral and proximal-distal axes of transgenic lamina were highly affected (Fig. 5d-i) as compared to *stf* mutant (control) (Fig. 5c). These results suggest that ectopic expression of *MtWOX9*-1 may have adverse effect on leaf lamina development in STF leaf pathway of leaf blade outgrowth.

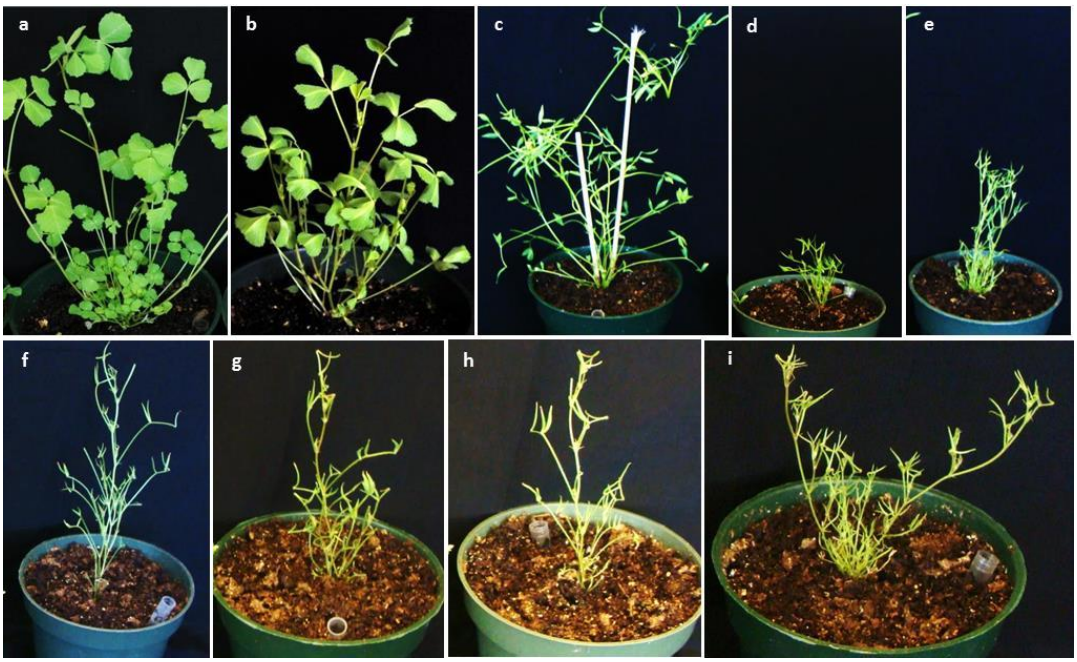

Fig. 5. Phenotype of ectopic expression of 35S*::MtWOX9-1* genes in transgenic *Medicago* WT and *stf* mutant. (a) R108 WT at vegetative stage (b) Phenotype of *35S::MtWOX9-1/*WT transgenic plant at vegetative stage (c) Phenotype of *stf* mutant plant (control) (d-i) Different phenotype of *35S::MtWOX9-1/stf* transgenic plants with worsen and arrested growth condition.

MtWOX9 protein interacts with STF and its partner proteins.

To understand the molecular mechanisms underlying MtWOX91 and STF mediated transcriptional effect in blade outgrowth, yeast two-hybrid (Y2H) assays was performed for STF and STF partner proteins using the Y2H Gold system. In this study, protein-protein interactions have tested between MtWOX9-1 and STF using full-length sequences of MtWOX9-1 fused to the GAL binding domain or bait (BD-MtWOX9-1) and STF fused to the activation domain or prey (AD-STF). Based on the conserved domain analysis, STF protein was divided into four major parts: the N-terminal domain, NTD (amino acids 1-90); homeodomain, HD (amino acids 91-163); middle domain, MD (amino acids 164-300); and C-terminal domain, CTD (amino acids 301-358). Further truncation also made for each domain in combination of one another. Then, Y2H interaction assay using these truncated versions of STF including AD-STF-1-153, AD-STF-1-163, AD-STF-1-300, AD-STF-154-300, AD-STF-164-300, AD-STF-154-Stop,

AD-STF-164-Stop and AD-STF-301-Stop; representing combined and specific domains of NTD, HD, MD, *WUS*-box and STF-box (Fig. 6a). Strong protein interactions under stringent conditions were detected between BD-MtWOX9-1 and AD-STF for the following truncations: AD-STF-1-300, AD-STF-154-Stop, AD-STF-164-300 and AD-STF-164-Stop. AD-STF-154-300 showed weak interaction while no interaction was detected between BD-MtWOX9 and AD-STF-1-153, AD-STF1-163 and AD-STF-300-Stop domains (Fig. 6a). Taken together, the analysis of STF truncation regions showed that the middle domain (AD-STF-164-300) truncated region may be responsible for MtWOX9-1 and STF protein interaction. Similarly, conserved MtWOX9-1 domains truncated into four regions such as MtWOX9-1-153 (NTD), MtWOX9-140-368 (MD) and MtWOX9-369-458 (CTD) and tested for their interaction with full-length STF protein (Fig. 6c). The result revealed that the middle domain of MtWOX9-1 also strongly interact with STF protein under stringent conditions, whereas, the rest of three domains of MtWOX9-1 have no interaction with STF (Fig. 6c). Unlike STF, specific domain of MtWOX9-1 (NTD, HD, MD and CTD) never showed interaction with full-length MtLUG and MtSPW73B. However, full-length MtWOX9-1 and combination of MD and CTD of MtWOX9-1 strongly interact with full-length MtLUG and MtSPW73B showing the requirement of different domains combination of proteins interactions (Fig. 6d). These results suggest that the middle domains (MD) of both MtWOX9-1 and STF genes may be required for strong protein interaction and might be the most important region for their biological function and interrelationship. The interaction was confirmed by BiFC assay using split YFP complementation in *Nicotinia benthamiana* leaf cells (Fig. 6e) showing that MtWOX9-1 interacts with STF in the nucleus of living cells.

Other proteins including transcriptional co-repressors, transcriptional co-activators, histone deacetylases, and polarity factors represented by MtTPL, MtLUG, MtAN3, MtHDA6, MtHDA19, MtFIL, MtAS2 and MtKAN have been identified to interact with *STF* in Y2H and are supposed to be important players in the *STF*-mediated leaf blade development pathway (Zhang et al., 2014). These proteins were fused to gal AD domain and tested their interaction with BD-MtWOX9-1. The results showed strong interaction of MtWOX9-1 with MtHDA6, MtHDA19, MtLUG and MtKAN but no interaction was detected with MtTPL and MtAN3 whereas, moderate interaction was

detected for MtWOX9-1 with MtFIL and MtAS2 (Fig. 6b). These interactions suggest that histone modification enzymes, co-activators, and both adaxial-abaxial specific genes may be involved in the process with MtWOX9 genes in *STF* pathway of leaf blade outgrowth.

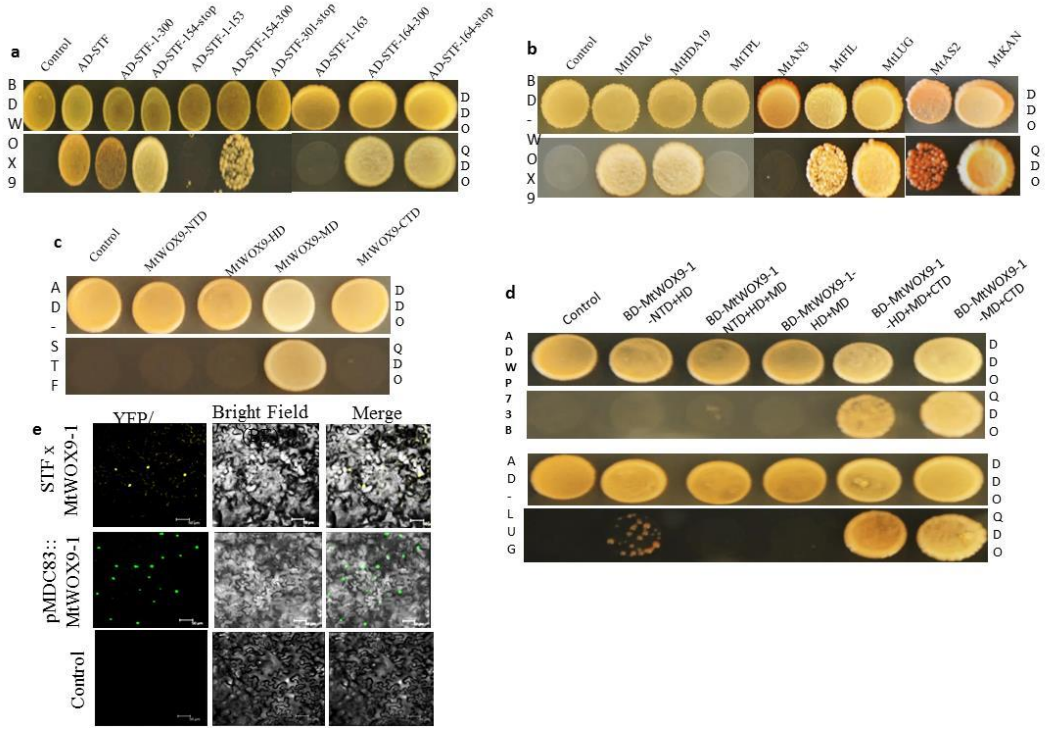

Fig. 6. Yeast-Two-Hybrid (Y2H) and BiFC protein interaction assay of MtWOX9-1 with STF and STF partner proteins. (a) BD-WOX9-1 protein interaction with AD-STF and its truncated regions (b) BD-WOX9-1 protein interaction with AD of MtHDA6, MtHDA19, MtTPL, MtAN3, MtLUG, MtAS2 and MtKAN proteins (c) BD-WOX9-1 specific domain interaction with full-length of AD-STF protein interaction (d) BD-WOX9-1 combined different domains interaction with full-length of AD-MtSWP73B and AD-MtLUG protein interaction. Yeast growth in QDO media indicates positive interaction. DDO, double dropout medium; TDO, triple dropout medium; QDO, quadruple dropout medium. (e) BiFC interaction between the MtWOX9-1-YC, and STF-YN (1st panel). MtWOX9-1 is nucleus localized (2nd panel). Yellow/Green fluorescence shows interaction in the nucleus. The control (bottom panel) shows absence of interaction between MtWOX9-1 and YC alone in the vector.

Effect of *MtWOX9-1* on tobacco wild type leaf blade and *lam1* mutant

To investigate the functional effects of *MtWOX9* gene in tobacco leaf blade development, the ectopic expression *MtWOX9-1* was analyze in *Nicotiana sylvestris* wild type (WT) and *lam1* mutant background, as tobacco is faster to transform and also representing simple leaf as compared to *M. truncatula* (Lin et al., 2013; Tadege et al., 2011a; Tadege et al., 2011b). Therefore, *MtWOX9-1* gene with 1374bp cloned into binary vector pMDC32 with 35S promoter (*35S::MtWOX9)* and was transferred into *Agrobacterium tumefaciens* strains, *AG2260* and transformed into tobacco plant by AMT. Twenty transgenic lines were generated for each background and positive lines were confirmed by DNA extraction and RT-PCR genotyping. Then, ten *MtWOX9-1/*WT transgenic lines of T2 generations were selected and evaluated for different leaf features and related phenotype. Normally, the leaf blade of tobacco WT is well expanded lamina with smooth margin and organized venation patterns (Fig. 7a-c and 8a). However, the generated transgenic lines of *MtWOX9-1* overexpressing plants showed various leaf phenotypes which can be categorized into two major classes; severe (Fig. 7d-f) account for about 50% of the transgenic lines and mild (Fig. 7g-i) phenotype accounting for the other 50%. Interestingly, severe phenotypes were characterized by narrow leaf blade, down curling, wavy margin, and arrested bushy growth with 2-5 tillers at the base of the plant (Fig. 7e & f). At seedling stage the leaves were highly twisted with distorted and coiled blade shape (Fig. 8g). In general, severe phenotype lines have small and numerous leaves ranging from 124-257 per plant with mean of 157 ±15 leaf number (fig 7e, f & m), whereas mild phenotypes leaf numbers range 20-32 per plant with mean of 27 ±1.2 (Fig. 7g-i & m) as compared to wild type 12 ±0.3 (Fig. 7a-c & m). Thus, leaf shape, size and number were among the most important features that are significantly affected by *MtWOX9-1* ectopic expression. In addition, the defects of leaf lamina with narrow cotyledon and first leaf also observed in seedling of severe phenotype of T2 generation at early stage (Fig. 7d) which indicates that *WOX9-1* is already active during the early stages of leaf organ development. Severe phenotype also showed thick and leathery leaf structure with outward teeth like projects at the leaf margin (Fig 8b-d & g). In severe phenotypes, the vein patterning was also abnormal with diminished mid and lateral vein formation (Fig. 8f & g). On the other hand, the leaf feature of mild phenotype was

characterized by semi-circular leaf blade with unsmooth and undulated surface (Fig. 7g-i and Fig. 8e). The leaf margin is also abnormal as compared to WT. However, overall mild phenotypes lamina expansion, leaf size and shape were partially affected as compared to severe phenotype. In general, both mediolateral and proximodistal leaf blade of severe phenotype were markedly affected as indicated in quantitative analyses. The average leaf blade width record of severe phenotype was 2.2 ±0.2 whereas for the mild phenotype width was 5.0 ±0.7 as compared to WT 11.6 ±0.2 that worsen fivefold for severe and twofold for mild phenotype (Fig. 7k). Similarly, not only the leaf blade width, but also the length was significantly affected with 7.3 ±0.6 for severe phenotype and 14.3 ±0.4 for mild phenotype, which is three and two fold less than the WT 23.0 ±0.3, respectively.

To get insight for the distinct leaf phenotypic classes of severe verses mild phenotype, semi-quantitative real time PCR performed to determine *MtWOX9-1* transcript accumulation in each phenotype. The expression analysis confirmed that the higher expression level of *MtWOX9-1* was associated to severe phenotype and lower expression with mild phenotype (Fig. 7j). This dosage dependent effect showed great impact on both leaf blade width and length. Taken together, ectopic expression of *MtWOX9-1* in wild type tobacco displayed distinct leaf morphological features suggesting the involvement of the gene in leaf blade outgrowth.

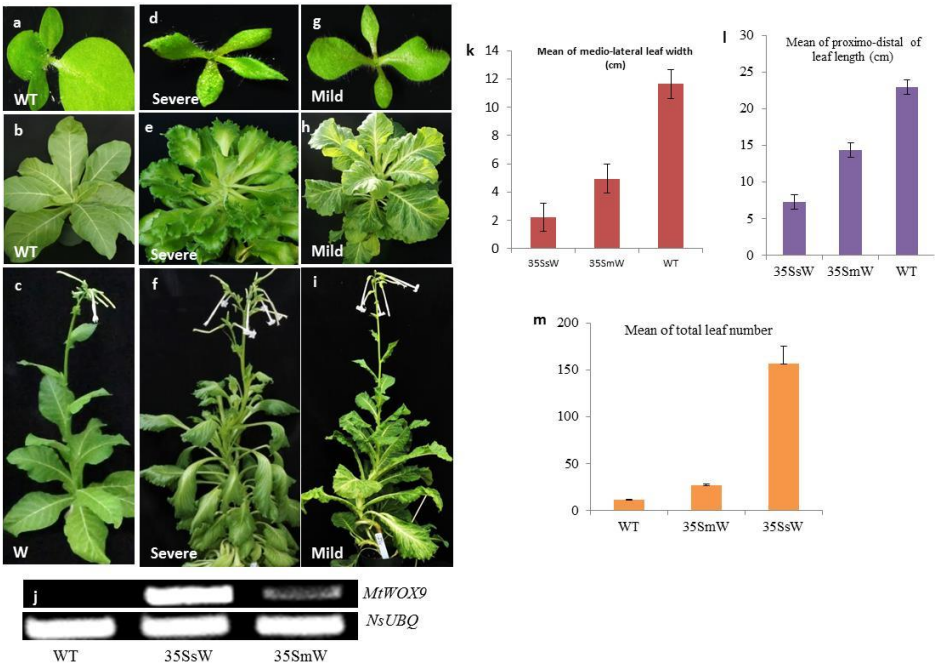

Fig. 7. Phenotypes of *35S::MtWOX9-1* expression in transgenic tobacco wild type (WT) background. (a-c) untransformed *N. sylvestris* wild type at seedling, vegetative & flowering stages. (d-f) Severe phenotype of *35S::MtWOX9-1* transgenic plants from seedling to flowering stag. (g-i) Mild phenotype of *35S::MtWOX9-1* transgenic plants from seedling to flowering stag. (j) Expression of *MtWOX9-1* transcripts in the leaves of WT, severe & mild Phenotype. Gene expression levels were determined by semi-quantitative RT-PCR using *NsUBQ* as loading control. (k) Mean of mediolateral leaf width of severe (35SsW), mild (35SmW) & WT phenotypes. (l) Mean of proximodistal leaf length of severe (35SsW), mild (35SmW) & WT phenotypes. (m) Mean of total leaf number of severe (35SsW), mild (35SmW) & WT phenotypes. Error bars show SE.

Fig. 8. Leaf phenotype of *35S:MtWOX9-1/WT* Transgenic lines (a) leaf of WT at vegetative (b-d) leaf phenotype of *35S::MtWOX9-1* of severe phenotype at vegetative stage (e) leaf phenotype*35S::MtWOX9-1* of mild phenotype at vegetative stage (f & g) leaf phenotype of *35S::MtWOX9-1* of severe phenotype at seedling stage.

MtWOX9-1 ectopic expression worsen *lam1* mutant phenotype

In previous studies, it has shown that ectopic expression of *Arabidopsis WOX9* derived by *STF* promoter *(STF::WOX9)* exhibited an enhanced *lam1* blade phenotype with thinner and shorter leaf blades compared with *lam1* mutant (Lin et al., 2013).

Therefore, I was interested to investigate whether *MtWOX9* function is conserved among species in worsening *lam1* phenotype. Then, in this study, *Medicago trancatula WOX9-1* derived by 35S promoter (*35S::MtWOX9-1*) was introduced into *lam1* mutant background. Similarly, two major classes such as nine severe (45%) phenotype and eleven mild (55%) phenotype for leaf were observed. Severe phenotypes were characterized by worsen cylindrical leaves forming a more radial features as well as arrested plant growth condition (Fig. 9b). The quantitative analysis made by measuring leaf length showed that severe phenotype leaf reduced by fivefold, 5.5 ±0.5 and mild phenotype reduced by twofold, 12.9 ±0.9 as compared to the control, *STF::GUS* 24.0 ±1.3 (Fig. 9a, b, c & e). Transcript accumulation of *MtWOX9-1* was also quantified the in severe verses mild phenotype by semi-quantitative RT-PCR and the result showed that the highest transcript level was found in severe phenotype, compared to the mild phenotype with lower levels of *MtWOX9-1* expression (Fig. 9d). Similarly, this result suggests that *MtWOX9-1* effect on blade outgrowth is dose dependent. Taken together, these results suggest that *MtWOX9-1* affects leaf blade outgrowth and its effect is conserved among *Medicago* and tobacco species.

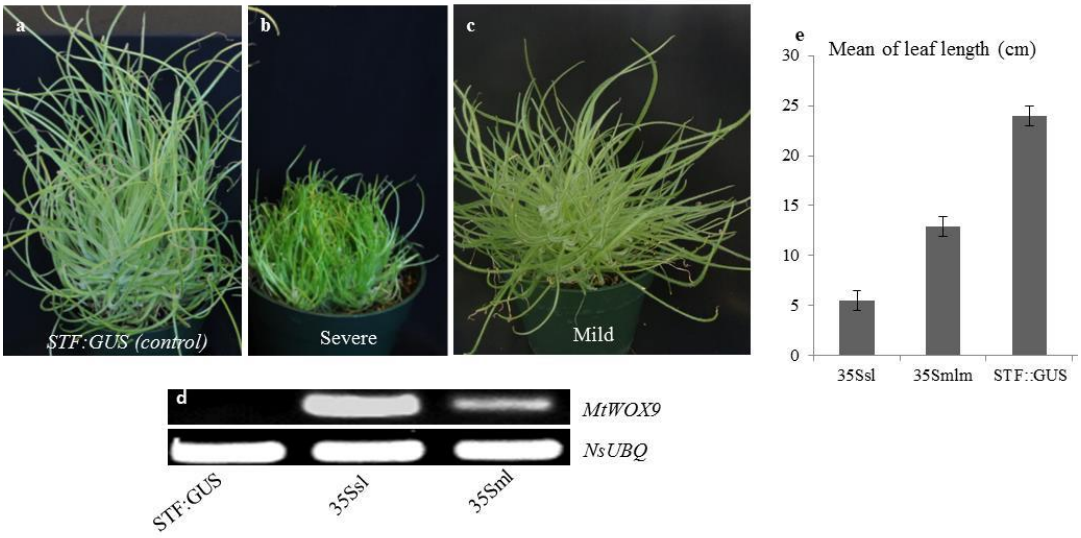

Fig. 9 Phenotype of *MtWOX9-1* gene in *lam1* tobacco mutant background transgenic lines. (**a**) *lam1* mutant with *STF::GUS* (control). (b) Severe phenotype of *35S::MtWOX9-1* transgenic plants. (c) Mild phenotype of *35S::MtWOX9-1* transgenic plants. (d) Expression of *MtWOX9-1* transcripts in the leaves of SFT::GUS, severe & mild Phenotype. Gene expression levels were determined by semi-quantitative RT-PCR

using *NsUBQ* as loading control. (e) Mean of leaf length of severe (35Ssl), mild (35Sml) & *STF::GUS* phenotypes. Error bars show SE

MtWOX9-1 ectopic expression in wild type tobacco delay flowering by repression of *FLOWERIGN LOCUS T*1 (*NsFT1*)

Further characterization of the severe phenotype of *MtWOX9-1* ectopic expression revealed significant delay of flowering in WT background transgenic lines. Normally, under greenhouse LD conditions, the flowering time of wild type *N. sylvestris* is about 72 days. However, for severe 35S:: M*tWOX9-1* transgenic lines, flowering time has taken 320 days, six time longer period to flower than the wild type (Fig. 10d-h). Based on this preliminary observation, we were interested to investigate the cause of delay of flowering in ectopically expressed *WOX9-1* transgenic lines. Then, ten severe plants from T2 generation and ten wild type plants were selected and grown under LD condition for the flowering time evaluation and expression pattern analysis of *Nicotiana sylvestris FLOWERING LOCUS T1* (*NsFT1)* in both 35S::*WOX9-1* transgenic lines and wild type. Among several *Nicotiana sylvestris FT*-like genes, *NsFT1* as one of the highly expressed at the floral transition period of tobacco wild type was selected. Fully expanded leaf samples were harvested at every 20 days interval for RNA extraction and cDNA synthesis. *NsFT1* transcript accumulation analysis was carried out at early seedling stages of 22 days old and continued up to first flower initiation of WT using semi-quantitative RT-PCR. Interestingly, *NsFT1* transcript accumulation was detected in WT from low to highest level, starting at 42, 62 and 72 days of vegetative, bolting and first flower initiation stage, respectively (Fig. 10a-c & i). On the contrary, there was no *NsFT1* transcript signal detected in *MtWOX9-1/*WT severe phenotype of same age of WT (Fig 10d-f & i). However, *NsFT1* expression observed in *MtWOX9-1/*WT lines at later growth stage (after 320 days) (Fig. 10g). Thus, this result suggests that the cause of delay flowering of *35S:MtWOX9-1/*WT transgenic lines may be due to the repression of tobacco *FT* gene (*NsFT1*).

Previous studies of *lam1* mutant showed that it rarely bolt and make flower under hot condition (Lin et al., 2013; Tadege et al., 2011a; Tadege et al., 2011b). Therefore, the expression of *NsFT1* in *lam1* and WT of same age under LD condition was also checked.

Similar to previous studies, *NsFT1* expression was detected in *lam1* mutant while strong expression of *NsFT1* observed in WT (fig 11a-c). Taken together, the absence of *NsFT1* expression in *lam1* mutant and *MtWOX9* overexpression transgenic lines led to speculate that the overexpression of *MtWOX9-1* in transgenic lines and the absence of *LMA1* in *lam1* mutant may have directly or indirectly effected for the repression of *NsFT1* that may control flowering time in both phenotypes. On the other hand, exogenous GA3 applications for two consecutive weeks induced bolting in both *lam1* mutant and *MtWOX9-1* transgenic lines, but not induce flowering (Fig. 13d & e).

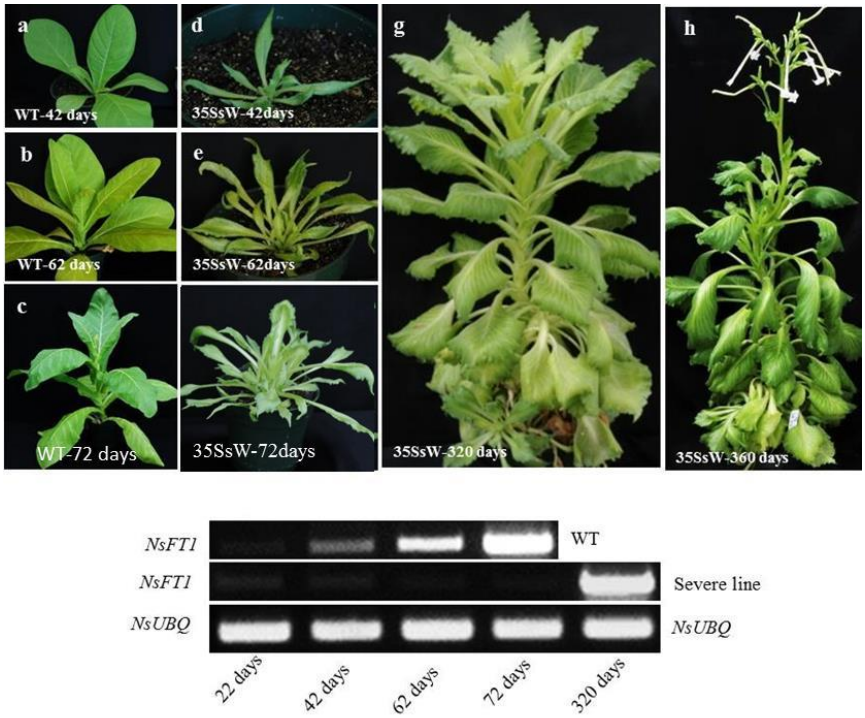

Fig. 10. Phenotype of *35S::MtWOX9-1/*WT and *NsFT1* expression pattern analysis in wild type and transgenic lines. (**a-c**) untransformed *N. sylvestris* wild type at vegetative, bolting & flowering stages. (d-h) Severe phenotype of *35S::MtWOX9-1* transgenic plants at vegetative, bolting & flowering stages. (i) Expression of *NsFT1* transcripts in the leaves of WT & severe phenotype of *35S::MtWOX9-1/*WT transgenic lines with delay flowering time. Gene expression levels were determined every 20 days interval, starting at seedling to first flower initiation stages by semi-quantitative RT-PCR using *NsUBQ* as loading control. *35S::MtWOX9-1/*WT transgenic lines delay flowering up to 320 days whereas WT flowers at 72 days

NsFT1 overexpression induce early flowering in wild type, *lam1* mutant and *MtWOX9-1*/WT late flowering transgenic lines

To confirm the biological function of *NsFT1* in promoting flowering, tobacco wild type was transformed using 35S promoter. Using leaf number as a flowering time measure, ten plants of T2 generation of transgenic line were evaluated under LD conditions, and all *NsFT1* transgenic lines at most had five leaves (5.2±0.2) at flowering compared to 12.±0.3 in wild type (Fig. 12c & g). This indicates that *NsFT1* ectopic expression induces early flowering in the wild type background and *NsFT1* is a promoter of flowering in tobacco.

Similarly, 35S::*NsFT1* overexpressed in late flowering severe phenotype of 35S::*MtWOX9-1* transgenic lines and generated nine T1 transgenic lines. We used three controlling systems (transgenic lines with empty vector, *MtWOX9-ox* transgenic lines passes through tissue culture and WT) for leaf and flower complementation comparison (Fig. 12a, b & d). Out of nine transgenic lines five, two and one plants flower at five, seven and nine leaves number (Fig.12e) which was less than the number of wild type at flowering time (Fig. 12a & 14a). Flowering time evaluation of transgenic line also at the T2 generation showed that 5.8±0.2 leaf number were formed, with very early flowering time to wild type 12±0.3 leaf number (Fig. 12f). On the other hand, the controls (*MtWOX9-ox* transgenic lines with empty vector and that passes through tissue culture) showed similar narrow leaf blade with down curled and late flowering as same as its progenitor background (Fig. 12b & d). This result indicates that *NsFT1* ectopic expression indeed complemented the flowering time of *MtWOX9* overexpression. Interestingly, *NsFT1* ectopic expressions relieves or normalizes the leaf blade defects of *MtWOX9-1* overexpressed transgenic lines with well expanded leaf lamina with smooth margin and normal venation pattern similar to WT (Fig. 12e & f, 13a & b, & 14e, f, h & i).

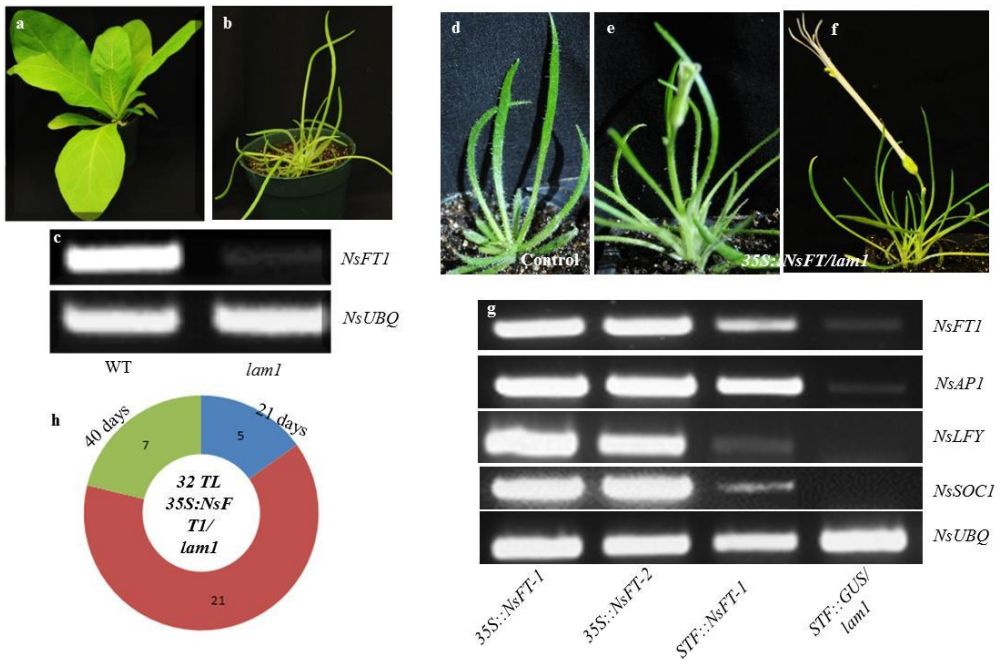

Fig. 11. Phenotype of WT, *lam1*, and *35S::NsFT1/lma1* ectopic expression and floral identity genes expression analysis in lam1 transgenic lines. (**a**) untransformed *N. sylvestris* wild type at bolting stage (8weeks old). (b) untrasformed *lam1* mutant of 8 weeks old plant. (c) Expression of *NsFT1* transcripts in the leaves of WT & *lam1* mutant 8 weeks old. (d) Phenotype *STF::GUS/lam1* of 4 week old. (e&f) Phenotype of *35S::NsFT1/lam1* transgenic lines with early flowering. (g) Induction of floral meristem identity marker genes, *NsAP1* and *NsLFY* in *35S::NsFT1/lam1* transgenic lines. Note the high level expression of *AP1* and *LFY* in *35S::NsFT/lam1* transgenic lines compared to none flowering *STF::GUS* (control). (h) Number of *35S::NsFT1/lam1* transgenic lines corresponding to number of days to flower. Gene expression levels were determined by semi-quantitative RT-PCR using *NsUBQ* as loading control.

As indicated in previous studies, *lam1* mutant rarely can spontaneously bolt and initiate flowers under high temperature (Lin et al., 2013; Tadege et al., 2011a; Tadege et al., 2011b) which might be caused by the low expression of *NsFT1*. To confirm this speculation, *NsFT1* gene derived by 35S promoter was introduced into *lam1* to rescue the non-flowering in *lam1* mutant. Through Agrobacterium transformation, thirty-two independent transgenic T1 lines of *lam1* background were generated (Fig. 11e&f). STF promoter also fused with *GUS* and transformed into *lam1* and ten lines were generated as

a control (Fig. 11d). Interestingly, all *35S::NsFT1* transgenic lines fully complemented *lam1* mutant flowering within 21-45 days (Fig. 11e, f & h) which is even less than the normal flowering time of WT (~70 days). Of the total 32 transgenic lines generated; five (16%), twenty (62%) and seven (22%) lines were flower at 21, 30 and 45 days difference, respectively (Fig. 11h).

To further, determine if the *NsFT1* ectopic expression activate flowering in *lam1* transgenic lines by activating the expression of the downstream floral meristem identity genes, transcript levels of *NsAP1, NsLFY* and *SOC1* were analyzed by using semi-quantitative RT-PCR. The results showed that the transcripts of *AP1, LFY* and *SOC1* were dramatically up-regulated in *NsFT1* transgenic lines at the early stage of floral initiation (Fig. 9g), indicating that tobacco *NsFT1* promotes flowering in *lam1* by activating floral meristem identity genes. These results indicate that *NsFT1* ectopic expression not only complements the none-flowering *lam1* mutant phenotype but also cause significantly early flowering in *lam1* mutant background.

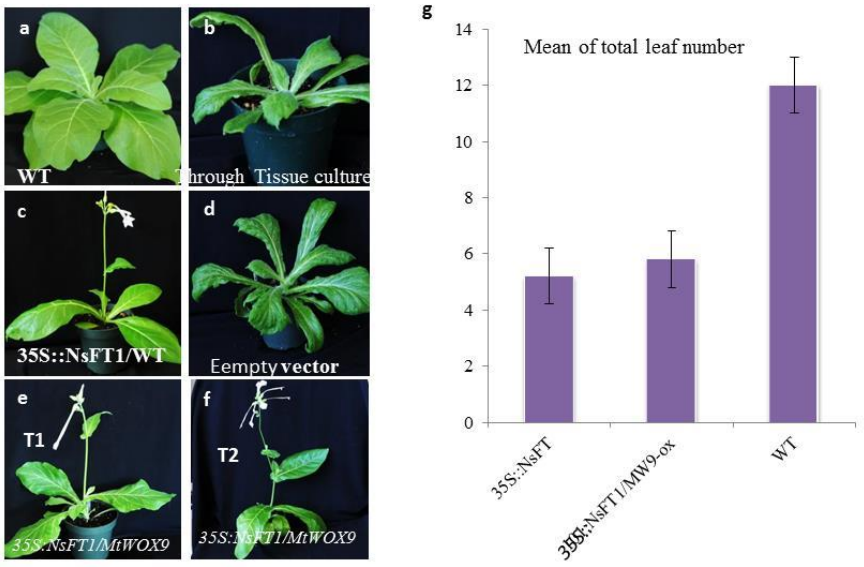

Fig. 12. Phenotype of *35S::NsFT1*ectopic expression in wild type and *35S::MtWOX9-1/*WT transgenic lines. (**a**)untransformed WT (control). (b) Phenotype of *35S::MtWOX9-1* transgenic lines passes through tissue culture (control) . (c) Phenotype of *35S::NsFT1/*WT transgenic with early flower. (d) Phenotype of *35S::MtWOX9-1* transgenic lines with 101 empty vector (control). (e&f) Phenotype of *35S::NsFT1 in 35S::MtWOX9-1* transgenic lines background of T1 & T2 generation with very early

flowering plants. (g) Mean of total leaf number of *35S::NsFT1*/WT, *35S::NsFT1/MtWOX9*-ox & WT plants

Fig. 13. Phenotype of *35S::NsFT1* in *35S::MtWOX9-1* transgenic lines and tobacco wild type (WT) transgenic lines. (a&b) Phenotype of *35S::NsFT1* ectopic expression in delay flowering transgenic lines of 35S::*MtWOX9-1/WT* (c) Phenotype of *35S::NsFT1* in wild type with early flowering phenotype (d) Phenotypic expression of *lam1* mutant after GA3 application cause early bolting but not induce early flowering. (e) Phenotypic expression of delay flowering transgenic lines of *35S::MtWOX9-1/*WT without vs with GA3 application which cause the line early bolting but not induce early flowering. Note: ectopic expression of *NsFT1* not only promote early flowering but also induce tillering in both transgenic lines background as opposed to the nature wild type.

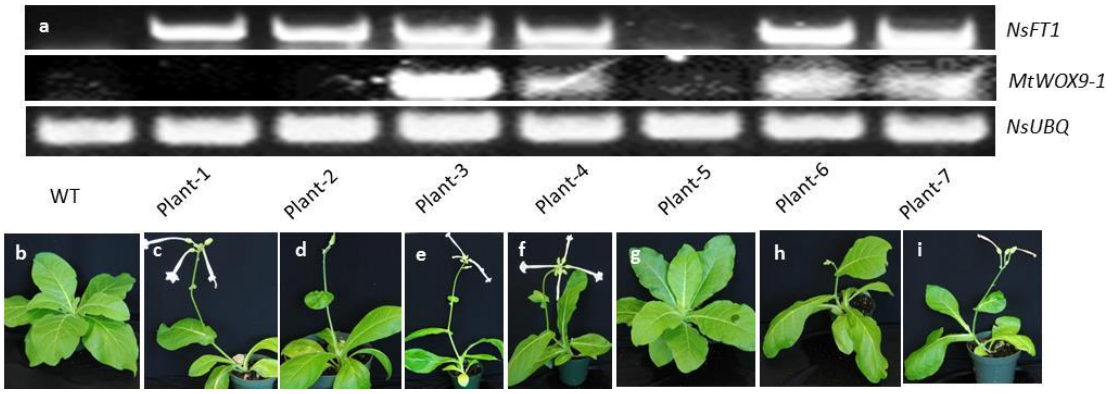

Fig. 14. Expression pattern of *NsFT1* and *MtWOX9* and phenotype in transgenic lines. (a) *NsFT1* and *MtWOX9-1* genes expression analysis by RT-PCR genotyping which corresponds to each plant in figures b-i. (b) Phenotype of untransformed wild type of seven week old (c-f, h & i). Phenotypeof *35S::NsFT1* in 35S::*MtWOX9-1* transgenic lines with early flowering plant. (g) Phenotype of wild type segregating out from the transgenic lines.

In general, the results suggest that the *NsFT1* gene is one of the key elements in controlling flowering time in *N. sylvestris*. Taken together, this result suggests that repression of *NsFT1* in *35S::MtWOX9-1*/WT and *lam1* mutant could be one of the main reasons for the late flowering phenotype of *MtWOX9* overexpression lines and non-flowering of *lam1* mutant plant. It is likely that the upregulation of *WOX9* in the *lam1* mutant is responsible for the repression of *NsFT1* and subsequent late flowering phenotype of the *lam1* mutant. However, understanding the exact mechanistic relationship of *NsFT1*, *WOX9* and *LAM1* genes in relation to leaf blade development and flowering time control needs further investigation.

DISCUSSION

Previous studies of *Arabidopsis* have shown that *WOX9* (*STIMPY*) expressed in embryo, proliferating tissues, vegetative SAM, leaf primordia, floral and inflorescence meristem, epidermal layer of placenta and upper portion of root meristematic zone. Moreover, *WOX9* is functionally involved in the process of vegetative growth of SAM, stem cell maintenance, maintaining growth in root and shoot apexes and promote cell division (Haecker et al., 2004; Wu et al., 2005). Here, two *Medicago WOX9*-like genes have been found (*MtWOX9-1*& *MtWOX9-2*) with more or less similar level of expression pattern with different level of expression level in different organs. Spatiotemporal expression pattern analysis shows that *MtWOX9*-1and *MtWOX9-2* in young leaf, shoot apex, stems, pod, and flower at different developmental stages of *Medicago* wild type. Similar expression pattern was also confirmed by using GUS reporter system which suggest the biological function of *WOX9* genes in plant development in general and leaf lamina development in particular. It could be assumed that the different level of expression in different organs of *Medicago* plant might have certain correlation with their specific and combined functional importance. It is common to have multiple copies of *WOX9* family as *WOX9* and *WOX8* in *Arabidopsis* (Haecker et al., 2004; Wu et al., 2005); evergreen (*EVG*) and sister of evergreen (*SOE*) in *petunia* (Rebocho et al., 2008). In *Arabidopsis*, gain-of-function of *WOX9/STIMPY* (*stip*-D) showed the wavy margins of leaves, which is the general indication of misregulated cell division and the plants have also increased number of axillary shoots that creates bushy phenotype (Brand et al., 2002; Gallois et al., 2002; Nath et al., 2003; Palatnik et al., 2003; Wu et al., 2005) similar to the observation in *35S::MtWOX9-1* transgenic lines with wavy margins of leaves and 2-5 branches (Fig. 12a-c) at the base tobacco transgenic lines as opposed to non-tiller nature of wild type. On the contrary, loss of function of *WOX9* in *Arabidopsis* leads to an arrested embryonic growth, failure in axillary meristem formation, failure of leaf primordial initiation, which indicates the importance of *WOX9* for maintaining of cell division in the leaf primordia which promote the growth of vegetative SAM and also affects the growth of many tissues such as leaf margin, axillary shoot and root shoot (Wu et al., 2005). Similarly, overexpression of *MtWOX9-1* in *Medicago* induced remarkable

leaf blade defect with downward curling suggesting the functional involvement of *MtWOX9-1* in leaf lamina outgrowth. Similarly, ectopic expression of *MtWOX9-1* in *N. sylvestris* reveals strong effects in forming wavy margin leaf blade development, as it is sufficient to modify the leaf features with the generation of unique leaf blade defects. Dose dependent response of *MtWOX9-1* was also observed in tobacco where the high dosage intensively altered leaf features including shape, size, margin, venation and numerous leaf numbers. Ectopic expression of *35S::MtWOX9-1* in *stf* (*Medicago*) and *lam1* (tobacco) also worsen the leaf phenotype by forming a more radial leaf blade with added proximodistal defects. Interestingly, the highest expression of *MtWOX9-1* in *stf* mutant was observed (Fig. 4) suggests that expression of *MtWOX9-1* is probably repressed by *STF*, which has been shown to modulates blade outgrowth by promoting cell proliferation at the adaxial–abaxial junction of leaf primordia (Tadege et al., 2011a). As reported in Lin et al., (2013), *Arabidopsis WOX9/STIMPY* acts as a strong transcriptional activator in luciferase assays and enhances the *lam1* phenotype with additional proximodistal defects, indicating the conserved function of *WOX9* across species. Therefore, phenotypic analysis of several transgenic lines of *MtWOX9-1* in *Medicago* and tobacco studies supports the previous speculation about the critical requirement of *WOX9* genes for the development of lateral organs, including leaves and inflorescences. More recently, *WOX9* has been shown to promote the establishment of cotyledon boundary along with the expression of *WOX2, CUC2* and *CUC3* (Lie C et al., 2012). The detail phenotypic characterization of severe phenotype of *35S::MtWOX9-1* in tobacco revealed not only distinct leaf feature defects but also significantly delay flowering time with reduced pod and seed setting potential compared to wild type. Generally, the flowering time of wild type under greenhouse LD condition is about ten weeks. However, severe *35S::MtWOX9-1* transgenic lines require about 45 weeks to flower i.e. six fold delay of flowering time compared to wild type. Thus, we were interested to know the cause of delay in flowering in ectopically expressed *35S::WOX9-1* transgenic lines and *lam1* mutant. Interestingly, tobacco *FLOWERING LOCUS T1* (*NsFT1)* transcript accumulation was significantly reduced in transgenic lines as well as *lam1* mutants compared to the critical stage of wild type, which leads to hypothesize that the lateness of transgenic lines and *lam1* mutant is lack of *NsFT1* activity. Therefore, to confirm whether

the lower expression of *NsFT1* is responsible for these late flowering, we ectopically expressed *35S::NsFT1* into *35S::MtWOX9-1/WT* severe phenotype and *lam1* mutant. Interestingly, *35S::NsFT1* fully complemented and significantly promoted early flowering in *35S::MtWOX9-1/WT* severe phenotype with the formation of fewer leaf than wild type. This result indicated that *35S::NsFT1* ectopic expression not only complemented the flowering time of *35S::MtWOX9-1*, but also recued the leaf blade defects. Similarly, overexpression of *35S::NsFT1* in the *lam1* mutant did not only rescue very late flowering of *lam1* mutant but also significantly enhanced early flowering i.e. 3-6 weeks. However, the *lam1* mutant leaf phenotypes were not complemented, suggesting that the leaf and flowering defects in the *lam1* mutant are controlled genetically distinct pathways.

Several studies indicated that the finely tuned balance of cell division and cell differentiation and expansion decisions is very crucial for leaf blade development in controlling both proximodistal and mediolateral axes and several genes have been identified that control the balance between cell division and cell differentiation in the proximal versus distal parts of the leaf (Kalve et al., 2014; Tadege, 2013; Tadege et al., 2011a; Tadege et al., 2011b). It is also interesting to note that ectopic expression *NsFT1* rescued the distorted tobacco leaf blade that affected by ectopic expression *MtWOX9-1* in severe transgenic lines indicating the functional interaction of *NsFT1* in leaf blade out growth. Similarly, the longer vegetative growth with numerous leaf production of *35S::MtWOX9-1*/WT in severe transgenic lines have shown that which might cause by cell over proliferation probably checked by *35S:NsFT1* ectopic expression to normalize the leaf size and shape. Several recent findings showed the pleiotropic functions of *FT*-like genes as general growth hormone affecting the various physiological process in monocots and dicots (Böhlenius et al., 2006; Guo et al., 2015; Hiraoka et al., 2013; Krieger et al., 2010; Lee et al., 2013; Li et al., 2015; McGarry and Ayre 2012; Melzer et al., 2008; Navarro et al., 2011; Niwa et al., 2013; Shalit et al., 2009; Smith et al., 2011). Thus, this transgenic lines phenotype data is in agreement with the above reports and very similar to the current ectopic expression effects *GhFT1* in tobacco wild type showed pleiotropic functions such as lateral shoots outgrow at the base, axillary buds at rosette axil, altering leaves morphology (Guo et al., 2015; Li et al., 2015). Taken, together these

results suggest the existence of direct or indirect functional interconnection among *WOX9, LMA1* and *FT* genes in leaf blade development and flowering time control. The effect of *WOX9* that shares common aspect with cytokinin that upregulates CycD3 expression to promote undifferentiated cell growth, and regulates organ initiation in shoot apex (Giulini et al., 2004; Riou-Khamlichi et al., 1999) should be investigated to answer the cause and effect of the observed phenotype in such transgenic lines' leaf structure defects and other related growth phenotypes. Furthermore, the complementation of *stip* mutant in *Arabidopsis* by exogenous application of sucrose indicates the functional role of *WOX9* in stimulating the cell cycle (Wu et al., 2005). Several genes were identified in *Petunia* inflorescence development and subsequent sympodial meristem formation; among which *EVG* was expressed in sympodial meristem and mechanically the model assumes that *EVG* counteracts the effect of unknown mobile factors that inhibits the expression of genes involved in florescence meristem, possibly indirectly by promoting proliferation of the lateral inflorescence meristem (Rebocho et al., 2008). Consistent with this assumption, the preliminary results of MtWOX9-1 protein interaction with several partners proteins such as STF, MtHDA6, MtHDA19, MtLUG, MtFIL, MtKAN, MtAS2 and MtWP73B in Y2H assay and in *stf, lam1* mutant and transgenic lines of *Medicago* and tobacco plants suggest the wide range involvement of *WOX9* in different developmental process in general and leaf blade development in particular that needs further genetic and molecular studies. Therefore, these scenarios suggest that *WOX9* has a broader role in maintaining growth in vegetative shoot apex that might be directly affect the lateral outgrowth (leaves and flowers) of the plant. Taken together, overexpression result is in agreement with the report of Lin et al., (2013) that *WOX9* exhibited the greatest activation activity among the other *WOX* gene family and might account for the enhanced narrow leaf phenotype and proximal-distal defects in *WOX9*-expressing *lam1* plants. These results strengthen the speculation that meristematic activity and lateral organ development in higher plants requires a balance between the activation and repression functions of *WOX9* as an activator role that counterbalances repression to modulate growth in both mediolateral and proximodistal planes during leaf morphogenesis. However, further molecular and genetic studies should be carried out to define the existing functional interconnection between *WOX9* and *STF* in leaf blade development

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CHAPTER III

THREE *FLOWERING LOCUS T*-LIKE GENES FUNCTION AS POTENTIAL FLORIGENS AND MEDIATE PHOTOPERIOD RESPONSE IN SORGHUM.

INTRODUCTION

Floral transition is a major phase change in flowering plants where developmental programs switch from vegetative growth to reproductive growth in which gametes are formed to ensure continuity to the next generation. Thus, plants coordinate the timing of their flowering with environmental changes to achieve reproductive success. Several environmental factors and endogenous developmental signals converge to determine reproductive competence and flowering. At the heart of this competence is a flowering hormone called florigen, originally proposed by Chailakhyan about 80 years ago (Chailakhyan, 1936). Florigen is a leaf-derived, graft transmissible signal that under inductive conditions moves from the leaf to the shoot apex through the phloem to induce transition to the reproductive phase (Chailakhyan, 1936; Chailakhyan, 1937; Zeevaart, 1976). The long sought after florigen has now been widely accepted to be the protein encoded by the *FLOWERING LOCUS T* (*FT*) gene of *Arabidopsis* and its orthologues (Lifschitz *et al.*, 2006; Corbesier *et al.*, 2007; Jaeger & Wigge, 2007; Tamaki *et al.*, 2007; Giakountis & Coupland, 2008; Zeevaart, 2008). *FT* encodes a protein with similarity to the mammalian phosphatidylethanolamine binding protein (PEBP) (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). In long days (LDs), *Arabidopsis FT* is up-regulated in the leaf by *CONSTANS* (*CO*) (Samach *et al.*, 2000), which encodes a B-box zinc finger transcription factor with a CCT domain (Putterill *et al.*, 1995). *CO* is diurnally regulated by photoreceptors and the circadian clock, and its protein accumulates towards the end of day in LDs but is degraded in short days (SDs) (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). In response to CO accumulation at dusk, *FT* transcript level peaks at the end of

day in LDs but not in SDs (Kardailsky *et al.*, 1999; Suarez-Lopez *et al.*, 2001). The FT protein is then transported from the leaf through the vasculature to the shoot apex (Corbesier *et al.*, 2007; Jaeger & Wigge, 2007; Lin *et al.*, 2007; Mathieu *et al.*, 2007), where it forms a complex with FLOWERING LOCUS D (FD) to activate floral transition (Abe *et al.*, 2005; Wigge *et al.*, 2005). FD is a bZIP transcription factor that in complex with FT activates the transcription of floral meristem identity genes such as *APETALA1 (AP1)* and *LEAFY (LFY)*. *FT* homologues have been reported from several species of both dicots and monocots with LD, SD or day neutral requirements for floral induction.

In the SD plant rice, *Heading date 3a* (*Hd3a*), the orthologue of *FT*, is activated by *Heading date1 (Hd1)*, the orthologue of *CO*, in SD photoperiods to induce flowering (Kojima *et al.*, 2002) analogous to the *CO-FT* activity in *Arabidopsis*. A second florigen, *Rice flowering locus T1 (RFT1)* has also been identified to promote flowering in LDs (Komiya *et al.*, 2008). *Rice indeterminate1 (RID1)* also called *OsID1* or *Ehd2*, a C2H2 zinc finger transcription factor homologous to the maize *ID1* (Colasanti *et al.*, 1998) gene, promotes flowering by activating *Early heading date1 (Ehd1)* independent of day length (Matsubara *et al.*, 2008; Park *et al.*, 2008; Wu *et al.*, 2008). *Ehd1* is a B-type response regulator induced by SD photoperiods and promotes flowering by activating *Hd3a/FT*-like genes independent of *Hd1* (Doi *et al.*, 2004). On the other hand, *Grain number, plant height and heading date7 (Ghd7)* (Xue *et al.*, 2008), encoding a CCT domain protein homologous to wheat VRN2 (Yan *et al.*, 2004), represses flowering in LDs by down-regulating *Ehd1* and *Hd3a* (Itoh *et al.*, 2010). Thus, the promoter and repressor activities of Ehd1 and Ghd7, respectively, enable the control of *Hd3a* transcription with a critical day-length threshold in rice to a resolution of 30 min (Itoh *et al.*, 2010). The proteins of both Hd3a and RFT1 are shown to move to the shoot apex (Tamaki *et al.*, 2007; Komiya *et al.*, 2009). In the shoot apex, 14-3-3 proteins bind to Hd3a as intracellular receptors, and the resulting complex is translocated to the nucleus to bind to OsFD1, homologue of FD, forming a ternary florigen activation complex (FAC), which induces transcription of *OsMADS15*, a homologue of *AP1*, leading to flowering (Taoka *et al.*, 2011).

In maize, *INDETERMINATE1* (*ID1*), a C2H2 zinc finger transcription factor, and *DELAYED FLOWERING1* (*DLF1*), a homologue of FD, activate flowering in the leaf and shoot apex, respectively (Colasanti *et al.*, 1998; Muszynski *et al.*, 2006). *ZMM4*, a

homologue of *AP1* promotes flowering downstream of *DLF1* in the shoot apex (Danilevskaya *et al.*, 2008a). Out of 25 *FT*-like genes, *ZCN8* (*Zea mays CENTRORADIALIS 8*) has been identified as the best maize florigen candidate activating flowering (Danilevskaya *et al.*, 2008b; Lazakis *et al.*, 2011; Meng *et al.*, 2011). *ZCN8* is diurnally regulated and its transcript is induced after 7-days of exposure to SD conditions in tropical maize but photoperiod sensitivity is attenuated in day-neutral temperate maize, activating flowering independent of day length (Danilevskaya *et al.*, 2011). *ZCN8* functions downstream of *ID1* and upstream of *DLF1* and *ZMM4*, analogous to the rice flowering pathway.

In sorghum (*Sorghum bicolor* L. Moench), flowering time is a key agronomic trait that determines whether it can be used as a grain or biomass crop. Sorghum is a multipurpose crop grown in many parts of the world, especially in arid and semi-arid regions for food, feed, fuel and fiber. Sorghum is a typical SD plant with substantial photoperiod sensitivity. However, like maize, photoperiod insensitive genotypes have been selected by breeders for grain production in temperate regions. As a result, temperate sorghum can be classified as: grain sorghum with attenuated photoperiod response, biomass sorghum (includes forage and energy sorghum) and sweet sorghum. Biomass and sweet sorghums require SD photoperiod for early flowering and flower very late in LDs, and were selected for increased biomass yield through longer duration of vegetative growth in temperate regions (Rooney *et al.*, 2007; Olson *et al.*, 2012). Grain sorghums, on the other hand, were selected for early flowering irrespective of day length to optimize grain yield production.

Despite this critical importance of flowering time for sorghum agronomy and the existence of more than 40 flowering time QTL (Mace *et al.*, 2013), very little is known about the molecular mechanism of flowering time control in sorghum. Six maturity loci named *Ma1-Ma6* that modify photoperiod sensitivity have been identified by genetic analysis (Quinby & Karper, 1945; Quinby, 1966; Rooney & Aydin, 1999; Morgan & Finlayson, 2000) in which dominance at each locus delays flowering in LDs. *Ma1* was identified as *SbPRR37*, a pseudoresponse regulator orthologue of rice *OsPRR37* (Koo *et al.*, 2013) and barley *Ppd-H1* (Turner *et al.*, 2005) that represses flowering in LDs (Murphy *et al.*, 2011). *Ma3* encodes Phytochrome B (Childs *et al.*, 1997), while *Ma6*

corresponds to *SbGhd7* (Murphy *et al.*, 2014), orthologue of the rice floral repressor *Ghd7*. Thus *SbPRR37, SbPhyB* and *SbGhd7* are floral repressors and confer photoperiod sensitivity upstream of the floral activators, orthologue of *Ehd1* and *FT*-like genes *SbCN8* and *SbCN12* (Murphy *et al.*, 2011; Yang *et al.*, 2014b).

Here 13 *FT*-like genes from sorghum were cloned and characterized to establish a molecular road map for understanding the mechanism of flowering time control in sorghum. With comprehensive analysis of spatial and temporal expression pattern, genotype specific expression patterns including commonly used cultivars and natural mutants, photoperiod response, protein-protein interaction patterns and transgenic analysis, 19 PEBP genes were identified that out of in the sorghum genome, three genes designated here *SbFT1*, *SbFT8* and *SbFT10* behave as functional *Hd3a/RFT1/ZCN8* orthologs, suggesting that sorghum probably has three florigens. Of these, three *SbFT* genes are induced by one week SD treatment in photoperiod sensitive genotypes and mediate photoperiod response, but the SD induction can be reversed by transition to LDs.

Research goals and objectives

The goal of this research was:

To clone and characterize sorghum *FT*-like genes to establish a molecular road map for understanding the mechanism of flowering time control in sorghum.

Specific objectives

To clone and study the evolutionary relationship of sorghum PEBPs (*SbFT/TFL1/MFT*-like) family through their proteins and DNAs sequence alignment and phylogenetic analysis

To determine temporal and spatial expression pattern of *SbFT/TFL1/MFT*-like genes in different sorghum developmental phases and identify the possible sorghum florigen candidates and

To identify, putative sorghum florigens by evaluating their interaction with SbFD and Sb14-3-3 proteins and their effect on flowering time by overexpressing in transgenic *Arabidopsis* and by complementation of the *ft-1* mutant.

MATERIALS AND METHODS

Plant materials and growth conditions

Sorghum bicolor (L.) Moench genotypes; grain sorghum (BTx623 & Tx430), sweet sorghum (Theis and Rio) and forage sorghum (FS000504 and FS000991) with wide differences in photoperiod response and flowering time were grown in one gallon pots in greenhouse under long day (LD) conditions with 16/8 hours light/dark cycle at 27-30^{0}C temperature, and in growth chamber of short day (SD) condition with 8/16 hours light/dark cycle at 24-27^{0}C temperature with 70-80% relative humidity and 150 µmol.m2 light intensity. In addition, early flowering mutants of sorghum, 38M *(ma1,ma2, ma3^R)*, 44M (*ma2, ma3^R)*, 90M *(ma3)* and the control 100M *(Ma1,Ma2, Ma3,Ma4)* were grown in LD greenhouse conditions. *Arabidopsis* plants, Landsberg *erecta* (L*er)* ecotype and *ft-1* mutant were grown in growth room under long day conditions of 16/8 hours light /dark cycle at 23-25^{0}C.

Plant tissue sample collection

The developmental stages of sorghum in relation to timing of morphological changes during the course of plant development have been well described (Vanderlip & Reeves, 1972). Six growth stages (S0 – S6) were selected to collect samples for transcript analyses. S0 represents seedling emergence, where coleoptile is just visible; S1 is 10 days after emergence when collar of the 3rd leaf is visible; S2 is when collar of the 5th leaf is visible 20 days after emergence; S3 is growing point differentiation with 7-10 leaf collars about 30 days after emergence; S5 is booting stage 50 days after emergence where head is extended into flag leaf sheath; and S6 is half blooming (anthesis) stage 60 days after emergence (Vanderlip and Reeves, 1972). Five samples were collected and pooled from fully expanded top leaf or other tissues as specified.

For diurnal expression analysis, young fully expanded leaf samples were harvested from three randomly selected BTx623 plants every 4 hrs during 52 hrs in the 24 hrs diurnal cycle. Samples were analyzed by real time qRT-PCR using Actin as control. Leaf samples from grain, sweet and forage sorghum genotype were also separately collected for varietal based analysis of *SbFT* transcript abundance. Samples

were collected after mid-night when the highest transcript accumulation of *SbFT* genes was found. Collected samples were immediately snap-frozen in liquid nitrogen and stored at -80^{0}C until processing.

RNA extraction, gene cloning and transformation

Total RNA was isolated using TRIzol Reagent (Invitrogen) for cDNA synthesis. Reverse transcription (RT) was performed using RNA treated with DNase I (Invitrogen), an oligo(dT) primer and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instruction. Expression pattern analyses were performed using semi-quantitative RT-PCR with specific forward and reverse primers (supporting information Table S1). Full-length *SbFT1*, *SbFT2*, *SbFT6*, *SbFT8*, *SbFT9* and *SbFT10* coding sequences were amplified by RT–PCR using total RNA extracted from leaf and apex tissues. Cloning was performed in pMDC32 gateway destination vector with 2x35S promoter for *SbFT1* and *SbFT8* while leaf specific STF promoter (Tadege et al., 2011) was used for *SbFT10* construct and the resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 by freeze-heat shock method. *Arabidopsis* was transformed using the floral dipping method (Clough & Bent, 1998).

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of 19 PEBP proteins of sorghum and homologs from other related species was performed using BioEdit software and the ClustalW program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Neighbor-joining phylogenic tree was constructed using MEGA6.0 default settings with 1000 bootstrap replications (http://www. megasoftware.net/).

Yeast-two-hybrid assays

Yeast two-hybrid analysis was performed using the ProQuest Two-Hybrid system (Invitrogen) following the manufacturer's instructions. The full-length proteins of AtFD and SbFD1 were cloned in pGBKT7-GW as bait, while the full-length proteins of SbFT1, SbFT8 and SbFT10 were cloned in pGADT7-GW as prey, and sets of constructs were co-transformed into Y2H Gold yeast strain (Clontech). Yeast transformants were selected on

synthetic minimal double dropout medium (DDO) deficient in *SD/-Leu/-Trp* and protein interactions were assessed on quadruple dropout medium (QDO) deficient in *SD/-His/-Trp/-Leu/-Ade*.

BiFC analysis and confocal microscopy

BiFC assays were conducted according to Lu *et al.*, (2010). Briefly, SbFT1, SbFT8, and SbF10 were cloned into pEARLEYGATE201-YN, while AtFD and Sb14-3-3 were cloned into pEARLEYGATE202-YC, by LR reaction. For SbFD1 BiFC assay, SbFT1, SbFT8, SbF10, and Sb14-3-3 were cloned in pEARLEYGATE202-YC while SbFD1 was cloned into pEARLEYGATE201-YN. Each construct was introduced into *Agrobacterium* by freeze-heat shock method. Pairs of combinations were co-infiltrated into four weeks old *N. benthamiana* leaves. P19 was used to inhibit transgenic silencing. YFP signal was observed after 48 to 60hrs of infiltration using a TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems).

RESULTS

Multiple sequence alignment and phylogenetic analysis of sorghum FT/TFL1/MFT-like proteins with homologs from other related species

BLAST search of the *Sorghum bicolor* genome v2.1 using *Arabidopsis FT* and rice *Hd3a* sequences identified 19 phosphatidylethanolamine binding protein (PEBP) family genes showing significant homology to both *FT* and *Hd3a* at the DNA and amino acid (aa) sequence level. Of these 19 PEBP sequences, 13 are *FLOWERING LOCUS T* (*FT*)-like, 4 are *TERMINAL FLOWER LIKE1* (*TFL1*)-like and 2 are *MOTHER OF FT* and *TFL1* (*MFT*)-like. On the basis of sequence homology to *Hd3a*, designated the 13 *FT*-like genes *SbFT1* to *SbFT13*, the 4 *TFL1*-like genes *SbTFL1-1* to *SbTFL1-4* and the 2 *MFT*-like genes *SbMFT1* and *SbMFT2* (Table 1). SbFT1, SbFT8 and SbFT10 correspond to SbCN15, SbCN12 and SbCN8, respectively, reported in previous studies (Yang *et al.*, 2014a; Yang *et al.*, 2014b). The 19 sorghum PEBP genes show limited homology to each other (Table 2), but have close homologues in both the maize and rice genomes (Table 3). The conserved PEBP domain displayed very high homology among sorghum PEBPs and *FT*-like genes from other species (Fig. S1) in which the functionally important Tyrosine (Y) at position 87 in SbFT1 was conserved in all FT-like proteins but substituted by a conserved Histidine (H) residue (Hanzawa *et al.*, 2005) in TFL1-like proteins (Fig. 1a & 7). Among the 13 SbFT proteins, only SbFT5 had an Asparagine (N) substitution at the equivalent position (Fig. 1a). This position also appeared to be not conserved in MFT-like proteins (Fig. 1a).

Table 1. Summary of 19 sorghum PEBP genes compared with *FT* genes from *Arabidopsis*, maize, rice and wheat.

Designation		Alias	Protein	Full-length amino acid identity (%)					
		Name	Size	*AtFT*	*ZCN8*	*ZCN12*	*ZCN15*	*Hd3a*	*VRN3*
1	*SbFT1*	(Sb10g003940)	179	72	62	65	97	93	92
2	*SbFT2*	(Sb03g001700)	173	72	58	60	82	79	82
3	*SbFT3*	(Sb06g020850)	174	73	56	57	70	70	71
4	*SbFT4*	(Sb04g025210)	174	70	57	56	69	68	70
5	*SbFT5*	(Sb0010s003120)	177	70	57	57	70	70	71
6	*SbFT6*	(Sb02g029725)	178	67	52	54	62	63	61
7	*SbFT7*	(Sb04g008320)	182	63	58	62	64	64	65
8	*SbFT8*	(Sb03g034580)	177	59	79	94	65	64	64
9	*SbFT9*	(Sb10g021790)	173	62	59	60	63	62	62
10	*SbFT10*	(Sb09g025760)	175	53	97	83	66	69	66
11	*SbFT11*	(Sb08g008180)	177	62	51	51	62	60	62
12	*SbFT12*	(Sb06g012260)	185	58	57	59	59	59	61
13	*SbFT13*	(Sb03g002500)	187	61	55	56	63	62	62
14	*SbTFL1-1*	(Sb04g021650)	173	56	49	53	58	60	58
15	*SbTFL1-2*	(Sb08g003210)	173	43	39	57	50	62	61
16	*SbTFL1-3*	(Sb05g003200)	173	54	47	55	63	60	59
17	*SbTFL1-4*	(Sb06g015490)	173	54	51	53	60	60	60
18	*SbMFT1*	(Sb03g008270)	171	57	46	47	55	57	56
19	*SbMFT2*	(Sb10g013070)	181	43	37	39	37	50	51

Table 2. Full-length amino acid sequence identify of sorghum PEBPs with Maize and Rice homologs.

	Designation	*Maize Homologous*	Score	e-value	aa Identity (%)	*Rice Homologous*	Score	e-value	aa Identity (%)
1	*SbFT1*	*ZCN15*	350	1e-123	97	*Hd3a*	337	3e-118	93
2	*SbFT2*	*ZCN14*	351	6e-124	99	*OsFTL1*	335	2e-117	94
3	*SbFT3*	*ZCN25*	353	2e-124	98	*OsFTL6*	345	2e-121	98
4	*SbFT4*	*ZCN16*	347	2e-124	96	*OsFTL5*	332	3e-116	99
5	*SbFT5*	*ZCN17*	359	4e-127	94	*OsFTL11*	332	4e-116	96
6	*SbFT6*	*ZCN18*	360	2e-127	98	*OsFTL4*	262	2e-88	93
7	*SbFT7*	*ZCN13*	353	1e-126	95	*OsFTL13*	315	3e-109	84
8	*SbFT8*	*ZCN12*	348	1e-122	94	*OsFTL9*	318	1e-110	87
9	*SbFT9*	*ZCN26*	346	1e-121	95	*OsFTL12*	323	6e-113	89
10	*SbFT10*	*ZCN8*	345	2e-121	97	*OsFTL10*	287	1e-98	75
11	*SbFT11*	*ZCN20*	311	3e-108	94	*OsFTL7*	296	4e-102	89
12	*SbFT12*	*ZCN21*	359	9e-127	96	*OsFTL12*	302	4e-104	79
13	*SbFT13*	--	--	--	--	*OsFTL8*	283	2e-96	78
14	*SbTFL1-1*	*ZCN2*	347	3e-122	97	*RCN2*	333	1e-116	92
15	*SbTFL1-2*	*ZCN3*	345	1e-121	96	*RCN1*	342	4e-120	95
16	*SbTFL1-3*	*ZCN1*	337	2e-118	94	*RCN3*	333	7e-117	92
17	*SbTFL1-4*	*ZCN4*	335	1e-117	94	*RCN4*	327	4e-114	91
18	*SbMFT1*	*ZCN10*	339	2e-119	96	*OsMFT2*	296	4e-102	84
19	*SbMFT2*	*ZCN11*	300	1e-103	85	*OsMFT1*	293	6e-101	79
20	*SbFD1*	*DLF1*	263	4e-93	80	OsFD1	123	5e-39	45

Table 3. Amino acid sequence identity within sorghum PEBP family proteins.

Designation	Sb FT1	Sb FT2	Sb FT3	Sb FT4	Sb FT5	Sb FT6	Sb FT7	Sb FT8	Sb FT9	Sb FT10	Sb FT11	Sb FT12	Sb FT13	SbTFL 1-1	SbTFL 1-2	SbTFL 1-3	SbTFL 1-4	SbM FT1	SbM FT2
SbFT1	--	81	70	69	70	62	63	66	63	62	61	60	63	57	62	60	59	54	48
SbFT2	81	--	66	68	68	63	64	60	63	60	62	60	63	58	60	59	60	58	48
SbFT3	70	66	--	90	84	67	60	57	58	57	70	57	60	54	54	53	54	53	46
SbFT4	69	68	90	--	82	66	60	56	56	56	71	55	62	53	53	53	53	53	44
SbFT5	70	68	84	82	--	67	58	58	56	59	71	54	61	55	56	55	55	57	47
SbFT6	62	63	67	66	67	--	57	54	57	54	61	56	58	48	51	50	50	53	45
SbFT7	63	64	60	60	58	57	--	59	62	55	62	58	61	56	58	56	54	49	43
SbFT8	66	60	57	56	58	54	59	--	60	81	51	59	56	51	55	53	51	48	40
SbFT9	63	63	58	56	56	57	62	60	--	58	51	73	61	52	54	54	54	48	44
SbFT10	62	60	57	56	59	54	55	81	58	--	51	57	52	51	52	51	51	48	38
SbFT11	61	62	70	71	71	61	62	51	51	51	--	49	58	46	47	47	48	54	46
SbFT12	60	60	57	55	54	56	58	59	73	57	49	--	60	49	51	51	50	48	45
SbFT13	63	63	60	62	61	58	61	56	61	52	58	60	--	53	53	55	54	52	47
SbTFL1-1	57	58	54	53	55	48	56	51	52	51	46	49	53	--	87	86	86	54	48
SbTFL1-2	62	60	54	53	56	51	58	55	54	52	47	51	53	87	--	95	82	55	49
SbTFL1-3	60	59	53	53	55	50	56	53	54	51	47	51	55	86	95	--	80	54	49
SbTFL1-4	59	60	54	53	55	50	54	51	54	51	48	50	54	86	82	80	--	55	48
SbMFT1	54	58	53	53	57	53	49	48	48	48	54	48	52	54	55	54	55	--	65
SbMFT2	48	48	46	44	47	45	43	40	44	38	46	45	47	48	49	49	48	65	--

Phylogenetic analysis revealed that sorghum PEBPs are grouped into 3 major clades: the FT clade containing SbFT1to SbFT13, the TFL1 clade containing SbTFL1-1 to SbTFL1-4 and the MFT clade containing SbMFT1 and SbMFT2 (Fig. 1b). The FT clade could be further subdivided into three groups. The functionally characterized monocot florigens, except maize ZCN8, clustered together in the Hd3a subclade. SbFT1 and SbFT2 belong to this group. The second subgroup, represented by AtFT, included SbFT3-6 and SbFT11. The third subgroup represented by ZCN8, included SbFT7-10, 12 and 13 (Fig. 1b). Since *AtFT, Hd3*a and *ZCN8* are well characterized major components of the florigen activation complex and the *SbFT* genes are distributed in all of these three subclades, it is not a trivial task to determine which of these *SbFT* genes function as activators of floral transition in sorghum.

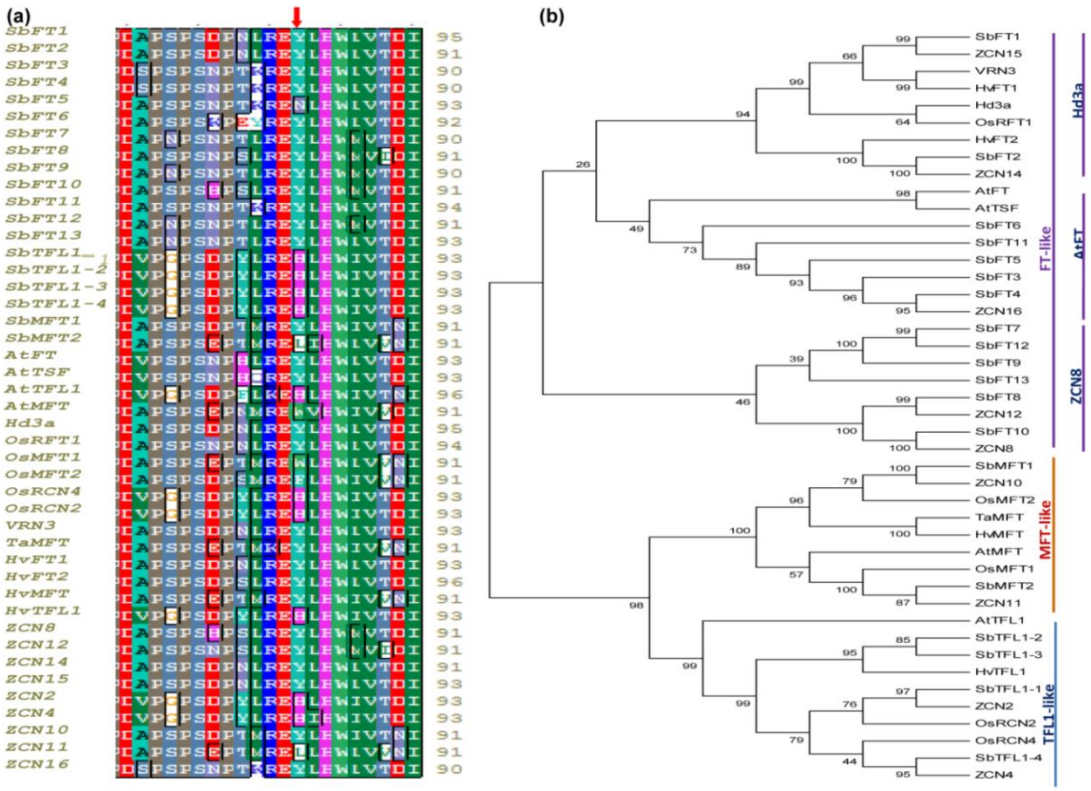


Fig. 1 Amino acid sequence alignment and phylogenetic analysis of 19 sorghum PEBPs and related PEBP proteins. (a**)** Multiple sequence alignment showing a portion of the PEBP domain where key differences were observed between FT and TFL1-like sequences. The red arrow points to conserved *Tyrosine* (*Y*) residue in FT-like or *Histidine* (*H*) in TFL1-like sequences. (b) Phylogenetic analysis of *SbFT*, *SbTFL1* and *SbMFT* genes based on the amino acid sequence of the full length protein. Thirteen sorghum FT-like, four TFL1-like and two MFT-like proteins group into three clades with the corresponding proteins from maize, wheat, barley, rice and *Arabidopsis*, each node indicated by solid lines of different color. The FT-like clade is further subdivided into ZCN8, AtFT and Hd3a subclades. Numbers on branches indicate bootstrap values for 1000 replicates.

Three sorghum *FT* genes are highly expressed in grain sorghum leaves at the floral transition stage

To understand which sorghum *FT-like* genes play functional roles in regulating flowering time, temporal and spatial expression patterns of the 19 PEBP genes were assessed in different tissues at different developmental stages in grain sorghum BTx623 genotype grown under long day (LD) conditions. The developmental stages of grain sorghum in relation to timing of morphological changes have been well described (Vanderlip & Reeves, 1972). Six growth stages (S0 – S6) were selected to collect samples for transcript analyses (see materials and methods). Expression analysis in different tissues using semi-quantitative RT-PCR revealed that most *SbFT/TFL1*-like genes were expressed amongst many of the different tissues tested (Fig. 2a). *SbFT3, SbFT4* and all the four *SbTFL1*-like gene transcripts were detected in root at variable expression levels. *SbFT1, SbFT3, SbFT6, SbFT8, SbFT9* and *SbFT10* genes were variably expressed in leaf from seedling stage (S0) to transition stage (S3). Of these six genes, five (*SbFT1, SbFT6, SbFT8, SbFT9 and SbFT10*) showed strong transcript accumulation during the critical floral transition period (S3). However, *SbFT6* and *SbFT9* also showed strong expression in the leaf at earlier developmental stages (S0-S2), leaving *SbFT1, SbFT8* and *SbFT10* as the only candidates that were specifically and strongly induced near the floral transition stage (Fig. 2a). Expression of *SbFT1* and *SbFT3* in the shoot apex at stage S3 was very weak but *SbFT2, 4, 5, 9, 11* and all of *SbTFL1s*, except *SbTFL1-4*, were strongly expressed in the shoot apex (Fig. 2a). On the other hand, *SbFT2, SbTFL1-1* and *SbTFL1-3* were detected in the stem at variable levels. *SbTFL1-4* was only very weakly expressed in root and shoot apex. *SbFT1, SbFT2, SbFT6, SbFT8, SbFT10, SbFT11, SbTFL1-1* and *SbTFL1-2* transcripts were also detected in the floral head at booting (S5) and in florets at blooming (S6) stages at various levels (Fig. 2a). The remaining five genes, *SbFT7, SbFT12, SbFT13, SbMFT1* and *SbMFT2*, were not detectable in the tissues analyzed. The strong and specific transcript accumulation of *SbFT1, SbFT8* and *SbFT10* in the leaf near the time of the critical floral transition period suggests that these three genes could be the sources of sorghum florigen, although this does not exclude the possibility that others may also have a contribution. For this reason,

SbFT1, *SbFT8* and *SbFT10* were focused to further characterize their involvement in flowering.

To determine if sorghum *FT* genes expression follows diurnal cycling, diurnal expression patterns of *SbFT1*, *SbFT8* and *SbFT10* in grain sorghum BTx623 in LDs was assessed using real time qRT-PCR. These results indicated that accumulation of transcripts of *SbFT1*, *SbFT8* and *SbFT10* started to increase at approximately 4 hrs after the light was off, peaked within 2 hrs and started to decline gradually, reaching basal levels in approximately 2 hrs after the light was on (Fig. 2b-d). All the three genes showed a similar pattern but *SbFT10* showed the strongest while *SbFT1* showed the least induction in the dark. In fact, the *SbFT1* induction was so low that it appeared almost flat compared to the other two (Fig. 2b-d) but it was induced by the dark at a lower scale with a slightly broader peak extending into dawn. These results suggest that all the three *SbFT* genes are regulated with a similar diurnal pattern but with different induction strength.

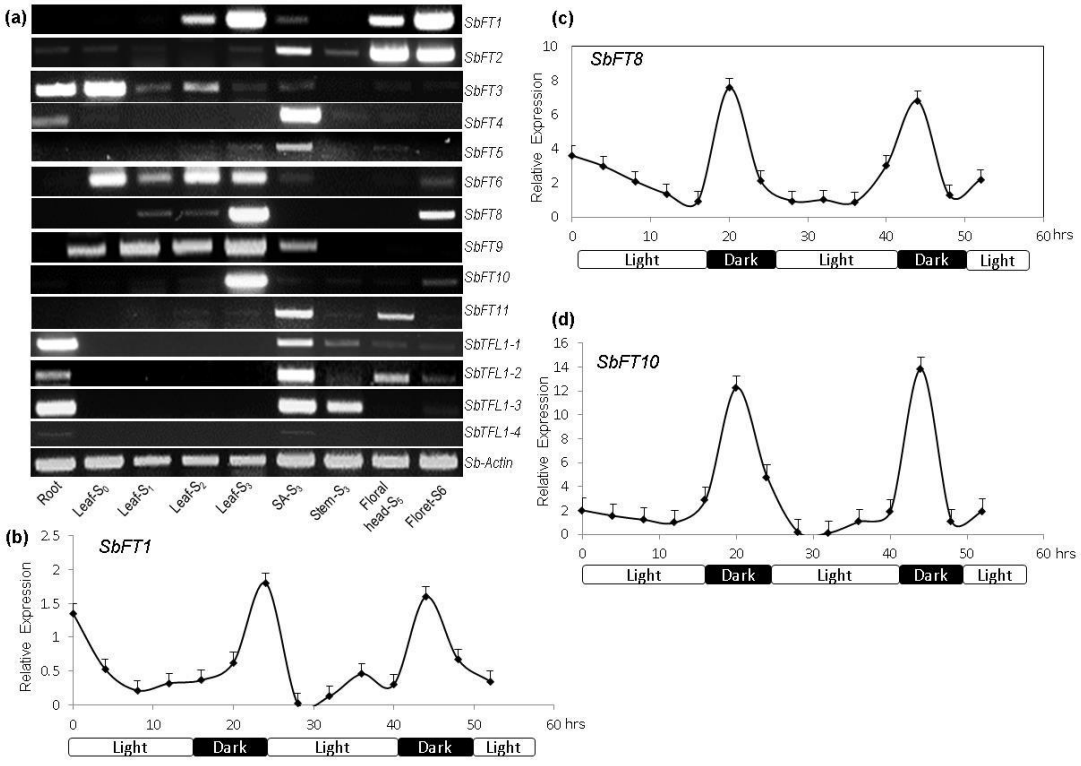


Fig. 2 Spatial and diurnal expression pattern analyses of sorghum PEBP genes. (a) Expression pattern analysis of sorghum SbFT/SbTFL1-like genes in different tissues of grain sorghum BTx623 at different growth stages analyzed by semi-quantitative RT-PCR. S0-S6 refer to the sorghum developmental stages. SA, shoot apex. (b-d) Diurnal

expression patterns of three selected SbFT-Like genes (SbFT1, SbFT8 and SbFT10) in fully expanded leaf blades of grain sorghum of BTx623 under LD conditions near the floral initiation period. Transcripts were analyzed by real time qPCR during 52 hrs in the 24 hrs diurnal cycle relative to ACTIN control. The light periods are shown in white, while the dark periods are shown in black.

SbFT1, SbFT8 and _SbFT10_ are expressed in grain sorghum and early flowering mutants but not in sweet sorghum and forage sorghum genotypes in LDs

Grain sorghum genotypes Tx430 and BTx623 are photoperiod insensitive and flower early irrespective of day length, whereas sweet sorghum genotypes Theis and Rio as well as commercial forage sorghum hybrids FS000504 and FS000991 are photoperiod sensitive and flower early in SDs but flower late in LDs. The, the expression patterns of *SbFT1, SbFT8* and *SbFT10* were tested in these six different genotypes grown under LDs to evaluate if sorghum *FT* genes account for variation in flowering time and photoperiod sensitivity. Fully expanded top leaf samples were collected from each genotype at dawn at a growth stage corresponding to the transition period in grain sorghum. The result showed that the transcripts of *SbFT1, SbFT8* and *SbFT10* were highly abundant in grain sorghum genotypes, but consistently very weak or absent in sweet and forage sorghum genotypes (Fig. 3a). On the other hand, expression of a non-florigen candidate, *SbFT9*, showed no difference between photoperiod sensitive and insensitive genotypes (Fig. 3a).

Further expression patterns of these three *SbFT*s determined in the available sorghum classical flowering mutants. Sorghum flowering mutants have long been identified by genetic selection based on the presence (*Ma*) or absence (*ma*) of one or more maturity loci that modify photoperiod sensitivity (Quinby, 1973). The early flowering mutants of sorghum, 38M (*mal, ma2, ma3*^{R}), 44M (*ma2, ma3*^{R}), near control 90M (*ma3*) and the control 100M (*Ma1, Ma2, Ma3, Ma4*) were grown in LD greenhouse conditions. *ma3*^{R} is a strong allele of *ma3.* Leaf samples were collected from each line at 4 and 5 weeks after emergence for transcript analysis. We found that the transcripts of *SbFT1, SbFT8* and *SbFT10* were highly abundant in the earliest flowering line 38M, followed by 44M, but consistently below detection in 90M and 100M at this developmental stage (Fig. 3b). 38M is slightly earlier heading than 44M and even this

difference was reflected by the expression levels of the three *SbFT* genes. In contrast, *SbFT9* expression showed no difference in all the tested lines (Fig. 3b). These results together demonstrate that expression of the three sorghum *FT* genes *SbFT1*, *SbFT8* and *SbFT10* is additively repressed by maturity loci, suggesting that these *SbFT* genes are involved in promoting flowering in sorghum and may account for differences in flowering time and photoperiod sensitivity between sorghum genotypes.

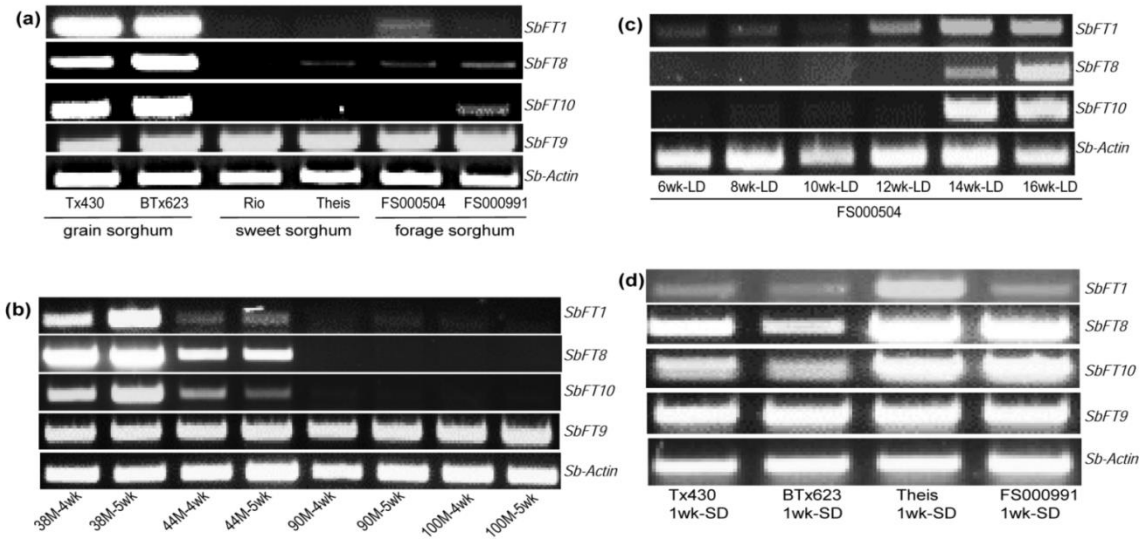


Fig. 3 Expression level of four *SbFT-Like* genes in leaves of different sorghum genotypes under SD and LD conditions. (a) Expression levels of *SbFT1, SbFT8, SbFT9* and *SbFT10* in the leaves of grain, sweet and forage sorghum genotypes 4wks after germination in LD conditions. (b) Expression levels of *SbFT1, SbFT8, SbFT9* and *SbFT10* in the leaves of different sorghum natural mutant lines and a parental control at 4wks and 5wks after germination in LDs. (c) Expression patterns of *SbFT1, SbFT8* and *SbFT10* in the leaves of late flowering forage sorghum (FS000504) at different stages of development under LD conditions.(d) Expression levels of *SbFT1, SbFT8, SbFT9* and *SbFT10* in the leaves of grain, sweet and forage sorghum at the seedling stage in response to one week SD treatment. Transcript expression levels were determined by semi-quantitative RT-PCR using sorghum Actin as loading control. SD, short day; LD, long day; wk, week.

Expression of three sorghum *FT* genes is induced by plant age and SD photoperiod in late flowering genotypes

The forage hybrid FS000504 is the latest flowering line which takes 22 weeks to heading in LD (16/8 hrs light/dark cycle) conditions. Therefore, the time at which the *SbFT* genes are expressed in this genotype under LD conditions was determined by collecting leaf samples at dawn in two week interval. The result showed that *SbFT1* was first detected at 12 weeks after emergence and *SbFT8* and *SbFT10* were also clearly induced by 14 weeks (Fig. 3c). Since this genotype heads in 22 weeks, 14 weeks probably represents the initiation of the floral transition period and suggests that the three sorghum *FT* genes are induced at the time of floral transition in FS000504 although this occurred 10 weeks later than that of grain sorghum.

To determine if SD photoperiod can induce the expression of sorghum *FT* genes in both photoperiod insensitive and sensitive genotypes were examined for the expression patterns of *SbFT1, SbFT8* and *SbFT10* in grain, sweet and forage sorghum genotypes grown under SD (8/16hr light/dark cycle) conditions. Leaf samples were collected from each genotype at dawn at one week after emergence. Interestingly, the transcripts of *SbFT1, SbFT8* and *SbFT10* were detected in most genotypes with the highest induction observed in the sweet sorghum Theis (Fig. 3d). Induction of *SbFT1* appeared to be the weakest and *SbFT8* the strongest in all the genotypes tested but generally the sweet and forage sorghums appeared to induce all the three *FT* genes stronger than the grain sorghums (Fig. 3d), suggesting that *SbFT1, SbFT8* and *SbFT10* promote flowering in photoperiod sensitive genotypes in response to short days, and SD induction of these genes, especially that of *SbFT1* is attenuated in grain sorghum genotypes. Rio and FS000504 require three weeks to induce at least *SbFT8* and *SbFT10* in response to SDs (Fig. S2), suggesting a minimum SD saturation requirement for *SbFT* gene induction depending on genotype.

Next investigation followed was to insight whether the SD treatment can be remembered and the one week high induction of *FT* genes in Theis is sufficient for floral promotion after transferring to LDs. To address this, Theis was grown in SDs for one to several weeks and at the end of each treatment, plants were transferred to LDs. Leaf samples were collected at the end of each treatment while plants were still in SD and after

transfer to LD at the specified period, and *FT* transcript levels were compared with controls that have been grown in LDs continuously. Expression of *SbFT1*, *SbFT8* and *SbFT10* genes was highly induced in Theis within one week of SDs as above and remained high at least in the second week of SDs (Fig. 4a). However, when SD treated plants were transferred to LDs for one week, the expression level of all three genes reduced dramatically to undetectable levels (Fig. 4a), indicating that one week growth in LDs was sufficient to completely reverse induction even after six weeks of SD photoperiods. This suggests that there is no long term memory for *SbFT* induction by SDs and the three *SbFT* transcripts were probably quickly destabilized by LD photoperiods in Theis.

However, the growth response appeared to be more complex. Theis grown constantly in LD took approximately 140 days to heading, while this was achieved in about 70 days when grown constantly under SD conditions (Fig. 4d). When plants were transferred from 1-4 weeks growth in SDs to LDs, vegetative growth continued with the same shoot (Fig. 4c), but when plants were transferred at the 5^{th} week and after, growth of the original shoot was arrested and new shoot growth was initiated from a lower node (Fig. 4d-h). This happened even when plants were transferred after heading (Fig. 4h). Interestingly, plants transferred after 7 weeks in SDs started heading at the 9^{th} week in LDs (Fig. 4f) while the control was still vegetative (Fig. 4b). These results suggest that LD photoperiod is dominant in reversing *SbFT* induction and growth stages established in SDs but some aspects of the floral transition may still be activated by the SD treatment provided that the SD lasts 7 or more weeks in the case of Theis.

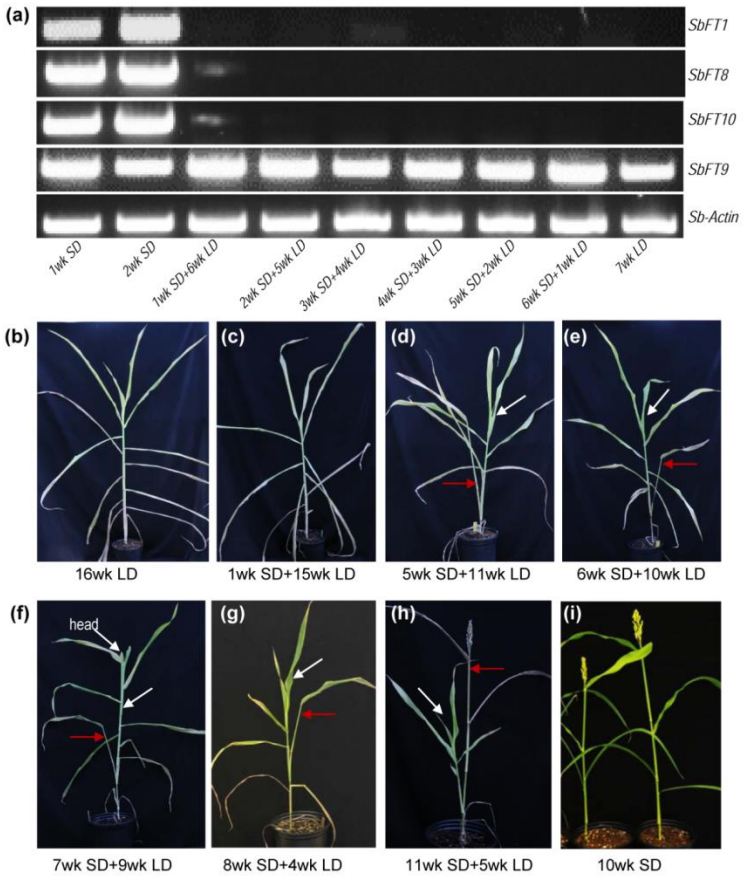


Fig. 4. *SbFT* gene expression and growth response of Theis to SD and LD treatment. (a) Transcript accumulation of *SbFT1*, *SbFT8*, *SbFT9* and *SbFT10* in the leaves of Theis after exposure to SDs, LDs, or SDs plus LDs for specified periods. For SD plus LD treatment, plants were grown in SDs for 1-6 week and transferred to LDs for 6-1 week before sampling. (b) and (i) Phenotype of Theis plants grown continuously in LDs and SDs for 16 weeks and 10 weeks, respectively. (c) – (h) Phenotype of Theis plants first grown in SDs for the specified 1-11 week and shifted to LDs for the specified 4-15 week. Note that in (d)-(h), the first shoot grown in SDs is arrested (red arrow) and a new shoot developed from the lower node (white arrow) after transfer to the LDs. SD, short day; LD, long day; wk, week.

Functional analyses of sorghum *FT* genes in transgenic *Arabidopsis*

To confirm that the biological function of *SbFT1*, *SbFT8* and *SbFT10* is indeed activation of flowering, we transformed each of these genes into L*er* driven by the 2x35S promoter. Under LD conditions, all *SbFT1* transgenic lines at most had two rosette leaves

(1.1 ±0.9) at flowering compared to eight to nine (8.4 ±1.0) in L*er* (Fig. 5a-c, i), where 36% flowered without forming any rosette leaves, indicating strong activation of flowering. In addition, ten of these transgenic lines were evaluated in the T2 generation for early flowering under SD conditions. All the ten T2 lines flowered with 2-3 rosette leaves compared to 10-12 in L*er* (Fig. 5d), indicating activation of flowering in transgenic *Arabidopsis* both in LD and SD conditions. The ability of *35S:SbFT1* to complement the late flowering *Arabidopsis ft-1* mutant was further tested. Complemented transgenic plants flowered early with 0-2 rosette leaves at bolting under long day conditions compared to the *ft-1* mutant, which produced 12-15 rosette leaves at bolting (Fig.5i & S3a, c). This indicates that *SbFT1* ectopic expression not only complements the *ft-1* late flowering phenotype but induces early flowering in the mutant background.

On the other hand, all *35S::SbFT8* transgenic lines in L*er* flowered before bolting with 2 tiny up-curled cauline leaves (Fig. 5e, i) and died without setting seeds, suggesting that *SbFT8* is probably more active and its high expression is lethal for the plants. Alternatively, *SbFT8* may regulate vegetative growth and plant architecture in addition to activation of flowering in sorghum. Furthermore, *35S::SbFT8* expression in the *ft-1* mutant resulted in very early flowering with zero to two rosette leaves at bolting under long day conditions (Fig. 5f, i & S3d, e), suggesting that *SbFT8* is, indeed, a strong activator of flowering. The fact that *SbFT8* expressing *ft-1* mutant plants survived better than *SbFT8* expressing L*er* plants suggest that the very high expression of *SbFT8* is most likely the cause for the observed defects.

As I used a vector (pMDC32) that drives very high expression with 2x35S promoter in all transformations, high expression of *SbFT10* led to even worse phenotype and resulted in lethality, and therefore I wase unable to obtain enough *SbFT10* transgenic lines for further analysis. Consequently, I resorted to a weaker promoter, *STENOFOLIA* (*STF*) from *Medicago truncatula* to obtain transgenic lines for *SbFT10*. The *STF* promoter drives expression in the leaf margin and middle mesophyll at the adaxial-abaxial junction including the vascular region but not in the shoot apical meristem (Tadege et al., 2011). *STF*::*SbFT10* expression resulted in early flowering with four phenotypic classes: flowering without bolting with 2 up-curled leaves (17%) which died without setting seeds (Fig. 5g), bolting with 2-3 up-curled leaves and terminal flower

(50%) (Fig. S3f), bolting with 4 rosettes leaves (23.5%) (Fig. S3g), and later flowering, bushy plants bolting with 9 or more rosette leaves (9.5%). The *STF::SbFT10* construct also complemented the *ft-1* mutant in LDs with an average of 1.8 rosette leaves at bolting (Fig. 5h, i). These results suggest that *SbFT10* activates flowering in *Arabidopsis* perhaps stronger than *SbFT8* because a weaker promoter (*STF*) was required to obtain *SbFT10* expressing transgenic *Arabidopsis* plants. Alternatively, *SbFT10* may have other functions in addition to activation of flowering that became detrimental in *Arabidopsis* with a very strong constitutive promoter. These transgenic analyses are consistent with the spatial, temporal and diurnal expression patterns, as well as the day length response and genotype dependent expression of *SbFT* genes, and together suggest that sorghum has at least three functional *FT* genes that can promote floral activation.

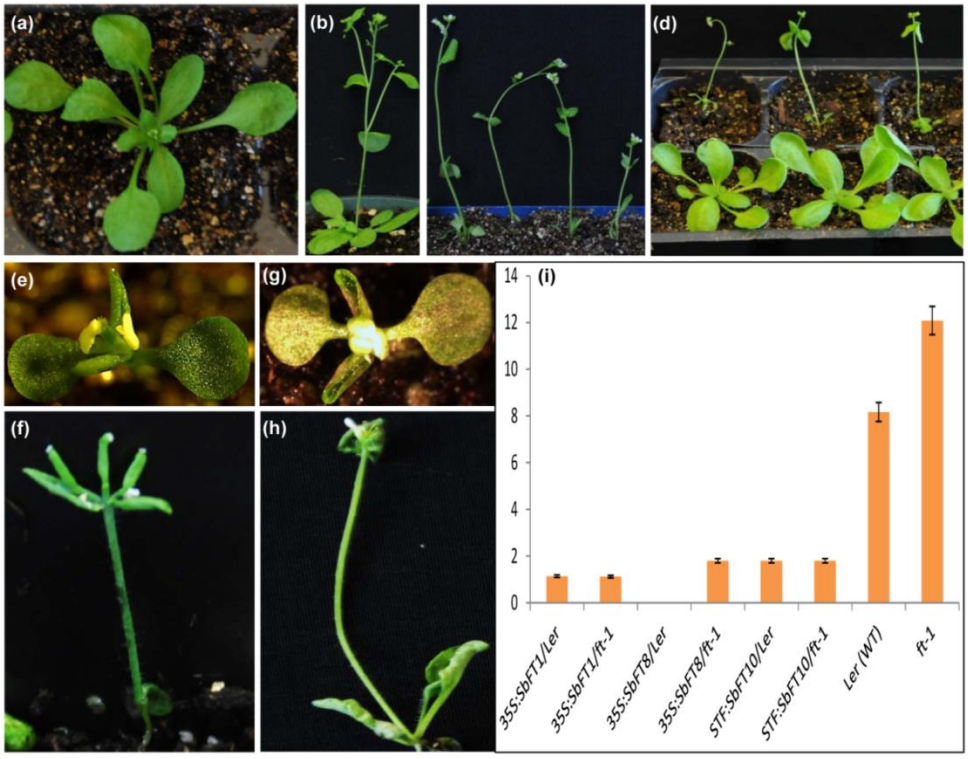


Fig. 5 Phenotype of transgenic *Arabidopsis* plants transformed with sorghum *FT* genes. (a & b) untransformed L*er* control. (c) Phenotype of *35S::SbFT1* transgenic plants under LD conditions. (d) Phenotype of *35S::SbFT1* transgenic plants (upper panel) vs L*er* (lower panel) under SD conditions. (e) Phenotype of *35S::SbFT8* transgenic plant flowering at the cotyledon stage without bolting under LD conditions. (f) Phenotype of *ft-1* mutant transformed with *35S::SbFT8* showing early flowering with a terminal flower

phenotype under LD conditions. (g) Strong early flowering Phenotype of transgenic L*er* plant transformed with *STF::SbFT10* under LD conditions. (h) Phenotype of *ft-1* mutant transformed with *STF::SbFT10* showing early flowering under LD conditions. (i) Flowering time of *SbFT1, SbFT8* and *SbFT10* transgenic *Arabidopsis* plants compared to L*er* and *ft-1* mutant in LDs measured by rosette leaf number at bolting. Error bars show SE

SbFT2, SbFT6* and *SbFT9* genes do not induce flowering in transgenic *Arabidopsis

Since *SbFT2* is expressed in the shoot apex at the floral transition stage and *SbFT6* and *SbFT9* are expressed in leaves at all stages of development (Fig. 2a), these three genes were further tested for their potential candidacy. The three genes were introduced into L*er* and *ft-1* mutant individually driven by the 35S promoter. These results revealed that none of these genes activated flowering in L*er* or rescued the late flowering *ft-1* mutant (Fig. S4), suggesting that *SbFT2, SbFT6* and *SbFT9* are likely not involved in the activation of flowering in sorghum.

SbFT1, SbFT8 and SbFT10 proteins may interact with SbFD1 and/or Sb14-3-3

We performed yeast-two-hybrid (Y2H) and bimolecular florescence (BiFC) assays to determine whether the *SbFT* encoded proteins are capable of interacting with AtFD, SbFD1 and Sb14-3-3. The results showed that SbFT1 but not SbFT8 and SbFT10 proteins interact with AtFD and SbFD1 in Y2H under stringent conditions (Fig. S5a). SbFT1, SbFT8 and SbFT10 were also tested whether interact with each other. SbFT1 interacted with both SbFT8 and SbFT10 and also showed self-interaction while SbFT8 and SbFT10 neither interacted with each other nor showed self-interaction in Y2H (Fig. S5b). However, BiFC assay using split YFP complementation in *N. benthamiana* leaf cells identified that the three sorghum FTs can interact with AtFD and SbFT1 interacted with SbFD1 (Fig. 6a). However, SbFT8 and SbFT10 did not show interaction with SbFD1 in BiFC (Fig. S6a). Sorghum homologue of rice GF14C, Sb14-3-3 was also cloned and tested its interactions with SbFTs in Y2H and BiFC. In Y2H, only SbFT1 but not SbFT8 and SbFT10 interacted with Sb14-3-3 (Fig. S5a), but in BiFC all SbFT1, SbFT8 and SbFT10 interacted with Sb14-3-3 in the cytoplasm, and SbFD1 interacted

with Sb14-3-3 in the nucleus (Fig. 6b). SbFT1, SbFT8 and SbFT10 were localized both in the nucleus and cytoplasm while Sb14-3-3 was exclusively localized in the cytoplasm (Fig. S6b) but Sb14-3-3 interaction with SbFD1 occurred in the nucleus, suggesting that all the three SbFT proteins are potentially capable of forming a floral activation complex with Sb14-3-3 and SbFD1 in the nucleus of living plant cells. Consistent with this, ectopic expression of *SbFT1*, *SbFT8* and *SbFT10* but not *SbFT9* induced expression of floral meristem identity genes *AP1* and *LFY* in transgenic *Arabidopsis* (Fig. S7).

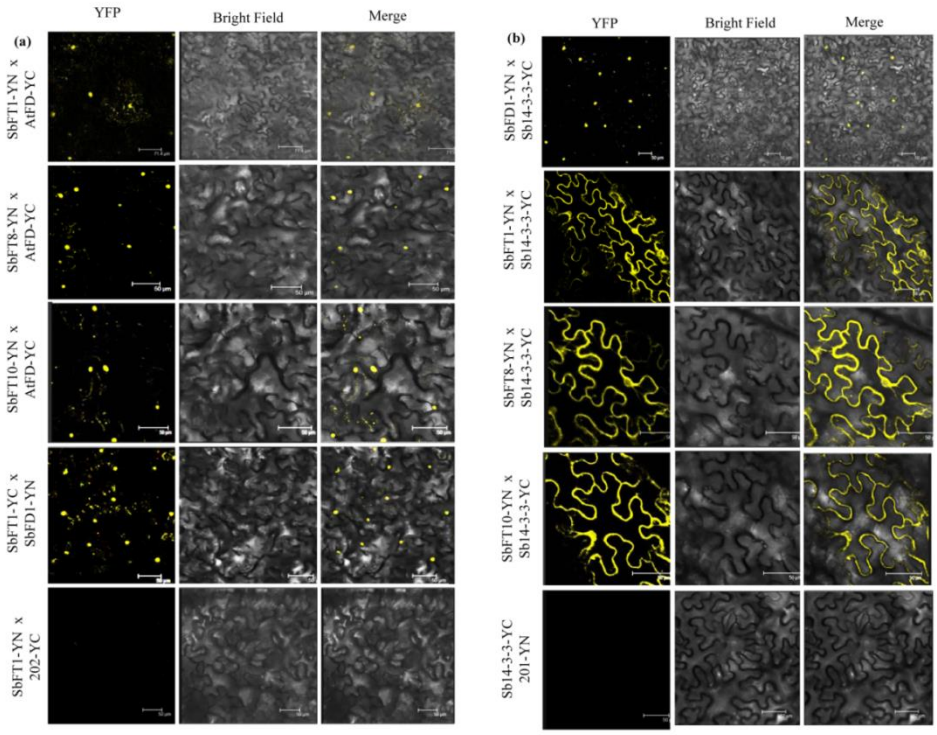


Fig. 6 Bimolecular fluorescence complementation (BiFC) assay in tobacco leaf cells testing protein-protein interaction between SbFT and AtFD, SbFD1 and Sb14-3-3 proteins. (a) Split YFP BiFC assay between *Arabidopsis* FD and sorghum FT proteins, as well as between SbFT1 and SbFD1. SbFT1 with AtFD (top panel), SbFT8 with AtFD (2nd panel), SbFT10 with AtFD (3rd panel) and SbFT1 with SbFD1 (4th panel) where the FTs were fused to N-terminal half while FD was fused to the C-terminal half of YFP. Reconstitution of yellow fluorescence shows positive interaction in the nucleus and absence of fluorescence in the negative control (bottom panel) shows no interaction

between SbFT1 fused to YN and YC alone of YFP. (b) BiFC interaction assay of Sb14-3-3 with SbFD1 and SbFT proteins. SbFD1-YN and Sb14-3-3 (top panel), SbFT1-YN and Sb14-3-3-YC (2^{nd} panel), SbFT8-YN and Sb14-3-3-YC (3^{rd} panel), SbFT10-YN and Sb14-3-3-YC (4^{th} panel). Reconstitution of yellow fluorescence shows positive signal in the nucleus for SbFD1 interaction with Sb14-3-3 and in the cytoplasm for Sb14-3-3 interaction with SbFTs. The control (bottom panel) shows absence of interaction between Sb14-3-3-YC and YN alone.

DISCUSSION

Control of flowering time is a major agronomic trait in sorghum that dictates its usage in grain or biomass production in temperate regions. Owing to its original domestication in tropical East Africa, sorghum exhibits strong photoperiod response. However, sorghum has been introduced into temperate agriculture where photoperiod insensitive varieties have been developed for optimum seed production in the warm summer months. Nineteen PEBP sequences from the sorghum genome (version 2.1) were examined and 13 FT-like (*SbFT1-SbFT13)* genes were identified, in an effort to understand the mechanistic control of flowering time in sorghum. Out of the 13 *FT*-like genes, *SbFT1, SbFT8* and *SbFT10* were identified as potential candidates for encoding florigens that activate floral transition and mediate photoperiodic responses. *SbFT1* is most closely related and syntenic to rice *Hd3a*, and was previously reported as sorghum *FT* (Murphy et al., 2011), whereas *SbFT8* and *SbFT10* were reported as *SbCN12* and *SbCN8*, respectively, analogous to the maize *ZCN12* and *ZCN8* genes (Yang *et al.*, 2014a; Yang *et al.*, 2014b). Since ZCN8 appeared to be the only florigen candidate in maize (Lazakis *et al.*, 2011; Meng *et al.*, 2011) but there are three candidates in sorghum, which retained the original naming "*FT*" to avoid confusion. These results indicated that although *SbFT1* exhibits the lowest transcript accumulation under SD conditions, all three genes *SbFT1*, *SbFT8* and *SbFT10* are induced by SDs especially in photoperiod sensitive genotypes, expressed in leaves at the time of floral transition irrespective of genotype and day length, expressed early in early flowering lines and late in late flowering lines under LD conditions, interact with sorghum 14-3-3 in BiFC assays, and strongly activate flowering in transgenic *Arabidopsis*. These observations are consistent with all three genes being floral transition activators, and may function redundantly to control flowering in sorghum.

Having two florigens is not uncommon as exemplified by *FT* and *TSF* in *Arabidopsis* (Yamaguchi *et al.*, 2005) and *Hd3a* and *RFT1* in rice (Komiya *et al.*, 2008). However, it is intriguing that sorghum may have at least three functional *FT*s, while maize, with a much larger genome, appears to have one functional *FT, ZCN8* (Lazakis *et al.*, 2011; Meng *et al.*, 2011). Sorghum is a close relative of maize and the two are

assumed to have shared a common ancestor as recently as 24 million years ago (Thomasson *et al.*, 1986); therefore a one-to-one correspondence of sorghum to maize genes might be expected. Indeed, all of the sorghum PEBP genes, except *SbFT13*, have close homologues in maize (Table 3) (Danilevskaya *et al.*, 2008b). But, despite the existence of *SbFT1* homologue with 97% aa identity, *ZCN15*, at the syntenic region on Chromosome 6 of maize, this gene appears to be not involved in maize flowering (Meng *et al.*, 2011). However, *SbFT8* and *SbFT10* are more closely related to the maize *ZCN8* rather than rice *Hd3a* or *RFT1* (Fig. 1b, Table 1), and are syntenic to *ZCN12* and *ZCN8*, respectively. It appears that sorghum has retained floral activation function for three genes *SbFT1*, *SbFT8* and *SbFT10* equivalent to maize *ZCN15*, *ZCN12* and *ZCN8* in which *SbFT1* is also collinear to rice *Hd3a* and maize *ZCN15* (Danilevskaya *et al.*, 2008a), although *ZCN15* appears not to play a role in floral activation in maize. However, not all *ZCN* genes are completely excluded from potential candidacy for florigen. For example, *ZCN12* is expressed in leaf and induced by SD treatment (Danilevskaya et al., 2008; Meng et al., 2011), suggesting a potential for floral activation in maize. Thus, with the possibility of additional functional *ZCN* genes in maize, the regulation of floral transition in maize and sorghum may not be that different after all. Further experiments with mutants or reduced expression lines will be required to quantitatively determine the contribution of each of the three *SbFT* genes to sorghum flowering and photoperiod response.

Sorghum *FT* genes are additively repressed by maturity loci in LDs but repression is overcome by SD treatment.

In the photoperiod insensitive grain sorghum genotype, BTx623, the expression of *SbFT1, SbFT8* and *SbFT10* in LDs is regulated by plant age in which expression of all three genes is barely detectable at the seedling stage but sharply increases near the floral transition period (Fig. 2a), suggesting that endogenous factors regulate *FT* expression and flowering in this genotype. *SbFT1, 8* and *10* are also highly expressed in leaf at the S3 stage in other early flowering genotypes such as 38M and Tx430 but not in 100M, forage and sweet sorghums (Fig. 3), suggesting repression by maturity loci. This repression appeared to be additive. In 38M (*ma1, ma2, ma3*^{R}), all the three *SbFT* genes are highly

expressed 5 weeks after germination but in 44M (*ma2, ma3*^{R}) expression of especially *SbFT1* and *SbFT10* is highly reduced, and in 90M (*ma2*) or 100M (*Ma1, Ma2, Ma3, Ma4*), expression of the three genes is not detectable (Fig 3b). This shows that the maturity loci, at least *Ma1, Ma2,* and *Ma3,* may cooperatively repress the expression of *SbFT1, 8, 10* genes in LDs, consistent with the genetic data showing maturity loci together have the strongest effect on flowering (Quinby & Karper, 1945; Quinby, 1966; Quinby, 1973; Rooney & Aydin, 1999; Morgan & Finlayson, 2000). The two loci that have major effects on flowering *Ma1* and *Ma3* encode PRR37 and PHYB proteins, respectively (Foster *et al.*, 1994; Childs *et al.*, 1997; Murphy *et al.*, 2011). *PHYB* is an upstream activator of *PRR37* and represses *SbFT* expression by repressing the *SbFT* activator *SbEHD1* through *PRR37* (Murphy *et al.*, 2011; Yang *et al.*, 2014a; Yang *et al.*, 2014b). But, there is significant difference in *SbFT* expression between 38M and 44M (Fig. 3b), which suggests that *PHYB* affects *SbFT* expression in addition to its effect through *PRR37* accounting for cooperative repression. This may be through activating other repressors such as *SbGHD7* (Yang *et al.*, 2014a), other phytochromes, the circadian clock, or directly repressing *SbEHD1* or *SbFT*s. Further experiments will establish if two or more of these possibilities are correct and whether the three *SbFT*s are differentially regulated by these upstream repressors.

Nevertheless, repression is fully overcome by growth under SD conditions. The results with SD to LD shift experiments suggest that there may not be intact long-term memory for SD treatment since *SbFT* expression in SDs was completely repressed by just one week LD photoperiods (Fig 4a). However, the SD memory is not all lost as it has some contribution to the early flowering of plants shifted to LDs (Fig. 4f) depending on the length of the SD treatment prior to shifting. A phenomenon related to this called night break is known in rice and other species, where a short light break during the long night interrupts the SD response in a dosage dependent manner (Ishikawa *et al.*, 2004; Ishikawa *et al.*, 2005; Ishikawa *et al.*, 2009; Higuchi *et al.*, 2012). This situation in rice favors the possibility for the presence of a separate maintenance factor of Hd3a induction by SDs, which would be sensitive to dosage dependent exposure to light. On the other hand, the strict day length control in rice mediated by repressor *GHD7* and activator *EHD1*functions on *Hd3a* expression (Itoh et al, 2010) could tip the balance to the *GHD7*

side with additional light exposure without necessarily having a separate factor for maintenance of *Hd3a* induction. *GHD7* and *EHD1* orthologues have been reported in sorghum (Murphy *et al.*, 2011; Yang *et al.*, 2014a; Yang *et al.*, 2014b) and in fact, SbGHD7 is encoded by *Ma6*, one of the maturity loci that modifies the photoperiod response (Murphy *et al.*, 2014). Further molecular analyses would be needed to understand the mechanism with which reversion of *FT* induction by LDs, memory of exposure to SDs, as well as de-repression of axillary meristem during photoperiod switching are achieved in sorghum, which will provide novel mechanistic insight in the response of sorghum developmental programs to environmental signals.

All three SbFT proteins physically interact with Sb14-3-3 but not necessarily with SbFD1

In *Arabidopsis*, rice and maize, the FT-FD protein interaction in the shoot apical meristem is required to directly activate transcription of floral meristem identity target genes. In rice, Hd3a interacts with OsFD1 via a 14-3-3 protein (Taoka *et al.*, 2011; Hiroyuki *et al.*, 2013), which appears to be a cytoplasmic receptor for Hd3a (Taoka *et al.*, 2011; Taoka *et al.*, 2013). The 14-3-3-Hd3a complex translocates to the nucleus to bind with OsFD1 and thereby activate OsMADS15 transcription (Taoka *et al.*, 2011; Hiroyuki *et al.*, 2013; Taoka *et al.*, 2013). This analysis suggests that this type of interaction may also be conserved in sorghum. SbFT1 but not SbFT8 and SbFT10 interact with SbFD1 in Y2H and BiFC assays (Fig. 6, S5, & S6). However, SbFT1 interacts with SbFT8 and SbFT10 in Y2H, and all three SbFTs interact with Sb14-3-3 in BiFC assays in the cytoplasm while SbFD1 interacts with Sb14-3-3 in the nucleus (Fig. S6b). It is thus likely that SbFT8 and 10 interact with SbFD1 in the nucleus through Sb14-3-3 analogous the situation in rice. The significance of SbFT1 interaction with SbFT8 and 10 is unclear at this point but it could be possible that SbFT1 may facilitate or stabilize SbFT8 and 10 interaction with SbFD1 in the nucleus. These observations together suggest that SbFT8 and SbFT10 may interact with SbFD1 through Sb14-3-3 and it is likely that a SbFT-Sb14-3-3-SbFD1 floral activation complex could form in the nucleus although this remains to be demonstrated. Consistent with these, all three *SbFT*s induced expression of *AtLFY* and *AtAP1*, and activated early flowering in transgenic *Arabidopsis*.

These results together demonstrate that sorghum has at least three *FT* genes that could potentially form a florigen activation complex and mediate genotype dependent photoperiod response and flowering time variation in sorghum. Since flowering is a key agronomic trait in sorghum, the three *SbFT* genes identified here can be used as valuable molecular markers in sorghum breeding programs.

Supporting Information Figures and Tables

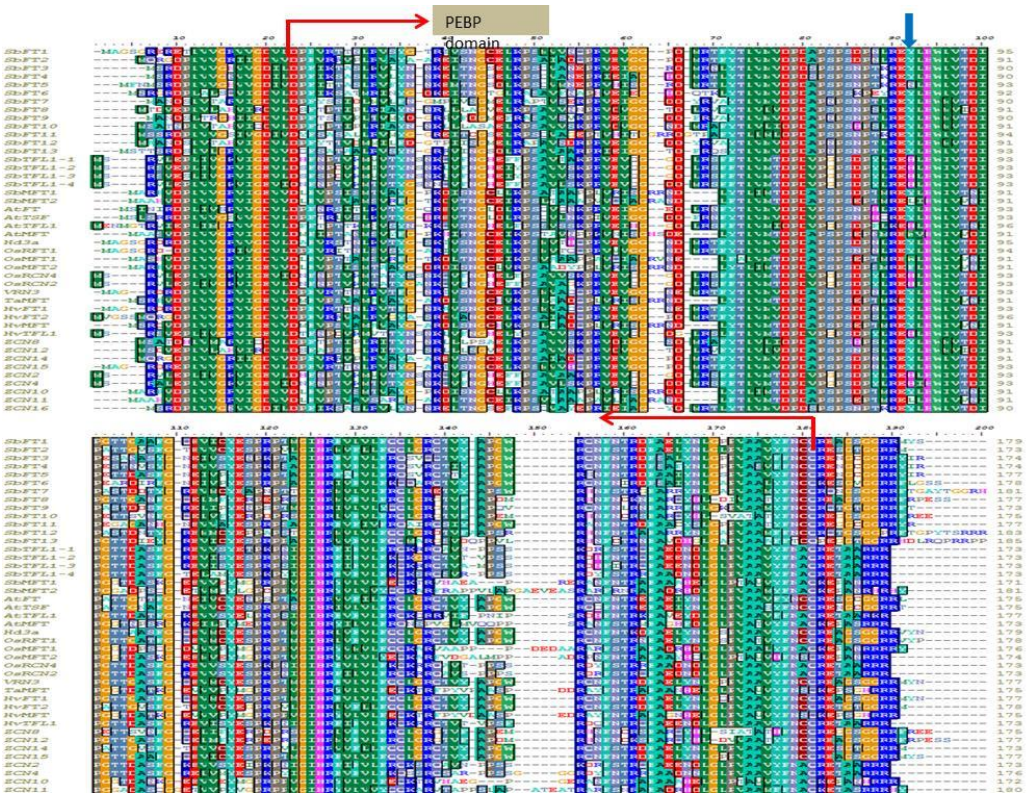


Fig. S1. Sequence alignment of SbFT/TFL1/MFT-like and related PEBP proteins. Multiple protein alignment of full-length amino acid sequences of 13 SbFT, four SbTFL1 and two SbMFT-like PEBP proteins with related *Arabidopsis*, maize, wheat, barley and rice PEBP proteins is shown. The PEBP domain boundaries are marked by red arrows. The blue arrow points to the Tyrosine (Y) residue conserved in FT proteins and Histidine (H) residue conserved in TFL1 proteins.

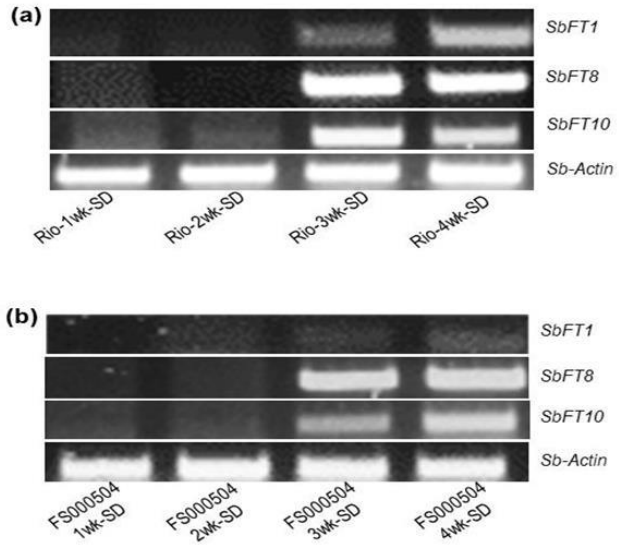


Fig. S2. Expression levels of *SbFT* genes in the leaves of late flowering sweet sorghum Rio and forage sorghum FS000504 (FS) under SD conditions. (a) Expression of *SbFT1*, *SbFT8* and *SbFT10* transcripts in the leaves of Rio during 1-4 week of seedling growth in short days. (b**)** Expression of *SbFT1, SbFT8* and *SbFT10* transcripts in the leaves of FS000504 during 1-4 weeks of seedling growth in short days. Gene expression levels were determined by semi-quantitative RT-PCR using sorghum Actin as loading control. SD, short day; wk, week.

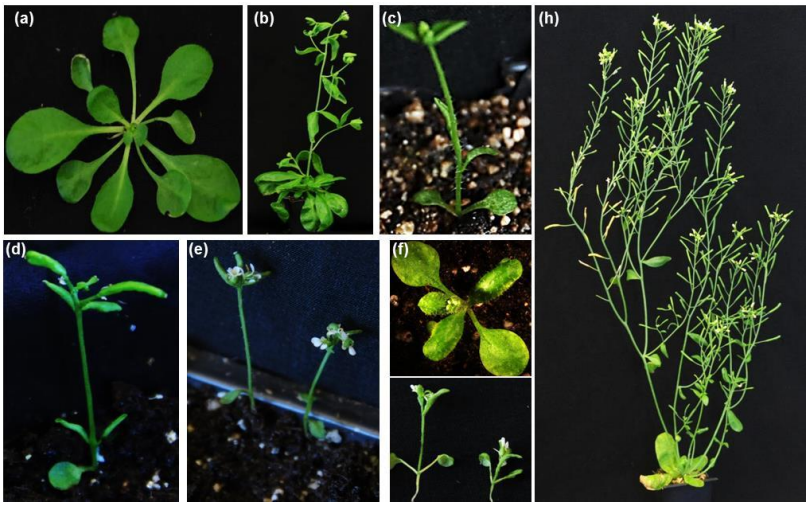


Fig. S3. Flowering phenotypes of *SbFT* transgenic *Arabidopsis* lines. (a) and (h) *ft-1* mutant showing rosette leaves at vegetative stage (a) and siliques in mature plant (h). (b) L*er* (WT) adult plant. (c) *ft-1* transformed with *35S::SbFT1*. (d) and (e) *ft-1* transformed

with *35S::SbFT8*. (f) L*er* transformed with *STF::SbFT10* and (g) *ft-1* transformed with *STF::SbFT10* showing weak early flowering phenotype.

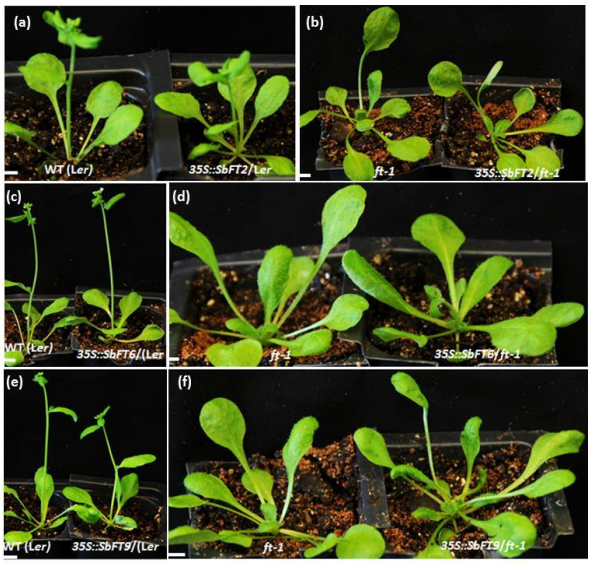


Fig. S4. Overexpression of *SbFT2, SbFT6* and *SbFT9* genes in *Arabidopsis Ler* and *ft-1* mutant plants does not alter flowering time. (a) Phenotype of WT (L*er) vs* transgenic line of *35S::SbFT2 /Ler* at bolting. (b) Phenotype of *ft-1* mutant (control) *vs* transgenic line of *35S::SbFT2 /ft-1*. (c) Phenotype of WT (L*er) vs* transgenic line of *35S::SbFT6 /Ler* . (d) Phenotype of *ft-1* mutant (control) *vs* transgenic line of *35S::SbFT6 /ft-1*. (e) Phenotype of WT (L*er) vs* transgenic line of *35S::SbFT9 /Ler*. (f) Phenotype of *ft-1* mutant (control) *vs* transgenic line of *35S::SbFT9 /ft-1*.

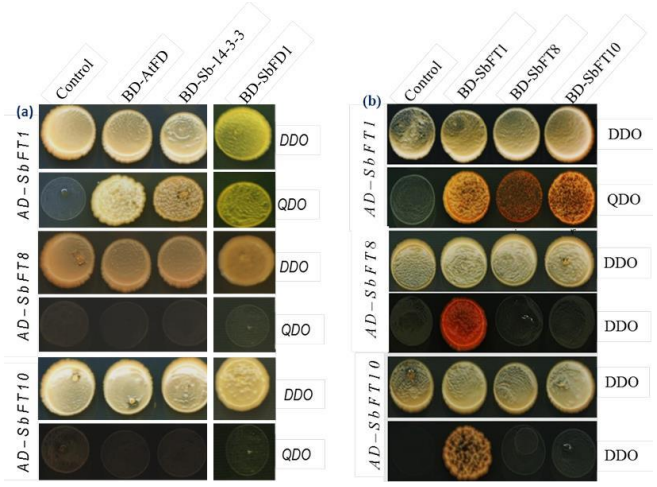


Fig. S5. Yeast-Two-Hybrid (Y2H) protein interaction assay of sorghum FT proteins with AtFD, SbFD1, Sb14-3-3, and with each other. (a) AD fused SbFT1, SbFT8 and SbFT10 interaction with AtFD, SbFD1 and Sb14-3-3 fused to the GAL4 BD. Yeast growth on

QDO media indicates positive interaction. (b) SbFT proteins interaction with each other. DDO, double dropout medium; QDO, quadruple dropout medium.

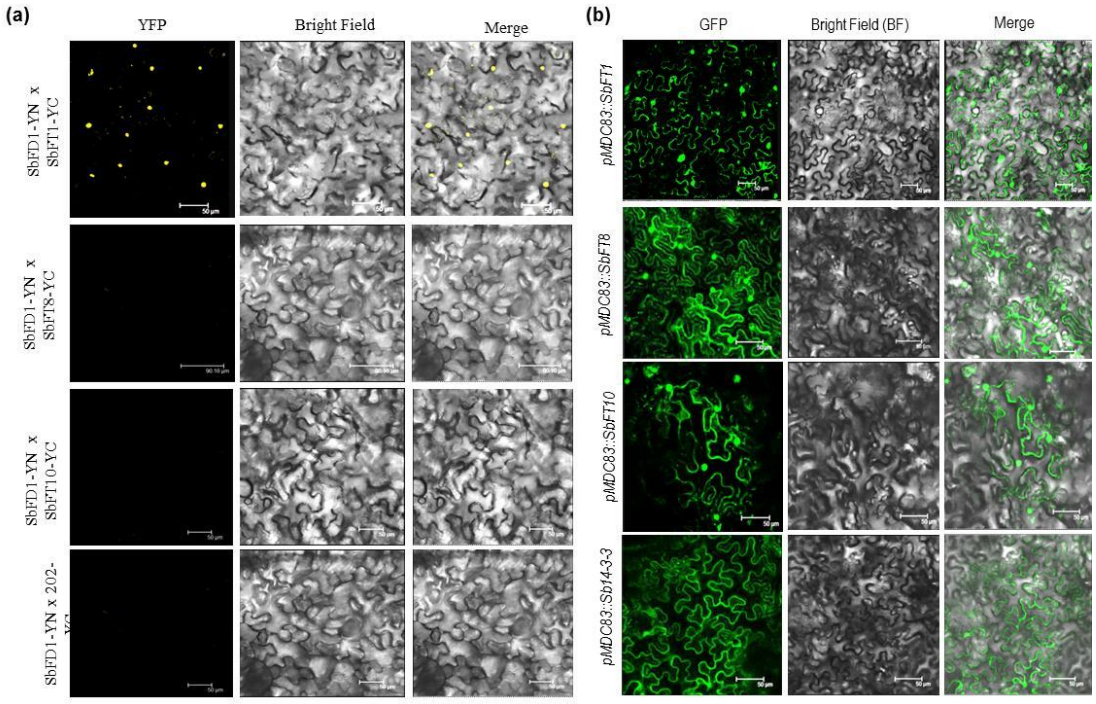


Fig. S6. BiFC assay and localization of SbFT and Sb14-3-3 proteins. (a) Split YFP BiFC assay showing SbFD1 interacting with SbFT1 but not with SbFT8 and SbFT10 proteins. The bottom panel is a negative control showing absence of interaction between SbFD1-YN and YC alone. (b) GFP fusion fluorescence showing both cytoplasmic and nucleus localization of SbFT1, SbFT8 and SbFT10 proteins and exclusively cytoplasmic localization of Sb14-3-3 in living *Nicotianal bethamiana* epidermal cells. The SbFT proteins were fused to GFP in pMDC83 vector.

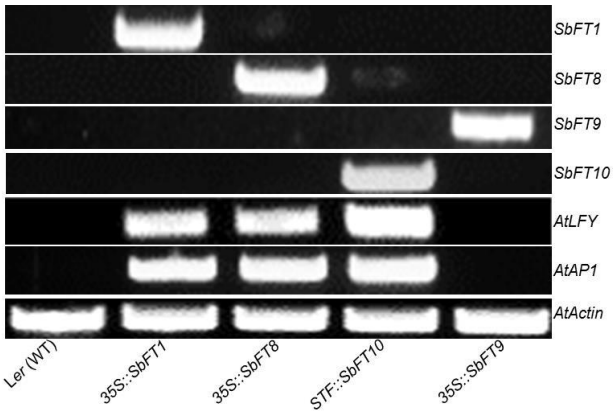


Fig. S7. Induction of floral meristem identity marker genes, *AtAP1* and *AtLFY* in *SbFT* expressing transgenic *Arabidopsis* lines. Note the high level expression of *AP1* and *LFY* in *SbFT1*, *SbFT8* and *SbFT10* ectopic expression lines compared to none in the WT and *SbFT9* expressing negative control, which does not activate flowering.

Table S1. Lists of Primers used in this study

Name	Forward Primer	Reverse Primer
MtWOX9-1	GCAACTGTGCCCTAATCTTCCTT	GTGATGAGGGTTATTATTCAATAGAGG
MtWOX9-2	AGGTTTACGGTTTCAACTTCTC	ATCGACAAATTATGAGAAATTATTG
MtActin	TCAATGTGCCTGCCATGTATGT	ACTCACACCGTCACCAGAATCC
MtWOX9-1-qRT	CCAGAACAAGAATCAGAACCAGAAC	TTAGGGAAACCAAGGGAAAATAC
MtWOX9-2-qRT	TTCAACCCTTACCTCAACAGAATG	ATGGTAGTAGGAGGAAGAGGAGC
promMtWOX9-1	CTCTTCACACACACTCAAAGAT	TCAGTGATTCTTCATCATCATC
NsFT1	ATGCCAAGAGAACGTGAACC	TCAATCGGCAGACCTTCTACG
NsAP1	CTTACTGCTACTGATGATGAAACCC	TGTTCGCTTCACGCTTATACTAAAT
NsLFY	CATCCTTTTATAGTGACGGAGCCA	GGAACATACCAAATGGCGAGTC
NsSOC1	AGGAACTTGAATCACTTGAGAGGCA	ACAATAACGGGTCTGTCTTATGGAT
NsUBQ	GGGAAGACCATCACTTTGGA	GCCACTAAAGGAAAGCACAGA
SbFT1	ATGGCCGGCAGCGGCAG	TCATGAGTACATCCTCCTTCCCCC
SbFT2	TCGGGACCCATTATTATATTCCTGT	ATACCCATCATCTTATTTCACCC
SbFT3	CGACATCGTGGACTACTTCTCAGC	GCATTGTTTCTATGCTGTCGCTTG
SbFT4	CTGCATCACTGAGGGTGTTCTAC	CCTTATGTACCTTCTTCCACCACAG
SbFT5	CGACATCGTGGACTACTTCTCAGC	GCATTGTTTCTATGCTGTCGCTTG
SbFT6	CAAATGGCACTGGACTACGAGC	AGTTGAAGTATAATGCAGCAACTGG
SbFT7	TGGTGCTCTTCAATGGTATGCC	GCTGGCAGTTGAAGTACATGGC
SbFT8	CCAGACAAGACCAGCAATAAGTTG	CTGTATTAGACGACGATATTCATGC
SbFT9	AGCAATGGCTAATGACTCTCTGAC	AGGGGAAGGTTAAAATTACTCAAGT
SbFT10	CAATGACATGAGGGCTTTCTACAC	AGTCTATGGTCTCTACTCTTCCCGA
SbFT11	CGACATCGTGGACTACTTCTCAGC	GCATTGTTTCTATGCTGTCGCTTG
SbFT12	ACCGTTGATATGATGATCCTATTCG	ATTAGACGTTTTGTCACCGATCCTT
SbFT13	AGGCAGTCCTACACGCTTGTTATG	ATCATCGTGCTGGTAAGCTGAGT
SbTFL1-1	ATGTCAAGGGTGTTGGAGCCTC	CTTAGGGTCGTTGGTTGGTCTTG
SbTFL1-2	TTCAGGTTATGACGGACCCAGATGT	ATCTCTGGAGGATGGCACAGTTACA
SbTFL1-3	TACCTTCCCTAATCGCCATAGTTCC	ACTGTCGTGTGATGAAATGGTCCCT
SbTFL1-4	ATGTCTAGGGTGTTGGAACCTCTAG	ATGGGAGTAGGGCTGAGGATTGA
SbMFT1	ATAGTGACCAACATACCAGGAGGAA	TAGAACATTATGAGACAGCCACACT
SbMFT2	ACCTCTTCACCCTGGTTATGACTG	TGAGACACCACAAGTTGAGAGAATG
Sb-14-3-3	GTAGCGTGTTCAGATATTTGATGTG	CTAAAACAATGGGCTCAGACAACA
AtAP1	GGAGAAGATACTTGAACGCTATGA	TGTAAGGATGCTGGATTTGG
Sb-Actin	ACATTGCCCTGGACTACGAC	CTTTCTGGAGGAGCAACCAC
At-Actin	CTCAGCACCTTCCAACAGATGTGGA	CCAAAAAAATGAACCAAGGACCAAA
AtLFY	GAAGAAATCAGGAGCGAGTTACA	CAGCTAATACCGCCAACTAAAGC
AtFD	ATGTTGTCATCAGCTAAGCATCAG	TCAAAATGGAGCTGTGGAAGAC
SbFT1-qRT	GCTAGCTTATCCCGCATATTACCC	CCACCCAAACTGCATCCACTCTTGAA
SbFT8-qRT	TGCATGCATGAATATCGTCGTCT	CCCGGGTAGTACATATAAGGTGGT
SbFT10-qRT	AACTGTCAAAGGGAAGGTGGATCG	GACTAAGCTCTCAACCCTTCAAGTC
Sb-β-actin-qRT	TGGCATCTCTCAGCACATTCC	AATGGCTCTCTCGGCTTGC
SbFD1	ATGGCGGCCATAGACATGGAG	TCAGAATGGCGCGGACGGCA

Accession numbers or gene identifiers of sequences used for multiple sequence alignment and phylogenetic tree construction in this study.

MtWOX9-1 (XP_003593686.1), MtWOX9-2 (AGX26723.1), NsFT (XP_009776089.1), NsAP1 (NP_001289508.1), NsLFY (XP_009780579.1), NsSOC1 (NP_001289529.1), NsWOX9 (XP_009793302.1), PcWOX9 (ACL11801.1), GmWOX9 (XP_006594207.1), VvWOX9 (XP_002273188.1), PhSOE (ABO93067.1), AtWOX9 (NP_180944.2), SbFT1 (XP_002436509.1), SbFT2 (XP_002457125.1), SbFT3 (XP_002446704.1), SbFT4 (XP_002454134.1), SbFT5 (XP_002489297.1), SbFT6 (XP_002462655.1), SbFT7 (XP_002451827.1), SbFT8 (XP_002456354.1), SbFT9 (XP_002438551.1), SbFT10 (XP_002456354.1), SbFT11 (XP_002443085.1), SbFT12 (XP_002446272.1), SbFT13 (XP_002457162.1), SbTFL1-1 (XP_002453931.1), SbTFL1-2 (XP_002442808.1), SbTFL1-3 (XP_002450283.1), SbTFL1-4 (XP_002447782.1), SbMFT1 (XP_002457494.1), SbMFT2 (XP_002436981.1), Sb-14-3-3 (XP_002445779.1), SbFD1 (XP_002460587.1), ZCN1 (NP_001105959), ZCN2 (NP_001106241), ZCN3 (NP_001106242), ZCN4 (NP_001106243), ZCN8 (NP_001106247), ZCN10 (NP_001106249), ZCN11 (NP_001106264), ZCN12 (NP_001106250), ZCN13 (XP_008646959), ZCN14 (NP_001106251), ZCN15 (NP_001106252), ZCN16 (NP_001106253), ZCN17 (NP_001106254), ZCN18 (NP_001106255), ZCN20 (NP_001296779), ZCN21 (XP_008671372), ZCN25 (NP_001106257), ZCN26 (NP_001106265), AtFD (AT4G35900.1), AtFT (AT1G65480.1), AtTSF (AT4G20370.1), AtTFL1, (AT5G03840.1), AtMFT (AT1G18100.1), Hd3a (AB052941.1), OsRFT1 (NP_001056859), OsMFT1 (NP_001057701), OsMFT2 (NP_001041806), OsRCN2 (NP_001173006), OsRCN4 (NP_001052733), OsFTL6 (NP_001053150), OsFTL5 (NP_001053150), OsFTL11 (AAX96155), OsFTL4 (AAX96155), OsFTL13 (BAD27710), OsFTL9 (NP_001044242), OsFTL12 (NP_001057842), OsFTL10 (EEC71474), OsFTL7 (EEE52982), OsFTL8 (EEC70127), RCN1 (NP_001066172), RCN3 (NP_001065771), VRN3 (AAW23034), TaMFT (BAK78908), HvFT1 (AAZ38709), HvFT2 (ABB99414), HvMFT (BAK06374) and HvTFL1 (BAH24197)

CHAPTER IV

GENERAL SUMMARY AND PROSPECTIVES

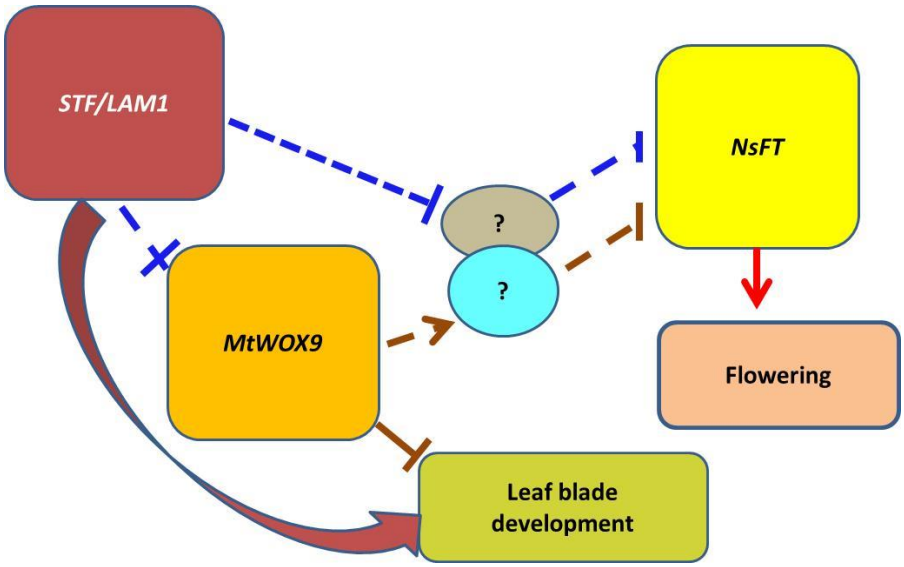
Summary of leaf blade development

The critical requirement of *WOX* genes for the development of lateral organs, including leaves and flowers, has become the focal point of current research based on the identification of several mutant phenotypes in angiosperms. It has also been indicated that the finely tuned balance between cell division, differentiation and expansion are very crucial for leaf blade development in controlling both proximodistal and mediolateral axes where the role of several genes have been identified. In this study, spatial and temporal expression of *MtWOX9*-1 and *MtWOX9-2* have shown in young leaf, shoot apex, stem, pod, and flower at different developmental stages of *Medicago* and a similar pattern was confirmed using *GUS* reporter, suggesting the involvement of *WOX9* genes in leaf blade development. Furthermore, overexpression of *MtWOX9-1* in *Medicago* induced remarkable leaf blade defect with downward curling leaf phenotype, suggesting the functional involvement of *MtWOX9-1* in leaf lamina outgrowth. Similarly, ectopic expression of *MtWOX9-1* in *N. sylvestris* reveals strong effects in leaf blade development, as it is sufficient to modify the leaf features with the generation of unique leaf blade defects. The altered leaf structure including shape, size, margin, venation and total leaf number of severe transgenic phenotype was related to the dose dependent expression of *MtWOX9-1*. The higher expression of *MtWOX9-1* was correlated with the higher degree of leaf lamina twists and distorted leaf size and shape. In addition, ectopic expression of *MtWOX9-1* in transgenic plants in *stf* (*Medicago*) and *lam1* (tobacco) dramatically enhanced leaf phenotype compared with the original *stf* and *lam1* mutants by forming a more compacted radial blade with added proximodistal defects. Interestingly, the highest expression of *MtWOX9-1* in *stf* mutant suggests that STF may have repression effect on *MtWOX9-1*. Taken together, these observations are consistent with the views of previous

studies that the meristematic activity and lateral organ development in higher plants require a balance between genes that modulate growth in both mediolateral and proximodistal planes during leaf morphogenesis.

Furthermore, detail phenotype evaluation of severe phenotype of 35S::*MtWOX9-1* in tobacco wild type revealed not only remarkable leaf defects and arrested growth but also showed significant late flowering time with poor pod and seed setting as compared to wild type. The flowering time of wild type under LD condition requires about ten weeks whereas ectopic expression of *35S::MtWOX9-1* in transgenic lines delayed flowering by 45 weeks i.e. six fold delay. Interestingly, the result have found that the cause of delay flowering in ectopically expressed 35S::*WOX9-1* transgenic line and rarely flowering *lam1* mutant, which appeared to be due to repression of *FLOWERING LOCUS T1* (*NsFT1)*, a key floral transition activator in tobacco. These data shows that the expression patterns of *NsFT1* consistently reduced in *35S::MtWOX9-1*/WT in severe transgenic lines and *lam1* mutant and have also shown the role of ectopic expression of *NsFT1* that fully rescued flowering time in both late flowering *35S::MtWOX9-1*/WT in severe transgenic lines and rarely flowering *lam1* mutant even earlier than wild type. It is also interesting to note that ectopic expression of *NsFT1* fully rescued the twisted and distorted tobacco leaf blades affected by ectopic expression of *35S::MtWOX9-1/WT* in severe transgenic lines indicating the functional interaction of *NsFT1* with *MtWOX9-1* in leaf blade out growth. Similarly, the results have shown that the longer vegetative growth with numerous leaf production of *35S::MtWOX9-1*/WT in severe transgenic lines was confirmed by the ectopic expression of *35S:NsFT1* to normalize the leaf number, size and shape and activate flowering time. Taken, together these results suggest the existence of functional interconnection among *WOX9, STF/LMA1* and *FT* genes in leaf blade development and flowering time control in tobacco. However, the exact connection between *WOX9*, *STF* and *FT* is still unclear. These data suggests that STF represses *WOX9* in the leaf, based on the upregulation of *WOX9* transcript in the *stf* mutant. *WOX9* in turn represses *FT* and appeared to be the cause of the late flowering phenotype in both the *lam1* mutant and the *MtWOX9* overexpression tobacco transgenic lines. But these are associations rather than direct evidences. STF may or may not directly repress *WOX9* and WOX9 may or may not directly repress *FT*. In fact, since WOX9 is a transcriptional

activator, a more likely scenario is that WOX9 activates another repressor of FT in tobacco which probably is not functioning or controlled by another factor in *Medicago*. Future experiments can address these issues. EMSA and ChIP assays, as well as luciferase assays using *WOX9* promoter will establish if *STF* directly represses *WOX9*. Similar assays need to be performed on the FT promoter to determine if WOX9 directly represses *FT*. Alternatively, *WOX9* can be expressed under the glucocorticoid (GR) inducible system in tobacco in the presence and absence of cyclohexamide (CHX), a protein synthesis inhibitor, and transcripts compared by RNA-seq. This experiment will tell if FT is directly repressed by *WOX9* without the need for new protein synthesis (in the CHX treatment) or indirectly repressed (only in samples without CHX treatment). Moreover, the effect of FT on leaf development needs to be worked out. The FT-STF interaction can be characterized by deleting the interaction domains and transforming the mutated *FT* into *WOX9* overexpressors to see if the *STF-FT* interaction is required by complementing the leaf phenotype of the *WOX9* transgenic plants. Other experiments could include evaluating the effect of *STF* and *WOX9* in the *FT* mutant background and that of *STF* and *FT* in the *WOX9* mutant background. These mutants are not available in tobacco at the moment but it should be possible to generate them in the future using the CRISPR/Cas9 technology.

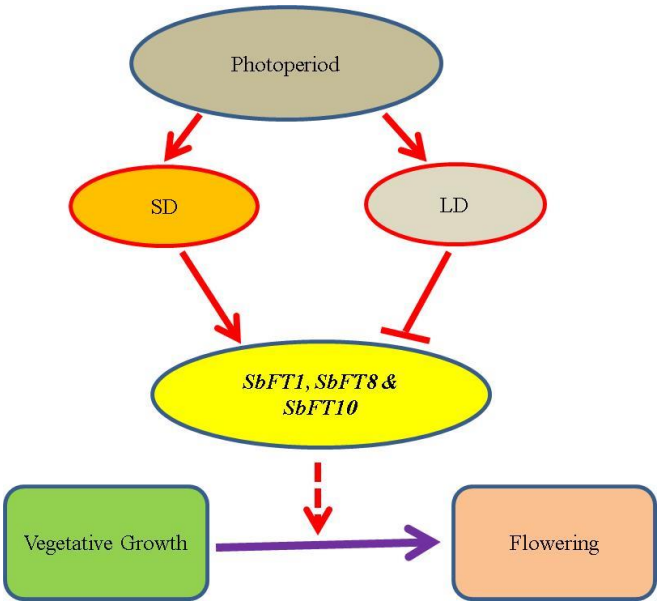


Model 1. ***STF/LAM1, WOX9* & *FT* may be interconnected in the control of flowering time and leaf blade development**

Summary of sorghum florigens

Control of flowering time is a major agronomic trait in sorghum that defines its usage in grain or biomass production in temperate regions. Owing to its original domestication in tropical East Africa, sorghum exhibits strong photoperiod response. However, sorghum has been introduced into temperate agriculture where photoperiod insensitive varieties have been developed for optimum seed production in the warm summer months. Nineteen PEBP sequences from the sorghum genome (version 2.1) were examined and 13 *FT*-like (*SbFT1-SbFT13)* genes were identified in an effort to understand the mechanistic control of flowering time in sorghum. Out of the 13 *FT*-like genes, *SbFT1, SbFT8* and *SbFT10* were identified as potential candidates for encoding florigens that activate floral transition and mediate photoperiodic responses. Further was made for the characterization of three sorghum candidate florigens to establish a molecular roadmap for mechanistic understanding using multiple approaches such as phylogenetic analysis, temporal and spatial expression study, ectopic expression and molecular interactions. These three genes are highly expressed in the leaf at the initiation stage of floral transition, expressed early in grain sorghum genotypes but late in sweet and forage sorghum genotypes, induced by SD treatment in photoperiod sensitive genotypes, cooperatively repressed by the classical sorghum maturity loci, interact with FLOWERING LOCUS D and 14-3-3 proteins and activate flowering in transgenic *Arabidopsis*, suggesting florigenic potential in sorghum. These results together demonstrate that sorghum has at least three *FT* genes that could potentially form a florigen activation complex and mediate genotype dependent photoperiod response and flowering time variation in sorghum. SD induction of these three genes in sensitive genotypes is fully reversed by one week long day treatment, and yet, some aspect of the SD treatment may be remembered to activate flowering after transfer to long days, indicating a complex photoperiod response mediated by *SbFT* genes. Further molecular analyses would be needed to understand the mechanism with which reversion of *FT* induction by LDs, memory of exposure to SDs, as well as de-repression of axillary meristem during photoperiod switching are achieved in sorghum, which will provide novel mechanistic insight in the response of sorghum developmental programs to environmental signals. Further experiments with mutants or reduced expression lines will

be required to quantitatively determine the contribution of each of the three *SbFT* genes to sorghum flowering and photoperiod response. The *SbFT* genes need to be expressed in sorghum individually and in combination to see their actual effects in the native plant. Expressing them in photoperiod sensitive genotypes under LD conditions will enable to evaluate if the three SbFTs are sufficient to overcome photoperiod response. Current transformation technology needs to be optimized to enable testing *SbFT*s in forage and sweet sorghum genotypes, which are more recalcitrant to transformation and regeneration compared to some of the photoperiod insensitive grain sorghum genotypes. It will also be interesting to investigate how these three *SbFTs* are regulated by the circadian clock (*GI* and *CO*), photoreceptors (*PHYB, PHYC*, *Cryptochromes*) and transcriptional regulators downstream of photoreceptors. Another interesting aspect to investigate will be whether *SbWOX9* and *SbNS/WOX3* interact with and/or regulate *SbFT* genes to affect leaf size and flowering time in sorghum. Such experiments will not only enlighten my understanding of the relationship of leaf development and floral transition but also in the long run will enable me to design plants that are rapidly responsive to the changing environment and minimize yield cost. Since flowering is a key agronomic trait in sorghum, the three *SbFT* genes identified here can be used as valuable molecular markers in sorghum breeding programs.



Model. 2. Short day photoperiod induces sorghum florigins to promote flowering while LD represses to enhance vegetative growth.

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VITA

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Doctor of Philosophy

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Major Field: Crop Science

Biographical

Personal Data: I was born in Ethiopia, Shoa Region. I have got my BSc. and MSc. in plant science and agronomy from Haramaya University, Ethiopia in 1991 and 2000, respectively. I served in Ethiopian Agricultural Research Institute (EIAR) working as Associate Researcher in field crops improvement program since 2010. I attended my graduate school at Oklahoma State University, Stillwater, Oklahoma in August 2010 majoring in Crop Science in Plant and Soil Sciences Department, where I will graduate from, in December, 2015 with a Doctor of Philosophy in Crop Science. I married Serkalem Kebede Laki on 9th February 1995 and my elder son Firaol T. Wolabu, my daughter Kebene T. Wolabu and my second son Fayera T.Wolabu were born.

Professional Experience: My work experience includes different research capacities as Junior/Assistant/Associate Researcher (I-III) on different highland field crops (legumes & cereals) in Ethiopia. Since I joined Oklahoma State University (OSU) as graduate student, I served as assistant researcher (AR) and gain work experience in Plant Functional Genomics (in Dr. Million Tadege's) laboratory on dicot leaf blade development genes and sorghum florigenes.

Professional Affiliations: Member of American Association for the Advancement of Science (AAAS)
Member of Plant and Soil Sciences Graduate Student Organization (PaSS-GSO) of Oklahoma State University.