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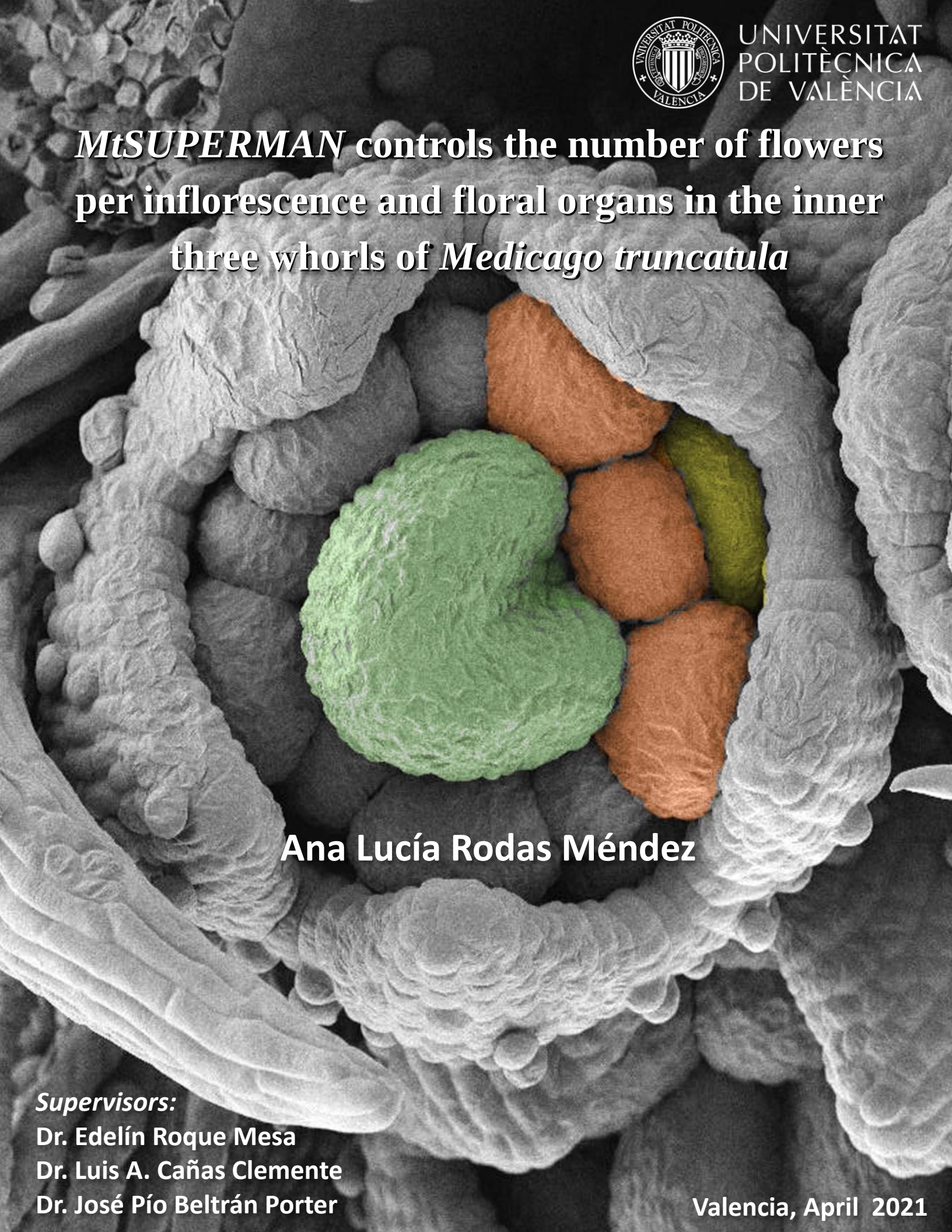
MtSUPERMAN controls the number of flowers per inflorescence and floral organs in the inner three whorls of *Medicago truncatula*



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Valencia, April 2021

UNIVERSITAT POLITÈCNICA DE VALÈNCIA
DEPARTMENT OF BIOTECHNOLOGY

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This thesis is presented to obtain the tittle of Philosophe Doctor on Biotechnology

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MINISTERIO DE CIENCIA E INNOVACION

ibmcp Instituto de Biología Molecular y Celular de Plantas

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CERTIFICAN

Que **Dª ANA LUCÍA RODAS MÉNDEZ** ha realizado bajo nuestra dirección el trabajo que con el título de *MtSUPERMAN* controls the number of flowers per inflorescence and floral organs in the inner three whorls of *Medicago truncatula,* presenta para optar al grado de Doctor por la Universitat Politècnica de València. El trabajo ha sido realizado en el Instituto de Biología Molecular y Celular de Plantas (IBMCP), Instituto mixto de la UPV y el CSIC.

Y para que así conste a los efectos oportunos firman la presente certificación en Valencia a 16 de Marzo de 2021.

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To Jeshúa
To Manuel
To my family

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Summary

Summary

Legumes are a large group of plants considered of great importance for their nutritional value in human and livestock nutrition. Besides, legume families are characterized by distinctive developmental traits as their compound inflorescence and complex floral ontogeny. For a better understanding of these distinctive features is important to study key regulatory genes involved in the inflorescence and floral development. The *SUPERMAN* (*SUP*) gene is a zinc-finger (Cys_2-Hys_2) transcriptional factor considered to be an active repressor that controls the number of stamens and carpels in *A. thaliana.* Moreover, *SUP* is involved in the floral meristem termination and the development of the carpel marginal derived tissues. The main objective of this work was the functional characterization of the *SUP* orthologue in the model legume *Medicago truncatula* (*MtSUP*). We achieved this objective based on a reverse genetic approach, gene expression analysis, and complementation and overexpression assays. Our results show that *MtSUP* is the orthologous gene of *SUP* in *M. truncatula*. *MtSUP* shares some of the roles already described for *SUP* with variations. Interestingly, *MtSUP* controls the determinacy of the secondary inflorescence (I2) meristem and the common primordia (CP). Thus, *MtSUP* controls the number of flowers and petal-stamens produced by the I2 meristem and the common primordia respectively. *MtSUP* displays novel functions for a *SUP*-like gene, playing key roles in the meristems that confer developmental complexity to this angiosperm family. This work allowed to identify *MtSUP,* a key gene that participates in the genetic regulatory network underlying compound inflorescence and flower development in the model legume *M. truncatula.*

Resumen

Las leguminosas son un grupo de plantas consideradas de gran importancia por su valor nutricional para la alimentación humana y ganadera. Además, las familias de leguminosas se caracterizan por rasgos distintivos de desarrollo como su inflorescencia compuesta y su compleja ontogenia floral. Para comprender mejor estas características distintivas, es importante estudiar los genes reguladores clave involucrados en el desarrollo de la inflorescencia y la flor. El gen *SUPERMAN* (*SUP*) es un factor transcripcional de dedos de zinc (Cys_2-Hys_2) considerado como un represor activo que controla el número de estambres y carpelos en *A. thaliana*. Además, *SUP* está involucrado en la terminación del meristemo floral y el desarrollo de los tejidos derivados del carpelo. El objetivo principal de este trabajo fue la caracterización funcional del ortólogo de *SUP* en la leguminosa modelo *Medicago truncatula* (*MtSUP*). Logramos este objetivo en base a un enfoque de genética reversa, análisis de expresión génica y ensayos de complementación y sobreexpresión. Nuestros resultados muestran que *MtSUP* es el gen ortólogo de *SUP* en *M. truncatula*. *MtSUP* comparte algunos de los roles ya descritos para *SUP* con algunas variaciones. Curiosamente, *MtSUP* controla la determinación del meristemo inflorescente secundario (I2) y de los primordios comunes (CP) a pétalos y estambres. Por tanto, *MtSUP* controla el número de flores y de pétalos-estambres que producen el meristemo I2 y los primordios comunes, respectivamente. *MtSUP* muestra funciones novedosas para un gen de tipo *SUP*, desempeñando papeles clave en los meristemos que confieren complejidad de desarrollo a esta familia de angiospermas. Este trabajo permitió identificar a *MtSUP*, un gen clave que forma parte de la red reguladora genética que subyace al desarrollo de la inflorescencia compuesta y de las flores en la leguminosa modelo *M. truncatula*.

Resum

Les lleguminoses són un gran grup de plantes considerades de gran importància pel seu valor nutricional per a l'alimentació humana i ramadera. A més, les famílies de lleguminoses es caracteritzen per trets distintius de desenrotllament com la seua inflorescència composta i la seua complexa ontogènia floral. Per a comprendre millor estes característiques distintives, és important estudiar els gens reguladors clau involucrats en la inflorescència i el desenrotllament floral. El gen *SUPERMAN* (*SUP*) és un factor transcripcional de dits de zinc (Cys_2-Hys_2) considerat com un repressor actiu que controla el nombre d'estams i carpels en *A. thaliana*. A més, *SUP* està involucrat en la terminació del meristemo floral i el desenrotllament dels teixits derivats del carpel. "L'objectiu principal d'este treball va ser la caracterització funcional de l'ortòleg de *SUP* en la lleguminosa model Medicago truncatula (*MtSUP*) . Aconseguim l'objectiu amb base en un enfocament genètic invers, anàlisi d'expressió gènica i assajos de complementació i sobreexpressió. Els nostres resultats mostren que *MtSUP* és el gen ortòleg de *SUP* en *M. truncatula*. *MtSUP* compartix alguns dels rols ja descrits per a *SUP* amb variacions. Curiosament, *MtSUP* està involucrat en la determinació del meristemo de la inflorescència secundària (I2) i els primordios comuns (CP). Per tant, *MtSUP* controla el nombre de flors i pètals-estams que produïxen el meristemo I2 i els primordios comuns, respectivament. *MtSUP* mostra funcions noves per a un gen tipus *SUP*, exercint papers clau en els meristemos que conferixen complexitat de desenrotllament a esta família d'angiospermes. "Este treball va permetre identificar a *MtSUP*, un gen clau que forma part de la xarxa reguladora genètica darrere de la inflorescència composta i el desenrotllament de flors en la lleguminosa model *M. truncatula*.

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Abbreviations

Abbreviations

A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
At	*Arabidopsis thaliana*
BAP	6-Bencylaminopurine
BCIP	5-bromo-4-chloro-3-indolylphosphate
bp	basepair
BSA	bovine serum albumine
C	carpel medial meristem
Carb	carbenicillin
Cas9	CRISPR associated protein 9
CDS	coding sequence
CG	Cytosine-Guianidine
CM	carpel margin
CP	common primordia
CRISPR	clustered regularly interspaced short palindromic repeats
DAP	days after pollination
DIC	differential interface contrast
DIG	digoxigenin
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDFS	ethylenediaminetetraacetic acid ferricsodium salt
FA	flower at anthesis
g	grams
gDNA	genomic DNA
hCas9	humanized *Cas9*
Hyg	Hygromycin
I1	primary inflorescence meristem
I2	secondary inflorescence meristem
kb	kilobase
Krpm	kilo revolution per minute
L	liter
l	liquid
LB	Luria-Bertani
M	Molar

M. truncatula	*Medicago truncatula*
mg	miligrams
$MgCl_2$	magnesium chloride
mL	mliliter
mM	milimolar
Mt	*Medicago truncatula*
MtAGa	*MtAGAMOUSa*
MtAGb	*MtAGAMOUSb*
MtFULc	*MtFRUITFULLc*
MtPI	*MtPISTILATA*
MtPIM	*MtPROLIFERATING INFLORESCENCE MERISTEM*
MtSGL1	*MtSINGLE LEAFLET1*
MtSHP	*MtSHATTERPROOF*
MtSUP	*MtSUPERMAN*
MtWUS	*MtWUSCHEL*
NBT	nitroblue tetrazolium
ng	nanogram
No.	number
O.D.	optical density
ºC	degree Celsius
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	potential of hydrogen
PhSUP	*Petunia hybrida SUPERMAN*
QSP	quantity sufficient
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-qPCR	reverse transcription - quantitative PCR
s	solid
SAM	shoot apical meristem
SDS	sodium dodecyl sulfate
SR2200	SCRI Renaissance Stain
SSC	saline-sodium citrate
SUP	*SUPERMAN*

TBE	Tris-HCl - Boric acid- EDTA
BS	Tris-buffered saline
Tim	Timentin
TM	trademark
URO	*UPRIGHT-ROSETTE*
UV	ultraviolet
V	Volts
VM	vegetative meristem
W/V	weight / volume
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
(R)	registred trademark
%	percentage
2,4D	2,4-Dichlorophenoxy acetic acid

Introduction

Introduction

1. Legumes

1.1. General characteristics

Legumes constitute a large group of plants that are valued for their nutritional characteristics and their importance in food security and sustainable agriculture (Cañas and Beltrán, 2018b). Legumes family, also known as Fabaceae or Leguminosae, evolved 60 million years ago and diversified into six subfamilies: Cesalpinioideae (mimosoid clade included), Cercidoideae, Detarioideae, Duparquetioideae, Dialoideae and Papilionoideae (Azani *et al.*, 2017). The Papilonoideae, which includes 445 genera and 14,000 species, is the most widely studied subfamily of legumes (Cañas and Beltrán, 2018a), in this clade are included crops as chickpea, peanut and soybean; which together with other grain legumes constitute more than one quarter of the world crop production (Schaefer *et al.*, 2012). After Asteraceae and Orchidaceae, Leguminoseae is the largest angiosperm family based on the number of species. Legumes occupy a great variety of habitats that range from aquatic to forest (De Faria *et al.*, 1989; Lewis *et al.*, 2005; Capoen *et al.*, 2010; Tabosa *et al.*, 2012). Legumes are characterized by distinctive traits as their capacity to symbiotically fix nitrogen, compound leaves and inflorescences, and a complex floral development (De Faria *et al.*, 1989; Ferrándiz *et al.*, 1999; Singer *et al.*, 1999; Benlloch *et al.*, 2003; Cañas and Beltrán, 2018a). All these traits make them of special interest to study unique developmental processes (Cañas and Beltrán, 2018a).

1.2. Nitrogen fixation

Legumes can harbour symbiotic nodules that are capable to fix atmospheric nitrogen. Nodules are specialized root organs that are formed based on a symbiotic

interaction between the plant and nitrogen-fixing bacteria called rhizobia (Oldroyd *et al.*, 2011).

1.3. Compound leaves

Leaves can be classified as simple or compound according to their complexity. A simple leaf has a single blade while a compound leaf has multiple units or leaflets. Leaves are formed from the periphery of shoot apical meristem (SAM). It has been shown that the class I *KNOTTED-LIKE* homeobox genes (*KNOXIs*) are required to maintain active the SAM and once the leaf primordia are initiated these genes are downregulated (Clark *et al.*, 1996; Long *et al.*, 1996). In compound leaf species, as tomato (*Solanum lycopersicum*) and *Cardamine hirsuta, KNOXIs* genes are reactivated to initiate the leaflets primordia (Bharathan, 2002; Hay and Tsiantis, 2006). In legume species that have compound leaves, such as pea and Medicago species, the *KNOXIs* genes do not seem to be involved in the initiation of leaflets (Hofer *et al.*, 2001; Peng *et al.*, 2011). Instead, the genes *UNIFOLIATA* (*UNI*) and *SINGLE LEAFLET1* (*SGL1*) are the ones that play a role in compound leaves development (Hofer *et al.*, 1997; Wang *et al.*, 2008).

1.4. Compound inflorescences

Inflorescence meristems are developed from the shoot apical meristem after the plant changes from a vegetative to a reproductive stage based on a group of environmental and developmental cues.

1.4.1. Types of inflorescences

Inflorescences can be determined or undetermined according to the determinacy of the primary inflorescence (I1). If the I1 terminates its meristematic activity after producing flower meristems or secondary inflorescences (I2), then the inflorescence

is considered as determined (i.e. panicle). Otherwise, if the I1 indefinitely produces floral meristems or I2 meristems, then the inflorescence is indeterminate (i.e. raceme). Cyme, raceme and panicle are the three mayor types of inflorescences architecture described (Figure 1). Inflorescence architecture is crucial for reproductive success and crop productivity, being a relevant trait in agriculture because it strongly influences fruit and seed production (Prusinkiewicz *et al.*, 2007).

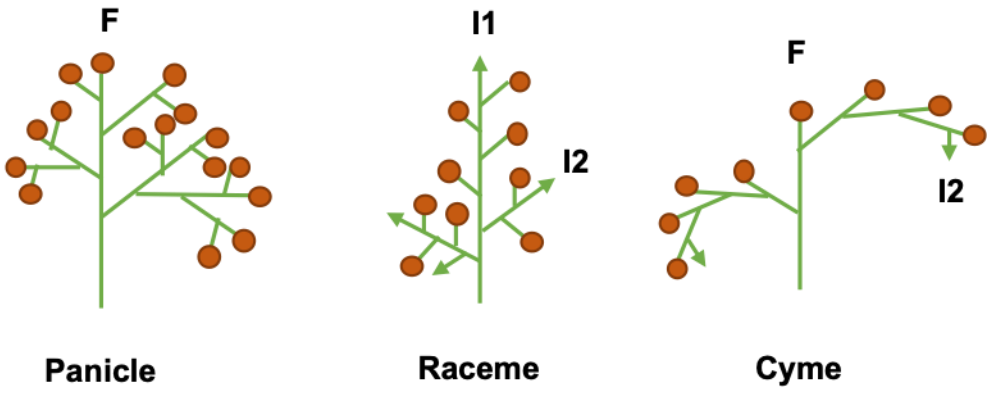


Figure 1. Schematic inflorescence architectures. Red circles represent flowers and arrows meristems. F: floral meristem, I1: primary inflorescence meristem, I2: secondary inflorescence meristem. Adapted from Prusinkiewicz *et al.*, 2007.

1.4.2. Secondary inflorescence meristem

Most legumes show complex raceme inflorescences as well as compound and double racemes with more than one branching. In legumes as *Pisum sativum* and *Medicago truncatula* the primary inflorescence meristem (I1) produces the secondary inflorescence meristems (I2). The existence of the I2 meristem is linked to the compound inflorescence development (Tucker, 2003; Benlloch *et al.*, 2007). The I2 meristem is a transient meristem that is intercalated between the I1 meristem and the floral meristem (FM). The I2 meristem identity is given by a genetic function derived from the sub-functionalization of the *AGL79* MADS-box gene clade within the AP1/SQUA/FUL family (Berbel *et al.*, 2012). In the model legume *Medicago*

truncatula the I2 meristem remains active to produce a fixed number of floral meristems (1 to 3) and then terminates as a residual organ or stub (Benlloch *et al.*, 2003, 2015).

1.4.3. Genetic regulatory network

In some legumes, during compound inflorescence development three meristems are involved: the primary inflorescence meristem (I1), the secondary inflorescence meristem (I2) and the floral meristem (FM) (Singer *et al.*, 1999; Benlloch *et al.*, 2003; Tucker, 2003). The identity and activity of these meristems is tightly controlled by a group of key regulatory genes. The compound inflorescence development in legumes has been linked to the I2 meristem identity gene *VEGETATIVE1* (*VEG1*); which encodes a transcription factor without a functional homologue in *A. thaliana* (Berbel *et al.*, 2012; Cheng *et al.*, 2018). In pea, recessive mutations in the genes *Fn* and *Fna* have been described to lead an increase number of flowers/pods produced from the I2 meristem, resulting in the multipod trait (White, 1917; Lamprecht, 1947). A similar situation occurs in *Cicer arietinum* (chickpea) when the genes *CYM* (*CYMOSE*) and *SFL* (*SINGLE FLOWER*) have recessive mutations which lead to the formation of a larger number of flowers by the secondary inflroescence meristems (Srinivasan *et al.*, 2006; Caballo *et al*., 2021). In the model legume *M. truncatula* the identity of the I1, I2 and FM is given by the genes *MtTFL1*/*MtAP1*, *MtFULc* and *MtPIM* respectively (Berbel *et al*., 2001; Benlloch *et al*., 2006; Cheng *et al*., 2018). The gene *MtSGL1* also plays a reiterative role in the identify determination of the floral meristem and the common primordia. The spatial and temporal expression of these genes together with their mutual repression controls the compound inflorescence development in *M. truncatula* (Figure 2) (Cheng *et al.*, 2018).

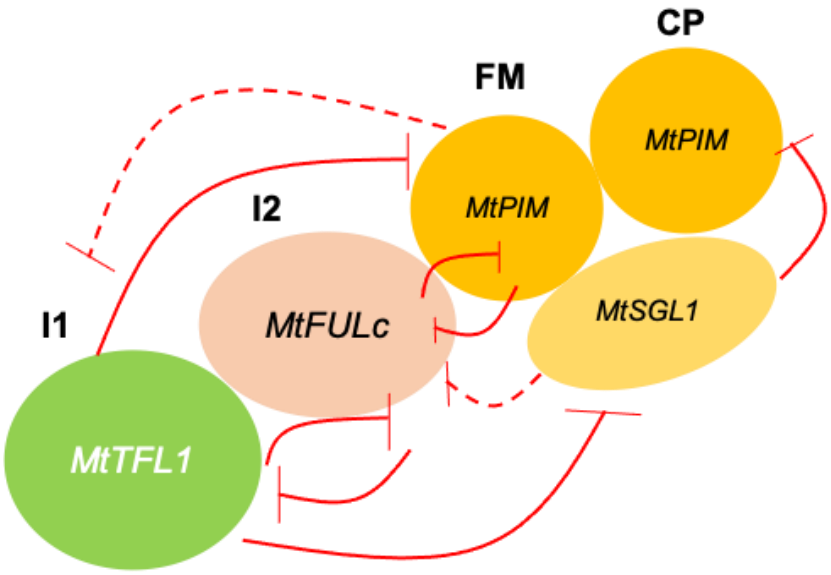


Figure 2. Genetic regulatory network in compound inflorescence in *M. truncatula*. The model includes the genes *MtTFL1*, *MtFULc*, *MtPIM* and *MtSGL1* which are the responsible to confer identity to the I1 mersitem, I2 meristem, floral meristem and common primordia respectively. I1: primary inflorescence meristem; I2: secondary inflorescence meristem; FM: floral meristem; CP: common primordia. Continuous lines represent direct repression, dashed lines indirect repression. Adapted from Cheng *et al.*, 2018.

1.5. Floral development

1.5.1. Floral organogenesis

Flowers are formed by four types of floral organs: sepals, petals, stamens and carpels which respectively constitute the first to the fourth whorls (Krizek and Fletcher, 2005; Prunet *et al.*, 2009). Each floral whorl produces a fixed number of floral organs from a pool of stem cells, these cells are transiently maintained until their determination or identity specification (Bowman *et al.*, 1989; Schultz and Haughn, 1991; Bossinger and Smyth, 1996). During floral organogenesis it is required a tight control of the stem cells proliferation in the floral meristem (FM). Moreover, after the cell identity specification it is important that the floral homeotic genes maintain their

expression domains for a proper organ development (Aida *et al*., 1997; Aida and Tasaka, 2006). In *A. thaliana* floral meristem termination occurs at stage 6 of flower development when the stem cells changes into a female program. This occurs when the C-class gene *AGAMOUS* (*AG*) turns off *WUSCHEL* (*WUS*) expression (Clark *et al.*, 1996; Prunet *et al.*, 2009). *AG* represses *WUS* directly by recruiting the polycomb group (PcG) and indirectly through *KNUCKLES* (*KNU*). *AG* also activates *CRABS CLAW* (*CRC*) a TF of the family YABBY for the FM termination and carpel organogenesis through the set of an auxin maximum (Prunet *et al.*, 2009). Flower initiation is an auxin dependent process in which the floral patterning is strictly affected by an auxin gradient (Krizek and Fletcher, 2005; Xu *et al.*, 2018).

1.5.2. Floral organogenesis in *M. truncatula*

The typical papilionoid flowers of *Medicago truncatula* (Figure 3), *Pisum sativum* and *Lotus japonicus,* as many legume flowers, display pentamerous floral organs per whorl (Figure 3) (Tucker, 2003; Lewis *et al.*, 2005). A typical wild type flower in *M. truncatula* has five sepals in the first whorl (W1); a keel petal formed by two fused petals, two wing petals and one standard petal in the second whorl (W2); nine fused stamens and one free stamen in the third whorl (W3); and a single carpel in the fourth whorl (W4) (Benlloch *et al.*, 2003).

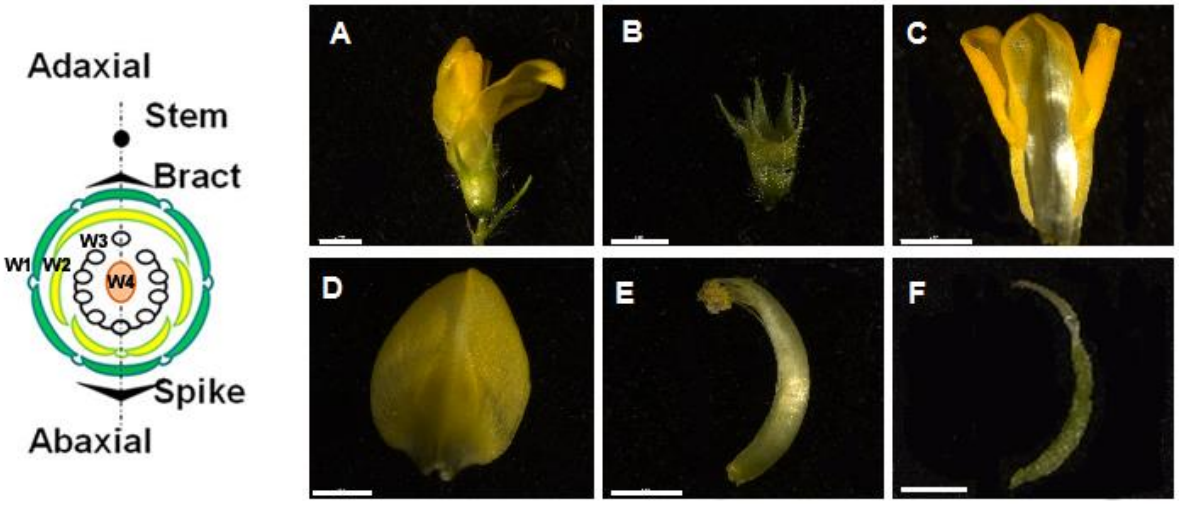


Figure 3. Flower and floral organs in *M. truncatula* R108. A. Wild type flower at anthesis. **B.** First whorl (W1): 5 sepals. **C-D** Second whorl (W2): 5 petals; one keel petal (two fused petals) and two wing petals (**C**); a standard or vexillum petal (**D**). **E.** Third whorl (W3): 10 stamens, 9 fused (staminal tube) and one free. **F**. Fourth whorl (W4): a single carpel. Scale bar: 2 mm.

There have been defined eight developmental stages in Medicago flowers (Benlloch *et al*., 2003). In *M. truncatula,* as in other papilionoid flowers, floral organ initiation goes unidirectionally from the abaxial to the adaxial position of the flower. In contrast to Arabidopsis, the organ differentiation shows a high degree of spatial and temporal overlapping. Even more characteristic is the existence of common primordia (CP) to petals and stamens, and the early carpel differentiation (Figure 4) (Ferrándiz *et al*., 1999; Benlloch *et al*., 2003). The common primordia are ephemeral meristems from which petals and stamens will differentiate (Ferrándiz *et al.*, 1999; Benlloch *et al.*, 2003; Weng *et al.*, 2011; Roque *et al.*, 2013). The model species Arabidopsis and Antirrhinum (Figure 4, left) show a centripetal and sequential organ differentiation. First are differentiated the sepal primordia, then the petal primordia, then the stamen primordia and finally the carpel primordium. In contrast, Pisum and Medicago plants (Figure 4, right) show unidirectional differentiation of the organ primordia with a high degree of overlapping. The most unique differences are the presence of common

primordia and the early carpel primordia differentiation in Pisum and Medicago (Benlloch *et al*., 2003)

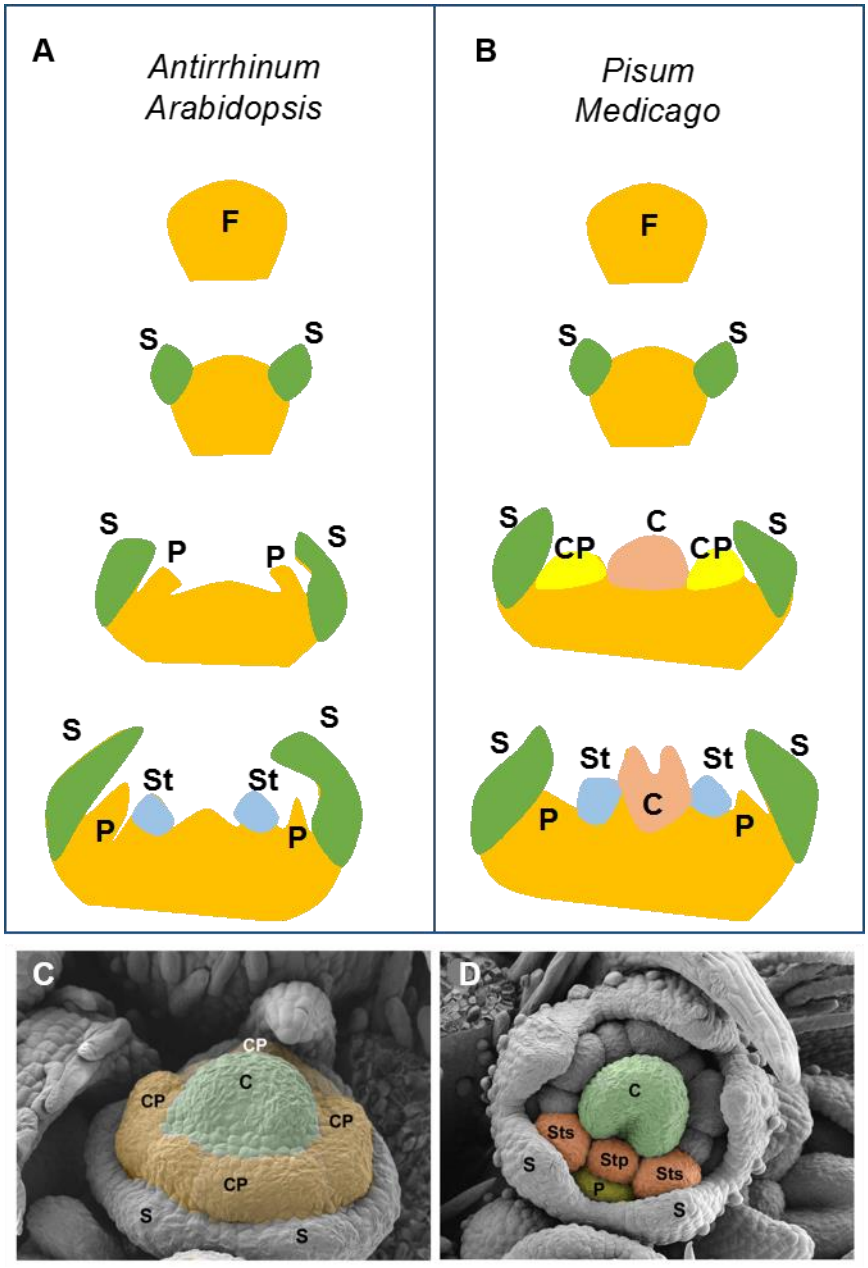


Figure 4. Comparative floral ontogeny between Antirrhinum/Arabidopsis and Pisum/Medicago. **A.** In the model species Arabidopsis and Antirrhinum organ differentiation is centripetal and sequential. First are differentiated the sepal primordia and at the end the carpel primordium. **B.** In the legumes *Pisum sativum* and *Medicago truncatula,* the four common primordia differentiate petals and stamens in the second and third whorl respectively. **C.** In *M. truncatula* the floral meristem shows the early carpel primordium (C) in the centre,

the four common primordia (CP) and the sepal primordial (S). **D.** Each common primordia differentiates petals (P) in the second whorl and antepetal (Stp) and antesepal (Sts) stamens in the third whorl. F: floral meristem; S: sepal primordium; CP: Common primordium; P: petal primordium; St: stamen primordium; C: carpel primordium. F: floral meristem; S: sepal primordium; P: petal primordium; St: stamen primordium; C: carpel primordium.

1.5.3. ABC**DE** model

The classical model for floral organ identity specification only includes the A, B and C homeotic genes. While the ABCDE model includes the D and E homeotic genes (Rijpkema *et al.*, 2010). In *A. thaliana*, all these genes encode MADS-box transcription factors with the exemption of the A-class gene *APETALA2* (AP2) (Weigel and Meyerowitz, 1994; Smaczniak *et al.*, 2012). MADS-box proteins establish higher order complexes to determine the identity of the floral organs (quartet model) (Melzer and Theißen, 2009). According to studies in *A. thaliana* the model proposes that the A and E protein complex specify the first whorl (sepals); the A,B and E proteins complex specify the second whorl (petals); the B, C and E protein complex specify the third whorl (stamens); and the C and E protein complex specify the fourth whorl (carpels) (Smaczniak *et al.*, 2012). The D class genes together with the E class genes specify the ovule identity (Pelaz *et al.*, 2000; Pinyopich *et al.*, 2003). The great diversity among angiosperms flowers has been influenced by the diversification of MADS-box genes during evolution (Litt and Kramer, 2010).

Floral development in legumes fits the classical ABC model and it has been shown that the A, B, C-class MADS-box genes identified in Arabidopsis are conserved in *M. truncatula* (Hecht *et al.*, 2005). Nevertheless, there are variations between these two species (Figure 5). In *M. truncatula* there are one A-class gene (*MtPIM*), four B-class genes (*MtTM6*, *MtNMH7*, *MtPI* and *MtNGL9*) and three C-class genes (*MtAGa*, *MtAGb* and *MtSHP*) implying duplication events and functional divergence during evolution (Benlloch *et al*., 2006, 2009; Roque *et al*., 2013, 2016; Serwatowska *et al*., 2014). The C-function activity in *M. truncatula* is completely performed by *MtAGa* and

MtAGb, while *MtSHP* has a role in ovule development and the fruit morphology (Colombo *et al*., 2010; Fourquin *et al*., 2013; Roque *et al*., 2018)

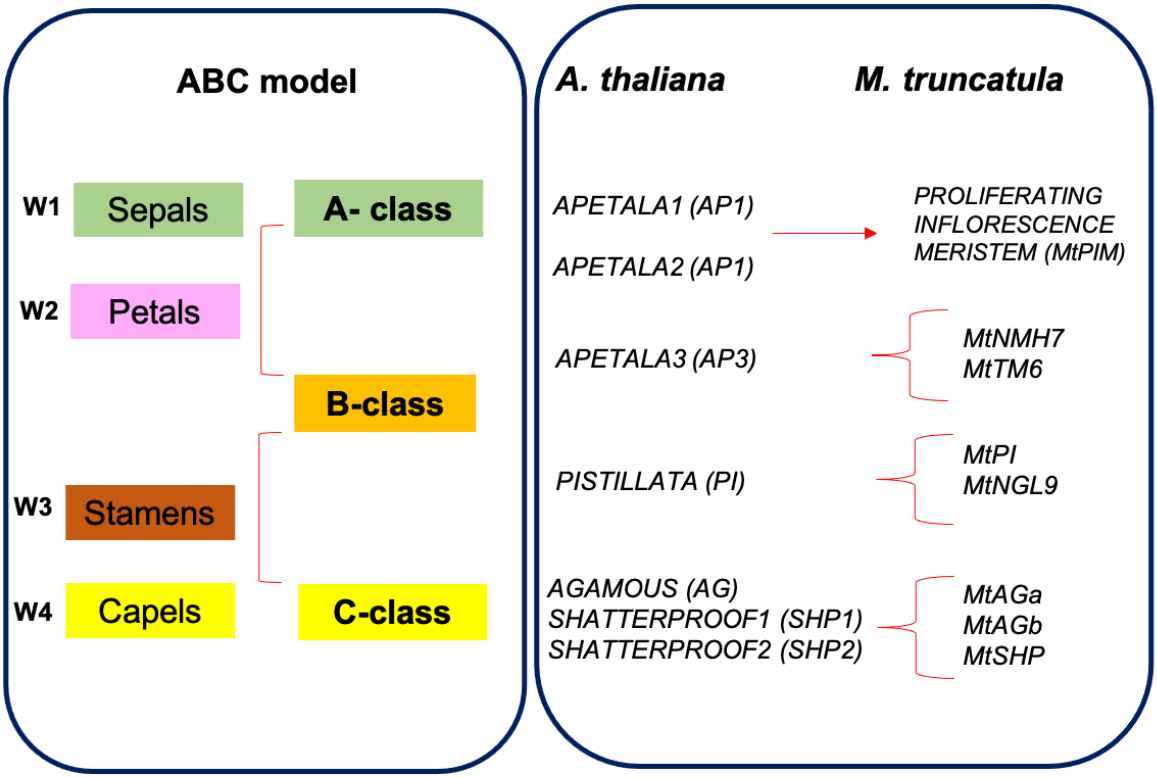


Figure 5. Classical ABC model for *A. thaliana* and *M. truncatula*. The classical ABC model states that the A-class genes specify the identity of the sepals, the A+B class genes the petals identity, the B+C class genes the stamens identity, and the C class genes the carpels identity. The ABC homeotic genes for *A. thaliana* and their homologous genes in *M. truncatula* are shown in Figure 5. Each B-class genes (*AP3* and *PI*) is duplicated in *M. truncatula.* The C-class genes includes members from the *euAG* and *PLENA* (*PLE*) lineages. Medicago harbors two genes for the *euAG* sub-clade (*MtAGa* and *MtAGb*) and one member of *PLENA* (*MtSHP*). W1: whorl 1 (sepals), W2: whorl 2 (petals), W3: whorl 3 (stamens), W4: whorl 4 (carpels) (Roque *et al*., 2018)

1.6. *Medicago truncatula* as a model legume

The Arabidopsis genome might not be a good model to understand how legumes genomes are arranged (Cannon, 2013). Since it is known that there is a poor (8%) microsynteny conservation between *A. thaliana* and *M. truncatula* genomes (Zhu *et*

al., 2003). Moreover, as mentioned previously, legumes have distinctive developmental traits that indeed require a more suitable model plant. For example, the study of compound inflorescence development cannot be performed in *A. thaliana* due to the lack of a secondary inflorescence meristem that confers the complexity. Since 1990 *Medicago truncatula* was proposed as a model system due to its relatively small genome (~375 Mbp), short generation time, autogamous nature, diploid genome (2n=16) and the availability of genetic transformation methods.

Nowadays, the whole genome of *M. truncatula* is sequenced and annotated. Besides there are succesful functional genomics tools (Cañas and Beltrán, 2018b). The transformation of *M. truncatula* requires *in vitro* tissue culture regeneration which is a longer process compared to the floral dip transformation followed for *A. thaliana.* In line with this, efforts have been made to produce large-scale mutant collections as the *Tnt1* mutants in the *M. truncatula* R108 genotype (Cheng *et al.*, 2011; Cheng *et al.*, 2014). More recent tools as CRISPR/*Cas* technology allow targeted mutagenesis. Moreover, functional genomics in *M. truncatula* has proven to be a powerful strategy to study the developmental and metabolic novelties in legumes (Cañas and Beltrán, 2018b).

2. *SUPERMAN* as a key regulatory gene

Floral homeotic genes play a crucial role in the floral organ identity specification. Nevertheless, there are other type of genes that also are key players during floral development. Transcription factors are the main regulators of gene expression, so their study is important for the understanding of development, differentiation, environmental responses and their role in evolution (Ramalingam *et al.*, 2003). A good example of a key transcription factor is the *SUPERMAN (SUP)* gene in *A. thaliana*. *SUPERMAN* encodes a protein that has a zinc-finger (Cys_2-Hys_2) motif and a C-terminus EAR-like motif which is proposed to be an active repressor (Sakai *et al.*,

1995; Hiratsu *et al.*, 2002, 2003, 2004). This gene was named as *SUPERMAN* (*SUP*) because its classical mutant (*sup-1*) has more stamens that the normal at expenses of the carpel (Bowman *et al.*, 1992). Within the functions described for *SUP* are included its role in floral meristem termination (B-class genes repression and cell proliferation control), outer integuments differentiation and its contribution to the formation of the carpel margin (Schultz *et al.*, 1991; Bowman *et al.*, 1992; Gaiser *et al.*, 1995; Breuil-Broyer *et al.*, 2016).

2.1. Functional models

Based on the study of different *SUP* mutants there have been proposed two functional models for *SUP*.

2.1.1. Functional *SUP* model

The first model proposes that *SUP* promotes floral meristem termination (FMT) indirectly through the B-class genes repression from the fourth whorl. This model is supported by the observation that *SUP* delimitates the expression domain of the B-class genes (*AP3* and *PI*) to the third whorl (Schultz *et al.*, 1991; Bowman *et al.*, 1992). In other words, it could be said that *SUP* indirectly promotes the determinacy of the floral meristem center (FMC).

2.1.2. Second or alternative functional *SUP* model

The second or alternative model states that *SUP* controls the cell proliferation between the third and the fourth floral whorls (Sakai *et al.*, 1995; Sakai *et al.*, 2000). This model is supported by the effects observed when *SUP* and other *SUP*-like genes are ectopically expressed (Nandi *et al.*, 2000; Bereterbide *et al.*, 2001; Hiratsu *et al.*, 2002; Kazama *et al.*, 2009; Nibau *et al.*, 2011; Zhao *et al.*, 2014). Both models are feasible and non-exclusive as both can explain the floral phenotypes of *SUP* mutants. Recent studies demonstrated that *SUP* bridges the floral meristem

determinacy and floral organogenesis through the fine-tuning of auxin biosynthesis (Xu *et al.*, 2018).

2.2. Classification of the *SUPERMAN* mutant alleles

SUPERMAN gene has been studied for more than two decades, giving place to the term cadastral gene when Bowman in 1992 studied *sup-1* mutant. This mutant was characterized for an increased number of stamens. Interestingly, the *SUP* mutant alleles that have a 'superman' phenotype is just a simplified version as there are alleles showing different phenotypes. On an allelic series study done by Breuil-Broyer *et al.* (2016) the *SUPERMAN* mutants were classified in three groups: according to their floral indeterminacy. The groups are: *superman*, *superwoman* and *supersex* (Figure 6). The classification basically relies on the number of floral organs in the third and fourth whorl of the flower (Breuil-Broyer *et al*., 2016).

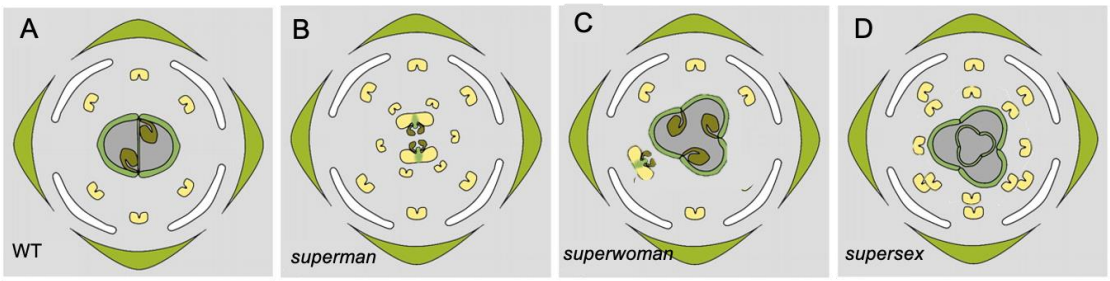


Figure 6. Classification of mutant alleles of *SUPERMAN*. A. Wild type flower with 4 sepals (green), 4 petals (white), 6 stamens (yellow) and two fused carpels (center). **B**. The 'superman' class of mutants with a limited number of supernumerary stamens and a reduced or absent carpel. **C**. The 'superwoman' class of mutants with more carpels and less stamens. **D.** The 'supersex' class of mutant with more carpels and more stamens than the wild type. Adapted from Prunet *et al*., 2009.

2.2.1. ´The 'superman' class

In the 'superman' class are included the *sup-1*, *sup-3* and *sup*-6 alleles. All of them are characterized by an increased number of stamens at expenses of the carpels. The

sup-3 and *sup-6* alleles present an alteration in the Zn finger motif and Ser/Pro rich domain respectively, while *sup-1* produces a possibly truncated protein (Bowman *et al*., 1992; Sakai *et al.,* 1995; Breuil-Broyer *et al.,* 2016). All together leads to conclude that the role of *SUPERMAN* in the 3rd and 4th whorl requires an intact protein *(*Bowman *et al*., 1992; Sakai *et al*., 1995; Breuil-Broyer *et al*., 2016).

2.2.1. ´The 'superwoman' class

The 'superwoman' class includes the epiallele *sup-eA31* which is characterized by an increased number of carpels. This class of mutants show a delay in the floral meristem termination and the phenotypes might be explained by the alternative or second model (section 2.1.2) (Breuil-Broyer *et al*., 2016).

2.2.2. The 'supersex' class

In the 'supersex' class is included the *sup-5* allele which shows one of strongest *SUP* mutant phenotype with an increased number of carpels and stamens. Studies with *sup-5* revealed that *SUP* also participates during late ovule development in the outer integuments asymmetrical growth. In addition to the ovule defects, *sup-5* mutant also shows a reduced fertility due to the abnormal positioning of the micropyle (Gaiser *et al.*, 1995; Jacobsen and Meyerowitz, 1997).

2.3. Functional studies of *SUP*-like genes

SUPERMAN has been widely studied in *A. thaliana* revealing crucial roles that *SUP* performs during floral development. Despite the important roles of *SUP*, until recently, there was only another *SUP*-like gene (*PhSUP*) functionally characterized on its own species (*Petunia hybrida*) (Nakagawa *et al.*, 2004). However, there are studies of *SUP*-like genes in tobacco, cucumber and rice based on gene expression analysis, ectopic expression and complementation assays (Nandi *et al.,* 2000; Nibau *et al.*, 2011; Zhao *et al.*, 2014). Interestingly, until now there are no published studies for

SUP-like genes in any legume family. Flowers are the most complex structure in plants and specially in legume species (Roque *et al.*, 2018). The characterization of a *SUP*-like gene in a model legume would be crucial for a better understanding of the novelties in flower development.

3. Background

Our research group is focused in understanding the genetic control of flower development in legumes, one of the crops of special importance. In the last decades our group has provided important contributions to this field using *Medicago truncatula* as a model system. The group has used the model legume to unveil the importance of duplicated genes, focused on the MADS-box genes, and their evolutionary fate in floral patterning in this species. However, floral organogenesis also requires a tight control of the cell proliferation and the cell identity specification. The *SUPERMAN* (*SUP*) gene from *Arabidopsis thaliana* has been characterized as a key player during floral organogenesis*. SUP* has been widely studied in *A. thaliana* but *SUP*-like genes in legume species remain uncharacterized. Here is presented the characterization of the *SUP* orthologous gene in *Medicago truncatula* (*MtSUPERMAN*; *MtSUP*).

Objectives

4. General objective

Legumes represent the third most important crop for human and livestock food so the knowledge generated could be used to improve their yield production. A better understanding of reproductive development in legumes is important for breeding purposes. Legumes have unique developmental novelties as their compound inflorescence and complex floral ontogeny that makes them of special interest to study the regulatory network behind these characteristics. *SUPERMAN* is a floral boundary gene of *Arabidopsis thaliana* that plays crucial roles during floral organogenesis controlling the number of stamens and carpels produced. Despite key regulatory genes, as *SUPERMAN*, have been thoroughly studied in *A. thaliana,* little is known in legumes. As mention before, legumes have different morphological traits which requires a more suitable model plant as *Medicago truncatula.*

The **general objective** of this work was to determine the roles of *MtSUPERMAN* during reproductive development in *M. truncatula.*

5. Specific objectives

To determine the roles of *MtSUPERMAN* during reproductive development in *M. truncatula* it was proposed to perform the molecular and functional characterization of *MtSUPERMAN.*

Objective 1. Molecular characterization

1.1. To identify the putative *SUPERMAN* ortholog in *Medicago truncatula* (*MtSUPERMAN*) based on phylogenetic analysis and sequence homology.
1.2. To analyse the expression pattern of *MtSUPERMAN* during inflorescence and flower development in *M. truncatula*.

Objective 2. Functional characterization

2.1. To evaluate the effects of ectopically express *MtSUPERMAN* in *A. thaliana*.

2.2. To evaluate the ability of *MtSUP* to replace SUP function through a complementation assay with *MtSUP* in the strong *sup-5* mutant allele of *A. thaliana*.

2.3. To functionally characterize a *Tnt1* insertional mutant for *MtSUP (mtsup-1)* in order to analyse *MtSUP* loss-of-function.

2.4. To characterize a *MtSUP* mutant allele generated by CRISPR/*Cas9* technology.

2.5. To analyze the expression pattern of various key regulatory genes which might interact with *MtSUP* during compound inflorescence and flower development in the wild type and mutants.

Materials and methods

1. Biological material

1.1. Plant material and growth conditions

In this study were used *Medicago truncatula* and *Arabidopsis thaliana* plants. The *M. truncatula* alleles included were wild type R108 and a *Tnt1* insertional mutant *(mtsup-1*) provided by the Noble Research Institute and a CRISPR/*Cas9* edited line (*mtsup-2*). The Arabidopsis lines used were *Landsberg erecta* (Ler) and *sup-5* mutant, ordered from the NASC stock centre. Detailed information is shown in Table 1.

Table 1. Plant material description

Specie	Allele	Mutation	Purpose
Medicago truncatula	R108	Wild type	*MtSUP* expression analysis and stable transformation.
	mtsup-1	*Tnt1* insertion 50 nt after ATG	*MtSUP* functional characterization
	mtsup-2	CRISRP-*Cas9* edited line	
Arabidopsis thaliana	Ler	Wild type	Stable transformation and overexpression assay
	sup-5	-54 +23 deletion	Complementation assay
	p*SUP*::*MtSUP*	*MtSUP* expression in *sup-5*	Complementation assay
	35S:: *MtSUP*	*MtSUP* overexpression	Overexpression assay

In this table are summarized all the plants used in this study including the allele, mutation and their purpose.

1.1.1. *Medicago truncatula* plants

To extract the seeds from Medicago, the dried fruits were mechanically scarified by rubbing them with sandpaper until its abrasion. Seeds were collected in a 50 mL conical tube to be treated with 7 mL of sulphuric acid during 5 min. Acid was eliminated

in the residue container No. 7 for acids. Under sterile conditions, seeds were rinsed five times with water, the last rinse lasted 2 hours. Seeds were set in a petri dish with humid filter paper sealed with film and covered with aluminium foil from light during two days at room temperature. For synchronization, seeds were moved to cold (4ºC) for one week. Seedlings around 4 cm were planted in 12 cm plastic pots with a mixture of peat:perlite:vermiculite (1:1:1) watered with Hoagland solution No.1. Each pot was covered with a plastic cup or film to maintain high humidity. After the first leaves emerged the cup or film was released. Plants were grown in greenhouse cabs or in a phytotron at 22ºC / 18ºC (day/night) with a photoperiod of 16 h light and 8 hours dark. Natural light was supplemented by mercury vapour lamps (400W).

1.1.2. *Arabidopsis thaliana* plants

Seeds were sterilized with ethanol and tween (0.01%) during one minute. Seeds were washed with ethanol (70%) for three minutes, and then placed on MS medium for germination one week at 22ºC. Alternatively, seeds could be directly placed on the soil without the sterilization and germination stages. Seedlings were sowed in the same mixture used for Medicago in pots (several seeds) or trays (individual seeds) and during 4 days were covered with film to maintain high humidity. Plants were grown at 22ºC / 19ºC (day/night) under long day conditions 16h/8h (light/dark), mercury vapour lamps (400W) were used to supplement natural light. Plants were irrigated with Hoagland's solution.

1.2. Bacterial material and growth conditions

1.2.1. Bacteria strains

In this study were included bacterial strains from the species *Escherichia coli* and *Agrobacterium tumefaciens*. Table No. 2 summarizes all the strains included and their purpose.

Table No. 2. Bacterial strains used in this study

Species	Strain	Purpose
Escherechia coli	DH5α	Bacterial transformation
	DH10B	Bacterial transformation
Agrobacterium tumefaciens	C58	*A. thaliana* transformation
	EHA105*	*M. truncatula* transformation

*EHA105 strain requires to be growth with rifampicin 20 ng/ µL to maintain plasmid integrity.

1.2.2. Growth conditions

E. coli and *A. tumefaciens* strains were grown at 37ºC / 28ºC for 24 / 72 hours respectively to obtain independent colonies. Same temperature conditions were followed for bacterial broth, incubation was overnight with agitation (225 rpm).

1.2.3. Media preparation

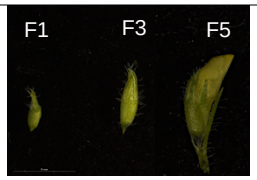
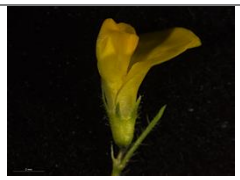
Bacterial strains were grown on Luria-Bertani (LB) media that contains tryptone 1%, yeast extract 1% and sodium chloride 1%; all adjusted to a pH of 5.7. For solid media 1.5% (weight/volume) of agar-agar (Pronadisa) was added to the media. Whenever a selection agent was required it was added to the liquid media when it reached a temperature around 55ºC after it was autoclaved.

2. Sample collection and fixation

Sample collection and fixation was performed according to which technique were required. Table No. 3 presents a summary of all the tissues collected whose

destination could be for nucleic acid extraction, *in situ* hybridization, histological analysis or microscopic techniques.

Table No. 3. Sample collected description

Plant material	Image
Floral apices	
Flowers before anthesis (F1, F3, F5)	
Flower at anthesis (FA)	
Carpels (FA)	
Fruits at 6 Days after pollination (DAP)	

Images intend to represent the tissue collected, details regarding the morphological variations between wild type and mutant tissues are in the results.

2.1. Sample collection for nucleic acids extraction

The RNA and DNA were obtained from fresh vegetative and reproductive tissues. The root and shoot apical meristem (SAM) were collected from seedlings and the stem and leaves from mature plants. The floral apices and flowers at

developmental stage 1, 3, 5 and at anthesis (FA) (Table No. 3) were collected from six weeks old plants. Plant material collected for RNA isolation was immediately frozen with liquid nitrogen. For DNA isolation the fresh tissue was kept on ice up to 20 minutes, otherwise it was frozen with liquid nitrogen. Tissues could be stored at -80ºC up to 6 months.

2.2. Sample collection and fixation for other techniques

All plant tissues intended to *in situ* hybridization, resin cross-section and scanning electron microscopy techniques were collected in a tube with FAE. FAE solution was freshly prepared with 50% ethanol, 5% glacial acetic acid and 4% formaldehyde. Samples were subjected to six vacuum pulses of 10 minutes each. FAE solution was changed every two pulses. Samples were incubated at 4ºC overnight, FAE was replaced with ethanol 70%. Samples could be stored up to six months at 4ºC. Before processing the samples, these were dehydrated with an ethanol gradient (85%, 90%, 95% and 100% ethanol) that lasted 10 minutes per change. The sample processing is detailed in the section of each technique.

3. Microscopic techniques

In this study the microscopic techniques included were light microscopy and macroscopy and scanning electron microscopy.

3.1. Light microscopy: differential interference contrast (DIC) optics

This technique was employed to capture the results from the *in situ* hybridization (ISH), seed and carpel clearing, and the resin cross section. The ISH produced a blue-violet color when transcript is detected and for this the DIC microscopy was useful. The microscope employed was a Leica DM5000 using the software NIS-Elements F3.0 with auto white option and manual control of contrast and brightness of the image.

3.2. Macroscopy

The fresh plant material was maintained in a petri dish with humid paper to avoid loss of turgidity. The equipment used was a Macroscope (Leica DMS1000) with a diascopic base for illumination.

3.3. Scanning electron microscopy (SEM)

3.3.1. Critical point processing

After dehydration process described on section 2.2, samples were dried using an Automated Critical Point Dryer (Leica EM CPD300) – program 8. For this process the equipment was filled with ethanol 100% that gradually was replaced for CO_2. Samples were managed in metallic baskets, always in anhydrous environment.

3.3.2. Sample dissection

A metallic plate was used as a base to place, dissect, coat and photograph samples. Carbon tape was used to clamp the samples and using forceps tweezers, a tweezer No. 3 and an awl. The carpels and floral apices were dissected through dry bumps to eliminate the undesired tissues maintaining the integrity of the structures.

3.3.3. Sample coating and image acquisition

Samples were sputter coated with gold during 90s pulse. The photographs were taken with an electronic microscope (Zeiss Germin). Images were acquired using a 20 µm aperture and 2 KV exposition.

3.4. Image processing

Adobe Photoshop® was used for the improvement of image quality and assembly of figures. ImageJ was used for quantitative purposes and image processing.

4. Molecular techniques

4.1. Plasmid DNA isolation

For plasmid recovery an individual colony was picked with a sterile toothpick and placed into 3 mL of LB media. This is incubated at 37ºC – 28ºC with agitation (225 rpm) during 24 – 48 hours for *E. coli* and *A. tumefaciens* respectively. Bacterial broth is centrifuged at 5,000 rpm during 5 minutes. The pellet is proceeded with the E.Z.N.A Plasmid DNA Mini Kit I (OMEGA – Bio-TEK) following manufacturer´s instructions.

4.2. Genomic DNA isolation

Two different DNA extraction procedures were followed according to the plant species.

4.2.1. Genomic DNA isolation for Medicago plants

Extraction was done with DNazol® Reagent according to the instructions of the manufacturer (Thermo Fisher) using 0.3 g of fresh tissue. DNA pellet was resuspended in 60 µL of buffer TE (Tris-HCL 10 mM and 1 mM EDTA at pH 8), gDNA was used for PCR or stored at -20ºC up to three months.

4.2.2. Genomic DNA isolation for Arabidopsis plants

Extraction was done from 100 mg of fresh tissue that was pulverized with a pestle in 500 µL of extraction buffer (0.2 M Tris-HCl pH 9.0; 0.4 M LiCl; 25 mM EDTA and 1% V/V SDS). Mixture was centrifuged during 5 min. at 13000 rpm, 350 µL of supernatant was transferred to a new Eppendorf with 350 µL of isopropanol, mixture was

centrifuged as before during 10 min. Supernatant was eliminated and the pellet was washed twice with ethanol 70%. Pellet was let dry and resuspended in 100 µL of buffer TE (Tris-HCL 10 mM and 1 mM EDTA at pH 8).

4.3. Total RNA isolation

RNA isolation was performed from 0.1 g of frozen tissue according to E.Z.N.A® Plant RNA kit recommendations (OMEGA bio-tek). One microgram of total RNA was treated with 1 µL Fermentas DNase I and 1 µL of buffer 10x during 30 min at 37ºC. For RNA precipitation were added 6 volumes of ammonium acetate (7.5 M) and 3 volumes of cold ethanol 100%. Mixture was incubated at -20ºC overnight and then centrifuged in cold (4ºC) at 15 krpm for 15 min. RNA pellet was washed with 500 µL of cold ethanol (70%) and centrifuged as before. The supernatant was eliminated, and the pellet was let dry for 5 min. RNA was resuspended in 10 µL of DECP water and quantified for reverse transcription (RT).

4.4. Nucleic acid quantification

Total RNA or DNA were quantified through spectrophotometry using a NanoDrop® ND-1000. The ratios 260/230 and 260/230 were used to examine quality of the samples.

Table 4. Nucleic acids purity ratios

Nucleic acid	260/280	260/230
DNA	~1.8	2.0-2.2
RNA	~2.0	2.0-2.2

4.5. Polymerase chain reaction techniques (PCR)

For these techniques specific primers were designed using primer3 considering a length between 16 - 25 nucleotides and a blast was done to evaluate non-specific

amplifications. Oligo Calc (http://biotools.nubic.northwestern.edu/OligoCalc.html) was used to evaluate CG content higher than 40% and potential hairpin formation.

Table No. 5. Primers list

Name	Primer Sequence	Use
MtACT-F	ATGTTGCTATTCAGGCCG	For RT-PCR
MtACT-R	GCTCATAGTCAAGGGCAAT	
Tnt1-F	ACAGTGCTACCTCCTCTGGATG	*mtsup-1* mutant genotyping
MtSUP-F	GTGAGAGTTAATGGTTGGATC	*mtsup-1* mutant genotyping
MtSUP-R	GAAAGTAAACATAAACTCTGACA	
MtSUP in situ F	GACAATCATCACCACCAACAACTC	*In situ* hibridization probe
MtSUP in situ R	GGGTACTACTACTTACTCTAATTAG	
Geno-CRIS-DIR	CCTCTTCAGCAACATCTCTCAAGCC	To genotype CRISPR/*Cas9* edited plants
MtSUP-ATG	ATGATGAAGAGGAACAATATGAACAC	RT-PCR and CDS of *MtSUP*
MtSUP-556-R	AGATGGATGGTTTTGAATGTGAAG	RT-PCR and to genotype CRISPR/*Cas9* edited plants
MtSUP-STOP	GGGTACTACTACTTACTCTAATTAG	CDS *MtSUP*
CRISPR_GS1-F	GTGCATCAGAAGATTACATGATGAA	To generate CRISPR *MtSUP* guide 1
CRISPR_GS1-R	AAACTTCATCATGTAATCTTCTGAT	
ProAtSUP5,1-F	GACGTTTGTCTGGAGAGAGGAATTGTG	Complementation Construct
ProAtSUP5,1-R	ATGTGCAAGCTCTTTCTTTTTGGCTATG	
3UTR_AtSUP-F	TTAGATGGTAATAACTTTATCCATAAAG	Complementation construct
3UTR_AtSUP-R	GAAGATTCATACTCCATCAAATAACAT	
MtWUS_in situ-F	GAGTGTACAATTTCAGCTGGATG	*In situ* hibridization probe
MtWUS_in situ-R	TTAATTAGCATAATCTGGTGACCTA	
MtPIM_in_situ-F	GTATACGCGACTGAAGGCAAAG	*In situ* hybridization probe
MtPIM_in_situ-R	TGGCAGGTATACAATGGTTCC	
MtPI-504	GGCACTTGAAGGTGTGGGAAA	*In situ* hybridization probe
MtPI-801	GAAACCAAATTCAATCACTTCATA	
MtFULc_in_situ-F	TGGAGCTACCAATGAAACACAG	*In situ* hybridization probe
MtFULc_in_situ-R	ACCTGCTTCTTCAAATTCCAATG	
MtSUP qPCR-F	GACCACAAAGACCTCAGAGAAA	RT-qPCR
MtSUP qPCR-R	TAGTACCCAAACGAAGCTCAAG	
TIP41 qPCR FOR	GTGAAAACTGTTGGAGAGAAGCAA	
TIP41 qPCR REV	TCAACTGGATACCCTTTCGCA	
MtFULc qPCR-F:	TGCAGGAGCAAAACAGCAAG	

MtFULc qPCR-R:	AGCACCTCTGGCTGACAAAT	
MtPIM qPCR-F	TATACGCGACTGAAGGCAAA	
MtPIM qPCR-R	CCTGGTTCTGCGTGTACGA	
MtAP1b-qPCR-F	TGTTGCACAAGAGGCTGC	
MtAP1b-qPCR-R	GGAAGAAGGGGATCTTGTAGTAAG	
MtWUS qPCR-F	AAACCCTCCCTTATATCCCTATG	
MtWUS qPCR-R	TCCAGCATCTTCAGCTTGATAC	
MtNGL9-qRTDIR	5GTTATTTCAATTCCCAGATGCCA	
MtNGL9-qRTREV	GCTGCATAGGCTGAACACGAA	
MtPI-qRTDIR	CATATGGCACTTGAAGGTGTGG	
MtPI-qRTREV	TGGTTAAATCCATTGTCCATGC	
MtNMH7-qRTDIR	TTAGGTCCACGCATGTTTGC	
MtNMH7-qRTREV	AGGATTAGGATGAGTAGGCTGTAAGC	
MtTM6-qRTDIR	CGAAGGAGACGAGGAATCAG-3’	
MtTM6-qRTREV	AGAGTGGAAGCACCATTGGC	
MtSecAg-qRTDIR	TGGCTACTAGGGTTGCTGGC	
MtSecAg-qRTREV	CCTCACCCAGTCCAGTGGAA	
MtAct11-F	ATGTTGCTATTCAGGCCG	RT-PCR
MtAct11-R	GCTCATAGTCAAGGGCAAT	

Primers concentration for PCR was 10 µM; for qPCR 300 mM; and for sequencing 5 µM.

4.5.1. Reverse transcription (RT)

One microgram of treated RNA (section 4.3) was retrotranscribed using the PrimeScript™ RT-PCR kit (Takara, Japan) according to the manufacturer instructions. cDNA was quantified and used for RT-PCR, quantitative PCR or stored at -80ºC up to 6 months.

4.5.2. Standard DNA PCR

To generate DNA fragments to genotype or for cloning purposes a standard PCR procedure was performed changing the specific primers (See Table No. 5), template DNA and the type of DNA polymerase. Long fragments and fragments used for cloning were amplified with TaKaRa Ex Taq™ (TaKaRa), a high-fidelity polymerase.

4.5.3. Reverse transcription PCR (RT-PCR)

Two microliter of reverse transcription reaction (section 4.4.1) were used to perform the RT-PCR analysis using the Speedy Supreme NZYTaq 2X green master mix (NZY tech), with *MtACTIN* (Medtr7g026230) as control gene. PCR products contain loading buffer from the master mix used.

4.5.4. Quantitative PCR (RT-qPCR)

In a final volume of 10 µL, twenty nanograms of cDNA (Section 4.5.4) were used for RT-qPCR with the respective primers (300 nM) that were mixed with EvaGreen® Master Mix (Cultek). Reactions were performed using a 7500 Fast Real Time PCR System (Applied Biosystems) in 96 well-optical plates. 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). Primer Express Software (Applied Biosystems, USA) with the default parameters was used to design the primers listed in Table No. 5.

4.6. PCR products visualization and purification

4.6.1. Agarose gel electrophoresis

Agarose gels were prepared at 1% (W/V) in buffer TBE 1% (Tris-HCl 0.89 M, boric acid 0.89 M and EDTA 2 mM). Samples were loaded with loading buffer 6X to a final concentration of 1X. Five microliters of the 1 Kb DNA ladder molecular weight markers was also loaded. The gel was run during 30 - 45 min. at a constant voltage (120V).

4.6.2. PCR product purification

Purification could be done directly from PCR product or from a gel band that contains the desirable product. In any case the NZYGelpure was used following the manufacturer indications (Nzytech, Lisboa).

4.7. Sequencing

The sequencing service from the IBMCP ("*Instituto de Biología Molecular y Celular de Plantas*") carried out all the fragments sequencing using a capillary sequencer ABI 3130 XL based on Sanger Technology. PCR products and plasmids were sent at a concentration of 10 ng/µL and 100 ng/µL respectively in a total volume of 10 µL.

5. Cloning techniques

5.1. Ligation reaction

The ligation reaction depends on which kind of vector you are using and the technology behind. Plasmids lists are summarized on Table No. 6.

Table No. 6. Plasmids list

Vector	Characteristics	Reaction	Use
pCR™8/GW/TOPO®	attL1 and attL2 sites, Spec^{R*}	Invitrogen™	Entry vector for Gateway technology
pENTR™/D-TOPO®	attL1 and attL2 sites, Kan^{R*}	Invitrogen™	Entry vector for Gateway technology
pGEM® T-easy vector	T7 and SP6 sites, Amp^{R*} and lacZ	Promega	Fragments cloning
pH7WG2	35S constitutive promoter from CaMv, Spec^{R*}	Invitrogen™	*MtSUP* overexpression

	BsaI reaction, Kan^{R*}	Multicloning assembly using Golden Braid 2.0
	BsmBI reaction Spec^{R}	

*Spec^{R}: spectinomycin resistance (100 µg/mL); Kan^{R}: kanamycin resistance (50 µg/mL); Amp^{R}: ampicillin resistance (100 µg/mL).

5.1.1. DNA ligation reaction

Ligation reactions mediated by a T4 ligase were done keeping a 3:1 molar relation between insert and vector. In the reaction were included ligation buffer 1X (Invitrogen®) and one unit (1U) of T4 ligase to a final volume of 10 µl. Ligation was carried out during 16 hours at 16ºC. Fragments for *in situ* hybridization probes or assemblies for other cloning steps were done in pGEM®-T easy (Promega) vector. Equation 1 was used to calculate the amount of insert required.

$$[\text{Insert (ng)}] = \frac{[vector\ (ng)] * insert\ size\ (kb)}{vector\ size\ (kb)} * molar\ relation\ (insert{:}\ vector)$$

Equation No. 1.

5.2. Gateway technology cloning system

Gateway technology is based on the lambda (λ) phage recombination properties. Gateway cloning vectors (see Table No. 6) have specific sites for recombination (att - site specific attachment). Entry Gateway vectors as pCR^{TM}8/GW/TOPO® (attL1 – attL2) and pENTR^{TM}/D-TOPO® (attL1 – attL4) introduce within the *att* sites the insert. The orientation of the insertion can be determined by sequencing using the T7 primer and reverse transcribed using an SP6 primer. A destination vector (*e.g.* pH7WG2) has two recombination sites attR1 and attR2, reaction is catalysed by a LR clonase

(Invitrogen™). This technology was used to generate the construct *pSUP*::*MtSUP* for the complementation assay (section 6.2).

5.3. GoldenBraid: multicloning system

GoldenBraid (GB) multicloning assembly was performed using two levels of plasmids alpha (α) and omega (Ω) in which only plasmids from the same level are compatible for the assembly ending in a plasmid from the other level. For example, alpha plus alpha end into an omega level, and otherwise. GB reaction was done using the enzymes *BsaI* or *BsmBI* and the ligase T4 in a digestion program of 25 cycles of digestion (2 min at 37ºC) and ligation (6 min. at 16ºC). Intermediate cloning products were used to transform *E. coli* DH5α in order to be verified or isolated for next step. Only verified and final constructs were transformed into *A. tumefaciens* for plant transformation.

5.4. Digestion with restriction enzymes

To verify if the cloning or multicloning was as expected digestion with restriction enzymes were performed with the buffer required following the incubation temperature and time indicated by the manufacturer. For multicloning were used 2-3 different restriction enzymes that cut in different positions and different pieces of the construct. An *in silico* digestion were used to list the number and size of the expected fragments. Digestions were run on an electrophoresis gel (section 4.6.1) to analyze the number and size of the bands. Additional to this verification in some intermediate and in all final constructs were partially sequenced (section 4.7).

5.4.1. Electroporation

Aliquots (50 µL) of electrocompetent bacterial cells were placed on ice for a gradual thaw. The cells plus 50 µL of LB broth and 0.8 µL of plasmid were added into an electroporation cuvette (Biorad). Electroporation were performed at 200 Ω, 25 µF and

1.8 kV for *E. coli* strains; and at 400 Ω, 25 µF and 1.8 kV for *A. tumefaciens* strains. Electroporation product was resuspended in 200 µL of LB. All was transferred into a sterile tube for recovery at 37ºC (*E. coli*) / 28ºC (*A. tumefaciens*) during 1 hour with agitation (225 rpm). Bacterial broth (100 µL) was plated on an LB agar plate with the selection agent. Independent colonies were used for plasmid DNA isolation (section 4.4).

6. Complementation and overexpression assays in *A. thaliana*

6.1. Overexpression assay of *MtSUP* in *A. thaliana*

The coding sequence (CDS) of *MtSUP* was amplified from gDNA of *M. truncatula* R108. The PCR product was cloned into the pCR™8/GW/TOPO® vector. An LR recombination reaction (Section 5.2) was performed using the pH2GW7.0 as destination vector. Final construct was cloned into *E. coli* (DH10b) to be verified and then into *A. tumefaciens* (C58) to transform *A. thaliana* (Ler) plants by floral dipping according to standard procedure. Seeds were recovered and germinated on media with hygromycin (20 mg/L) for selection. Resistant seedlings were transplanted to soil and 34 days old plants were used for molecular and phenotypic characterization.

6.2. Complementation assay of the *sup-5* mutant with *MtSUP*

To construct the g*AtSUP-MtSUP* transgene we used Gateway cloning technology based on LR recombination reaction between multiple entry clones. Coding sequence of *MtSUP* was amplified (section 6.1) and cloned between the L1 and L4 *attL* sites of the entry vector pENTR™/D-TOPO®. The 3'UTR sequence was amplified from *AtSUP* using the primers 3'SUP-FOR and 3'SUP-REV (Table No. 5); fragment was cloned into the pCR™8/GW/TOPO® entry vector. An LR recombination reaction between the two entry clones was done to have the first assembly CDSMtSUP::At3'UTR in the destination vector pH7m24GW.3. This first construct was used as a template to amplify the CDSMtSUP::At3'UTR fragment using the primers MtSUP-ATG and 3'SUP-

REV. This fragment was cloned into the pCR™8/GW/TOPO®. The 5' genomic sequence of *AtSUP* (5.1 kb) was amplified using the primers -5.1 pSUP and pSUP-REV (Table No. 5) and cloned between the L1 and L4 *attL* sites of the entry vector pENTR™/D-TOPO®. Another LR recombination reaction between the two last entry clones was done having the pH7m24GW.3 as destination vector to generate the g*AtSUP-MtSUP* construct. Final construct was cloned as described in overexpression assay (section 6.1). Transgenic lines g*AtSUP-MtSUP* were used as the pollen donor for crosses to the homozygous mutant *sup-5*. The resulting progeny were allowed to self-fertilize; next generation of plants resistant to hygromycin and homozygous for *sup-5* allele were used for molecular and phenotypic characterization of the complementation assay.

7. Generation of *MtSUP* mutants by CRISPR/*Cas9* system

7.1. Constructs assembly

The software ARES-GT (https://github.com/eugomin/ARES-GT) was used to design and select the guide (sgRNA) to target *MtSUP* gene using CRISPR/*Cas9* system as described by Minguet, 2020. Figure 7 shows a schematic representation of the guide positioning within the gene *MtSUP* and the transcriptional units of the CRISPR/*Cas9* system. The intermediate and final constructs were design using the GoldenBraid 2.0 assembly as described by Sarrion-Perdigones *et al*., 2013 (https://gbcloning.upv.es/). We generated an alpha vector named R1 (Figure 8) containing the h*Cas9* under the promoter AtUBQ (GB2478) and the hygromycin resistance genes (GB0235) for *in vitro* selection. The transcriptional unit named R2 (Figure 9) was generated with the sgRNA multiplexed (3x) under control of the promoter AtU6-26 (GB1001). The final construct named as R3 (Figure 10) was used to transform *M. truncatula* mediated by Agrobacterium.

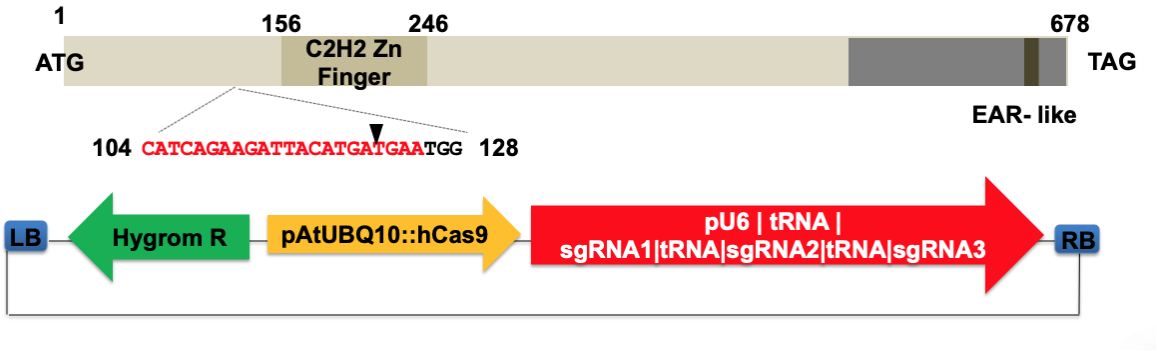


Figure 7. CRISPR/*Cas9* system used to target *MtSUP* Figure 7 shows a schematic representation of the transcriptional units used to target the gene *MtSUP.* Note that the guide is multiplexed three times. This strategy was followed to enhance the positioning of the guide in the target.

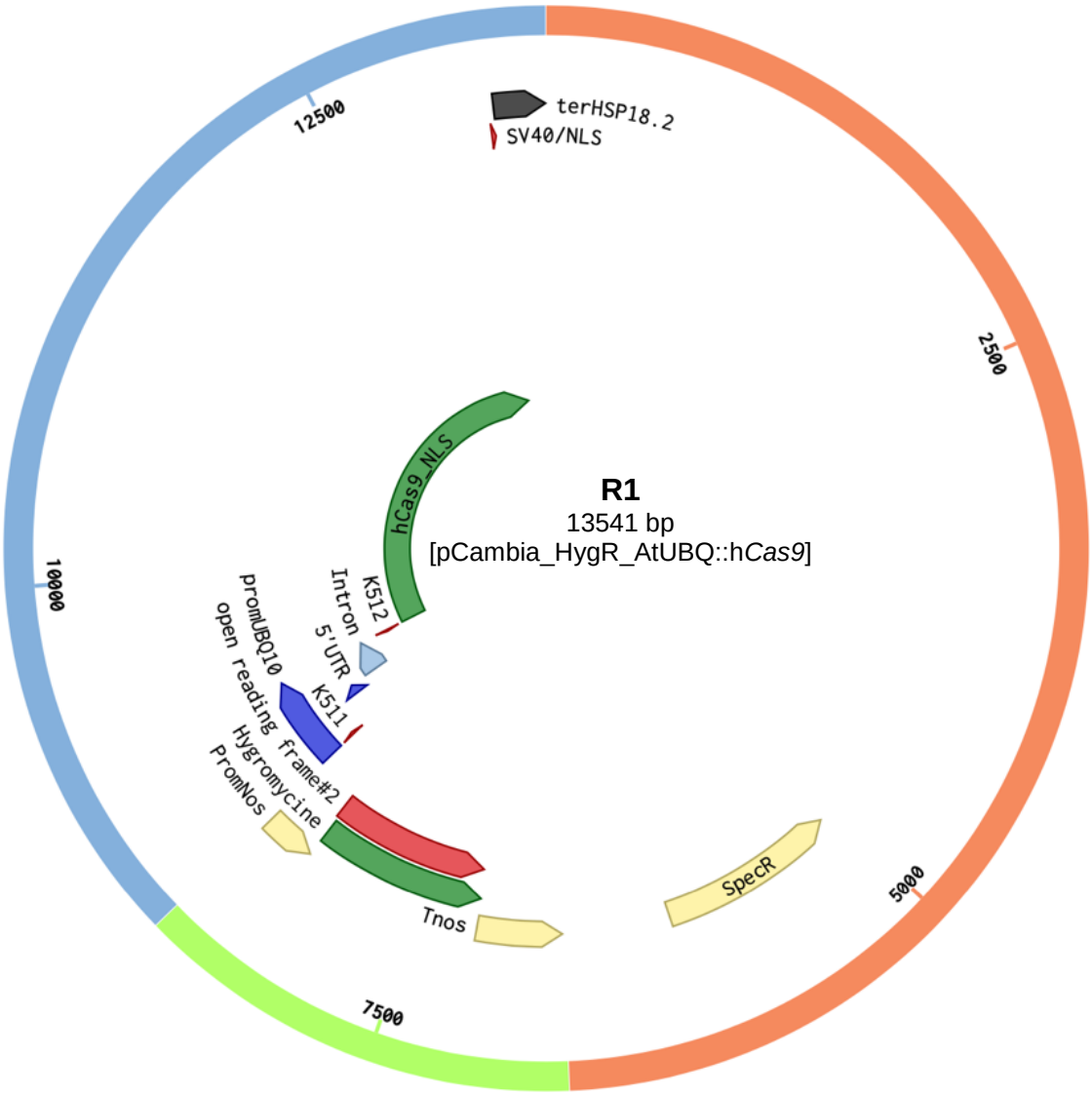


Figure 8. Schematic representation of R1 (α 1). This plasmid was obtained from the LR reaction between the plasmids GB2478 (Ω1) and GB0235 (Ω2) that ended in an alpha vector. R1 vector contains the expression module for hygromycin resistance genes and for the *Cas9* that is directed by the AtUBQ promoter.

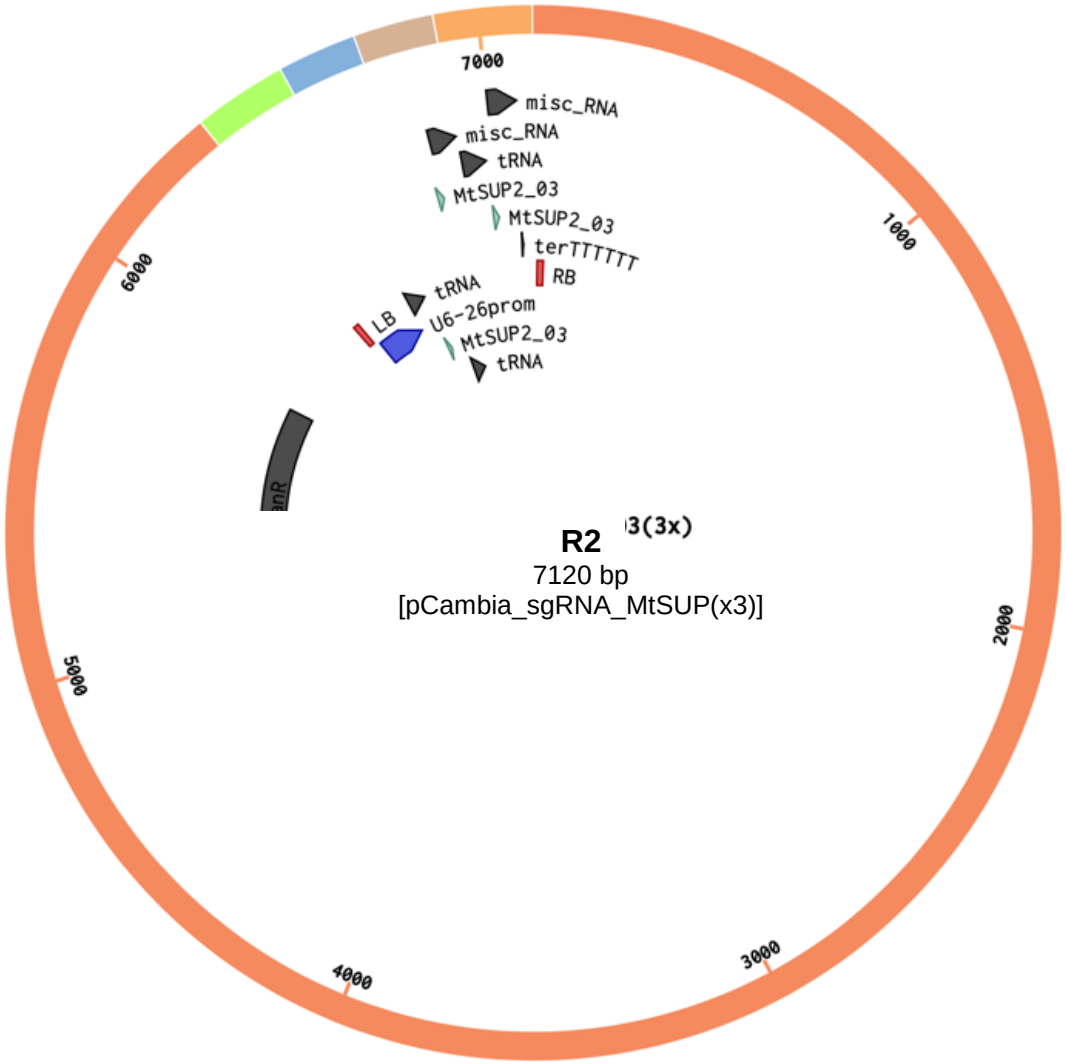


Figure 9. Schematic representation of R2 (α 2).R2 is and alpha vector that contains the sgRNA (3x) that will direct the *Cas9* to the target gene *MtSUP*.

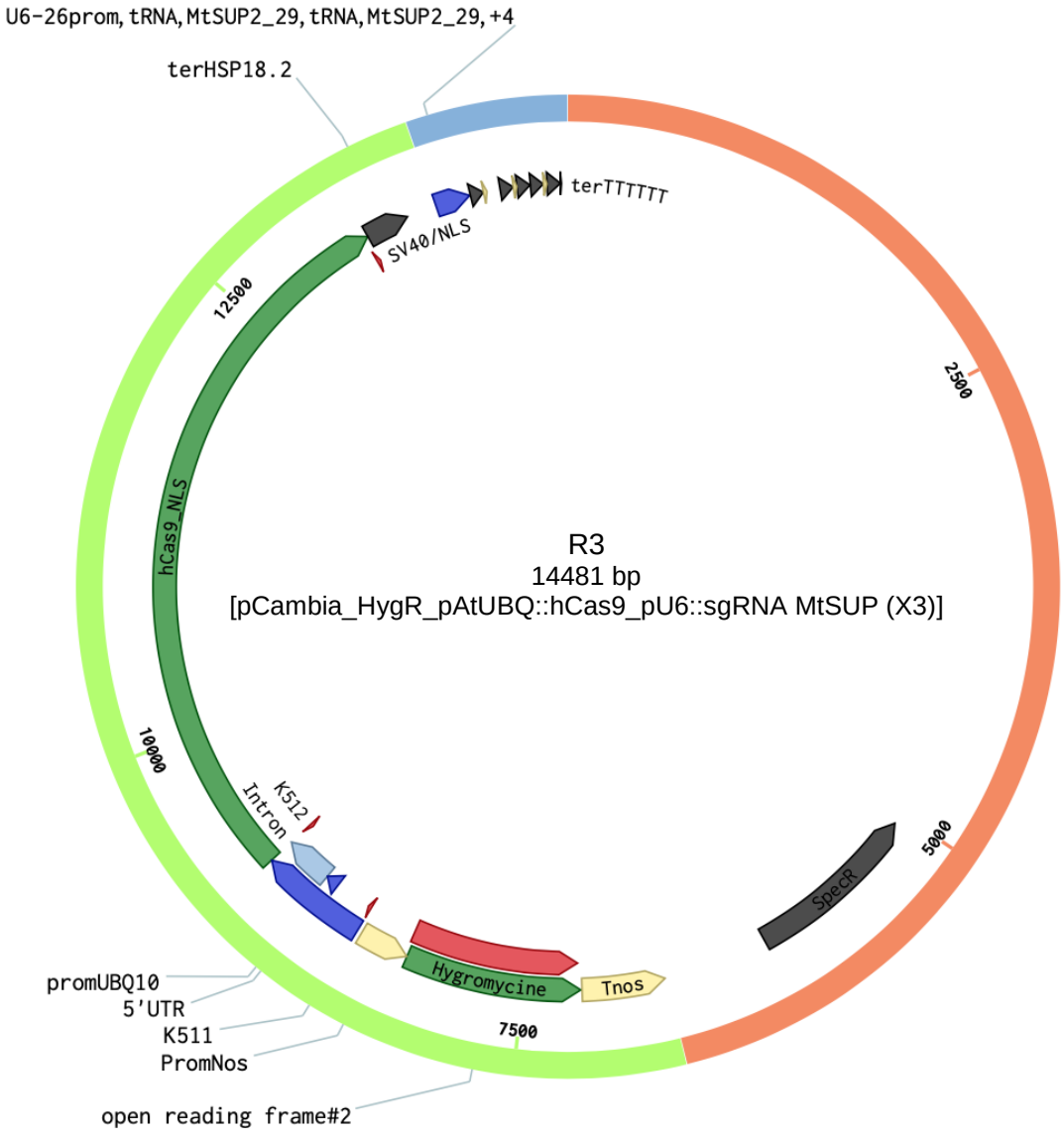
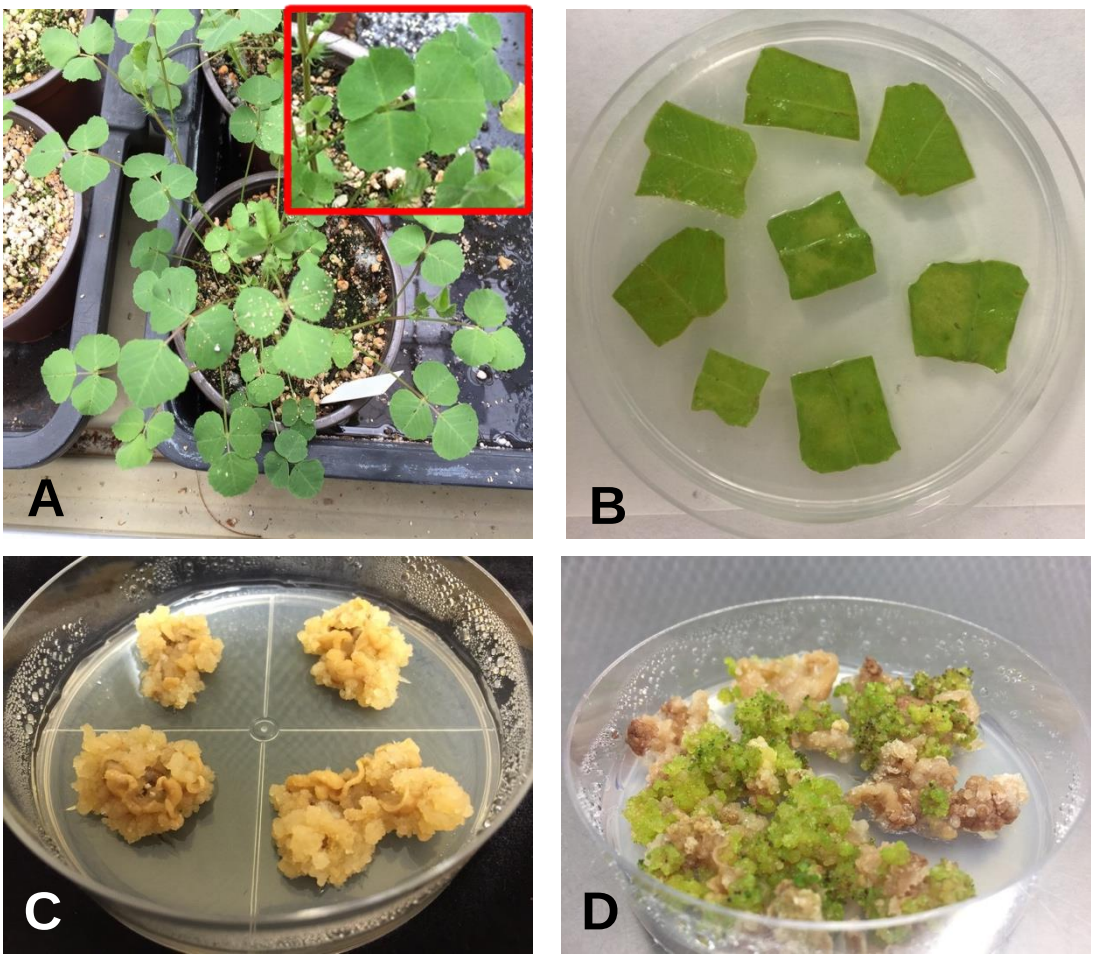


Figure 10. Schematic representation of R3 (Ω3). The R3 corresponds to the final plasmid that has an omega level. This was obtained from a LR reaction between the plasmids R1 (α 1) and R2 (α 2).

7.2. *M. truncatula* transformation using leaf explants

Medicago truncatula R108 transformation was performed according to Cosson *et al.*, 2014 using hygromycin 15 mg/L (Hyg15) for mutants' selection and timentin 200

mg/L] (Tim200) plus carbenicillin 200 mg/L (Carb200) for agrobacterium control. The strain *A. tumefaciens* EHA105 carrying the final CRISPR/*Cas9* construct was the vector that mediated the transformation. Thirty independent transformations were performed to obtain 15 transgenic plants. Main changes and annotations to the transformation procedure are listed below. Figure 11 shows the different steps of the regeneration process.



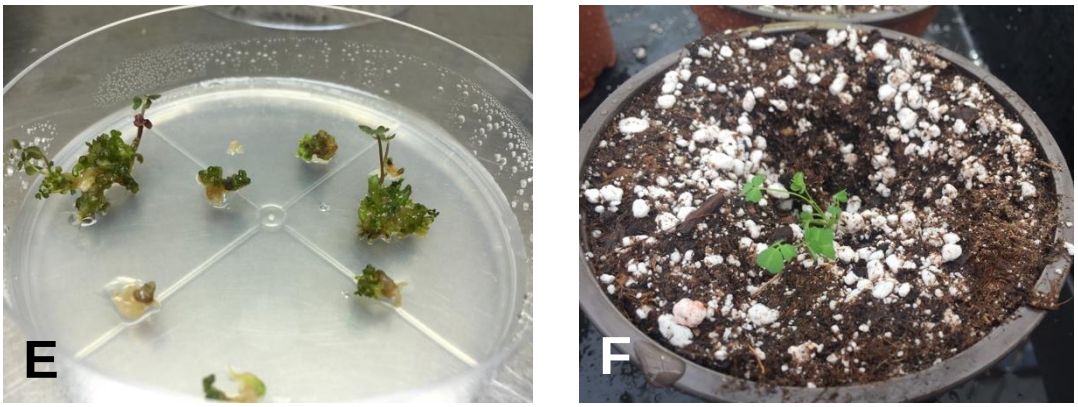


Figure 11. Regeneration steps of *M. truncatula* R108. A. *M. truncatula* R108 plants used for the transformation; a trifoliate leaf is shown in the red square. **B.** Leaf explants during co-culture step. **C**. Callus-mediated regeneration **D**. Somatic embryogenesis mediated regeneration **E**. Seedlings developing roots and aerial tissues. **F.** Seedlings transferred to the phytotron.

7.2.1. Media preparation

Table No. 7 N6 major salts stock composition

Compound	Quantity for 1L	Final concentration (mM)
$MgSO_4*7H_2O$	1.85 g	7.5
KNO_3	28.30 g	280
$(NH_4)_2SO_4$	4.63g	35
$CaCl_2*2H_2O$	1.66g	11
KH_2PO_4	4.00 g	30
H_2O	QSP 1 L	----

Store at 4ºC without autoclaving. Aliquots of 500 mL were frozen at -20ºC.

Table No. 8. SH minor salts stock composition

Compound	Quantity for 1L	Final concentration (mM)
$MnSO_4*H_2O$	1 g	60
H_3BO_3	500 mg	80
$ZnSO_4*7H_2O$	100 mg	3.5
KI	100 mg	6
$Na_2MoO_4*2H_2O$	10 mg	1
$CuSO_4*5H_2O$	20 mg ($CuSO_4$ = 12.8 mg)	0.8

$CoCl_2$*6H_2O	10 mg	0.4
H_2O	QSP 100 mL	----

Store at 4ºC without autoclaving.

Table No. 9. SH vitamins stock composition

Compound	Quantity for 1L	Final concentration (mM)
Nicotinic acid	500 mg	40
Thiamine HCl (B1 vitamin)	500 mg	15
Pyridoxine HCl (B6 vitamin)	500 mg	24
H_2O	QSP 100 mL	----

Store at 4ºC without autoclaving. Fifty milliliter aliquots were frozen at -20ºC.

Table No. 10. Antibiotics and hormones stock

Compound	Quantity for 10 mL	Final concentration
Hygromicyn	---	50 mg/mL
Timentin	2 g	200 mg/mL
Carbenicillin	3 g	300 mg/mL
2,4-Dichlorophenoxy acetic acid (2,4 D)	10 g	1 mg/mL
6-Benzylaminopurine (BAP)	10 g	1 mg/mL

Hygromycin was bought at the stock concentration (HygroGold™, InvivoGen). All antibiotics were filtered (0.25 µm filter) and divided in aliquots of 1 mL. 2,4D was dissolved in ethanol and stored at 4ºC. BAP was dissolved in a small volume of NaOH 2M and brought to final concentration with water; stock solution was stored at -20ºC.

Table No. 10. Media preparation

Compound or stock solution	SH3a (l)	SH3a (s)	SH9	1/2 SH9
	For 1L			
N6 major salts (mL)	100	100	100	50
SH minor salts (mL)	1	1	1	0.5
SH vitamins (mL)	1	1	1	0.5
EDFS [7 g/L] (mL)	20	20	20	10
Myo-inositol (mg)	100	100	100	50
Sucrose (g)	30	30	20	10
2-4 D [4 mg/L] (mL)	1.6	1.6	-	-

BAP [0.5 mg/L] (mL)	0.2	0.2	-	-
pH	5.8	5.8	5.8	5.8
H2O	1L	1L	1L	1L
Phytagel (g)	-	8.5	-	-
Kalys' agar (g)	-	-	7.5	7
Antibiotics	**SH3a (l)**	**SH3a (s)**	**SH9**	**1/2 SH9**
Hygromycin [50 mg/mL] (µL)	300	300	300	-
Timentin [200 mg/mL] (µL)	1000	1000	1000	-
Carbenicillin [300 mg/mL] (µL)	1000	1000	1000	-

EDFS -ethylenediaminetetraacetic acid ferric-sodium salt- (E6760 Sigma-Aldrich) stock solution was prepared adding 7 g of the compound in 1 L of water, this was stored at 4ºC up to three months. Antibiotics were added after autoclaving the media.

7.2.2. Agrobacterium preparation

All steps listed below were done in duplicated.

7.2.2.1. Pre-culture

Two days before the transformation an independent colony from a fresh culture of agrobacterium was inoculated into 2 mL of LB plus rifampicin (50 mg/L) and kanamicyn (50 mg/L) to be incubated overnight at 28ºC with agitation (200 rmp).

7.2.2.2. Culture

The day before the transformation similar procedure to pre-culture was followed at large scale (100 mL of LB). For the inoculation, pre-culture was diluted 1/500. In the transformation day, once the cultures had an $O.D._{(600\ nm)}$ of 0.6 were centrifuged at 3,000 rpm x 20 min in cold (4ºC). Pellets were resuspended in same initial volume with $SH3a_{(l)}$ media and were kept on ice until infiltration step (section 7.2.3).

7.2.3. Leaf explants preparation

Leaf explants were obtained from 42 – 50 days old plants grown in the greenhouse, which seedlings were not subjected to vernalization. Per each independent transformation 100 young trifoliate leaves (300 leaves) were cut. Leaves were washed during 7 min. with a sodium hypochlorite solution (10%) plus two drops of dish soap. Under sterile conditions leaves were washed four times with water and were kept humid for the infiltration step.

7.2.4. Infiltration and co-culture

Infiltration was performed cutting three rectangles from each leaf while these were soaked in bacterial broth. After infiltration leaf explants were co-cultivated on $SH3a_{(s)}$ without antibiotics during 18 hours at 24ºC in darkness. Afterward, explants were washed with liquid media (SH3a) plus antibiotics (Carb200 + Tim200) to eliminate the excess of agrobacterium. Regeneration and transformation controls were soaked in sterile liquid media.

7.2.5. Callus formation

Leaf explants were set on SH3a(s) media with all the antibiotics (Hyg15+Tim200+Carb200) around 6-8 weeks at 24ºC in darkness to induce callus formation. Explants were turned every two weeks to enhance the production of callus. During this step and forward, regeneration control was grown in media without hygromycin; while transformation control only with hygromycin.

7.2.6. Somatic embryogenesis

Callus were transferred into $SH9_{(s)}$ media at 24ºC with light (130 µE/m2/s) in a 12 hours photoperiod. Media refreshment was every three weeks or whenever it was a contamination. Pre-embryos started to appear around the fourth week; 20-30 days

later embryos were developed. Three weeks later embryos developed cotyledons and a small radicle (seedlings).

7.2.7. Seedlings

Seedlings were transferred into Magenta^{TM} B-cap 330 mL vessels with ½ $SH9_{(s)}$ media for aerial (stem and leaves) and root development. Four to six weeks later seedlings were transferred to the greenhouse. Some seedling did not develop a rooting system so the media was supplemented with indole-3-acetic acid (0.5 mg/L).

7.2.8. Phytotron transfer

Seedlings were transferred to a phytotron chamber as described for seedlings becoming from dried fruits (section 1.1.1). No specific requirements were followed despite the growth conditions at 22ºC / 18ºC (day/night) with a photoperiod of 16 h light and 8 hours dark. The most important consideration for this step is to have a clean phytotron without plagues.

8. RNA *in situ* hybridization

RNA digoxygenin-labelled probes were *in situ* hybridized as described by Gómez-Mena and Roque, 2018. Sense and antisense probes were generated by the T7 and SP6 polymerases with specific fragments of the genes *MtSUP, MtPIM, MtFUlc, MtPI* and *MtWUS*. Fragments were cloned into pGEM® T-easy vector (Promega). Primers used to generate these fragments are listed in Table 1.

8.1. Solutions preparation

The majority of the steps are RNase sensitive. Accordingly, all the material and solutions were kept in separate storage and handled under the cleanest conditions as possible. Carbonate buffer had 80 mM $NaHCO_3$ and 120mM Na_2CO_3 (pH 10). PBS

10 x contained 1.3 M NaCl, 0.03 M Na_2HPO_4 and 0.03 M NaH_2PO_4. Saline sodium citrate solution (20x) was prepared with 3 M NaCl in 0.3 M sodium citrate adjusted to a pH 7.0. Hybridization buffer contained 5x SSC, 3% SDS, 50% formamide. Washing buffer was prepared with 50% formamide and 2x SSC. Tris-buffered saline stock (TBS 10x) was prepared with 1 M Tris-HCl pH 7.0 and 5.4 M NaCl. Blocking buffer was prepared with 0.5% blocking reagent (Roche) in TBS 1x. BSA buffer had 1% bovine serum albumin (BSA), 0.3% triton x-100 in TBS (1x). Alkaline buffer has 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$. Detection buffer was prepared with alkaline buffer, 150 µL of BCIP (50 mg/mL) and 150 µL NBT (100 mg/mL) per 100 mL of buffer.

8.2. Samples embedding in paraffin

Samples were fixed and dehydrated as described in section 2.2. Ethanol was gradually replaced by Histo-Clear® (National Diagnostics) at room temperature incubations. First two incubations of 30 min. each in ethanol 100%, then three incubations of 2 hours each in ethanol : Histo-Clear® solutions (3:1, 1:1, 1:3 v/v). Another two incubations of 2 hours each with 100% Histo-Clear® were performed. A similar procedure was followed to replace Histo-Clear® by paraffin Paraplast® Plus (McCormick Scientific) starting with an over-night incubation at 58ºC, paraffin replaced one third of the Histo-Clear® volume. Same incubation was followed the next 2 days replacing the mix with liquid paraffin every 4 hours. All these incubations were performed with an automatic tissue processor in which all the process lasted 24 hours.

8.3. Paraffin block sectioning

Once the samples were embedded in liquid paraffin, these needed to be individually positioned into metallic molds and let solidify. Blocks could be stored at 4ºC. Paraffin blocks were cut in 8 µm sections with a microtome (Microm HM330).

Strips of 8 sections were floated into warm (37ºC) water to spread and transfer them into a glass microscope slide. Slides were placed over a hot plate (40ºC) to let dry.

8.4. Gene fragment cloning

A specific region around 400 bp of the gene of interest was chosen to be amplified by PCR using cDNA as a template. Fragments were cloned into pGEM® T-easy vector containing the T7 and SP6 RNA Polymerase promoters to be cloned in antisense orientation and generate the probe. For the negative control the same was followed using the cloned amplified in sense orientation.

8.5. RNA probe transcription and labelling

The vector containing the gene fragment (1 µg), 2 µL of DIG RNA labeling mix (Roche), 2 µL of RNA polymerase buffer (10x), 1 µL of RNase inhibitor and 2 µL of RNA polymerase (T7 or SP6) were mixed in a total volume of 20 µL to be incubated during 2h at 37ºC. Add 1 µL of DNase I (Fermentas) and incubate other 15 min. Stop the reaction adding 2 µL of 0.2 M EDTA (pH 8.0). The probe was precipitated adding one volume of ammonium acetate (7.5 M) and 6 volumes of cold ethanol (100%). This was incubated at -80ºC over-night, followed by a cold (4ºC) centrifugation for 10 min. at 5,000 rpm. Pellet was washed with 500 µL of ethanol (70%) and centrifuged for 5 min. at 5,000 rpm to remove ethanol. Pellet was let dry and then dissolved in 50 µL of RNase free water. Probe was run in a 1.5% agarose gel to check integrity.

8.6. DIG-labelled probes quantification

RNA probes were serial diluted (1/10, 1/100, 1/1000 and 1/10000) as the DIG-labelled control (100 ng/µL). One µL of each dilution was blotted into a charged (+) nylon membrane to be UV-cross linked. Membrane faced up was incubated 5 min. in TBS (1x), 10 min. in blocking buffer, 5 min. in BSA buffer, 2 min. in Anti-DIG-AP antibody (1:3000 in BSA buffer), 5 min. in BSA buffer (twice), 2 min. in alkaline buffer

and 12 min. in detection buffer (in darkness). Membrane was rinsed in water to stop the reaction and compare the intensity of the spots with the control and estimate the concentration to be adjusted at 0.3 ng/µL/kb.

8.7. Pre-hybridization

Slides obtained from section 8.2 were place in zigzag in a rack that was placed into a glass container of 250 mL with the required solution. For the deparaffination and rehydration the racks were immersed twice in Histo-Clear® (10 min) and followed by incubations of 2 min. each with ethanol series (100%, 80%, 95%, 70%, 50%, 30% and water). For the hydrolysis, samples were incubated 20 min. in HCl (0.2 M), 5 min. in water, 5 min. in 2x SCC (repeat) and 5 min. in water. For proteinase K treatment, samples were incubated 17 min. in proteinase K (1µg/mL in 100 mM Tris; 50 mM EDTA pH 8.0), 2 min in glycine (2mg/mL in PBS) and two washes in PBS 2 min. each. For fixation, samples were incubated 10 min. in formaldehyde (4% in PBS), two washes in PBS 5 min. each. To rehydrate samples were incubated 2 min. in water and then in ethanol series (30%, 50%, 70% and 95%, twice in 100%) to let them dry completely before the hybridization.

8.8. Hybridization and washes

Hybridization buffer was used to dilute the probes considering that each slide requires 60 µL. Probe was denaturized with a heat block at 80ºC during 2 min. then transferred to a thermo block at 50ºC. Sixty microliters were added to each slide and covered with a plastic coverslip avoiding air bubbles. Slides were transferred to a humid chamber that was sealed to avoid evaporation. Chambers were incubated over-night at 50ºC (oven). Slides were transferred to the rack and incubated 30 min. in washing solution to facilitate coverslips fall off. Slides were washed twice at 50ºC during 90 min. in washing solution (50% formamide, 2xSCC).

8.9. Immunodetection of DIG-labelled probes

Slides were transferred facing up to square petri dishes with TBS. Following incubations replaced the previous solutions. Slides were incubated in blocking buffer 1 h, BSA buffer for 30 min. and in Anti-DIG-AP antibody (1:3000 in BSA) for 90 min. to be washed during 5min. Slides were three times washed with BSA buffer (30 min. each). For the detection slides were transferred to clean square Petri dishes to be incubated 5 min. in alkaline buffer and up to 72 h. in detection buffer with NBT/BCIP substrates (in darkness). Reaction was stopped by immersing the slides in water. To eliminate background samples were incubated 2 min. in water and ethanol series (70&, 95%, 100%) and let dry.

9. Resin cross-sections

Samples were pre-infiltrated and infiltrated in synthetic resin (Leica®) according to manufacturer´s instructions in darkness and agitation. In the process, the samples were incubated (1h) in solutions with different proportions of ethanol 100% and resin I (ethanol: resin I: 2:1, 1:1, 1:2). The final incubation was in pure resin and lasted 48 hours, resin was refreshed once. Resin I was prepared adding 0.250 mg of hardener I to the synthetic resin. Resin II (hardener II) was used for the final inclusion in which the samples were set 2 days for complete solidification. Material was sectioned (1 µm thickness) with a rotary ultramicrotome (Reichert-Jung, Ultracut E) and stained with toluidine blue solution (1%) during 3 min. Sectioned and stained material was imaged using light microscopy (Leica, DM5000).

10. Ovule clearing

Ovule clearing was performed according to Noguero *et al*., 2015. First, fresh tissue was incubated one hour in a solution of methanol and acetic acid (9:1). Then, samples were incubated in chloral hydrate solution from 4 – 6 hours. Chloral hydrate solution

was prepared mixing 40 g of chloral hydrate in 5 mL of glycerol (100%) and 10 mL of water. Solution was kept at 4ºC in darkness.

11. Functional characterization of *MtSUP mutants*

11.1. *Tnt1* insertional mutant's genotyping

Tnt1 insertion lines for *MtSUP* ordered from the Noble Research Institute were genotyped by PCR using two sets of primers in order to differentiate homozygous from heterozygous plants for the insertion. A trifoliate leaf from two weeks old plants was used to extract genomic DNA and perform the PCR.

Table No. 11. Tnt1 insertional lines for *MtSUP.*

Line	Tnt1 line	Set of primers I	Set of primers II	Gene accession number	Insertion position (nt)
1	NF11278	Tnt1-F + MtsupF	MtsupF + 556rev	*MtSUP* Medtr2g076060	50
2	NF6111	Tnt1-F + MtsupF	MtsupF + 556rev		164
3	NF15766	Tnt1-F + MtsupF	MtsupF + 556rev		198
4	NF5413	Tnt1-F + MtsupF	MtsupF + 556rev		319

Set of primers I indicated the presence of the retrotransposon in the target gene, while the second set of primers indicated if the insertion was in one or both copies of the gene.

The line 1 had the expected segregation 1:2:1 and only homozygous plants had a phenotype associated to the gene function. The other lines were related to embryonic lethality, no homozygous plant was obtained.

11.2. CRISPR/*Cas9* mutant's genotype

All the *M. truncatula* regenerated plants (N=15) subjected to the transformation process were considered as putative CRISPR/*Cas9* mutants. Floral apices of 40 days

old plants were used to extract genomic DNA and perform two PCRs using specific primers for *hCas9* and *MtSUP* (Table No. 5). For *hCas9* was also analysed the expression through a RT-PCR. For *MtSUP* were used primers flanking 300 bp upstream and 300 bp downstream from the expected edition position. The fragment generated for *MtSUP* was directly sequenced to analyse plausible editions using the web platforms TIDE (Brinkman *et al.*, 2014) and DSDecode (Liu *et al.*, 2015) with default settings. Transcripts analysis was performed through RT-PCR with primers for the complete coding sequence (Table No. 5).

11.3. Phenotypic characterization of *MtSUP* mutants

MtSUP mutants were phenotypically characterized during different stages of reproductive development including compound inflorescence development; floral organogenesis; ovule and carpel development; and embryo development. The analysis was always performed in parallel with the wild type *M. truncatula* R108. Samples analysed by *in situ* hybridization, scanning electron microscope (SEM) or resin sectioning was fixed and dehydrated as described in section 2.2. Sample processing was according to the technique used, which is described in their corresponding section.

11.3.1. Macroscopic observations

For each allele (mutants and wild type) were analysed 120 inflorescences from 12 independent plants (10 inflorescences per plant). For the analysis were counted the number of flowers per inflorescence; from each flower a macroscopic observation was made to describe any abnormality (*e.g*. absence of residual organ or spike). To determine the number of organs per whorl, 100 flowers (10 flowers / plant) of each allele were dissected separating the sepals, petals, stamens and carpel. To determine the fruit yield 100 flowers (10 flowers / plant) were labelled at anthesis. After 20 days it was determined the percentage of flowers that produced fruits. A similar procedure

was performed to determine the number of seeds per fruit extracting and counting the seed from 100 dried fruits (10 fruits / plant).

11.3.2. Scanning electron microscopy (SEM) analysis

Young floral apices from 35 days old plants and carpels from flowers at pre-anthesis were examined by scanning electron microscopy. Floral apices were analysed to determine defects during floral organogenesis and compound inflorescence development.

11.3.3. *In situ* hybridization assays

For each allele (wild type and mutants) the first floral apices found after floral transition were collected from 12 plants and processed for *in situ* hybridization. This was performed to have a detailed *MtSUP* spatial and temporal expression pattern. This technique was also used to compare the expression pattern from genes of interest as *MtWUS, MtPIM, MtFULc;* always comparing the wild type with the mutant.

11.3.4. Resin cross-section analysis

For each allele (wild type and *mtsup-1*) carpels from flowers at anthesis were sectioned and stained in order to perform a histological examination of the gynoecium. Equivalent sections from three different carpels were used to calculate the ovary and ovule areas using ImageJ (section 3.2).

11.3.5. Ovule clearing analysis

One hundred carpels from each allele (wild type and *mtsup-1)* were treated according to Noguero *et al*., 2015 (section 10) with chloral hydrate solution for 6 h and observed with a Microscope (Nikon, Eclipse E600) equipped with Nomarski DIC optics.

11.3.6. Transcripts analysis

Total RNA was isolated from floral apices in order to analyse transcripts generated from *MtSUP*. For *mtsup-2* RT-PCR product was directly sequenced to confirm the edition.

12. Bioinformatic analysis

12.1. Sequence analysis and phylogenetic tree

In order to identify all the putative orthologs of *SUPERMAN* in *M. truncatula* the nucleotide sequence of *SUP* (AT3g23130) was set as the query against the Legume Information System (https://legumeinfo.org/). From all the sequences retrieved the ones that had an e-value up to E-18 were chosen to construct the phylogenetic tree. In the tree were also included the *Petunia hybrida* (petunia) orthologs of SUP. It was used MEGAX software (Kumar *et al.*, 2018) based on Neighbor-Joining method (Saitou and Nei, 1987) and the Jones–Taylor–Thornton matrix-based model (Jones *et al.*, 1992). All ambiguous positions were removed for each sequence pair with the pairwise deletion option. The accession numbers of the genes are shown on the right of the branches. The tree was rooted using the Arabidopsis gene *UPRIGHT ROSETTE* (*URO*) (AT3G23140) as outgroup. Reliability of internal nodes was assessed using bootstrap (Felsenstein, 1985) with 1000 pseudo-replicates.

12.2. Microsynteny conservation

VISTA program http://www-gsd.lbl.gov/vista/ (Frazer *et al.*, 2004) was used to evaluate microsynteny conservation between *MtSUP* and *SUP.*

12.3. Protein alignment

The protein alignment of AtSUP, PhSUP and MtSUP was conducted using the multiple sequence alignment program PRALINE (http://www.ibi.vu.nl/programs/pralinewww/). We used the Color Align Conservation program (https://www.bioinformatics.org/sms2/color_align_cons.html). Residues that are identical among the sequences have a black background, and those that are similar among the sequences have a grey background. The remaining residues receive a white background.

13. 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).

14. Statistical analysis

All the statistical analyses were performed using SPSS® Software and Microsoft Excel considering the parameters and requirements for each test. The significance value for all the cases was of 0.05 with a minimum of three biological replicas.

Results

Characterization of *MtSUPERMAN* in *M. truncatula*

1. Identification of the putative *SUPERMAN* ortholog in *M. truncatula* (*MtSUPERMAN*)

In order to retrieve all the *SUP*-like genes from *Medicago truncatula* the *SUPERMAN* sequence (AT3G23130) was set as query against the Legume Information System (https://legumeinfo.org/). From all the sequences retrieved with an e-value up to E-18 a phylogenetic analysis (Figure 12) was performed including other *SUP* orthologs. In the *SUP* clade only one gene (*Medtr2076060.*1) from *M. truncatula* falls along with *SUP,* other SUP homologs from other legume species, and *PhSUP* the *SUPERMAN* ortholog in *Petunia hybrida* (Sakai *et al.*, 1995; Nakagawa *et al.*, 2004).

SUP-like genes form a well-defined clade different from other zinc-finger protein members of the Cys_2His_2 family (Figure 12). For example, the *RABBIT EARS (RBE)* gene from *A. thaliana* forms a separate clade from the *SUP* clade (Takeda *et al.*, 2011). Studies in *A. thaliana* have revealed that *SUPERMAN* is a single copy gene which is exclusively expressed in the floral apices (Sakai et al., 1995). In contrast with legumes as soybean (*Glycine max*) and lupin (*Lupinus angustifolius*), which harbor two *SUP*-like genes. In *M. truncatula* a single gene is the homolog of *SUP* that was renamed as *MtSUPERMAN* (*MtSUP*).

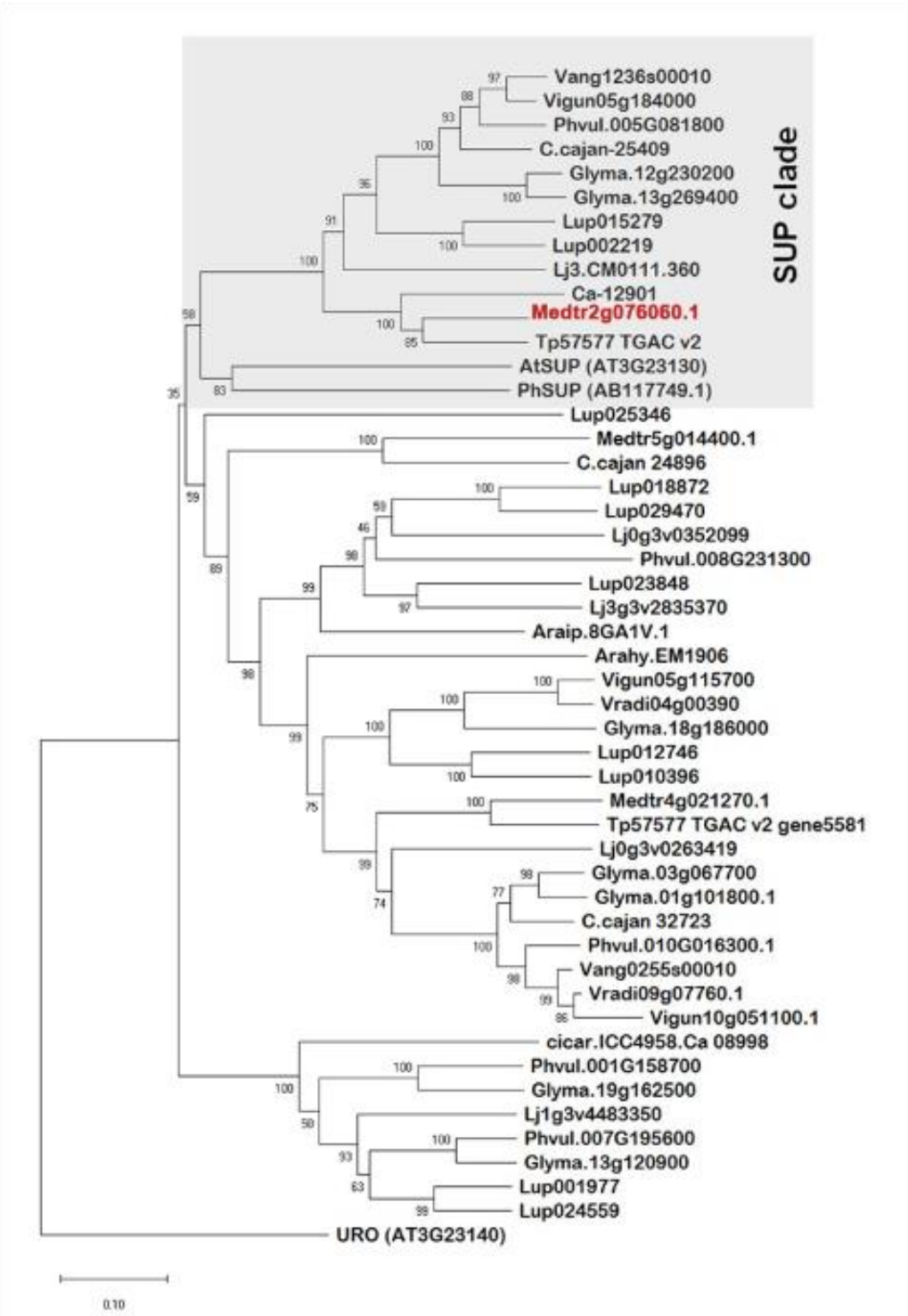


Figure 12. Phylogenetic analysis of *SUPERMAN*-like genes. This phylogenetic tree was constructed based on the Neighbor-Joining method using the *SUP*-like sequences of *Medicago truncatula* (Medtr), *Arachis ipaensis* (Araip), *Arachis hypogaea* (*Arahy*), *Cajanus*

cajan (C.cajan), *Cicer arietinum* (Ci/Cicar), *Glycine max* (Glyma), *Lotus japonicus* (Lj), *Lupinus angustifolius* (Lup), *Phaseolus vulgaris* (Phvul), *Trifolium pratense* (Tp), *Vigna angularis* (Vang), *Vigna radiata* (Vrad), *Vigna unguiculata* (Vigun), *Arabidopsis thaliana* (At) and *Petunia hybrida* (Ph). The number next to the branches represents the percentage in which the associated taxa are clustered together based on the bootstrap test (10,000 replicates). The tree was drawn to scale to the units of the evolutionary distances that were computed using the JTT matrix-based method. Evolutionary analysis was conducted in MEGA X.

2. Sequence and microsynteny analysis

MtSUP, *SUP* (*AtSUP*) and the *SUP* ortholog in *Petunia hybrida* (*PhSUP*) sequences were included in the alignment analysis. Besides *SUP*, *PhSUP* is the only ortholog that has been functionally characterized in its own species (Nakagawa *et al.*, 2004). Sequence alignment between *SUP* (*AtSUP*), *PhSUP* and *MtSUP* shows two highly conserved domains: the zinc-finger domain (QALGGH) and the EAR motif (DLELRL) (Figure 13, red squares).

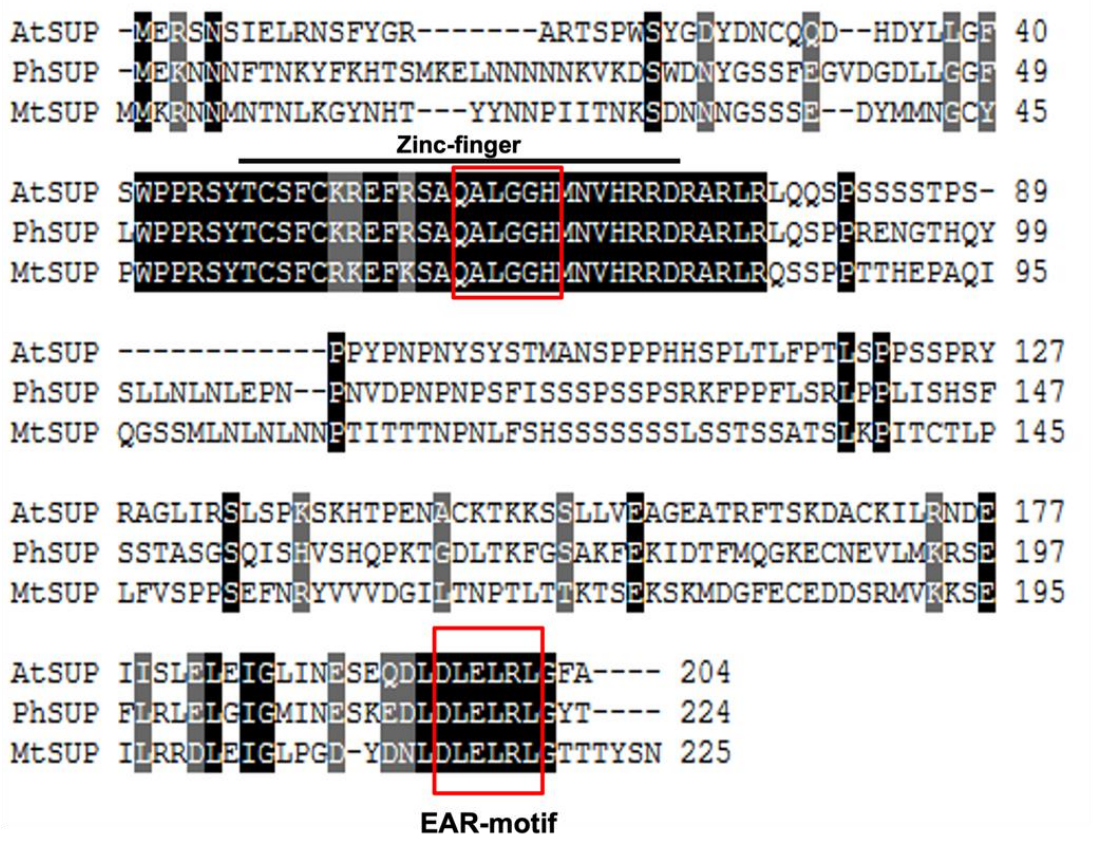


Figure 13. Sequence alignment between *SUP* orthologs. *In silico* alignment of the tree

AtSUP, PhSUP and MtSUP proteins show a highly conserved sequence in the zinc-finger domain and the EAR-motif. Conserved amino acids are enclosed in the red squares. The identical residues are shaded in black and in gray the ones that are only conserved in two of the three sequences. Gaps (-) correspond to absent amino acids that at least exist in one of the proteins included in the alignment.

Figure 14 shows a schematic representation of the 225 amino acids protein that *MtSUP* encodes. The zinc-finger domain is located between the amino acids 56 to 77 (nucleotides 168 – 231), while the EAR motif is situated within the amino acids 213 – 218 (nucleotides 639 – 654). Based on *SUP* studies in *A. thaliana* the EAR motif is considered to be an active repressor (Hiratsu *et al.*, 2002) while the Zn finger domain has specific DNA binding properties (Takatsuji, 1999).

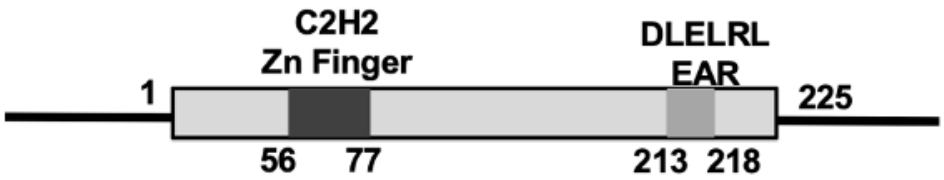


Figure 14. Schematic representation of *MtSUPERMAN*. The scheme of MtSUP protein was based on the graphical structure retrieved from www.uniprot.org (UniProtKB-Q2HSC7). The zinc-finger domain and EAR motif are represented the positions among the amino acids sequences. The number represent the number of amino acids.

Microsynteny analysis of *MtSUP* on the VISTA alignment (Figure 15) shows that only the adjacent genes to *MtSUP* located in the chromosome 2 from *M. truncatula* (*chr2*: 31,772,701-31,916,100) find certain collinearity with the chromosome 1 (*chr1*: 1-30,427,671) from *A. thaliana* (Figure 15, red square). However, the *SUPERMAN* gene is located in the chromosome three from *A. thaliana*, where no collinearity is found. These results show that there is no microsyntey conservation between *MtSUP* and *SUP*.

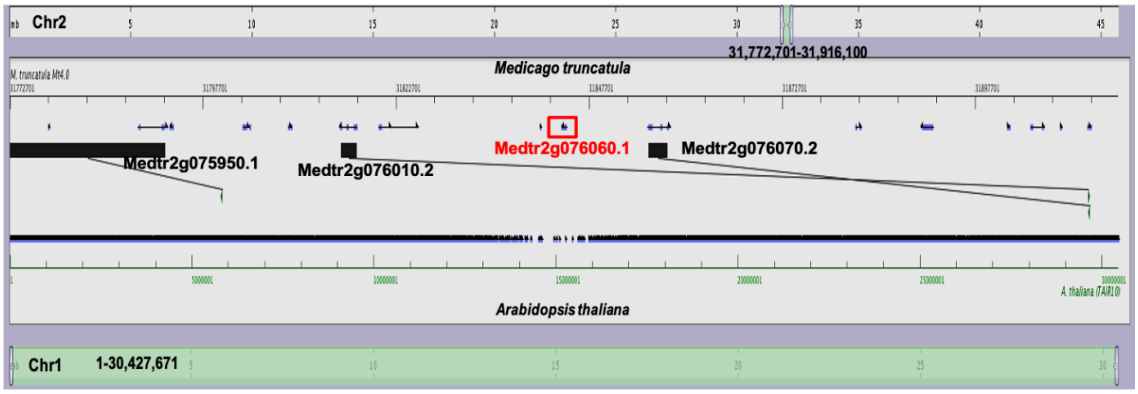


Figure 15. VISTA alignment between *M. truncatula chr 2* and *A. thaliana chr1.* A snapshot from the VISTA alignment constitutes Figure 15. The alignment was between the chromosome 2 from *M. truncatula* and the chromosome 1 from *A. thaliana.* The positions in the chromosome of each species are shown in the parenthesis. Data from Medicago and Arabidopsis were obtained from Mt4.0 and TAIR release respectively. The red square annotates the position in which *MtSUP* (Medtr2g076060.1) is located in Medicago.

The web-based approach indicate that there is no synteny conservation between *MtSUP* and *SUP* suggesting independent *SUP*-like gene translocations. Functional studies included in this work would test the hypothesis that *MtSUP* is the *SUP* ortholog in *M. truncatula*.

3. *MtSUPERMAN* expression analysis

The first experiment to analyze the expression of *MtSUP* in *M. truncatula* was a RT-PCR from different vegetative tissues and from floral apices. As expected *MtSUP* is exclusively expressed in floral apices (Figure 16).

Figure 16. Tissue expression analysis of *MtSUP* by RT-PCR. The gene *MtACTIN* was used as control since it is a gene of constitutive expression. Amplifications were performed for 30 and 35 cycles for the control and *MtSUP* respectively. Melting temperature was of 57ºC for both amplifications. The roots and SAM (shoot apical meristem) tissues were obtained from sterile seedlings. For leaves a young trifoliate leaf was used. Pieces of 4 cm from the second internode were used for stems. Around 30 floral apices were used for RNA extraction. For all tissues one microgram of total RNA was used for reverse transcription.

In situ hybridization assays were performed in order to elucidate the temporal and spatial expression of *MtSUP* during compound inflorescence and flower development. *MtFRUITFULLc* (*MtFULc*) and *MtPROLIFERATING INFLORESCENCE MERISTEM* (*MtPIM*) are MADS-box genes which confer identity to the secondary inflorescence meristem (I2) and floral meristem (FM) respectively (Cheng *et al.*, 2018). Thus, *MtPIM* and *MtFULc* were used as marker genes to identify these meristems. *MtSUP* was firstly detected in the secondary inflorescence (I2) meristem (Figure 17 A), a similar expression to *MtFULc* (Figure 17 E). *MtSUP* expression in the I2 falls down as this meristem matures producing a FM in which *MtSUP* accumulates (Figure 17 B). *MtPIM* accumulates in the entire developing FM (Figure 17 D,F,G), while *MtSUP* locates in the entire FM and the remnant cells of the I2 (Figure 17 C).

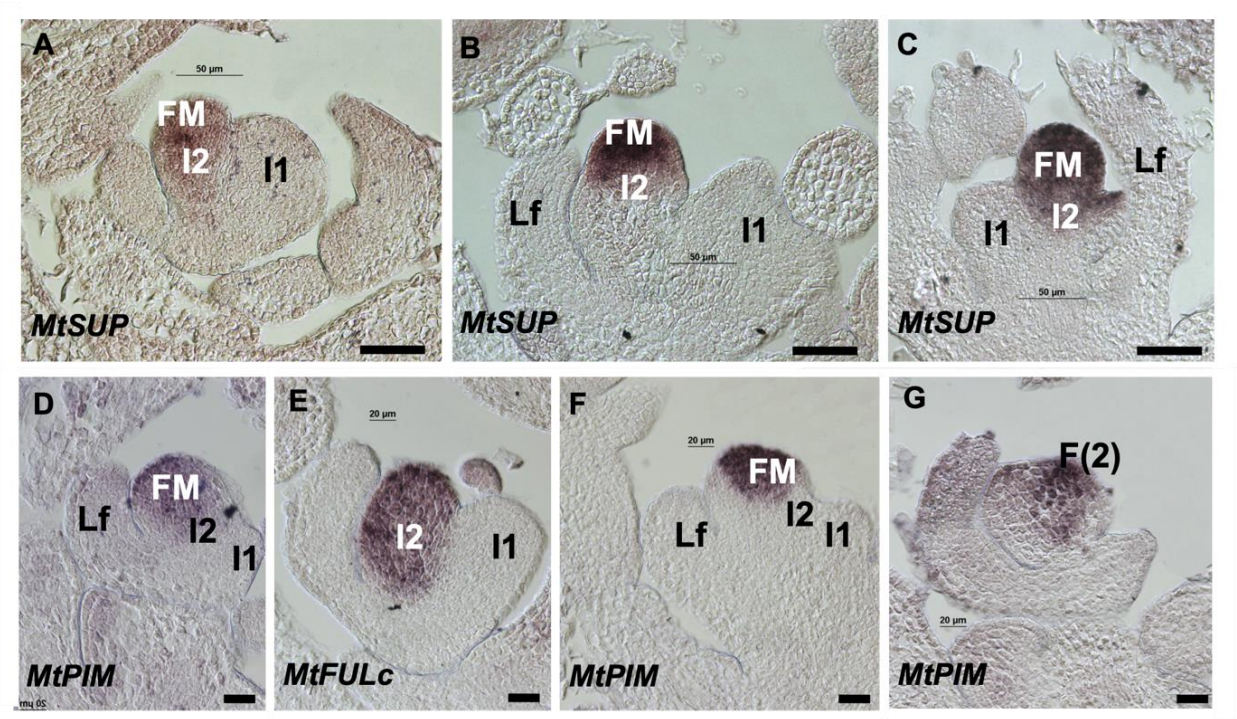


Figure 17. Expression patterns of *MtSUP* and key regulatory genes during compound inflorescence development in *M. truncatula*. *MtSUP* transcript was first detected in the secondary inflorescence (I2) meristem and in the emerging floral meristem (FM). **B.** Strong *MtSUP* transcript detection in the arisen FM and in some of the I2 remnant cells. **C.** Continued strong expression of *MtSUP* in the FM and cells that will become the spike. **D.** *MtPIM* transcript localization is used as a marker of the FM. **E.** *MtFULc* transcript detection is used as a marker of the I2 meristem. In **D** and **E** is shown an equivalent stage to that shown in panel **A**. **F.** *MtPIM* is expressed in floral meristem that is produced from the I2 meristem. **G.** *MtPIM* is expressed at stage 2 of floral development after the floral apex flattens out. I1: Primary Inflorescence meristem; I2: Secondary Inflorescence meristem; Lf: Leaf; FM: Floral meristem. From A to C scale bar= 50 µm; From D to G scale bar= 20 µm.

At the first stages of floral development *MtSUP* transcript locates in the cells that will become the common primordia (Figure 18 A-C). After common primordia differentiate, *MtSUP* is detected within these ephemeral meristematic cells (Figure 18 D). *MtSUP* expression is restricted to stamen primordia once these arise (Figure 18 E), continuing while stamens are developed (Figure 18 F). Besides, *MtSUP* is expressed in the carpel marginal tissue (Figure 18 F, G, H) that will develop the parietal placenta (Figure 18 I). The expression pattern of *MtSUP* suggests that it

might play a role during compound inflorescence development and flower organogenesis in *M. truncatula*.

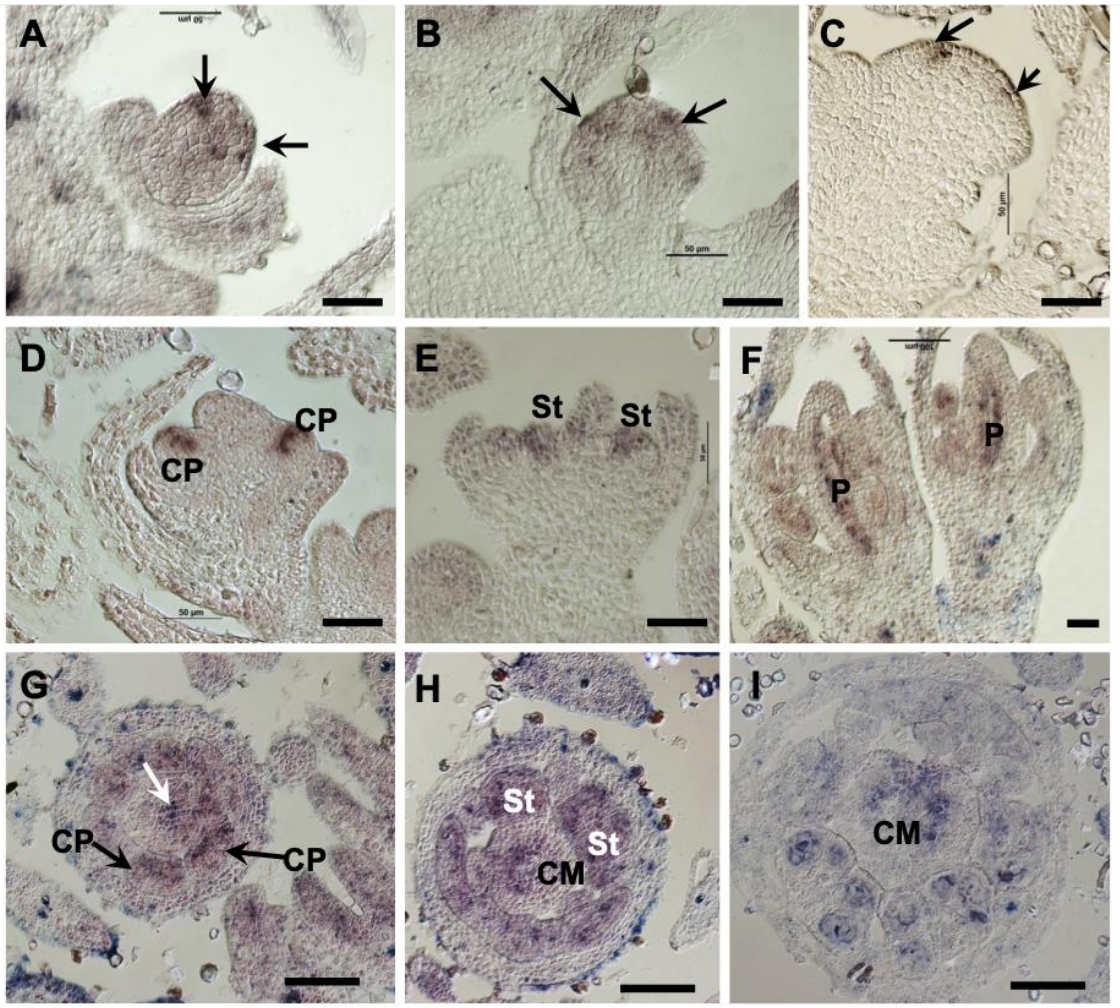


Figure 18. *MtSUP* expression pattern during floral development in *M. truncatula*. *MtSUP* transcript accumulates in the floral primordium in the cells that will differentiate the common primordia (CP) (arrows), this continues until late stage 2 (**B**) and stage 3 (**C**) of floral development. **D.** At late stage 4 of floral development *MtSUP* transcript locates in a portion of the common primordia. **E.** At stage 5 of floral development *MtSUP* is expressed in the stamen primordia. **F.** At late stage 7 the *MtSUP* transcript is detected in the placenta and developed stamens. **G.** Cross-section at late stage 4 shows that *MtSUP* is expressed within the common primordia and in the carpel margins (white arrow). **H.** Cross-section stage 5 shows that *MtSUP* transcript accumulates stamen primordia and carpel marginal (CM) tissues. **I.** Cross-section of late stage 7 shows that *MtSUP* transcript accumulates in the carpel margin and in the developed stamens. CP: common primordia; P: placenta; St: stamens; CM: carpel margins;

Floral developmental stage was defined according to Benlloch *et al*., 2003. Scale bar = 50 µm.

4. Complementation of *sup-5* mutant in *A. thaliana* by *MtSUPERMAN*

To evaluate the capability of *MtSUP* to replace *SUP* in *A. thaliana* a complementation assay was performed. This was achieved using the allele *sup-5* as background, one of the strongest *SUP* mutants (Breuil-Broyer *et al*., 2016). This mutant is characterized by a strong indeterminacy. Flowers produce from 2 to 5 carpels (3.5±1.30, Table 12) and from 8 to 14 stamens (12.0±1.80; Table 12). The construct introduced into *sup-5* had all the genomic context from *SUP* (*pSUP*) and the coding sequence from *MtSUP*. Thirteen transgenic plants carrying the construct (Figure 19) were phenotypically characterized and compared to the wild type (Ler. Figure 20 A) and the mutant (*sup-5*, Figure 20 B-C).

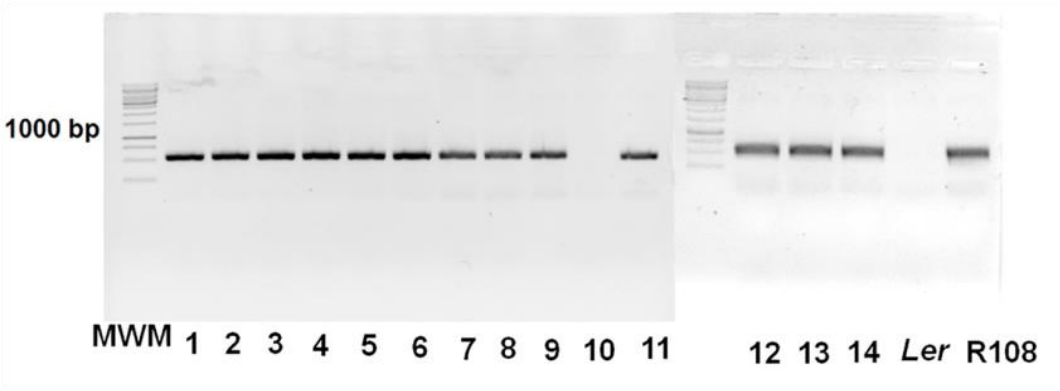


Figure 19. Genotyping for *MtSUP* in p*SUP*::*MtSUP*;*sup*-5 lines. Total gDNA was isolated from young leaves of p*SUP*::*MtSUP plants, M. truncatula* (R108), and *A. thaliana* (Ler) to perform a PCR with specific primers for *MtSUP* CDS (MtSUP-ATG/MtSUP-556R). Wild type Arabidopsis and Medicago plants were used as negative and positive controls respectively. A band means that *MtSUP* was incorporated in the genome of *sup-5* plants. From the 14 plants analysed only the plant number 10 did not any amplicons. The remaining 13 plants that have the expected amplicon were used for further analysis.

Complementation was from partial (Figure 20 D-E) to full (Figure 20 F) rescue of the floral phenotype. Lines 1, 3, 4, 5, 7, 8 and 13 had almost a full complementation level. In average the complemented plants had 6.52±0.33 stamens and 2.31±0.31

carpels (Table 12). These results indicate a conservation of the biochemical function between *MtSUP* and *SUP*.

Table 12. Phenotypical characterization of *pSUP*::*MtSUP*; *sup-5* transgenic plants

Allele	Line	Stamen No. (mean±SD)	Carpel No. (mean±SD)
Wild-type		6.0±0.00	2.0±0.00
sup-5		12.0±1.80	3.5±1.30
pSUP*::*MtSUP*;*sup-5	1	6.6±0.55	2.2±0.45
	2	6.8±0.45	2.8±0.45
	3	6.4±0.55	2.4±0.55
	4	6.2±0.45	2.2±0.45
	5	6.0±0.00	2.0±0.00
	6	6.6±0.55	2.8±0.45
	7	6.0±0.00	2.4±0.55
	8	6.4±0.55	2.0±0.00
	9	6.6±0.89	2.6±0.89
	10	7.0±0.00	2.0±0.00
	11	6.8±0.45	2.0±0.00
	12	7.0±0.71	2.6±0.89
	13	6.4±0.89	2.0±0.71
	Average	6.52±0.33	2.31±0.31

For each line is shown the mean and the standard deviation of the carpel and stamen numbers. Data for p*SUP*::*MtSUP*;*sup-5* lines were obtained from the dissection of 10 flowers per transgenic line. For the wild type and for the *sup-5* mutant were dissected 60 flowers from 6 plants (10 flowers per plant).

Figure 20. Complementation of *sup-5* mutant in *A. thaliana* by *MtSUP.* A. Wild type flower from *A. thaliana* (Ler). **B.** A flower from the mutant *sup-5* that has more stamens (13) and carpels (4). **C.** Another *sup-5* mutant flower with more stamens (9) and carpels (3). **D-F** Transgenic plants carrying the *pSUP::MtSUP* construct in *sup-5* as background. Plants show partial recovery of stamen and carpel number (**D**); plants with full recovery of stamen number and partial recovery of carpel number (**E**); and full recovery of flower phenotype (**F**). Bars = 0.75 mm.

5. Ectopic expression of *MtSUP* in *A. thaliana*

MtSUPERMAN was overexpressed to evaluate and compare its effects with the ectopic expression of *SUP* in *A. thaliana*. In 2002, Hiratsu and collaborators revealed that the carboxyterminal domain in the SUP protein acts as an active repressor. When *SUP* was ectopically expressed in Arabidopsis the whole plant experimented dwarfism without affecting cells size. Nevertheless they suggested that *SUP* could regulate cell proliferation (Hiratsu *et al.*, 2002). *MtSUP* was overexpressed in *A. thaliana* under de control of the constitutive promoter CaMV35S. Transgenic plants (35S::*MtSUP*) showed a significant plant height reduction (Figure 21 A-B). The cauline leaves were also smaller in overexpressing lines than in the wild type leaves (Figure 21 C).

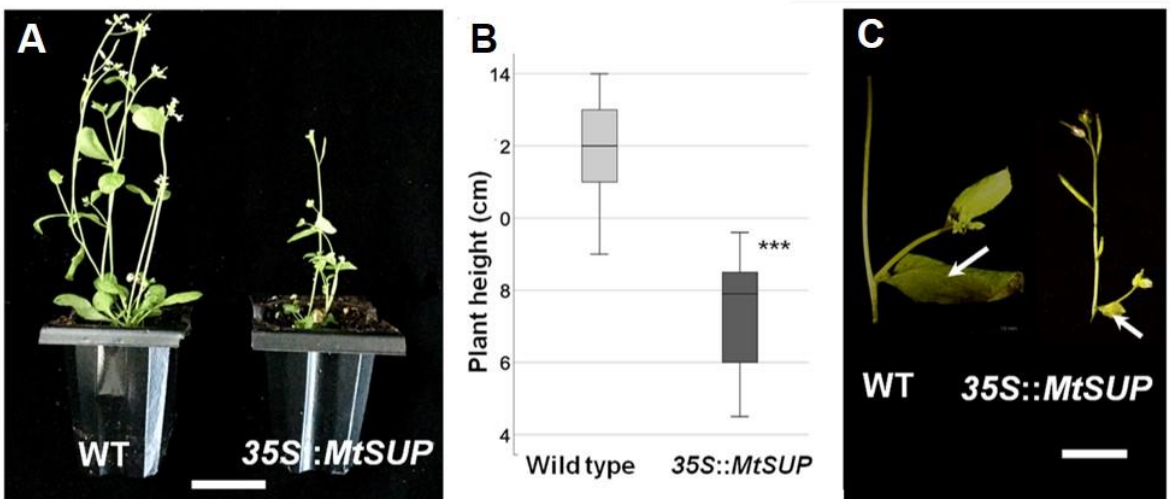


Figure 21. Plant measurements of the *35S:MtSUP* lines. A. Plant height differences between 34-days-old wild type and 35S::*MtSUP* plants. **B.** Box-plot of the plant height measurements in the wild type and *35S::MtSUP* plants. A Kurskal-Wallis test for independent samples (n=17) indicated significant (P-value < 0.001***) differences in plant height. **C.** Cauline leaf (white arrow) from a wild type (left) and a 35S::*MtSUP* plant (right). Scale bars A = 2.5 cm (A) and C =10 mm.

A RT-qPCR was performed to compare the expression levels of *MtSUP* (Figure 22) with respects to the height reduction (Figure 21 B). A correlation test between plant height and *MtSUP* level of expression indicated that there is an inverse relation between these variables (Figure 23). This means that the plants that experienced a more severe dwarfism had higher expression levels of *MtSUP*. The effects described for *MtSUP* overexpression are similar to the ones for Arabidopsis 35S::*SUP* plants (Hiratsu *et al.*, 2002) and other *SUP*-like genes overexpressed (Nibau *et al.*, 2011; Nakagawa *et al.*, 2004; Zhao *et al.*, 2014). This indicates a conserved role of controlling cell proliferation for *SUP*-like genes.

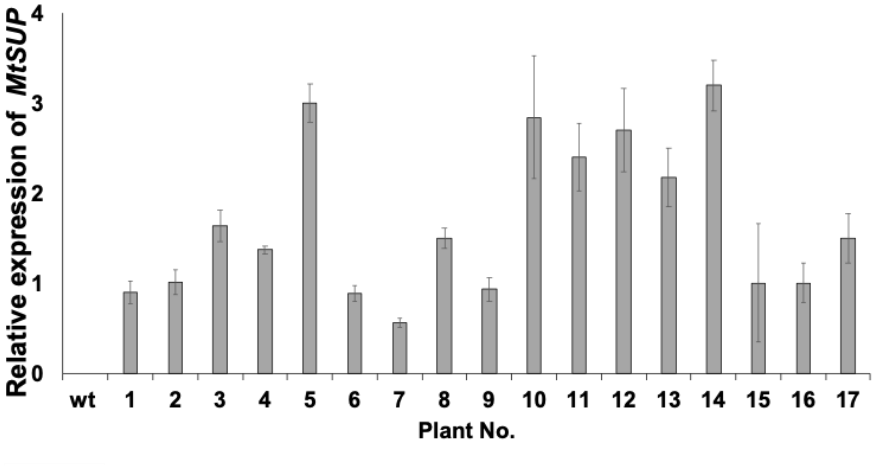


Figure 22. Expression levels of *MtSUP* in 35S::*MtSUP* plants. Each value represents the relative expression levels of *MtSUP* (mean), the bars represent the standard deviation of three biological replicates.

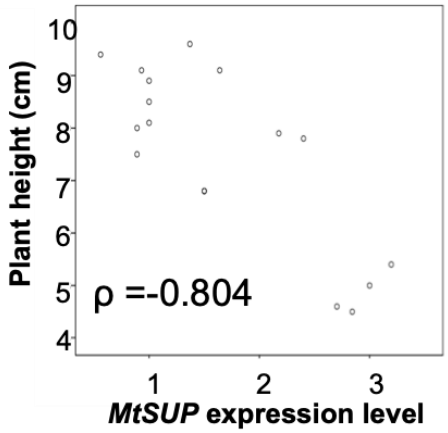


Figure 23. Scatter plot between plant height and *MtSUP* expression levels in 35S::*MtSUP* plants. Pearson Correlation Coefficient (ρ) shows a significant negative correlation (-0.804) between the plant height and expression levels with a p-value less than alpha (0.05). A total of 17 independent plants carrying the 35S::*MtSUP* construct were analyzed.

Besides plant height reduction, Figure 24 shows that petals (B), flowers (B) and siliques (C) were also smaller in 35S::*MtSUP* plants compared to the wild type ones (A, C). Moreover, 35S::*MtSUP* plants showed floral defects regarding the number and

size of the floral organs in the whorls 2, 3 and 4 (Figure 24 E-G) compared to the wild type ones (Figure 24 D). Some overexpressing plants showed flowers with less or absent stamens, while the number of carpels was higher (Figure 24 E-G). This suggests that some of the stamens were transformed into carpels. These floral defects are similar to the ones observed in *A. thaliana* and *N. tabacum* when *SUP* was ectopically expressed under the *API* promoter (Yun *et al.*, 2002).

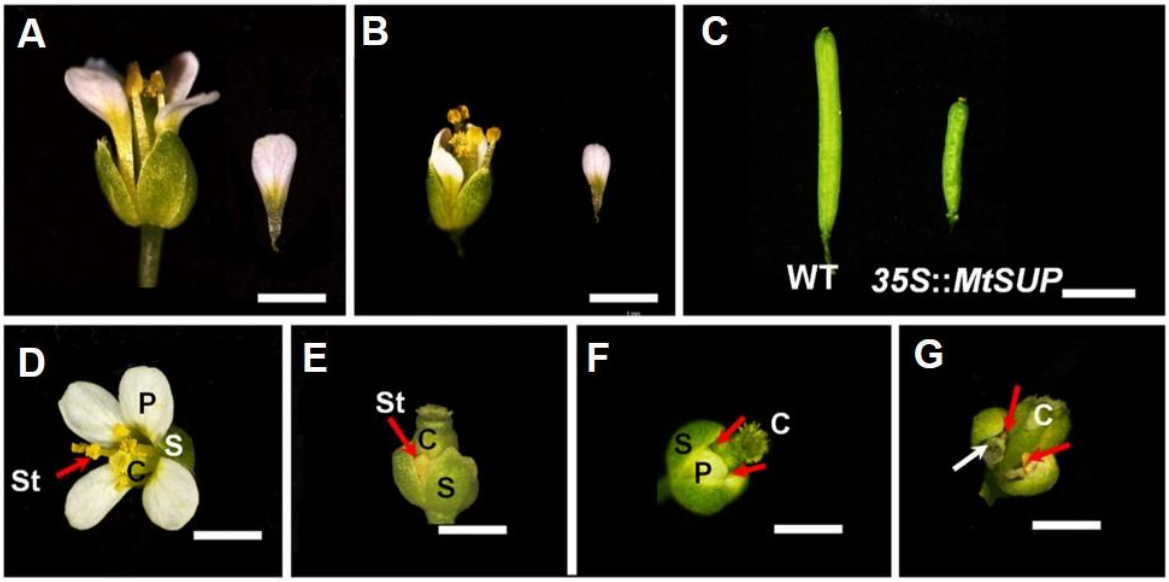


Figure 24. Phenotypic effects of *MtSUP* ectopic expression in *A. thaliana.* A. Wild type flower. Petal size is showed. **B.** *35S::MtSUP* flower. Petal size is petal. **C.** Siliques size comparison between the wild type and *35S::MtSUP* plant. **D.** Wild type flower. **E-F.** Abnormal 35S::*MtSUP* flowers showing absent petals and undeveloped stamens **(E)**, sepals transformed into petals **(F)**, and undeveloped stamens and petals with less sepals **(G)**. Undeveloped stamens and petals are indicated by a red and white arrow respectively. S: sepal; P: petal; St: stamen; C: carpel. Scale bars 10 mm (A, B, D-G), 5 mm (C).

6. Molecular characterization of *MtSUP Tnt1* insertional mutants

Tnt1 insertional mutants for *MtSUP* were screened by the Noble Research Institute from their insertion library for *M. truncatula* R108. *Tnt1* insertional lines were ordered and characterized to identify at least one *MtSUP* loss of function mutant. The line 1 (NF11278) was of interest to study the role of *MtSUP*. Homozygous plants from the

line 1 constitute the *mtsup-1* mutant allele. This mutant harbours the retrotransposon at 50 bp downstream of the start codon of *MtSUP* gene (Figure 25).

Figure 25. Schematic representation of the *Tnt1* insertion in *mtsup-1*. Schematic representation of the *MtSUP* gene, including the start codon, coding sequence and the stop codon. The red wedge is a graphical representation of the retrotransposon position (50 bp after the ATG). Dimensions are related to the number of nucleotides.

It was verified that this line had a normal segregation (Table 13, Figure 26) and that only the homozygous plants had a mutant phenotype.

Table 13. Segregation analysis of *mtsup-1*

Genotype	Wild type	Heterozygous	Homozygous	Segregation
T0 generation (N= 8)	2	4	2	Normal
T1 generation (N=22)	7	11	4	Normal

A 25% of homozygous, 25% of wild type and 50% of heterozygous plants was considered as a normal segregation. Slight variations are accepted due to the reduced number of plants.

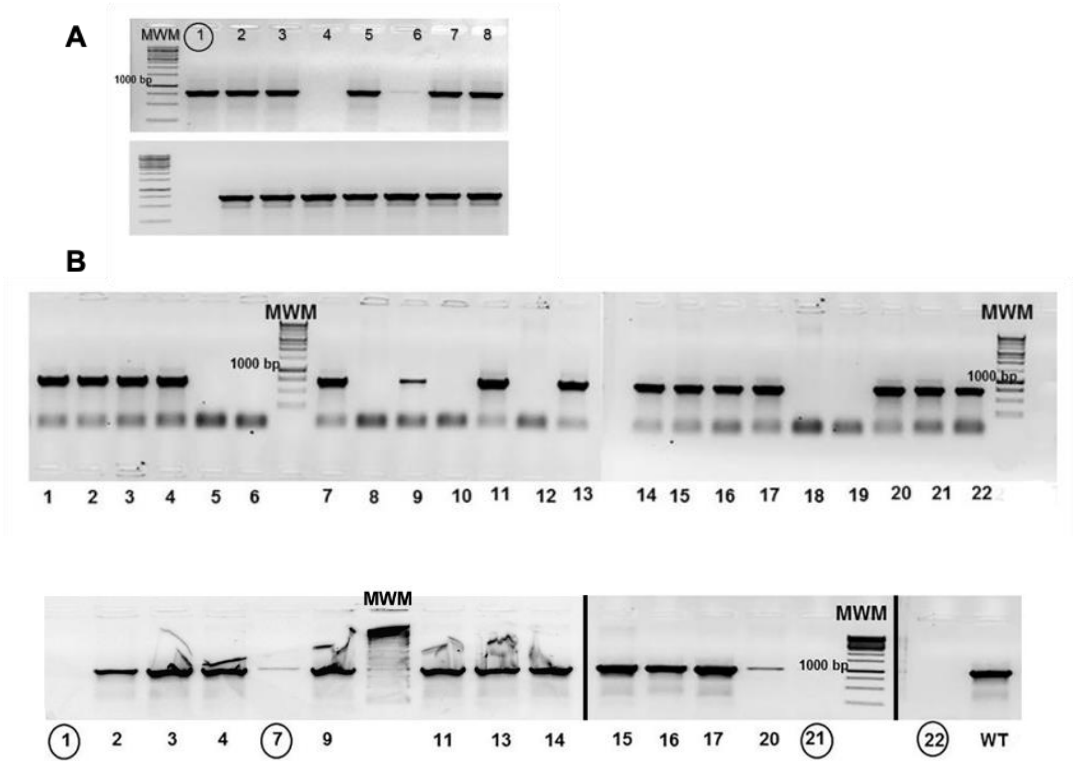


Figure 26. Genotyping of *mtsup-1* plantsGenotyping of *mtsup-1* plants. (**A**) R0 and (**B)** R1 *mtsup-1* progeny. Gel shows the PCR amplification using MtSUP-F/Tnt1-F (upper part) and MtSUP-F/MtSUP-556R (lower part) primers. A band in the upper gel means positive amplification for the *Tnt1* retrotransposon. A band in the lower gel means that *MtSUP* was amplified. The combination of bands means: -/+ that the plant is wild type, +/+ *Tnt1* insertion in heterozygosis and +/- *Tnt1* insertion in homozygosis. Circled number represent the plants that are homozygous for *MtSUP* mutation. Segregation was as expected and only the homozygous plants showed a mutant phenotype. MWM: molecular weight market (1 kb). WT: wild type.

RT-PCR analysis revealed that no full transcript is produced in *mtsup-1* (Figure 25). Detailed characterization of this mutant is shown in the next sections.

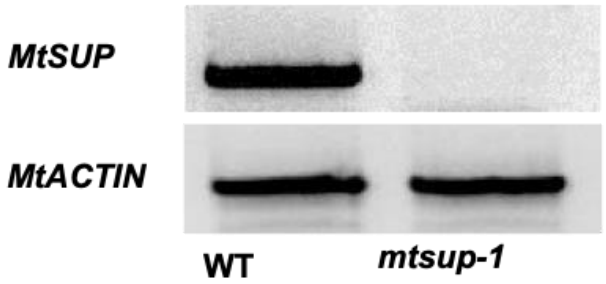


Figure 27. Transcript analysis in *mtsup-1*. The gene *MtACTIN* was used as control for being a gene of constitutive expression. Amplifications were performed for 30 and 35 cycles for the control and *MtSUP* respectively. Melting temperature was of 57ºC for both amplifications. Around 30 floral apices were used for RNA extraction. One microgram of total RNA treated with DNaseI was used for reverse transcription. In *mtsup-1* no full *MtSUP* transcript is detected.

7. Molecular characterization of *MtSUP* CRISPR/*Cas9* mutants

Based on the use of CRISPR/*Cas9* technology an additional mutant allele was generated. Fifteen transgenic plants (T0) expressing the *Cas9* were regenerated after the transformation process (Figure 28). Only one plant (No. 8) had an edition in heterozygosis (Figure 29B). The edition consisted in a four-nucleotide deletion at the expected location, 5 nucleotides before the PAM sequence (Figure 29B, black line). Descendants of this line (T1) had a normal segregation for the *Cas9* and the edition. Homozygous plants for the deletion constituted the *mtsup-2* mutant allele (Figure 29C).

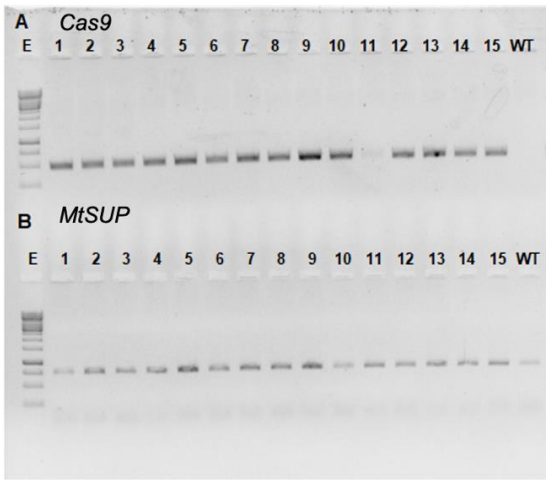


Figure 28. Genotyping of *CRISPR/Cas9* regenerated plants. The 15 regenerated plants (T0) from the transformation process were analysed to determine the *Cas9* expression (**A**) and to amplify part of the gene *MtSUP* (**B**). **A**. The 15 regenerated plants expressed the *Cas9*, the pale band in the plant 11 was also considered as positive. The region amplified from the gene *MtSUP* includes 300 bp upstream and 300 bp downstream from the position in which the *Cas9* is expected to cut. All the fragments amplified in B were sent for sequencing. Only the plant number 8 had a four-nucleotide deletion in *MtSUP* CDS. The homozygous descendants of plant 8 constitute the *mtsup-2* allele.

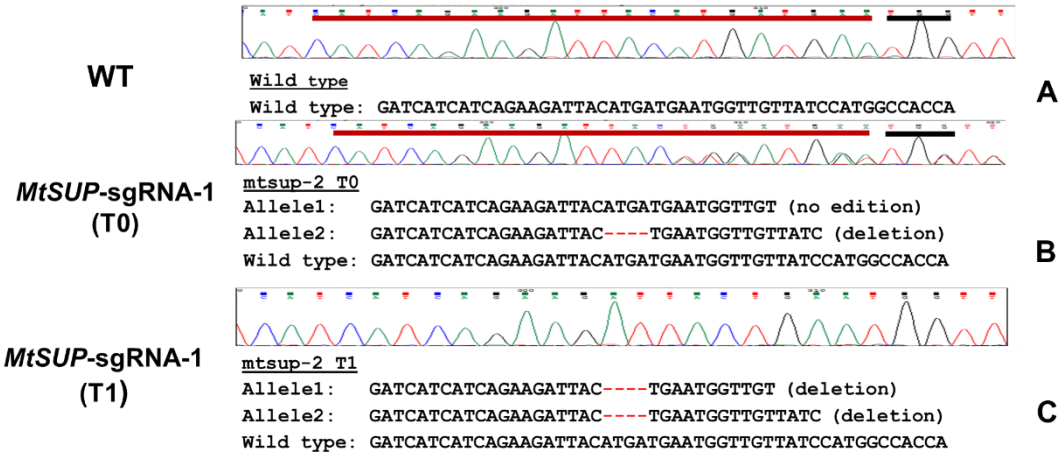


Figure 29. Sequences analysis for CRISPR/*Cas9* lines T0 and T1. Chromatograms include a window of 26 nucleotides in which the red line corresponds to the sgRNA used to target *MtSUP* in the CRISPR/*Cas9* construct. The black line corresponds to the PAM (NGG) sequence. *Cas9* was expected to cut 5 nucleotides before the PAM sequence. **A.** Wild type was used as reference. **B.** *MtSUP*-sgRNA-1 (T0) shows a four-nucleotide deletion in

heterozygosis, that explains the overlapping picks in the chromatogram. **C.** In the next generation (T1) a quarter of the descents were homozygous for this four-nucleotide deletion.

Transcripts were analysed by RT-PCR and then sequenced. *In silico* translation of the sequence of *mtsup-1* transcript resulted in a truncated protein of 39 amino acids (Figure 30). Functional characterization of this mutant is in the next sections.

```
MtSUP    MMKRNNMNTNLKGYNHTYYNNPIITNKSDNNNGSSSEDYMMNGCYPWPPR 50
MtSUP-2  MMKRNNMNTNLKGYNHTYYNNPIITNKSDNNNGSSSEDY---------- 39

MtSUP    SYTCSFCRKEFKSAQALGGHMNVHRRDRARLRQSSPPTTHEPAQIQGSSM 100
MtSUP-2  ---------------------------------------------- 39

MtSUP    LNLNLNNPTITTTNPNLFSHSSSSSSSLSSTSSATSLKPITCTLPLFVSP 150
MtSUP-2  ---------------------------------------------- 39

MtSUP    PSEFNRYVVVDGILTNPTLTTKTSEKSKMDGFECEDDSRMVKKSEILRRD 200
MtSUP-2  ---------------------------------------------- 39

MtSUP    LEIGLPGDYDNLDLELRLGTTTYSN 225
MtSUP-2  ------------------------ 39
```

Figure 30. Alignment between MtSUP and the *in silico* translated protein from *mtsup-2* transcript. Total RNA was extracted from *mtsup-2* floral apices to be reverse transcribed and sequenced. The sequence of *mtsup-2* transcript was translated with Expasy webtool and then aligned to MtSUP. The mutant allele *mtsup-2* produced a truncated protein of 39 amino acids.

MtSUP mutants showed defects during reproductive development. Detailed characterization of the effects related to the impairment of *MtSUP* function is described in the following sections.

8. *MtSUP* mutants show defects in the inflorescence architecture in *M. truncatula*

The first phenotypical observation was that none of the mutants produced the residual organ or spike in the inflorescences (Figure 31B-H). Besides, compared to the typical wild type inflorescence of a single flower per peduncle (Figure 31A) the mutants produced from two (Figure 31B) to four (Figure 31H) flowers per peduncle.

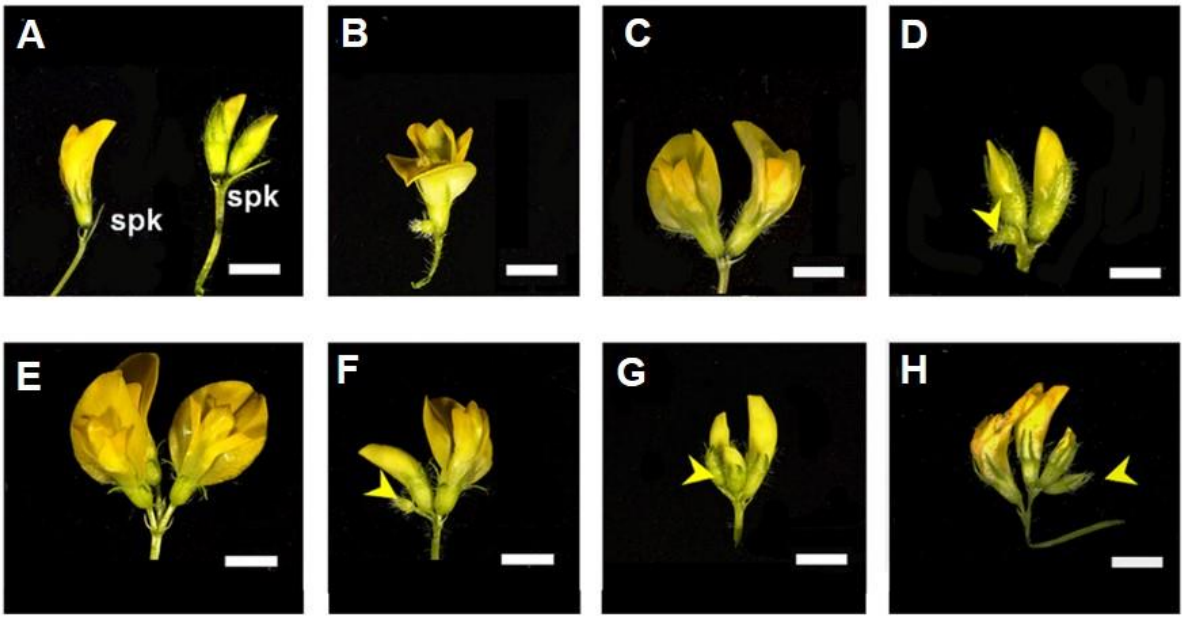


Figure 31. Inflorescence architecture defects in *MtSUP* mutants. A. Wild type (R108) with one (singlet) or two (doublet) flowers per peduncle, each type has a spike or residual organ (spk). **B.** A singlet without spike observed in *mtsup-2* mutant. **C.** A doublet without spike. **D-G**. Different examples of three flowers per peduncle (triplet) observed in *mtsup-1*. **H.** The maximum number of flowers per peduncle observed in *mtsup-1*, four flowers per peduncle (quadruplet). Scale bars= 2 mm. Spk = spike.

In the wild type the 90% of the inflorescence were singlets (Figure 32), while two flowers per peduncle (double or duplet,) were a minority (10%) (Figure 31A – 32). In contrast, the 86% of *msup-1* inflorescences carried two (double) to three (triplet) flowers per peduncle (Figure 31C-G; 32), while singlets were only in the 3.4% of the inflorescences (Figure 32). The remaining 10 % of *mtsup-1* inflorescences produced four flowers per peduncle (quadruplet, Figure 31H; 32) resembling a multiflowered phenotype. In *mtsup-2* the majority of the inflorescences (76%) produced duplets (Figure 32), the remaining inflorescences were singlets without spike (Figure 31 B; 32).

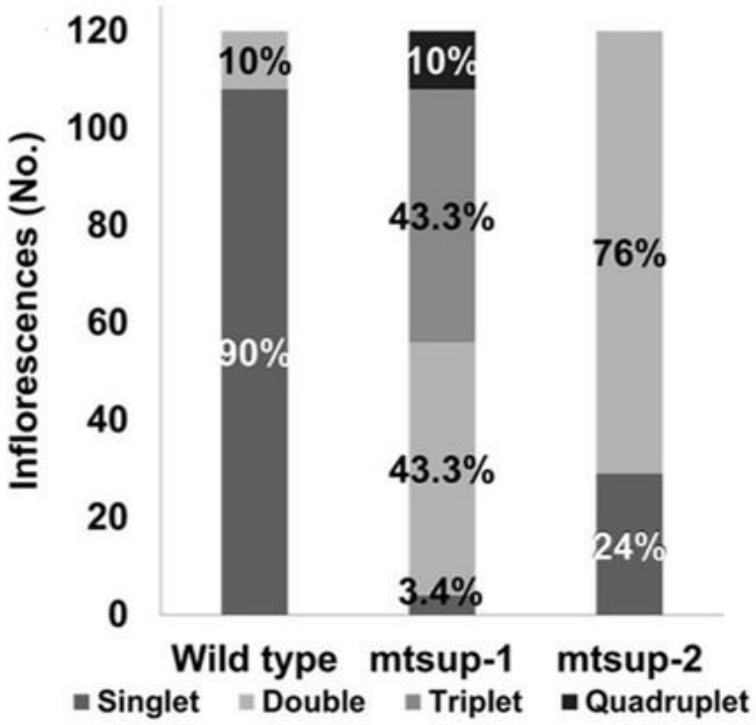


Figure 32. Number of flowers per peduncle in *MtSUP* mutants and the wild type. A total of 120 inflorescences per each line were analysed to count the number of flowers per peduncle. Average number are represented by the bars and the numbers are the percentage of each type. Singlet = one flower/peduncle; double: two flowers/peduncle; triplet: three flowers/peduncle; quadruplet four flowers/peduncle.

MtSUP mutants showed an increased number of flowers per peduncle (Table 14) compared to the wild type (1.1±0.32 flowers/peduncle, Table 14). The allele *mtsup-1* shows the strongest phenotype in this regard (2.6±0.73 flowers/peduncle, Table 14), therefore *mtsup-1* was chosen to be thoroughly analysed by Scanning Electron Microscopy (SEM) and *in situ* hybridization assays.

Table 14. Number of inflorescences per peduncle

Allele	Number of flowers / peduncle
Wild type (N=120)	1.1±0.32

mtsup-1 **(N=120)**	2.6±0.73
mtsup-2 **(N=120)**	1.8±0.44

The number of flowers per peduncle represents the mean ± the standard deviation. For each allele a total of 120 inflorescences from 12 plants (10 inflorescences/plant) were analysed.

MtSUP mutants produced multi-flowered peduncles (Figure 31 B-H), thus the characterization of the inflorescences development by Scanning Electron Microscopy (SEM) would give information about the origin of these extra flowers. To achieve this, the first 10 floral apices from 10 different plants for the wild type and *mtsup-1* were dissected. SEM images indicated that there is an over-proliferation of flower primordia in the mutant (Figure 33 B) compared to the wild type (Figure 33 A). The wild type follows an acropetal succession in which the oldest flowers are at the bottom and the inflorescence meristems at the top. Each of the two flowers observed (Figure 33 A) has a bract and a spike. The mutant in contrast produced more flowers from the same secondary inflorescent meristem. Besides, after each floral meristem (FM) is developed from the I2 meristem no spike is produced. Instead, the I2 meristem terminates as a FM (Figure 33 B, D).

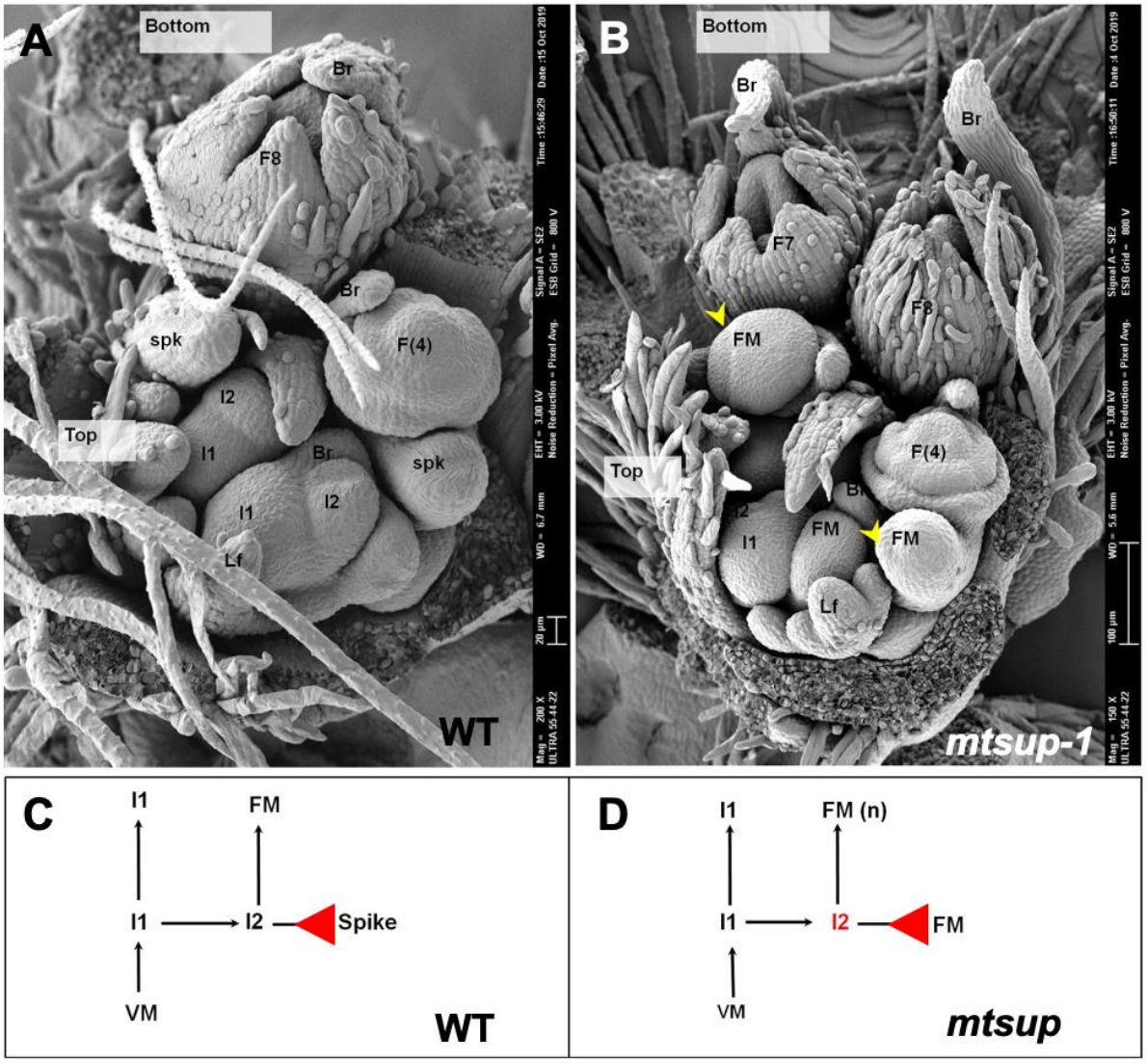


Figure 33. Inflorescence architecture analysis in the wild type and *mtsup-1*. A. Inflorescent apices in the wild type (R108) in which each of the two flowers at stage 8 (F8) and 4 (F4) have a bract and a spike. In acropetal succession the oldest flowers are at the bottom and the inflorescence meristems (I1 and I2) and the top. **B.** Inflorescent apices in *mtsup-1*, six flowers at different developmental stages (F2, F3, F4, F7 and F8) are observed. The flowers have a bract but lack the spike. Instead, a spike another floral meristem is produce (yellow arrow). Floral developmental stages F(N) were defined according to Benlloch *et al*., 2003. **C.** Wild type inflorescence architecture, the vegetative meristem (VM) divides to produce a primary inflorescence (I1) meristem. The I1 meristem divides to produce a secondary inflorescence (I2) meristem. The I2 meristem produces from 1 to 2 flowers and ends as a spike. **D.** Inflorescence in *MtSUP* mutants. The development is similar to the WT

until the I2 meristem prolongs its activity (in red) producing more flowers and terminates as a floral meristem instead a spike. VM: vegetative meristem; I1: primary inflorescence meristem; I2: secondary inflorescence meristem; FM: floral meristem; Br: bract; spk: spike; Lf: leaf. Scale bar 20 µm (A) and 100 µm (B).

MtSUP expression analysis (section 3) indicates that this gene is expressed in the secondary inflorescence meristem (I2) and in the floral meristem (FM). Besides, *MtSUP* mutants show defects in the inflorescence architecture. These observations suggest that *MtSUP* is part of the genetic regulatory network that controls compound inflorescence development in *M. truncatula*. Part of this network are *MtPIM* and *MtFULc*, MADS-box genes that confer identity to the I2 and the FM respectively, that mutually repress each other (Cheng *et al.*, 2018). We studied the expression pattern of *MtFULc* and *MtPIM* in the wild type and the *mtsup-1* mutant by *in situ* hybridization (Figure 34). In the wild type the expression pattern of *MtFULc* is as described in the previous chapter, it accumulates in the I2 meristem until the I2 divides to produce a FM. *MtFULc* expression decreases in the remaining cells of the I2 (Figure 34 A), these cells will produce the spike or residual organ that is observed in the wild type inflorescences (Figure 34 A, 35 A). In *mtsup-1* plants, *MtFULc* prolongs in the I2* meristem (Figure 34 B). Instead of one FM the I2 meristem produces more FMs than the expected ones (Figure 34 C, E). In the wild type *MtPIM* restricts its expression to the emerging FM (Figure 34 D, 35 B), while in the mutant expands its expression to the territory of the remaining cells of the I2 (Figure 34 E, F), as a result these cells acquire floral identity (Figure 35 C, D)

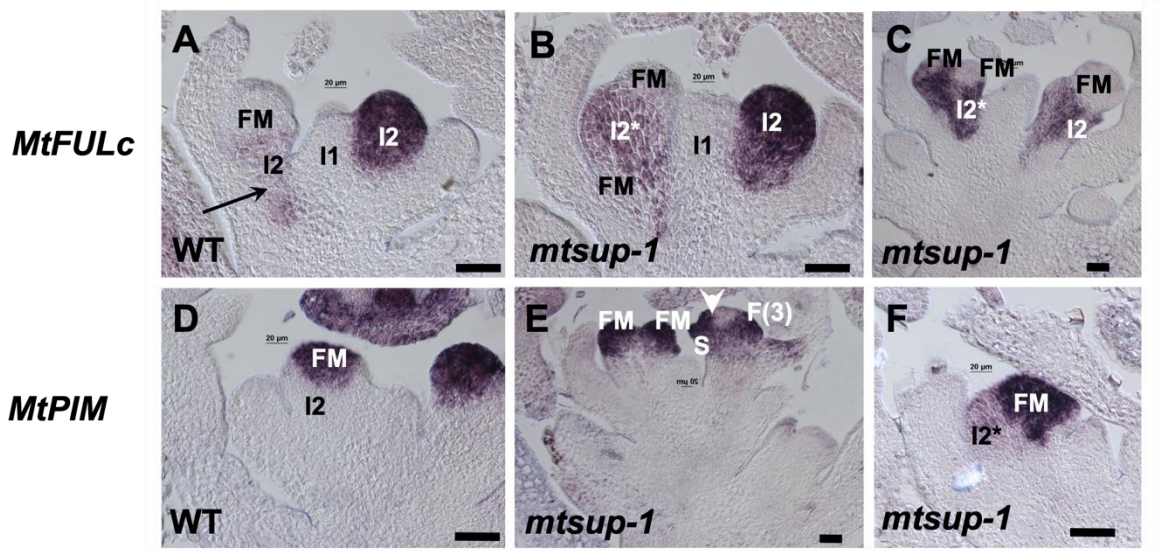


Figure 34. ***MtFULc*** **and** ***MtPIM*** **transcripts detection by in situ hybridization**. **A-C.** *MtFULc* transcript localization in the secondary inflorescence meristem (I2). **A** In the wild type a strong signal can be detected in the young I2, while the signal decreases in the mature I2 (arrow). **B**. In *mtsup-1* a similar signal in the young I2 is observed, while in the other I2* *MtFULc* prolongs its expression. **C.** In *mtsup-1,* transcript accumulates in the I2 and in I2* meristem. **D-F**. *MtPIM* transcript localization in the floral meristem (FM). **D.** In the wild type the transcript is restricted to the emerging FM. **E**. In *mtsup-1*, transcript accumulates in the two FM's that were originated from the I2* meristem; in a floral primordium at stage 3 (arrow) transcript accumulates in the sepal primordia and in a portion of common primordia. **F.** In *mtsup-1*, transcript signal is strong in the FM but also a weak signal is observed in the remaining cells of the I2. FM: floral meristem; I1: primary inflorescence meristem; I2: secondary inflorescence meristem; I2*: I2 meristem that ends as a FM; I2; S: sepals; CP: common primordia; C: carpel. F(N): flower at N developmental stage defined by Benlloch *et al.*, 2003. Scale bars: 10 µm.

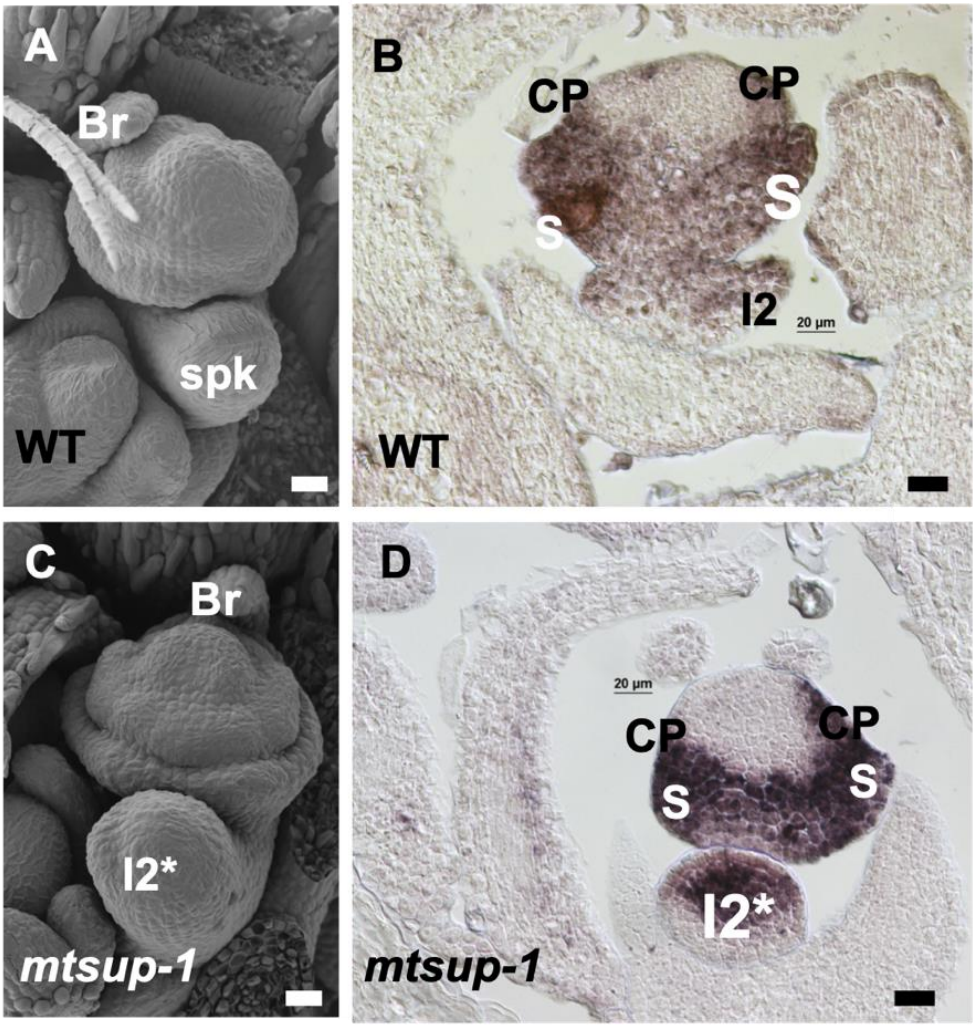


Figure 35. Floral meristem termination instead a spike in *mtsup-1*. **A.** SEM image of a wild type floral primordium with its respective bract and spike. **B.** In the wild type, *MtPIM* transcript localization in the sepal primordia and in a portion of the common primordia. **C.** In *mtsup-1*, the I2* (future spike) acquires floral identity. **D.** In *mtsup-1, MtPIM* transcript besides its detection in the floral primordia it is also detected in the remaining cells of the I2* conferring them floral identity. Br: bract; Spk: spike; I2: secondary inflorescence meristem; CP: common primordia; S: sepals. Scale bars: 50 µm.

A quantitative PCR (RT-qPCR) was also performed to compare the expression levels of marker genes. *MtPIM* expression levels are upregulated in *mtsup-1* (Figure 36), this is expected as more floral primordia are produced. In contrast, in the mutant the expression levels of *MtFULc* are down-regulated (Figure 36). An important

observation is that *MtFULc* transcript occupied a wider area of expression (Figure 34 B, C). In turn, in the mutant the prolonged activity of *MtFULc* gives place to more floral meristems (Figure 34 E). Another gene analysed by RT-qPCR was *MtSINGLE LEAFLET1* (*MtSGL1*), the *LEAFY* (*LFY*) ortholog in *M. truncatula*. *MtSGL1* plays important roles as conferring identity to the common primordia and with *MtPIM* is synergistically involved in the FM identity acquisition (Cheng *et al.*, 2018). In the mutant the expression levels of *MtSGL1* were as the wild type (Figure 36), suggesting that this gene acts upstream to *MtSUP*.

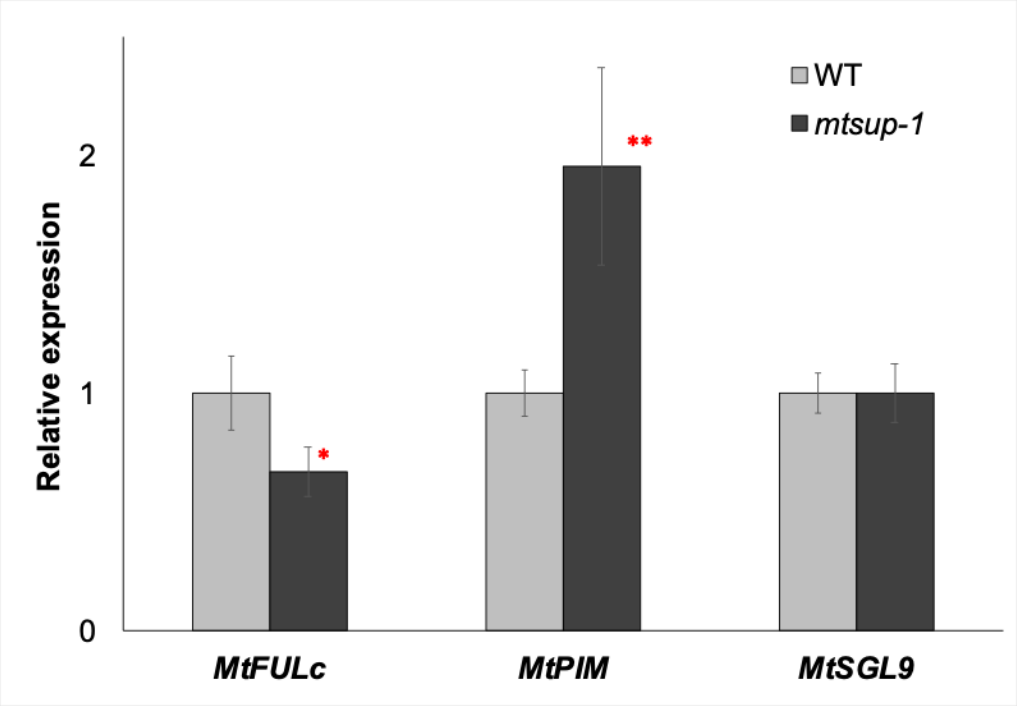


Figure 36. Relative expression levels of *MtFULc*, *MtPIM* and *MtSGL9*. Relative expression levels of *MtFULc* (Medtr7g16630), *MtPIM* (Medtr8g066260) and *MtSGL1* (Medtr3g098560) in *mtsup-1* were plotted relativized to their expression in the wild type which values were set to 1.00. Values represent the mean ± standard deviation (SD) of 3 biological replicates. A two tailed T-test indicated if the difference between the expression levels was significative. If p-value <0.05 (*) and <0.01 (**). Inflorescences characterization by visual observation, scanning electron microscopy and expression analyses support that *MtSUP* regulates compound inflorescence development in *M. truncatula*.

9. *MtSUP* mutants show defects in the floral organogenesis in *M. truncatula*

Medicago truncatula produces flowers arranged in pentamerous clusters of organs, producing five sepals, five petals, ten stamens and a single carpel (Figure 37 A,E) The *MtSUP* mutants (*mtsup-1* and *mtsup-2*) produced flowers with a range of floral phenotypes that were classified according to the abnormalities in the floral organ number (Table 15, Figure 37 A).

The class one of flowers was characterized by having a normal number of organs but a reduced number of ovules (Figure 37 A, Table 15). In the class two were included the flowers with an increased number of carpels, displaying from two to three carpels fused at their base with less ovules that were exposed (Figure 37 A-D, Table 15). Flowers of class one and two were observed in both mutant alleles, *mtsup-1* and *mtsup-2*. The third class of flowers had from 6 to 8 petals and from 6 to 9 stamens (Table 15, Figure 37 A). This class of flowers in which the extra petals are produced at expenses of the stamens were only observed in the CRISPR/*Cas9* mutant allele (*mtsup-2*, Figure 37 F). The fourth class of flowers were the ones that had supernumerary organs, 6 to 8 petals, 12 to 16 stamens, and two carpels with less ovules (Figure 37 A,G; Table 15). These flowers had the strongest phenotype and were only observed in the *Tnt1* insertional mutant, *mtsup-1*.

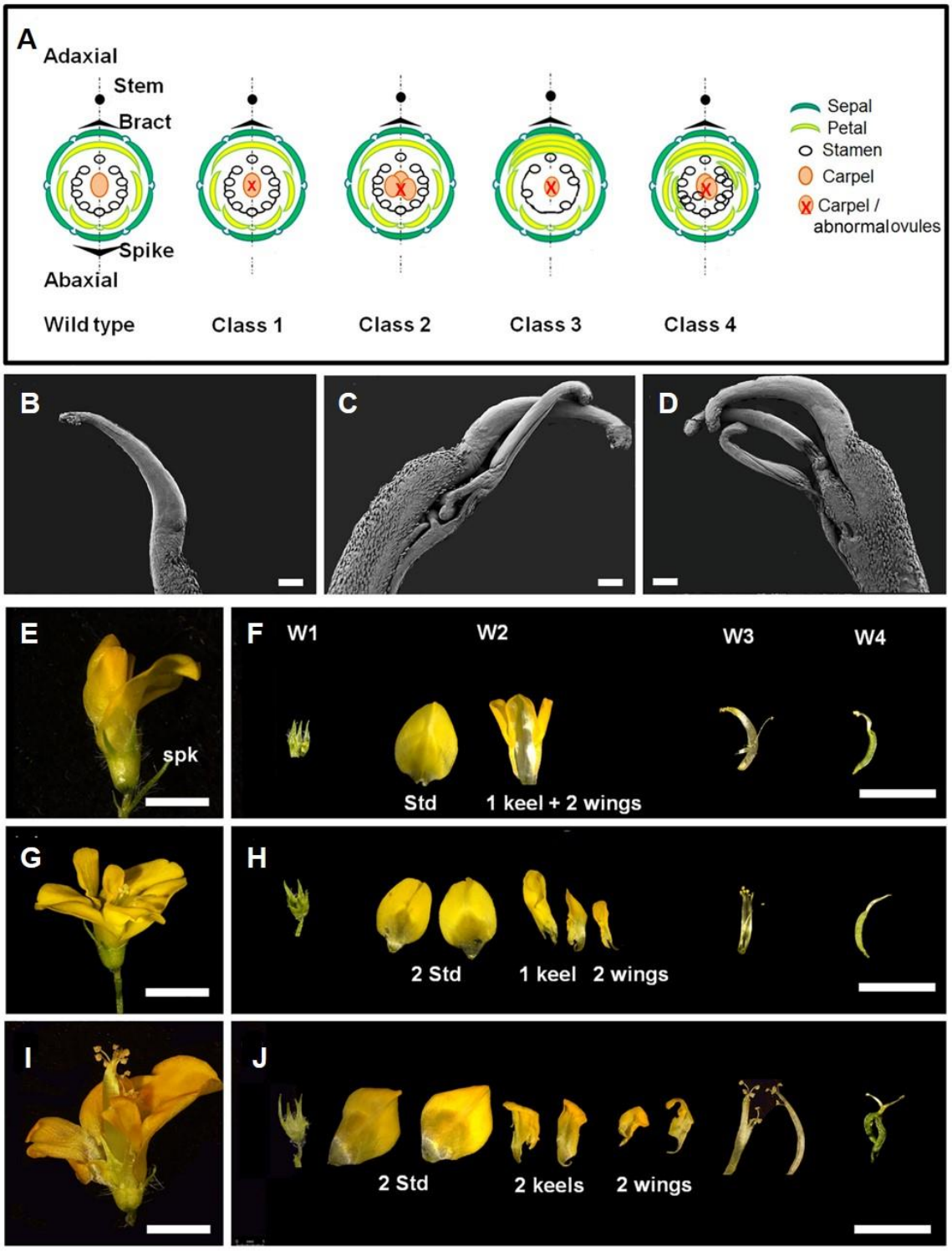


Figure 37. Flowers characterization of the wild type and *MtSUP* mutants. A. A schematic representation of the floral phenotypes observed in *mtsup-1* and *mtsup-2,* compared to the wild type. Phenotypes were classified in four classes according to the abnormalities in the

floral organ number. Class 1: less ovules; Class 2: more carpels (2-3); Class 3: more petals at expenses of the stamens; Class 4: more petals, stamens and carpels. In all classes the ovules were affected. **B-D.** Scanning electron microscope of carpels of flowers at anthesis. **B.** Wild type single carpel. A double (**C**) and triple (**D**) carpel fused in the base from *MtSUP* mutants (class 2). **E-G**. Dissection of flowers showing the number of sepals (whorl 1, W1); petals (whorl 2, W2); stamens (whorl 3, W3) and carpels (whorl 4, W4). **E.** Wild type flower with 5 sepals, 5 petals (1 standard, 2 wings and 1 keel- two fuse petals), 10 stamens (9 fused +1 free) and 1 carpel. **F,** Typical flower from a mutant class 3 with more petals (6) and less stamens (7). **G.** Typical flower from a mutant class 4 with 8 petals, 14 stamens and 2 carpels. Scale bars = 50 µm (B, C, D) and 0.5 mm (E, F, G).

Table 15. Floral phenotypes classification in *MtSUP* mutants

Allele	Class	Stamen No.	Carpel No.	Petals No.	Ovule No.	Floral level
Wild type (N=100)	WT	10.00±0.10	10±0.10	5.00±0.00	9.10±0.50	100%
mtsup-1 **(N=100)**	1	10.00±0.15	1.02±0.15	5.00±0.00	7.80±0.80	71%
	2	10.00±0.19	2.40±0.49	5.00±0.00	7.40±0.70	18.7%
	4	14.00±1.64	2.20±0.40	7.60±0.51	6.15±2.10	10.3%
mtsup-2 **(N=100)**	1	9.95±0.21	1.00±0.00	5.00±0.00	6.10±1.24	69%
	2	9.63±0.67	2.24±0.48	5.00±0.00	5.41±1.61	19%
	3	5.40±2.04	1.09±0.39	5.97±0.43	4.39±1.62	12%

Numbers represent the mean ± the standard deviation. Data was obtained from the dissection of 100 flowers per allele (10 flowers/plant X 10 plants = 100 flowers).

9.1. *MtSUP* mutants show a delayed floral meristem termination

In *A. thaliana SUP* mutants showed floral indeterminacy that was related to a prolonged *WUSCHEL* expression (Prunet *et al.*, 2009; Breuil-Broyer *et al.*, 2016). *WUSCHEL* is a gene that maintains active the stem cells of the floral meristem centre (FMC) until these are set into a female program by *AGAMOUS* (AG) to give place to the carpel primordia. *MtWUSCHEL* expression was tracked by *in situ* hybridization. *MtWUS* was firstly detected after floral transition in the I1 and I2 meristems in the wild type (Figure 38 A) and *mtsup-1* (Figure 38 E). In the wild type *MtWUS* continues its expression in the emerging floral meristem (FM) (Figure 38 B) until the stage 3 of flower development when it is no longer detected (Figure 38 C-D). While in *mtsup-1,*

MtWUS has a similar expression to the wild type during early stages (Figure 38 F) but later on prolongs its expression until stage 4 (Figure 38 G-H). These results indicated that floral indeterminacy exhibited by *MtSUP* mutants could be linked to a prolonged *MtWUS* expression as occurs for *SUP* mutants in A*. thaliana*.

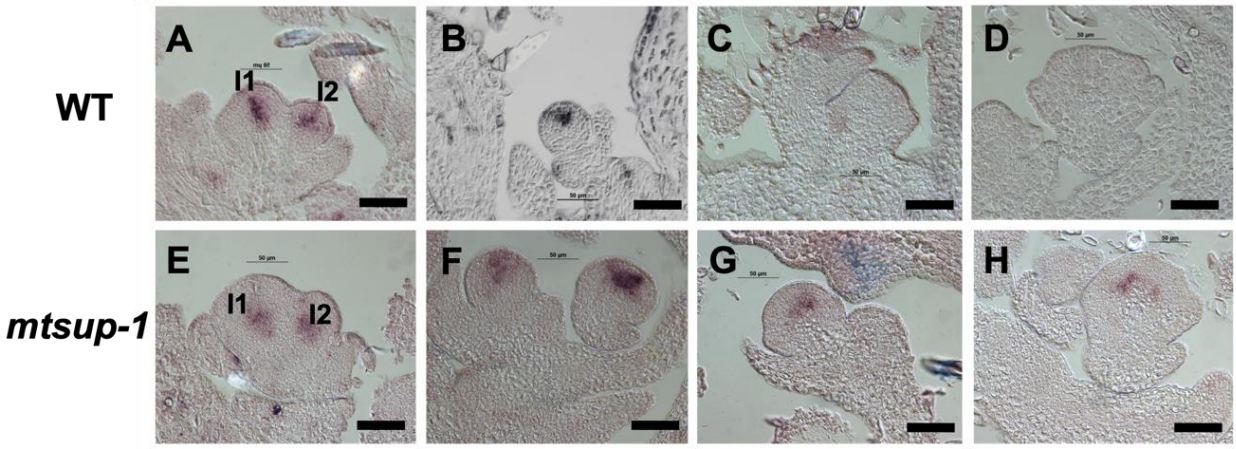


Figure 38. *MtWUS* expression in *mtsup-1* and the wild type. A-D. *MtWUS* expression in the wild type (R108). **E-H.** *MtWUS* expression in *mtsup-1* **A,E.** *MtWUS* transcript is detected in the I1 and I2 in the wild type and *mtsup-1*. **B, F.** *MtWUS* transcript is detected in the floral meristem centre in the wild type and *mtsup-1*. **G, H.** *MtWUS* is detected in the floral primordia at stage 3 (**G**) and 4 (**H**) in *mtsup-1*, while is no longer detected in the wild type (**C, D**). Scale bars= 50µm.

9.2. B-class MADS-box genes are up regulated in *MtSUP* mutants

SUPERMAN has been thoroughly studied using the *flo-1* or *sup-1* mutant which has supernumerary stamens at expenses of the carpel (Bowman *et al*., 1992; Sakai *et al*., 1995). It is proposed that *SUP* acts as repressor of B-class MADS box genes (*AP3* and *PI*) in the fourth whorl (Prunet *et al.*, 2017). In *sup-1, AP3* and *PI* expand their expression to the fourth whorl. Based on this information the expression of the master regulator of the B-function in *M. truncatula* (*MtPI*) was analysed by *in situ* hybridization. *MtPI* restricts its expression to the common primordia in the wild type (Figure 39 A). In the mutant *MtPI* has a similar expression to the wild type not being detected in the

centre of the floral primordia (Figure 39 B-C). However, in the mutant *MtPI* expands its expression to the new the FM (Figure 39 C) that was originated from the cells that should turn into a spike but instead acquired floral identity. In line with this the expression levels of all B-class MADS-box genes (*MtPI*, *MtNMH7*, *MtTM6* and *MtNGL9*) were analysed by RT-qPCR. All B-class genes were significantly up-regulated in *mtsup-1* (Figure 39) with the exception of *MtNGL9* (Figure 39) who has been described to have the minor contribution to the B-function in *M. truncatula* (Roque *et al*., 2016).

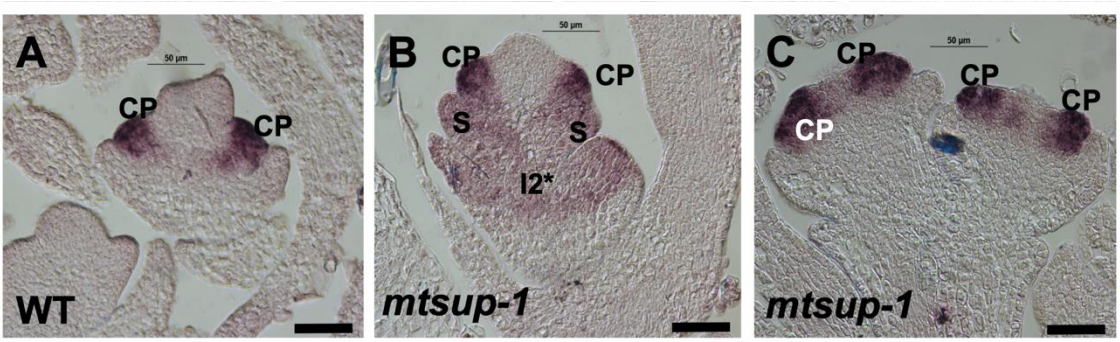


Figure 39. *MtPI* expression in the wild type and *mtsup-1.* **A.** In the wild type (WT), *MtPI* transcript is localized in the common primordia (CP). **B.** In msup-1, *MtPI* transcripts accumulates in the common primordia and is also detected in the sepals and in the remaining cells of the I2* that acquired floral identity. **C.** In *mtsup-1,* a double flower in which *MtPI* transcript accumulates in the common primordia. Scale bars = 50 µm.

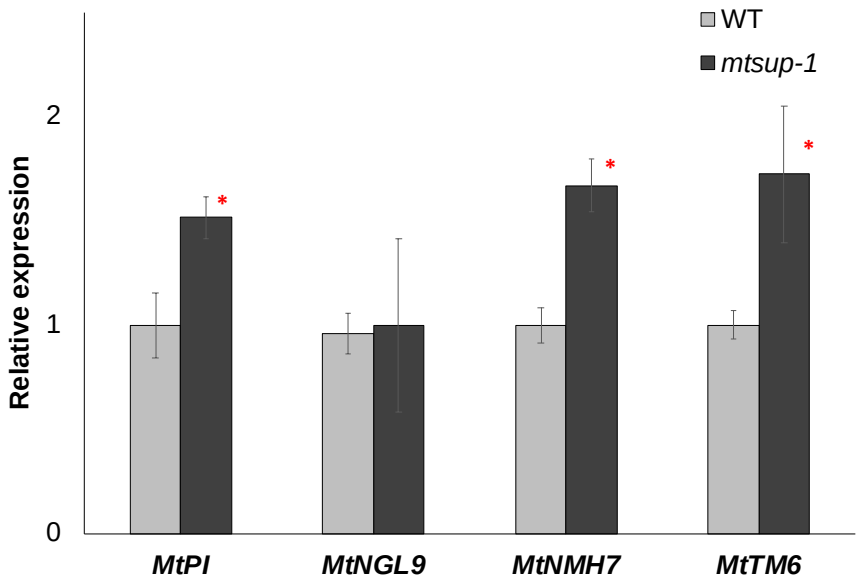


Figure 40. B-class genes relative expression in the wild type and *mtups-1*. Relative expression of the Medicago B-class MADS-box genes: *MtPI* (Medtr3g088615); *MtNGL9* (Medtr1g029670); *MtNMH7* (Medtr3g113030); *MtTM6* (Medtr5g021270). Wild type values were set to 1.00. Gene expression levels in *mtsup-1* were relative to their expression in the wild type. Columns represent the mean and the bars the standard deviation of three biological replicates. A two-tailed T-Test was performed to determine if there was a significative difference between expression values. A p-value <0.05 (*) and <0.01 (**).

10. *MtSUP* mutants show defects in gynoecium and fruit development

The impairment of *MtSUP* functions produces a range of phenotypes that includes changes in compound inflorescences architecture and floral abnormalities. The floral phenotype of the mutants leads to classify them in four classes (section 9, Figure 37 A). The class 1 had a reduced number of ovules (Table 14) and ovules seem abnormal. An histological examination of the gynoecia was performed to analyze the gynoecia anatomy. The analysis revealed that *MtSUP* mutants produced less and smaller ovules, which also lacked the funiculi (Figure 41 B).Moreover, in the mutants the gynoecium area was significantly reduced when compared to the wild type ones (Figure 41 C-D, 42). Not only was the gynoecium affected, but also the number (Table 16) and quality (Figure 41 F) of the fruits produced. The wild type fruits had a typical

coiled barrel-shaped structure (Figure 41 E) while in the mutants the pods were lopsided (Figure 41 F) with an abnormal surface. All the gynoecia defects and abnormalities in the fruits in the mutants lead to defective or absent seeds (Figure 41 E).

Table 16. Fruit and seed production in the wild type and *MtSUP* mutants

Allele	Fruit yield production	Seed production
Wild type (N=100)	77%	9.50±0.67
mtsup-1 (N=100)	58%	3.54±0.51
mtsup-2 (N=100)	46%	3.91±0.63

The fruit yield production shows the percentage of flowers that were able to produce a mature fruit. For this, 100 flower per allele were labelled at anthesis and tracked until the fruit was mature or aborted. For the seed production is shown the average number of seeds per dried fruit. A total of 100 dried fruits per allele were used for the counting.

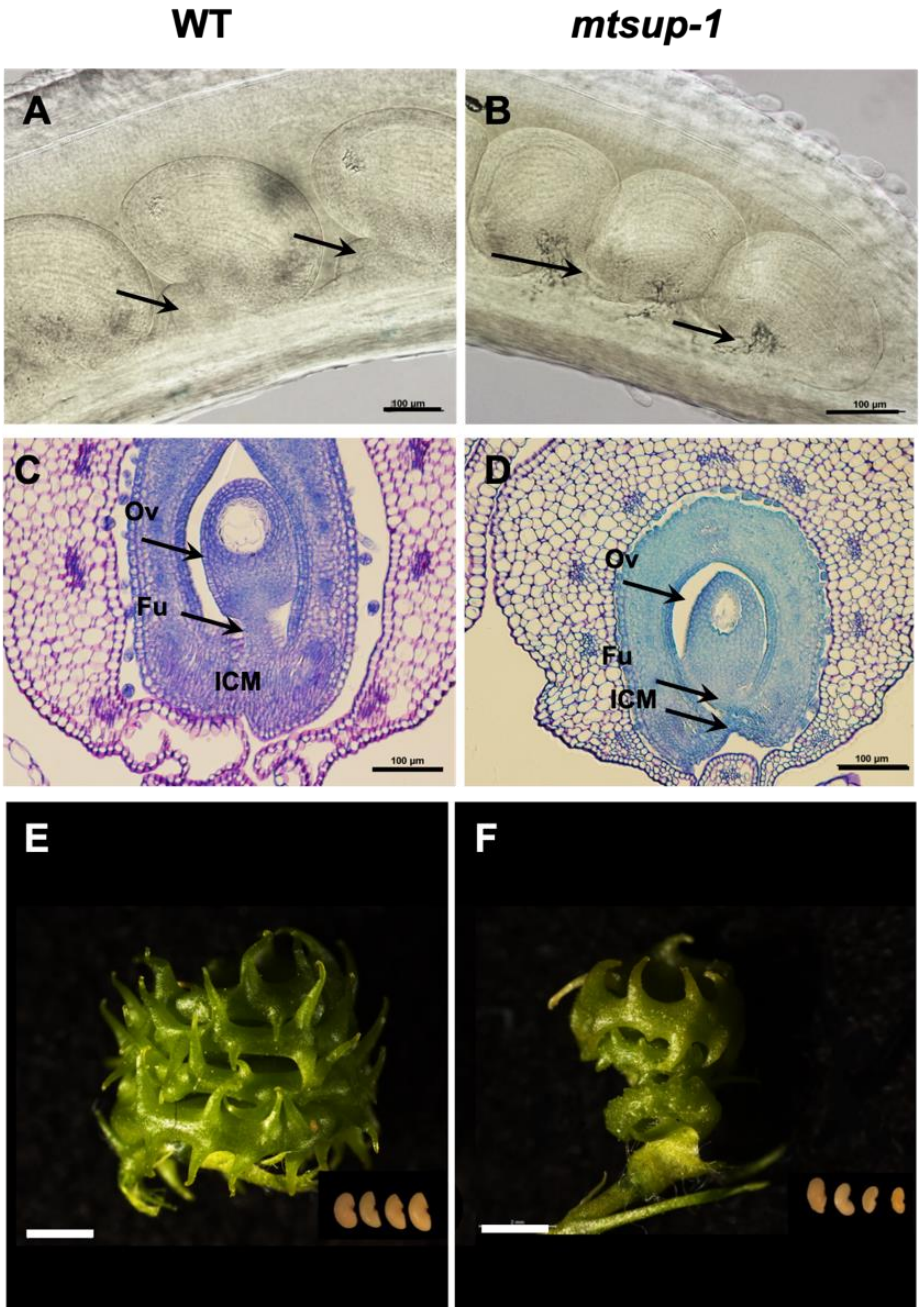


Figure 41. Defects in gynoecium and fruit development in *MtSUP* mutants. **A-B.** Cleared carpels from flowers at anthesis visualized by differential interference contrast (DIC) microscopy. **A.** Wild type carpel with its ovules of normal size with their funiculi (arrow). **B.** A *mtsup-1* carpel with smaller ovules that lack the funiculi (arrows). **C-D**. Cross-sections of gynoecium from flowers at pre-anthesis. **C**. Wild type gynoecium with a normal sized of tissues. **D.** mtsup-*1* gynoecium with a reduction of the medial tissues. **E.** A typical wild type

fruit 6 days after pollination, at the right bottom are shown mature and dry seeds **F**. An abnormal fruit 6 DAP typically observed in *MtSUP* mutants. At the right bottom are the typical mature and dry seeds obtained from this fruit. Ov=ovule; Fu=funiculi; ICM=inner carpel margin. Scale bars = 100 µm (A-D) and 2 mm (E-F).

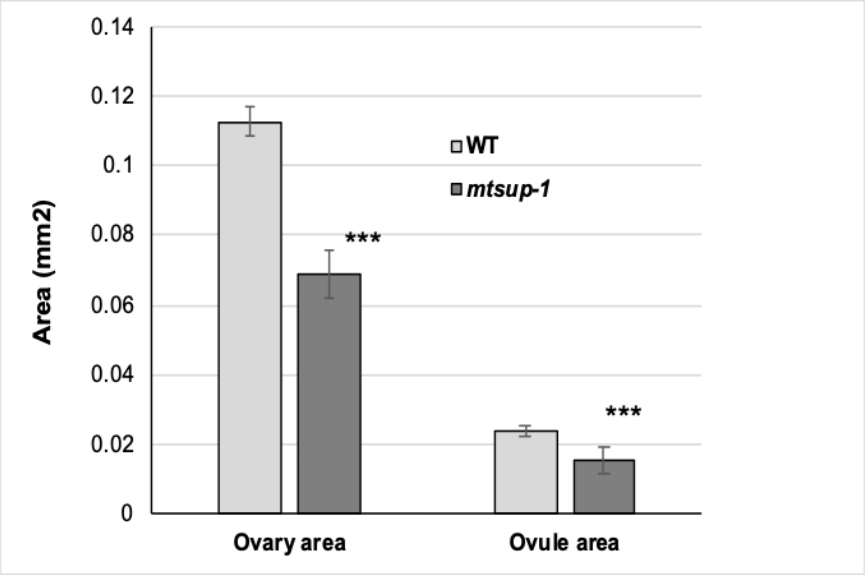


Figure 42. Gynoecium areas in the wild type and *mtsup-1.* Three cross-sections at the same level of gynoecia from three different flowers from independent plants were used to calculate the ovary and ovule area (ovule + funiculus). In Figure 41 C-D are examples of the images used for this purpose. The software Fiji was used to calculate the area in square millimetres according to Schindelin *et al*., 2012. A Kruskal-Wallis test for independent samples (n=3) was performed to determine if there was a significative area reduction in *mtsup-1* compared to the wild type. If p-value < 0.001, ***.

Based on the results presented in the previous sections, an impairment of *MtSUP* functions results in defective carpel medial tissues development. In other words, derived tissues from the carpel margin like the funiculi and ovules are affected or collapsed. As consequence, fruit and seed development is affected. Previous results implicate a reduced fruit production and fertility in *MtSUP* mutants.

Discussion

1. Characterization of *MtSUPERMAN*

The main objective of this thesis is to functionally characterize the *SUPERMAN* ortholog in the model legume *Medicago truncatula*. In order to achieve this objective a first step was to identify the putative *SUP* ortholog. Based on the phylogenetic analysis for Medicago a single gene (*MtSUP*) falls in the clade of *SUP*-like genes. This is in contrast with other legumes such as *Glycine max* (soybean) and *Lupinus angustifolius* (lupin) that harbour two *SUP*-like genes. *Lupinus* and *Glycine* lineages have experienced a whole genome duplication event (WGD) independent to the previous WGD in the common ancestor of Papilionoidade clade (Cannon *et al.*, 2006, 2015). The *in silico* sequence analysis showed that *MtSUP* has a conserved sequence. Besides, as its homolog (*SUP*), *MtSUP* is exclusively expressed in the floral apices (Sakai *et al.*, 1995). These results support that *MtSUP* is the putative ortholog of *SUP*. There is no shared synteny between the chromosome regions containing *SUP* and *MtSUP,* indicating independent *SUP*-like gene translocations from the last common ancestor of both species. These results fit the model of degenerate microsynteny between the Arabidopsis and Medicago genomes (Zhu *et al.*, 2003).

The complementation of *sup-5*, one of the strongest *SUP* mutants, with the *pSUP*::*MtSUP* construct was an important experiment to demonstrate the ability of *MtSUP* to rescue the floral phenotypes. Complemented lines (pSUP:*MtSUP*;*sup-5*) had a nearly wild type number of sexual organs (stamens and carpels). This is in contrast to the mutant (*sup-5*) that had supernumerary stamens and carpels. Slight variations in the level of complementation between the 13 transgenic lines studied carrying the pSUP:*MtSUP*;*sup-5* construct could be due to the background (*sup-5*) itself had variations in the degree of indeterminacy. However, the average number of organs in the complemented lines showed the ability of *MtSUP* to codify a protein that performed an almost equivalent biochemical function to SUP in *A. thaliana*.

The ectopic expression of *MtSUP* in *A. thaliana* had similar effects to the ones described when *AtSUP* (SUP) was overexpressed in *A. thaliana* (Hiratsu *et al.*, 2002). Plants carrying the 35S::*MtSUP* construct showed a generalized dwarfism, which is consistent with the role on the control of cell proliferation for *SUP*-like genes (Hiratsu *et al.*, 2002; Nakagawa *et al.*, 2004; Nibau *et al.*, 2011). Overall, these approaches support that *MtSUP* is the *SUP* ortholog in *M. truncatula*. The gene expression analysis and the characterization of *MtSUP* mutants revealed that *MtSUP* has conserved roles to *SUP* during the carpel marginal region development and floral meristem termination. But also, our results have shown that *MtSUP* plays novel functions that have not been described for any *SUP*-like gene, this is consistent with the fact that legumes have unique developmental features. Detailed discussion is in the next sections.

2. Characterization of *MtSUP* mutants during reproductive development

Taking advantage of the *Tnt1* insertional library, one of the mutants (*mtsup-1*) analysed in this thesis was obtained from the Nobel Research Institute facility. The other mutant (*mtsup-2*) was generated based on the use of gene editing CRISRPS/*Cas9* technology. The analysis of two *MtSUP* mutants was considered of great importance to support the described roles for *MtSUP* in *M. truncatula.*

2.1. *MtSUP* plays a role during floral meristem termination and carpel marginal tissue development

The expression pattern of *MtSUP* during floral organogenesis shows that even before the carpel primordium is initiated *MtSUP* transcript is already detected in the floral meristem centre (FMC). The increased number of carpels in *MtSUP* mutants (class 2) is related to a delay in floral meristem termination (FMT). This could be linked to the prolonged expression of *MtWUS* as the pool stem cells remain undifferentiated

for a longer time (Prunet *et al.*, 2009). In contrast, *SUP* in *A. thaliana* is not expressed in the FMC, instead plays a non-cell autonomous function in the FMT (Prunet *et al.*, 2017). *MtSUP* and *MtWUS* might interact as their temporal and spatial expression overlaps. This is the first work in which the expression of *MtWUS* was tracked during early stages of floral development. Results suggest that in *M. truncatula* the FMT occurs rapidly as *MtWUS* was no longer detected after floral apex flattens. This is consistent with an early carpel initiation in legumes (Ferrándiz *et al.*, 1999), as FMT occurs when the pool of stem cell of the FMC are set to a female fate (Prunet *et al.*, 2009). We have shown that *MtSUP* plays an important role during floral meristem termination; whether this is based on a direct interaction with *MtWUS* or not, needs further investigation.

It has been described that a proper carpel primordium formation requires a correct FMT (Sakai *et al*., 2000; Prunet *et al*., 2017). This is in agreement with the defects in the marginal derived tissues of the gynoecium in *MtSUP* mutants. The final effect of these affected or collapsed tissues is a reduced fertility compromising the reproductive success. Comparing with other *SUP*-like genes, in petunia *PhSUP* mutants showed similar defects in the placenta (Nakagawa *et al.*, 2004). Also, the strong *sup-5* mutant in *A. thaliana* shows a reduced ovule number and a shortening of funiculi (Gaiser *et al.*, 1995). The more severe defects in the derived tissues from the placenta in *MtSUP* mutants are consistent with alterations during early stages of carpel development. These results support that *MtSUP* is required for proper floral meristem termination and for the correct development of the carpel marginal tissues.

2.2. *MtSUP* controls the number of petals, stamens and carpels produced in *M. truncatula*

In *A. thaliana*, *SUPERMAN* is considered to be a gene that participates in the control of the number of carpels and stamens produced (Schultz *et al.*, 1991; Bowman *et al.*, 1992; Sakai *et al.*, 1995; Gaiser *et al.*, 1995; Jacobsen and Meyerowitz, 1997;

Breuil-Broyer *et al.*, 2016). Besides, the floral phenotypes in *MtSUP* mutants suggest that this function is conserved in *M. truncatula*. *MtSUP* mutants were classified according to their abnormalities in the floral organ numbers. The mutants from the class three (*mtsup-2*) had extra petals and a reduced number of stamens; this resembles the *sup-1* mutant in *A. thaliana* that has more stamens at expense of the carpels (Bowman *et al.*, 1992). Moreover, the class four of mutants (*mtsup-1*) have more petals, stamens and carpels resembling the Arabidopsis *sup-5* mutant that is defined as a 'supersex' mutant with more stamens and carpels (Gaiser *et al*., 1995; Breuil-Broyer *et al*., 2004;). The phenotypic differences between *mtsup-1* and *mtsup-2* might be due to the nature of their mutations. The allele *mtsup-1* has a retrotransposon insertion and in consequence no full *SUP* transcript could be detected, while the *mtsup-2* allele produced a transcript that had a four-nucleotide deletion resulting in a truncated protein due to a premature stop codon.

The Arabidopsis *sup-1* and *sup-5* mutants with which the *MtSUP* mutants bear similarity exhibit an ectopic expression of *PI* and *AP3* in the fourth whorl (Sakai *et al*., 1995; Breuil-Broyer *et al*., 2016; Prunet *et al*., 2017). Instead, in *mtsup-1, MtPI* and *MtPIM* expression was expanded towards the I2 meristem to specify the petal and stamens in the new floral meristem (FM). This FM is the one that is observed in the position of the spike and acquired floral identity. On the other hand a consistent result with the described for *SUP* mutants is that the B-class MADS box genes are upregulated in *mtsup-1*. *MtSUP* could be repressing the B-function as it has been proposed for *SUP* in Arabidopsis (Yun *et al.*, 2002; Prunet *et al.*, 2017). This is in agreement with the ability of *MtSUP* to replace SUP function in the strong *sup-5* mutants. Also, the floral abnormalities observed in the 35S::*MtSUP* plants are similar to the observed when *SUP* was overexpressed under the *AP1* promoter in Arabidopsis (Yun *et al.*, 2002). If the focus is set on the similarities between *mtsup-1* and *mtsup-2*, the supernumerary petals is a hallmark trait. This is consistent with *MtSUP* transcript localization in the common primordia where the meristematic cells that will produce petals and stamens coexist. A constant number of meristematic cells will give place to

the organ primordia (Bossinger and Smyth, 1996). As a role already described for *SUP*, *MtSUP* might also repress cell proliferation but in the common primordia. Thus, in *MtSUP* mutants the extra petals and stamens could be produced due to an over proliferation of the meristematic cells that express the A and B-class MADS-box genes. This fits well with the antiproliferative effect observed in the 35S::*MtSUP* plants that exhibit a general reduction in size and also with the upregulation of the transcript levels of the A and B-class MADS box genes in *mtsup-1* (Benlloch *et al*., 2006, 2009; Roque *et al*., 2013, 2016). Indeed *MtSUP* might be controlling the determinacy of the common primordia as a prolonged maintenance of these meristematic cells can give place to extra organs (Bowman *et al.*, 1989; Bossinger and Smyth, 1996). The supernumerary petals, stamens and carpels in *MtSUP* mutants could also be explained by a delay in the floral meristem termination as it was discussed in the previous section (section 2.1). The floral meristem termination is linked to the turn off of *MtWUS.* The precise timing of this process is not yet known; however, the results show that *MtWUS* is not detected after the floral apex flattens in the wild type. In contrast, *MtWUS* expression is prolonged maintaining during more time the pool of stem cells from the floral meristem centre (FMC) in *mtsup-1*. This could explain that a limited number of extra floral organs are produced as the floral meristem termination is delayed.

Taken together, these results support that *MtSUP* has a function during floral meristem termination, carpel marginal tissues development and the determinacy of the common primordia. Compared to *SUP*, *MtSUP* has a novel function in the common primordia determinacy. As other *SUP*-like genes *MtSUP* seems to conserve its cell antiproliferative role in the unique common primordia from legume species.

2.3. *MtSUP* controls the number of flowers produced in *M. truncatula*

One of the most interesting observations in *MtSUP* mutants is the multi-flower phenotype which is indicative of a change in the inflorescence development. This

multi-flower phenotype is similar to mutants reported in 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). An increased production of flowers is of agronomical interest in crops, as legume grains, since there could be an increased fruit production as more flowers are produced (Benlloch *et al*., 2015). However, *MtSUP* mutants do not produce more fruits due to the defects in the floral organs. In *MtSUP* mutants the carpel and the ovule show abnormalities that result in a reduced fruit yield.

In the model legumes, pea and Medicago, the number of flowers is determined by the activity of the secondary inflorescence (I2) meristem. However, the genetic regulatory network that controls the number of flowers produced per I2 was unknown (Benlloch *et al.*, 2015; Devi *et al*., 2018). The expression analysis of *MtFULc, MtPIM* and *MtSUP* together with inflorescence images support that *MtSUP* is required to control the activity of the I2 meristem. It has been reported that in *M. truncatula* (R108) the I2 meristem, derived from the primary inflorescence (I1) meristem, divides to produce from 1 to 2 floral meristems (FM) (Benlloch *et al*., 2003). The I2 meristem is a transient state between the vegetative and the reproductive tissue that remains immature until the floral identity acquisition (Prusinkiewicz *et al.*, 2007). After producing a FM the remaining cells of the I2 meristem enter in senescence and produce the residual organ or spike (Tucker, 1989; Benlloch *et al.*, 2007). The timing of this process condition the structures that could be formed (Prusinkiewicz *et al.*, 2007). In *MtSUP* mutants an I2 meristem gives place to more floral meristems than the wild type. Moreover, the residual cells of the I2 meristem terminate as a floral meristem instead a spike. The changes observed in the inflorescences in *MtSUP* mutants might be explained through the functional models proposed for *SUP* in Arabidopsis. It was discussed (section 2.2) that in *MtSUP* mutants the FMT is delayed. A prolonged expression of *MtWUS* was an indicator of the FMT delay. Therefore, the I2 meristem determinacy could also be linked to the gradual turn off of *MtWUS* in the I2 meristem. As *MtWUS* prolongs its expression the I2 meristem could prolong its

activity in *MtSUP* mutants. Another option is that *MtSUP* could influence the I2 meristem activity through the control of cell proliferation in this meristem. In *MtSUP* mutants, *MtFULc* transcript occupies a wider compared to the wild type. There might be more cells expressing the MADS-box gene *MtFULc*. On the other hand *MtSUP* seems to restrict *MtPIM* expression to the FM. This is consistent with the invasion of the expression domain of *MtPIM* to the I2 meristem. As a consequence to this invasion the remnant cells of the I2 meristem would lose their vegetative nature acquiring a floral identity. *MtSUP* controls the activity of the I2, in turn, also regulates the number of flowers produced per inflorescence. *MtSUP* could be considered one of the genes behind the multi-flower trait already observed in nature. The molecular mechanism of *MtSUP* to control the I2 meristem activity requires further research. However, this work provides valuable information about the genetic regulatory network behind compound inflorescence development in legumes.

Overall, we have shown that *MtSUP* plays a novel function during compound inflorescence development. *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. We propose a model in which *MtSUP* is part of the key regulatory network behind compound inflorescence and flower development in *M. truncatula* (Figure 43). Compared to the current model, in the model that we proposed *MtSUP* is involved in the determinacy of the I2 meristem, floral meristem and common primordia. Besides, we also propose that *MtSUP* controls the expression domain of *MtPIM* to avoid its invasion to the remaining cells of the I2 meristem (Figure 43).

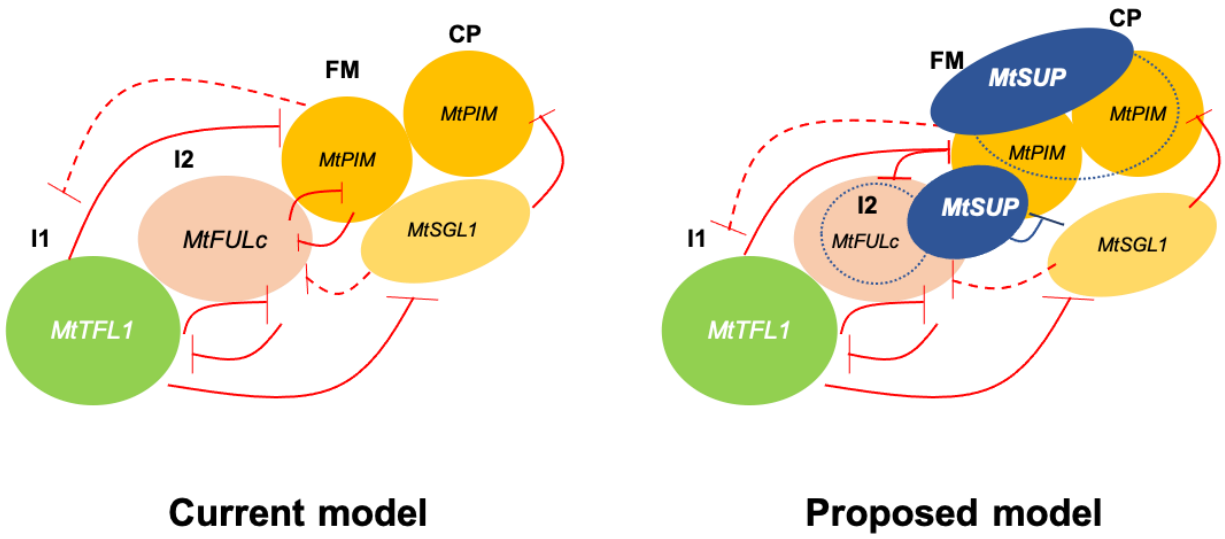


Figure 43. Model for *MtSUP* role during compound and flower development in *M. truncatula*. The current model includes the genes *MtTFL1, MtFULc*, *MtPIM* and *MtSGL1* which are responsible to confer identity to the I1 meristem, I2 meristem, floral meristem and common primordia respectively. The continuous red lines represent direct repression and the red dashed lines indirect repression. The proposed model is based on the functional characterization of *MtSUP*. All the new elements are in blue. We propose that *MtSUP* is a key gene for the determinacy of the I2 meristem, floral meristem and the common primordia. The determinacy control of these meristems is represented by the blue dashed curves. Besides *MtSUP* seems to repress *MtPIM* from the I2 meristem (blue line). I1: primary inflorescence meristem; I2: secondary inflorescence meristem; FM: floral meristem; CP: common primordia. The current model was adapted from Cheng *et al.*, 2018 Figure 8.

3. Novel functions for a *SUP*-like gene are linked to novelties during reproductive development in legumes

Through this work it has been described that *MtSUP* shows novel functions for a *SUP*-like gene. This is coincident with the fact that legumes have unique developmental novelties. Indeed, *MtSUP* plays a role in the most unique meristems: the common primordia (CP) and the secondary inflorescence meristem (I2). *MtSUP* and *SUP* have functionally diverged by transcriptional changes which is a mode of evolution usually observed in duplicated genes (Ohno, 1970; Kimura, 1983). However, most of *SUP*-like genes are single-copy genes (Sakai *et al.*, 1995; Nakagawa *et al.*, 2004; Kazama *et al.*, 2009; Nibau *et al.*, 2011; Zhao *et al.*, 2014; Ezura *et al.*, 2017),

with the exception of dioic plants in which *SUP*-like gene are duplicates and show sex-dependent expression (Song *et al.*, 2013).

Regulatory mutations such as point mutations, transposition, duplication, and rearrangements have a role in organismal evolution (King and Wilson, 1975; Carroll, 2005). These types of mutations usually take place after whole-genome duplication (WGD) events, such as the one that pre-dated the speciation of legumes around 60 million years ago. This WGD contributed to the degenerated synteny conservation between legumes and other plant families. Interestingly, these events played an important role to shape the genome of *M. truncatula* (Cannon *et al.*, 2006). Microsynteny analysis included in this work revealed that there is no collinearity between the flanking regions of *MtSUP* and *SUP*, which is in agreement with the model of degenated microsynteny between the Medicago and Arabidopsis genomes (Zhu *et al*., 2003).

Overall, the transcriptional innovations of *MtSUP* could affect the I2 meristem and common primordia, transitory meristems that are consumed during inflorescence and flower development. Compound inflorescence development in grasses also includes higher order inflorescence meristems (Bommert *et al.*, 2005; Gallavotti *et al.*, 2010). In contrast to legumes, the genetic control of compound inflorescence in grasses does not seem to include any *MtFULc* gene related function. Interestingly, in maize it is a Cys_2-His_2 zinc-finger protein of the class RA1 which promotes the determinacy in the inflorescence meristems (Vollbrecht *et al.*, 2005). MtSUP as Ra1 class protein in maize seem to have a similar role during inflorescence development. However, it is hard to assess the orthology relationship between these two Cys_2-Hys_2 zing finger proteins from phylogenetically distant plant families. It would be of interest to correlate changes in the spatial-temporal expression of *MtSUP* orthologues to more complex inflorescence architecture in legumes or other angiosperms.

The role of *MtSUP* during compound inflorescence development and floral organogenesis could be casually link to the existence of the secondary inflorescence meristem and the common primordia in legumes. It requires further investigation to link the developmental novelties of inflorescence architecture and flowers with the functional divergence of *MtSUP* in *M. truncatula.* This thesis unveils the function of a key regulatory gene (*MtSUP*) during compound inflorescence and flower development in this legume species.

Conclusions

Conclusions

The enclosed results in this Thesis about the functional characterization of *MtSUPERMAN* in the model legume *Medicago truncatula* lead to the following conclusions:

First. *MtSUPERMAN* is the orthologous gene of *SUPERMAN* in the model legume *Medicago truncatula*.

Second. *MtSUPERMAN* controls the secondary inflorescence (I2) meristem activity and regulates the number of flowers per inflorescence in *M. truncatula.*

Third. *MtSUPERMAN* has a role in the determinacy of both the floral meristem center and the common primordia, thus controlling the number of floral organs in the inner three whorls in *M. truncatula*.

Fourth. *MtSUPERMAN* seems to control the number of flowers and floral organs produced possibly through the control of cell proliferation in the common primordia and the secondary inflorescence meristem in *Medicago truncatula.*

Fifth. *MtSUPERMAN* 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.

Publications and awards

1. Publications

The enclosed results in this thesis are published in the Plant Journal volume 105, issue 03 from February 2021. A copy of the cover, graphical abstract and summary are included in the supplementary information. The article reference is:

Rodas, A. L., Roque, E., Hamza, R., Gómez-Mena, C., Minguet, E. G., Wen, J., Mysoure, K.S., Beltrán, J.P., Cañas, L. A. (2021). MtSUPERMAN plays a key role in compound inflorescence and flower development in Medicago truncatula. *The Plant Journal*, 105 (3): 816-830. tpj.15075. https://doi.org/10.1111/tpj.15075

2. Disclosure and Awards

2.1. Video presentation

2.1.1. 12th International Congress of Plant Molecular Biology (IPMB 2018)

The video was in the top three videos of all participants. **I was awarded with a free entrance to the congress (540 euros).**

2.2. Poster presentation

2.2.1. 12th International Congress of Plant Molecular Biology (IPMB 2018).

2.2.2. 9th International Conference on Legume Genetics and Genomics (ICLGG 2019). **I was awarded as the best poster presentation (300 euros).**

2.2.3. XXIII Meeting of the Spanish Society of Plant Physiology and the XVI Hispano-Portuguese Congress of Plant Physiology.

I was awarded as the best poster presentation in the congress (300 euros).

2.3. Oral presentation

2.3.1. 1st PhD Meeting in Plant Science in Pamplona, Spain.

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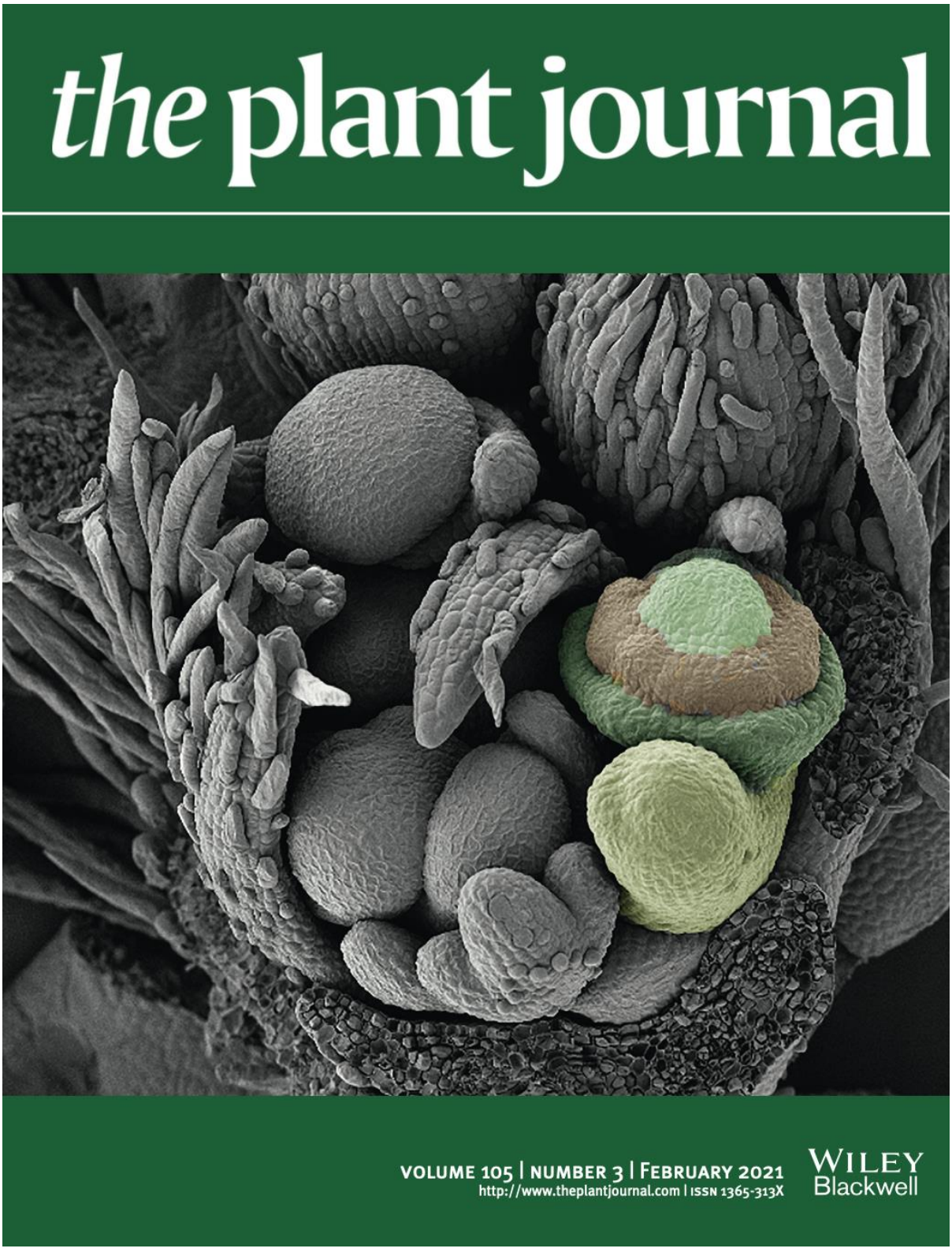
Supplementary information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Front cover: MtSUPERMAN controls the number of flowers produced per inflorescence in Medicago truncatula (Rodas *et al.*, 2020, pp. 816-830). SEM image of the MtSUP mutant inflorescence. The secondary inflorescence meristem terminates as an additional floral meristem (colored image) instead of a spike. Carpel primordium: light green; common primordia to petals and stamens: orange; sepals primordia: dark green; emergent floral meristem instead of a spike: yellow. Picture by Rodas *et al.*

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

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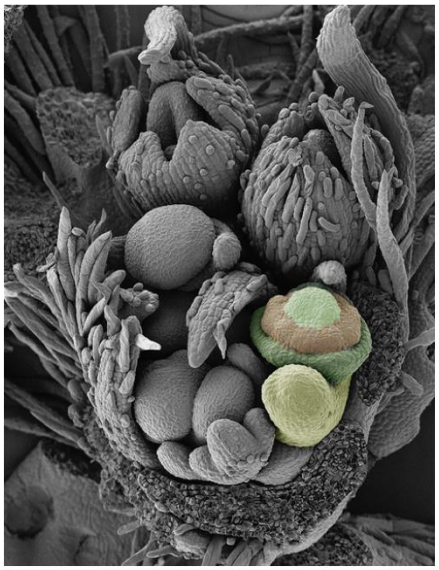

Significance Statement

MtSUPERMAN (*MtSUP*) is the orthologue 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* orthologue in eudicots.

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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.

Keywords: Legumes, *Medicago truncatula*, *MtSUPERMAN*, compound inflorescence, meristem determinacy, floral meristem termination, flower development, *CRISPR/Cas9* mutants, *Tnt1* mutants.