ORIGINAL RESEARCH ARTICLE

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

Ana Lucía Rodas[1], Edelín Roque[1], Rim Hamza[1], Concepción Gómez-Mena[1], Eugenio G. Minguet[1], Jiangqi Wen[2], Kirankumar S. Mysore[2], José Pío Beltrán[1] and Luis A. Cañas [1]

[1] Instituto de Biología Molecular y Celular de Plantas (CSIC-UPV). Ciudad Politécnica de la Innovación, Edf. 8E. C/ Ingeniero Fausto Elio s/n. E-46022 Valencia. Spain.
[2] Plant Biology Division. Noble Research Institute. 2510 Sam Noble Parkway, Ardmore, OK 73401. USA.

Authors for correspondence:
Edelín Roque
Tel: +34 96 3877879
Email: edroque@ibmcp.upv.es
Luis A. Cañas
Tel: +34 96 3877879
Email: lcanas@ibmcp.upv.es

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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;

2004). It has been proposed two functional models to explain the *SUP* function. The first model suggest 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 p*SUP*::*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 p*SUP*::*MtSUP*; *sup-5* transgenic plants were analysed (Figure S3). Most transgenic p*SUP*::*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*

(Cheng *et al.*, 2018). *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 *mtsup-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 *mtsup-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 *mtsup-1* (Figure 5o). Later on, a strong *MtWUS* signal occupies the wild-type floral meristem centre (FMC) and the nascent two floral meristems in *mtsup-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 *mtsup-1* mutant flower (Figure 5q, r). Our results revealed a prolonged *MtWUS* expression during floral development in *mtsup-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 *mtsup-1* floral primordia (Figure 5s, t). In *mtsup-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 *mtsup-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 *mtsup-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 *MtWUS*. The precise timing of *MtWUS* turn off during flower development is not yet known. Our results suggest that *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 *et al.*, 1999; Benlloch *et al.*, 2003). *MtSUP* and *MtWUS* expression patterns show a spatial overlap in the I2 and FM (Figure 1a - c; Figure 4k, l; Figure S9), while in *A. thaliana SUP* and *WUS* show almost no spatial overlap. Consequently, *SUP* does not directly regulate *WUS* expression. Therefore, the effect of *SUP* on *WUS* is largely non cell-autonomous (Prunet *et al.*, 2017). Our results provide evidence that FMT in *M. truncatula* requires a precise timing of *MtWUS* turn off, and that *MtSUP* is involved in this process. Whether the overlapping expression pattern of *MtSUP* and *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 *et al.*, 2000; Prunet *et al.*, 2008). *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 *phsup-1* mutant of petunia (Nakagawa *et al.*, 2004). Also, *mtsup-1*, *mtsup-2* and *sup-5* mutants share the shortening of funiculus and reduced ovule numbers (Gaiser *et al.,* 1995). Defects in different tissues of the gynoecium in *MtSUP* mutants are consistent with alterations at the early stages of the carpel medial region development. This fits with *MtSUP* early expression in the inner carpel region. Our findings support that *MtSUP* is required for proper medial tissue development in *M. truncatula*, a similar function assigned to *SUP* in *A. thaliana* (Breuil-Broyer *et al.*, 2016). In summary, our results show that known functions of *SUP* in the control of cell proliferation, the precise timing of FMT, and the carpel media region development are conserved in *M. truncatula*. However, *MtSUP* controls not only the number of floral organs in the inner two whorls, as *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 *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.*,

2012; Cheng *et al.*, 2018). 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 4ºC 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 aligment 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

(https://www.ebi.ac.uk/Tools/st/emboss_transeq/) to translate nucleic acid sequences to their corresponding peptide sequences.

Overexpression assay and complementation test of the *sup-5* mutant

MtSUP cDNA was cloned into the gateway vector pH7WG2 (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/). To perform the complementation assay, we used the construct (p*SUP*::*MtSUP*) to transform the *sup-5* mutant (Gaiser *et al.*, 1995) by floral dipping (Zhang *et al.*, 2006). The resulting transgenic p*SUP*::*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) (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/). 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) 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/mtsup-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 (*mtsup-1* and *mtsup-2*), transcript analysis was performed by RT-PCR with primers for the complete coding sequence. The PCR product from *mtsup-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 retrotranscribed 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 $2^{\Delta\Delta Ct}$ 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 CO_2 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.

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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 p*SUP*::*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.

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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 L*er* (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 p*SUP*::*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) Arabidopis 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 L*er* (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 stamens 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* mutans. 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; I1: Primary inflorescence meristem; I2: Secondary inflorescence meristem. Lf: leaf. 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 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. I1. Primary inflorescence meristem. I2. 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 perfomed to analyze significant differences between wild type and M*tSUP* 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.

Allele	Flowers / peduncle	Class	Stamen No.	Carpel No.	Petals No.	Ovule No.	Floral level	Fruit yield production	Seed production
Wild type	1.10±0.32	**WT**	10.0±0.10	1.0±0.10	5.00±0.00	9.10±0.50	100%	77%	9.5±0.67
mtsup-1	2.60±0.73	**1**	10.0±0.15	1.02±0.15	5.00±0.00	7.80±0.80	71.0%	58%	3.54±0.51
		2	10.0±0.19	2.40±0.49	5.00±0.00	7.40±0.70	18.7%		
		4	14.0±1.64	2.20±0.40	7.60±0.51	6.15±2.10	10.3%		
mtsup-2	1.80±0.44	**1**	9.95±0.21	1.00±0.00	5.00±0.00	6.10±1.24	69.0%	46%	3.91±0.63
		2	9.63±0.67	2.24±0.48	5.00±0.00	5.41±1.61	19.0%		
		3	5.40±2.04	1.09±0.39	5.97±0.43	4.39±1.62	12.0%		

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.

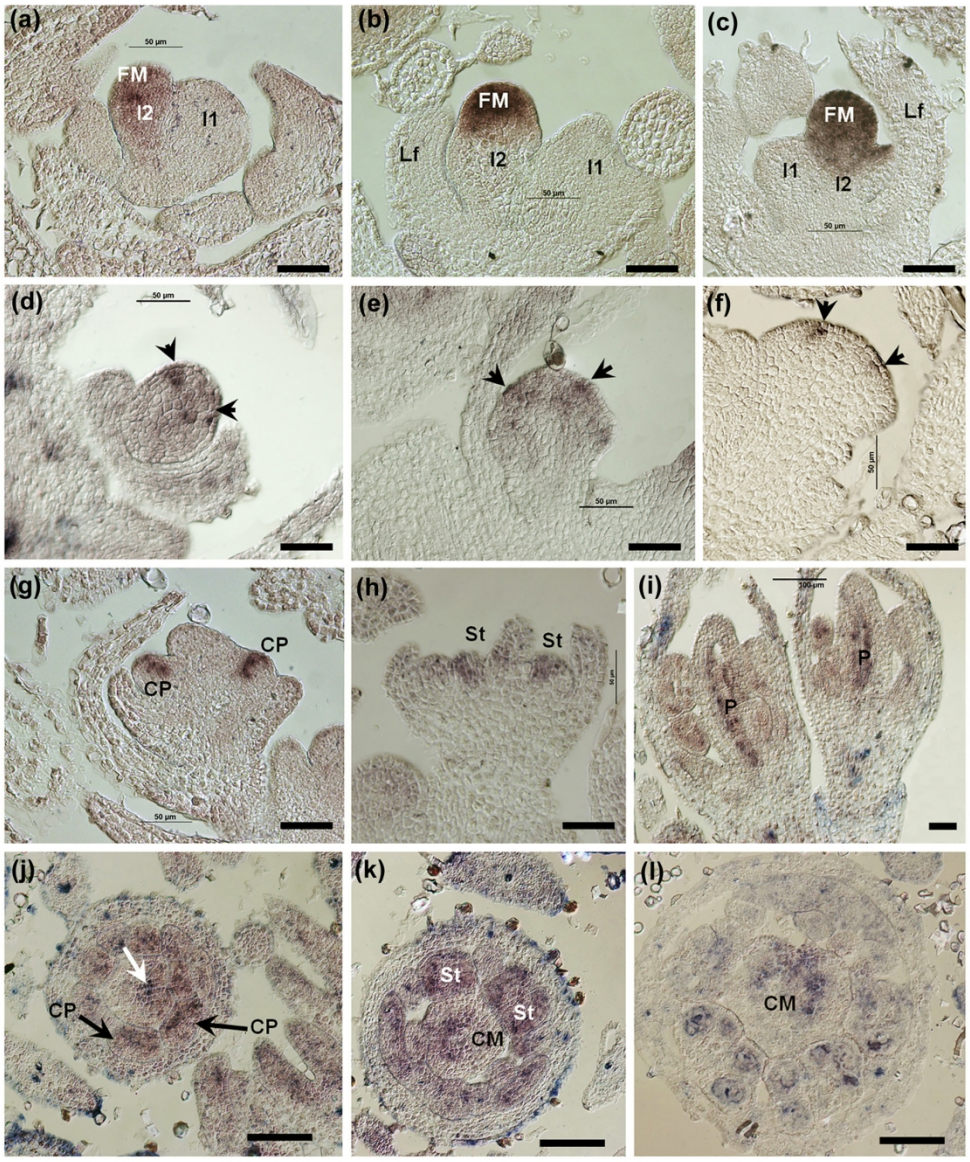

Figure 1

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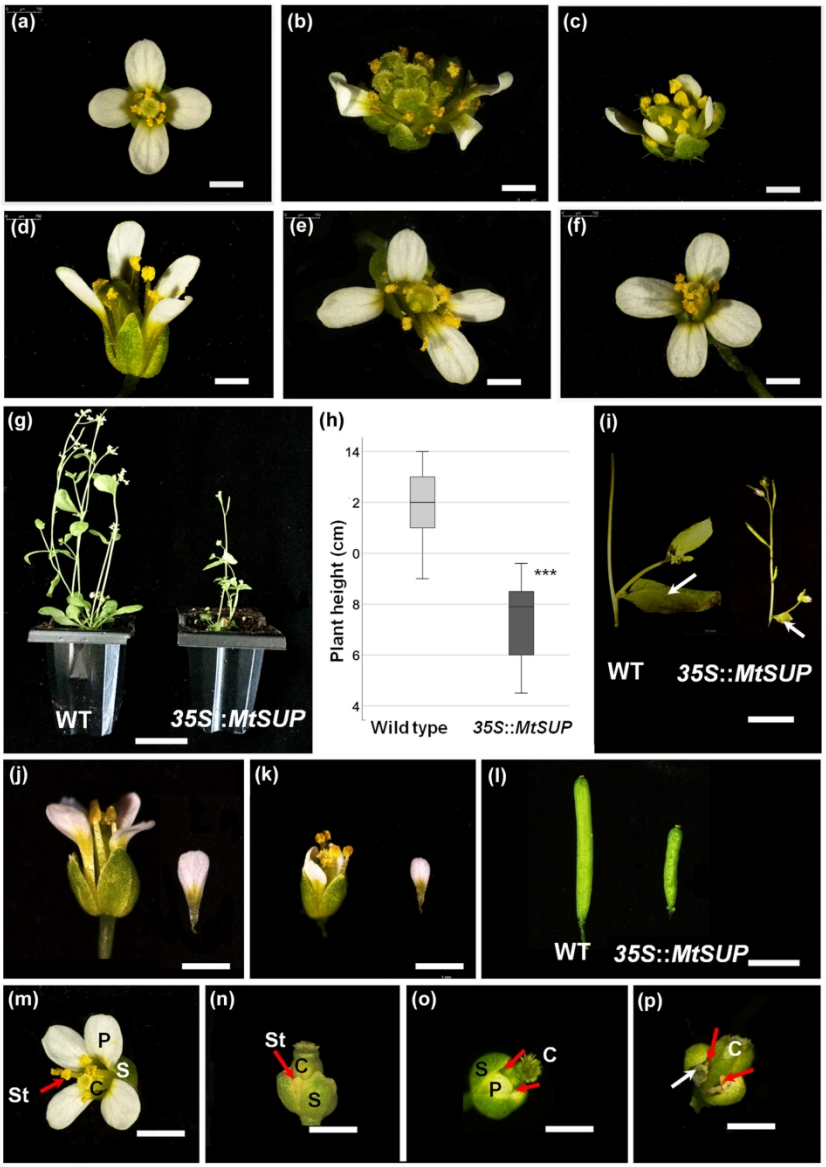

Figure 2

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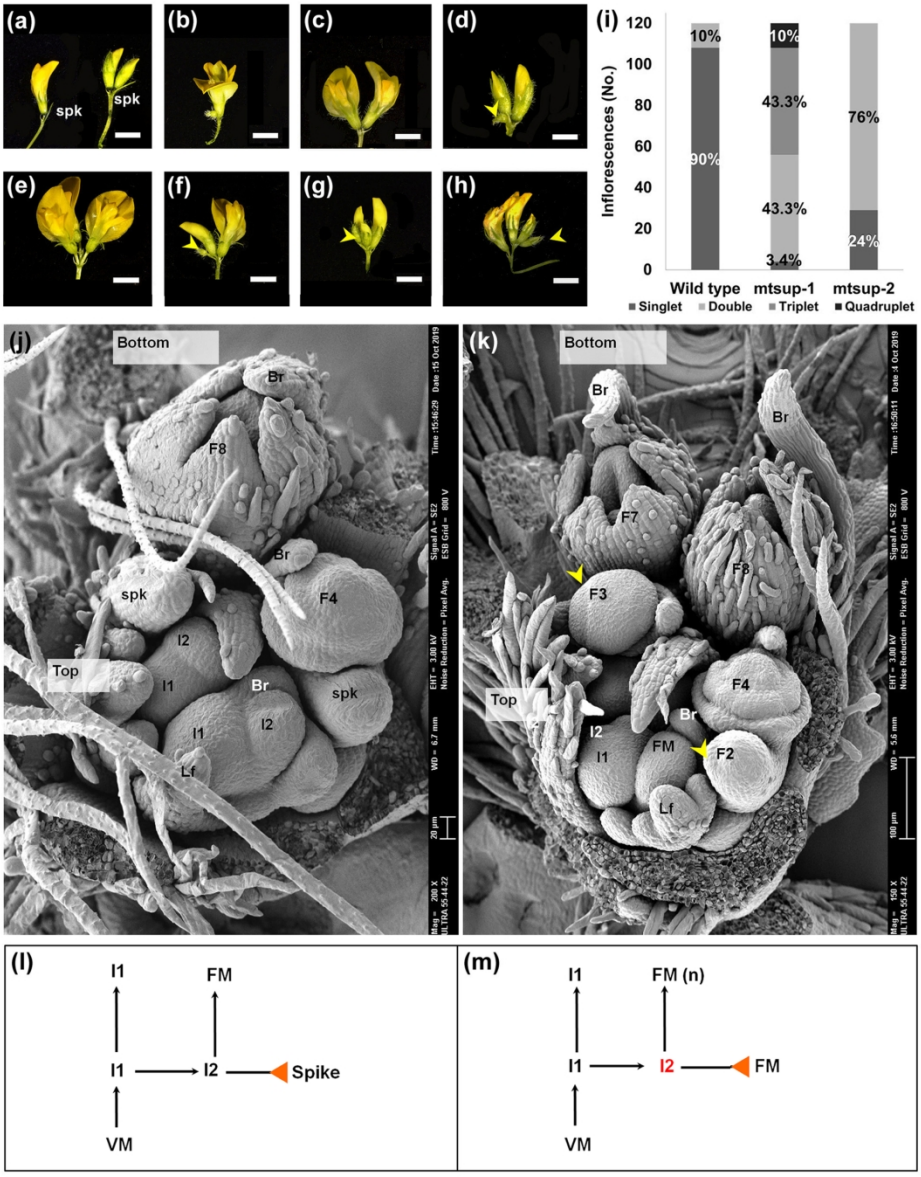

Figure 3

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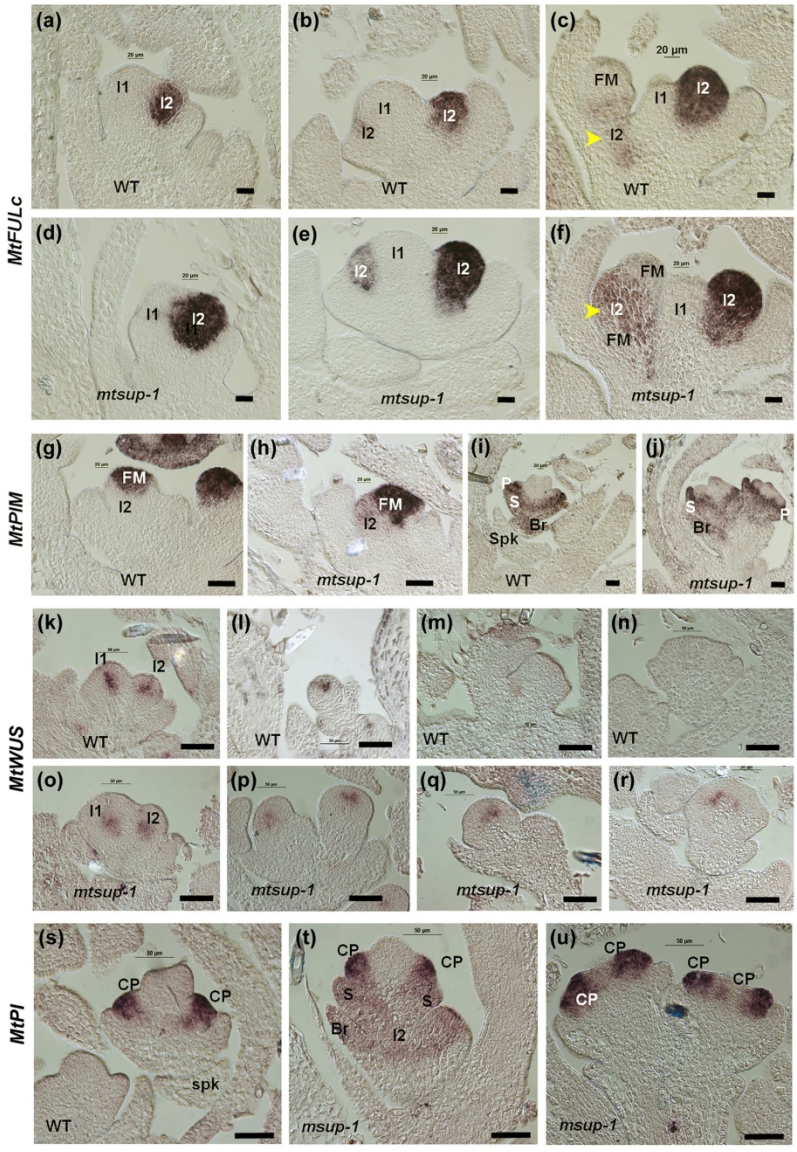

Figure 4

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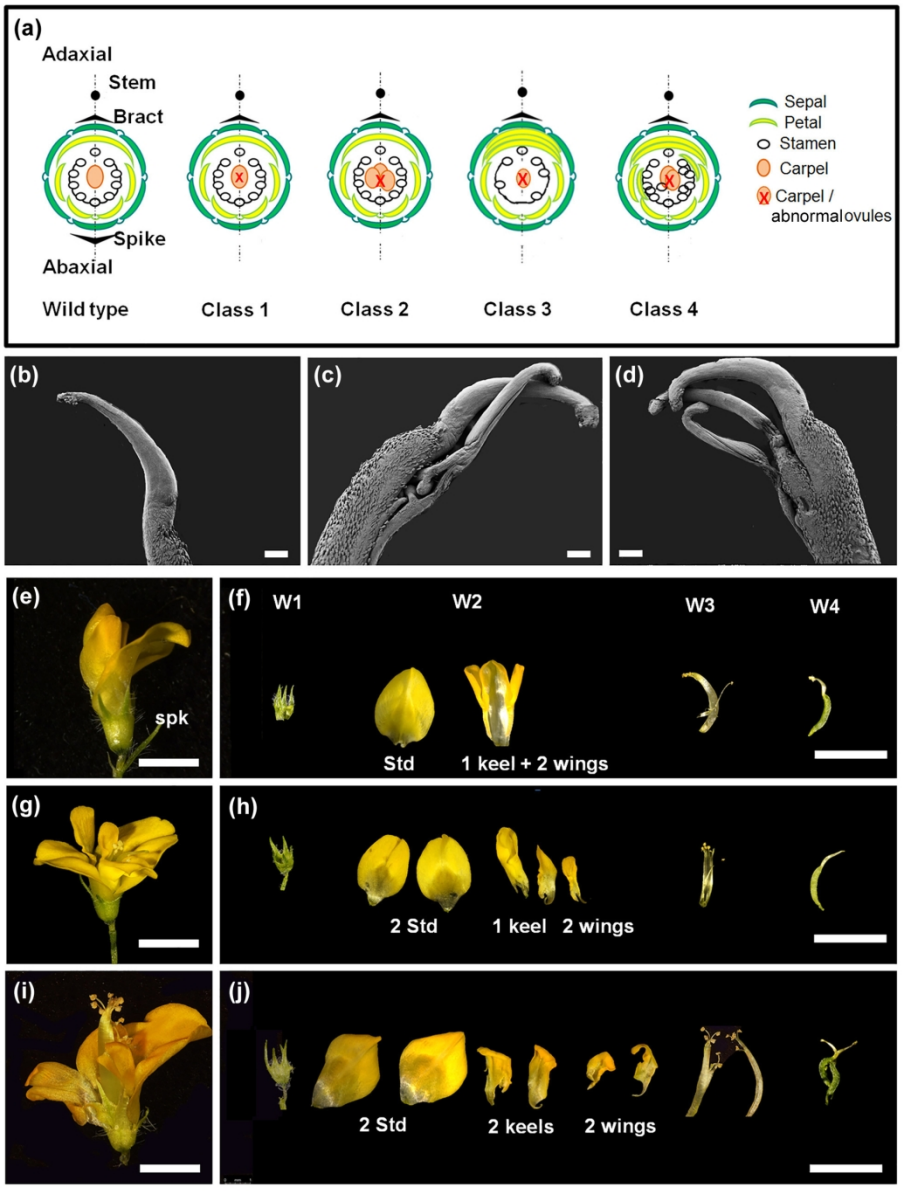

Figure 5

168x220mm (300 x 300 DPI)

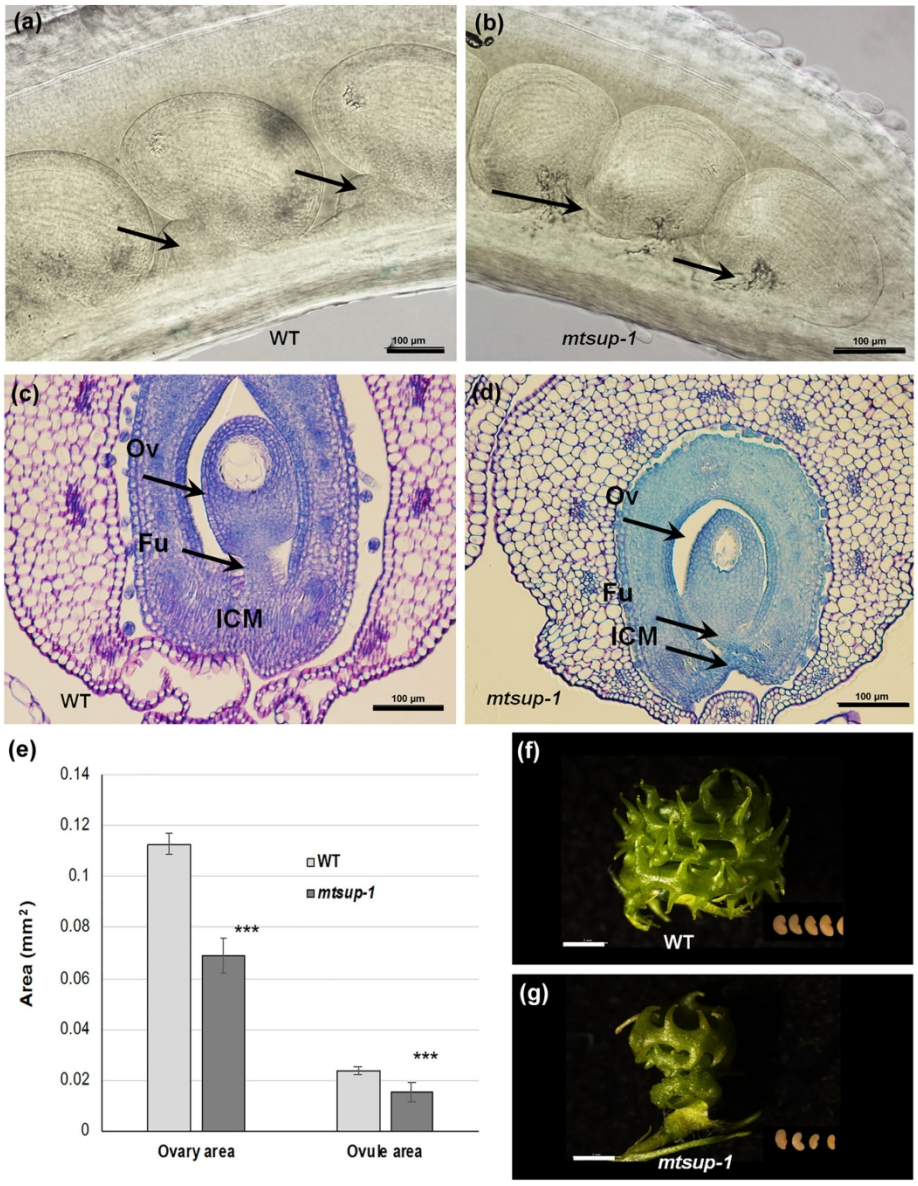

Figure 6

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.