Genetic regulation of flowering time and inflorescence architecture by MtFDa and MtFTa1 in Medicago truncatula

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Abstract

Regulation of floral transition and inflorescence development is crucial for plant reproductive success. FLOWERING LOCUS T (FT) is one of the central players in the flowering genetic regulatory network, whereas FLOWERING LOCUS D (FD), an interactor of FT and TERMINAL FLOWER 1 (TFL1), plays significant roles in both floral transition and inflorescence development. Here we show the genetic regulatory networks of floral transition and inflorescence development in Medicago truncatula by characterizing MtFTa1 and MtFDa and their genetic interactions with key inflorescence meristem (IM) regulators. Both MtFTa1 and MtFDa promote flowering; the double mutant mtfd Δ mtfta1 does not proceed to floral transition. RNAseq analysis reveals that a broad range of genes involved in flowering regulation and flower development are up- or downregulated by MtFTa1 and/or MtFDa mutations. Furthermore, mutation of MtFDa also affects the inflorescence architecture. Genetic analyses of MtFDa, MtFTa1, MtTFL1, and MtFULc show that MtFDa is epistatic to MtFULc and MtTFL1 in controlling IM identity. Our results demonstrate that MtFTa1 and MtFDa are major flowering regulators in M. truncatula, and MtFDa is essential both in floral transition and secondary inflorescence development. The study will advance our understanding of the genetic regulation of flowering time and inflorescence development in legumes.

Introduction

When and where to form flowers are crucial for plant reproductive success. In order to flower at the right time and to develop suitable inflorescences, plants need to constantly and accurately monitor their local environments and internal signals. Understanding the mechanisms of flowering regulation and inflorescence development has significance for crop production by harnessing and manipulating these processes in breeding (Eshed and Lippman, 2019). Extensive genetic and molecular studies in model species have demonstrated that plants have intricate regulatory pathways in coordinating internal and external signals in floral transition (Koomneef et al., 1998; Simpson et al., 1999; Andrés and Coupland, 2012). These regulatory pathways are integrated by several flowering integrators, FLOWERING LOCUS T (FT), LEAFY (LFY), and SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1; Blázquez and Weigel, 2000; Araki, 2001; Wellmer and Riechmann, 2010; Posé et al., 2012). Among the three integrators, FT is the major integrator of the networks and channels flowering signals...
from light, temperature, vernalization, and hormone fluctuation into floral transition (Baurle and Dean, 2006). FT is a small protein that is synthesized in leaves and moves to shoot apices. In the shoot apex, FT interacts with FLOWERING LOCUS D (FD) to promote floral transition by upregulating other flowering genes and activating floral meristem (FM) identity genes APETALA 1 (AP1), CAULIFLOWER (CAL), and FRUITFULL (FUL; Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Turck et al., 2008). The function of FT as a mobile protein in triggering flowering is highly conserved across species (Lifschitz et al., 2006; Tamaki et al., 2007; Laurie et al., 2011).

Upon floral transition, the shoot apical meristem (SAM) is transformed from a vegetative meristem into an inflorescence meristem (IM). In addition to forming leaves and lateral branches (or axillary buds), IM laterally produces flowers to form a simple inflorescence, or produces secondary IMs (SM or I2) that bear flowers to form a compound inflorescence (Stebbins, 1973; Coen and Nugent, 1994). TERMINAL FLOWER 1 (TFL1), LFY, and AP1 are key regulators of the simple racemose inflorescence in Arabidopsis (Liljegren et al., 1999). TFL1 and FT are closely related but belong to different clades in the phosphatidylethanolamine binding protein (PEBP) family. Like FT, TFL1 is also a mobile protein (Goretti et al., 2020). TFL1 is upregulated upon floral transition and expressed at the center of the primary IM (I1), and the TFL1 protein moves from the center to the peripheral layer, where it is associated with FD to repress the expression of LFY, AP1, and some other flowering genes, therefore maintaining the shoot meristem indeterminacy (Hanano and Goto, 2011; Goretti et al., 2020). Meanwhile, AP1 is expressed in the FM and, together with LFY, restrains the TFL1 expression in the FM. The reciprocal repression loop dynamically controls the development of simple inflorescences (Ratcliffe et al., 1998; Liljegren et al., 1999; Hempel et al., 2000; Blázquez et al., 2006). During the development of compound inflorescences in legume species, whereas TFL1/DETERMINE (DET), AP1/PROLIFERATING INFLORESCENCE MERISTEM (PIM), and LFY/SINGLE LEAFLET 1 (SGL1) retain the conserved functions for IM indeterminacy and FM identity, FRUITFULLc (FULc)/VEGETATIVE1 (VEG1) acquires the unique function for I2 meristem identity. FULc/VEG1 is specifically expressed in secondary IMs and confines the expression of TFL1/DET in I1 and AP1/PIM in FMs (Berbel et al., 2012; Benloch et al., 2015; Cheng et al., 2018). The modified reciprocal repression model of TFL1, FULc, and AP1/VEG1 elegantly elucidates the regulation of compound inflorescence development in legumes (Singer et al., 1999; Benloch et al., 2015; Cheng et al., 2018).

The timing of flowering and the inflorescence architecture are closely related. The inflorescence complexity has been delicately demonstrated through manipulating the expression of flowering-time and IM identity genes LFY and TFL1 in Arabidopsis (Bradley et al., 1997; Prusinkiewicz et al., 2007; Jaeger et al., 2013). In tomato (Solanum lycopersicum), the inflorescence architecture is also controlled by the rate of meristem maturation and its inflorescence complexity can be increased by delaying FM commitment (Park et al., 2012, 2014; Thouet et al., 2012). SINGLE FLOWER TRUSS (SFT), the ortholog of FT in tomato, controls the floral transition and also modulates shoot architecture by regulating meristem maturation (Lifschitz et al., 2006; Krieger et al., 2010). The local balance between SFT and SELF-PRUNING (SP), the ortholog of TFL1, regulates the growth/termination cycling in tomato (Krieger et al., 2010; Lifschitz et al., 2014). Both FT and TFL1 can form protein complexes with FD to regulate downstream targets. FD is a bZIP transcription factor and has conserved function in promoting flowering in several species (Wigge et al., 2005; Muszynski et al., 2006; Sussmilch et al., 2015). In Arabidopsis, FD interacts with FT to upregulate the floral identity genes in promoting floral transition, or with TFL1 to negatively modulate the FD-dependent transcription of target genes to fine-tune the flowering time and the development of IMs (Abe et al., 2005; Wigge et al., 2005; Ahn et al., 2006; Hanano and Goto, 2011; Tylewicz et al., 2015). In rice (Oryza sativa), Heading Date 3a (Hd3a) and RETICULOCALBIN 1 (RCN1), orthologs of FT and TFL1, respectively, both bind to OsFD1 through 14-3-3 proteins and form flowering-act-complex (FAC) or flowering repress complex (FRC). The equilibrium between FAC and FRC determines the indeterminate vegetative growth or flowering at SAMs (Taoka et al., 2011; Tsuji et al., 2013b). In pea (Pisum sativum), PsFDa/VEG2 interacts with all five PsFT proteins in vitro. Mutation of PsFDa/VEG2 significantly delays the floral transition and causes defects in inflorescence development (Sussmilch et al., 2015).

Legumes are second only to the Graminiae in their importance to humans. Understanding the regulatory mechanisms of flowering time and inflorescence development has great significance for grain and forage productivity improvement. Because of its small genome size and short life cycle, Medicago truncata has been selected as a model legume with extensive genomic and genetic resources. Medicago truncata is closely related to the most important forage crop, alfalfa (Medicago sativa), and shares similar inflorescence architectures with pulse legumes. Comparative phylogenetic analysis shows that most of the known flowering-related genes are present in M. truncata, though with significant differences. The key gene families, such as CONSTANS (CO), FT, FUL, SOC1, SHORT VEGETATIVE PHASE (SVP), and TFL1, have undergone genomic duplication and expansion (Hecht et al., 2005; Weller and Ortega, 2015; Nelson et al., 2017; Weller and Macknight, 2018). Due to the available genetic resources, several key flowering gene families have been functionally characterized in M. truncata (Jaudal et al., 2013, 2014, 2015, 2016, 2018; Weller and Ortega, 2015; Thomson et al., 2019; Zhang et al., 2019). Among the flowering-time genes, MtFTa1, out of the other five family members (MtFTa2 and 3, MtFTb1 and 2, and MtFTc), is the major regulator for floral transition in response to long day and vernalization, though how MtFTa1...
functions in flowering regulation is not well understood in *Medicago* (Laurie et al., 2011; Jaudal et al., 2013; Nelson et al., 2017). Our recent study demonstrated that MtTFL1, MtFULc, and MtAP1/SGL1 are essential for inflorescence and FM identity, but have no significant effects on floral transition. The genetic regulation model among the genes for inflorescence development is conserved with that in pea (Berbel et al., 2012; Benloch et al., 2015; Cheng et al., 2018).

To further understand the genetic regulatory network from floral transition to inflorescence formation in *M. truncatula*, we isolated late-flowering mutants with or without defects in inflorescence development in the Tnt1-insertion population. Flanking sequence analysis revealed that the mutants with only late-flowering phenotype have Tnt1 insertions in MtFTa1, whereas the mutants exhibiting both late flowering and defects in inflorescence development have Tnt1 insertions in MtFDa. Here, we report the functional characterization of MtFDa and MtFTa1, and their genetic interactions with key inflorescence regulators during flowering regulation and inflorescence development in *M. truncatula*.

**Results**

**Both MtFDa and MtFTa1 play a role in floral transition in *M. truncatula***

From a forward screen of the Tnt1-insertion population, we isolated three mutants exhibiting a significantly delayed flowering phenotype. Genetic analysis showed that the late-flowering phenotype of two of the lines, NF11200 and NF11802, is caused by Tnt1 insertions in the 5′-untranslated region (UTR) and the first exon in the gene MtFTa1, respectively (Figure 1A; Supplemental Figure S1, A and C). The two lines were named mtfta1-1 and mtfta1-2, respectively. The late-flowering phenotype in the third line (NF11119) is caused by a Tnt1 insertion in the first exon of the gene MtFDa (Medtr5g022870; Figure 1B; Supplemental Figure S1B) and was named mtfda-1. MtFDa shares 67% amino acid identity with the pea ortholog VEG2/PsFDa. To further confirm if the phenotype is caused by the mutation of MtFDa, we isolated two additional Tnt1 insertion lines, NF9972 (mtfda-2) and NF10125 (mtfda-3), for MtFDa through polymerase chain reaction (PCR)-based reverse screening (Cheng et al., 2018). Both mtfda-2 and mtfda-3 have Tnt1 insertions in the first exon of MtFDa (Supplemental Figure S1B). Homozygous mutant plants of mtfda-2 and mtfda-3 also showed late-flowering phenotypes (Supplemental Figure S1D).

In order to compare the flowering time, germinated seeds of mtfta1, mtfda, and wild-type (WT, ecotype R108) with or without vernalization at 4°C for 10 d were transferred into soil and grown in the greenhouse under long days (LDs). With vernalization treatment, flower buds were visible at nodes 3–5 or after 25–28 d of growth in the greenhouse for WT plants, at nodes 13–15 or after 55–60 d for mtfda plants, and at nodes 20–23 or after 75–80 d for mtfta1 plants (Figure 1, A and B; Supplemental Figure S1, C and D; Table 1). Without vernalization treatment, WT plants flowered at nodes 10–12, whereas mtfda plants flowered at node 23. Interestingly, un-vernalized mtfda plants flowered at a similar time as vernalized plants, i.e. 75–80 d of growth in the greenhouse (Table 1). The results indicate that mutation in either MtFDa or MtFTa1 delays floral transition and that mutant mtfda remains responsive to vernalization treatment, whereas mutant mtfta1 does not. In addition, it was noticeable that mtfda plants have more tertiary branch elongation, which results in bushy shoots relative to mtfta1 and WT plants (Figure 1C; Supplemental Figure S1, E and F), indicating that mutation of MtFDa may promote lateral growth of shoots.

**Double mutation of MtFDa and MtFTa1 blocks floral transition**

FD and FT interact to form a flowering-signaling complex at the shoot apex to promote floral transition (Abe et al., 2005; Wigge et al., 2005). To understand the essential roles of MtFDa and MtFTa1 in controlling floral transition in *M. truncatula*, we generated the double mutant mtfda mtfta1 and compared the growth and development of mtfda mtfta1 with single mutants and WT. While single mutants mtfta1 and mtfda flowered late as described above (Figure 1, A and B; Supplemental Figure S1H), the double mutant mtfda mtfta1 never formed flowers, even after 8 months of growth in the greenhouse, and maintained continuous vegetative growth (Figure 1D and Supplemental Figure S1I). It has been reported that PsTFL1/DET expression increased upon floral transition, and the upregulation in I meristem is used as a developmental marker for floral transition in pea (Berbel et al., 2012; Sussmilch et al., 2015). We then examined the expression of MtTFL1 in the shoot apex of 90-d-old double-mutant plants by both reverse transcription–quantitative PCR (RT-qPCR) and *in situ* hybridization. The expression of MtTFL1 was very low or undetectable at the center of SAM (Figure 7D), suggesting no floral transition in mtfda mtfta1 plants.

Mutant mtfulc is a nonflower mutant with normal floral transition (Cheng et al., 2018). Because of the transformation of secondary inflorescences (I2) into vegetative shoots, two lateral shoots were observed in axillary positions in mtfulc instead of one compound flower and one lateral shoot in WT after floral transition (Figure 1, F and H). Therefore, we considered two lateral shoot elongation in axillary positions as an indicator of flowering in *M. truncatula* plants. At the vegetative stage, mtfda mtfta1 plants developed normally as WT, with one lateral shoot at each leaf axillary (Figure 1, E and G). Whereas WT or mtfulc plants transitioned to flowering with flower or extra lateral shoot formation (Figure 1, F and H), mtfda mtfta1 plants remained in the vegetative status with one lateral shoot in leaf axillary positions (Figure 1, D and E), indicating that floral transition did not occur in mtfda mtfta1. In addition, mtfda mtfta1 plants also showed more tertiary branch growth as observed in mtfda (Supplemental Figure S1G). Since the original mtfda mtfta1 plants became too big to be maintained in pots, plantlets were regenerated by...
cutting. The regenerated mtfd1 mtfta1 plants were grown for another 4 months, and floral transition was not observed. Taken together, we concluded that MtFDa and MtFTa1 are key flowering regulators in the MtFD and MtFT family and that both MtFDa and MtFTa1 are required for floral transition. Double mutation of MtFDa and MtFTa1 completely blocks the floral transition in M. truncatula.

**Mutation of MtFDa affects inflorescence development**

In addition to delayed floral transition, mtfd1 plants also had defective secondary inflorescences (I2). In WT plants, I2 was initiated laterally from the primary inflorescence (I1), producing one small bract and one to three flowers before termination as a small spike (Figure 2A; Supplemental Figure S2A). In mtfd1, I1 showed normal indeterminate growth, whereas I2 was transformed into inflorescence branches, which laterally produced single flowers subtended by normal or defective compound leaves (Figure 2, B and C; Supplemental Figure S2, B and C). After forming four to six floral nodes, the apex arrested as a defective flower or floral bud (Figure 2D; Supplemental Figure S2D); usually, only the first two flowers developed into pods (Figure 2C; Supplemental Figure S2C). The phenotypes of extended

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**Figure 1** Phenotypes of mutants of mtfd1, mtfta1, and mtfd1 mtfta1 grown under vernalization and long day (VLD). Two-month-old WT plants have pods (arrows), whereas mtfta1 (A) and mtfd1 (B) plants are not flowering. (C) Three-month-old bushy plant of mtfd1. (D) Left, 5.5-month-old mtfd1 mtfta1 plant, not flowering; right, 2-month-old WT plant with pods. (E) Shoot from 5.5-month-old mtfd1 mtfta1 plant, showing one lateral branch (arrow) at the leaf axillary position. (F) Shoot from 3-month-old mtfulc plant, showing two lateral branches (arrow) at the leaf axillary position. (G and H) Shoots from WT plants at vegetative stage (G) and flowering stage (H), showing one lateral branch (arrow) (G), one lateral branch, and one compound flower (or pods, red arrow) at each leaf axillary position (H).

**Table 1** Comparison of flowering time in WT, mtfd1, and mtfta1 with or without vernalization

| Genotype | With vernalization | Without vernalization |
|----------|--------------------|-----------------------|
|          | Number of plants (n) | Days to flowering | Number of nodes | Number of plants (n) | Days to flowering | Number of nodes |
| WT       | 17                 | ~25                  | 3.5 ± 0.87     | 14                 | ~40                  | 12.3 ± 1.73     |
| mtfd1    | 14                 | ~55                  | 13.7 ± 2.49    | 12                 | ~75                  | 23.5 ± 1.29     |
| mtfta1   | 10                 | ~70–80               | 20.4 ± 4.84    | 12                 | ~70                  | 20.3 ± 1.54     |
growth of I₂ and transformation of bracts into compound leaves indicated that the I₂ identity is partially impaired in mtfda, suggesting that MtFDa is required for secondary inflorescence identity and development. In WT, each plant developed three to five primary inflorescence shoots, which all produced flowers. However, in mtfda, some primary shoots at lower parts of the plants only produced secondary shoots without flower formation (Figure 2E). The phenotype indicates that the identities of both I₂ and flowers are not acquired in these inflorescence shoots.

MtFDa was expressed at both vegetative and floral stages. The expression was low in young plants, yet was increased prior to floral transition (Figure 2F). In situ hybridization showed that MtFDa expression was detected in the SAM and axillary meristems (AMs), but not in leaf primordia at the vegetative stage (Figure 2G). Upon floral transition, MtFDa was detected in all meristems, including I₁, I₂, FMs, and AMs (Figure 2, H–J; Supplemental Figure S4A). Again, the expression was neither detected in leaf primordia nor in floral organ primordia (Figure 2, I and J). The expression pattern of MtFDa is consistent with its functions in regulating flowering time and inflorescence development.

Genetic interaction between MtFDa, MtTFL1, and MtFULc during inflorescence development

We previously reported that MtTFL1 is crucial for maintaining the I₁ indeterminacy and MtFULc is required for I₂ identity (Cheng et al., 2018). Mutation of MtTFL1 led to the termination of the primary inflorescence, whereas mutation of MtFULc transformed I₂ into I₁-like vegetative shoots and...
the double mutant *mtfulc* *mttf1* produced simple flowers instead of compound inflorescences with determinate *I*₁ (Figure 1F; Supplemental Figure S2, E and F). To investigate how the flowering genes *MtFDa* and *MtFTa1* genetically interact with inflorescence identity genes *MtFULc* and *MtTFL1* during floral transition and inflorescence development, we performed a cross between *mtfulc mttfl1* and *mtfda* plants and obtained double mutants *mtfda mtfulc* and *mtfda mttfl1* and triple mutant *mtfda mtfulc mttfl1*. Flowering time and inflorescence development were examined in these mutants.

In *mtfda mttfl1* plants, the *I*₁ inflorescence showed normal indeterminate growth comparable to WT and *mtfda* instead of being terminated as compound flowers seen in *mttf1*; *I*₂ inflorescences developed similarly as *mtfda*, laterally forming single flowers and compound leaves (Figure 3, A–C; Supplemental Figure S2, G and H), and the apex arrested as flowers or floral buds after several floral nodes (Figure 3, D and E; Supplemental Figure S2I). The phenotype of the double mutant *mtfda mttfl1* is similar to that of the single mutant *mtfda*, indicating that *MtFDa* is epistatic to *MtTFL1* in regulating inflorescence development.

In *mtfda mtfulc* plants, the development of *I*₁ inflorescences remained unaffected as in single mutants *mtfda* and *mtfulc*, whereas the development of *I*₂ inflorescences was different from both single mutants. Unlike *mtfulc* plants wherein *I*₂ was transformed into *I*₁-like vegetative shoots without flower formation (Figure 1F), the *I*₂ of

![Figure 3](https://academic.oup.com/plphys/article/185/1/161/5985539)
MtFDa mtfulc plants was transformed into I1-like shoots, laterally producing compound inflorescences and compound leaves, and the apex remained indeterminate (Figure 3, F–H; Supplemental Figure S3, A and B). The compound inflorescence consisted of two compound leaves and two to three flowers (Figure 3I). Unlike mtfda plants that formed flowers at the third branch order, mtfda mtfulc plants developed flowers at a higher order, the fourth branch order. The further transformation of I2 to I1-like inflorescence shoots in mtfda mtfulc plants indicated that both MtFDa and MtFULc are required to ensure normal I2 development, and the flower formation indicates that MtFDa is epistatic to MtFULc in promoting flower development.

The triple mutant mtfda mtfulc mtfta1 exhibited similar inflorescence phenotypes as the double mutant mtfda mtfulc, i.e., indeterminate I1 and I2, and compound flowers at a higher order (Figure 3, J–M; Supplemental Figure S3, D–F). The results further indicate that MtFDa and MtFULc are essential for I1 identity and MtFDa is epistatic to MtTFL1 for I1 indeterminacy. The plants of mtfda mtfta1, mtfulc, and the triple mutant transitioned to the floral stage at a similar time as mtfda plants and some of the primary inflorescence shoots in these mutants produced flowers at late stages or no flowers, which is also similar to mtfulc (Supplemental Figure S3G). The mutation of MtFULc and/or MtTFL1 had no further effect on the floral transition of mtfda, which is in agreement with the previous report on the functions of MtFULc and MtTFL1. Taken together, we concluded that MtFDa plays a key role in floral transition, functions coordinately with MtFULc for I2 identification, and is epistatic to MtTFL1 for I1 indeterminacy.

**Changes of global gene expression during floral transition in mtfta1 and mtfda**

Mutations in MtFTa1 and MtFDa delay floral transition by different lengths of time; the double mutation of MtFTa1 and MtFDa shows additive effects and blocks the floral transition. The results imply differential functions of MtFTa1 and MtFDa in flowering-time regulation. To obtain insights into how MtFTa1 and MtFDa regulate flowering time mutually and differentially, we carried out gene expression profiling in these mutants between vegetative and floral stages by RNA sequencing. The shoot apices from WT-L, mtfta1, mtfulc, and mtfda mtfta1 plants, which were sampled from the same segregating F2 population grown in vernalization and long day conditions, were dissected at the vegetative stage and at the early floral stage, when flower buds were barely visible, for RNA isolation and subsequent RNA sequencing. There were four genotypes, two developmental stages, and three biological replicates for each sample. WT-L flowering plants were used as the control for the floral stage and mtfda mtfta1 plants were used as the control for the vegetative stage, and the samples of WT-L plants at the vegetative stage were not included (not sampled; Supplemental Table S1). Totally, 7,750 genes were differentially regulated across genotypes and developmental stages by pairwise comparisons with q-value < 0.05 (Supplemental Table S2). The data of the differentially expressed genes were uploaded to Web MeV (Multiple Experiment Viewer) for further analysis. Two-way ANOVA was used to analyze the effect of genotypes and developmental stages on gene expression with $p \leq 0.005$ (Supplemental Table S3A and B). From the analysis, we found that 1,158 genes were commonly regulated across genotypes upon floral transition, of which 657 genes were downregulated and 501 genes were upregulated (Supplemental Table S3A). Both up- and downregulated genes were loaded into MapMan software for functional characterization. Results showed that in the downregulated gene group, protein-related genes were highly represented (247 out of 657), most of which are involved in protein synthesis; genes in the RNA category were also highly represented, most of which are involved in RNA binding, processing and general regulation (Figure 5A). In the upregulated gene group, genes in various functional categories were more or less evenly represented. Most of the upregulated protein-related genes are involved in protein degradation and post-translation, and the upregulated genes in the RNA category were enriched by transcription factors, such as MADS, HLH, MYB, and WRKY (Figure 5B; Supplemental Table S3A). Furthermore, genes involved in the processes of metabolisms and development were also enriched in the upregulated gene cluster (Figure 5B; Supplemental Table S3A). The results indicate that the shoot apex experiences a switch into a very active status for transcription and metabolism along with floral transition.
Figure 4 Phenotypes of mtfta1 mtfulc and mtfta1 mttfl1 grown under VLD. (A), inflorescence of mtfta1 mtfulc, showing two lateral branches without flower formation. (B and C), inflorescences of mtfta1 mtfl1 (B) and MtFTa1/fta1 mtfl1 (C), showing terminated flowers. (D and E), inflorescences with pods in mtfta1 mtfl1 (E) and MtFTa1/fta1 mtfl1 (D), arrows show new flower position. (F) MtFTa1/fta1 mtfl1 plant is flowering. (G) mtfta1 mtfl1 plant flowers late.

Figure 5 Functional categorization of commonly differentially expressed genes from vegetative stage to floral stage under VLD condition in WT-L, mtfda mtfta1, mtfda, and mtfta1 by MapMan. (A) The downregulated gene categorization showing that protein-related genes (dark green) are highly over-present. (B) The upregulated gene categorization showing that genes in various functional categories were more or less evenly represented.
Expression change of flowering-time and floral-development genes in mfta1 and mtfda

From the global expression analysis, we were particularly interested in the differential expression of MADS-box genes, which are known for their extensive involvement in flowering and floral-development regulation, and other known floral-development genes (Supplemental Table S4A and B; Figure 6). There were 29 differentially regulated MADS-box genes, including flowering-promoting gene SOC1, flowering-repressing gene SVP-LIKE, inflorescence-development gene FUL, FM identity genes AP1 and CAL-LIKE, and floral homeotic genes SEP and AGL (Supplemental Table S4; Figures 6 and 7, B–D). While SVP genes and AGL19 were commonly downregulated, most of the other MADS genes were commonly upregulated in all genotypes, except that MtSOC1a and two AGL genes, AGL11 and AGL80, were downregulated in mtfda (Supplemental Table S4A; Figures 6, 7, G and H). In addition, the expression of FM developmental genes SGL1 and WUS were also increased upon floral transition in all genotypes (Supplemental Table S4A; Figures 6 and 7, F).

Furthermore, we examined known or potential flowering-time genes that may be involved in different genetic pathways. These include photoperiod pathway genes, plant age-related SQUAMOSA PROMOTER-BINDING-LIKE (SPL), gibberellin-related genes, vernalization-related FRIDIGA-like (FRI), carbohydrate status-related TREHALOSE-6-PHOSPHATE SYNTHASE (TPS), and FLOWERING-PROMOTTING FACTOR-LIKE (FPF; Supplemental Table S4, A and B; Figure 6). Upon floral transition, TPS and FPF were commonly downregulated in mtfta1, but upregulated in WT-L plants. Genes from the circadian and photoperiod pathways, including EARLY FLOWERING-LIKE 3 and 4 (EFL3 and 4), FKF, and CONSTANS-LIKE (COL), showed differential regulation between mtfta1 and WT-L. In mtfta1, except for EFL3 and one COL gene, other genes showed either a similar changing pattern as WT-L plants or no significant changes upon floral transition (Supplemental Table S4A; Figures 6 and 7, E). Most GA-related genes also showed opposite expression patterns between mtfta1 and WT-L plant; whereas most SPL genes (except SPL1b) were downregulated in mtfta1 but upregulated in mtfda, showing opposite regulation between mtfta1 and mtfda upon floral transition (Supplemental Table S4A; Figures 6 and 7, I). Only FRI genes were commonly upregulated upon floral transition in all three genotypes (Supplemental Table S4A; Figure 6).

Therefore, gene expression profiling revealed that the genes involved in FM and floral organ development are commonly regulated upon floral transition, and that mutation of MtfDa or MtFTa1 only delays the expression changes of the genes, suggesting that the floral developmental genes are not exclusively or directly regulated by MtfDa or MtFTa1. However, most of the flowering-time genes are differentially regulated between mtfta1, mtfda, and WT-L upon floral transition, especially in mtfta1. The results suggest that MtfDa and MtFTa1 have both common and distinct roles in different flowering pathways and that MtFTa1 has broader functions in flowering regulation. None of the floral-development genes were upregulated in the double mutant mtfda mtfta1 (Supplemental Tables S2–4; Figure 6), which is in agreement with the vegetative-growth phenotype, indicating that both MtfDa and MtFTa1 are required for the upregulation of inflorescence and FM identity genes.

Expression domains of MtTFL1, MtFULc, and MtAP1 in the inflorescence apices of mutants

As described above, MtfDa is expressed in shoot apical and AMs (Figure 2, G–I). RNAseq analysis showed that upregulation of the inflorescence and FM-related genes MtFULc, MtFULb, MtAP1/CAL, and SGL1 were delayed in both mtfda and mtfta1, though only mtfda displayed inflorescence architecture defects. To further understand the underlying mechanism of how MtfDa affects the inflorescence development, we compared the expression domains of inflorescence identity genes MtTFL1, MtFULc, and MtAP1 in shoot apices of mtfda to WT and/or mtfta1. MtTFL1 expression was increased upon floral transition and the expression domain was confined to the center of I1 meristems and AMs in WT and mtfta1 (Figure 8, A and B), but it was low and not increased upon floral transition and was undetectable in I1 meristems and AMs in mtfda (Figure 7, A and B; Supplemental Figure S4B). The results indicate that upregulation of MtTFL1 in I1 meristem may distinctly depend on the functionality of MtFDa.

MtFULc was specifically expressed in I2 meristems and inflorescence stems, but was absent in the initiated FMs in WT (Figure 8, E and F). In mtfda, MtFULc was also detected in I2 meristems and inflorescence stems as in WT (Figure 8, G and I). However, different from WT, the expression in mtfta1 was retained in I2 meristems and present in newly formed FMs and was detectable in floral primordia (Figure 8, H and I).

MtAP1 was detected in FMs, following which expression was detected in sepal and common primordia, and then confined to petals in WT (Figure 8, J and K). In mtfda, the expression pattern of MtAP1 was similar to that of WT (Figure 8, L; Supplemental Figure S4, C and D). Additionally, MtAP1 was also detected in lateral FMs formed from I2 (Figure 8, L and M; Supplemental Figure S4D), indicating the continuous formation of flowers on I2 shoots and the consistency with the inflorescence phenotype in mtfda.

In summary, mutation of MtfDa suppresses MtTFL1 expression in both I1 and AMs, which further supports the conclusion that MtFDa is epistatic to MtTFL1 in inflorescence development. Mutation of MtfDa does not change the expression patterns of MtFULc in I2 meristems and MtAP1 in FMs, but the retained MtFULc expression in I2 may postpone the termination of I2 meristems and lead to the formation of more flowers. The expression patterns of MtTFL1, MtFULc, and MtAP1 are consistent with the inflorescence phenotypes in mtfda. In mtfta1, the localization of MtFULc and MtAP1 is similar to that in WT.
Figure 6 Expression changes of selected flowering and development-related genes from vegetative to floral stages in mutants and WT-L plants. All plants are from the same F2 segregation population grown under VLD and genotyped. Genotypes: WT-L: MtFDa MtFTa1; fd: mtfda MtFTa1; ft: MtFDa mtfta1; fdft: mtfda mtfta1. Days 30, 63, and 90 are sampling days after planting. Vegetative stage: fd1, ft1, ft2, and fdft; floral stage: WT-L, fd2, and ft3. See Supplemental Table S4A for gene description. Color shows the expression level of individual transcript in FPKM transformed to log2; green to red color indicates expression levels from low to high.
Discussion

This study elucidated the essential functions of MtFDa in flowering time control and inflorescence development. Both MtFDa and MtFTa1 are essential for floral transition, playing mutual and specific roles in flowering regulation. The mutation of both MtFDa and MtFTa1 results in a complete block of floral transition. MtFTa1 has no significant roles in inflorescence development. Genetic analyses among MtFDa, MtTFL1, and MtFULc further demonstrated that MtFDa is essential for the development of secondary inflorescences and functions epistatically to MtTFL1 and MtFULc. A schematic working model was proposed to depict the effects of mutations of MtFDa, MtFTa1, MtFULc, and MtTFL1 on floral transition and IM development (Figure 9, A and B).

Both MtFDa and MtFTa1 are important for floral transition

MtFTa1 is a key flowering regulator in the long-day and vernalization pathways in M. truncatula (Laurie et al., 2011; Jaudal et al., 2013). The late-flowering phenotype and non-responsiveness to cold treatment of mtfta1-1 and mtfta1-2 in this study are in agreement with the exclusive role of MtFTa1 in the vernalization and photoperiod pathways and with the common function integrating several flowering pathways. FTs are upregulated by CO in the photoperiod pathway but repressed by FLOWERING LOCUS C (FLC) in the vernalization pathway in Arabidopsis (Baurle and Dean, 2006). In M. truncatula, MtCOL genes unlikely play a central role in the photoperiod pathway; FLC homolog is absent in M. truncatula and other temperate legumes; therefore, the direct upstream regulators of MtFTa1 are largely unknown (Wong et al., 2014; Weller and Macknight, 2018). However, its expression pattern (increasing with cold and long-day treatments and high in monofoliate leaf without diurnal change) and the strong flowering phenotype in mtfta1 indicate that MtFTa1 plays a more significant role in flowering regulation (Laurie et al., 2011; Jaudal et al., 2013; Thomson et al., 2019). RNAseq analysis in the current study did show that a broad range of flowering genes, from known targets SOC1 and FUL to genes in photoperiod, age-related, and GA-related pathways, are differentially regulated by the mutation of MtFTa1 upon floral transition, further confirming the essential role of MtFTa1 in flowering time regulation.
Figure 8 Gene expression at floral stage in WT and mutants by in situ hybridization. (A–D) MtTFL1 expression is detected at the center of l1 meristem and AM in WT (A), in l1 meristem of mtfal1 (B), but undetectable in mtfda (C), and not detectable in SAM in mtfda mtfal1 (D). (E and F) MtFULc expression is detected in l2 meristems and inflorescence stem in WT (E and F); (G–I) MtFULc expression in mtfal1, showing signal is detected in l1 meristem (G), FMs (H), and inflorescence stems (I). (J–M) MtAPI expression. (J and K), signal is detected in FM, sepal, and petal in WT; (L and M) signal is also detected in FMs, sepals, and petals in mtfda. Bar: 50 μm in B, D, F, G–H, K, and M; 100 μm in A, C, E, I, J, and L. FM FP, floral primordia; FW, flower.

Figure 9 (A) Illustration of effects of gene mutation on IM development. Yellow boxes indicate mutant genotypes. Red italicized font indicates the specified gene mutation. Different oval shapes filled with different colors indicate different genes. If an oval shape has the gene name, then it means this gene is expressed in this genotype at the specified meristem stage. Green oval indicates vegetative stage. Different darkness of orange oval indicates floral transitioned stage. (B) Genetic regulation of floral transition and inflorescence development. Red line shows inflorescence development in WT and mtfal1; Green line shows inflorescence development in mtfda; Blue line shows inflorescence development in mtfal1 and mtfal2; VM, vegetative meristem; I1, primary inflorescence; I1', I1-like inflorescence; I2, secondary inflorescence; I2', I2-like inflorescence.
FD is a mediator of FT signaling in promoting floral transition (Abe et al., 2005; Wigge et al., 2005; Collani et al., 2019). In mtfda, the late-flowering phenotype and the regulation of the common targets in flowering and inflorescence development, such as LFY, SOC1, and FULc, reveal that MtFDa shares the conserved function as a central player in flowering time control (Muszynski et al., 2006; Sussmilch et al., 2015; Collani et al., 2019; Romera-Branchat et al., 2020). The large amount of commonly regulated flowering genes in mtfda and mtf1a1 mutants upon floral transition suggests that MtFDa and MtF1Ta1 are interdependent regarding functionality. However, the fact that mtfda flowers earlier than mtf1a1 and retains responsiveness to vernalization implies distinctive functions for MtFDa and MtF1Ta1. Additionally, there were more differentially expressed genes in mtf1a1 than in mtfda during floral transition, suggesting that MtF1Ta1 has broader functions in flowering time regulation than MtFDa. The differential regulation of SOC1a and SPL genes in mtfda and GA-related and photoperiod pathway genes in mtf1a1 supports the independent functions of MtFDa and MtF1Ta1 during floral transition. Furthermore, the complete block in floral transition by double mutation of MtFDa and MtF1Ta1 indicates that MtFDa and MtF1Ta1 have complementary roles in floral transition.

Flowering signals can be initiated from plant age, photoperiod, vernalization, and hormones, and regulated by a large amount of genes in an intricate genetic network that converge to regulate a small number of floral integrator genes. Individual mutants only affect flowering time quantitatively by either accelerating or delaying flowering. The model that FT interacts with FD to regulate floral genes is conserved in several species (Abe et al., 2005; Wigge et al., 2005; Taoka et al., 2011; Tsuji et al., 2013b; Sussmilch et al., 2015). Both FT and FD belong to gene families with other members. The number of FT or FD homologs varies in different plant species, and the functions of individual FT or FD genes and their essentiality in controlling flowering have diverged across different species (Laurie et al., 2011; Tsuji et al., 2013a; Tylewicz et al., 2015). TWIN SISTER OF FT (TSF) and FD PARALOG (FDP) are the closely related genes of FT and FD, respectively, in Arabidopsis; however, these genes play minor roles in flowering regulation (Yamaguchi et al., 2005; Romera-Branchat et al., 2020). The FT/TSF floral signal is largely transduced through association with FD/FDP. The mutants of ft tsf, fd fdp, and ft tsf fd only delay flowering, indicating that no FT or FD gene is absolutely crucial and the FT/FD module is just one of the flowering signal modules in Arabidopsis (Jang et al., 2009; Jaeger et al., 2013). In rice, 2 out of 13 FT-LIKE genes, namely Hda3 and RFT1, are essential for flowering, and the FD genes OsFD1 and OsFD2, respectively, promote floral transition and affect leaf development (Komiyama et al., 2008; Tsuji et al., 2013a). There are six and five FT genes in M. truncatula and pea, respectively. Each FT gene shows a different expression pattern and responsiveness to environmental signals (Hecht et al., 2005, 2011; Weller and Macknight, 2018). Whereas MtF1Ta1 plays a key role in vernalization and long-day flowering pathways, mutation of PsFTA/GIGAS does not delay floral transition under LD and vernalization conditions but with no flower formation, suggesting that other FT genes, such as PsFTb, may play a key role in flowering (Hecht et al., 2011; Laurie et al., 2011; Putterill et al., 2013). PsFDa/VEG2 is the only ortholog of FD in pea, and it interacts with all five PsFT proteins. The mutant veg2 shows a strong late-flowering phenotype, indicating its essential role in flowering time control (Sussmilch et al., 2015). In M. truncatula, there are two FD genes, MtFDa and MtFDb (Sussmilch et al., 2015). As described above, mtfda flowers late, whereas mtfdb flowers normally. Despite the multiple family members and potential functional diversification of MtFT and MtFD, the complete blocking of floral transition in the double mutant mtfda mtf1a1 suggests that MtFDa and MtF1Ta1 are the key players for floral transition in the MtFD and MtFT families, indicating that flowering signals pass through either the MtFDa–MtFTs or the MtF1Ta1–MtFDs module. Therefore, compared to other model species, the function of FD and FT has diverged among the family members in legumes; MtFDa and MtF1Ta1 have acquired broader functions in controlling flowering and are the central switches for floral transition in M. truncatula.

**MtFDa plays a primary role in controlling the inflorescence architecture**

In addition to the conserved function in promoting flowering, MtFDa plays an essential role in secondary inflorescence development. Mutant mtfda flowers late and transforms l2 into l1-like meristem, which is similar to the mutant veg2-2, a weak allele of PsFDa mutations in pea. The null mutant veg2-1 delays floral transition and further prevents flower formation (Sussmilch et al., 2015). In mutant mtfda, some inflorescence shoots also do not produce flowers. MtFD has another family member, MtFDb, which has no corresponding ortholog in pea (Sussmilch et al., 2015). The difference of the phenotype severity between mtfda and veg2-1 may be caused by functional redundancy of MtFDa and MtFDb, though the Tnt1 insertion mutant mtfdb does not show noticeable phenotypes in either flowering time or inflorescence development under long-day conditions. The functions of individual FD genes are diverged in different species and range between the conserved flowering time regulation, ABA responses in Arabidopsis seedlings, leaf development in rice, and seasonal growth control in poplar (Abe et al., 2005; Jaeger et al., 2013; Tsuji et al., 2013a; Tylewicz et al., 2015; Romera-Branchat et al., 2020). Except for the pleiotropic effects on inflorescence development of delayed flowering1 in maize (Zea mays), no other inflorescence defects have been described in other species (Muszynski et al., 2006). Therefore, in addition to the conserved function in flowering time regulation, FD genes in legumes have acquired a new function in controlling secondary inflorescence development.
The reciprocal repression models of TFL1, LFY, and AP1 in simple racemose, or TFL1, FULc, and AP1/LFY in compound racemose, address the fundamental mechanisms in maintaining IM indeterminacy and lateral flower differentiation during inflorescence development (Liljegren et al., 1999; Berbel et al., 2012; Benlloch et al., 2015; Cheng et al., 2018).

However, the ab normal inflorescence architectures in mutants veg2 and mtfda indicate the essential function of MtFDa/PsFDa in compound inflorescence regulation in legume species (Sussmilch et al., 2015). The prevailing phenotype of mtfda in double mutants mtfda mtftf1 and mtfda mtftfc or in the triple mutant mtfda mtftfc mtftf1 further demonstrates the key roles of MtFDa in both primary and secondary IM identity and in regulating downstream components of inflorescence development. When the MtFDa function is lost, the MtTFL1 expression is not detectable in inflorescence apices, indicating that MtFDa is required for the upregulation of MtTFL1 upon floral transition, or that MtFDa directly or indirectly regulates the MtTFL1 expression in apical meristems. In a flowering regulatory network model, it is proposed that FT/FD should upregulate TFL1 expression (Jaeger et al., 2013). Since FD is expressed at both vegetative and reproductive stages, upregulation of TFL1 by FD upon floral transition may be achieved through FD interacting with gradually increased FT proteins. In M. truncatula, the MtTFL1 expression is only suppressed in mtfda, but not in mtfta1. Therefore, it is unlikely that up-regulation of MtTFL1 is achieved through the association of MtFDa with MtFTa1. There are another five members in the MtFT family. MtFTc is induced upon floral transition and highly expressed in flower buds, but the mutant mtfc shows neither later-flowering nor defective-inflorescence phenotypes (Laurie et al., 2011). Therefore, MtFTc may not be the interaction partner of MtFDa, or at least not the only one, in regulating the expression of MtTFL1. In the inflorescence development of pea, it is proposed that VEG2/PsFDa is associated with PsFTb2 to regulate DET/PsTFL1 expression (Sussmilch et al., 2015). However, DET/PsTFL1, as the marker of floral transition, is still induced upon floral transition in veg2, though the induction is delayed (Sussmilch et al., 2015). The difference of MtTFL1 and DET expression in mutants of mtfda and veg2 indicates the regulation complexity among FDs, FTs, and TFL1 in different legumes.

In addition to TFL1, other genes, such as AP1, CAL, and FUL, are also direct targets of the FT/FD complex in floral transition (Abe et al., 2005; Wigge et al., 2005; Jaeger et al., 2013). The delayed upregulation of MtAP1, MtCAL, and MtFUL in mutants mtfda and mtfta1 is consistent with the conserved functions of MtFDa and MtFTa1. The reciprocal repression model of TFL1, FULc, and AP1 elegantly explains the genetic regulation of compound inflorescence development in pea, and the model is further verified in M. truncatula as well (Jaeger et al., 2013; Benlloch et al., 2015; Cheng et al., 2018). In mtfda, even though MtTFL1 is not upregulated in I1 meristems, MtFULc and MtAP1 are still confined in the original expression domains of I2 and FMs, respectively, but not expanded to I1 meristem. The expression analysis in the current study showed that SPL genes are differentially regulated in mtfda. It has been reported that FD upregulates the expression of SPL3/4/5 by direct binding to their promoters. Furthermore, the binding of SPL3/4/5 to the FM identity genes facilitates the DNA binding of the FT/FD complex to promote flowering (Formara and Coupland, 2009; Wang et al., 2009; Yamaguchi et al., 2009; Jung et al., 2012, 2016). Taken together, we propose that when MtFDa is functional, MtFDa associates with MtFTa1 to promote the vegetative growth to I1 transition of SAM. Meanwhile, MtFDa associates with MtFTb/MtFTc to upregulate the floret development genes, including MtTFL1 and MtSPL in SAMs to promote the I2 to I3/FM transition. When MtFDa is not functional, the upregulation of IM genes is aborted, therefore, I1 partially loses the capacity to form normal I2 or I3, is transformed into I1-like. Analysis of the double mutant mtfda mtfd will further confirm the hypothesis, though a similar model has been proposed in pea and supported by nonflowering phenotypes in mutants veg2/pstfda and GIGAS/pfsta (Benlloch et al., 2015; Sussmilch et al., 2015).

MtFTa1 has no significant roles in inflorescence architecture development and the primary inflorescence indeterminacy by MtTFL1 is dependent on MtFDa

Both FT and TFL1 encode closely related PEBPs. Whereas FT promotes flowering as a universal florigen, TFL1 represses flowering as an anti-florigen and regulates inflorescence development via formation of competitive or antagonistic transcription complexes with FD (Ahn et al., 2006; Jaeger et al., 2013). Recent reports have shown that TFL1 is recruited to chromatin by FD and upregulation of FT competes TFL1 from chromatin-bound FD, whereas FT enhances FD genome-wide DNA binding (Collani et al., 2019; Zhu et al., 2020). The balance between florigen and anti-florigen, such as FT and TFL1 in Arabidopsis, SFT/SP in tomato, and Hd3a/RCN1 in rice, regulates the fate, growth, or termination of the SAM (Kobayashi et al., 1999; Jaeger et al., 2013; Lifschitz et al., 2014; Kaneko-Suzuki et al., 2018). In Arabidopsis, ft mutants flower late, whereas FT-overexpressing plants flower early with terminal flowers, which are similar to tfl1. The double mutant ft tfl1 shows some additive phenotypes, i.e. intermediate late flowering and determinate inflorescences, indicating that in addition to the antagonistic roles in floral transition, FT and TFL1 also have unique functions (Kardailsky et al., 1999; Kobayashi et al., 1999; Hanzawa et al., 2005). Mutation of SP in tomato does not affect the floral transition of primary meristems. Instead, it only affects the sympodial unit growth, gradually producing a few compound leaves and terminated with consecutive inflorescence (Krieger et al., 2010; Lifschitz et al., 2014). There
are three TFL1 orthologs in pea, DET/PsTFL1a, PsTFL1b, and LATE FLOWERING (LF)/PsTFL1c. LF/PsTFL1c controls floral transition, whereas DET/PsTFL1a maintains indeterminacy of apical meristems, indicating divergent functions of PsTFL1s in two distinct developmental processes (Foucher et al., 2003). MtTFL1 is the ortholog of PsTFL1a with similar functions. Mutant mttfl1 has normal flowering timing and shows gradual reduction of compound leaves, internodes, and pedicels until termination of the primary IM (Cheng et al., 2018). The double mutant mtfta1 mttfl1 only shows additive phenotypes of late flowering and termination of 1, meristems. MtFTa1 overexpression does not lead to the termination of primary IM (Putterill, personal communication). Therefore, the antagonistic function between FT and TFL1 may be shared across the family members. Further genetic and molecular dissections are necessary to fully understand the functions of individual member genes in these two families.

Materials and methods

Plant materials and growth

Tnt1 insertion lines for MtFDa (NF11119) and MtFTA1 (NF11200 and NF11802) were identified from a forward screen carried out at the Noble Research Institute. PCR-based reverse screening for additional insertion lines (NF9972 and NF10125) for MtFDa was preceded as previously described (Cheng et al., 2014). To obtain the double mutant mtfta1 mttfl1, mtfta (NF11119) was cross-pollinated with mtfta1 (NF11200); to obtain the double mutants mtfta mttfl1, mtfta1 mttfl1, and mttfl1 mtfta1, and the triple mutant mtfta1 mttfl1 mtfta1, heterozygous mtFDa/ fda mttfl1 was cross-pollinated with mtfta1 mttfl1 from our previous study (Cheng et al., 2018). The mutants from the F2 segregation population were confirmed by PCR genotyping using different primer combinations (Supplemental Table S5). The expression of MtFTA1 and/or MtFDa in the single mutants mtfta and mtfta1 and the double mutant mtfta1 mttfl1 was confirmed by RT–PCR (Supplemental Figure S6).

Seeds of M. truncatula WT R108 and Tnt1 insertion mutants were scarified with concentrated sulfuric acid for eight minutes, rinsed with water, and put on filter paper for 10 d at 4°C. Germinated seeds were transferred into one-gallon pots with Metro-Mix 350 (Scotts) composite soil and grown under conditions of 16 h/8 h day/night light cycle, 150 μE m⁻² s⁻¹ light intensity, 22°C/18°C day/night temperature, and 70% humidity.

RNA extraction, RT–PCR, and RT-qPCR

Vegetative and inflorescence shoots were collected at ZT 5–6 (11:00–12:00 a.m.) from WT and mutant plants. Total RNA was extracted using the Tri-Reagent (Gibco-BRL Life Technologies) and treated with Turbo DNase I (Ambion). For RT–PCR and RT-qPCR, 3 μg of total RNA was used for reverse transcription using the SuperScript III Reverse Transcriptase (Invitrogen) with the oligo (dT)₂⁰ primer. Two microliters of 1:20 diluted cDNA was used as templates. Three biological replicates for each sample and three technical replicates for each biological replicate were used. RT-qPCR was carried out using a 7900HT Fast Real-time PCR System (Applied Biosystems), and the data were analyzed using SDS 2.2.1 software (Applied Biosystems). The transcript levels were determined by relative quantification using the M. truncatula ubiquitin (MtUBQ) gene as the internal reference. Gene-specific primers used for RT–PCR and RT-qPCR are listed in Supplemental Table S5.

RNA-seq analysis

Shoot apices from mtfta1 MtFTA1, MtFDa mtfta1, and WT-L plants, which were genotyped from the same F2 population, were sampled at approximately ZT 6 in the following date points: (1) Day 30 when floral buds were visible in WT-L plants for samples WT-L, fd1, and ft1; (2) Day 63 when floral buds were visible in mtfta plants for samples fdtt, fd2, and ft2; (3) Day 90 when floral buds were visible in mtfta1 plants for sample ft3. Three to four shoot apices were collected from each plant as one sample, and three biological replicates were used for each treatment (Supplemental Table S1). Total RNA was isolated from shoot apex samples using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich), and the residual genomic DNA was removed from RNA samples using Turbo DNase I (Thermo Fisher Scientific) and then purified using the Qiagen MinElute Kit (Qiagen). RNA-seq libraries were prepared using TruSeq Stranded mRNA Sample Preparation kits (Illumina). Briefly, mRNA was purified from 1 μg of total RNA, fragmented and converted to double-stranded DNA for sequencing. Individual libraries were uniquely indexed using TruSeq Single Indexes (Illumina), and pooled in equal molar ratio. The pooled libraries were sequenced on a NextSeq 500 Sequencing system (Illumina).

The raw RNA-seq reads were first trimmed for quality using a custom Perl script that removed bases from the end of each read until two consecutive bases with quality scores greater than 30 were found. Any read less than 30 bp long after trimming was discarded, along with its mate. The trimmed reads were then mapped to the M. truncatula genome version 4.0 using TopHat version 2.1.1 (http://ccb.jhu.edu/software/tophat/index.shtml). The mapped reads were then assembled into transcripts and quantified using Cufflinks version 2.2.1 (http://cole-trapnell-lab.github.io/cufflinks/). Differential expression testing was performed with Cuffdiff, with q-value (adjusted p-value) < 0.05, which is part of the Cufflinks software package. The SRA metadata was deposited to National Center for Biotechnology Information (NCBI) under the accession number PRJNA649443.

In situ hybridization

Inflorescence shoot apices from WT and mutant plants were sampled for in situ hybridization. Sample fixation/dehydration/paraffin embedding in tissue preparation was performed according to Long’s protocol (http://www.its.cal
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