Genome-wide characterization of SPL family in Medicago truncatula reveals the novel roles of miR156/SPL module in spiky pod development

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Abstract

Background: SQUAMOSA Promoter Binding Protein-Likes (SPLs) proteins are plant-specific transcription factors that play many crucial roles in plant growth and development. However, there is little information about SPL family in the model legume Medicago truncatula.

Results: In this study, a total of 23 MtSPL genes were identified in M. truncatula genome, in which 17 of the MtSPLs contained the putative MtmiR156 binding site at the coding or 3′ UTR regions. Tissue-specific expression pattern analysis showed that most MtmiR156-targeted MtSPLs were highly expressed in seed and pod. The observation of MtmiR156B-overexpressing plants reveals that MtmiR156/MtSPL modules are not only involved in the development of leaves and branches, but also in the seed pod development, especially the formation of spine on pod.

Conclusion: The spines on pods are developed in many plant species, which allow pods to adhere to the animals, and then be transported on the outside. This study sheds light on the new function of SPL family in seed dispersal by controlling the formation of spiky pod, and provides insights on understanding evolutionary divergence of the members of SPL gene family among plant species.

Keywords: SPL genes, miR156, Seed development, Spiky pod formation, Medicago truncatula, Legume

Background

Transcription factors (TFs) play an essential role in regulatory networks of many important developmental processes by activating or repressing the transcription of downstream target genes [1]. The SQUAMOSA Promoter-Binding Protein-Likes (SPLs) proteins are plant-specific TFs and all of them have a highly conserved SBP domain with proximately 78 amino acids in length [2–4]. The SBP domain contains two zinc-finger like structure (Cys-Cys-Cys-His and Cys-Cys-His-Cys) and one nuclear localization signal (NLS) motif [5, 6]. SPL proteins specifically recognize and bind to the cis-element TNCGTACAA, which is located at the promoters of target genes with GTAC as its core sequence [5, 7]. The initial two SPL genes, AmSBP1 and AmSBP2, are firstly identified from the Antirrhinum majus, and they act as transcriptional activators and regulate the expression of floral meristem identity gene SQUAMOSA [3]. The SPL genes have been reported to play the important role in regulation of multiple aspects of plant growth and development, including leaf and shoot development [8, 9], vegetative phase change [10, 11], flowering [12], sporogenesis [13], male fertility [14], plant hormone signal transduction [15], and copper homeostasis [16]. So far, the SPL families have been identified and characterized in several plant species, such as Arabidopsis thaliana [2], Oryza sativa [17], Solanum lycopersicum [18], Gossypium raimondii [19], Vitis vinifera [20], Brassica napus [21], Glycine max

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Many microRNAs have been identified, which regulate gene expression by binding and cleaving their target mRNAs [27, 28]. miRNA156 is one of the highly conserved miRNA families in plants [29]. In Arabidopsis, in total 16 SPLs are identified and termed as AtSPL1 to AtSPL16, respectively [5]. Among them, 10 are targets of AtmiR156, which are AtSPL2-AtSPL6, AtSPL9-AtSPL11, AtSPL13, and AtSPL15 [9, 12, 30–32]. In rice, 19 OsSPL genes have been identified, and 11 of them are targeted by OsmiR156 [17]. Most Arabidopsis miR156 binding sites of the targeted-SPL genes are located in the downstream of the SBP domain at the coding sequences, while in AtSPL3, AtSPL4, and AtSPL5, they are located in the 3′ UTR of the mRNAs [12].

These miR156-targeted SPL genes play redundant roles in plant morphology and development among different species. Several studies show that SPL genes are involved in the regulation of flower and inflorescence development. In Arabidopsis, AtSPL3, AtSPL4 and AtSPL5 have similar functions and play vital roles in vegetative phase change and floral transition [7, 9, 12]. Furthermore, AtSPL3 can bind directly on the promoter regions of AP1, LFY and FUL to activate their expression in controlling the timing of flower formation [33]. In addition, SOC1 and FT regulate the expression of AtSPL3, AtSPL4 and AtSPL5 in response to photoperiod and gibberelin (GA) signals to promote flowering [34].

In wheat, two paralogous SPL genes, TaSPL20 and TaSPL21, are highly expressed in the lemma and palea, and ectopic expression of them in rice can promote panicle branching [35]. SPL genes also play important roles in regulating lateral organ and shoot development. In Arabidopsis, AtSPL2, AtSPL10 and AtSPL11 redundantly regulate proper development of lateral organs in association with shoot maturation in the reproductive phase [36]. Besides, maternal phenotype analysis shows that AtSPL9 and AtSPL15 act redundantly in regulation of the juvenile-to-adult phase transition [11]. In maize, SPL transcription factor TASSELSHEATH4 plays an essential role in bract development and the establishment of meristem boundaries [37]. Two duplicated loci, UNBRANCHED2 and UNBRANCHED3, affect crop productivity traits by regulating the rate of lateral primordia initiation [38]. In addition, a series of studies show that SPL genes are involved in the regulation of seed and fruit development. In rice, the OsmiR156-targeted SPL gene, OsSPL14/IDEAL PLANT ARCHITECTURE 1 (IPA1), regulates shoot branching in the vegetative stage and enhances rice grain yield at the productive stage [39, 40]. OsSPL16/GRAIN WIDTH 8 (GW8) acts as a positive regulator of cell proliferation and is involved in control of grain size, shape and quality [41]. In tomato, the SPL gene COLORLESS NON-RIPENING (CNR) is critical for normal fruit ripening, and mutation in CNR results in colorless fruits with a substantial loss of cell-to-cell adhesion [42].

Medicago truncatula is a fast-emerging model legume for functional genomics study. However, the information and function of SPL gene family in M. truncatula are largely unknown. In this study, we reported the genome-wide identification and characterization of SPL genes in M. truncatula. Totally, 23 MtSPL genes were identified, and their phylogenetic relationship, protein motifs, gene structures, and chromosomal locations were analyzed. Furthermore, we found that most MtmiR156-targeted MtSPL genes were highly expressed in pod and seed. Overexpression of MtmiR156B transgenic plants displayed the small seeds and pods, especially the loss of pod spine. These findings demonstrate that MtmiR156-targeted MtSPL genes play the novel roles in pod and seed development in M. truncatula.

**Results**

**Genome-wide identification of MtSPL genes**

In order to identify SPL genes in M. truncatula genome, we executed a genome-wide search of MtSPLs by protein BLAST using the 16 AtSPLs and 19 OsSPLs sequences against the Medicago truncatula Genome Database. Initially, a total of 24 putative MtSPL sequences were obtained. Medtr8g066240 was excluded from the MtSPL gene family due to the absence of a complete SBP domain. Therefore, totally 23 MtSPLs with the conserved SBP domain were characterized in genome of M. truncatula (Additional file 1). The MtSPL genes were named according to their closest Arabidopsis orthologs (Fig. 1). The protein lengths of MtSPLs ranged from 143 to 1025 amino acids, and the gene locus, isoelectric point, intron number, and chromosome location of the MtSPL genes were shown in Table 1.

**Phylogenetic analysis and chromosomal localization of MtSPL genes**

To further achieve the evolutionary relationship between MtSPL genes and other SPLs, a phylogenetic tree containing 16 AtSPLs, 19 OsSPLs, and 23 MtSPLs was constructed using MEGA7.0 with Neighbor-Joining method (Fig. 1). According to the phylogenetic analyses, the 58 SPL proteins were clustered into seven distinct groups (named G1-G7), and each group contained at least one SPL from A. thaliana, rice, and M. truncatula (Fig. 1). To determine the chromosome distribution of MtSPL genes in M. truncatula, the 23 MtSPL genes were located on each chromosome based on the M. truncatula genome data. These MtSPL genes showed uneven distribution on the M. truncatula chromosomes (Fig. 2a). Chromosome 8, 2, 7, and 1 contained 6, 5, 4, and 3
MtSPL genes, respectively. Both chromosome 3 and 4 contained 2 MtSPL genes, chromosome 5 contained only one MtSPL gene, and no MtSPL gene located on chromosome 6.

Conserved motifs and gene structure analysis of MtSPL genes
To further understand the structural diversity of the MtSPL genes, gene exon/intron structure analysis was carried out using online Gene Structure Display Server tool. The exon/intron structures of the 23 MtSPL genes were generated by alignment of MtSPL gene coding sequences with their corresponding genomic sequences. Gene structure illustrations displayed the high variation in the number of introns, from 0 to 10 (Fig. 2b). To gain a better understanding of the protein sequence characteristics of the MtSPLs, online MEME search was performed to analyze the conserved domains and motifs. Besides the conserved SBP domain (motif 4), in total 20 conserved motifs were identified in MtSPLs (Fig. 3a, Additional file 2). The conserved SBP domain consisted of three motifs: zinc finger 1 (Zn-1, C3H), zinc finger 2 (Zn-2, C2HC), and a nuclear localization signal (Fig. 3b, c).

Analysis of MtmiR156 and its target sequences in MtSPLs
In order to understand the function of miR156 and miR156-targeted MtSPL genes, we searched the miRBase
Database and found 10 MtmiR156 genes, MtmiR156A-MtmiR156J, in *M. truncatula* genome (Fig. 4a, Additional file 3a). Based on the MtmiR156 precursor sequences, the stem-loop structures of MtmiR156 were found (Additional file 3a). Previous studies showed that miR156 could bind to the coding or 3′ UTR region of the SPL genes and reduce gene activity. Then, we used the online psRNATarget tool to find MtmiR156 complementary sequences in the MtSPL transcripts. By comparison of the MtmiR156 mature sequences and the MtSPL transcript sequences, we found that total 17 MtSPL genes have the MtmiR156 binding sites, 13 of which were located in coding regions and 4 in 3′ UTR regions, respectively (Fig. 4b, Additional file 3b).

### Expression profile of MtSPL genes in different organs

The expression pattern of a gene is often correlated with its function. In order to understand the developmental functions of the MtSPL genes, we investigated the expression profiles of the 23 MtSPL genes by quantitative real-time PCR (qRT-PCR) in six different organs, including roots, stems, leaves, flowers, pods, and seeds. qRT-PCR results showed that the relative expression levels and patterns of the MtSPL genes were varied in these organs (Fig. 5). For example, the non-MtmiR156-targeted MtSPLs (MtSPL1, MtSPL7, MtSPL12, MtSPL14 and MtSPL16) were expressed in all of the organs tested, while MtSPL8 was highly expressed in flower and pod (Fig. 5a). The MtmiR156-targeted MtSPL genes also showed differential expression profiles (Fig. 5b). Most MtmiR156-targeted MtSPLs, such as MtSPL4, MtSPL5A, MtSPL5B, MtSPL6B, MtSPL6C, MtSPL11, MtSPL13B and MtSPL15A, were highly detected in pod, implying their specific roles in pod development. MtSPL2, MtSPL5B, MtSPL10A and MtSPL13A were expressed at high levels in seed, indicating the possible involvement in seed development.

### MtmiR156-targeted MtSPLs play important roles in pod and seed development

To investigate the possible roles of the MtmiR156-targeted MtSPLs in growth and development of *M. truncatula*, the genomic sequence of MtmiR156B was cloned and introduced into wild type plants under the regulation of the cauliflower mosaic virus 35S promoter (Fig. 6a). Seven positive transgenic lines were obtained based on the PCR results (Additional file 4). The MtmiR156B was highly expressed in two transgenic

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### Table 1 The SPL gene family in *M. truncatula*

| Name  | Gene ID      | CDS (bp) | Introns | Length (aa) | pI  | Location   | miR156 target |
|-------|--------------|----------|---------|-------------|-----|------------|---------------|
| MtSPL1| Medtr1g086250| 3012     | 9       | 1003        | 5.99| chr1:38604282..38611936+ | NO            |
| MtSPL2| Medtr3g085180| 1305     | 3       | 434         | 8.33| chr3:38492623..38497699+ | YES           |
| MtSPL3| Medtr4g088555| 435      | 1       | 144         | 6.97| chr4:35174504..35179012- | YES           |
| MtSPL4| Medtr2g014200| 432      | 1       | 143         | 6.75| chr2:3964615..3967481+ | YES           |
| MtSPL5A| Medtr2g078770| 516      | 1       | 171         | 8.31| chr2:32971840..32974296- | YES           |
| MtSPL5B| Medtr8g463140| 543      | 0       | 180         | 8.95| chr8:22196925..22198280- | YES           |
| MtSPL6A| Medtr5g046670| 1503     | 4       | 500         | 7.43| chr5:20459089..20465349- | YES           |
| MtSPL6B| Medtr2g461920| 1620     | 2       | 539         | 6.78| chr2:25606881..25611792+ | YES           |
| MtSPL6C| Medtr4g109770| 1413     | 2       | 470         | 6.44| chr4:45646472..45650584- | YES           |
| MtSPL7| Medtr2g020620| 2238     | 10      | 745         | 6.63| chr2:6886415..6893572+ | NO            |
| MtSPL8| Medtr8g005960| 984      | 0       | 327         | 8.48| chr8:419202..421415- | YES           |
| MtSPL9| Medtr7g444860| 945      | 0       | 314         | 8.58| chr7:15012335..15016587+ | YES           |
| MtSPL10A| Medtr8g080680| 1197     | 0       | 398         | 7.55| chr8:34725302..34727462- | YES           |
| MtSPL10B| Medtr8g080670| 1239     | 0       | 412         | 7.93| chr8:34719933..34722421- | YES           |
| MtSPL11| Medtr8g080690| 1131     | 0       | 376         | 8.65| chr8:34729479..34731697- | YES           |
| MtSPL12| Medtr7g110320| 3006     | 0       | 1001        | 6.13| chr7:45210190..45220920- | NO            |
| MtSPL13A| Medtr8g096780| 1173     | 0       | 390         | 8.19| chr8:40622636..40626632+ | YES           |
| MtSPL13B| Medtr3g099080| 1131     | 2       | 376         | 7.04| chr3:45410078..45413482+ | YES           |
| MtSPL13C| Medtr7g028740| 1104     | 0       | 367         | 8.2 | chr7:9871981..9875095+ | YES           |
| MtSPL14| Medtr1g035010| 3012     | 8       | 1003        | 7.5 | chr1:12692334..12698670+ | NO            |
| MtSPL15A| Medtr7g029390| 1014     | 0       | 337         | 8.91| chr7:38693347..38697295- | YES           |
| MtSPL15B| Medtr1g053715| 1053     | 2       | 350         | 8.86| chr1:22678198..22682537- | YES           |
| MtSPL16| Medtr2g046550| 3078     | 9       | 1025        | 6.64| chr2:20453789..20462574+ | NO            |
plants (Fig. 6b). The MtmiR156B-overexpressing plants exhibited small leaves, increased lateral branches and reduced plant height, indicating that MtmiR156B-targeted MtSPLs play important roles in leaf morphogenesis, branching and stem elongation (Fig. 6c-e). Moreover, reduced function of the MtmiR156B-targeted MtSPLs also led to the defects in reproductive development. Compared with wild type, the spikes of the flowers in MtmiR156B-overexpressing plants were absent (Fig. 6f), however, the development of stamen and carpel was normal (Fig. 6g).

Most MtmiR156-targeted MtSPLs were highly expressed in pod, implying that they may play redundant roles in pod development. Compared with the long slender spines developed on the pod surface in wild type, overexpression of MtmiR156B led to the reduction in pod size and the loss of spines on pod surface (Fig. 7a-d). The seed number in each pod, seed size and weight were significantly reduced in the MtmiR156B-overexpressing plants, compared with those in wild type (Fig. 7e-g). These results demonstrate that the MtmiR156/MtSPL regulation module is critical for the pod and seed development.

To further determine which MtSPL genes are involved in pod wall and seed development, the expression levels of all the MtmiR156-targeted SPL genes were analyzed in the pod wall and seed of the wild type and MtmiR156B-overexpressing plants. qRT-PCR results demonstrated that the expression of nine MtSPLs was significantly reduced in the pod wall of the MtmiR156B-overexpressing plants.
Fig. 3 Conserved domains and motifs in MsSPL proteins. a The full length MsSPL protein sequences were used to execute the motif search on MEME tool. Domains and motifs were represented by the boxes with different numbers and colors.

b Alignment of the conserved SBP domain in MsSPL proteins. Multiple SBP domain sequences alignment was performed using Jalview software. Two Zn-finger structures (Zn-1, Cys3His and Zn-2, Cys2HisCys) and one NLS are indicated.

c Sequence logo of the conserved SBP domain of MsSPLs. Sequence logo was obtained from Weblogo online software. The overall height of each stack indicates the sequence conservation at each position, while the height of the letters within each stack indicates the relative frequency of the corresponding amino acid.
overexpressing plants (Fig. 8a). The expression of MtSPL5A, MtSPL5B, MtSPL15A, and MtSPL15B showed over two-fold decrease in the transgenic plants, indicating that those MtSPL genes play crucial roles in spiky pod wall development (Fig. 8a). While, eleven MtSPLs, especially MtSPL5A, MtSPL6B, MtSPL6C, MtSPL10A and MtSPL13B, were downregulated in seed of the MtmiR156B-overexpressing plants, indicating the functional redundancy among those MtSPL genes during seed development (Fig. 8b).

**Discussion**

Transcription factors play important roles during the processes of plant growth and development. The SPL genes encode a family of plant-specific transcription factors that contain the conserved SBP domain [2, 43]. In this study, through a genome-wide identification, we obtained 23 MtSPL genes from *M. truncatula* genome. Phylogenetic analysis showed that the MtSPL genes were more closely related to *Arabidopsis* than rice SPL genes, indicating that eudicots SPL genes may diverge from a common ancestor [44]. However, the number of MtSPL genes in *M. truncatula* is greater than that in *Arabidopsis* and rice, which contain 16 and 19 SPLs, respectively. Sequence homologous analysis suggested that faster gene duplication rates or species-specific gene duplication manners might play important roles in SPL gene expansion in *M. truncatula*. Gene structure and motif composition analyses showed that MtSPL genes within the same group shared similar motifs and exon/intron organization, suggesting that the functional evolution of SPL genes may be tightly correlated with the diversification of gene structure and conservation of motifs [6, 45].

Some SPL genes were posttranscriptionally regulated by miR156 and involved in multiple developmental processes [4, 9, 11, 32, 36, 39–41, 45–49]. Based on the miRNA database information, ten MtmiR156 genes were found in *M. truncatula* genome. The mature sequences of the miR156 genes between *A. thaliana* and *M. truncatula* are similar, indicating the functional conservation of the miR156 in different plant species [29]. It has been reported that 11 of 16 SPLs in *Arabidopsis* and 11 of 19 SPLs in rice contained the putative miR156 binding sites [2, 7, 11, 12, 17, 36, 46]. By searching the MtmiR156 complementary sequence in MtSPLs mRNAs, we found that 17 MtSPLs out of 23 contained the putative MtmiR156 binding sites, suggesting the conservation of miR156-mediated posttranscriptional regulation in plants.

In *Arabidopsis*, loss-of-function of multiple SPL genes or overexpression of AtmiR156A resulted in the decreased rosette leaf area [46]. In tomato and petunia, overexpressing AtMIR156B or PhSBP1code-silenced plants produced the higher number of small leaves and branches [47, 50]. Furthermore, overexpression of OsmiR156B in switchgrass and loss-of-function in IPA1 in rice and down-regulation of MsSPL13 in alfalfa also led to the increased number of branches [39, 40, 51, 52]. In accordance with these reports, the similar phenotypes, including more branches and small leaves, were also displayed in MtmiR156B-overexpressing plants. These observations indicate that MtmiR156/MtSPL regulation module plays the conserved roles in vegetative development.

The seeds of legume are developed within an ovary-derived pod, whose walls provide numerous functions,
Fig. 5 Expression patterns of MtSPL genes in six different organs. a Expression patterns of non-MtmiR156-targeted MtSPL genes. b Expression patterns of MtmiR156-targeted MtSPL genes
such as the protection of the seeds and the production of photosynthates [53–55]. Seed dispersal is the transport of pods/seeds away from the parent plant and has been implicated in rapid plant migration and the spread of invasive [56–59]. The pods in many plant species develop the spines or stiff hairs, such as *Trifolium angustifolium*, *M. polymorpha* and *M. truncatula* [60, 61]. Such structures are very important for seed migration, because seed pods can adhere to animals by means of spines or hairs, and be transported on the outside [60, 62]. So, the proper development of spines/hairs on pod is critical for seed dispersal, along with species diversity or ecological invasion. The pod and pod wall of *M. truncatula* are helical and thick with spines [63–65]. In this study, we found that most *MtmiR156*-targeted *MtSPL* genes were highly expressed in pod and seed. Moreover, seed size and number in *MtmiR156B*-overexpressing plants were decreased. Importantly, the development of spines on pod was also defective, due to the downregulation of several *MtmiR156*-targeted *MtSPLs*. These observations indicate that the *MtmiR156/MtSPL* regulation module may contribute to the genetic variability through the regulation of pod morphology.

In *Arabidopsis*, ectopic expression of the *TaSPL16* results in early flowering and increase of organ size and yield [66]. This finding implies that *SPL* is possible for the improvement of seed production in legume species. In this study, *35S: MtmiR156B* plants show defective spines on pod in *M. truncatula*. However, the number of lateral branches is increased dramatically. The biomass
is a critical trait in evaluation of forage grass. Therefore, downregulation of targeted SPL genes by overexpression of miR156 in legume forage, such as alfalfa, may provide a helpful tool to improve forage production.

**Conclusion**

In this study, we performed genome-wide analyses and identified SPL genes in *M. truncatula*. The genetic redundancy of *MtSPL* genes hinders the discoveries of their potential functions. However, the phenotypes of *MtmiR156B*-overexpressing plants reveal that *MtmiR156/MtSPL* modules are not only involved in the development of leaves and branches, but also indirectly contribute to seed dispersal by controlling the formation of spine on pods. Characterization of the loss-of-function *MtSPL* mutants may help to provide insight into the roles of *MtmiR156/MtSPL* module in the development of spine of pod, and shed light on the new function of SPL family among plant species.

**Methods**

**Plant materials and growth conditions**

*Medicago truncatula* R108 ecotype was used as the wild type, which is obtained from the Noble Research Institution, USA. The seeds of 35S:*MtmiR156B* and wild type were scarified with sandpaper and treated at 4 °C for 5 days. The germinated seeds were transferred to nursery seedling plate (4 × 4 × 5 cm Length, Width, Height) for 2 weeks. Then, the seedlings were transferred to Luing soil mix (soil: vermiculite = 3:1) and grown in the greenhouse at 22 °C (day) / 22 °C (night) with 16 h (day) / 8 h (night) photoperiod, and relative humidity at 70 to 80%.
Identification and phylogenetic analysis of SPL genes in *M. truncatula*

To identify the SPL genes in *M. truncatula* genome, firstly, we used 16 AtSPL and 19 OsSPL protein sequences to execute BLASTP search the Medicago truncatula Genome Database (http://www.medicagogenome.org/). The AtSPL protein sequences were obtained from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/). The OsSPL protein sequences were obtained from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). Totally, 24 putative MtSPL genes were identified in *M. truncatula* genome using blast with a cut-off E-value > 1e−3. Secondly, we searched the Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/) and confirmed the blast search result. Thirdly, the 24 MtSPL protein sequences were further analyzed on the NCBI Conserved Domain Search website (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and found that Medtr8g066240 lost the conserved SBP domain. Therefore, Medtr8g066240 was excluded from the putative MtSPL genes, and total 23 MtSPL genes were used for study.

Multiple protein sequence alignment was performed using Jalview software (http://www.jalview.org/). The phylogenetic tree for Arabidopsis, rice, and Medicago SPL gene family members was constructed using MEGA 7.0.

Chromosome location and gene structure of MtSPL genes

The informations of MtSPL genes on chromosome location, including chromosome length, gene direction, and gene start and stop position, were obtained from the *M. truncatula* genome database. Exon / intron structures of MtSPL genes were determined by aligning the coding sequences and their corresponding genomic sequences using the online Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) website.

The identification of conserved domain and the prediction of MtmiR156A-targeted MtSPLs prediction

The online MEME tool (http://meme-suite.org/) was used to predict both conserved domains and potential motifs in the 23 MtSPL proteins with the following parameters: maximum number of motifs to find, 20; minimum width of motif, 6; maximum width of motif, 100; minimum number of sites for each motif, 2. The mature sequences of *M. truncatula* MtmiR156A to MtmiR156J were obtained from miRBase database (http://www.
mirbase.org). The MtmiR156-targeted MtSPL genes and their binding sites were obtained by searching the gene coding and UTR regions on the psRNATarget (http://plantgrn.noble.org/psRNATarget/home) website.

RNA extraction and gene expression analysis

The samples in 60-day old wild-type plants were used for RNA extraction. For gene expression pattern analysis, the roots, leaves, flowers, pods and seeds samples were harvested from primary roots, fully expanded leaves, fully opened flowers, 20-day post-pollination pods and seeds. To analyze the relative expression levels of MtmiR156-targeted MtSPL genes in the MtmiR156B-overexpressing plants, 20-day post-pollination seeds and pod walls were collected from wild type and 35S:MtmiR156B transgenic plants. To analyze the relative expression levels of MtmiR156B in the MtmiR156B-overexpressing plants, 60-day old fully expanded leaves were collected from wild type and 35S:MtmiR156B transgenic plants.

Total RNA of these organs was extracted using the Trizol-RT Reagent (Molecular Research Center, INC) following the manufacturer’s instructions. The quality and quantity of the extracted RNA were analyzed using Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). Reverse transcription PCR was performed with 2.5 μg total RNA using Roche First Strand cDNA Synthesis Kit (Roche, USA). Then, the cDNA was diluted to 20 ng/μl with DEPC treated H2O. For quantitative real-time PCR (qRT-PCR) analysis, the primers of the 23 MtSPL genes were designed by Primer Express 3.0 software (Additional file 5). qRT-PCR was executed in triplicate for each organ on CFX Connect™ (Bio-Rad, USA) using FastStart Essential DNA Green Master Kit (Roche, USA). The MtUBI gene was selected as an internal control, and the relative expression levels of different MtSPL genes were calculated using 2^{-ΔΔCT} method [67].

Plasmid construction and plant transformation

To obtain the MtmiR156B overexpression construction, the 830 bp DNA sequence containing the MtmiR156B stem-loop structure was amplified using primer pair MtmiR156B-F/MtmiR156B-R. The MtmiR156B sequence was transferred into the pEarleyGate 100 vector by Gateway LR reaction (Invitrogen, USA) [68]. Then 35S:MtmiR156B destination construct was introduced into Agrobacterium strain EHA105. For stable transformation, leaves of wild type were transformed with EHA105 strain containing the 35S:MtmiR156B vector [69].

Additional files

- **Additional file 1:** The sequences of all genes involved in this study. (DOCX 31 kb)
- **Additional file 2:** Multiple amino acid sequences alignment of MtSPL proteins using full-length amino acid sequences. Sequences were aligned using Jalview software. (DOCX 575 kb)
- **Additional file 3:** Secondary structure of MtmiR156 and regulation of MtSPLs by MtmiR156. RNA secondary structures of the MtmiR156A-MtmiR156A J were predicted by the online mfold Web Server. The nucleotides with light green color in stem-loop structures indicate the mature MtmiR156 sequences. Multiple MtSPL genes were regulated by MtmiR156. The deoxyribonucleotide with shaded color indicates the conserved sequences targeted by MtmiR156. (DOCX 548 kb)
- **Additional file 4:** Molecular characterization of MtmiR156B overexpression lines. PCR analysis was performed using primer pair 35S-F/MtmiR156B-R for regenerated transgenic plants together with the positive control (35S:MtmiR156B), and negative control (Wild-type). (DO CX 168 kb)
- **Additional file 5:** Primers used in this study. (DOCX 23 kb)

**Abbreviations**

AP1: APETALA1; FT: FLOWERING LOCUS T; FUL: FRUITFULL; GA: Gibberellin; LFY: LEAFY; NJ: Neighbor-Joining; NLS: Nuclear localization signal; qRT-PCR: Quantitative real-time PCR; SBP: SQUAMOSA PROMOTER BINDING PROTEIN; SOC1: SUPPRESSOR OF OVEREXPRESSION OF CO 1; SPL: SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; TFs: Transcription factors; UTR: Untranslated region; Zn1: Zinc finger 1; Zn2: Zinc finger 2

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**Authors’ contributions**

HW, CZ, and LH conceived the study and designed the experiments. HW, ZL, YX, LK, JS, YL, CF, XW, Z-YW, CZ, and LH performed the experiments and analyzed the data. HW, CZ, and LH wrote the article. All authors have read and approved the final version of the manuscript.

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**Availability of data and materials**

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**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
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