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RESEARCH ARTICLE

Dissection of genetic regulation of compound inflorescence development in *Medicago truncatula*

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ABSTRACT

Development of inflorescence architecture is controlled by genetic regulatory networks. *TERMINAL FLOWER1* (*TFL1*), *APETALA1* (*AP1*), *LEAFY* (*LFY*) and *FRUITFULL* (*FUL*) are core regulators for inflorescence development. To understand the regulation of compound inflorescence development, we characterized mutants of corresponding orthologous genes, *MtTFL1*, *MtAP1*, *SINGLE LEAFLET1* (*SGL1*) and *MtFULc*, in *Medicago truncatula*, and analyzed expression patterns of these genes. Results indicate that *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* play specific roles in identity determination of primary inflorescence meristems, secondary inflorescence meristems, floral meristems and common primordia, respectively. Double mutation of *MtTFL1* and *MtFULc* transforms compound inflorescences to simple flowers, whereas single mutation of *MtTFL1* changes the inflorescence branching pattern from monopodial to sympodial. Double mutant *mtap1sgl1* completely loses floral meristem identity. We conclude that inflorescence architecture in *M. truncatula* is controlled by spatiotemporal expression of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* through reciprocal repression. Although this regulatory network shares similarity with the pea model, it has specificity in regulating inflorescence architecture in *M. truncatula*. This study establishes *M. truncatula* as an excellent genetic model for understanding compound inflorescence development in related legume crops.

KEY WORDS: Development, Inflorescence, *Medicago truncatula*, Genetic regulation, Legume

INTRODUCTION

During vegetative growth, the plant shoot apical meristem (SAM) initiates leaf primordia and axillary shoot meristems on meristem flanks. Upon sensing inducible environmental and/or developmental cues, plants transition from vegetative to reproductive growth stages and the SAM transforms into an inflorescence meristem. Depending on inflorescence meristem activities of when and where floral meristems are initiated, a wide variety of inflorescence structures arise in the plant kingdom. Based on the determinacy of primary inflorescence meristem (I_1), two types of inflorescences are defined: (1) determinate inflorescence, in which I_1 meristem terminates as a floral meristem or secondary inflorescence meristem (I_2); and (2) indeterminate inflorescence, in which I_1 indefinitely grows to produce lateral floral meristems or I_2. Based on inflorescence architectures, three major types of inflorescences are defined: raceme, cyme and panicle (Prusinkiewicz et al., 2007; Fig. S1). I_2 activities further enrich the complexity of inflorescence architectures by reiterating I_1, leading to compound inflorescences, which evolved from simple inflorescences (Stebbins, 1973; Coen and Nugent, 1994; Ma, 1998; Park et al., 2014; Teo et al., 2014).

Plant inflorescence architectures are essential for reproductive success in nature and crop productivity in agriculture. The development of inflorescence architectures is tightly controlled by genetic regulatory networks. Prusinkiewicz et al. postulated a transient model, in which the newly formed meristem is in a transient status (immature) and obtains its identity (mature) to form a flower or a branch depending on its vegetative level (Prusinkiewicz et al., 2007). The model can address the evolution and development of different inflorescence architectures, though the key regulators are different among raceme, cyme and panicle inflorescences (Prusinkiewicz et al., 2007; Lippman et al., 2008; Thompson and Hake, 2009; Park et al., 2014).

In the model species *Arabidopsis* and *Antirrhinum*, simple raceme inflorescences are developed. *TERMINAL FLOWER1* (*TFL1*)/*CENTRORADIALIS* (*CEN*), *LEAFY* (*LFY*)/*FLORICAULA* (*FLO*) and *APETALA1* (*AP1*)/*SQUAMOSA* are key players in inflorescence development (Ratcliffe et al., 1998; Liljegren et al., 1999; Davies et al., 2006; Denay et al., 2017). *Arabidopsis TFL1* is closely related to the florigen gene *FLOWERING LOCUS T* and is upregulated upon floral transition in the apical meristem to repress the expression of floral genes, therefore maintaining the indeterminacy of apical inflorescence meristem. Mutation of *TFL1* transforms an indeterminate inflorescence apical meristem to a determinate floral meristem (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1996, 1997; Blazquez et al., 1997). *AP1* and *LFY* are floral meristem identity genes and play synergistic roles in maintaining floral meristem identity (Irish and Sussex, 1990; Huijser et al., 1992; Mandel et al., 1992; Gustafson-Brown et al., 1994; Moyroud et al., 2009, 2010). Double mutation of *LFY* and *AP1* enhances inflorescence phenotypes, developing inflorescence shoots instead of flowers in *Arabidopsis* (Huala and Sussex, 1992; Weigel et al., 1992).

Based on extensive genetic and molecular studies in *Arabidopsis*, a genetic regulatory model of inflorescence development has been proposed. In this model, *TFL1* is expressed in the center of apical meristems and TFL1 moves to outer layers of apical meristems to repress the expression of floral meristem identity genes *AP1* and *LFY*, thereby maintaining the indeterminacy of apical meristems (Pidkowich et al., 1999; Ratcliffe et al., 1999; Wagner et al., 1999; Hempel et al., 2000; Blázquez et al., 2006; Conti and Bradley, 2007). This model defines the development of simple raceme inflorescences.

Some legume species are characterized by having a compound type of inflorescence development. In these species, I_1 laterally produces I_2 meristems and each I_2 meristem further develops into one to three lateral flowers before it terminates as a stub (Singer et al., 1999; Benlloch et al., 2003). *PROFERATING*

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INFLORESCENCE MERISTEM (*PIM*) and *UNIFOLIATA* (*UNI*)/*SINGLE LEAFLET1* (*SGL1*) are orthologs of *AP1* and *LFY*, respectively, in legumes. *PIM* has conserved functions in floral meristem identification. The *pim* mutants exhibit both higher-order secondary inflorescences and abnormal flowers in pea and *M. truncatula* (Berbel et al., 2001; Taylor et al., 2002; Benlloch et al., 2006). *UNI*/*SGL1* plays a specific role in compound leaf regulation, in addition to its conserved function in flower development. The *uni/sgl1* mutants transform compound leaves into simple leaves and produce cauliflower-like floral structures (Hofer et al., 1997; Wang et al., 2008). There are three *TFL1* orthologs in pea: *DETERMINATE(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 different *TFL1* orthologs in two distinct developmental processes in pea (Foucher et al., 2003).

By studying the non-flowering mutant *vegetative1* (*veg1*) in pea, Berbel et al. demonstrated that *PsFULc* is crucial for secondary inflorescence development (Berbel et al., 2012). Therefore, they proposed a modified model in which *DET*/*PsTFL1a* maintains I_1 indeterminacy and *PIM/AP1* defines the floral meristem identity, whereas *VEG1*/*PsFULc* specifies I_2 identity, a new regulatory layer added between *DET* and *PIM* to accommodate compound inflorescence development (Benlloch et al., 2007, 2015).

Medicago truncatula is a model legume species with compound leaves and compound inflorescences like pea. Upon floral transition, the shoot apical meristem transforms into I_1. In addition to continuously producing vegetative organs, the I_1 meristem laterally initiates I_2, which produces one bract, one to three flowers and a spike with an elongated petiole. Each floral meristem sequentially develops five sepals and four common primordia, which further differentiate into five petals, 10 stamens and one carpel (Benlloch et al., 2003). By taking advantage of *Tnt1* mutant population in *M. truncatula*, we obtained *Tnt1* insertion mutants for *MtTFL1*, *MtFULc*, *SGL1* and *MtAP1*. By characterizing mutant phenotypes and analyzing gene expression, we established the genetic relationship of the four genes in regulating compound inflorescence development in *M. truncatula*.

RESULTS

Isolation and characterization of inflorescence mutants *mttfl1* and *mtfulc*

MtTFL1 (Medtr7g104460), which shares 94% identity in amino acids with PsTFL1a, was retrieved from the *M. truncatula* genome database as the *TFL1* orthologous gene, with three other *TFL1* homologous genes. Three insertion lines of *MtTFL1* were identified from the *Tnt1* insertion population by BLAST searching the flanking sequence tag (FST) database (Fig. S2A). Mutant *mttfl1* plants showed no defects during vegetative growth and no difference with wild-type plants in flowering time (Table S1). After floral transition, *mttfl1* mutants developed one or two wild-type-like inflorescences, followed by one or two defective inflorescences with shortened pedicels, and then the I_1 meristem abruptly terminated as abnormal flowers subtended with or without defective leaves (Fig. 1A-D). The wild-type-like inflorescences consisted of one to two normal fertile flowers, whereas the defective and terminated inflorescences usually produced abnormal and sterile flowers with short pedicles, loose floral structures, and reduced numbers of petals and stamens (Fig. S2C,D). After termination of the apical meristem, the upmost axillary meristem reiterated the I_1 developmental pattern. Lost dominancy of the primary shoot changed the growth pattern of inflorescence shoots from monopodial to sympodial, resulting in extensively branched *mttfl1* plants.

MtFULc (Medtr7g016630) was identified in the *Medicago* genome database as the ortholog of *VEGETATIVE 1* (*VEG1*)/*PsFULc* in pea, along with the homologous genes *MtFULa* and *MtFULb* (Berbel et al., 2012; Jaudal et al., 2015). By searching the *Tnt1* FST database, we obtained one line, *mtfulc-1*, with a *Tnt1* insertion in exon 2, and two lines, *mtfulc-2* and *mtfulc-3*, with *Tnt1* insertions in intron 4 and 5, respectively (Fig. S2B). At the vegetative stage, *mtfulc-1* plants showed no morphological difference with heterozygous or wild-type plants (Fig. 1E). However, when heterozygous or wild-type plants transitioned to the floral developmental stage, *mtfulc-1* mutants continued vegetative growth and produced no flowers. Upon close examination, a small bud was observed in the axillary position beside the outgrowing lateral shoot (Fig. 1F), indicating that the floral transition does occur in *mtfulc-1* mutants. Instead of producing one secondary inflorescence and one lateral vegetative branch at the axillary position as in wild type, *mtfulc-1* mutants developed two vegetative shoots, which became more apparent after two to three node development (Fig. 1G,H; Fig. S2E). The *mtfulc-1* mutants remained vegetative, showed no sign of senescence and never produced flowers, resulting in greater biomass production (Fig. S2F). These data indicated that *MtFULc* is required for the I_2 identity. In contrast to *mtfulc-1*, *mtfulc-2* and *mtfulc-3* did not show any noticeable phenotypes (data not shown).

Double mutant *mttfl1 mtfulc* changes compound inflorescences into simple flowers

As described above, mutation of *MtTFL1* leads to determination of the primary inflorescence, whereas mutation of *MtFULc* blocks the floral development of secondary inflorescence, indicating significant roles of *MtTFL1* and *MtFULc* in I_1 and I_2 meristem identity, respectively. To understand the genetic regulation of *MtTFL1* and *MtFULc* during inflorescence development, we generated the *mttfl1 mtfulc* double mutant by crossing *mttfl1-3* with *MtFULc/mtfulc-1*. The double mutant and single mutant *mttfl1* plants developed flowers at a similar time. Instead of developing compound inflorescences, which consist of one to two flowers, one bract and one spike in wild type, the double mutant developed single flowers with short pedicles in leaf axils (Fig. 1I-K, Fig. S2G). After flower formation at two to three nodes, the I_1 meristem abruptly terminated and developed a single flower without bract and spike subtended (Fig. 1J, Fig. S2H). Some double mutant flowers developed into pods with very short pedicles (Fig. 1L), which were in contrast to the long pedicle pods in *mttfl1* and wild-type plants (Fig. 1M). Similar to the single mutant *mttfl1*, the shoot growth pattern in the double mutant changed from monopodial to sympodial after apical meristem termination (Fig. 1N,O). These results indicated that simultaneous loss of *MtTFL1* and *MtFULc* transforms compound inflorescences into simple flowers, indicating that *MtFULc* is crucial for I_2 meristem development, and that *MtTFL1* is epistatic to *MtFULc* in the regulation of inflorescence development.

Mutants *mtap1*, *sgl1* and *mtap1 sgl1* lose floral meristem identity

It has been reported that mutations of *MtAP1* (Medtr8g066260, *MtPIM*) and *SGL1* (Medtr3g098560) affect floral development in *M. truncatula* (Hofer et al., 1997; Benlloch et al., 2006; Wang et al., 2008). One mutant line (NF11426) with abnormal inflorescences was identified from a forward screen of the *Tnt1* insertion

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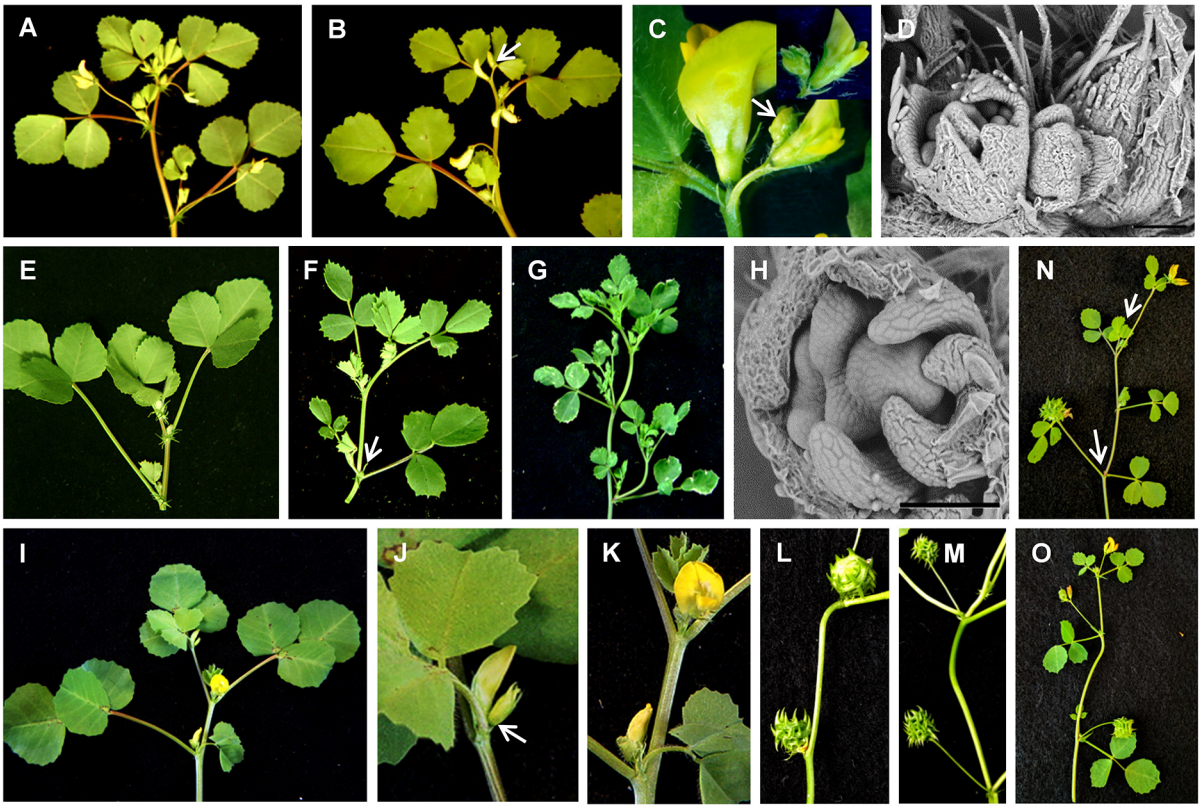

Fig. 1. Phenotypes of the *mttfl1*, *mtfulc* and *mttfl1 mtfulc* mutants. (A) Inflorescence shoot of wild type. (B-D) *mttfl1*. (B) A shoot apex terminated as a one-flower inflorescence (arrow) with a two-leaflet leaf. The first inflorescence is normal; the second and third are defective with short pedicles. (C) Terminated shoot apex with a two-flower inflorescence; one flower is smaller and abnormal (arrow); inset provides a magnified view of the inflorescence, showing the bract. (D) SEM of the terminated apex with two developing flowers. (E) Vegetative shoot of wild type, showing one axillary branch at each node. (F-H) *mtfulc*. (F) A shoot with one axillary branch and one very small bud (arrow) at each node. (G) A shoot with two outgrowing axillary branches at each node at advanced stages. (H) SEM of the *mtfulc* shoot apex, showing vegetative-like status without flower formation. (I-L,N) *mttfl1 mtfulc*. (I) Inflorescence shoot, showing that the shoot apex terminates as a single flower after the formation of three single flowers. (J) Magnified view of the determinate flower (arrow). (K) Magnified view of simple flowers with short pedicels at each node. (L) Single pods with short pedicels formed. (M) Pods with long pedicels in *mttfl1*. (N) *mttfl1 mtfulc* shoot growth pattern, showing axillary shoot growth (arrows) after primary apex determination. (O) Wild-type shoot. Scale bars: 100 µm.

population. Genetic analysis showed that the phenotype was caused by a *Tnt1* insertion in exon 2 (at 3538 bp from ATG) of *MtAP1*. Mutation of *MtAP1* had no effects on vegetative growth and primary inflorescence development but did affect floral development (Fig. S3A). Instead of producing one or two flowers as in wild type, the *mtap1* mutant developed proliferating mixtures of abnormal flowers, vegetative shoots or leaf-like structures at the end of pedicels (Fig. 2A,B). At the early flowering stage, *mtap1* developed vegetative shoots instead of flowers (Fig. S3B). Gradually, more meristems gained floral identity and produced abnormal flowers with fused sepals and petals, and fewer stamens (Fig. S3C,D). At late stages, some flowers produced fertile pods (Fig. 2C, Fig. S3E). Scanning electron microscopy (SEM) showed that I_2 meristems in *mtap1* did not acquire floral meristem identity for further floral organ development. Instead, the meristems reiterated bracts and I_2-like meristems and spikes (Fig. 2F,J).

Mutant *sgl1* exhibited simple leaves and cauliflower-like structures consisting of reiterated sepals and carpels (Fig. 2B). Close-up observation of the mutant revealed that its I_2 meristems produced normal-appearing floral meristems that initiate sepal primordia, common primordia and carpel primordium (Fig. 2G). The sepal and carpel primordia developed into sepals and carpel-like structures. The common primordia, however, did not differentiate into petal and stamen primordia; instead, they acted as floral meristems to reiterate floral primordium initiation, resulting in cauliflower-like structures (Fig. 2K, Fig. S3F). Normal flowers were never observed on *sgl1* plants.

It has been reported that *LFY* plays a key role in floral meristem identity but *mtap1* only partially loses floral meristem identity. To further elucidate the function of *SGL1* in floral development, we generated the double mutant *mtap1 sgl1* by crossing heterozygous plants of *MtAP1* and *SGL1*. The double mutant showed additive morphological phenotypes of *mtap1* and *sgl1*, with simple leaves and branched secondary inflorescences (Fig. 2B,D,E). SEM observation revealed that I_2 meristems in the double mutant gave rise to bracts and floral meristems, and the floral meristems repeated the formation of bracts and meristems. No floral organs were observed (Fig. 2H,L; Fig. S3G). These results indicated that the double mutant completely loses floral meristem identity and the floral meristems act as I_2 meristems.

In summary, mutations of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* affected the identity acquisition of I_1 meristem, I_2 meristem, floral meristem and common primordia, respectively. *SGL1* played a synergistic role with *MtAP1* in floral meristem identity.

Expression and localization of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1*

To better dissect genetic regulation of inflorescence development genes, we analyzed the temporal and spatial expression patterns of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* using quantitative real-time

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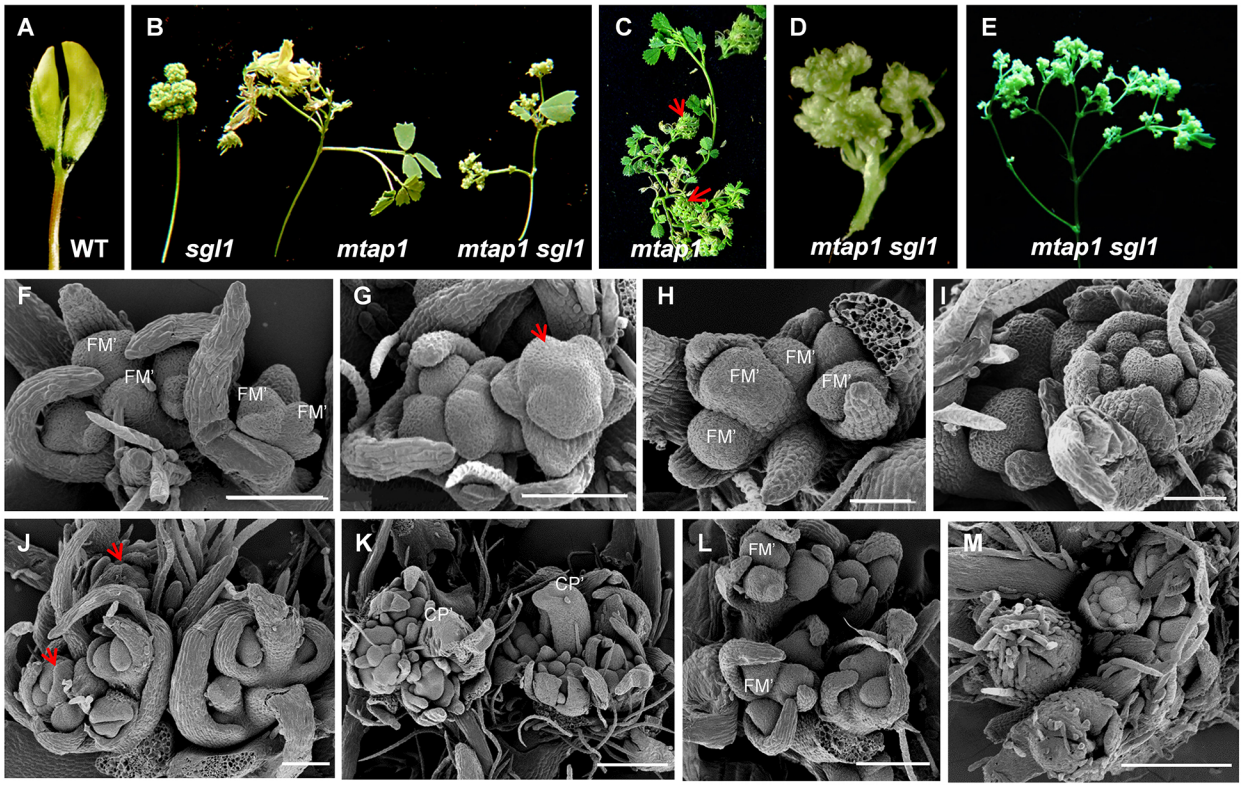

Fig. 2. Inflorescence phenotypes of *mtap1*, *sgl1* and *mtap1 sgl1*. (A) A two-flower inflorescence in wild type. (B) Proliferating inflorescences in the three mutants, showing a vegetative shoot conversion in *mtap1* and leaf-like conversion in *mtap1 sgl1*. (C) One inflorescence at late stages in *mtap1*, showing two pods (arrows). (D,E) Proliferating secondary inflorescences in *mtap1 sgl1*. (F,J) *mtap1*. (F) A secondary inflorescence with two defective florets, showing proliferating floral meristems. (J) A secondary inflorescence at the late stage, showing developing abnormal flowers (arrows). (G,K) *sgl1*. (G) Primary inflorescence apex, showing one floral primordium with developing sepals, common primordia and center carpel primordium (arrow). (K) Secondary inflorescence with two clusters of florets, each with center carpel surrounded by reiterated differentiating florets. (H,L) *mtap1 sgl1*. (H) A secondary inflorescence, showing proliferating floral meristems. (L) A secondary inflorescence at late stage. (I,M) Primary inflorescence apices in wild type. FM′, proliferating floral meristems; CP′, carpel. Scale bars: 100 µm in F-H,K,M; 50 µm in I and L; 200 µm in J.

PCR (qRT-PCR) and *in situ* hybridization. Shoot apices of wild-type plants were sampled at 10, 17, 24 and 30 days after germination. The expression of *MtAP1*, *SGL1*, *MtFULc* and *MtTFL1* were very low at day 10, increased significantly at day 17 and increased further at day 24. At day 30, floral buds were visible at shoot apices and the expression of these genes decreased, but were maintained at relatively high levels (Fig. 3A). No temporal expression differences were detected among the four genes in the current sampling intervals; they are all upregulated upon floral transition.

The spatial expression patterns of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* in wild-type inflorescence apices were analyzed using *in situ* hybridization. *MtTFL1* was highly expressed in the central part of I_1, but was not detected in I_2 and floral primordia (Fig. 4A-C). *MtTFL1* was also expressed in the center of axillary meristems (Fig. 4D). *MtFULc* was highly expressed in I_2 meristems, but not detectable in I_1 (Fig. 4E). *MtFULc* was not expressed in newly arising floral meristems and floral organs (Fig. 4F-G), but was detectable in I_2 meristems of axillary shoots (Fig. 4H) and inflorescence stems (Fig. 4F). Similar to the results observed by Benlloch et al. (2006), *MtAP1* was first detected at the side top of I_2 and then in the floral meristem and bract primordia (Fig. 4I,J). Later, *MtAP1* was confined to the area where sepal primordia and petal primordia will arise (Fig. 4K). During floral organ development, *MtAP1* was detectable in sepals and petals, but not in stamen primordia and carpel primordia (Fig. 4I,L). At late floral development stages, *MtAP1* was expressed only in petals (Fig. 4L). *SGL1* was detected in the rachis of compound leaves, and a low expression level of *SGL1* was also detected in I_1, I_2 and leaf primordia (Fig. 4M). High *SGL1* expression was detected in floral meristems and floral organ primordia (Fig. 4N-P). In floral organs, *SGL1* was mainly expressed in petals and developing carpels (Fig. 4Q).

In summary, the expression of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* was all upregulated upon floral transition. *MtTFL1*, *MtFULc* and *MtAP1* were specifically expressed in I_1, I_2 and floral meristem, respectively; *SGL1* showed a broad expression pattern in leaf primordia, floral meristem and all floral organ primordia. The expression domains of each gene were coherent to the inflorescence phenotypes when the corresponding gene was impaired.

Gene expression in inflorescence development mutants

To further decipher the genetic regulatory network in inflorescence development, we investigated the expression levels and patterns of the four genes in inflorescence apices of *mttfl1*, *mtfulc*, *mtap1*, *sgl1* and *mtap1sgl1* mutants by qRT-PCR and *in situ* hybridization.

In *mttfl1*, the expression level of *MtFULc* was slightly decreased, whereas the expression of *MtAP1* and *SGL1* was similar to that of wild-type-like plants (Fig. 3B). In *mtfulc*, the expression of *MtTFL1* was increased by approximately twofold. The expression of *MtAP1* was very low or undetectable and the expression level of *SGL1* was also lower than that of wild-type-like plants (Fig. 3B), which was in agreement with the no-flower phenotype of *mtfulc*. In *mtap1*, no

Development (2018) 145, dev158766. doi:10.1242/dev.158766

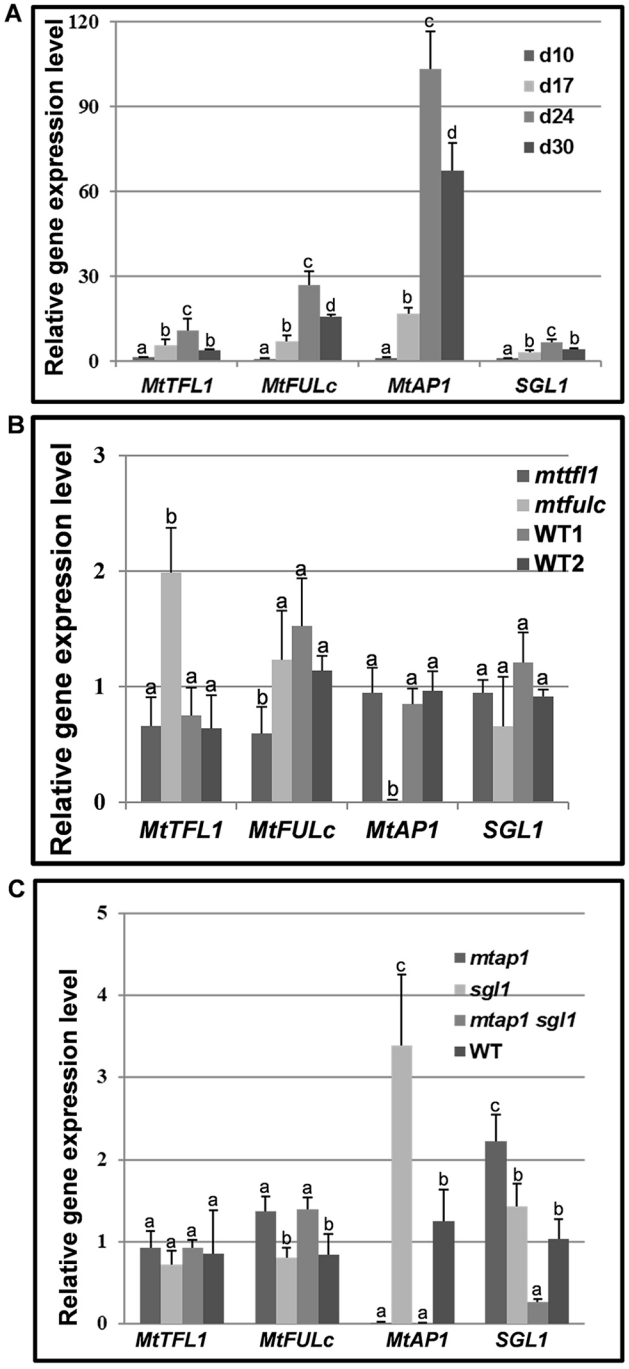

Fig. 3. Relative expression of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* by quantitative real-time PCR in shoot apices of wild type and mutants. (A) Gene expression in wild type at 10, 17, 24 and 30 days after germination. (B) Gene expression in inflorescence apices of *mttfl1*, *mtfulc* and wild-type-like plants. WT1: wild-type-like plants from the *MtTFL1* insertion line. WT2: wild-type-like plants from the *MtFULc* insertion line. (C) Gene expression in inflorescence apices of *mtap1*, *sgl1*, *mtap1 sgl1* and wild-type-like plants. Three biological replicates for each sample and three technical repeats for each replicate were performed. Mean±s.d. is shown. For comparison within each group (for the same gene), the same letters in different samples indicate no statistical differences, whereas different letters indicate significant differences, with $P<0.05$ as determined by one-way ANOVA. The primer pair for *MtAP1* spans the *Tnt1* insertion.

significant expression change was observed for *MtTFL1*, but the expression of *MtFULc* and *SGL1* was increased (Fig. 3C). In *sgl1*, the expression of *MtTFL1* and *MtFULc* showed no significant difference from wild type, whereas the expression of *MtAP1* was dramatically increased compared with that of control plants (Fig. 3C). In the double mutant *mtap1 sgl1*, no significant expression changes were detected for *MtTFL1*, but the expression of *MtFULc* was increased, which was similar to that in the *mtap1* single mutant (Fig. 3C).

The spatial expression of these genes in mutants was further examined by *in situ* hybridization. First, the expression domains of *MtFULc*, *MtAP1* and *SGL1* were explored in *mttfl1*. Before the I_1 meristem was terminated, the expression patterns of the three genes were similar to those in wild type (Fig. 5A,B,E,H). Besides the normal expression domain in I_2 meristems (the same as in wild type), a weak signal of *MtFULc* was detected in the I_1 meristem upon I_1 termination (Fig. 5C). Similarly, *MtAP1* was detected in I_2 meristems in addition to expression domains in floral meristems, sepal and petal primordia (Fig. 5F,G). Strong *SGL1* signals were detected in all meristems, including axillary meristems and floral organ primordia (Fig. 5H-J).

Next, we examined the expression of *MtTFL1* in *mtfulc*. Besides the normal expression in I_1 meristems, *MtTFL1* was also detected in defective secondary inflorescence (I'_2) meristems and axillary meristems in *mtfulc* plants (Fig. 5K-M). Fig. 5N showed that *MtTFL1* was detected in both I_1 and I'_2 meristems in two consecutive sections.

Furthermore, the expression domains of *MtTFL1*, *MtFULc* and *SGL1* were examined in *mtap1*. *MtTFL1* showed normal expression patterns in I_1 and axillary meristems, but not in I_2 meristems. It was not detected in the defective floral (FM′) meristems (Fig. 6A,B), but was detected in some secondary lateral meristems in proliferating inflorescences (Fig. 6C,D), which was in agreement with the phenotype of vegetative shoot formation in *mtap1*. *MtFULc* was normally detected in I_2 meristems, but the expression was maintained in initiated meristems and reiterating meristems (Fig. 6E,F). *MtFULc* was also detectable in floral primordia and inflorescence stems but absent in developing floral organs (Fig. 6F,G); sometimes *MtFULc* was detectable in secondary lateral meristems of proliferating inflorescences (Fig. 6H). *SGL1* was detected in most reiterating meristems and floral organ primordia in *mtap1* (Fig. 6I-K).

We also investigated the expression of *MtTFL1*, *MtFULc* and *MtAP1* in *sgl1*. *MtTFL1* and *MtFULc* showed similar expression domains as in wild type and no ectopic expression in reiterated floral meristems (data not shown). *MtAP1* was expressed in floral meristems, sepal primordia and common primordia as in wild type (Fig. 6M,N). However, unlike in wild type, in which the expression of *MtAP1* was restrained in petal primordia but absent in stamen primordia during common primordium differentiation (Fig. 4I,K,L), *MtAP1* was detected in common primordia reiterated meristems of *sgl1* plants (Fig. 6L,N,P). *MtAP1* was absent in developing sepals and carpel-like structures (Fig. 6L,O,P).

Finally, we examined the expression domains of *MtTFL1* and *MtFULc* in *mtap1 sgl1*. *MtTFL1* was detected in the center of the I_1 meristem and axillary meristems as in wild type (Fig. 7A,B). It was barely detectable in reiterated meristems, but was detected in some secondary lateral meristems (Fig. 7C,D). *MtFULc* showed normal expression in I_2 meristems as in wild type (Fig. 7E), but was maintained in the reiterated floral meristems, including bract primordia (Fig. 7F,G). In the double mutant, *MtTFL1* and *MtFULc* showed wild-type-like expression patterns in some lateral meristems, which were consistent with the observed vegetative shoot formation from the secondary inflorescence (Fig. 7D,H).

Development (2018) 145, dev158766. doi:10.1242/dev.158766

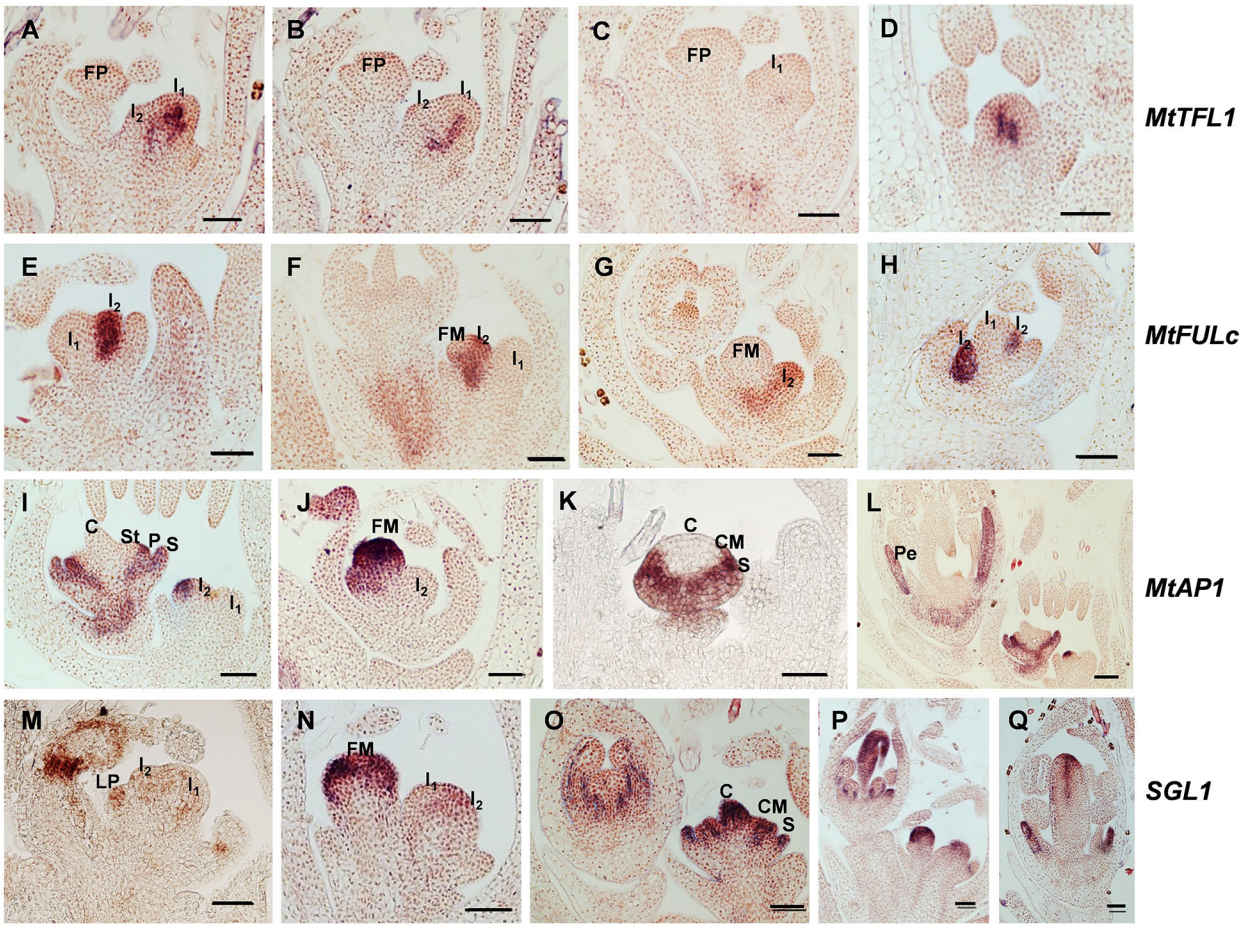

Fig. 4. Spatial expression of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* in wild type by *in situ* hybridization. (A-D) *MtTFL1*. (A-C) Consecutive sections, showing that *MtTFL1* is expressed in the center of the primary meristem (I_1), but not in I_2 and floral primordia. (D) *MtTFL1* is also expressed in axillary meristem. (E-H) *MtFULc* is exclusively expressed in I_2 meristem (E,H) but is absent in emerging floral meristems and developing floral organ primordia (F). *MtFULc* is restricted to I_2 meristem (G). (I-L) *MtAP1* is expressed in the emerging floral meristem, sepal and petal primordia, but is absent in stamen and carpel primordia (I). *MtAP1* is expressed in the bract and floral meristem, but absent in I_2 meristem (J). In a flower primordium, *MtAP1* is present in the outer part but absent in the inner part where stamen primordia and carpel will develop (K). *MtAP1* is only expressed in petals in a maturing flower (L). (M-Q) *SGL1* is expressed in developing compound leaves and weak signals in I_1 meristem (M). Strong signal in floral meristems (N). *SGL1* is expressed in all floral organ primordia (O) and in I_2 meristem and floral meristem with strong signal in developing carpels (P). *SGL1* signal stays in petals and carpels (Q). C, carpel; CM, common primordium; FM, floral meristem; FP, floral primordium; LP, leaf primordium; P, petal primordium; Pe, petal; S, sepal primordium; St, stamen primordium. Scale bars: 50 µm.

In summary, *MtTFL1* was ectopically expressed in I_2 meristems in *mtfulc*. *MtFULc* showed ectopic expression in floral meristems in *mtap1* and *mtap1 sgl1*. *MtAP1* and *SGL1* were ectopically expressed in reiterated meristems in the reciprocal mutants. *MtFULc*, *MtAP1* and *SGL1* were ectopically expressed in determinate apical meristems of *mttfl1*, indicating a repression loop in the sequential control of inflorescence meristem identity.

Global gene expression change in *mtap1*, *sgl1* and *mtap1 sgl1*

The global gene expression patterns in inflorescence apices of *mtap1*, *sgl1* and *mtap1sgl1* were compared using microarray analysis. Eight-hundred and twenty-eight genes (probesets with gene ID) showed significant expression changes between the mutants and wild-type-like plants (Table S2). Among them, 246 genes were up- or downregulated (with two-fold changes) in the three mutants. However, when the expression levels of individual samples were examined, 445 genes showed a similar expression change trend at various levels (less than twofold) in the three mutants, including 435 genes that were downregulated. Seventy-four genes were regulated only in *mtap1*, of which 64 were downregulated. Sixty-seven genes were regulated only in *sgl1*, most of them were upregulated (Fig. S4, Table S2).

We specifically analyzed all MADS-box genes and some known floral development-related genes in the microarray results (Table S3). Sixteen MADS-box and three other genes were regulated, including three class A genes, three class B genes, six *AGAMOUS-like* genes to class C and three *SEP-like* genes to class E (Table 1). As demonstrated above, *MtAP1* and *SGL1* were upregulated reciprocally in *mtap1* and *sgl1*. The other two class A genes were also upregulated in *sgl1* but were not significantly changed in *mtap1* and *mtap1 sgl1*. All class B and class C genes were downregulated in the three mutants, but class B genes were dramatically downregulated in *sgl1*. Class E genes were also downregulated in *mtap1* and *mtap1 sgl1*, but were not significantly changed in *sgl1*. Noticeably, *SHORT VEGETATIVE PHASE*, a flowering repressor, was significantly downregulated in all three mutants. Therefore, in agreement with the phenotypes of defective floral organs in all three mutants, the majority of the impaired genes, including most floral organ identity MADS-box genes, were downregulated.

Taking all the results together, a series of schematic diagrams has been drawn to illustrate the inflorescence phenotypes of individual

Development (2018) 145, dev158766. doi:10.1242/dev.158766

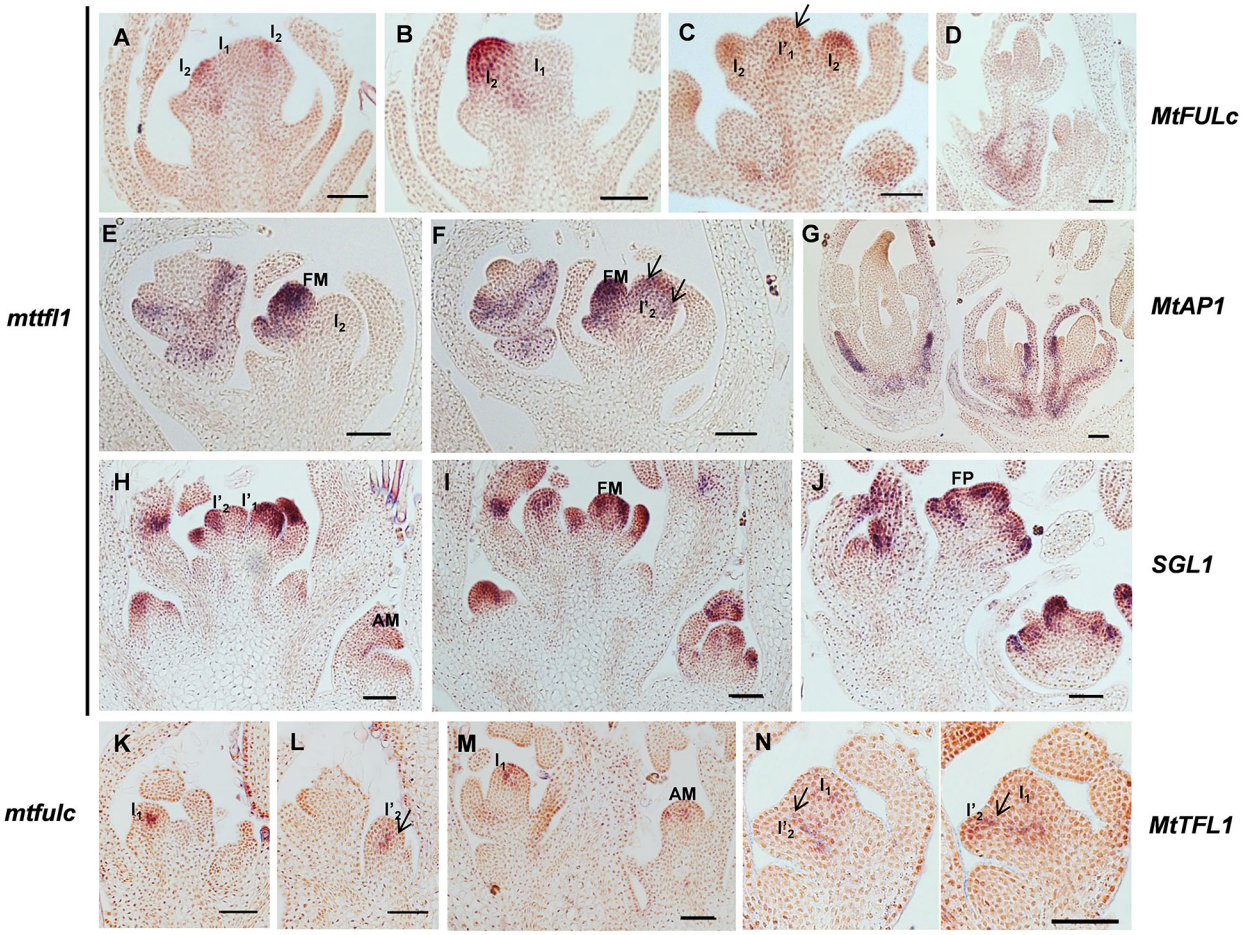

Fig. 5. Gene expression in *mttfl1* and *mtfulc* by *in situ* hybridization. (A-D) *MtFULc* is expressed in I_2 meristems (A,B). *MtFULc* is present in both I_1 and I_2 meristems in the determinate shoot apex (C) and in inflorescence stem (D). (E-G) *MtAP1* is expressed in bract, floral meristem, and sepal and petal primordia (E), and in terminating I_2 meristem (I'_2,F). *MtAP1* normal expression pattern in terminal flowers (G). (H-J) *SGL1* is expressed in all inflorescence meristems, leaf primordia and axillary shoot meristems (H,I), and in all floral organ primordia of terminal flowers (J). *MtTFL1* is expressed in I_1 and I_2 meristems of *mtfulc* (K,L). *MtTFL1* is also expressed in I_1 meristem and axillary shoot meristem (M). (N) Two consecutive sections showing *MtTFL1* expression in I_1 and I_2 meristems. AM, axillary meristem; FM, floral meristem; FP, floral primordia. Scale bars: 50 µm. Arrows indicate ectopic expression sites.

mutants and the associated gene expression patterns (Fig. 8A). Furthermore, the effects of gene mutation on inflorescence development are summarized in one simple scheme, and the genetic regulatory network of inflorescence architecture has been established in *M. truncatula* (Fig. 8B).

DISCUSSION

MtTFL1 regulates I_1 meristem indeterminacy, maturation rate and shoot architecture

Flowering involves two sequential events: floral transition and floral meristem identity determination. *TFL1* genes play essential roles in both events by repressing floral transition and maintaining inflorescence meristem indeterminacy (Shannon and Meeks-Wagner, 1991). The function of *TFL1* in maintaining inflorescence meristem indeterminacy is well conserved in orthologous genes (Liu et al., 2010). The expression pattern of *MtTFL1* and the formation of determinate I_1 meristems in *mttfl1* plants indicate that *MtTFL1* shares the conserved function in maintaining primary inflorescence meristem indeterminacy, but not in regulating flowering time in *M. truncatula*. The flowering time may be regulated by other *MtTFL*-like genes. The consistency of the ectopic expression of *MtTFL1* and the vegetative shoot growth of *mtfulc* and *mtap1* mutants further confirm the function of *MtTFL1* in inflorescence meristem indeterminacy. The ectopic expression of *MtFULc*, *MtAP1* and *SGL1* in the apical meristem of *mttfl1* and *mttfl1 mtfulc* suggests that *MtTFL1* represses both *MtFULc* and the floral meristem identity genes *MtAP1* and *SGL1* in I_1 meristems. The conclusion is in agreement with the genetic regulation models proposing that *TFL1*/*CEN* plays antagonistic roles with floral meristem identity genes to maintain dominancy in primary inflorescence development (Fig. 8A,B) (Bradley et al., 1997; Blázquez et al., 2006; Benlloch et al., 2015).

Although it does not affect flowering time, mutation of *MtTFL1* does result in abrupt termination of inflorescence meristems with shortened internodes and pedicles, and degenerative compound leaves and flowers, indicating that *MtTFL1* is involved in the regulation of meristem maturation. The process shares great similarity with the precocious maturation of sympodial growth, which is caused by the mutation of the *TFL1* orthologous genes *SELF PRUNING* (*SP*) in tomato or *FASCICULATE* (*FA*) in wild pepper (Pnueli et al., 1998; Elitzur et al., 2009). The evidence is in agreement with the suggested common mechanism by which *TFL1* genes regulate the progression rate of shoot apices through different developmental phases, which integrates the functions of *TFL1* orthologs across species with different inflorescence architectures (Ratcliffe et al., 1998).

Development (2018) 145, dev158766. doi:10.1242/dev.158766

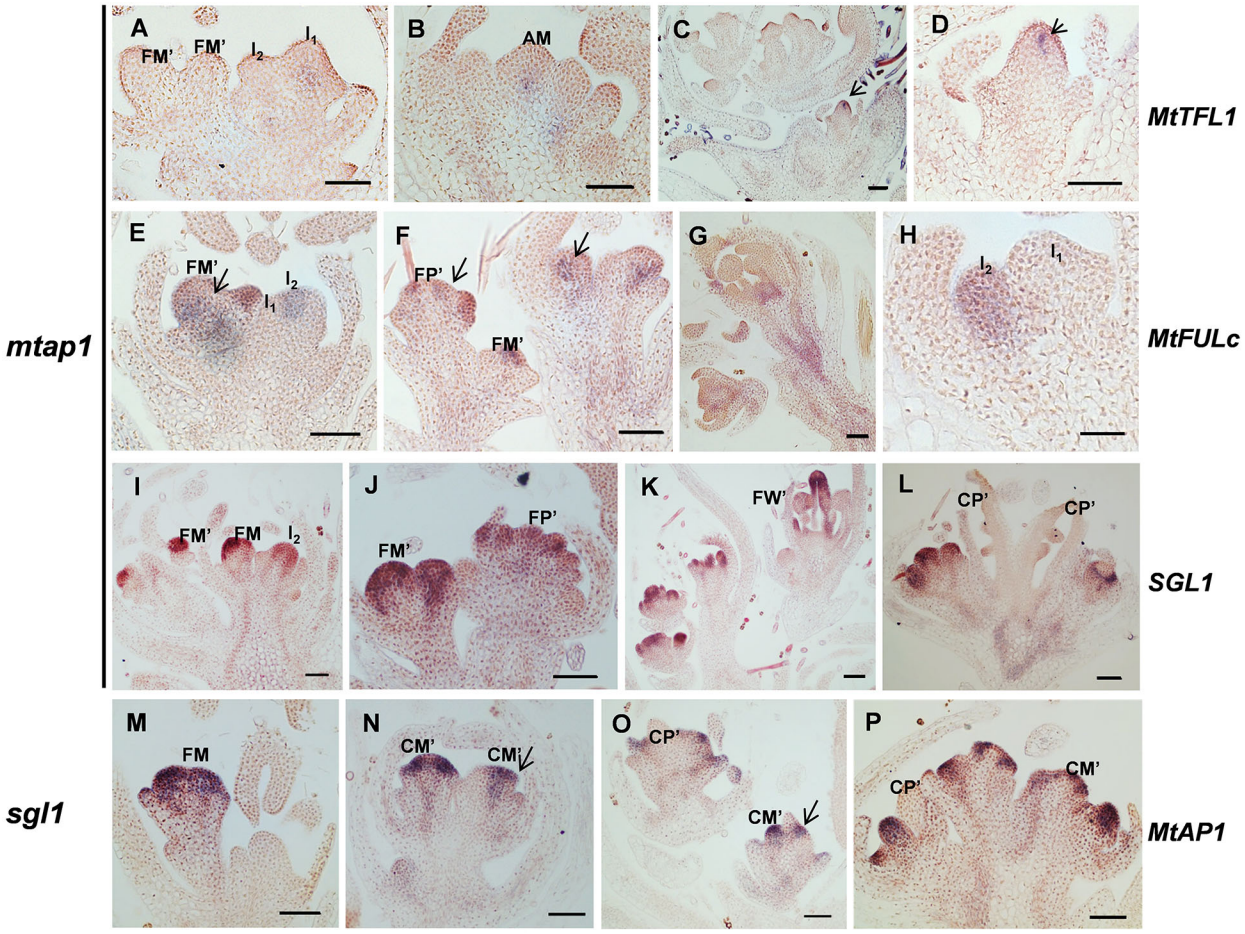

Fig. 6. Gene expression in *mtap1* and *sgl1* by *in situ* hybridization. *MtTFL1* is expressed in the center of I_1 meristem (A), axillary shoot meristem (B) and secondary lateral meristem (C) of *mtap1*. (D) Higher magnification view in C. *MtFULc* is expressed in I_2 meristem and retained in floral meristem (E), proliferating meristems (F) and inflorescence stem, but not in developing floral primordia and flower (G) or in I_1 meristem of secondary lateral inflorescence shoot (H) of *mtap1*. *SGL1* is expressed in floral meristems (I), proliferating meristems and floral organ primordia (J), and floral organs (J,K) of *mtap1*. *MtAP1* is expressed in bract and floral meristems (M), in common primordia (N), and in reiterated floral meristems and floral primordia (P), but absent in carpels (L) and in the carpel (O) of *sgl1*. CM, common primordium; CM′, defective common primordium; CP, carpel; FM, floral meristem; FM′, defective floral meristem; FP, floral primordia. Scale bars: 50 µm in A-G,I-P; 25 µm in H. Arrows indicate ectopic expression sites.

Plant growth habit, or branching pattern, determines shoot architecture. There are two basic growth patterns: monopodial growth, as in *Arabidopsis*, in which the primary apical meristem remains indeterminate and develops a main axis; and sympodial growth, as in *Solanaceous* plants, in which the primary apical meristem terminates and further development is reiterated by upmost axillary meristems. In pea and *M. truncatula*, inflorescence shoots display monopodial growth. Mutation of *MtTFL1*, however, changes the growth pattern of inflorescence shoots from monopodial to sympodial in both *mttfl1* and *mttfl1 mtfulc*, resulting in short and extensively branched shoot architecture. However, mutation of DET does not change the growth pattern of pea, thus no extensively branched shoots are observed (Singer et al., 1999). Tomato and pepper plants have typical sympodial growth during the reproductive stage. *SP* in tomato and *FA* in pepper are not expressed in shoot apical meristem, but are expressed in sympodial inflorescence meristems and auxiliary meristems, and decrease with the meristem outgrowth (Thouet et al., 2008; Elitzur et al., 2009). Mutations of *SP* or *FA* result in the precocious maturation of sympodial growth without affecting the inflorescence development in *Solanaceous* (Pnueli et al., 1998; Elitzur et al., 2009). The similarity of inflorescence shoot architectures between mutants *sp* or *fa* and *mttfl1* or *mttfl1 mtfulc* indicates that *TFL1* genes may be also involved in the genetic regulation of shoot growth patterns and that controlling the expression pattern of *TFL1* could be a very useful tool for manipulating crop shoot architecture.

MtFULc maintains secondary inflorescence meristem identity by repressing the expression of *MtTFL1* and *MtAP1*

FUL belongs to the *AP1*/*SQUA* gene family. *FUL* genes show diverse expression patterns and functions in different species (Immink et al., 1999; Litt and Irish, 2003; Pabón-Mora et al., 2012; Jaudal et al., 2015). *FUL* plays significant roles in carpel/fruit development, and is also involved in flowering time regulation, floral meristem identity, cauline leaf development and meristem determinacy in *Arabidopsis* (Gu et al., 1998; Ferrándiz et al., 1999, 2000; Melzer et al., 2008). Even though *FUL* shows transient specific expression in inflorescence meristems, mutation of *FUL* has no visible impact on the inflorescence architecture in *Arabidopsis*. Only during the development of compound inflorescences does *VEG1*/*PsFULc* acquire significant functions in the identity of secondary inflorescence meristems in pea (Berbel et al., 2012). The specific expression pattern and no-flower phenotype in *mtfulc* confirm that *MtFULc* shares similar functions

Development (2018) 145, dev158766. doi:10.1242/dev.158766

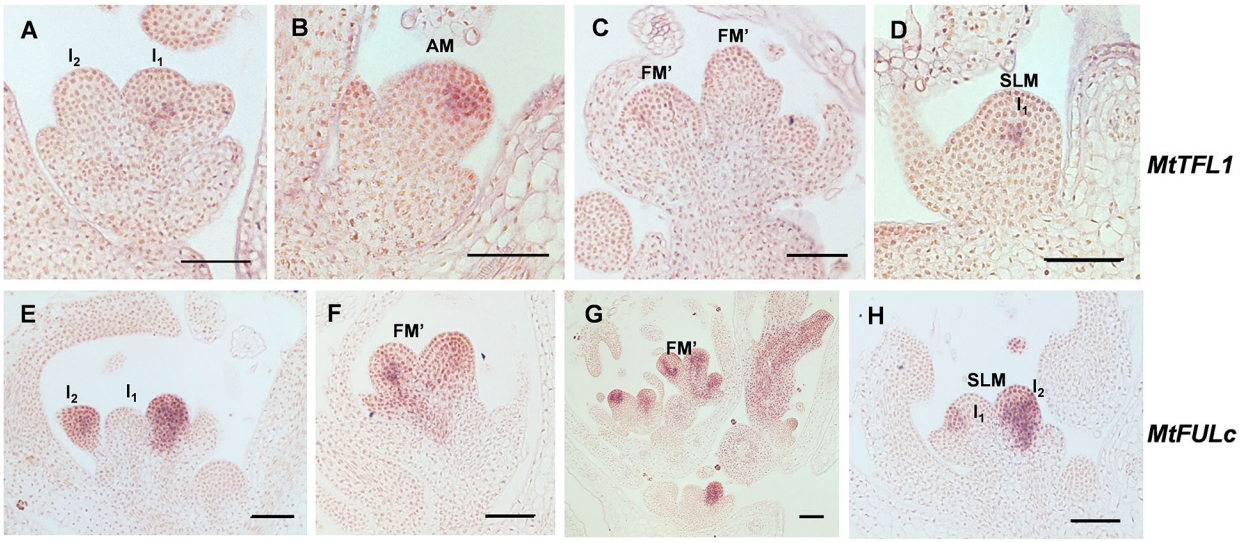

Fig. 7. Gene expression in *mtap1 sgl1* by *in situ* hybridization. (A-D) *MtTFL1* is expressed in I_1 (A) and axillary meristem (B), with weak signal in reiterated meristems (C) and in secondary lateral inflorescence meristem (D). (E-H) *MtFULc* is expressed in I_2 of the primary inflorescence apex (E); the expression remains in floral meristems derived from I_2 (F), in reiterated meristems and in inflorescence stem (G), and in I_2 of the secondary lateral inflorescence meristem (H). AM, axillary meristem; FM′, defective floral meristem; SLM, secondary lateral meristem. Scale bars: 50 µm.

in I_2 meristem identity with *VEG1*. Development of normal pods in *mttfl1 mtfulc* indicates that *MtFULc* may not share the function of *FUL* in carpel and fruit development in *Arabidopsis* (Gu et al., 1998; Ferrándiz et al., 1999).

Compared with the compound inflorescence in *mttfl1*, *mttfl1 mtfulc* produces simple flowers, indicating that *MtFULc* is essential for compound inflorescence development. The expanded expression domain of *MtFULc* in proliferating inflorescences of *mtap1* and *mtap1 sgl1* further supports its role in I_2 meristem identity. On the other hand, it may also indicate the correlation between the expansion of *MtFULc* spatiotemporal expression and the increase of inflorescence complexity (Figs 2B-E, 6F and 7C). Inflorescence architecture is controlled by the rate of meristem maturation and the inflorescence complexity can be increased by delaying floral meristem commitment. This notion is supported by the effect of manipulating *TFL1* and *LFY* expression on inflorescence structures in *Arabidopsis*, and is shown by the effect of *COMPOUND INFLORESCENCE*(*S*) and *ANATHA* on the cyme inflorescence complexity in tomato (Bradley et al., 1997; Prusinkiewicz et al., 2007; Lippman et al., 2008; Park et al., 2012). Results from the present study support the notion that *MtFULc*, in coordination with *MtTFL1* and *MtAP1*, regulates inflorescence complexity by controlling the commitment of secondary inflorescence meristems. It also supports the theory that regulation of floral meristem commitment is a common mechanism for defining inflorescence complexity, but the key regulators are different in different inflorescence architectures (Bradley et al., 1997; Park et al., 2012, 2014). Whether simple flowers can be changed to compound inflorescence by extending *FUL* expression in the inflorescence meristems in *Arabidopsis* is an interesting topic that will shed light on further understanding the formation of compound racemose inflorescence.

Table 1. Expression changes of floral identity genes in wild type and mutants

			Average signal				Ratio		
Probe set ID	Gene ID	Description	Wild type	*mtap1*	*sgl1*	*mtap1 sgl1*	*mtap1*/wild type	*sgl1*/wild type	*mtap1 sgl1*/wild type
23308036	Medtr8g066260	AP1	184.96	157.76	773.28	134.41	0.85	4.18	0.73
23058848	Medtr5g046790	CAL-A	97.55	139.43	192.62	149.20	1.43	1.97	1.53
23319641	Medtr6g464720	CAL-D	56.46	60.47	99.38	50.87	1.07	1.76	0.9
23173686	Medtr3g113030	AP3	146.35	33.69	14.03	14.35	0.23	0.1	0.1
23129269	Medtr3g088615	PI	158.81	38.49	7.77	8.54	0.24	0.05	0.05
23192726	Medtr5g021270	Unavailable	87.95	44.64	13.65	8.40	0.51	0.16	0.1
23090390	Medtr3g452380	AGA	22.74	6.48	11.32	5.18	0.28	0.5	0.23
23270852	Medtr5g061740	AGAb	11.28	4.06	3.93	3.93	0.36	0.35	0.35
23105449	Medtr8g087860	AGAb-L	128.32	43.56	24.40	16.83	0.34	0.19	0.13
23090390	Medtr3g452380	AGL-5	22.74	6.48	11.32	5.18	0.28	0.5	0.23
23224166	Medtr8g033270	AGL6	36.91	9.64	17.57	7.86	0.26	0.48	0.21
23295705	Medtr3g084980	AGL9	83.84	31.45	38.47	12.46	0.38	0.46	0.15
23210946	Medtr6g015975	SEP1	61.51	29.02	89.54	15.68	0.47	1.46	0.25
23191764	Medtr7g016600	SEP1-L	87.87	40.51	137.24	18.95	0.46	1.56	0.22
23095998	Medtr8g097090	SEP3	128.84	46.11	133.69	9.71	0.36	1.04	0.08
23257455	Medtr5g032150	SVP	87.95	44.64	13.65	8.40	0.51	0.16	0.1
23147778	Medtr4g094748	UFO-L	9.46	17.16	57.49	7.03	1.81	6.07	0.74
23238410	Medtr7g100590	AP2	36.65	54.90	99.75	55.18	1.5	2.72	1.51
23259286	Medtr3g098560	SGL1	23.26	41.28	33.30	15.50	1.77	1.43	0.67

Data are mean signal intensities from three biological replicates.

Development (2018) 145, dev158766. doi:10.1242/dev.158766

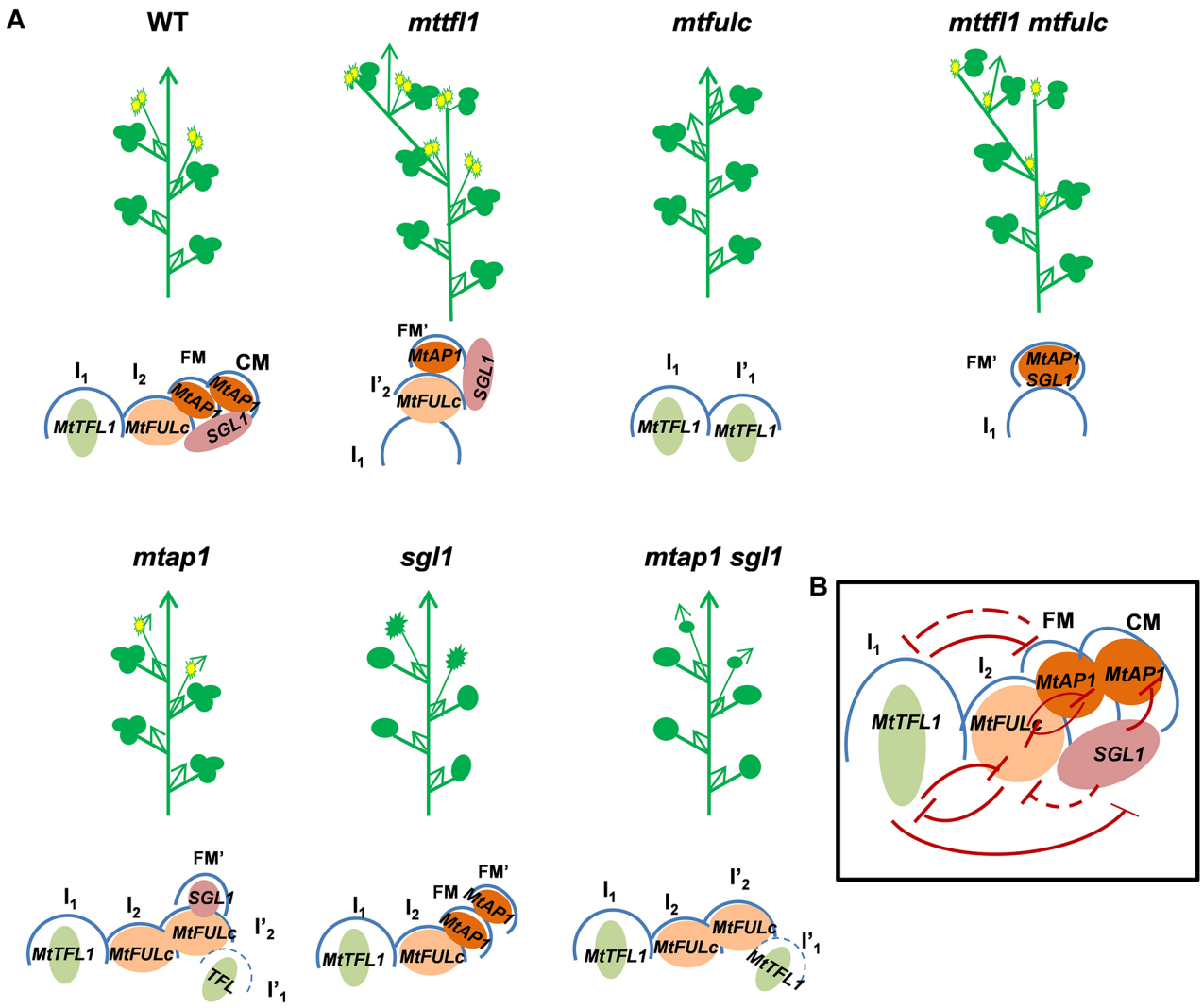

Fig. 8. Schematics of genetic interaction and regulation of inflorescence development genes. (A) Illustrations of inflorescence phenotypes and gene expression patterns in wild type and mutants. Arrows indicate indeterminate shoots in the apex or axils. Yellow circles represent flowers (a pair of flowers in wild type and *mttfl1*; one flower in *mtap1* and *mttfl1 mtfulc* double mutant). Green circles and ovals represent leaflets (*sgedl1* and *mtap1 sgl1* have single leaflets; all other mutants and wild type have three leaflets). Spiky green structures denote cauliflower-like flowers in *sgl1*. (B) Genetic regulation network of the four genes during inflorescence development. Each colored region represents corresponding gene expression localization. Solid lines indicate direct repression; dashed lines indicate indirect repression. I_1, primary inflorescence meristem; I'_1, I_1-like meristem; I_2, secondary meristem; I'_2, I_2-like meristem; FM, floral meristem; FM′, FM-like meristem; CM, common primordia.

Mutant *mtfulc* shows transformation of I_2 to lateral I_1-like meristems and no-flower phenotype. *MtTFL1* is ectopically expressed in I_1-like meristems, which, on the one hand, indicates that *MtTFL1* expression is repressed by *MtFULc* in I_2 meristems, and on the other hand, further confirms the function of *MtTFL1* in inflorescence meristem indeterminacy. The no-flower phenotype is in agreement with the low expression level of floral meristem identity genes in *mtfulc*, which is in contrast to the dramatically increased expression upon floral transition in wild-type plants. Does mutation of *MtFULc* directly block the upregulation of floral meristem genes and result in no flower formation? Or does ectopic expression of *MtTFL1* inhibit the expression of floral meristem genes to prevent flower initiation? If the first explanation were true, *MtTFL1*, *MtFULc* and *MtAP1/SGL1* would act in a linear mode and the double mutant *mttfl1 mtfulc* would show a similar no-flower phenotype to *mtfulc*. In fact, the double mutant displays flower formation and a determinate primary inflorescence meristem, which is similar to *mttfl1* mutant. This observation suggests that mutation of *MtFULc* releases only the inhibition of *MtTFL1* expression in I_2 meristems, but does not directly affect the expression of *MtAP1/SGL1* to prevent floral development. However, in mutant *mttfl1*, *MtAP1* is still absent in I_2 meristems, even though the repression of *MtTFL1* is abolished, indicating that *MtFULc* does repress the *MtAP1* expression in I_2 meristems. Therefore, the reciprocal repression between *MtTFL1* and *MtFULc* is similar to that of *PsTFL1* and *VEG1* in pea (Benlloch et al., 2007, 2015; Berbel et al., 2012). Both *MtTFL1* and *MtFULc* repress the expression of *MtAP1/SGL1*, and *MtTFL1* is epistatic to *MtFULc* in the regulation of floral development, partially maintaining the antagonistic mode between *TFL1* and *AP1/LFY* in *Arabidopsis*

MtAP1 plays a major role in floral meristem identity and represses *MtFULc* expression in floral meristems

The specified expression domain of *MtAP1* and the conversion of floral meristems into secondary inflorescences, leaf-like bracts and sepals, and defective petals in the *mtap1* mutant, confirm its

Development (2018) 145, dev158766. doi:10.1242/dev.158766

conserved function with orthologous genes in floral meristem and floral organ identity (Mandel et al., 1992; Taylor et al., 2002; Benlloch et al., 2006). The complete conversion of floral meristems into I_1 in the basal part and the absence of wild-type-like secondary inflorescence even at late stages in the *mtap1* mutant indicate that *MtAP1* is also involved in secondary inflorescence development. The complementary expression domains between *MtAP1* and *MtFULc* in wild-type plants and the ectopic expression of *MtFULc* in *mtap1* suggest that *MtAP1* represses *MtFULc* to acquire floral meristem identity, which agrees with studies in pea and *Arabidopsis* (Ferrándiz et al., 2000; Berbel et al., 2012). In *mtap1* and *mtap1 sgl1*, some I_1-like inflorescence shoots are produced at early reproductive stages and the expression of *MtTFL1* is detectable in some secondary lateral meristems, indicating that some of the meristems regain I_1-like meristem identity. Secondary I_1-like meristem formation is not fully understood. However, we speculate that *MtFULc* represses *MtTFL1* in I_2 meristems, whereas *MtAP1* represses *MtTFL1* expression in floral meristems, which is possibly reminiscent of the reciprocal repression between *TFL1* and *AP1* in *Arabidopsis* (Liljegren et al., 1999).

SGL1 plays synergistic role with *MtAP1* in floral meristem identity

LFY/FLO/UNI is another major floral meristem player in plants. Mutation of *LFY/FLO* causes complete or partial conversion of floral meristems into inflorescence meristems (Coen et al., 1990; Schultz and Haughn, 1991). *UNI* is involved in the regulation of I_1 meristem identity in pea as *uni* mutant plants terminate with the formation of a stub at the apical meristem (Singer et al., 1999; Berbel et al., 2012). Unlike *lfy/flo/uni*, *sgl1* never shows the replacement of flowers with inflorescences or termination of I_1 meristem; instead, it exhibits reversion of common primordia into floral meristems without further differentiation of petal and stamen primordia. *MtAP1* maintains its expression in reversed and reiterated meristems, and the expression of class B function genes is dramatically reduced in *sgl1*, indicating that *SGL1* is required for petal/stamen specification from common primordia by repressing *MtAP1* expression and upregulating class B function genes (Wagner et al., 1999). However, the double mutant *mtap1 sgl1* enhances *mtap1* phenotypes and leads to the complete conversion of floral meristems into I_2-like meristems. This observation suggests that *SGL1* has synergistic function with *MtAP1* in floral meristem identity. The large number of commonly regulated genes among *mtap1*, *sgl1* and *mtap1 sgl1* further indicate the conserved functions of *MtAP1* and *SGL1* in floral meristem identity and floral organ development in *M. truncatula*.

Besides the conserved functions in flower development, *SGL1*/*UNI* plays significant roles in compound leaf development. Mutation of *SGL1*/*UNI* leads to simple leaf formation in some legume species (Hofer et al., 1997; Wang et al., 2008). Additionally, mutation of *RFL*, the ortholog of *LFY* in rice, leads to simplification of panicles in rice (Rao et al., 2008). These results indicate that *LFY* genes are required for maintaining meristem transient indeterminacy to form determinate structures, i.e. leaflets and panicles (Hofer et al., 1997; Rao et al., 2008; Wang et al., 2008). The formation of common primordia is a unique transition phase during flower development in legumes. The process resembles the formation of compound leaves from leaf primordium to leaflets. The mutation of *SGL1* results in the reversion of common primordia to floral meristems. Therefore, one of the *SGL1*/*UNI* functions in flower development is to promote or maintain a transient phase of indeterminate growth, as suggested in compound leaf development (Hofer et al., 1997).

In conclusion, we deciphered the genetic regulation of inflorescence development in *M. truncatula* via comprehensive and systematic expression and genetic analyses of four key genes. Our data show that inflorescence architecture is controlled by spatiotemporal expression of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* through reciprocal repression, which is not well defined in pea. The genetic regulatory network involving the four genes shares similarity with the modified model in pea, but also shows specificity in compound inflorescence development in *M. truncatula*. The study establishes *M. truncatula* as an excellent genetic model for understanding compound inflorescence development in related important crop species.

MATERIALS AND METHODS

Plant materials and growth

Seeds of *Tnt1* insertion lines for *MtTFL1* (NF7897, NF17535 and NF18476), *MtFULc* (NF10716, NF15285 and NF18126), *SGL1* (NF0740) and *MtAP1* (NF11426) were requested from Noble Research Institute. The insertion homozygosity of individual genes was identified by PCR with combinations of gene-specific primers and *Tnt1* primers. PCR-based reverse screening for additional insertion lines for *MtFULc* was carried out as previously described (Cheng et al., 2014), though no additional line was obtained. For obtaining double mutant *mttfl1 mtfulc*, *mttfl1-3* (NF18476) flowers were cross-pollinated with heterozygous *MtFULc* flowers (NF10716); F1 plants with *Tnt1* insertions in both *MtTFL1* and *MtFULc* were identified by PCR genotyping, and F2 seeds were harvested from confirmed F1 plants. The *mttfl1 mtfulc* double mutant plants were identified from the F2 segregating progenies by PCR genotyping. To obtain double mutant *mtap1 sgl1*, heterozygous *MtAP1* flowers were cross-pollinated with heterozygous *SGL1* flowers. The F2 seeds were collected from F1 plants and germinated for F2 segregating progenies. The double mutants from the F2 segregation population were confirmed by PCR genotyping. The primer sequences of each gene for genotyping are listed in Table S4.

Seeds of *M. truncatula* wild type (R108) and *Tnt1* insertion mutants were scarified with concentrated sulfuric acid for 8 min, rinsed with water and put on filter paper for 7-10 days at 4°C. Germinated seeds were transferred into one-gallon pots with Metro-Mix 350 (Scotts) composite soil and grown at 16 h/8 h day/night cycle at 150 µE/m^2/s light intensity, 22°C/18°C day/night temperature and 70% humidity for sampling, phenotype observation and pod collection.

In situ hybridization

Inflorescence shoot apices from six-week-old wild-type and mutant plants were fixed and the *in situ* hybridization was performed as described by Zhou et al. (2010). cDNA fragments (400-600 bp) from non-conserved regions of *MtTFL1*, *MtFULc*, *MtAP1* and *SGL1* were used as the probes for *in situ* hybridization. The primer pairs of each probe are listed in Table S4.

Scanning electron microscopy

Inflorescence shoot apices from six-week-old wild-type and mutant plants were dissected and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.2) for 12 h at 4°C. After rinsing with PBS for 3 h, fixed samples were dehydrated in an ethanol series and critical point dried. The samples were mounted on metal stubs and sputter-coated with gold, observed under a Zeiss DSM-960A SEM (Carl Zeiss) at an accelerating voltage of 5 kV.

RNA extraction, RT-PCR and qRT-PCR

Shoot apices of wild type were collected at day 10, 17, 24 and 30 days after germination for expression analysis during floral transition. Inflorescence apices from six-week-old wild-type and mutant plants were sampled. Three biological replicates were used for each sample. Total RNA was extracted using Tri-Reagent (Gibco-BRL Life Technologies) and treated with Turbo DNase I (Ambion). For RT-PCR and qRT-PCR, 3 µg of total RNA were used for reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) with the oligo $(dT)^{20}$ primer. cDNA (2 µl diluted 1:20) was

used as a template. Three technical replicates were carried out for each template. Gene-specific primers, which span non-conserved regions and are used for RT-PCR and qRT-PCR, are listed in Table S4. All qRT-PCR 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-conjugating enzyme gene (Medtr3g110110) as the internal reference.

Transcriptome analysis

For transcriptome analysis, three biological replicates of the inflorescence shoots were sampled from six-week-old wild-type-like plants (including wild-type and heterozygous plants in the same segregating progeny), *mtap1*, *sgl1* and *mtap1 sgl1* homozygous plants in F2 segregation progenies. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen). Purified RNA was treated with Turbo DNase I (Ambion). Total RNA (500 ng) from each sample was used for labeling with the GeneChip WT PLUS Reagent Kit (WT PLUS Kit) (Affymetrix) based on the manufacturer's recommendation. Hybridization and scanning for microarray analysis were conducted according to the manufacturer's instructions (GeneChip Medicago Transcriptome, Affymetrix). Data normalization between chips was conducted using RMA (Robust Multichip Average) (Irizarry et al., 2003). Presence/absence calls for each probeset were obtained using dCHIP (Li and Wong, 2003). Gene selections based on the associative *t*-test were made using Metlab (MathWorks) (Dozmorov and Centola, 2003). A selection threshold of two for transcript ratios and a Bonferroni-corrected *P* value threshold of 2.19202E-06 were used.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.W.; Methodology: X.C., G.L., J.W.; Validation: X.C.; Formal analysis: X.C., G.L., J.W.; Investigation: X.C., G.L., J.W.; Resources: Y.T.; Data curation: Y.T.; Writing - original draft: X.C., J.W.; Writing - review & editing: J.W.; Supervision: Y.T., J.W.; Project administration: J.W.

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Data availability

Microarray data have been deposited in ArrayExpress with accession number E-MTAB-6462.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.158766.supplemental

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