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Genome-wide identification and analysis of abiotic stress responsiveness of the mitogen-activated protein kinase gene family in *Medicago sativa* L.

Hao Liu[1,2†], Xianyang Li[1†], Fei He[1†], Mingna Li[1], Yunfei Zi[3], Ruicai Long[1], Guoqing Zhao[3], Lihua Zhu[3], Ling Hong[3], Shiqing Wang[3], Junmei Kang[1], Qingchuan Yang[1] and Lin Chen[1*]

Abstract

Background The mitogen-activated protein kinase (MAPK) cascade is crucial cell signal transduction mechanism that plays an important role in plant growth and development, metabolism, and stress responses. The MAPK cascade includes three protein kinases, MAPK, MAPKK, and MAPKKK. The three protein kinases mediate signaling to downstream response molecules by sequential phosphorylation. The *MAPK* gene family has been identified and analyzed in many plants, however it has not been investigated in alfalfa.

Results In this study, *Medicago sativa MAPK* genes (referred to as *MsMAPKs)* were identified in the tetraploid alfalfa genome. Eighty *MsMAPKs* were divided into four groups, with eight in group A, 21 in group B, 21 in group C and 30 in group D. Analysis of the basic structures of the *MsMAPKs* revealed presence of a conserved TXY motif. Groups A, B and C contained a TEY motif, while group D contained a TDY motif. RNA-seq analysis revealed tissue-specificity of two *MsMAPKs* and tissue-wide expression of 35 *MsMAPKs*. Further analysis identified *MsMAPK* members responsive to drought, salt, and cold stress conditions. Two *MsMAPKs* (*MsMAPK70* and *MsMAPK75*) responds to salt and cold stresses; two *MsMAPKs* (*MsMAPK60* and *MsMAPK73*) responds to cold and drought stresses; four *MsMAPKs* (*MsMAPK1*, *MsMAPK33*, *MsMAPK64* and *MsMAPK71*) responds to salt and drought stresses; and two *MsMAPKs* (*MsMAPK5 and MsMAPK7)* responded to all three stresses.

Conclusion This study comprehensively identified and analysed the alfalfa *MAPK* gene family. Candidate genes related to abiotic stresses were screened by analysing the RNA-seq data. The results provide key information for further analysis of alfalfa *MAPK* gene functions and improvement of stress tolerance.

Keywords *Medicago sativa* L., *MsMAPK* gene family, Genome-wide analysis, Tissue-specific expression, Abiotic stress response

†Hao Liu, Xianyang Li and Fei He contributed equally to this work.

*Correspondence:
Lin Chen
chenlin@caas.cn

[1]Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[2]College of Grassland Science, Qingdao Agricultural University, Qingdao 266109, China
[3]Institute of Forage Crop Science, Ordos Academy of Agricultural and Animal Husbandry Sciences, Ordos 017000, China

Background

Plants are exposed to various biotic or abiotic stresses during different phases of growth and development [1]. To cope with these stresses, plants harbour multiple regulatory mechanisms [2–4]. Signal transduction plays an important role in stress responsive mechanism [5]. The MAPK cascade, common in eukaryotes and important component of signalling [6, 7], is central to plant growth and development [8], hormone signal transduction [9], and response to biotic or abiotic stresses [10, 11].

The MAPK cascade is composed of three sequential protein kinases: MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MAPKKs), and MAP kinases (MAPKs) [12, 13]. MAPKKKs activates downstream MAPKKs by phosphorylating serine/threonine residues in the MAPKK activation loops S/T-xxxx-S/T [14]. MAPKKs present in the centre of the cascade are protein kinases with dual specificity [15] that can not only accept the activation signal of upstream MAPKKKs but also activate downstream MAPKs by phosphorylating tyrosine/threonine residues in the TXY motifs of the MAPK activation loops [16, 17]. Activated MAPKs can be transported to the cytoplasm or nucleus to phosphorylate other proteins (kinases, transcription factors, cytoskeleton-binding proteins) for regulation of various cellular activities [18–20], and hence these downstream MAPKs are crucial for signal transduction.

The MAPK cascade was first identified as a microtubule-associated protein kinase in animal cells in 1986 [21, 22]. This enzyme is called "mitogen-activated protein kinase" due to their response to mitogen phosphorylation at tyrosine residues [23, 24], which is associated with growth and development, hormonal responses, and stress [25–27]. The *MAPK* gene family members of many species have been identified; for example, 20, 38, 54, 15, 19, and 54 *MAPKs* have been identified in *Arabidopsis thaliana* [28], *Glycine max* [29], *Triticum aestivum* [30, 31], *Oryza sativa* [32], *Zea mays* [33] and *Gossypium hirsutum* [34], respectively. The function of the MAPK cascade pathway has been the subject of recent studies in many species. the *MEKK1-MKK4/5-MPK3/6* cascade was the first signalling module identified in *A. thaliana*, which up-regulates the expression of the *WRKY22/29* transcription factor, while enhancing resistance to fungal and bacterial pathogens [35]. In *Hordeum spontaneum*, three enriched MAPK cascades (MEKK1-MKK2-MPK4/6, MEKK17/18-MKK3-MPK1/2/7/14, MKK3-MPK8) can effectively participate in in salt stress adaptation and tolerance as well as homeostasis of reactive oxygen species (ROS). [36]. In *A. thaliana*, *MPK3* and *MPK6* play role in growth and development. For example, *MPK3* and *MPK6* mediate the guidance response in pollen tubes [37], and *MPK3* and *MPK6* and their upstream *MAPKKs* (*MKK4* and *MKK5*) serve as regulators of stomatal development [38]. Further, *MPK3* and *MPK6* control salicylic acid signaling by upregulating NLR receptor expression in pattern and effector-triggered immune processes [39]. 1-amino-cyclopropane-1-carboxylic acid synthase (ACS) catalyzes the committing and rate-limiting steps in the ethylene biosynthesis pathway. *MPK3* and *MPK6* can phosphorylate and stabilize ACS2 and ACS6, and regulate the expression of ACS2 and ACS6 genes through another *MPK3*/*MPK6* substrate *WRKY33*, thereby regulating ethylene synthesis [40]. *MPK3*/*MPK6* can also degrade and destroy the stability of ICE1 (CBF expression inducer 1), which regulates C-repeat-binding factor (CBF) transcription factors associated with cold stress, thereby negatively regulating CBF expression and freezing tolerance in plants [41]. *AtMPK6* is found to phosphorylate *AtMYB15* to reduce the binding affinity of *AtCBF3* and freezing tolerance [42]. In *G. max*, *GmMPK4* is a negative regulator of the defense response and a positive regulator of growth and development, like the function of *ATMPK4* in *A. thaliana*, suggesting functional conservation across plant species, during evolution [43]. GMK1 is regulated by both phosphatidic acid and hydrogen peroxide (H_2O_2) and is translocated to the nucleus during salt stress [44]. In *Z. mays*, the transcriptional level of *ZmMPK3* was significantly increased when maize seedlings were subjected to exogenous signal molecules such as ABA, H_2O_2, jasmonic acid and salicylic acid, as well as various abiotic stresses such as salinity. At the same time, it was found that ABA and H_2O_2 induced a significant increase in *ZmMPK3* activity [45]. *ZmMPK3* and *ZmMPK5* were induced by drought and cold stress [46, 47]. In *O. sativa*, *OsMAPK6* can phosphorylate the *OsLIC* (zinc finger protein), which regulates the transcription of *OsWRKY30* gene and enhancing the response to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xoc*) [48]. *OsMAPK2* seems to play roles in stress signal transduction pathways and panicle development in *O. sativa* [49].

Alfalfa (*Medicago sativa* L.) is a high-quality legume forage (high nutritional value and good palatability), cultivated in China for more than 2,000 years [50]. However, the *MAPK* gene family has not been investigated in alfalfa. In this study, the *MAPK* gene family of alfalfa was comprehensively analyzed via genome-wide screening, phylogenetic analysis, gene structure and conserved motif analysis, and chromosome localization and collinearity analysis. The RNA-seq analysis of alfalfa *MAPK* genes in different tissues and under different stresses was carried out. The results of this study provide key information for further analysis of the function of *MAPKs* and their utility in molecular breeding of alfalfa.

Results

Diversity of *MAPK* genes in *M. sativa* genome

Using a combination of *in silico* approaches including Hidden Markov model and domain-based search methods a total of 80 *MsMAPK* genes were identified from the "Xinjiangdaye" reference genome. The important characteristics of gene and protein sequences are shown in Table 1 and Table S1.

Among all the MsMAPK members, the longest and shortest proteins were MsMAPK27 (716 aa. MW: 81.36 kDa) and MsMAPK13 (137 aa, MW: 15.78 kDa), respectively. The highest and lowest isoelectric points (pIs) were found for *MsMAPK7* (9.41) and *MsMAPK16* (4.97), respectively, and the instability index ranged from 29.98 (*MsMAPK21*) to 49.48 (*MsMAPK34*). The *MsMAPK* members showed divers localization in the cell, with fifty-six members in the cytosol, five in the chloroplast, three in the cytoskeleton, seven in the mitochondria, eight in the nucleus, and only one in the endoplasmic reticulum (Table S1).

Of the total eighty, 76 *MsMAPK* genes (*MsMAPK1-76*) were located on 23 chromosomes (none on chr1.1, chr1.2, chr1.3, chr1.4, chr6.2, chr6.4, chr7.1, chr7.3, or chr7.4), whereas four genes (*MsMAPK77*, *MsMAPK78*, *MsMAPK79*, *MsMAPK80*) were identified on the unanchored 50,223–50,226. Each chromosome contained various genes, ranging from 1 to 9 (Fig. 1). Finally, 80 *MsMAPK* genes were renamed according to their chromosomal locations (*MsMAPK1-MsMAPK80*).

Phylogenetic analysis of *MAPK* genes in *M. sativa*

To further explore the evolutionary relationships between 80 *MsMAPK* members in *M. sativa*, a phylogenetic tree was constructed, including 20 sequences from *A. thaliana*, 32 from *G. max* and, 15 from *O. sativa* (Fig. 2). According to the classification of *MAPK* gene family in *A. thaliana* [51], and based on the conserved phosphorylation motifs (TEY, TDY) in the activation loop [52], the *MsMAPK* family genes were divided into A, B, C and D subgroups. Among them, *MsMAPK* members in groups A, B and C have TEY motifs, while *MsMAPK* members in group D have TDY motifs (Fig. S1), which is consistent with the findings of previous studies. Groups A, B, C and D contained 8, 21, 21 and 30 members, respectively, and some of the results were consistent with the information in previous reports [53].

Analysis of the *MAPK* gene basic structures and conserved domains of MAPK proteins in *M. sativa*

To study the structural characteristics of the *MAPK* family members in *M. sativa*, the presences of conserved motifs were analyzed using the online tool MEME. Ten motifs were predicted, and the basic information is shown in Fig. S2. All MsMAPK proteins contained Motif 1 (Fig. S1), and several other members contained Motif 2, Motif 3, Motif 4, Motif 5, Motif 6, Motif 7, and Motif 10. Except for MsMAPK29, all group D members contained Motif 8. Except for MsMAPK58, all group A and group B members contained Motif 9 (Fig. 3A). Motif 2 contains a TXY structure. As shown in Fig. S1, all MsMAPK members contained a TXY structure. The C-terminus of the MsMAPK proteins in group A and group B contain a –(LH)DxxDE(P)xC- motif, which is defined as the CD domain and is a site for identifying substrate proteins (Fig. S3) [54].

Gene structure analysis revealed that in addition to *MsMAPK79*, *MsMAPK77* (containing 4 introns) and *MsMAPK58* (containing 6 introns) in group A and group B, the other members contained 5 introns each. In group C, 7 members contained 2 introns each, and 14 members contained only 1 intron each. The number of introns in the group D genes significantly differed from that in the genes of groups A, B, and C, ranging from 7 to 14 (Fig. 3B).

Gene duplication events and collinearity analysis of *MAPK* genes in *M. sativa*

The gene duplication events of *MAPK* genes in alfalfa were analyzed. As shown in Fig. 1, a total of 12 tandem duplication events were found, involving 32 *MsMAPKs*, such as the tandem duplication event *MsMAPK10*/*MsMAPK11*/*MsMAPK12*/*MsMAPK13* located on chr3.1 and another tandem duplication event *MsMAPK41*/*MsMAPK42*/*MsMAPK43*/*MsMAPK44* located on chr4.3 (Table S2). Moreover, a total of 131 segmental duplication events involving 64 *MsMAPK* genes were detected (Table S3). As can be seen in Fig. 4, the *MsMAPK* genes in most of the segmental duplication events are located at similar positions on each chromosome in the homologous chromosome. For example, *MsMAPK2*/*MsMAPK4*/*MsMAPK6*/*MsMAPK8* are located on chr2.1, chr2.2, chr2.3 and chr2.4, respectively. There were significantly more segmental duplication events than tandem duplication events. In summary, it can be speculated that the development and evolution of the alfalfa *MAPK* family genes mainly rely on segmental duplication events.

Next, to clarify the potential evolutionary relationships of the *MAPK* genes family in different crop species, the evolutionary relationships and collinearity between *M. sativa* and *A. thaliana* and between *G. max* and *Medicago truncatula* were predicted (Fig. 5). The results showed that 46 *MsMAPK* genes were collinear with those of *A. thaliana*, 55 *MsMAPK* genes were collinear with those of *G. max*, and 56 *MsMAPK* genes were collinear with those of *Medicago truncatula*. At the same time, there were 80, 180 and 90 collinear gene pairs in *A. thaliana*, *G. max* and *Medicago truncatula*, respectively.

Table 1 Basic information of *MAPK* genes family in *M. sativa*

Gene Name	Gene ID	Chr Location	Protein Length (aa)	MW (kDa)	pI	Instability Index	Subcellular Location
MsMAPK1	MS.gene87007	chr2.1:25642402–25,646,669	627	70.94	9.32	35.15	Endoplasmic Reticulum
MsMAPK2	MS.gene002842	chr2.1:66455889–66,461,661	564	64.25	8.67	39.45	Cytoplasm
MsMAPK3	MS.gene039306	chr2.2:19816049–19,820,859	608	68.98	9.37	38.16	Cytoplasm
MsMAPK4	MS.gene01817	chr2.2:63772671–63,778,434	564	64.25	8.67	39.45	Cytoplasm
MsMAPK5	MS.gene000227	chr2.3:23343065–23,347,884	608	68.90	9.38	35.78	Cytoplasm
MsMAPK6	MS.gene00918	chr2.3:66052563–66,058,332	564	64.25	8.67	39.45	Cytoplasm
MsMAPK7	MS.gene96008	chr2.4:23417091–23,422,487	608	68.80	9.41	35.55	Cytoplasm
MsMAPK8	MS.gene004529	chr2.4:65912941–65,919,598	564	64.25	8.67	39.45	Cytoplasm
MsMAPK9	MS.gene69580	chr3.1:34255271–34,259,598	602	68.54	9.17	33.78	Cytoplasm
MsMAPK10	MS.gene04176	chr3.1:44918584–44,920,225	368	42.60	8	32.62	Mitochondrion
MsMAPK11	MS.gene04173	chr3.1:44931377–44,932,931	195	23.23	5.7	30.37	Cytoplasm
MsMAPK12	MS.gene04169	chr3.1:44960507–44,962,110	322	37.29	6	31.31	Cytoplasm
MsMAPK13	MS.gene04164	chr3.1:44997092–44,997,890	137	15.78	5.73	30.62	Cytoplasm
MsMAPK14	MS.gene019256	chr3.2:40400581–40,404,890	602	68.55	9.17	33.39	Cytoplasm
MsMAPK15	MS.gene05668	chr3.2:51498733–51,500,376	368	42.54	7.58	32.45	Mitochondrion
MsMAPK16	MS.gene05677	chr3.2:51554289–51,555,971	218	25.09	4.97	48.13	Cytoplasm
MsMAPK17	MS.gene05679	chr3.2:51572655–51,574,258	322	37.28	6.11	30.06	Cytoplasm
MsMAPK18	MS.gene42314	chr3.2:89502194–89,503,724	372	42.68	6.12	34.43	Cytoplasm
MsMAPK19	MS.gene070315	chr3.3:48590345–48,591,990	368	42.59	8.26	32.07	Mitochondrion
MsMAPK20	MS.gene070312	chr3.3:48604077–48,605,830	286	33.56	6.26	32.32	Mitochondrion
MsMAPK21	MS.gene070308	chr3.3:48629033–48,630,636	322	37.30	6.05	29.98	Cytoplasm
MsMAPK22	MS.gene070303	chr3.3:48665676–48,666,474	137	15.78	5.66	30.08	Cytoplasm
MsMAPK23	MS.gene22119	chr3.4:43049814–43,054,549	602	68.61	9.17	34.72	Cytoplasm
MsMAPK24	MS.gene04319	chr3.4:56220664–56,222,305	368	42.53	7.98	33.99	Cytoplasm
MsMAPK25	MS.gene04326	chr3.4:56265102–56,266,765	342	39.60	6.92	31.64	Cytoplasm
MsMAPK26	MS.gene064692	chr3.4:97218763–97,220,320	372	42.68	6.12	34.43	Cytoplasm
MsMAPK27	MS.gene94543	chr4.1:488940–498,277	716	81.36	6.66	47.03	Nucleus
MsMAPK28	MS.gene040139	chr4.1:506305–510,440	505	57.74	6.34	43.44	Cytoskeleton
MsMAPK29	MS.gene040136	chr4.1:537100–539,902	350	39.50	6.09	38.64	Cytoplasm
MsMAPK30	MS.gene06000	chr4.1:5435232–5,438,156	384	43.97	6.54	42.91	Chloroplast
MsMAPK31	MS.gene26583	chr4.1:22650720–22,656,759	387	44.40	5.52	44.13	Nucleus
MsMAPK32	MS.gene08564	chr4.1:37986714–37,990,587	374	43.02	4.99	41.54	Cytoplasm
MsMAPK33	MS.gene08572	chr4.1:38156436–38,159,400	371	42.86	5.6	34.51	Cytoplasm
MsMAPK34	MS.gene93771	chr4.2:414669–419,179	583	66.75	6.55	49.48	Cytoplasm
MsMAPK35	MS.gene048860	chr4.2:5762908–5,765,832	384	43.97	6.54	42.91	Chloroplast
MsMAPK36	MS.gene39573	chr4.2:23842355–23,848,496	387	44.40	5.52	44.13	Nucleus
MsMAPK37	MS.gene004136	chr4.2:42889970–42,893,908	374	43.01	4.99	40.42	Cytoplasm
MsMAPK38	MS.gene004143	chr4.2:43009514–43,012,509	371	42.86	5.6	34.51	Cytoplasm
MsMAPK39	MS.gene046981	chr4.3:510404–514,660	511	58.46	6.43	43.34	Nucleus
MsMAPK40	MS.gene55731	chr4.3:5493992–5,497,119	384	43.97	6.54	42.91	Chloroplast
MsMAPK41	MS.gene023551	chr4.3:14663300–14,664,497	369	42.46	6.52	32.22	Cytoplasm
MsMAPK42	MS.gene023550	chr4.3:14670634–14,671,978	368	42.61	6.46	30.97	Cytoplasm
MsMAPK43	MS.gene08659	chr4.3:14756701–14,757,895	368	42.33	6.56	33	Cytoplasm
MsMAPK44	MS.gene08660	chr4.3:14761327–14,762,681	368	42.62	6.5	31.34	Nucleus
MsMAPK45	MS.gene47530	chr4.3:25669261–25,675,368	387	44.40	5.52	44.13	Nucleus
MsMAPK46	MS.gene65257	chr4.3:41078319–41,082,249	374	43.01	4.99	40.42	Cytoplasm
MsMAPK47	MS.gene65266	chr4.3:41265955–41,268,959	371	42.88	5.6	34.51	Cytoplasm
MsMAPK48	MS.gene89722	chr4.4:5341253–5,344,177	384	43.97	6.54	42.91	Chloroplast
MsMAPK49	MS.gene09073	chr4.4:14082432–14,083,626	368	42.33	6.56	33	Cytoplasm
MsMAPK50	MS.gene09075	chr4.4:14094130–14,095,474	368	42.61	6.86	30.74	Cytoplasm
MsMAPK51	MS.gene008714	chr4.4:25174043–25,180,123	387	44.40	5.52	44.13	Nucleus
MsMAPK52	MS.gene049834	chr4.4:43623794–43,627,670	374	43.02	4.99	41.54	Cytoplasm
MsMAPK53	MS.gene049843	chr4.4:43797410–43,800,733	371	42.88	5.6	33.47	Cytoskeleton

Table 1 (continued)

Gene Name	Gene ID	Chr Location	Protein Length (aa)	MW (kDa)	pI	Instability Index	Subcellular Location
MsMAPK54	MS.gene024644	chr5.1:3567752–3,569,897	375	43.10	5.86	43.22	Cytoplasm
MsMAPK55	MS.gene062353	chr5.1:73313721–73,320,314	533	60.87	9.15	30.64	Cytoplasm
MsMAPK56	MS.gene21637	chr5.2:3333349–3,335,531	375	43.10	5.86	42.05	Cytoplasm
MsMAPK57	MS.gene030055	chr5.2:77576055–77,582,649	533	60.87	9.15	30.64	Cytoplasm
MsMAPK58	MS.gene072770	chr5.3:3790113–3,792,460	348	39.83	5.63	46.34	Cytoplasm
MsMAPK59	MS.gene017127	chr5.3:73741189–73,747,597	533	60.87	9.15	30.89	Nucleus
MsMAPK60	MS.gene019321	chr5.4:4498192–4,500,369	375	43.10	5.86	41.65	Cytoplasm
MsMAPK61	MS.gene038360	chr5.4:72661842–72,667,094	526	60.09	9	31.63	Cytoskeleton
MsMAPK62	MS.gene58902	chr6.1:71271709–71,278,049	571	64.48	6.71	34.14	Mitochondrion
MsMAPK63	MS.gene37814	chr6.3:73652539–73,658,812	571	64.44	6.71	34.4	Mitochondrion
MsMAPK64	MS.gene05280	chr6.3:73788859–73,795,218	571	64.44	6.71	34.4	Mitochondrion
MsMAPK65	MS.gene85441	chr7.2:26996423–27,000,130	372	42.94	6.28	38.9	Cytoplasm
MsMAPK66	MS.gene58718	chr8.1:9852080–9,855,687	559	63.54	8.65	40.2	Cytoplasm
MsMAPK67	MS.gene007822	chr8.1:53741564–53,747,170	580	65.87	9.1	36.1	Cytoplasm
MsMAPK68	MS.gene84270	chr8.1:72833369–72,838,261	371	42.81	5.98	46.77	Cytoplasm
MsMAPK69	MS.gene041459	chr8.2:11929650–11,933,269	559	63.54	8.65	40.2	Cytoplasm
MsMAPK70	MS.gene032355	chr8.2:48669355–48,674,954	580	65.87	9.1	36.1	Cytoplasm
MsMAPK71	MS.gene019674	chr8.2:67902869–67,907,719	371	42.82	5.98	47	Cytoplasm
MsMAPK72	MS.gene036594	chr8.3:8740823–8,744,347	531	60.28	8.91	40.85	Cytoplasm
MsMAPK73	MS.gene32270	chr8.3:48841978–48,847,590	580	65.84	9.1	36.1	Cytoplasm
MsMAPK74	MS.gene068705	chr8.3:64676224–64,681,053	371	42.84	5.98	45.25	Cytoplasm
MsMAPK75	MS.gene38970	chr8.4:49180683–49,186,346	579	65.74	9.1	36.48	Cytoplasm
MsMAPK76	MS.gene056822	chr8.4:66951918–66,956,761	371	42.83	5.98	46.07	Cytoplasm
MsMAPK77	MS.gene058518	50223:46950–50,974	258	29.57	6.33	30.78	Cytoplasm
MsMAPK78	MS.gene023959	50224:32231–35,868	372	42.94	6.14	39.36	Cytoplasm
MsMAPK79	MS.gene51857	50225:8582–11,787	254	29.16	6.5	30.8	Cytoplasm
MsMAPK80	MS.gene058519	50226:2160–5837	362	41.60	7.17	43.19	Chloroplast

chr: chromosome; aa: amino acid; MW: molecular weight; pI: isoelectric point

Analysis of *cis*-acting elements in the promoter regions of *MAPK* genes in *M. sativa*

PlantCARE database was used to identify the *cis*-acting elements in the promoter of the *MsMAPK* genes. The *cis*-acting elements were divided into three categories: growth and development, hormone response, and stress response (Table S4). Ten *cis*-acting elements related to the hormone stress response were screened for analysis (Fig. 6). Among these, abscisic acid responsiveness (ABRE) was present in the most *MsMAPK* members (65), and flavonoid biosynthesis (MBSI) was present in the least members (7). The results showed that *MsMAPKs* may play corresponding roles in regulating growth and development, hormone response and stress response.

Expression of *MAPK* genes in different tissues of *M. sativa*

To clarify the expression patterns of the *MsMAPK* genes in different tissues, the transcriptome data of six different tissues (roots, elongated stems, pre-elongated stems, leaves, flowers, and nodules) of *MsMAPKs* were obtained from a public database (Table S5). The results showed that 58 *MsMAPK* genes were expressed in at least one tissue. Among them, two *MsMAPKs* showed tissue-specific expression (Fig. 7A), and 35 *MsMAPKs* were expressed in six tissues (Fig. 7D). For example, *MsMAPK18* was only expressed in elongated stems, and *MsMAPK7* was expressed in six different tissues. In addition, 3, 4, 7 and 7 *MsMAPK* genes were expressed in 2, 3, 4 and 5 different tissues, respectively. (Fig. 7A-C). Although some *MsMAPK* genes can be expressed in a variety of tissues, the expression patterns of these genes in different tissues vary greatly. For example, the expression levels of *MsMAPK7* in flowers and leaves were significantly greater than those in the other four tissues. It can be speculated that the *MsMAPK* genes may play roles in different growth and developmental stages.

Expression of *MAPK* genes in *M. sativa* under abiotic stress response

To explore the potential regulatory mechanisms of the *MsMAPK* genes under different stresses, the RNA-seq data of alfalfa plants under thre abiotic stresses (salt, drought, cold) were analyzed (Table S6). As shown in Fig. 8A-C, multiple *MsMAPK* genes exhibited responses to salt (11 genes), drought (10 genes), cold (11 genes), respectively. As shown in Fig. 8D, there were 10 *MsMAPK* genes that responded to only one stress, e.g., *MsMAPK3*, *MsMAPK36*, and *MsMAPK50*

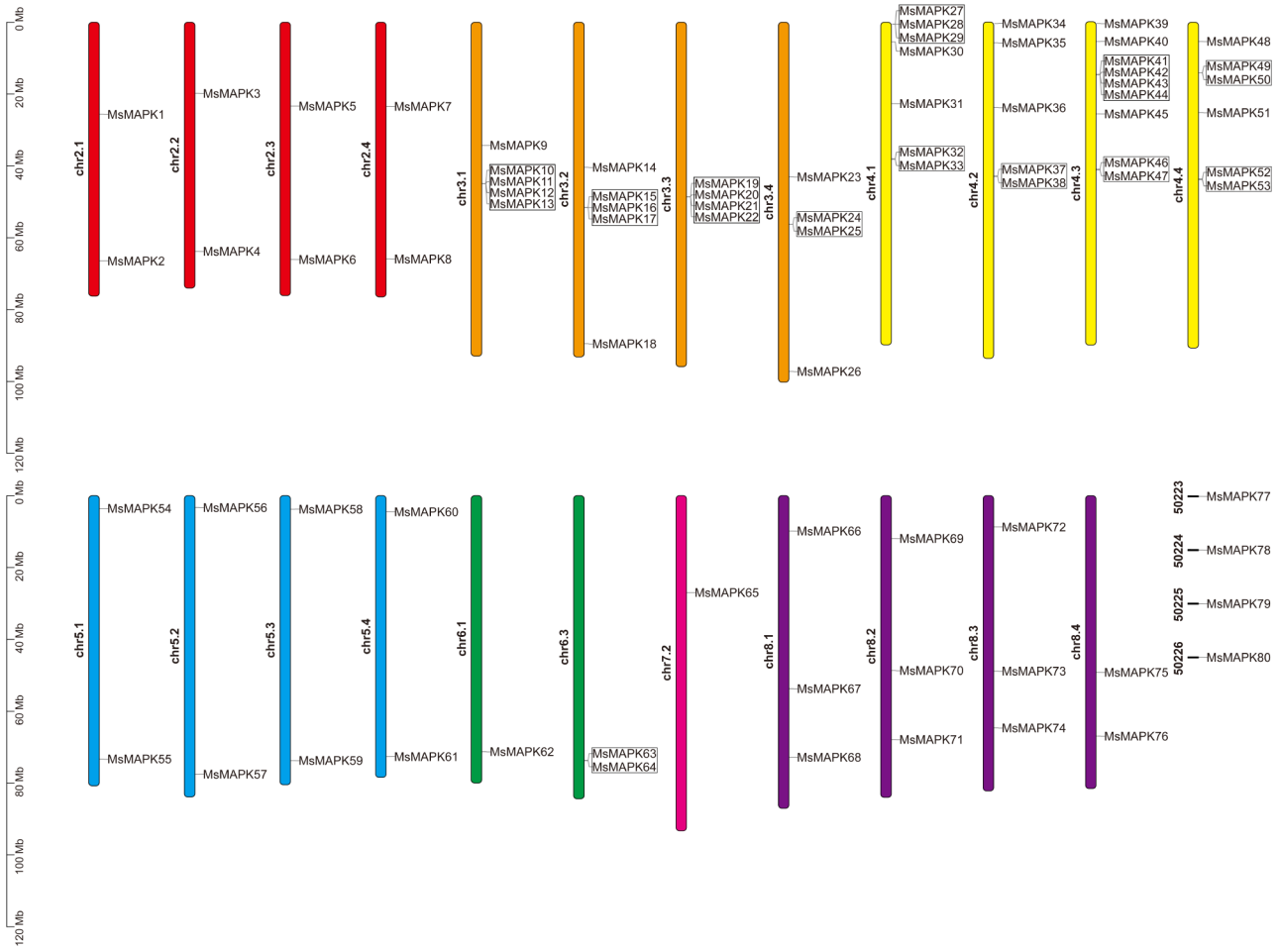

Fig. 1 Chromosome distribution of the *MAPK* genes in *M. sativa*. Each color represents different groups of chromosomes. *MsMAPK* genes were renamed as *MsMAPK1-MsMAPK80* according to the order of chromosomes and the position of *MsMAPK* gene on chromosomes. Rectangle represents a tandem duplication event

responded only to salt, drought, and cold stress, respectively. Eight *MsMAPK* genes can respond to two stresses, e.g., *MsMAPK1*, *MsMAPK70*, *MsMAPK60*. However, two *MsMAPK* genes can respond to all three stresses (*MsMAPK5*, *MsMAPK7*).

To verify the RNA-seq data, several key genes were selected for RT−qPCR analysis. The experimental primers used are shown in Table S7. As shown in Fig. 9, under drought stress, the expression of the *MsMAPK7*/*33*/*36* increase first but never goes down the control. Under cold stress, the expression of *MsMAPK7* gradually increased over time while expression of *MsMAPK51* gradually decreased over time. The expression of the *MsMAPK53* increase first but never goes down the control. Under salt stress, the expression of the *MsMAPK7* and *MsMAPK33* increase first but never goes down the control. The RT−PCR results were consistent with the RNA-seq data.

Discussion

The mitogen-activated protein kinase (MAPK) cascade exists widely across eukaryotes and has been studied in many plants, such as *A. thaliana* [28], *G. max* [29], and *O. sativa* [32]. However, the *MAPK* genes family has not been described in alfalfa. In this study, a total of 80 *MAPK* genes were identified in the "Xinnjiangdaye" genome. Activated *MAPKs* can be transported to the cytoplasm or nucleus to phosphorylate other kinases, transcription factors and cytoskeleton-binding proteins to regulate various cellular activities [55]. Subcellular localization prediction of 80 *MsMAPKs* revealed that 64 genes were predicted to be located in the cytoplasm or nucleus, which was consistent with the findings of previous studies [53]. In *A. thaliana*, *MAPK* genes are divided into four different groups according to their evolutionary relationships and the presence of TDY and TEY phosphorylation motifs. Among them, groups A, B and C contain TEY motifs, and group D contains TDY motifs, which is consistent with the results of this study. In other

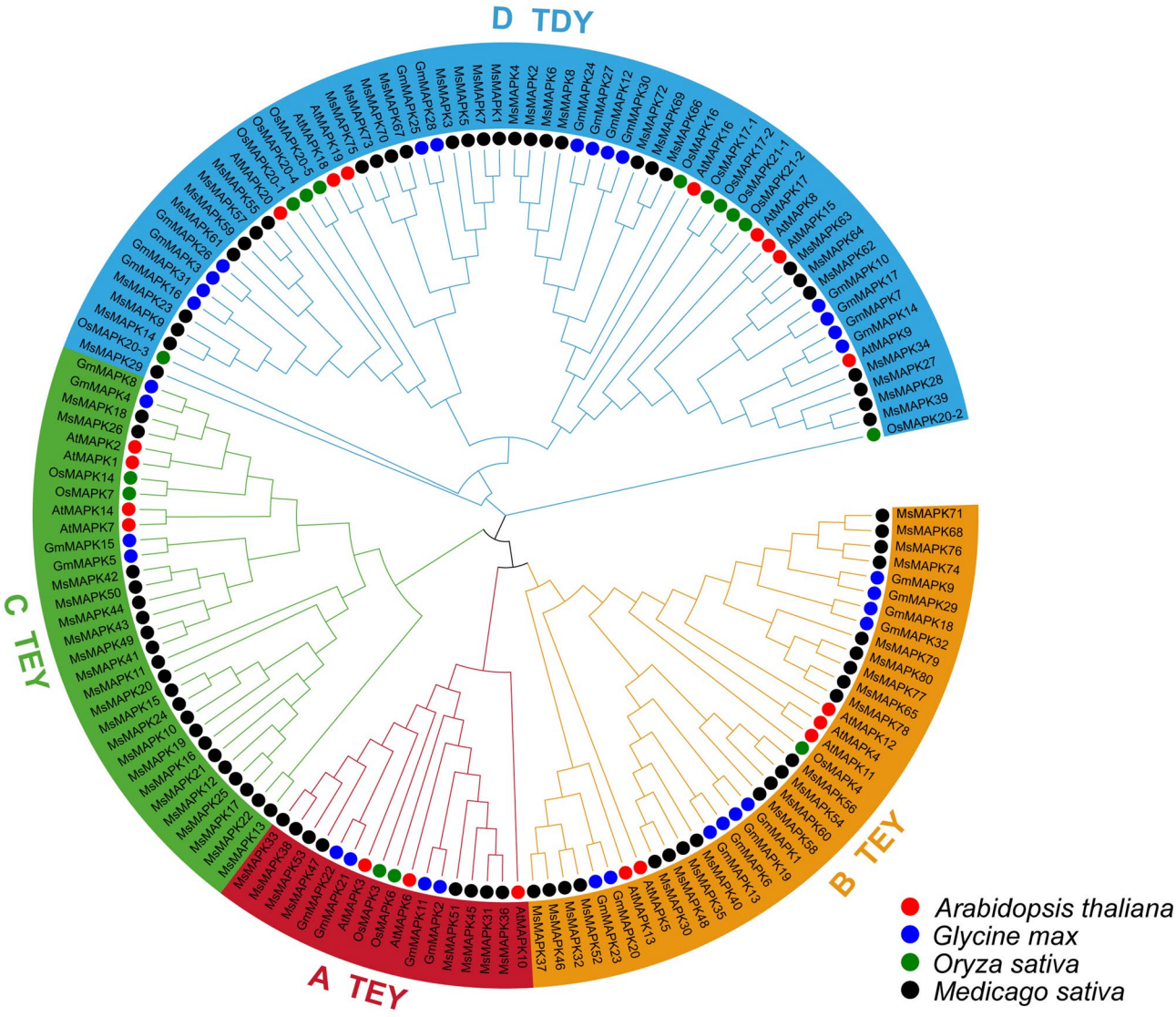

Fig. 2 Phylogenetic tree of *MAPK* genes in *M. sativa*, *A. thaliana*, *G. max* and *O. sativa*. Red, orange, green and blue represent the A, B, C and D subgroup, respectively. The red, blue, green and black circles represent *A. thaliana*, *G. max*, *O. sativa*, and *M. sativa*, respectively

studies, MAPK proteins include not only TEY and TDY motifs but also MEY, TEM, TSY, TEC, TVY, etc. [56]. Presence of only TEY and TDY motifs among alfalfa MAPKs indicate conservation of protein motifs.

The amplification of *MAPK* family members is essential for plant evolution [12]. Compared with the 20 *MAPK* genes in *A. thaliana*, there are 17 *MAPK* genes in *Medicago truncatula*, and there are far more *MAPK* genes in *M. sativa* than in *A. thaliana*. The reason may be that *A. thaliana* and *Medicago truncatula* use the haploid genome [57, 58], while this study used the "Xinjiangdaye" tetraploid genome [59]. Duplications of individual genes, chromosome segments, or entire genomes are common. In some cases, these duplications can facilitate the evolution of new functions that enable plants to better cope with stress [60]. Plant genomes tend to evolve at a higher rate than other eukaryotic genomes, leading to higher genome diversity [61]. Tandem duplication and segmental duplication are the two main forms of plant gene family expansion [62]. The analysis of gene duplication events revealed segmental duplication events were significantly more than tandem duplication events in *MsMAPKs*, indicating that segmental duplication is the main mechanism of *MAPK* gene family evolution and expansion in alfalfa. There were significantly more homologous gene pairs between alfalfa and leguminous plants than between alfalfa and *A. thaliana*, and the most homologous gene pairs were found in *G. max*. The homologous gene pairs between alfalfa and legumes were significantly more than those between alfalfa and *Arabidopsis*, and the most homologous gene pairs were found in soybean, indicating

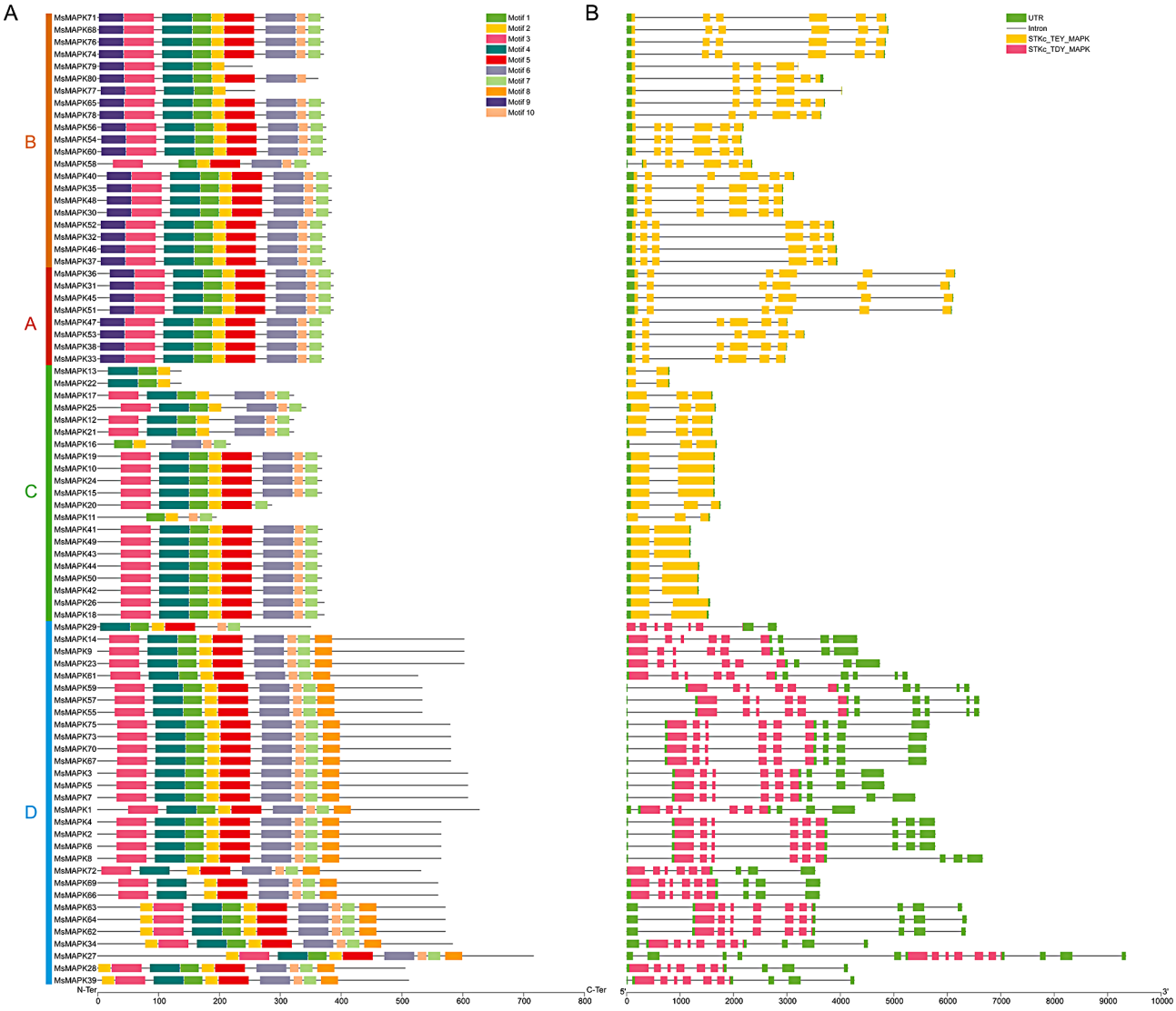

Fig. 3 Basic structures and motifs of the *MsMAPK* genes. (**A**) The structure of MsMAPK protein motif. (**B**) Basic structures of *MsMAPK* genes

that the distribution of *MAPK* genes in legumes was relatively conservative.

Cis-acting elements on the *MsMAPK* gene promoters are involved in a variety of cellular functions. The MAPK cascade is a highly conserved signaling pathway in higher plants that is involved not only in cell division, apoptosis and plant growth and development but also in plant responses to abiotic stress [1]. As mentioned above, *MPK3* and *MPK6* are related to the formation of pollen tubes and stomata in terms of growth and development [30, 31]. The hormone response is related to the synthesis of salicylic acid and ethylene [32, 33]. In terms of stress responses, they are not only related to cold stress [34] but can also interact with AtPFA-DSP5 to negatively regulate the salt responses of plants [63]. To a certain extent, they can also enhance salt tolerance through their negative regulation of *Arabidopsis* response regulator 1 (ARR1), ARR10 and ARR12 protein stability [64]. Through BLAST and phylogenetic analyses, the *MsMAPK* genes with the highest similarity between *ATMPK3* and *ATMPK6* in alfalfa were identified. Among them, *MsMAPK33*/*38*/*47*/*53* had the high similarity with *ATMPK3* (identity > 82%), and *MsMAPK31*/*36*/*45*/*51* had the high similarity with *ATMPK6* (identity > 88%). By analysing the RNA-seq data from different tissues, among them, 6 *MsMAPKs* were expressed in all six tissues, but at different levels. Except for *MsMAPK45*, the other five genes had relatively high expression levels in roots. According to previous studies, YODA and *MPK6* regulate cell division and mitotic microtubules through an auxin-dependent mechanism and are involved in the development of postembryonic roots [65], therefore, it can be speculated that they may have similar functions. The analysis of RNA-seq data for different stresses

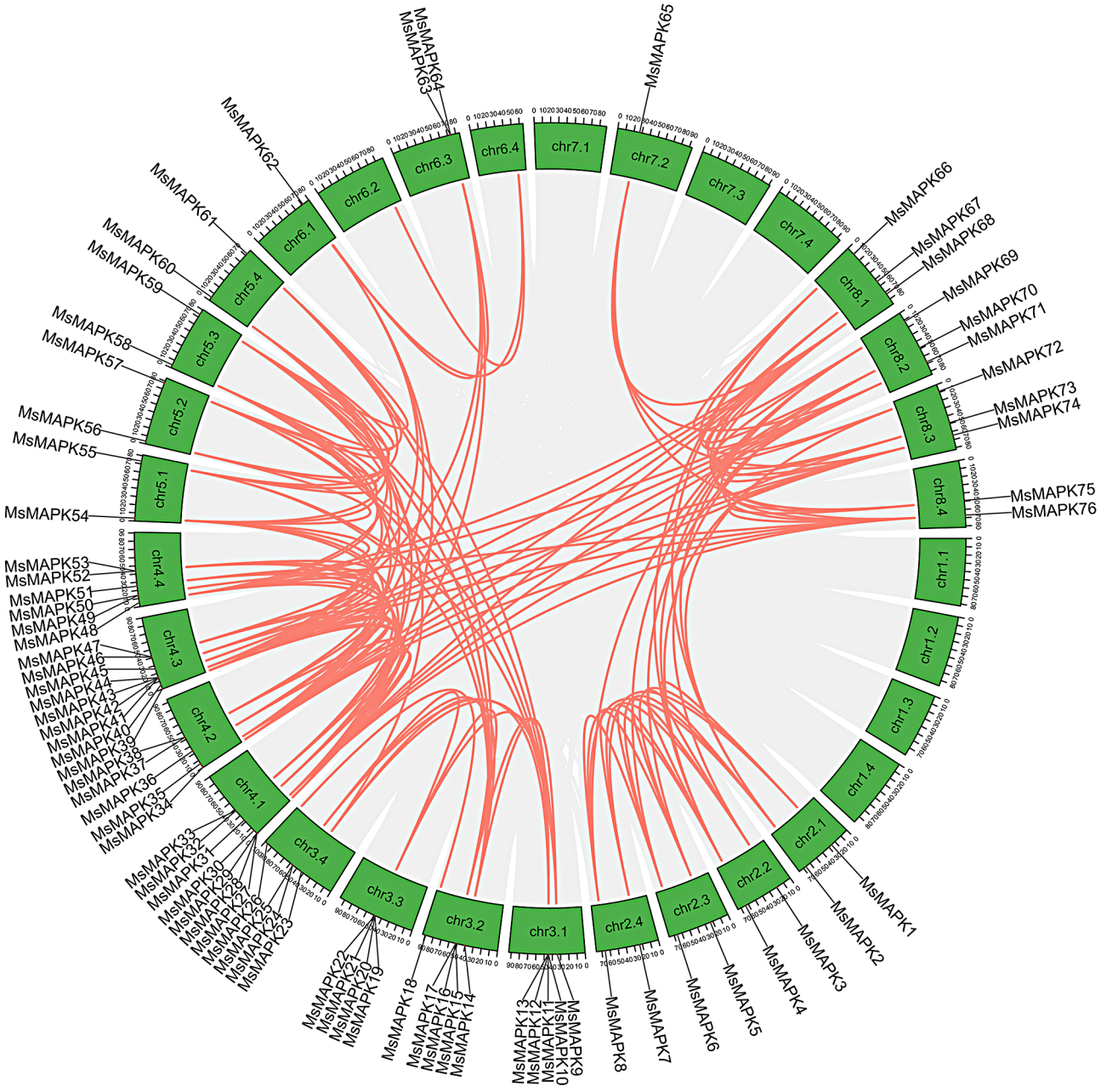

Fig. 4 Schematic diagram of the syntenic relationships of *MsMAPK* genes in *M. sativa*. The gray ribbons represent syntenic blocks in the alfalfa genome, and the segmental duplication events are marked in red

revealed that 11, 10 and 11 *MsMAPK* genes exhibit responses to salt, drought, and cold stress, respectively. Among them, *MsMAPK70* and *MsMAPK75* can exhibit responses to salt and cold stress. *MsMAPK60* and *MsMAPK73* can exhibit responses to cold and drought stress. *MsMAPK1*/*33*/*64*/*71* can exhibit responses to salt and drought stress. Notably, *MsMAPK5* and *MsMAPK7* can exhibit responses to four stresses. The above *MsMAPK* genes may be key for improving the abiotic stress resistance of alfalfa.

Alfalfa is an important forage crop. However, because most of the planting areas in China are located in areas with severe salinization, it is inevitable that many abiotic stresses are encountered during the growth of alfalfa, resulting in a decline in quality. Therefore, it is very important to cultivate new varieties of alfalfa with good stress resistance. In this study, several important stress-responsive *MsMAPK* genes were predicted by analysing RNA-seq data. In the next study, these several *MsMAPK* genes can be transgenic or gene edited to verify their functions. With the development of transgenic

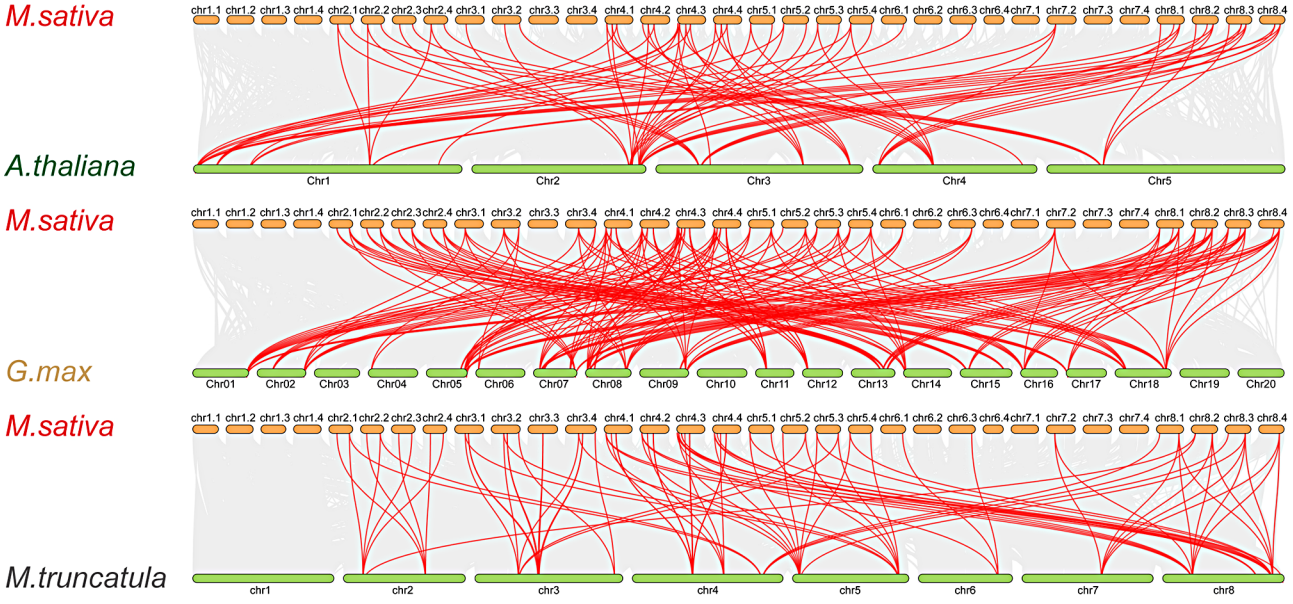

Fig. 5 Collinearity analysis of the *MsMAPK* genes with those of *A. thaliana*, *Medicago truncatula* and *G. max*. The gray ribbons represent syntenic blocks in the alfalfa genome, and the segmental duplication events are marked in red

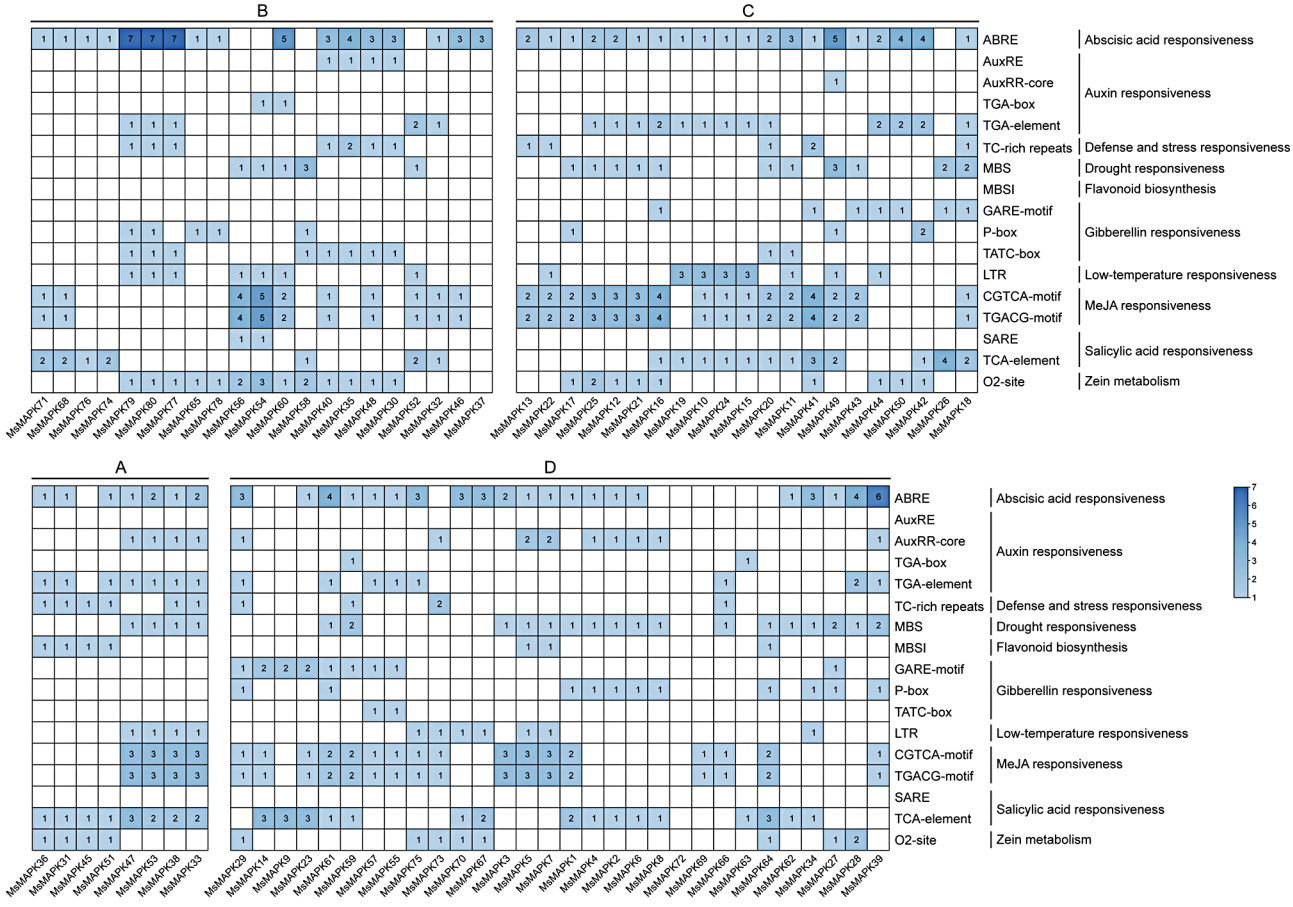

Fig. 6 Cis-acting elements of the *MAPK* gene promoters in *M. sativa*.**A-D**: *MsMAPK* genes were divided into four groups

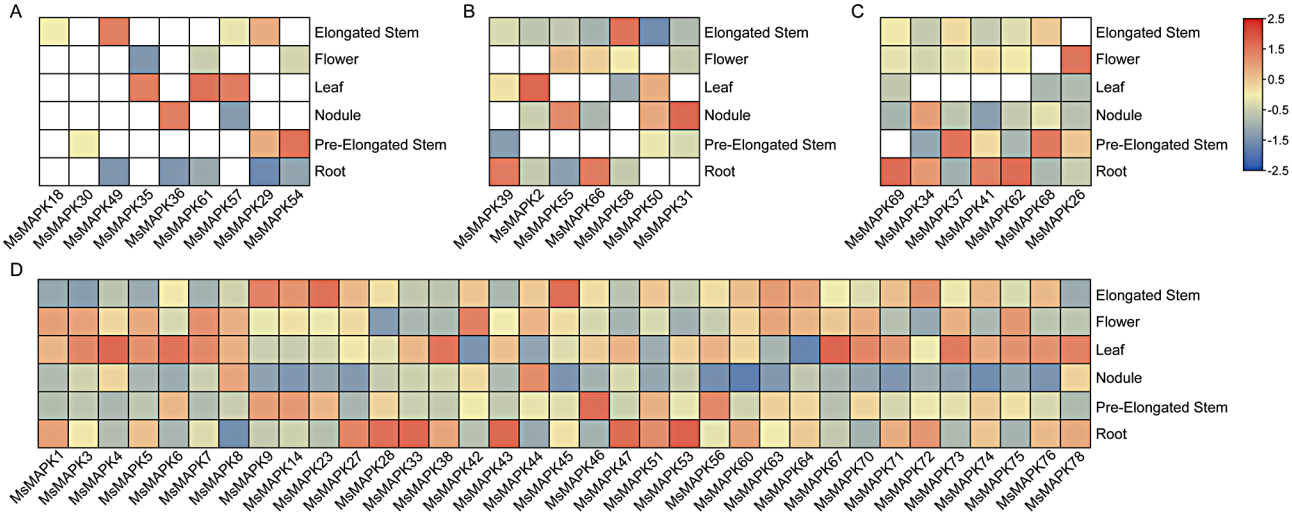

Fig. 7 Expression of 58 *MsMAPK* genes in different tissues. The expression levels were normalized by row using the Z – scores algorithm. The color scale on the right side of the heatmap indicates the relative expression levels, and the color gradient from blue to red indicates an increase in expression levels

technology and gene editing technology, it has become possible to breed new alfalfa varieties with high stress resistance through molecular breeding.

Conclusions

In this study, 80 *MsMAPK* genes were identified in the alfalfa genome; these genes were subdivided into groups A, B, C and D. Group D contained the most *MsMAPK* genes. All *MsMAPK* genes contained a TXY domain; *MsMAPK* genes in groups A, B, and C contained a TEY domain, and *MsMAPK* genes in group D contained a TDY motif. there were significantly more segmental duplication events than tandem duplication events for the *MsMAPK* genes. Tissue RNA-seq analysis revealed that 2 *MsMAPKs* exhibited tissue-specific expression, and 35 *MsMAPKs* exhibited pantissue expression. Abiotic stress RNA-seq analysis revealed that 11, 10 and 11 *MsMAPKs* exhibited responses to salt, drought and cold stresses, respectively. Among them, two *MsMAPKs* (*MsMAPK70* and *MsMAPK75*) responds to salt and cold stresses; two *MsMAPKs* (*MsMAPK60* and *MsMAPK73*) responds to cold and drought stresses; four *MsMAPKs* (*MsMAPK1*, *MsMAPK33*, *MsMAPK64* and *MsMAPK71*) responds to salt and drought stresses; and two *MsMAPKs* (*MsMAPK5 and MsMAPK7)* responded to all three stresses. In summary, we comprehensively described the *MAPK* genes family of *M. sativa*. The results lay a foundation for future exploration of the function of the *MsMAPK* genes and provide new ideas for molecular breeding of alfalfa.

Materials and methods

In silico identification and analysis of *MAPK* genes in *M. sativa* genome

The alfalfa genome used in this study was from the Alfalfa Genome Project (https://fgshare.com/projects/whole_genome_sequencing_and_assembly_of_Medicago_sativa/66380) [66]. The MAPK proteins of *A. thaliana* (TAIR database: https://www.arabidopsis.org/), *G. max* (*Glycine max* genome database: https://www.soybase.org/), and *O. sativa* (RGAP database: http://rice.uga.edu/) were used as BLAST templates. The hidden Markov model (HMM) was used to obtain the configuration file (PF00069) of the MAPK domain from the Pfam database to further remove redundancy [67]. The theoretical molecular weights (MWs), isoelectric points (pIs) and instability indices of the *MsMAPKs* were estimated using tools available at the ExPASy database (http://www.ExPASy.org/).

Phylogenetic and gene and protein structure analysis

Phylogenetic trees were constructed based on the MAPK protein sequences of *M. sativa*, *A. thaliana*, *G. max*, and *O. sativa* using MEGA11, the neighbor-joining (NJ) method and 1000 bootstrap repeats. DNAMAN9 software was used to compare the sequences of alfalfa MAPK proteins. The online MEME website (https://meme-suite.org/meme/tools/meme) was used to analyze the MsMAPK protein motifs, and the number of motifs was set to 10. The conserved domains of the MsMAPK protein were predicted by the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/cdd/). Structural information of alfalfa *MAPK* gene was obtained from alfalfa genome Gff annotation file. TBtools-II (v2.110) software was used to visualize the results of above-mentioned analysis.

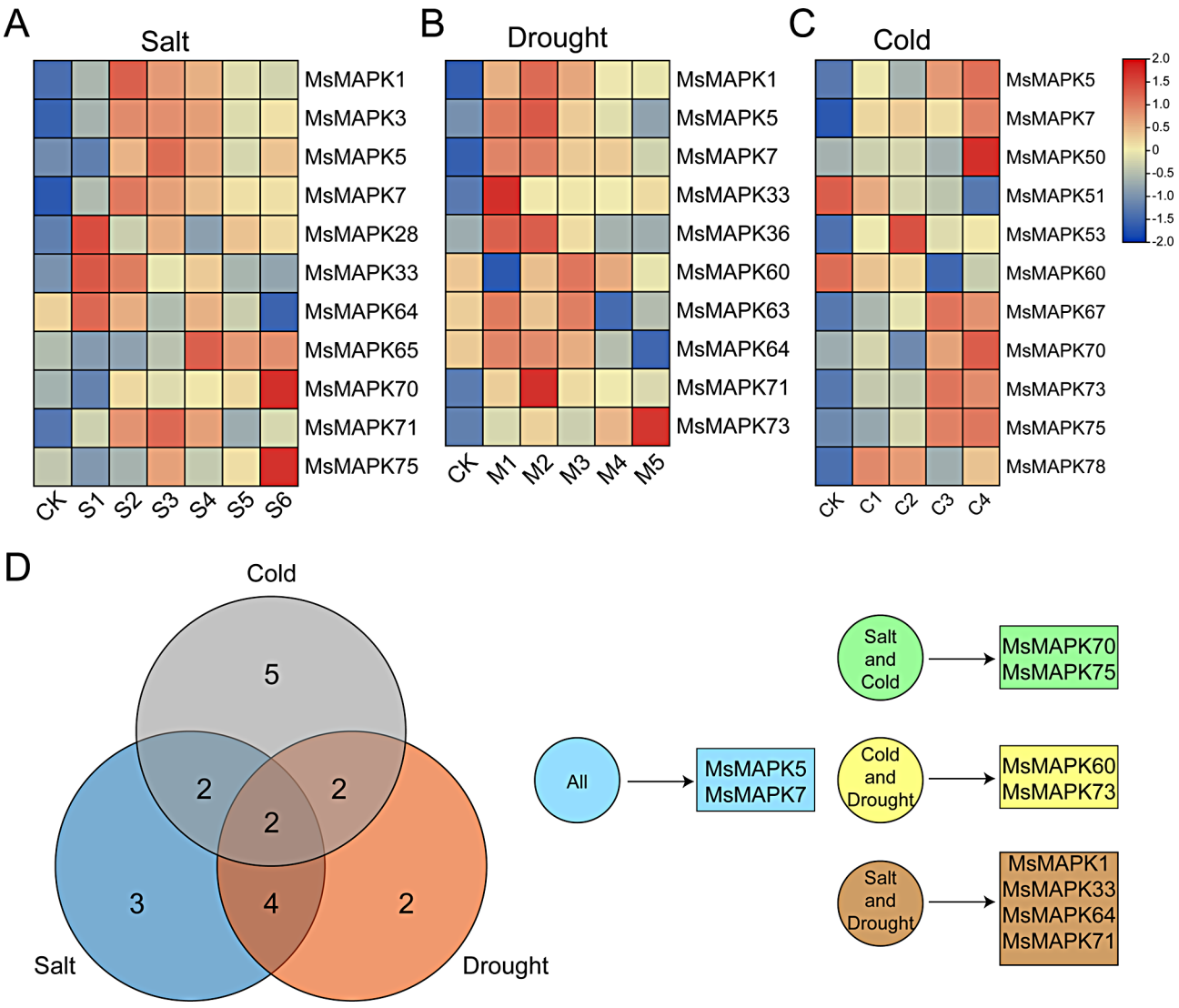

Fig. 8 Expression of *MAPK* genes in *M. sativa* under stress. (**A**) Expression of *MAPK* genes in *M. sativa* under salt stress. (**B**) Expression of *MAPK* genes in *M. sativa* under drought stress. (**C**) Expression of *MAPK* genes in *M. sativa* under cold stress. (**D**) Venn diagrams of *MsMAPK* genes responding to three stresses. Under salt stress: 0 h as CK and 0.5, 1, 3, 6, 12 and 24 h as S1 to S6, respectively. Under drought stress: 0 h as CK and 1, 3, 6, 12 and 24 h as M1, M2, M3, M4 and M5, respectively. Under cold stress: 0 h as CK and 2, 6, 24 and 48 h as C1, C2, C3 and C4, respectively

Chromosomal localization, gene duplication events and collinearity analysis

The chromosomal distribution information of the *MsMAPK* genes was obtained from the gff file of the alfalfa genome, and visualized by TBtools software [68]. The MCScanX tool was used to analyze the collinearity information of *MAPK* genes within and between species [69]. Tandem duplication occurs when two or more genes are located within 200 Kb of the same chromosome. Tandem duplication events were identified by comparing the location of the chromosome where the *MsMAPK* gene is located. The results were visualized with TBtools.

Promoter *cis*-acting element analysis

TBtools was used to extract 2000 bp fragments upstream of the *MsMAPK* gene promoters, and the *cis*-acting elements were identified through the online website PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and visualized using TBtools.

Expression profile analysis using RNA-seq data

RNA-seq data from six alfalfa tissues (roots, elongated stems, pre-elongated stems, leaves, flowers, and nodules) (SRP055547) and studies on response to salt, drought and cold stress (SRR7091780-SRR7091794 and SRR7160313-SRR7160357) were downloaded from the NCBI database for analysis [70, 71]. TBtools was used to visualize the data.

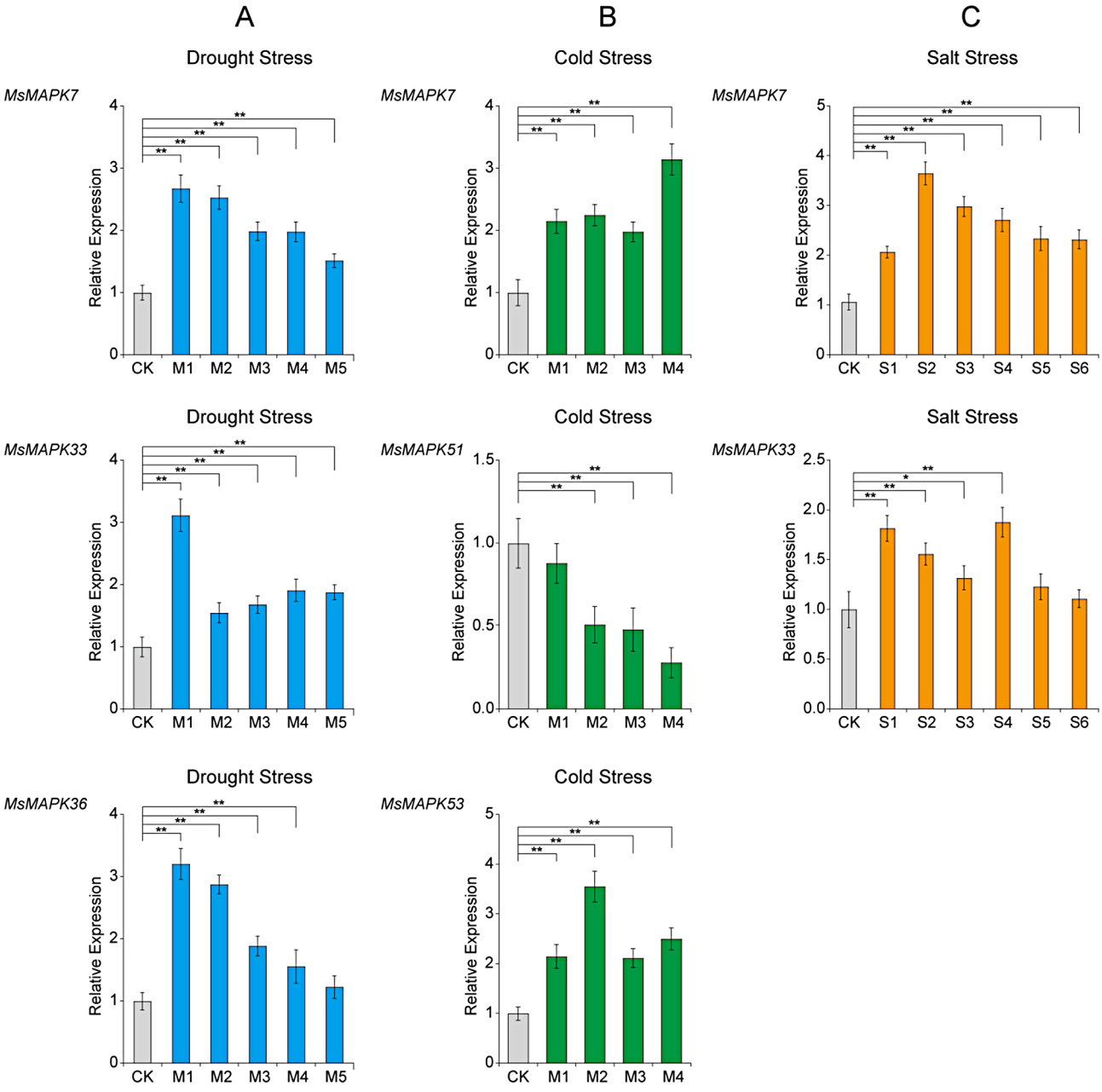

Fig. 9 *MsMAPK* expression under drought, salt and cold stress conditions according to RT–qPCR. (**A**) Expression of *MsMAPKs* under drought stress. (**B**) Expression of *MsMAPKs* under cold stress. (**C**) Expression of *MsMAPKs* under salt stress

Plant materials, growth and stress conditions, and RT–qPCR analysis

Alfalfa (Zhongmu No.1) seeds were obtained from the Institute of Animal Science of the Chinese Academy of Agricultural Sciences. Seeds were treated at 4 °C for 3 days and then cultured in a greenhouse for 2 weeks under a 16/8 hours light cycle, 70–80% relative humidity and 24 °C/20 ℃ day/night temperature conditions. Mannitol (400 mM) was used to simulate drought stress, and root tip samples were collected at 1, 3, 6, 12, and 24 h. Plants were subjected to cold stress treatment at 4 °C, 0 h was selected as the control group, and 2, 6, 24 and 48 h were selected as the sampling time points to take leaf samples. NaCl (250 mM) treatment was used to simulate salt stress. The root tip samples were collected, 0 h was used as the control group, and 0.5, 1, 3, 6, 12 and 24 h were used as the sampling time points. There were three replicates for each stress treatment, and five seedlings were pooled in each replicate. Untreated control plants were cultured normally.

Total RNA was extracted from all samples using TRIzol reagent according to the manufacturer's instructions.

The corresponding cDNA was obtained using an EasyScript First-Strand cDNA Synthesis Kit (random primer (N9)). The primers used in the study were designed using Primer 5.0 software. SYBR Premix Ex Taq (Takara, Japan) and a 7500 real-time fluorescent quantitative PCR system (Applied Biosystems, Foster City, CA, USA) were used for the RT−qPCR experiments. Three replicates were analyzed for each sample, and the data were standardized relative to alfalfa actin gene expression. Relative expression levels were calculated by using the $2-\Delta\Delta CT$ method with Actin2 used as the internal reference.

Abbreviations

MAPK/MPK	Mitogen-activated protein kinase
NLR	Nucleotide-binding domain leucine-rich repeat receptor
ACS	1-amino-cyclopropane-1-carboxylic acid synthase
CBF	C-repeat-binding factor
ICE1	Inducer of CBF expression 1
ABA	Abscisic acid
LIC	Leaf and tiller angle increased controller
MW	Molecular weight
pI	Isoelectric points
aa	Amino acid
PFA-DSP	Plant and fungi atypical dual-specificity phosphatases
ARR	Arabidopsis response regulator
HMM	Hidden Markov model

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05524-4.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Author contributions

Experimental design and planning and first draft writing, H.L., L.C.; preparation and modification of the images, F.H., X.L.; data processing, manuscript modification, Y.Z., G.Z.; data analysis and test data accuracy, L.Z., L.H. and M.L.; application and analysis of the software used in the experiment, S.W., R.L.; data and manuscript review, J.K., Q.Y.; funding acquisition, L.C. All the authors contributed to the article. All authors have read and agreed to the published version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Field and laboratory studies were conducted by local legislation. This article does not contain any studies with human participants or animals and does not involve any endangered or protected species. The plant materials sampled and experiments performed in this research complied with institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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