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MtSUPERMAN plays a key role in compound inflorescence and flower development in *Medicago truncatula*.

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SUMMARY

Legumes have unique features, such as compound inflorescences and a complex floral ontogeny. Thus, the study of regulatory genes in these species during inflorescence and floral development is essential to understand their role in the evolutionary origin of developmental novelties. The SUPERMAN (SUP) gene encodes a C2H2 zinc-finger transcriptional repressor that regulates the floral organ number in the third and fourth floral whorls of *Arabidopsis thaliana*. In this work, we present the functional characterization of the *Medicago truncatula* SUPERMAN (*MtSUP*) gene based on gene expression analysis, complementation and overexpression assays, and reverse genetic approaches. Our findings provide evidence that *MtSUP* is the orthologous gene of *SUP* in *M. truncatula*. We have unveiled novel functions for a *SUP*-like gene in eudicots. *MtSUP* controls not only the number of floral organs in the inner two whorls, but also in the second whorl of the flower. Furthermore, *MtSUP* regulates the activity of the secondary inflorescence meristem, thus controlling the number of flowers produced. Our work provides insight into the regulatory network behind the compound inflorescence and flower development in this angiosperm family.
INTRODUCTION

Legumes have distinctive morphological traits that make them of particular interest for the study of developmental regulatory networks. Differential traits include their capacity for symbiotic nitrogen fixation, the presence of compound leaves and inflorescences, and a complex floral ontogeny (De Faria et al., 1989; Weberling and Pankhurst, 1989; Ferrándiz et al., 1999; Singer et al., 1999; Benlloch et al., 2003; Cañas and Beltrán, 2018). Arabidopsis thaliana produces a unique inflorescence meristem (I₁), from which flowers are formed. However, in legumes after the floral transition, the shoot apical meristem (SAM) produces a primary inflorescences meristem (I₁) that generates secondary inflorescence meristems (I₂) from which flowers are formed (Tucker, 2003; Benlloch et al., 2007). In the model legume Medicago truncatula, the I₂ meristems remain active until the production of one to three flowers and a stub or spike (Benlloch et al., 2003; 2015). The intercalation of a new meristem (I₂) between the I₁ and the floral meristem (FM) is linked to compound inflorescence development in legumes. The existence of this intercalated meristem (I₂) is based on a novel genetic function derived from the sub-functionalization of the AGL79 MADS-box gene clade within the eudicot AP1/SQUA/FUL family (Berbel et al., 2012). There are few studies about the I₂ meristem activity despite the potential agronomical interest to produce more flowers (Benlloch et al., 2015).

Floral development in legumes shows early carpel initiation and the formation of common primordia that are ephemeral meristems from which petals and stamens will differentiate. Flowers produce four types of organs: sepals, petals, stamens and carpels, which are arranged in four floral whorls (Krizek and Fletcher, 2005; Prunet et al., 2009). Floral organs are formed from the floral meristem that consists of a pool of stem cells that are transiently maintained until its termination producing a fixed number of whorls and floral organs (Bowman et al., 1989; Schultz et al., 1991; Bossinger and Smyth, 1996). Superman (SUP) is a gene that participates in the control of the number of carpels and stamens produced in A. thaliana (Sakai et al., 1995). The SUP gene encodes a transcriptional repressor that contains a zinc-finger (Cys2His2 type) motif and a C-terminus EAR-like motif (DLELRL) that has been proposed to act as an active repressor (Sakai et al., 1995; Hiratsu et al., 2002; 2003;
It has been proposed two functional models to explain the SUP function. The first model suggests that SUP promotes floral meristem termination through the exclusion of B genes APETALA 3 (AP3) and PISTILLATA (PI) from the fourth whorl (Schultz and Haughn, 1991; Bowman et al., 1992). The second or alternative model stipulates that SUP controls the cell proliferation balance between the third and fourth whorls (Sakai et al., 1995; Sakai et al., 2000). These models are non-exclusive, since both are compatible to explain the floral phenotypes of the sup alleles (Breuil-Broyer et al., 2016) and the effects of the ectopic expression of SUP and SUP-like genes in different plant species (Nandi et al., 2000; Bereterbide et al., 2001; Yun et al., 2002; Kazama et al., 2009; Nibau et al., 2011; Zhao et al., 2014). Moreover, other functions described for SUP include its role in the integuments differentiation (Gaiser et al., 1995) and its contribution to the formation of the carpel medial region (Breuil-Broyer et al., 2016). Recent studies showed that, in A. thaliana, SUP bridges floral meristem determinacy and floral organogenesis through the fine-tuning of auxin biosynthesis (Xu et al., 2018). SUP has been thoroughly studied in A. thaliana. However, no other SUP homologues have been functionally characterized in other plant species except for Petunia hybrida (Nakagawa et al., 2004).

The evolutionary inventiveness in the inflorescence architecture and flower structure distinguishes the legume family (Hofer and Ellis, 2014). The formation of common primordia constitutes an additional step in floral organogenesis that, along with the existence of the intermediate I2, are structural reiterations that confer complexity to legumes inflorescence (Ferrándiz et al., 1999; Berbel et al., 2012; Hofer and Ellis, 2014). Transcriptional regulators are key players in plant development and can be considered evolutionary switches (Doebley and Lukens, 1998). In turn, functional studies of key regulatory genes in the development of inflorescences and flowers of legumes are crucial for a better understanding of their roles during evolution and their link with developmental novelties in this angiosperm family. Here, we report the functional study of MtSUP, a key gene involved in the determinacy of inflorescence and floral meristems in the model legume Medicago truncatula.

RESULTS
Identification of the SUPERMAN ortholog in *Medicago truncatula*

The phylogenetic analysis shown in Figure S1a groups *Medtr2g076060.1* in the SUP clade, along with the SUP, PhSUP and SUP homologs from other legume species (Table S1). The SUP homologs form a well-defined clade separated from other members of the legume Cys2His2 zinc-finger proteins (ZFPs) family (Figure S1a). Soybean (*Glycine max*) and lupin (*Lupinus angustifolius*) harbour two SUP-like genes in their genomes. This fact is consistent with additional whole-genome duplications (WGD) in the *Glycine* and *Lupinus* lineages after the WGD in the common ancestor of the Papilionoideae clade of legumes (Cannon et al., 2006, 2015; Schmutz et al., 2010; Kroc et al., 2014; Hane et al., 2017). We renamed the *Medtr2g076060.1* gene to *MtSUP*. The *MtSUP* gene encodes a protein of 225 amino acids (Figure S1b) containing a single zinc-finger motif with the highly conserved QALGGH motif characteristic of the Cys2His2 ZFPs family (Englbrecht et al., 2004), and an EAR-like motif (DLELRL) responsible for the repressive activity of SUP (Hiratsu et al., 2002, 2003, 2004). This suggested that *MtSUP* could be the putative SUP ortholog in *M. truncatula*. We used the OrthoVenn2 web platform (Wang et al., 2015) to identify putative ortholog genes from Arabidopsis and *M. truncatula* throughout comparing their genomes. We found that *MtSUP* and SUP do not appear in the orthologous clusters that maintain certain synteny between *M. truncatula* and *A. thaliana* (Table S2). These results fit the model of degenerate microsynteny between the Arabidopsis and *Medicago* genomes (Zhu et al., 2003). However, functional studies described below confirmed that *MtSUP* is the orthologous gene of SUP.

**Expression analyses of MtSUP**

In agreement with the expected role of *MtSUP* during flower development, RT-PCR analysis showed that *MtSUP* is expressed exclusively in floral apices (Figure S2). *In situ* hybridization (Figure 1) shows that *MtSUP* transcript began to accumulate in the secondary inflorescence meristems (I2) (Figure 1a). Later on, *MtSUP* transcript is detected in the floral meristem (FM) once this meristem emerges from the I2 (Figure 1b, c). Once the FM flattens, *MtSUP* transcript accumulates in the meristematic cells that will give place to the common primordia (Figure 1d - f). Once the common primordia have differentiated,
MtSUP is expressed within them (Figure 1g). Later on, MtSUP restricts its expression to the differentiated stamen primordia (Figure 1h), until the late stages of stamen development (Figure 1i - l). MtSUP is also expressed in the carpel margin that will develop a parietal placenta from which the funiculi, integuments, and ovules will differentiate (Figure 1i - l). The expression pattern of MtSUP suggests that it might play a role both during inflorescence and floral development in M. truncatula.

**Complementation of sup-5 floral phenotype by MtSUP**

To test the functional conservation between MtSUP and SUP, we performed a complementation assay. We introduced the pSUP::MtSUP construct into the sup-5 mutant background, one of the strongest SUP mutant alleles (Gaiser et al., 1995; Breuil-Broyer et al., 2016). The construct contains 5.1 kb of the 5′ upstream SUP region, the coding sequence of MtSUP and 0.6 kb of the SUP 3′ non-transcribed region. Our study revealed that the stamen and carpel numbers of the sup-5 vary between flowers (Figure 2b, c; Table S3). Stamen number ranged from 8 to 14 (12.3±1.80; Table S3) and carpel number ranged from 2 to 5 (3.5±1.30, Table S3). Thirteen independent T0 pSUP::MtSUP; sup-5 transgenic plants were analysed (Figure S3). Most transgenic pSUP::MtSUP; sup-5 flowers showed a nearly wild-type flower phenotype regarding stamen (6.52±0.33) and carpel number (2.31±0.31, Table S3; Figure 2d - f). These results demonstrate the ability of the MtSUP protein to replace the SUP function in A. thaliana.

**Ectopic expression of MtSUP in Arabidopsis thaliana**

We ectopically expressed MtSUP to evaluate the effects of its constitutive expression in the heterologous system A. thaliana. 35S::MtSUP plants were smaller than wild-type plants (Figure 2g), showing a significant plant height reduction (Figure 2h). Also, 35S::MtSUP plants produced smaller leaves (Figure 2i), flowers (Figure 2k), and siliques (Figure 2l). We analysed the expression levels of the MtSUP transgene in the overexpression lines by quantitative RT-PCR (RT-qPCR) (Figure S4a) and detected a negative correlation between the plant height and the expression levels of MtSUP (Figure S4b). The constitutive expression of MtSUP leads to dwarfism, the same phenotypical effect described
for Arabidopsis 35S::SUP plants (Hiratsu et al., 2002). Similar phenotypes have been described in plants that overexpress SUP-like genes confirming that the activity of SUP controlling cell proliferation is conserved among species (Nakagawa et al., 2004; Nibau et al., 2011; Zhao et al., 2014). Besides, we often found flowers with floral defects in the number and size of floral organs especially in whorls 2, 3, and 4 (Figure 2n - p). The lack of stamens linked to increased carpel numbers suggests that some third-whorl stamens were transformed into carpels and then fused in the fourth whorl (Figure 2n - p). Similar floral defects were observed in Arabidopsis and tobacco after the ectopic expression of SUP using the AP1 promoter (Yun et al., 2002).

Molecular characterization of MtSUP mutants

To investigate the function of MtSUP in M. truncatula, we took advantage of a Tnt1 transposon insertion library, constructed in the R108 line of M. truncatula (Cheng et al., 2011, 2014). Genotyping of the Tnt1 insertion line identified homozygous plants for this insertion, which we name from now as mtsup-1 (Figure S5b - d). The retrotransposon insertion was located 50 bp downstream of the ATG start codon of MtSUP (Figure S5a). Moreover, using the CRISPR/Cas-9 system (Jinek et al., 2013; Ran et al., 2013), we generated an additional mtsup mutant allele. The molecular analysis of several independent T0 transgenic lines revealed that the MtSUP-sgRNA line 1 (named as mtsup-2 allele) carried a four nucleotides deletion as heterozygous at the predicted Cas9 editing site (Figure S5f). T0 transgenic plants were self-pollinated and the genomic fragment of MtSUP from T1 transgenic plants was sequenced, thus identifying homozygous edited plants with the mtsup-2 allele (Figure S5f). We performed RT-PCR to analyse the expression of MtSUP in mtsup alleles. Absence of MtSUP full coding-sequence transcripts in mtsup-1 floral apices was confirmed by RT-PCR (Figure S6a), while mtsup-2 carrying a deletion in the gene sequence produced a transcript (Figure S6b) that putatively generates a truncated protein containing the first 39 amino acids (Figure S6c, d). Both mutant alleles showed defects in inflorescence architecture and floral development, which denotes an impairment of MtSUP function.

MtSUP mutations lead to a multi-flower phenotype in M. truncatula
Medicago truncatula ecotype R108 inflorescences produce from one to two flowers and a spike (Figure 3a). Most of the inflorescences in the wild type (90%) carried a typical single flower per peduncle, while two flowers per peduncle were scarcely observed (Figure 3a, i). In contrast, mtsup alleles produced from two to four flowers per peduncle at most of the flowering nodes (Figure 3b - h). While wild-type flowers carried the spike (Figure 3a), the mtsup flowers lacked this organ (Figure 3b - h). The majority of the mtsup-1 flowers showed two to three flowers (43% each), while only 3.4% of the peduncles showed single flowers (Figure 3c - g, i). The remaining flowering nodes produced four flowers per peduncle (10%), (Figure 3g - i). The mtsup-2 allele produced twin flowers (76%) in almost all flowering nodes, and the remaining were single flowers without spikes (Figure 3b, c, i). Therefore, mtsup alleles produced more flowers than the wild type (Table 1). We characterized the multi-flower phenotype of the strongest mtsup-1 allele using Scanning Electron Microscopy (SEM). A wild type inflorescence development follows an acropetal succession in which the oldest flower is at the bottom and the inflorescence meristems (I1 and I2) at the top (Figure 3j). Two flowers at different developmental stages are observed, each with a bract and a spike (Figure 3j). In contrast, mtsup-1 inflorescences harbour an increased number (6-7) of flowers at different developmental stages (Figure 3k, Figure S7). These observations indicate that the I2 meristems generate more flower primordia than the wild type. Also, after the floral meristem develops from the axil of each bract no spike is generated, instead the I2 meristem terminates as a flower meristem (Figure 3m). Besides, we examined the relationship between multi-flower phenotype and the expression patterns of inflorescence markers MtFRUITFULLc (MtFULc) and MtPROLIFERATING INFLORESCENCE MERISTEM (MtPIM) (Cheng et al., 2018) in mtsup-1 and wild-type plants by in situ hybridization experiments (Figure 4). We also analysed the effect of the mtsup-1 mutation on the expression levels of MtFULc, MtPIM and MtSINGLE LEAFLET 1 (MtSGL1) genes (Figure S8). MtFULc and MtPIM are MADS-box genes responsible for meristem identity acquisition of I2 and FM, respectively, and they mutually repress each other (Benlloch et al., 2006; Cheng et al., 2018). The MtSGL1 gene plays a synergistic role with MtPIM in floral meristem identity and it is required for petal/stamen specification from common primordia in M. truncatula.
In situ hybridization showed that MtFULc signal is restricted to the I2 meristem both in the wild type and mtsup-1 (Figure 4a - e). Once the FM is produced, a weak MtFULc signal was detected in the wild type I2 meristem (Figure 4c). In contrast, in mtsup-1, a stronger MtFULc signal occupies a wider area in the I2 meristem that gives place to two FMs (Figure 4f). In the wild type, MtPIM expression is restricted to the young FM (Figure 4g), while in mtsup-1, MtPIM expands its expression to the I2 meristem (Figure 4h). Later on, MtPIM was detected in the bract, sepal, and the common primordia of the two floral primordia generated by the I2 (Figure 4j), whereas a similar expression was detected in the single floral primordial of wild-type flowers (Figure 4i). Both the ectopic MtPIM expression and its detection in more flower primordia than in the wild type are consistent with the up-regulation of MtPIM in the mtsup-1 mutant (Figure S8). MtFULc mRNA levels decreased in the mtsup-1 lines, as compared to the wild type (Figure S8), despite MtFULc transcript displays a broader area of expression (Figure 4f). The mtsup-1 mutation does not change the expression levels of SGL1, suggesting that SGL1 acts upstream of MtSUP during inflorescence and flower development, as described for LFY in A. thaliana (Sakai et al., 2000). These results demonstrate that MtSUP participates in the development of the compound inflorescence in M. truncatula.

MtSUP mutations affect floral development.

The MtSUP mutants produce flowers displaying a range of floral phenotypes that we classified in four phenotypical classes from 1 to 4 based on abnormal floral organ number (Table 1; Figure 5). M. truncatula flowers show a pentamerous arrangement of sepals and petals, ten stamens and a central carpel (Benlloch et al., 2003). Flowers showing a class 1 floral phenotype had a standard number of floral organs, but fewer ovules (Figure 5a; Table 1). Flowers classified as class 2, displayed two or three carpels fused at their base, and often, exposed or absent ovules (Figure 5c, d; Table 1). Flowers with more petals (from 6 to 8), fewer stamens (from 6 to 9), and deformed carpels with less ovules were found only in the mtsup-2 allele (class 3 phenotype) (Table 1, Figure 5a, g, h). The class 4 phenotype corresponds to flowers with supernumerary organs: 6 to 8 petals, 12 to 16 stamens, and two independent carpels bearing less ovules (Table 1; Figure 5a, i, j). These flowers were only
observed in the mtsep-1 mutant allele (Table 1). In summary, MtSUP mutants frequently showed an increased number of floral organs in the second, third and fourth whorls (Figure 5). Similar supernumerary organs in the third and fourth whorls are found in A. thaliana sup mutants (Bowman et al., 1992; Gaiser et al., 1995; Sakai et al., 1995; Jacobsen and Meyerowitz, 1997; Breuil-Broyer et al., 2016). These floral phenotypes were linked to a delay in WUSCHEL (WUS) repression at floral meristem centre (Prunet et al., 2009, 2017; Breuil-Broyer et al., 2016) and expansion of AP3 and PI expression to the centre of the Arabidopsis flower primordia (Sakai et al., 1995; Breuil-Broyer et al., 2016; Prunet et al., 2017). Thus, we followed MtWUS (Chen et al., 2009) expression by in situ hybridization in floral apices in the mtsep-1 mutant. Our results showed that MtWUS is firstly expressed during floral transition since MtWUS accumulates in the wild type I1 and I2 meristems (Figure 5k). The same expression pattern was observed in mtsep-1 (Figure 5o). Later on, a strong MtWUS signal occupies the wild-type floral meristem centre (FMC) and the nascent two floral meristems in mtsep-1 (Figure 5l, p). We did not detect MtWUS expression in the wild-type floral primordia at stages 3 and 4 of floral development (Figure 5m, n), while MtWUS expression is maintained until stage 4 in the mtsep-1 mutant flower (Figure 5q, r). Our results revealed a prolonged MtWUS expression during floral development in mtsep-1. In addition, in situ hybridization was performed to analyse the expression of MtPI, the master regulator of B-function in M. truncatula (Benlloch et al., 2009; Roque et al., 2016). MtPI showed strong expression in the common primordia both in the wild type and in the mtsep-1 floral primordia (Figure 5s, t). In mtsep-1, MtPI showed weak ectopic expression in sepals and in the I2 meristem (Figure 5t). Later on, MtPI expression was detected in the common primordia of twin mtsep-1 flowers produced by the I2 meristem (Figure 5u). RT-qPCR experiments indicated that the expression of the B-function genes MtPI, MtNMH7 and MtTM6 (Benlloch et al., 2009; Roque et al., 2013, 2016) was significantly increased in mtsep-1, while MtNGL9 expression did not seem to be affected by the impairment of MtSUP function (Figure S8). This effect is consistent with the minor contribution of MtNGL9 to the B-function during flower development (Roque et al., 2016).

**MtSUP mutations affect gynoecium and fruit development.**
$mtsup$ mutants produced flowers with a reduced number of ovules (Table 1). Histological examination of gynoecia anatomy revealed that ovules from $mtsup$ mutants were smaller and lacked the funiculus compared to the wild type (Figure 6a - d). $mtsup$ mutants had a significantly reduced medial gynoecia area compared to the wild type (Figure 6e). $mtsup$ mutants also showed a reduced fruit production (Table 1) and their pods contained defective or absent seeds (Figure 6g bottom right; Table 1). Also, the $mtsup$ pods were lopsided, lacking the typical coiled barrel-shaped structure, and the expected immature spines in the pod surface (Figure 6g) that are observed in the wild-type pod at similar stage (Figure 6f). We conclude that $mtsup$ mutations affect the development of the carpel medial tissues, resulting in collapsed areas in the mature carpel. As a consequence, ovule, seeds and fruit development were affected, thus contributing to the reduced fruit production and fertility observed in the $mtsup$ mutants.

**DISCUSSION**

_MtSUP_ controls the number of floral organs in the inner three whorls of _M. truncatula_.

_SUPERMAN_ is a gene that participates in the control of the number of carpels and stamens produced in _A. thaliana_ (Schultz _et al._, 1991; Bowman _et al._, 1992; Gaiser _et al._, 1995; Jacobsen and Meyerowitz, 1997; Breuil-Broyer _et al._, 2016). This function is conserved in _M. truncatula_ according to the floral phenotypes displayed by _MtSUP_ mutants. The $mtsup$-2 (class 3) and $mtsup$-1 (class 4) floral phenotypes resemble to the _Arabidopsis sup-1_ and _sup-5_ alleles, respectively. The _sup-1_ flowers exhibit additional stamens at the expense of the carpel (Bowman _et al._, 1992; Breuil-Broyer _et al._, 2016), but in $mtsup$-2, additional petals at the expense of stamens are formed. The strong _sup-5_ allele produces more stamens and additional carpels (Gaiser _et al._, 1995; Breuil-Broyer _et al._, 2016), but in $mtsup$-1, also more petals are produced. The differences among the floral phenotypes of $mtsup$-1 and $mtsup$-2 alleles might be attributed to the different nature of their mutations. While no transcript of _MtSUP1_ could be detected by RT-PCR from floral apices of the $mtsup$-1 mutant, the $mtsup$-2 mutant putatively produced a non-functional MtSUP protein as a consequence
of a premature stop codon in the transcribed mRNA. However, it should not be ruled out that this defective transcript might generate a truncated protein with some function. These Arabidopsis sup alleles show ectopic expression of PI and AP3 in the fourth whorl region (Sakai et al., 1995; Breuil-Broyer et al., 2016; Prunet et al., 2017). However, in mtsup-1, the expression of MtPI was not detected in the fourth whorl. Instead, it expanded towards the indeterminate I2 meristem (Figure 4t, similarly to MtPIM (Figure 4h). MtPIM and MtPI seem to be recruited to specify petals and stamens in the new floral primordia derived from the I2 meristem. The Medicago B-function genes are upregulated in mtsup-1. Therefore, MtsUP could be repressing B-class MADS-box genes as it has been proposed for SUP in Arabidopsis (Yun et al., 2002; Prunet et al., 2017). This is in accordance with the conservation of the biological function of MtsUP demonstrated by its ability to complement the phenotype of the strong sup-5 mutant allele. Besides, the floral homeotic defects found in several 35S::MtsUP flowers were similar to those observed when the AP1 promoter drove the ectopic expression of SUP to the Arabidopsis flower (Yun et al., 2002).

Interestingly, the supernumerary petals phenotype exhibited by the mtsup alleles, is a distinctive trait in M. truncatula that correlates well with the early expression of MtsUP in the common primordia. It is assumed that floral organ primordia are initiated from a constant number of cells (Bossinger and Smyth, 1996). Thus, MtsUP might be performing a similar role to SUP repressing cell proliferation but in the common primordia. Production of extra petals and stamens in the mtsup flowers could be the consequence of an over-proliferation of the meristematic cells that express the Medicago floral organ identity A- and B-class of MADS-box genes. This effect is in agreement with the cell anti-proliferation effect of MtsUP found in 35S::MtsUP transgenic plants and the increase in the transcript levels of the M. truncatula A- and B-class MADS-box genes (Benlloch et al., 2006, 2009; Roque et al., 2013, 2016). Based on our results, we propose that MtsUP could regulate the termination of the common primordia meristem.

Prolonged maintenance of the stem cells in the floral meristem (FM) has been related to extra organs (Bowman et al., 1989; Bossinger and Smyth, 1996). The supernumerary organs in the inner three whorls in MtsUP mutants could also be explained based on a delay in floral meristem termination (FMT). In MtsUP
mutants, the delay in FMT was linked to prolonged expression of \textit{MtWUS}. The precise timing of \textit{MtWUS} turn off during flower development is not yet known. Our results suggest that \textit{MtWUS} expression ceases early after the floral apex flattens. An early FMT is consistent with one of the features of floral ontogeny in legumes: the early carpel primordium initiation (Ferrándiz \textit{et al.}, 1999; Benlloch \textit{et al.}, 2003). \textit{MtSUP} and \textit{MtWUS} expression patterns show a spatial overlap in the I2 and FM (Figure 1a - c; Figure 4k, l; Figure S9), while in \textit{A. thaliana} \textit{SUP} and \textit{WUS} show almost no spatial overlap. Consequently, \textit{SUP} does not directly regulate \textit{WUS} expression. Therefore, the effect of \textit{SUP} on \textit{WUS} is largely non cell-autonomous (Prunet \textit{et al.}, 2017). Our results provide evidence that FMT in \textit{M. truncatula} requires a precise timing of \textit{MtWUS} turn off, and that \textit{MtSUP} is involved in this process. Whether the overlapping expression pattern of \textit{MtSUP} and \textit{MtWUS} would allow a direct interaction between them or with other MADS-box genes would be involved in this process needs further investigation.

Proper FMT is required to form carpel primordia (Sakai \textit{et al.}, 2000; Prunet \textit{et al.}, 2008). \textit{MtSUP} mutants show defects in many structures derived from the marginal tissues of the gynoecium resulting in a reduced reproductive success. Similar defects in placental development were reported for the \textit{phsup-1} mutant of petunia (Nakagawa \textit{et al.}, 2004). Also, \textit{mtsup-1}, \textit{mtsup-2} and \textit{sup-5} mutants share the shortening of funiculus and reduced ovule numbers (Gaiser \textit{et al.}, 1995). Defects in different tissues of the gynoecium in \textit{MtSUP} mutants are consistent with alterations at the early stages of the carpel medial region development. This fits with \textit{MtSUP} early expression in the inner carpel region. Our findings support that \textit{MtSUP} is required for proper medial tissue development in \textit{M. truncatula}, a similar function assigned to \textit{SUP} in \textit{A. thaliana} (Breuil-Broyer \textit{et al.}, 2016). In summary, our results show that known functions of \textit{SUP} in the control of cell proliferation, the precise timing of FMT, and the carpel media region development are conserved in \textit{M. truncatula}. However, \textit{MtSUP} controls not only the number of floral organs in the inner two whorls, as \textit{SUP} in Arabidopsis, but also in the second whorl of the flower. This difference might fall into the existence of common primordia meristems, in which \textit{MtSUP} seems to control cell proliferation.
**MtSUP controls the activity of the secondary inflorescence meristem in M. truncatula**

*MtSUP* mutants not only produce a proliferation of organs in the inner three whorls but also more flowers per peduncle in most of the flowering nodes. The multi-flower phenotype exhibited by the *MtSUP* mutants share similarities with mutants reported in other legume species, such as chickpea (*Cicer arietinum*) and pea (*Pisum sativum*) (Lamprecht, 1947; Murfet, 1985; Singer *et al.*, 1999; Gaur and Gour, 2002; Srinivasan *et al.*, 2006; Devi *et al.*, 2018). This trait is of agricultural interest in grain legumes since it is expected that multi-flower plants would become multi-pod plants (Benlloch *et al.*, 2015). However, this is not the case of *MtSUP* mutants, as fruit yield was reduced because of an abnormal carpel and ovule development. In other legumes, it is common that natural mutations that produce multi-flower phenotype do not directly produce more pods (Gaur and Gour, 2002; Srinivasan *et al.*, 2006). No genes controlling the number of flowers per I2 have been identified in legumes (Benlloch *et al.*, 2015; Devi *et al.*, 2018).

*MtSUP* mutations do not affect the specification of the I2 meristem identity since these are typically produced from the I1 meristem, which was confirmed by the detection of the I2 meristem marker MtFULc (Figure 4a - f) and SEM images (Figure 3k, l; Figure S6). The impairment of *MtSUP* function leads to the production of additional flowers. Thus, *MtSUP* is controlling the activity of the I2 meristems, as it is known that their activity defines the number of flowers produced (Tucker, 2003; Benlloch *et al.*, 2007, 2015). This is consistent with the spatial and temporal expression patterns of *MtSUP* in the I2 and floral meristems during inflorescence development. The I2 meristem, after producing from one to two flowers, terminates in a residual organ or spike (Figure 3k) (Benlloch *et al.*, 2003; Tucker, 2003). In contrast, in *MtSUP* mutants, the I2 meristem terminates in a flower instead of a spike (Figure 3l). The origin of the inflorescence phenotype in *MtSUP* mutants might be explained by linking our results to the proposed models for SUP functions in *A. thaliana*. We propose that determinacy of the I2 meristem could be linked to a gradual extinction of MtWUS expression in the I2 since MtWUS expression ceases early, just before the floral apex flattens. *MtSUP* and MtWUS expression patterns show a spatial overlap in the I2 and FM (Figure 1a - c; Figure 4 k, Figure S9). There could be a
possible link between the delay in MtWUS expression turn off, and the delay in the I2 determinacy.

Alternatively, MtSUP might control the balance of cell proliferation in the I2, repressing cell divisions of meristematic cells that express the MADS-box gene MtFULc, since MtFULc transcript occupies a wider area in mtsup-1 than in the wild type once FMs arise from the I2. Besides this, mtsup phenotypes may be mediated by the repression of the MADS-box transcription factors that control the I2 and FM identity in M. truncatula. This prediction comes from the observation that MtSUP seems to restrict MtPIM expression to the FM, since the invasion of MtPIM to the I2 is produced in mtsup-1 apices (Figure 4e). As a consequence, the I2 meristems lose their vegetative nature and acquire floral identity instead of producing a spike (Figure 3k). Consequently, the mtsup-1 mutation leads to an increase in the transcript levels of MtPIM (Figure S8), triggering a strong repression of MtFULc (Figure S8) since the I2 transient meristem terminates as a FM. The spatial and temporal expression pattern of MtSUP in the I2 meristem, FM and common primordia could allow interactions with other target genes coordinately expressed, as the MADS-box genes MtPIM and MtFULc.

Further research is required to infer the molecular mechanism of MtSUP during this developmental process. Nevertheless, our work certainly provides valuable information about the genetic regulatory network behind compound inflorescence development in the model legume M. truncatula.

MtSUP and developmental specializations in inflorescence architecture and flowers in legumes

Genetic networks established between meristem identity genes during inflorescence development in the eudicot species Arabidopsis and M. truncatula share significant similarities (Blázquez et al., 2006). However, these eudicot species show different inflorescence architectures according to the complexity of their branching. Compound inflorescences in legumes are associated with high-order inflorescence meristems (I2) absent from the simple inflorescence of species such as Arabidopsis. The I2 identity specification mechanism is performed by MADS-box transcription factors belonging to the euFUL clade of the AP1/SQUA/FUL lineage in Pisum sativum and M. truncatula (Berbel et al.,
Our results add a new element to the genetic network that orchestrates compound inflorescence development in the model legume *Medicago truncatula*, the *MtSUPERMAN* gene. *MtSUP* participates in the process by which I2 meristems acquire a determinate fate controlling the number of flowers produced. This is a novel function for an ortholog of *SUP* in eudicots.

Interestingly, compound inflorescence formation in grasses involves high-order inflorescence meristems (Bommert *et al.*, 2005; Bortiri and Hake, 2007; Kellogg, 2007; Thompson and Hake, 2009; Gallavotti *et al.*, 2010). However, the genetic network controlling this process in grasses is different from that in legumes and it does not seem to involve an *euFUL* gene-related function. Nonetheless, in maize, the Cys2-His2 zinc-finger protein of the EPF class RA1, also promotes meristematic determinacy in the inflorescence (Vollbrecht *et al.*, 2005). The rapid evolution of C2H2 zinc-finger genes makes it difficult to assess the orthology relationships from phylogenetically distant plant families, but, intriguingly, these two related proteins seem to have similar roles in inflorescence meristem determinacy. Compound inflorescences have independently emerged several times during angiosperm evolution (Berbel *et al.*, 2012). Can changes in the spatial and temporal expression pattern of *SUP*-like genes be correlated with the differences in the inflorescence architecture among plant species? Regarding legumes, it would be interesting to know if higher levels of inflorescence complexity found in some species correlate with transcriptional differences of *MtSUP* orthologs in these species.

In *M. truncatula*, *MtSUP* has a role in the secondary inflorescence meristem and common primordia determinacy. Thus, *MtSUP* is linked to the unique and ephemeral meristems entirely consumed during inflorescence and flower development in legumes. One might speculate that transcriptional innovation of *MtSUP* could have been crucial for the developmental specialization in legumes. Further research is needed to determine how changes in the expression pattern of transcriptional repressors could explain a great part of the variation in the inflorescence architecture and flower morphology found among plant species.
EXPERIMENTAL PROCEDURES

Plant material and growth conditions
The wild type *Medicago truncatula* ecotype R108, *mtsup-1* and *mtsup-2* mutants were grown in the glasshouse at 22°C : 18°C (day : night) with a photoperiod of 16 h : 8 h (light : dark) in a mixture of soil : sand (3 : 1). Seeds were scarified with sulphuric acid during 5 min, rinsed in water and set on filter paper during 2 days at room temperature and then at 4°C during 9 days for germination. *Arabidopsis thaliana* cv. *Landsberg erecta* (Ler) and *sup-5* mutant ordered from the NASC stock centre were grown in the greenhouse at 21°C under long day conditions.

Sample collection and RNA and DNA isolation
Vegetative tissues of *M. truncatula* plants were collected from seedlings (root and shoot apical meristems) and from mature plants (stem and leaves). Reproductive tissues were collected from floral apices (6 weeks after germination) and flowers at anthesis. RNA isolation was performed from 0.1 g of tissue according to E.Z.N.A® Plant RNA kit recommendations (OMEGA bio-tek). Genomic DNA isolation was performed with DNazol® Reagent according to the instructions of the manufacturer (Thermo Fisher).

Sequence analysis
For phylogenetic tree, we used the retrieved sequences in the BLAST analysis against the Legume genomes database (https://legumeinfo.org/) (Table S1). We used Neighbor-Joining method (Saitou and Nei, 1987) conducted in MEGAX (Kumar et al., 2018), based on distance matrices from 10 000 bootstrap (Felsenstein, 1985) replicates and rooted to the EPF Zn finger protein *UPRIGHT ROSETTE* (*URO*) (Takatsuji, 1999). The conserved microsynteny between *MtSUP* and *SUP* was assessed using OrthoVenn web platform (https://orthovenn2.bioinfotoolkits.net). Protein alignment of *AtSUP*, *PhSUP* and *MtSUP* was performed using the PRALINE program (http://www.ibi.vu.nl/programs/pralinewww/) and Color Align Conservation program (https://www.bioinformatics.org/sms2/color_align_cons.html). *MtSUP* and *MtSUP-2* alignment used the same approach. We used EMBOSS Transeq
Overexpression assay and complementation test of the sup-5 mutant

*MtSUP* cDNA was cloned into the gateway vector pH7WG2 ([https://gatewayvectors.vib.be/](https://gatewayvectors.vib.be/)). We transformed Arabidopsis with *A. tumefaciens* strain GV3101::pMP90(RK) previously transformed with this construct (Bechtold and Pelletier, 1998). To perform the complementation assay we generated a gene construct containing the fragment of *SUP* genomic DNA sufficient for proper *SUP* expression (Ito *et al.*, 2003), taking advantage of the MultiSite Gateway LR recombination reaction between multiple entry clones and a Gateway Destination vector (pH7m24GW,3; [https://www.psb.ugent.be/](https://www.psb.ugent.be/)). To perform the complementation assay, we used the construct (pSUP::MtSUP) to transform the sup-5 mutant (Gaiser *et al.*, 1995) by floral dipping (Zhang *et al.*, 2006). The resulting transgenic pSUP::MtSUP; sup-5 plants were identified using specific primers for *MtSUP* (Table S4).

Generation of mutants by the CRISPR-Cas9 gene editing system

We used the ARES-GT software ([https://github.com/eugomin/ARES-GT](https://github.com/eugomin/ARES-GT)) (Minguet, 2020) to select the sgRNA CRISPR target. A schematic representation of the sgRNA is shown in Figure S5e. The CRISPR-Cas9 constructs were designed using the GoldenBraid 2.0 assembly approach (Sarrion-Perdigones *et al.*, 2013) ([https://gbcloning.upv.es/](https://gbcloning.upv.es/)). We generated a binary vector containing the hCas9 under the control of AtUBQ promoter, the hygromycin resistance and a transcriptional unit harboring three copies of the sgRNA downstream the AtU6-26 promoter (pAtU6-26). This construct was used to transform *M. truncatula* R108 (Cosson *et al*. 2015). Hygromycin [15 mg/l] was used for the selection of transformants and timentin [200 mg/l] plus carbenicillin [200 mg/l] for agrobacterium control.

Molecular and phenotypic characterization of *MtSUP* mutants

Genotyping of the Tnt1 insertion line (NF11278) ([https://medicago-mutant.noble.org/mutant/database.php](https://medicago-mutant.noble.org/mutant/database.php)) was performed by PCR using a primer specific for the target gene *MtSUP* (Table S1) and a primer specific for the LTR
border of the Tnt1 retroelement. The segregation analysis of heterozygous F1 and F2 MtSUP/mtsupsup-1 showed the expected 1 : 2 : 1 segregation, in which only the homozygous plants had a mutant phenotype. For the CRISPR-Cas9 mutant, the putative transgenic T0 and T1 plants were subjected to PCR using primers specific for hCas9 and MtSUP (Table S4). The amplicon generated for MtSUP by PCR was directly sequenced to analyse their plausible editions using the DSDecode (Liu et al., 2015) and TIDE (Brinkman et al., 2014) web-based tools. For both alleles (mtsupsup-1 and mtsupsup-2), transcript analysis was performed by RT-PCR with primers for the complete coding sequence. The PCR product from mtsupsup-2 was sequenced using the -52-MtSUP-F primer (Table S4). Phenotypical characterization of mtsup mutants was assessed using ten independent plants as biological replicates for each line. 120 inflorescences, from 50 day old plants, were analysed to characterize the multi-flower phenotype of each allele. The average floral organ number per whorl was determined by dissecting 100 flowers at anthesis (FA) for each line. The fruit yield production was calculated based on the percentage of FAs that produced a mature fruit (N= 100).

**RT-qPCR analysis**

Total RNA (2 µg) was treated with DNase I, according to the manufacturer (Thermo Scientific). One microgram of treated RNA was retrotransribed using the PrimeScript™ RT-PCR kit (Takara, Japan). Two µl of this reaction were used to perform the RT-PCR analysis using the Speedy Supreme NZYTaq 2X green master mix (NZY tech, Portugal), with MtACTIN (Medtr7g026230) as the control gene. Twenty nanograms of cDNA were used for RT-qPCR with the respective primers (300 nM) were mixed with the EvaGreen® Master Mix (Cultek). Reactions were performed in 96 well-optical plates using a 7500 Fast Real Time PCR System (Applied Biosystems). For relative expression, TIP41 (AT4G34270), Secret Agent (O-linked N-acetyl glucosamine transferase (Medtr1g079510) and MtACTIN (Medtr7g026230) were used to normalize with the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001) for Arabidopsis and Medicago respectively. Primer Express Software (Applied Biosystems, USA) with the default parameters was used to design the primers listed in Table S4.
Microscopy

Images of flowers, plants or fruits were obtained using a stereomicroscopes (Leica MZ28) or (Leica 1000). For histological examination, tissues were fixed in FAE (formaldehyde : acetic acid : ethanol, 10% : 5%: 50%) and embedded in paraffin or synthetic resin (Leica®). Resin-embedded flowers were sectioned and stained with toluidine blue solution (1%) and then were imaged using light microscopy (Leica, DM5000). For scanning electron microscopy (SEM), fixed samples were dehydrated in an ethanol series, critical-point dried in liquid CO₂ with an Automated Critical Point Dryer (Leica EM CPD300), sputter coated with gold in a 90s pulse, and photographed with an electronic microscope (Zeiss Gemini). Images were acquired using a 20 µm aperture and 2 KV of exposition.

In situ hybridization

RNA in situ hybridization with digoxigenin-labelled probes was performed as described previously (Gómez-Mena and Roque, 2018). The RNA antisense and sense probes were generated using the T7 and SP6 polymerases using specific fragments of the MtSUP, MtPIM, MtFULc, MtPI, MtWUS genes, and cloned into the pGEM-T Easy vector (Promega).

Carpels clearing

Carpels from wild type R108 and mtsup-1 plants were treated according to Noguero et al. (2015) with chloral hydrate solution for 6 h and observed with a Microscope (Nikon, Eclipse E600) equipped with Nomarski DIC optics.

Medicago truncatula Accession Numbers

M. truncatula gene sequences used this study are in the release Mt4.0v1 from the M. truncatula genome project under the following accession numbers: MtPIM (Medtr8g066260); MtSGL1 (Medtr3g098560); MtNMH7 (Medtr3g113030); MtTM6 (Medtr5g021270); MtNGL9 (Medtr1g029670); MtPI (Medtr3g088615); Medtr5g021930 (MtWUS); MtFULc (Medtr7g016630) and Secret Agent (O-linked N-acetyl glucosamine transferase (Medtr1g079510); MtACTIN (Medtr7g026230).
DATA AVAILABILITY STATEMENT
All relevant data can be found within the manuscript and its supporting materials.

ACKNOWLEDGEMENTS
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AUTHOR CONTRIBUTIONS
E.R., J.P.B and L.A.C conceived and designed the experiments. A.L.R., E.R., R.H, C.G-M, J.W and K.S.M performed the experiments. C.G.M, J.P.B and L.A.C contributed with valuable comments during the manuscript writing. E.G.M performed new analytic and computational tools. A.L.R and E.R analysed the data. E.R and A.L.R wrote the manuscript.

CONFLICT OF INTEREST STATEMENT
The authors declare no conflict of interests.

SHORT SUPPORTING MATERIAL LEGENDS
Figure S1. Sequence analysis of SUPERMAN-like genes.
Figure S2. RT-PCR analysis of the putative MtSUP in different tissues of M. truncatula.
Figure S3. Genotyping of transgenic pSUP::MtSUP; sup-5 plants.
Figure S4. Transcript levels of MtSUP in the 35S::MtSUP lines and their correlation with plant height.
Figure S5. Molecular characterization of MtSUP mutants.

Figure S6. Transcript analysis of MtSUP mutants.

Figure S7. SEM images of the wild type and mtsup-1 inflorescence apices.

Figure S8. qRT-PCR analysis of meristem identity genes MtFULc, MtPIM, MtSGL1 and the Medicago B-class genes.

Figure S9. Expression pattern of MtSUP and MtWUS during floral development in M. truncatula.

Table S1. Resulting BLAST hits for SUP-like genes in legumes.

Table S2. Orthologous gene clusters among A. thaliana and M. truncatula.

Table S3. Number of stamens and carpels in pSUP::MtSUP; sup-5 transgenic plants.

Table S4. Primers used in this work.

REFERENCES


pp. 43-56.


Lamprecht, H. (1947) The inheritance of the number of flowers per inflorescence and the origin of Pisum; illustrated by polymeric genes. *Agricultural and Horticultural Genetics, 1*, 16-25.


**FIGURE LEGENDS**

Figure 1. Expression pattern of *MtSUP* during inflorescence and floral development in *Medicago truncatula*. (a) *MtSUP* expression was first detected in the secondary inflorescence meristem (I2) and in the emerging floral meristem (FM). (b-c) *MtSUP* was strongly expressed in the emerging FM, similar to *MtPIM* expression. (d) At stage 2, *MtSUP* begins to accumulates in the meristematic cells of the floral
primordium from which will differentiate the common primordia. (e,f) At late stage 2 (e) and stage 3 (f), MtSUP mRNA accumulates in the cells of the floral primordium from which will differentiate the common primordia (arrow). (g) At late stage 4, MtSUP expression was detected in the common primordia that will differentiate the petals and stamens. (h) At stage 5, MtSUP was detected in the stamen primordia. (i) At stage 7, MtSUP mRNA accumulates in the placenta and also displayed a weak expression in the developed stamens. (j) Cross-section of floral primordia at late stage 4. MtSUP expression is detected in the common primordia (black arrow). Also, it is strongly expressed in the early carpel margins (white arrow). (k) Cross-section of floral primordia at stage 5. MtSUP is detected in the developed stamens and in the carpel margins. (l) Cross-section of floral primordia at late stage 7. MtSUP expression is detected in the stamens and in the carpel margins. I1: Primary Inflorescence meristem; I2: Secondary Inflorescence meristem; Lf: Leaf; FM: Floral meristem; CP: Common primordia; P: Placenta; St: Stamens; CM: carpel margins; F(N): Floral primordium at ‘N’ stages of development according to Benlloch et al., 2003. Scale bars: (a-i) 50 µm, (j-l) 20 µm.

Figure 2. Complementation of sup-5 floral phenotype and effects of MtSUP overexpression in Arabidopsis. (a) Arabidopsis wild type Ler (WT) flower with 6 stamens and one gynoecium consisting in two fused carpels. (b,c) sup-5 flowers exhibiting more stamens (12.0±1.80) and more carpels (3.5±1.30). (d,e,f) Flowers from transgenic pSUP::MtSUP;sup-5 plants with a partial recovery of stamen and carpel number (d); full recovery of stamen number and a nearly full recovery of sup-5 carpel number (e); full recovery of flower development (f). (g) Dwarf 35S::MtSUP plant compared with the wild type. (h) Quantitative differences in plant heights of 34-days-old wild type and 35S::MtSUP plants. Boxes represent the first, second (median) and third quartiles. Whiskers show 1.5 x interquartile range. A Kruskal-Wallis test for independent samples (n=17, *** when P-value < 0.001) was performed to analyse significant differences between wild type and 35S::MtSUP plants. (i) Differences in cauline leaf (arrow) in the wild type compared with the 35S::MtSUP plants (arrow). (j) Arabidopsis wild type flower (left) and a wild-type petal (right). (k) 35S::MtSUP flower (left) and its petal (right) displaying a size reduction compared with the wild type. (l) Siliques from wild type and 35S::MtSUP plants. (m) Arabidopsis wild type Ler (WT) flower. (n) 35S::MtSUP flower showing normal number of sepals. Most of petals and stamens are missing with the exception of an undeveloped stamen indicated with a red arrow. Carpel was wider than the wild type, likely as consequence of transformed stamens into carpels and their fusion with the fourth-whorl (yellow arrow). (o)
$35S::MtSUP$ flower showing normal number of sepals. Most of petals and stamens are
missing with the exception of undeveloped petals and stamens indicated with a white
and red arrow, respectively. (p) $35S::MtSUP$ flower showing a decrease of organ
number in whorl 1 and a lack of petals and stamens. Undeveloped petal and stamen
are indicated with a white and red arrow, respectively. P: petals; S: sepals; St:
stamens; C: carpel. Scale bars: (a-f) 0.75 mm, (g) 2.5 cm, (i) 10 mm, (l) 5 mm, (j, k, m-
p) 1 mm.

Figure 3. Inflorescence phenotypes in the wild type and $MtSUP$ mutants. (a)
Typical wild type (R108) inflorescence with one (singlet) or two (doublet) flowers per
peduncle with spike (spk). (b-c) Inflorescences of $mtsup-2$ with one (singlet) (b) or two
(c) flowers per peduncle without spike. (d-f) Inflorescences of $mtsup-1$ with three
flowers per peduncle (triplet) without spike. In (e) the three flowers are at a similar
stage. In (d) and (f) the third flower is very young (yellow arrows). (g-h) Four flowers
per peduncle (quadruplet) in $mtsup-1$ inflorescences. The third and fourth flowers are
younger than the other two (yellow arrows). (i) Percentage of each type of
inflorescence in the wild type and $MtSUP$ mutants. A total of 120 inflorescences were
analyzed. (j-k) SEM images of the wild type and $mtsup-1$ inflorescence apices. (j) In an
individual wild type inflorescence two flowers at different developmental stage (F8 and
F4) are observed with their bract and spike. Development follows an acropetal
succession in which the oldest flowers are at the bottom and at the top are the
inflorescence meristems (I1 and I2). (k) In an individual $mtsup-1$ inflorescence, 6
flowers at different developmental stages (F8, F7, F4, F3, F2) are observed. These
flowers have a bract but no spike is generated instead another flower is produced
(yellow arrow). Inflorescence meristems (I1 and I2) and FM are observed at the top.
(l,m) Schematic representation of the inflorescence meristems identity in the wild type
$M. truncatula$ and $MtSUP$ mutants. (l) In wild type, the vegetative meristem (VM)
converts to a primary inflorescence meristem (I1), which generates secondary
inflorescence meristems (I2) that produce the flowers. The I2 meristems produce one
to two flowers (FM) before generating a spike. (m) In $mtsup$ mutants, inflorescence
development is similar to the wild type until the I1 generates an I2. Unlike the wild type,
the I2 meristem prolongs its activity (in red) producing more flowers and terminates in a
flower meristem instead a spike. Br: Bract; FM: Floral meristem; F(N): Flower
primordium at “N” stages of development according to Benlloch et al., 2003. spk: Spike;
Scale bars: (a-h) 2 mm, (j) 20 µm, (k) 100 µm.
Figure 4. Expression patterns of MtFULc, MtPIM, MtWUS and MtPI genes. In situ hybridization in mtsup-1 compared with the wild type. (a-c) MtFULc mRNA localization is restricted to the I2 meristem in the wild type. In c, a weak MtFULc signal was detected in the I2 meristem once the FM has developed from the I2 (yellow arrow). (d-f) MtFULc mRNA localization in a young I2 meristem of mtsup-1. In f, A stronger and wider signal of MtFULc was detected in the I2 meristem once the two FMs has just emerged from the I2 (yellow arrow). (g) Restricted expression of MtPIM in the floral meristem in the wild type inflorescence. (h) MtPIM is strongly expressed in the floral meristem and also a weak signal was detected in the I2 meristem in mtsup-1. (i) MtPIM is expressed in the bract and in sepal and petal primordia. (j) MtPIM mRNA localization in the two flower primordia developed from the I2 in mtsup-1. MtPIM is expressed in the bract and in sepal and petal primordia. (k-n) MtWUS expression in the wild type. MtWUS mRNA was detected until floral meristem has fully arisen from the I2 (l). (o-r) MtWUS expression in mtsup-1 mutant. MtWUS expression was detected in the flower primordium until stage 4 of floral development (q,r). (s) MtPI expression is detected in common primordia in the wild type. (t) MtPI expression is detected in the common primordia in mtsup-1. Also, MtPI was weakly detected in the bract, sepals and in the I2 that should become a spike, but acquired floral identity. (u) MtPI mRNA accumulates in the common primordia, just before differentiated petal and stamen primordia in the mtsup-1 twin-flower primordia. (v) Primary inflorescence meristem. (w) Secondary inflorescence meristem. FM: Floral meristem. CP: common primordia. S: Sepals. C: carpel primordium. Floral developmental stages were defined according to Benlloch et al., 2003. Scale bars: (a-j) 20 µm, (k-u) 50 µm.

Figure 5. Floral phenotypes of MtSUP mutants. (a) Schematic representation of different flower phenotypes of mtsup alleles. According to the severity of the defects, flowers were classified in four classes (1 to 4). (b) SEM images of a wild type carpel. (c) SEM images of mtsup-1 flowers with two fused carpels (Class 2). (d) SEM images of mtsup-1 flowers with three fused carpels (Class 2). (e) Wild type M. truncatula flower. Five sepals in the first whorl (W1). (f) Floral organs in the wild type. In the whorl 1 (W1), the typical 5 sepals. In the second whorl (W2), one standard petal, two wing petals and a keel petal (formed by two fused petals), ten stamens in the third whorl (W3), nine fused and one free forming the staminal tube. One carpel in the fourth whorl (W4). (g) Floral phenotype of class 3 mtsup-2 flowers. (h) Floral organs in a typical class 3 (mtsup-2). In the whorl 1 (W1), the typical 5 sepals, In the whorl 2 (W2), an increased number of petals (6 petals: 2 standards (Std), 2 wings and the keel) and a reduced number of stamens in W3 (7 stamens). (i) Floral phenotype of a typical class 4
mtsup-1 flower. (j) Floral organs in a typical class 4 mtsup-1 flower. In the whorl 1 (W1), the typical 5 sepals are observed. In the whorl 2 (W2), an increased number of petals (8 petals: 2 standards, two keels and two wings), fourteen stamens in W3, and 2 carpels in W4. Scale bars: (b-d) 50 µm, (e-j) 0.5 mm.

Figure 6. MtSUP mutations affect gynoecium and fruit development. (a) Differential interference contrast (DIC) microscopic image of an optically cleared gynoecium of M. truncatula. Arrows indicate the funiculus. (b) DIC microscopic image of an optically cleared gynoecium of mtsup-1. Lacking of funiculus in mtsup-1 ovules (arrows). (c) Cross-section of M. truncatula wild type gynoecium (pre-anthesis stage). (d) Cross-section of mtsup-1 gynoecium (pre-anthesis stage). (e) Reduction of medial gynoecia tissues in mtsup mutants. Three cuts at the same level of the gynoecia from three different flowers (independent plants) were used to calculate the average area of the ovary and the ovule + funiculus (labeled as ovule area) area. Fiji software was used to calculate the area in square millimetres (Schindelin et al., 2012). A Kruskal-Wallis test for independent samples (n=3, *** when p-value < 0.001) was performed to analyze significant differences between wild type and MtSUP mutants gynoecium areas. (f) M. truncatula wild type fruit at stage 5 (4 DAP= four days after pollination). Wild type mature seeds (bottom right). (g) Typical abnormal fruit of MtSUP mutants at similar stage. Typical seeds from MtSUP mutants. mtsup-1 mature seeds (bottom right). ICM: Inner Carpel Margin; Fu: Funiculus; Ov: Ovule. Scale bars: (a-d) 100 µm, (f, g) 2 mm.

Table 1. Effect of MtSUP mutations on inflorescence, floral and fruit development.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Flowers / peduncle</th>
<th>Class</th>
<th>Stamen No.</th>
<th>Petal No.</th>
<th>Ovule No.</th>
<th>Floral level</th>
<th>Fruit yield production</th>
<th>Seed production</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>1.10±0.32</td>
<td>WT</td>
<td>10.0±0.10</td>
<td>1.0±0.10</td>
<td>5.00±0.00</td>
<td>9.10±0.50</td>
<td>100%</td>
<td>77%</td>
</tr>
<tr>
<td>mtsup-1</td>
<td>2.60±0.73</td>
<td>1</td>
<td>10.0±0.15</td>
<td>1.02±0.15</td>
<td>5.00±0.00</td>
<td>7.80±0.80</td>
<td>71.0%</td>
<td>58%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>10.0±0.19</td>
<td>2.40±0.49</td>
<td>5.00±0.00</td>
<td>7.40±0.70</td>
<td>18.7%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>14.0±1.64</td>
<td>2.20±0.40</td>
<td>7.60±0.51</td>
<td>6.15±2.10</td>
<td>10.3%</td>
<td></td>
</tr>
<tr>
<td>mtsup-2</td>
<td>1.80±0.44</td>
<td>1</td>
<td>9.95±0.21</td>
<td>1.00±0.00</td>
<td>5.00±0.00</td>
<td>6.10±1.24</td>
<td>69.0%</td>
<td>46%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>9.63±0.67</td>
<td>2.24±0.48</td>
<td>5.00±0.00</td>
<td>5.41±1.61</td>
<td>19.0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5.40±2.04</td>
<td>1.09±0.39</td>
<td>5.97±0.43</td>
<td>4.39±1.62</td>
<td>12.0%</td>
<td></td>
</tr>
</tbody>
</table>

Twelve plants per allele (10 inflorescences/plant, N=120) were used to count the number of flowers per peduncle. Ten plants per allele (10 flowers/plant, N=100) were used for floral organ, fruit and seed number counting. The data shows the mean ± the standard deviation.
Figure 1

168x200mm (300 x 300 DPI)
Figure 3

168x215mm (300 x 300 DPI)
Figure 4

168x245mm (300 x 300 DPI)
Figure 5

168x220mm (300 x 300 DPI)
Figure 6

168x216mm (300 x 300 DPI)
Significance statement

*MtSUPERMAN (MtSUP)* is the ortholog of the *SUPERMAN* gene of Arabidopsis in the model legume *Medicago truncatula*. *MtSUP* is the first gene identified that controls the number of flowers in the compound inflorescence in a legume species, being a novel function for a *SUPERMAN* ortholog in eudicots.