Genome-wide identification, phylogeny and expression analysis of the SPL gene family and its important role in salt stress in *Medicago sativa* L.

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**Abstract**

**Background:** SQUAMOSA promoter-binding protein-like (SPL) transcription factors are widely present in plants and are involved in signal transduction, the stress response and development. The SPL gene family has been characterized in several model species, such as *A. thaliana* and *G. max*. However, there is no in-depth analysis of the SPL gene family in forage, especially alfalfa (*Medicago sativa* L.), one of the most important forage crops worldwide.

**Result:** In total, 76 putative *MsSPL* genes were identified in the alfalfa genome with an uneven distribution. Based on their identity and gene structure, these *MsSPLs* were divided into eight phylogenetic groups. Seventy-three *MsSPL* gene pairs arose from segmental duplication events, and the *MsSPLs* on the four subgenomes of individual chromosomes displayed high collinearity with the corresponding *M. truncatula* genome. The prediction of the cis-elements in the promoter regions of the *MsSPLs* detected two copies of ABA (abscisic acid)-responsive elements (ABREs) on average, implying their potential involvement in alfalfa adaptation to adverse environments. The transcriptome sequencing of *MsSPLs* in roots and leaves revealed that 54 *MsSPLs* were expressed in both tissues. Upon salt treatment, three *MsSPLs* (*MsSPL17*, *MsSPL23* and *MsSPL36*) were significantly regulated, and the transcription level of *MsSPL36* in leaves was repressed to 46.6% of the control level.

**Conclusion:** In this study, based on sequence homology, we identified 76 SPL genes in the alfalfa. The SPLs with high identity shared similar gene structures and motifs. In total, 71.1% (54 of 76) of the *MsSPLs* were expressed in both roots and leaves, and the majority (74.1%) preferred underground tissues to aerial tissues. *MsSPL36* in leaves was significantly repressed under salt stress. These findings provide comprehensive information regarding the SPB-box gene family for improve alfalfa tolerance to high salinity.

**Keywords:** *Medicago sativa*, SPL gene family, Salt stress, Legume

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**Background**

Alfalfa (*Medicago sativa* L.) is the most widely grown forage legume crop worldwide [1]. Alfalfa has been widely used in animal feed because of its high biomass yield, good palatability, and strong adaptability. Currently, almost half of the world’s irrigated land and approximately 20.0% of cultivated land are affected by salinity [2].
However, many plant growth areas, such as China, are on saline-alkali soil, which seriously affects the growth and development of alfalfa. Transcription factors (TFs) play extremely important roles in controlling the growth and development of plants. TFs greatly affect plant development, secondary metabolism, and abiotic stress tolerance by binding cis-acting elements in the promoter regions of target genes [3, 4]. Although the SPL gene can regulate inflorescence formation and fruit development and enhance stress resistance, knowledge regarding the functions of these SPL proteins and named MsSPL genes in alfalfa is limited. Therefore, it is important to explore the possible functions of MsSPL genes to understand the regulation of growth, development, and abiotic stress in alfalfa. SPLs constitute a plant-specific family and are widely distributed in green plants. SPL is a general term for a type of transcription factor, and its structure is similar to the SBP box [5]. The SQUAMOSA promoter-binding protein (SBP) domain is highly conserved, with a length of approximately 76 amino acids [6]. The SBP domain contains two tandem zinc fingers (Cys-Cys-His-Cys and Cys-Cys-Cys-His) and one nuclear localization signal (NLS) motif [6, 7]. The members of this transcription factor family share a highly conserved DNA binding domain, the SBP. The SBP box was first isolated from the A. majus cDNA library, and because of its ability to recognize and bind the SQUAMOSA (SQUA) promoter, it was named SQUA [8]. An increasing amount of evidence suggests that TFs play an important role in the regulatory network of plant growth and development [9]. Various gene families, such as TEOSINTE BRANCHED 1, CYCLOIDEA, PCF1 (TCP) [10], and IQ67-Domain (IQD) [11], have been found in eukaryotes.

With the publication of many plant genomes, SPL gene family members have been identified and characterized in A. thaliana [12], O. sativa [13], and G. max [14]. According to the sequence homology and hylogenetic analyses of SPL genes, this family is usually divided into 6–9 subgroups. In P. trichocarpa, there are 28 PtSPL genes, and these genes are divided into 8 subgroups [15]. In A. thaliana, a total of 16 members have been identified as SPL proteins and named AtSPL1 to AtSPL16. They were divided into eight groups according to the amino acid sequence [16]. The functions of these SPL genes in A. thaliana have also been identified, and these genes play an important role in leaf, flower, and shoot development [17, 18].

MicroRNAs are small RNAs of approximately 16–26 nucleotides in length that regulate gene expression at the posttranscriptional level in a sequence-specific manner [19]. As a key regulatory factor in most biological processes, the miR156/SPL module participates in the transformation from the vegetative stage to the reproductive stage, fruit ripening, and yield improvement [20]. However, the miR156/SPL module also responds to abiotic stresses in many plant species [21, 22]. When A. thaliana is under heat stress, the SPL gene is posttranscriptionally downregulated by miR156, which is essential for adapting to repeated heat stress [23]. The overexpression of the SPL gene in B. platyphylla is also very obvious and can improve the scavenging of reactive oxygen species to enhance tolerance to salt and drought stress [24]. Some related studies have also been carried out in alfalfa. In alfalfa, the transcript abundance of the miR156-targeted SPL8 and SPL13 genes was related to salt and drought tolerance [25, 26]. Studies have shown that drought stress increases the expression of miR156 by increasing leaf gas exchange and abscisic acid (ABA) while reducing water loss, thereby increasing the resistance of alfalfa to this stress [25]. To date, SPL genes have been isolated and identified in many plants, such as A. thaliana and O. sativa, but few studies investigated SPL genes in alfalfa, and the function of these proteins is unclear.

Despite the support of physiological, biochemical, and molecular data, the biological function of SPL transcription factor genes is still unclear. In this study, the gene structure, motif composition, chromosome location, and gene duplication of 76 recently completed alfalfa genome sequences were analyzed, and the evolutionary relationship of M. sativa was compared with those of A. thaliana, M. truncatula, and G. max. A quantitative real-time PCR (qRT–PCR) analysis was performed to examine the gene expression patterns in different tissues and their responses to salt stress. Through an overall expression analysis in alfalfa, the role of the members of the specific SPL gene family in the different biological processes of alfalfa was determined. This study not only provided valuable information for screening SPL genes important for the growth and development of alfalfa but also provided a method for mining SPL gene families in other plants.

**Results**

**Sequence identification of the SPL genes in M. sativa**

As a plant-specific transcription factor, SQUAMOSA promoter-binding protein-like (SPL) genes are involved in the plant response to adverse environmental conditions. To obtain SPL genes in the forage legume alfalfa, the SBP domain, a canonical feature of SPL, was screened from a Chinese landrace (Medicago sativa L. Xinjiang-DaYe) genome using HMM and BLASTP [27]. The hits were then confirmed by Pfam (http://pfam.xfam.org/) and the Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/cdd). Ultimately, 76 genes were designated MsSPL and named MsSPL1—MsSPL76 (Table S1).
The prediction of the subcellular location showed that MsSPLs were localized in the nucleus and that 16 (21.1%) SPLs were also distributed in the cytoplasm, suggesting that the putative MsSPL transcription factors function mainly in the nucleus. The predicted MsSPLs vary in terms of the protein length and isoelectric point (pI), ranging from 100 (MsSPL42) to 1,026 (MsSPL76) amino acids (aa) and 5.24 (MsSPL42) to 9.65 (MsSPL49), respectively (Table S1).

Sequence alignment and phylogenetic analysis of MsSPLs
The multiple alignment showed that in addition to the conserved SBP domain (approximately 78 amino acid residues in length), most MsSPLs shared a highly conserved nuclear localization signal (NLS) and two zinc finger-like structures, namely, Zn-1 and Zn-2 (Fig. S1). In Zn-1, the CCCH is at positions 214, 219, 236, and 239, while in Zn-2, the CCHC is at positions 255, 258, 262, and 274 amino acids. Consistent with a previous report [28], the predicted NLS possesses conserved residues, such as lysine (K) and arginine (R), at positions 271–287.

To analyze the phylogenetic relationship, a neighboring tree of SPLs in M. sativa (76), M. truncatula (23) and A. thaliana (16) was constructed using MEGA (Fig. 1). The MsSPLs similar to their counterparts in M. truncatula and A. thaliana were clustered into eight groups (Groups I ~ VIII) with varying numbers of members. The largest group (Group II) contained 18 members, accounting for 23.7% of the MsSPLs, whereas the smallest groups (Group I and Group III) possessed four members. Relative to A. thaliana SPLs, the homologs in M. sativa and M. truncatula are closer. Interestingly, compared with MsSPL, multiple (2~6) MsSPL counterparts were grouped into the same cluster, indicating the expansion of MsSPL, probably due to genome duplication of the tetraploid forage.

Gene structure and motif composition of MsSPLs
A phylogenetic tree was constructed based on the predicted full-length MsSPL protein sequences and these proteins were also roughly divided into eight subgroups (Fig. 2A). An analysis of the gene structure of the SPL family in alfalfa revealed that the number of exons varied from 1–11. It seems that the MsSPL members in one group share a similar number of exons, with differences in intron sizes (Fig. 2B). Approximately half (52.6%) of the MsSPLs consist of 3–4 exons, of which 34.2% (26 out of 76) with 3 exons and 18.4% (14 out of 76) with 4 exons (Fig. 2B). The members of Groups I and II, excluding MsSPL30, contain relatively more exons (10–12) than the average number of exons in MsSPLs. The MsSPLs in Group VI comprise no more than three (1–3) exons. In addition to the conserved SBP domain at the N-terminus of MsSPLs, there is a conserved ankyrin (ANK) domain at the C-terminus of some Group II members (70.6% = 12/17), which is involved in protein–protein interactions in G. max [14]. The findings were consistent with a previous report in S. miltiorrhiza [29]. Therefore, MsSPLs from the same group share a similar gene structure, and the length of the exons is conserved correspondingly.

We examined the motif composition of the MsSPLs using MEME. In total, 20 motifs (motifs 1~20) were identified in the 76 putative MsSPLs ranging from 2 (MsSPL42) to 18 (MsSPL1, MsSPL2, MsSPL24, MsSPL38, MsSPL39 and MsSPL61) for individual proteins (Fig. 2C). On average, Groups II and VI contained the most and the fewest motifs, respectively. Among them, motifs 1, 2 and 8 were the top three motifs present in 97.4%, 89.5% and 96.1% of the MsSPLs, respectively, suggesting that these motifs are the most important components of MsSPL proteins. Similar to the gene structure, the MsSPL members from the same group, particularly Group I, shared similar motif compositions, including the motif type and number. Some motifs were present in certain groups. For example, motifs 3, 5 and 7 were present exclusively in the SPL members in Groups I and II (Fig. 2C). Among the Group II members, 13 contain a conserved motif, and 6 contain 18 of the 20 motifs, except for Motif 13 and Motif 20. Therefore, MsSPL members of the same group share a similar gene structure and motif composition, while SPLs from different groups are likely to have specific structures, implying that the functional conservation and diversity of the MsSPLs evolved during evolution.

Analysis of the distribution, gene duplication and syntenic of the MsSPLs
The predicted MsSPLs were mapped based on the genome database of M. sativa (XinJiangDaYe) [27]. Seventy-four of the 76 MsSPLs were unevenly distributed on 26 chromosomes, and the remaining two (MsSPL38 and MsSPL49) have not yet been assembled (Fig. 3). On average, there are approximately 2.8 SPL genes on each chromosome (Chr). Among them, no SPL was identified on Chr4.3, Chr5.2 or Chr6, while Chr4.2 possessed seven SPLs, probably due to gene duplication events.

The analysis of the duplication event in the MsSPL family showed that there were 73 pairs of segmental duplicates but no tandem duplications (Fig. 4). MsSPL homologs (such as MsSPL8 and MsSPL68) from different chromosomes share higher identity. This result suggests that segment duplication may contribute to MsSPL expansion.
Evolutionary analysis of the MsSPLs and orthologs from three model species

To explore the evolutionary origin of MsSPLs, we performed a syntenic analysis of SPLs from three model species *A. thaliana*, *M. truncatula* and *G. max*. In total, 16, 23, and 41 SPLs have been identified in *A. thaliana* (125 Mb) [30], *M. truncatula* (500 Mb) [31] and *G. max* (1.025 Gb) [32]. In total, 57 MsSPLs displayed syntenic relationships with *M. truncatula*, 56 MsSPLs displayed syntenic relationships with *G. max* and 33 MsSPLs displayed syntenic relationships with *A. thaliana* (Fig. 5). Among these MsSPLs, 131 pairs of orthologous genes were found with *G. max*, 79 pairs of orthologous genes were found with *M. truncatula*, and 40 pairs of orthologous genes were found with *A. thaliana*. Consistent with the MsSPL distribution on the chromosome,
chromosomes 1, 2 and 7 of alfalfa accounted for the top three SPL homologous gene pairs with the three model plants (Table S2). Syntenic blocks between the two Medicago species showed that the four subgenomes of alfalfa had high collinearity with the corresponding M. truncatula genome. Approximately 63.3% (50 of 79) of the MsSPLs paired with MtSPLs on the same chromosome, suggesting the relatively conserved distribution of SPLs between the two legumes. The pairing of 36.7% of the SPLs across chromosomes implies the occurrence of interchromosomal rearrangements, particularly between chromosome 4 and chromosome 8, as reported by Li et al. [33].

Prediction of the cis-acting elements in the promoter of MsSPLs
The cis-elements 2 kb upstream of the start codon (ATG) of MsSPLs were analyzed using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). According to the classification of wheat [34], 25 cis-elements were found for MsSPLs, with 11 (44.0%) related to hormone and stress responses, nine (36.0%) related to light responsiveness, and five (20.0%) related to plant growth and development (Fig. 6 and Table S3). Regarding the cis-acting elements predicted to be associated with hormone and stress responses, abscisic acid (ABA)-responsive elements (ABREs) and AU-rich elements (AREs) were predominant in MsSPLs, accounting for 89.4% and 86.8%, respectively. Both cis-elements are present in individual MsSPLs with an average of two copies. The presence of ABRE, the major cis-element in ABA-responsive genes, implies the potential involvement of MsSPLs in alfalfa resistance to osmotic stresses, including drought and salinity, as previously reported in O. sativa and A. thaliana [23, 35].

Comparison of the expression patterns of MsSPLs between roots and leaves
To compare the expression pattern of the MsSPLs between roots and leaves, 14-day-old alfalfa seedlings were used for RNA sequencing. Based on fragments per kilobase of transcript per million fragments mapped (FPKM), 68 MsSPLs were expressed in either tissue tested. Among them, 54 MsSPLs were detected simultaneously in both tissues, with 74.1% (40 of 54) showing higher expression in roots than in leaves (Fig. 7A). Nine MsSPLs were detected in roots rather than leaves, with four (SPL15, SPL55, SPL60 and SPL62) from Group V and two (SPL5 and SPL68) from Group VIII (Fig. 7B). In contrast, five MsSPLs exhibited the opposite expression pattern, showing expression in leaves but not roots (Fig. 7C). Our results show that approximately 72.1% (49 of 68) of MsSPLs had higher expression in roots relative to aerial
tissues, indicating that these MsSPLs are preferentially expressed in underground tissues at this stage.

To investigate the response of alfalfa to salt stress at the transcription level, we measured the expression of MsSPLs in roots and leaves from seedlings treated with NaCl (300 mM) for 2 weeks. The data were arbitrarily filtered by the absolute value of Log2(FoldChange) ≥ 1 and Padj < 0.05 (Fig. S2). Three MsSPLs (MsSPL17, MsSPL23 and MsSPL36) were differentially expressed under the salt treatment, with the former MsSPL upregulated in the roots by 17.3% and the latter two downregulated in the leaves by 48.0% and 46.6%, respectively. Although both MsSPL17 and MsSPL23 showed leaf-preference under normal conditions, upon salt stress, the expression of the two genes contrasted each other, suggesting that these genes likely play opposite roles in the alfalfa response to long-term high salinity. The results were confirmed by qRT–PCR verification (Fig. 8 and Table S4).

**Discussion**

**SQUAMOSA-promoter Binding** Protein Like (SPLs) encode a family of plant-specific transcription factors containing a conserved SQUAMOSA-promoter Binding Protein (SBP) domain and are involved in the regulation of the flowering time [36], plant development [37] and the stress response [38]. The SPL family has been mostly identified in model plants, such as *A. thaliana* [39], *O. sativa* [13] and *M. truncatula* [40]. The release of the alfalfa genome sequence in recent years [27, 41] has facilitated the identification of MsSPLs from the most valuable forage worldwide. The findings of this study could benefit alfalfa production and breeding, especially the generation of varieties with improved tolerance to environmental stresses, such as alkalinity and salinity.

*M. sativa* SPLs (MsSPLs) are canonical and highly conserved with SPLs from the model plant species. One line of evidence shows that similar to the orthologs from *M. truncatula* and *A. thaliana*, the 76 MsSPLs we identified here were phylogenetically clustered into eight groups based on their sequence identity, gene structure and motif composition. In individual groups, the number of MsSPLs was greater than that of MtSPLs or AtSPLs, with MsSPL members much closer to *M. truncatula* than *A. thaliana*. Moreover, the MsSPLs from the four subgenomes of alfalfa showed high collinearity with *M. truncatula* orthologs from the corresponding chromosome, and neither species had an SPL gene on chromosome 6 [40]. Multiple MsSPLs are probably attributed to the genome duplication of the tetraploid legume in comparison with MtSPLs. Interestingly, although there were 73 pairs of segment duplications among the MsSPLs, no tandemly duplicated MsSPL pairs were found (Fig. 4), indicating that the two legumes diverged prior to the occurrence of genome recombination for SPLs. Notably, in addition to the conserved motifs, such as Motif 1, Motif 2 or Motif 8, several unique motifs were present in certain groups of MsSPLs, suggesting a potential contribution to the specific biological functions of the dedicated MsSPL groups.

It appears that MsSPL36 is a crucial candidate for improving alfalfa tolerance to salt stress. Plant SPLs are well known as targets of microRNA156 in regulating phase change (from the vegetative to reproductive stage).
and the stress response [3, 42]. For example, miR156-mediated downregulation of three SPLs, i.e., SPL2, SPL9 and SPL11, enhanced *A. thaliana* tolerance to heat stress [23]. Thirty-one maize SPLs displayed variations in their expression behavior upon exposure to one or more stresses, such as dehydration, salinity, cold and ABA [38], and transgenic tobacco expressing ZmmiR156 exhibited improved performance against drought and salt [42]. In addition, the SPLs in *T. chinensis* and *D. glomerata* have been shown to respond to salt, drought and heat stresses via the negative control of miR156 [21, 43].

In China, most alfalfa plantations are located in regions with saline-alkali soil due to the lack of farmland [44]. An urgent and promising task for alfalfa production in the nation is to breed varieties with tolerance to high salinity [45]. Our prediction of the cis-elements present in the
putative promoter region of the MsSPLs revealed that 89.4% of MsSPLs possessed ABA-responsive elements (ABREs) [46], suggesting a possible contribution to the alfalfa abiotic stress response. Similar results have been documented in wheat and Betula luminifera [34, 47]. The transcriptomic analysis revealed that although 10.5% of the MsSPLs were undetected in our experiments, 89.5% of the detected MsSPLs were expressed in either the roots or leaves of 2-week-old plants, with 74.1% preferring roots to leaves. Under long-term high salinity (0.3 M NaCl) pressure, three MsSPLs were differentially expressed with MsSPL17 up-regulated in roots, while MsSPL23 and MsSPL36 down-regulated in leaves of two-week-old plants. Our verification by quantitative RT–PCR showed that MsSPL36 in leaves was repressed by the salt treatment to 46.6% of the control level. Experimental evidence is needed to support the notion that MsSPL36 is involved in the alfalfa response to environmental stresses, especially salt. The generation of transgenic alfalfa with MsSPL36 knockout via miR156 or overexpression could facilitate the elucidation of its functions in forage under adverse environmental conditions. A comprehensive analysis with multiple time points of salt treatment could be helpful for a dynamic illustration of MsSPL expression profiles in response to stress.

Conclusion
The phylogeny and diversification of SPL genes in alfalfa were investigated at different levels, including gene structures, evolutionary relationships, synteny analysis and expression patterns. All 76 MsSPL genes were divided into 8 groups, and genes in the same group shared similar evolutionary features and expression patterns, implying potentially similar functions for MsSPL genes. SPLs with a high identity shared similar gene structures and motifs. In total, 71.1% of the MsSPLs were expressed in both roots and leaves, and the majority (74.1%) preferred underground tissues to aerial tissues. The expression of MsSPL36 in leaves was significantly repressed by salt stress. Our findings provide comprehensive information regarding the SPB-box gene family in alfalfa and have a certain value for alfalfa to improve salt tolerance.

Methods
Plant materials and growth conditions
Alfalfa seeds (Cultivar Zhongmu No. 1) from the Institute of Animal Science of the Chinese Academy of Agricultural Sciences were germinated in a petri dish, treated at 4 °C for 3 days and then grown in a greenhouse at 24 °C (day)/20 °C (night) under a 16 h light/8 h dark photoperiod at a relative humidity of 70 to 80% for 4 days. The germinated seedlings were transferred to flowerpots placed in the greenhouse and developed for 7 days. The two-week-old seedlings were irrigated either with 20 ml 300 mM NaCl solution every two days or water as a control group. After 14 days of treatment, the roots and leaves of the alfalfa seedlings were sampled, immediately placed in liquid nitrogen, and stored at -80 °C until further use.

Identification of SPL genes in alfalfa
The alfalfa genome was downloaded from the alfalfa Genome Project (https://figshare.com/projects/whole_genome_sequencing_and[assembly_of_Medicago_
The A. thaliana protein sequences were obtained from A. thaliana Information Resource (TAIR) (https://www.arabidopsis.org/), and the M. truncatula genome was searched on a website (http://www.medicagogenome.org/). The largest number of SPL genes was screened from the alfalfa genome by two BLASTp methods, and the hidden Markov model (HMM) profiles corresponding to the SBP domain (PF03110) were downloaded from the Pfam protein family database (https://pfam.xfam.org/). In total, 76 MsSPL genes were identified in the M. sativa genome using BLAST with a cutoff E-value > 1e−9. We collected the amino acid sequence of A. thaliana SPL proteins from the TAIR library, which ranges from 131 to 1035 aa. Similar SPL genes from the alfalfa genome were identified by using the SPL gene sequence of A. thaliana as a target. Subsequently, we analyzed the conserved domain of the MsSPL genes and removed the gene that did not contain the SBP conserved domain. Finally, 76 genes containing the SBP domain were screened from the alfalfa genome. The ExPASy website (https://web.expasy.org/compute_pi/) was used to analyze the MsSPL gene sequences to obtain the theoretical isoelectric points (pIs) and molecular weights (MWs).
Fig. 7  FPKM of MsSPLs in roots and leaves using RNA sequencing. A  FPKM of MsSPLs expressed in both roots and leaves. The R/L ratio represents the ratio of FPKM in roots to that in leaves. B  FPKM of the MsSPLs detected in roots but not leaves. C  FPKM of the MsSPLs detected in leaves rather than roots. The color represents the FPKM normalized value. The blue and orange colors represent higher and lower expression, respectively.

Fig. 8  Expression levels analyses of three MsSPL genes under salt treatment by qRT-PCR. A Expression levels of three MsSPL genes in root. B Expression levels of three MsSPL genes in leaf. "CK" represents normal growth condition, "Salt" represents salt treatment. The levels in root and leaf of the CK were arbitrarily set to 1. Error bars represent the standard deviations of three technical replicates.
Phylogenetic analysis and intron–exon structure determination
The SPL protein sequences for the phylogenetic tree were obtained from the UniProt database (https://www.UniProt.org). The multiple amino acid sequences of identified MsSPL genes were aligned using Clustalx2.0 software with the default parameters. Phylogenetic trees comparing M. sativa, A. thaliana and M. truncatula were constructed with the NJ method, and the specific parameters were Poisson model and 1000 bootstrap replications by using the MEGA software. The SPL protein sequences from M. sativa, A. thaliana and M. truncatula were also aligned using the Clustalx2.0 program before the phylogenetic tree was constructed. Then, the MsSPL gene structure was predicted by an online gene structure editor (http://gsds.cbi.pku.edu.cn/) website to align the coding and genome sequences. The determination of the conserved motifs in the MsSPL proteins was conducted by the MEME online program (http://meme.nbcr.net/meme/intro.html), and the parameters were set to the optimum mode width of 6 to 200 and the maximum number of motifs of 20.

Chromosome location, gene duplication and synteny analysis
Information concerning the chromosomal location of MsSPL genes, including the chromosome length, gene direction, and gene start and stop positions, was obtained from the alfalfa genome database. MCScanX software was used to analyze the MsSPL replication events and detect collinear regions between MsSPLs and collinear blocks of MsSPL genes with A. thaliana, M. truncatula, and G. max. All function and chromosomal location information was obtained by TBtools software [48].

Cis-element analysis
The upstream 2 kb sequence was extracted as the promoter region for the prediction of cis-acting elements. The homeopathy components of the promoter sequence were predicted by the online tool PlantCARE, and the predicted results were drawn by GSDS online software.

Gene expression pattern of MsSPL gene families with RNA-seq data
The Illumina HiSeq 2500 platform was used to sequence the cDNA library based on synthetic sequencing technology, and a large amount of high-read data was obtained. Two replicates were prepared for the construction of a sequencing library per sample. We used RNA-seq data to analyze the gene expression patterns of MsSPL genes. The data were filtered and compared to the reference genome of XinjiangDaYe alfalfa. In addition, we applied FPKM (fragments per kilobase of transcript per million fragments mapped) to calculate the gene expression level according to the number of reads mapped to the reference sequence. The heatmap of the MsSPL gene expression profile was constructed by R software.

Gene pattern analyses of MsSPL genes by real-time quantitative RT-PCR
Total RNA was extracted from the roots and leaves of normally growing and salt-treated alfalfa seedlings with TRIzol reagent according to the manufacturer's instructions. Then, the cDNA library was constructed for the subsequent reactions using the Genesand Kit (UnionScript First-strand cDNA Synthesis Mix for qPCR). SYBR Premix Ex Taq II (TaKaRa) with a CFX96 real-time PCR system (Bio-Rad) was used to conduct the RT–PCR experiments. The qRT–PCR primers were designed on the NCBI website (https://blast.ncbi.nlm.nih.gov/) (Table S4). MsActin was used as the internal reference gene for data normalization. A total of four samples (CK_root, CK_leaf, Salt_root and Salt_leaf) were used in this study. Three independent biological replicates and three technical repeats were taken. Roots and leaves under control conditions were selected as the control samples for measuring gene expression under salt treatment. The data were quantified by the 2−△△CT method [49].

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12870-022-03678-7.

Additional file 1: Table S1. List of the 76 MsSPL genes identified in this study.

Additional file 2: Table S2. One-to-one orthologous relationships between Medicago sativa L. and other plants.

Additional file 3: Table S3. Cis_elements contained in the MsSPL genes promoter region.

Additional file 4: Table S4. The primer sequences of qRT-PCR.

Additional file 5: Fig. S1. Alignment of the conserved SBP domain in MsSPL proteins.

Additional file 6: Fig. S2. Expression profile MsSPL genes across different tissues.

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Authors' contributions
Fei He, Rucai Long, and Zhen Wang planned and designed the research and analyzed data. Zhen Wang, Fei He, Rucai Long wrote the manuscript. Chunxue Wei, Mingna Li, Yunxiu Zhang, Junmei Kang, Qingchuan Yang performed the experiments. Zhen Wang, Lin Chen supervised the research. Fei He, Rucai Long contributed equally. All authors read and approved the final manuscript.
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Availability of data and materials
RNA sequence data from roots and leaves after 14 days of salt treatment in a greenhouse has been submitted to The NCBI Sequence Read Archive (BioProject: PRJNA777963).

Declarations

Ethics approval and consent to participate
The cultivar Zhongmu No.1 used in the experiment is supplied by Institute of Animal Science of Chinese Academy of Agricultural Sciences. These plant materials are widely used in China. This article does not contain any studies with human participants or animals and did not involve any endangered or protected species. The collection of plant material and use comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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