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Genome-wide investigation of the PIF gene family in alfalfa (*Medicago sativa* L.) expression profiles during development and stress

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Abstract

Background Phytochrome-interacting factors (PIFs) plays an important role in plants as hubs for intracellular signaling regulation. The PIF gene family has been identified and characterized in many plants, but alfalfa (*Medicago sativa* L.), an important perennial high-quality legume forage, has not been reported on the PIF gene family.

Results In this study, we presented the identification and characterization of five *MsPIF* genes in alfalfa (*Medicago sativa* L.). Phylogenetic analysis indicated that *PIFs* from alfalfa and other four plant species could be divided into three groups supported by similar motif analysis. The collinearity analysis of the MsPIF gene family showed the presence of two gene pairs, and the collinearity analysis with *AtPIFs* showed three gene pairs, indicating that the evolutionary process of this family is relatively conservative. Analysis of *cis*-acting elements in promoter regions of *MsPIF* genes indicated that various elements were related to light, abiotic stress, and plant hormone responsiveness. Gene expression analyses demonstrated that *MsPIFs* were primarily expressed in the leaves and were induced by various abiotic stresses.

Conclusion This study conducted genome-wide identification, evolution, synteny analysis, and expression analysis of the *PIFs* in *alfalfa*. Our study lays a foundation for the study of the biological functions of the PIF gene family and provides a useful reference for improving abiotic stress resistance in *alfalfa*.

Keywords *Medicago sativa*, PIF transcription factors, Genome-wide, Abiotic stress

Introduction

At present, the *PIFs* has been identified in many plants such as *Arabidopsis thaliana* [1], tomato (*Solanum lycopersicum*) [2], apple (*Malus domestica*) [3], grape (*Vitis vinifera*) [4], carrot (*Daucus carota*) [5], peanut (*Arachis hypogea*), rice (*Oryza sativa*) [7], maize (*Zea mays*) [8], *Brachypodium distachyon* [9]. PIFs are a sub-family of basic helix-loop-helix (bHLH) transcription factors, so all PIFs contain conserved bHLH domains.The bHLH domain contains approximately 60 amino acids, including 15 DNA binding regions of amino acids, while the rest are HLH regions. The former is responsible for binding of *PIFs* with their target sequences at G-box (5'-CACGTG-3') or E-box (5'-CANNTG-3') [10, 11], thus regulating the expression of target genes.The latter participates in the homodimerization or heterodimerization [12]. The N-terminal region of PIFs contains Active Phytochrome-B (APB)-binding and/or Active Phytochrome-A (APA)-binding motifs, which are interaction

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sites for phyB and phyA [13]. Among the plants that have already been reported, most PIFs contain conserved APB domains (EL××××GQ), and some PIFs also contain APA domains that are not conserved.

Transcription factors are instrumental in monitoring environmental conditions and responses of plants under both normal and adverse conditions. Phytochrome-interacting factors (PIFs) is capable of coordinating environmental factors, such as light and temperature, with internal plant growth signals, thereby promoting plant growth and developmental processes. *PIFs* not only play an important role in light signal transduction, but also perceive environmental signals and integrate them with various hormones, circadian rhythms, and other internal signals. They have critical functions in regulating plant seed germination [14, 15], flowering [16], fruit development [17], and plant type regulation [18], among other growth and development processes. In biological stress response, PIF plays a dual role in plant defence. On the one hand, it regulates resistance genes, and on the other, it induces the production of phytohormones, including salicylic acid (SA), jasmonic acid (JA), and gibberellin (GA). These hormones help the plant to defend itself against pathogens. In *Arabidopsis*, many *PIFs* have been identified and functionally characterized. *PIF1* has been demonstrated to play a pivotal role in the inhibition of light-dependent seed germination [19], as well as in the regulation of key genes involved in chlorophyll biosynthesis, with the objective of optimising the greening process in a direct or indirect manner [20]. PIF3 has been demonstrated to promote hypocotyl elongation in response to ethylene and the biosynthesis of chlorophyll and anthocyanin accumulation under light conditions [21], negatively regulates seedling de-etiolation along with other *PIFs* [22, 23].

As a high-quality forage, alfalfa is widely used in the animal husbandry industry due to its high protein content and good palatability. In recent years, as the global population has grown and people's quality of life has improved, there has been a concomitant rise in demand for meat, egg and milk agricultural products. This, in turn, has led to a rise in demand for alfalfa. However, the competition between food crops and pasture grasses for the limited arable land resources is becoming increasingly evident. Therefore, the breeding of alfalfa varieties with high resistance that can be cultivated on marginal lands is of great significance for the sustainable development of alfalfa productivity and the utilisation of marginal lands [24]. Although the PIF gene family has been identified in many plants, it has not been reported in alfalfa. In light of the potential involvement of *PIFs* in alfalfa stress tolerance and the dearth of research on *MsPIFs*, the present study was undertaken to identify and examine the PIF gene family using alfalfa as the test material. In this study, we identified the presence of five *PIF* genes in alfalfa, named them by chromosomal location, and analysed their physicochemical properties, gene structure, motif composition, evolutionary relationships, *cis*-acting elements, as well as their specific expression in different tissues and expression patterns under different abiotic stresses. The results provide a genetic resource and theoretical basis for the molecular breeding of alfalfa resistance. Furthermore, they are of great significance for overcoming the constraints of unfavourable natural conditions on alfalfa cultivation, expanding the scope of alfalfa cultivation and improving productivity.

Results

Identification and chromosomal location analysis of the PIF gene family in alfalfa

A total of five *PIF* genes were identified in alfalfa through a blast search. and named *MsPIF1* to *MsPIF5* based on their positional arrangement on chromosomes. The distribution of *MsPIFs* was found to be uneven across chromosomes 1 to 8 of alfalfa, with *MsPIF5* located on chromosome 1 and *MsPIF1-4* located on chromosome 7(Fig. 1).

As shown in Table 1, The amino acid sizes of the five genes ranged from 239 to 680 aa, with corresponding molecular weights (MW) ranging from 26.48 to 74.7 kDa and isoelectric points (pI) ranging from 5.33 to 8.76. All of the *MsPIFs* analysed were located in the nucleus.

Multiple sequence alignment and phylogenetic analysis of the *MsPIF* genes

Comparison of PIFs in alfalfa and *Arabidopsis thaliana* showed that each *MsPIFs* has a bHLH domain, this sequence is approximately 60 amino acids in length, which is consistent from the length obtained in other studies. The glutamic acid (E) and arginine (R) residues responsible for binding downstream G-box and E-box in the bHLH domain are conserved in MsPIFs. All genes except MsPIF1 have an APB domain, but the APB domain in MsPIF5 is incomplete. Only MsPIF5 contains APA domain, this is consistent with AtPIF1 and AtPIF3 (Fig. 2).

To investigate the evolutionary relationships of the PIF gene family, a phylogenetic tree of the PIF gene family was constructed in this study based on *PIFs* identified from four other plant species (*Arabidopsis*, rice, maize, and peanut). Phylogenetic analysis showed that these genes could be categorized into three groups, PIF1/4/5, PIF2/3/6, and PIF7/8, and the five *MsPIFs* were dispersed in each of the three groups (Fig. 3). *MsPIF5* belonged to PIF2/3/6; *MsPIF2* and *MsPIF3* were in PIF7/8; *MsPIF1* and *MsPIF4* were assigned into PIF1/4/5. Moreover, the homology between the PIF gene family in alfalfa and

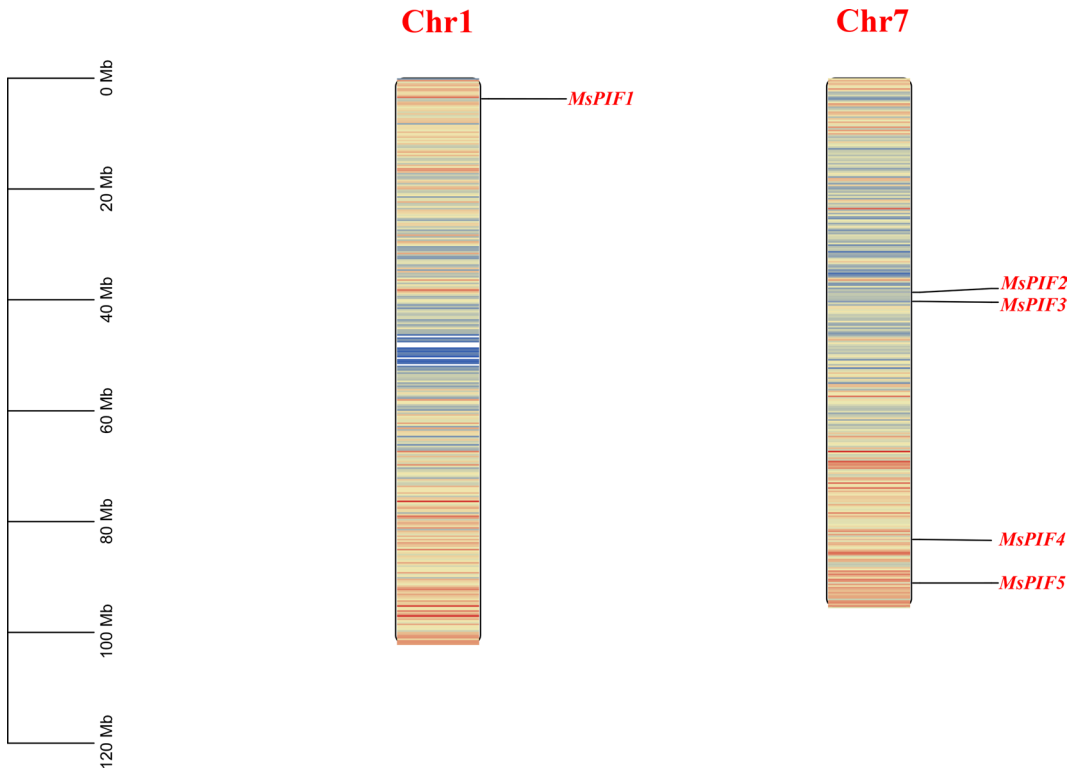

Fig. 1 Location of *MsPIFs* on alfalfa chromosomes

Table 1 Characterization of *PIF* genes in alfalfa

Gene name	Gene ID	Chromosome location	Gene location	Protein length(aa)	MW (kDa)	PI	Predicted localization
MsPIF1	MsG0180000277.01.T01	Chr1	3708808:3710783	239	26.48	6.29	Nucleus
MsPIF2	MsG0780038030.01.T01	Chr7	38650885:38658723	492	52.98	8.41	Nucleus
MsPIF3	MsG0780038108.01.T01	Chr7	40285873:40293893	492	53.06	8.76	Nucleus
MsPIF4	MsG0780040838.01.T01	Chr7	83219453:83223659	474	52.63	5.33	Nucleus
MsPIF5	MsG0780041453.01.T01	Chr7	91073876:91083647	680	74.75	6.11	Nucleus

peanut is higher than that in other plants, both of which are leguminous plants.

Motif composition and gene structure of *PIFs* in alfalfa and other plant species

According to Fig. 4C, it can be seen that all five identified MsPIFs have typical bHLH_AtPIF_like domains. This is consistent with the results in *Camellia sinensis* [6]. Through TBtools software, we predicted 10 motifs in PIF gene family, named motif 1–10. As shown in Fig. 4B, motif 1, 2, 3, and 7 are more conserved in the PIF family. Based on the results in Supplementary Material 1, motifs 1, 2, and 7 have conserved core sequences of the bHLH domain, possibly representing the bHLH domain, and motif 3 has a conserved core sequence representing the APB domain. In the group PIF1/4/5, MsPIF1 did not predict motif 3. In the PIF7/8 group, all genes except *AhPIF8B2* and *AtPIF7* have motif 10, which may be a characteristic of this group of members. In the PIF2/3/6 group, all genes except *AtPIF2* and *AtPIF6* contain motif 5, which can be used to distinguish this group. The resemblance in motif composition among members of the same group serves to substantiate the credibility of evolutionary tree grouping (Fig. 4A).

In order to investigate the characteristics of the gene structure of the PIF gene family, we mapped the CDS/UTR structure of PIF gene family using TBtools software (Fig. 4D). The majority of *PIF* genes were found to contain 6–7 intron regions, indicating that *PIF* genes have been subject to evolutionary conservation. *MsPIF1* has only 4 intron, proving its structural incompleteness.

Synteny analysis of the *MsPIF* genes

In order to determine whether replication events occurred in the PIF gene family of alfalfa during evolution, this study used TBtools for gene replication event analysis. As shown in Fig. 5A, two gene pairs were

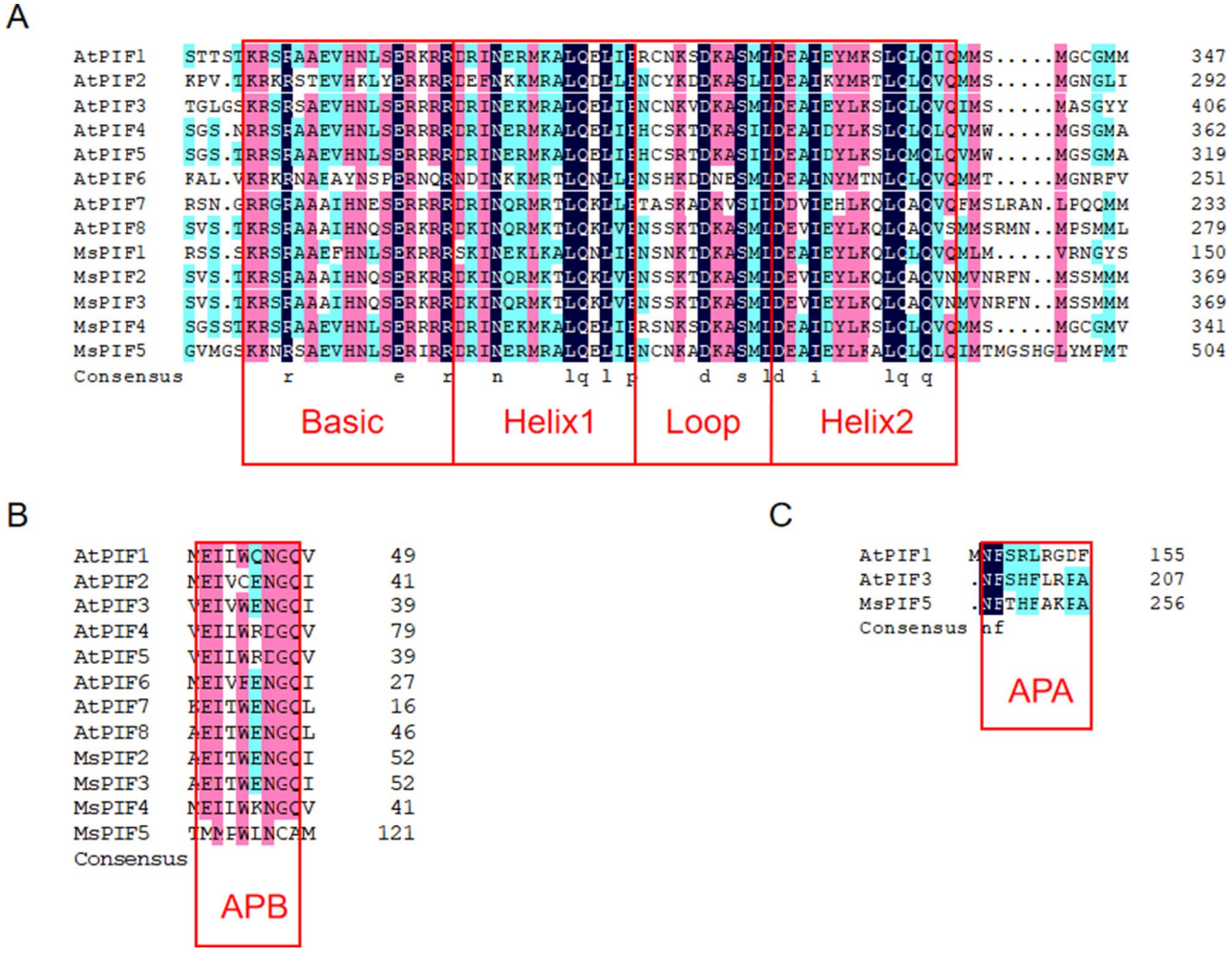

Fig. 2 Multiple sequence alignment of the conserved domains of PIFs from alfalfa and *Arabidopsis*

identified, there is no concatenated replication, but there are two instances of fragment replication.

In order to further elucidate the origin and evolutionary relationship of *PIF* genes, homologous comparisons were conducted between the *PIF* genes of alfalfa and *Arabidopsis.* The results in Fig. 4B showed that three *MsPIFs* and three *AtPIFs* together formed three gene pairs, indicating a close relationship between *MsPIFs* and *AtPIFs*. Indicating the existence of gene replication during the evolution of the gene, and the replication process is relatively conservative.

Cis-acting elements in alfalfa *PIF* genes

In order to further explore the potential function of *MsPIFs*, a regulatory region of 2000 bp upstream of the promoter was selected to analyze *cis*-acting elements. The *cis*-acting elements of the *MsPIFs* can be divided into three groups (Fig. 6), with stress response elements including. The results indicate that the main function of the PIF family is light response, while playing an important role in hormone response and stress response.

Tissue-specific expression of *MsPIFs*

To investigate the possible functions of *MsPIF* genes, we used qRT-PCR to measure the expression levels of *MsPIF* genes in different tissues of alfalfa, including the roots, stems, young leaves, mature leaves and flowers (Fig. 7). The results showed that all *MsPIFs* expressions can be detected and had similar expression patterns. The expression levels of *MsPIF1* and *MsPIF5* are low in all tissues. *MsPIF3* and *MsPIF4* are highly expressed in leaves and stems, especially in mature leaves. Except for *MsPIF1*, which has a higher expression level in flowers compared to other tissues, all other genes have the lowest expression level in roots and higher expression level in leaves compared to other tissues. The results indicate that the *PIF* genes plays different roles in alfalfa.

Expression of *MsPIFs* in response to abiotic stress in leaves

To evaluate the function of *MsPIFs* under abiotic stress, the expression patterns of *MsPIF* genes after drought, cold, salt and alkaline treatments were detected by qRT-PCR (Fig. 8). All *MsPIFs* are expressed under all abiotic

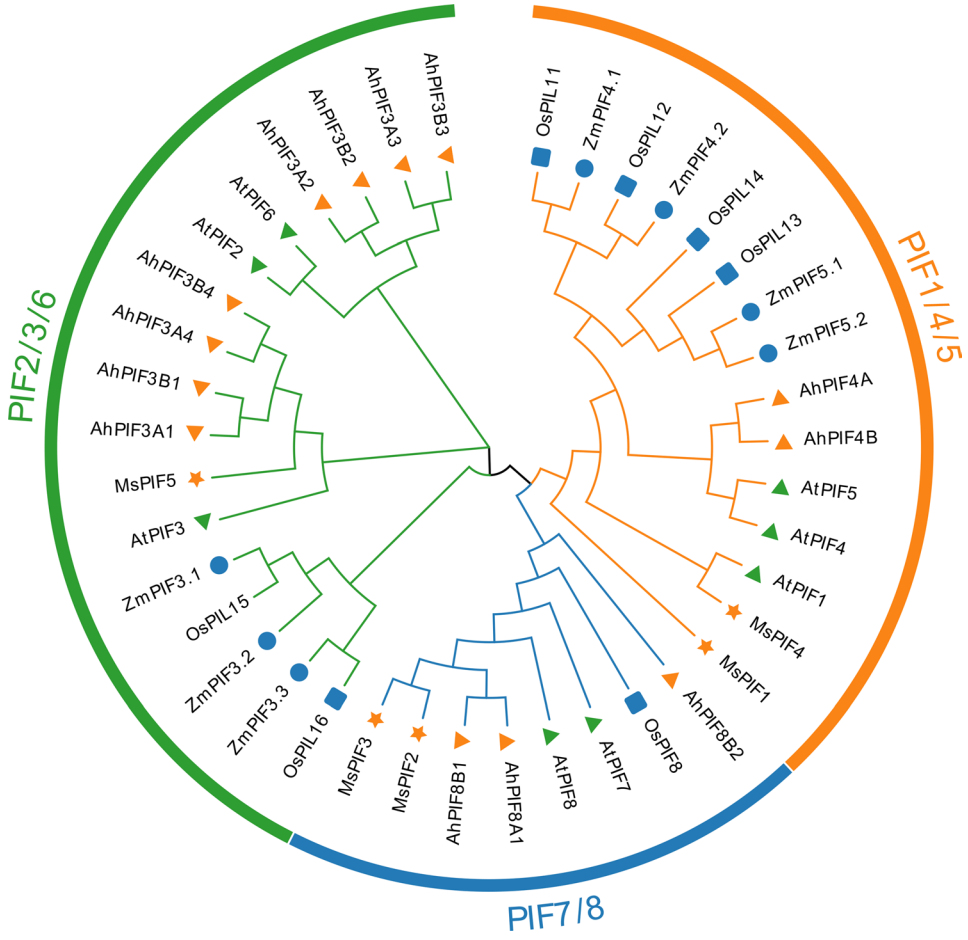

Fig. 3 Phylogenetic analysis of PIF proteins in alfalfa, *Arabidopsis*, rice, maize, and peanut. The tree was divided into PIF1/4/5, PIF7/8 and PIF2/3/6 sections by the colors orange, blue and green

stress treatments, with most genes having the highest expression levels at 6 h and 12 h.

Under drought treatment, *MsPIF2*, *MsPIF3*, and *MsPIF4* showed similar changing trends, reaching their maximum expression level at 1 h, followed by significant downregulation, and then rebounding at 24 h. Under cold stress, all five genes showed a significant upregulation followed by a significant downregulation trend. *MsPIF1* and *MsPIF3* reached their maximum values at 3 h, *MsPIF*2 reached their maximum values at 6 h, and *MsPIF4* reached their maximum values at 1 h, which was 3.9 times higher than before treatment. *MsPIF5* also reached its maximum value at 1 h. Under salt stress, the expression levels of *MsPIF1* and *MsPIF4* were low and did not show significant changes, while other genes showed significant changes. Among them, the expression levels of *MsPIF2* and *MsPIF3* reached their maximum levels after 1 h of treatment, about three times the level before treatment, and then significantly decreased to 0.1 times. Returned to the pre-treatment level at 24 h. Under alkaline stress, gene expression levels reached their maximum levels within 1 h, approximately three times higher than before treatment.

Discussion

Phytochrome-interacting factors (PIFs) represent signalling hubs in plants, capable of integrating a range of external environmental stimulus signals, which are then conveyed to other downstream factors. This enables plants to adapt to a variety of unfavourable conditions.

This study identified a total of 5 *PIFs* and named them *MsPIF1* to *MsPIF5* based on their chromosomal localization order in alfalfa (Table 1). Studies have found that there are 8 *PIF* genes in *Arabidopsis*, 6 in rice, 7 in maize, and 13 in peanut, indicating that different species have different numbers of *PIFs*.

Numerous studies have shown that all PIFs have conserved bHLH domains, with most genes having an APB domain and a few genes having an APA domain. bHLH domains have a basic region where they bind to the G-box or E-box of the promoter of a downstream gene [25, 26]. The bHLH domain was found in all *PIF* genes in *Arabidopsis*, rice, maize and peanut, and the same was

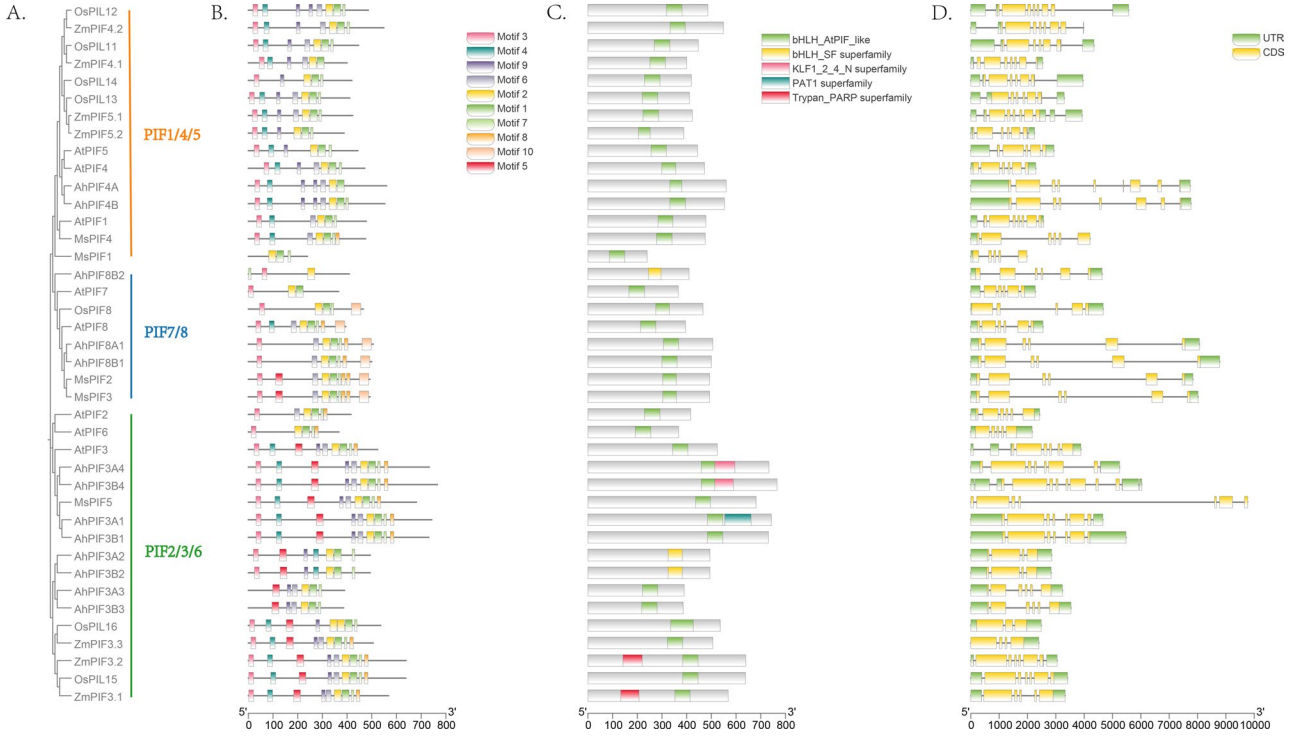

Fig. 4 Phylogenetic analysis, conserved motif analysis, and gene structure analysis of the PIF gene family from alfalfa, *Arabidopsis*, peanut, rice, and maize. (**A**) Phylogenetic analysis, (**B**) Motif composition analysis. The 10 different coloured squares indicate the 10 motifs predicted by TBtools software. (**C**) Conserved motif prediction, predicted by CD-Search website and visualised by TBtools software. (**D**) Gene structure analysis. Green squares represent UTR regions and yellow squares represent CDS regions

true for the five *PIF* genes in alfalfa. The APB domain is the site where PhyB binds to the PIFs [27], and PhyB is one of the major photoreceptors in plants. In peanut, AhPIF3A3 and AhPIF3B3 do not contain APB domain, and APB domain are also missing from VvPIF1 in grape. MsPIF1 in alfalfa also lacks this domain. It is speculated that this gene cannot interact with PhyB, possibly affecting light signalling. APA, on the other hand, is a PIF gene binding site for PhyA. The APA domain has been found in PIF1 and PIF3 in *Arabidopsis*. It is also present in Zm3.1-3.3 in maize and OsPIL15 and OsPIL16 in rice [28]. The APA domain was also found in MsPIF5 of alfalfa.

In order to facilitate a more precise classification of the PIF gene family, we conducted a phylogenetic analysis, integrating the *PIF* genes from *Arabidopsis*, maize, rice, peanut and alfalfa. The phylogenetic tree demonstrated that these genes can be classified into three distinct groups: PIF2/3/6, PIF7/8, and PIF1/4/5 (Fig. 3). Proteins in different groups exhibit variation in the number and type of conserved motifs, with conserved motifs displaying a degree of similarity within the same group. In the PIF2/3/6 group, both have motif 5, in the PIF7/8 group both have motif 10, and in PIF1/4/5 both motifs are absent (Fig. 4B). Gene structure analysis revealed that the number of exons and introns of *PIF* genes were similar in different species, but the length varied greatly, which was consistent with the findings of the study of grape *VvPIFs*.

The PIF gene family has two fragment repetitions in alfalfa itself, and three gene pairs between alfalfa and *Arabidopsis*, indicating the conservatism of the PIF gene family during evolution (Fig. 5). In addition, there are no gene pairs for *PIFs* in alfalfa and rice, which may be due to differences between dicots and monocots.

An understanding of the distribution of *cis*-acting elements in the promoter regions of genes may prove crucial in grasping how gene expression is regulated [29]. The prediction of *cis*-acting elements in alfalfa revealed that the majority of the elements were associated with light response, hormone regulation, and stress resistance. Notably, the highest number of light response elements was observed, indicating the potential involvement of *MsPIFs* in the light signalling pathway, which regulates photomorphogenic construction in plants (Fig. 6). These findings align with those of other studies. *MdPIF* genes have been demonstrated to be subject to light-mediated circadian regulation in apple [30]. Furthermore, the *OsPIL13*, *OsPIL15* and *OsPIF14* genes have also been shown to undergo circadian regulation [31]. Furthermore, the stability of the PIF gene family is influenced by light [32]. Additionally, ABA, GA, IAA and MeJA response elements, as well as various stress

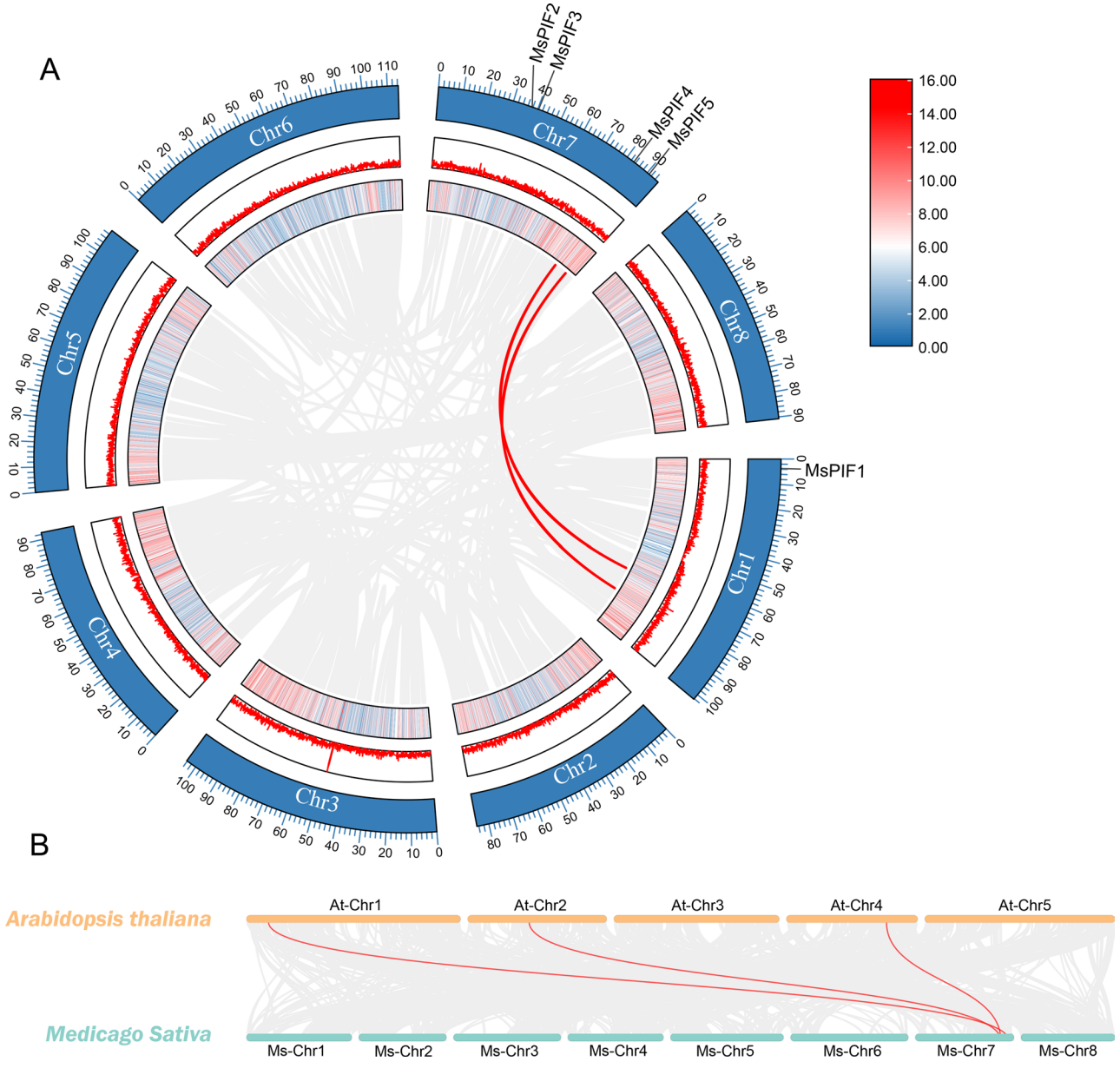

Fig. 5 (**A**) Synteny analysis of *PIF* genes in alfalfa. The gray lines indicate all synteny blocks in the alfalfa genome, (**B**) Synteny and collinearity of PIF genes between alfalfa and *Arabidopsis*. The synteny of *PIF* genes between alfalfa and *Arabidopsis*. are showed by red lines. Grey lines in the background indicate all the collinear blocks between alfalfa and *Arabidopsis*

response elements, were identified in the promoters of the majority of *MsPIF* genes. Phytohormones such as ABA, GA and JA have been demonstrated to regulate plant responses to abiotic stresses [33]. The *ZmPIF1* promoter region has been identified as a rich source of drought-responsive (MBS) and ABRE elements. Furthermore, *ZmPIF1* expression levels were elevated in *maize* following drought and abscisic acid treatments [34]. In alfalfa, eight abscisic acid-related ABRE elements were identified in *MsPIF5* and two drought stress-responsive elements were identified in *MsPIF4*, suggesting that these two genes may be associated with drought stress.

The expression of *PIF* genes are specific in different tissues of plants and have common and unique functions in various developmental and physiological processes. PIFs was initially considered a negative regulator of the light pathway, which promotes the morphological formation of dark growing plants [25]. The expression levels of six *AtPIFs* (*PIF1*, *PIF3*, *PIF4*, *PIF5*, *PIF7*, and *PIF8*) in seedlings and leaves are higher than those in roots, flowers, or fruits [35]. The four *VvPIFs* of grape are highly expressed in leaves, stems, and tendrils. In carrot, *Dcpifs* are also highly expressed in aboveground tissues (leaves and petioles) that directly receive light [36]. In this study,

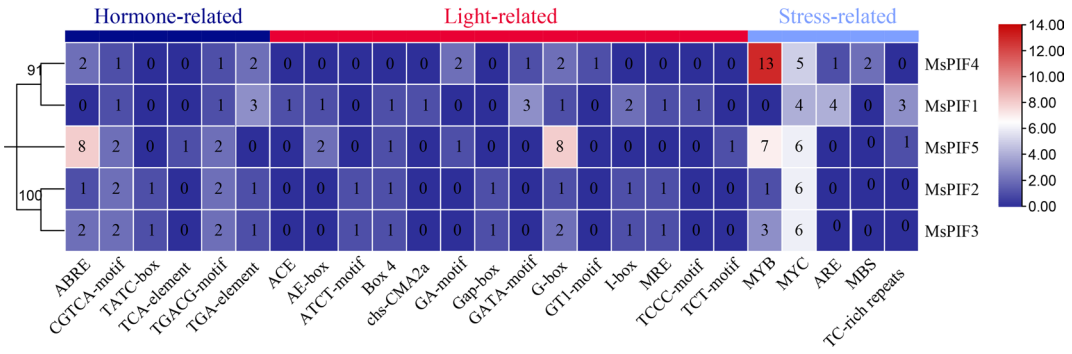

Fig. 6 The *cis*-acting elements in the promoters of *MsPIFs* predicted by PlantCARE. The red represents high expression levels, and the blue represents low expression levels

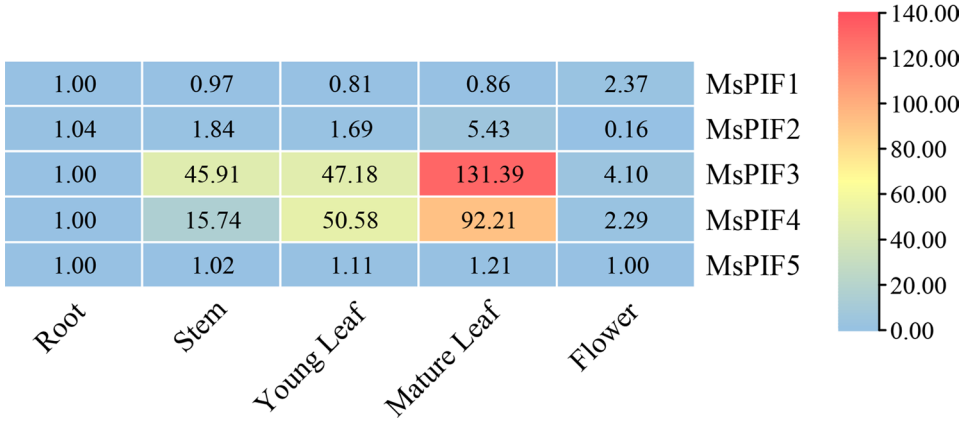

Fig. 7 Relative expression levels of *MsPIF* genes in different tissues by qRT-PCR. Heat map of *MsPIF* genes expression by TBtools. Red represents high expression level and blue represents low expression level

the expression levels of *MsPIFs* were also higher in young and old leaves than in roots, stems, and flowers. This may be related to the response of *PIFs* to light.

In accordance with the PIF genes, PIFs act as negative regulators of photomorphogenesis. However, they also play key roles in other processes, as core members of the photopigment-mediated light signalling pathway [37]. They facilitate adaptive and resistant responses of plants to environmental changes, including drought [38], salt [23], and cold [34], by interacting with other proteins or protein complexes or by regulating phytohormone levels and activating or repressing the expression of downstream genes [39, 40]. Prior research has demonstrated that under low-temperature stress, *PIF* genes can bind to the CBF transcription factor, thereby facilitating the interaction of the CBF transcription factor with the downstream cold-regulated gene (COR). This interaction ultimately confers a positive regulatory role in response to cold stress in diverse photoperiods [41–43]. In response to drought stress, *PIF* genes are primarily employed to enhance drought resistance by modulating stomatal opening and closure to reduce plant transpiration rate or regulating the level of ABA in the plant [44]. *PIF* genes have been less studied in salt stress, mostly in monocotyledonous plants. Rice transgenic plants overexpressing *ZmPIF3* had strong salt tolerance. Overexpression of *OsPIL14* also enhanced salt tolerance in rice and alleviated the inhibitory effect of NaCl on seedling growth [45, 46]. Our data can demonstrate that the expression of *MsPIFs* can be induced by several abiotic stresses. *MsPIFs* respond to drought, cold, salt, and alkaline stress.

Conclusion

In this study, we identified five *PIF* genes and classified into three main groups in alfalfa. *MsPIFs* are randomly distributed on two chromosomes, and two genes come from fragment replication. The *cis*-acting elements of *MsPIFs* genes are involved in hormone regulation, stress regulation, and light response. The expression pattern of *MsPIFs* in different tissues and under different treatment conditions suggests that it plays an important role in plant growth and development. The present study offers a comprehensive analysis of the involvement of PIF family members in alfalfa growth and development, as well as in response to corresponding abiotic stresses. This analysis

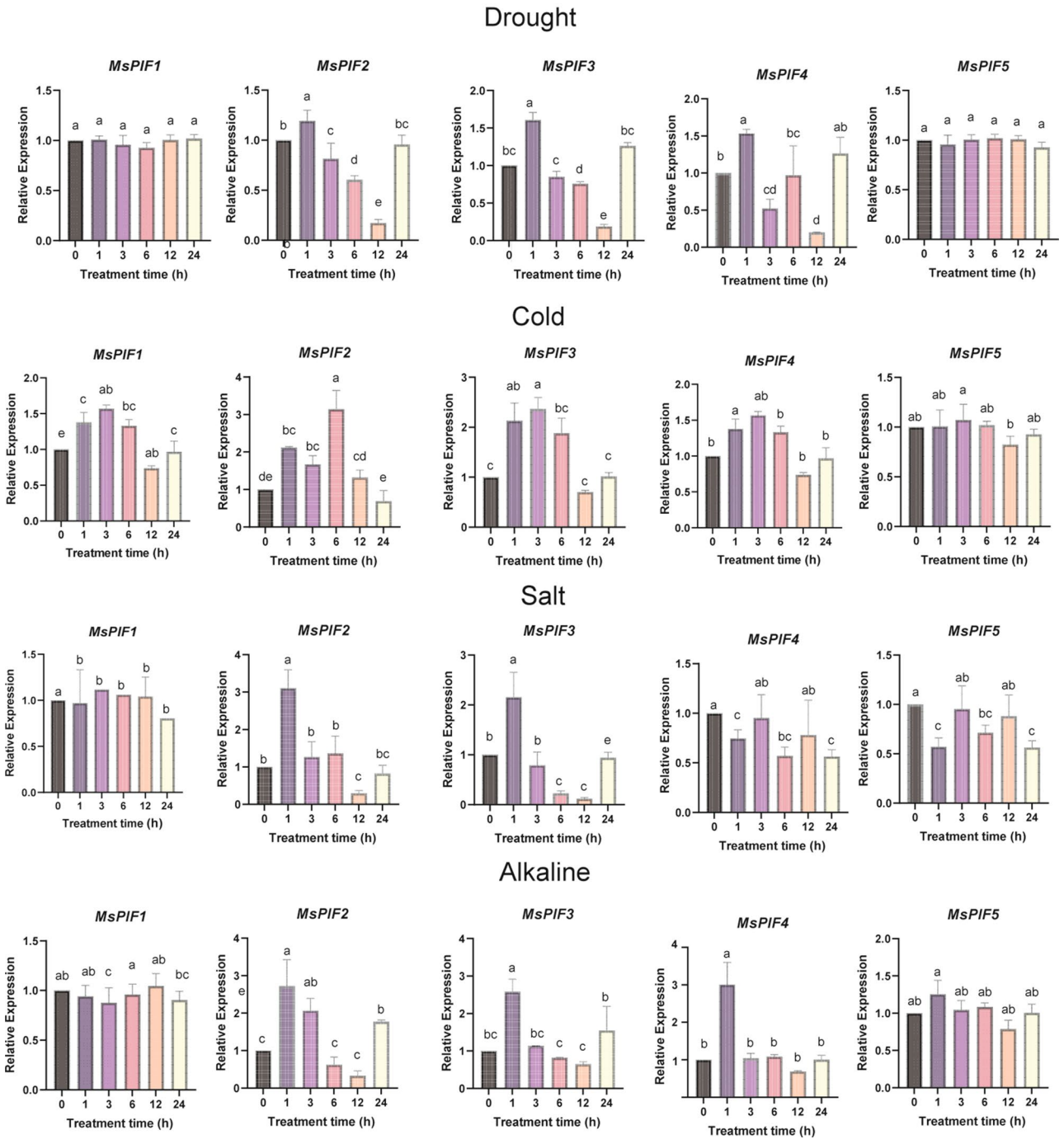

Fig. 8 The relative expression of *MsPIFs* under different abiotic stresses is illustrated in the accompanying chart. The letters above the bars represent the level of significant differences between treatments, with multiple comparisons made using Duncan's method ($P < 0.05$). The error bars represent the mean ± standard error of three independent biological replicates

provides a foundation for further research on the function of *MsPIFs.*

Materials and methods

Plant materials and treatments

This study used *Medicago sativa* L.'Zhongmu No.1' as the material. The alfalfa seeds were selected for their full and shiny appearance and subsequently cultivated with vermiculite and irrigated with a 1/10-strength Hoagland nutrient solution. The temperature was set at 22℃ during the day and 18 °C at night, with a 16-hour light period and an 8-hour dark period. The relative humidity was maintained at 70%.

Four weeks after sowing, healthy and uniformly growing alfalfa plants were selected and placed in treatments comprising 4℃, 15% PEG-6000, 150 mM NaCl, and 150 mM $NaHCO_3$ for 1, 3, 6, 12, and 24 h. Sampling was conducted at the corresponding time points. Samples of roots, stems, mature leaves, young leaves and flowers of 4-week-old alfalfa were taken. The aforementioned samples were obtained through the implementation of three biological and three technical replicates. The samples were stored at a temperature of -80℃ [47–49].

Identification and characterization of *PIF* genes in alfalfa

Download the genome and annotation files of *Medicago sativa* L.'Zhongmu No.1' from the website (https://figshare.com/articles/dataset/Medicago_sativa_genome_and_annotation_files/12623960) and the sequences of eight AtPIF proteins in *Arabidopsis thaliana* from the TAIR database (https://www.arabidopsis.org/). Eight AtPIFs were used as query sequences in a BLASTP search of the alfalfa genome, and candidate genes with E-values less than 1e-5 were submitted to the Interpro and SMART websites (http://smart.embl-heidelberg.de/) for verification of the presence of the bHLH domain. After removing duplicate sequences and redundant transcripts, five MsPIFs were finally obtained.

Chromosomal localization analysis of *MsPIFs* was performed using TBtools software, and the isoelectric points (pI) and molecular weights (MW) of MsPIFs were predicted using the online website ExPASy ProtParam (https://web.expasy.org/protparam/), and using WoLF PSORT (https://wolfpsort.hgc.jp/) to predict the subcellular localization.

Multiple sequence alignment and phylogenetic analysis

The identified PIFs protein sequence of alfalfa was compared with the PIF protein sequences of *Arabidopsis*, maize, rice, and peanut using DNAMAN tool. The genomes of *Arabidopsis* and other plants are sourced from the Tair and NCBI (https://www.ncbi.nlm.nih.gov/). Phylogenetic trees were plotted using the MEGA11 software with the NJ method and 1000 bootstrap replications and then visualised using Evolview (https://evolgenius.info//evolview-v2/#login) [50].

Motif composition and gene structure analysis of *PIF* genes in alfalfa

Motif composition of alfalfa, *Arabidopsis thaliana*, rice, maize, peanut was analysed using MEME Suite (https://meme-suite.org/meme/), where motif length was set to 6–50 and number was set to 10 [51]. Conserved domains were analyzed using CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd) [52–54]. The gene structures of different plants were predicted by their reference genome annotations Gff on GSDS 2.0 (https://gsds.gao-lab.org/) [55].

Synteny analysis of *MsPIF* genes

Gene synteny and collinearity within the alfalfa genome and between alfalfa and *Arabidopsis thaliana* using the MCScanX tool, and extracting and visualising information about PIF family members using TBtools.

Analysis of *cis*-element within the MsPIF gene promoter

Extracted the upstream 2000 bp sequence of the starting codon of five members of the PIF family in alfalfa using TBtools software, and then uploaded it to the PlantCARE online website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Predicted and analyzed all promoters to obtain *cis*-acting elements [56]. The *cis*-acting elements of each genes were arranged by type in Excel software and visualized through TBtools.

RNA extraction and gene expression analysis

RNA was extracted using CoWin Biotech, Beijing, China, and first stranded cDNA was synthesized using the reverse primer oligo (dT) 10 using the instructions of the HiScript II Q Select reverse transcriptase kit (Vazyme Biotech, Nanjing, China). Through ChamQ™ Universal SYBR qPCR Master Mix (Vazyme, China) was used for quantitative qRT-PCR. Using the *GAPDH* gene of alfalfa as an internal reference. The reaction process is as follows: react at 95℃ for 30 s; 40 cycles, 95℃ for 5 s, 60℃for 34 s; and 95℃ for 15 s [57, 58].

Statistical analyses

The data were collated using Excel 2010, an Student's t-test and one-way ANOVA using SPSS 22.0, and multiple comparisons were performed using Duncan's method. The graphical representation of the data was produced using the GraphPad Prism 5.0 software.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12863-024-01264-4.

Supplementary Material 1

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Author contributions

QL wrote the manuscript and JY revised it. QL, JY and GC designed the experiments. QL and BW conducted experiments. WX and YY prepared figures. All authors read and approved the final manuscript.

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Data availability
The data does not involve sequencing and are listed in the article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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