

Genome-wide Identification of MAPK- MAPKK Gene Families in *Juglans regia* and their Expression Profile under Chilling Stress

Kai Liu

walnut1k@163.com

BinZhou Polytechnic

Yang Li

BinZhou Polytechnic

Lianlian Ji

BinZhou Polytechnic

Guokang Zhang

BinZhou Polytechnic

Hongxia Wang

Hebei Agricultural University

Ruifen Zhang

Qingdao Academy of Agricultural Sciences

Research Article

Keywords: walnut, chilling stress, MAPK- MAPKK, RNA-sequencing, COR gene

Posted Date: September 24th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7568820/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Walnut is a temperate deciduous tree and late-spring frost often causes serious damage to its growth and development. MAPK- MAPKK are widespread in eukaryotes and play an important role in the stress response by regulating the expression of stress-related genes in response to environmental changes. In this study, we screened for the MAPK-MAPKK gene family in the walnut genome database. We found 16 MAPKs, 8 MAPKKs members. Subsequently, we analyzed the structure of each member and performed a phylogenetic analysis. The two families of MAPK-MAPKK were divided into four subgroups, and similar gene structures and conserved motifs were observed in each subgroup. Analysis of the promoter sequences of each member showed that they contained abundant hormone and stress response elements. Transcriptome sequencing showed that the expression of each family member was specific in different tissues and induced by chilling stress. Further, the transcription level of each family member under chilling stress showed a significant correlation in the correlation analysis, and *JrMAPK2*, *JrMAPK3*, *JrMAPK10*, and *JrMAPK15* were directly related to *COR1*, *COR4*, and *COR7* by qRT-PCR. Gene structure, genome evolution, and expression analysis uncovered a relationship between the MAPK-MAPKK cascade in walnut, and that *JrMAPK2*, *JrMAPK3*, *JrMAPK10*, and *JrMAPK15* genes might be pivotal for the walnut chilling response pathway. In this study, we systematically describe the MAPK, MAPKK families in walnut for the first time, and understanding the cascade network of MAPK, MAPKK in plants will help lay a foundation for elucidating their function in the walnut chilling stress response.

1. Introduction

Protein kinases, also known as protein phosphorylases, are a class of enzymes that catalyze protein phosphorylation by transferring the phosphate group on ATP to the amino acid residues of the substrate protein[1]. The mitogen-activated protein kinase (MAPK) cascade is a typical signal transduction pathway that responds to external stimuli, cascades signaling molecules, and amplifies biological signals. In plants, this pathway is critical for the plant signal transduction network, gene expression regulation, cytoplasmic function, biological and abiotic stress responses, hormone responses, cell differentiation, and developmental processes[2–3]. The basic composition of the MAPK signaling cascade includes MAPK kinase (MAPKK), and MAPK, and this tertiary kinase pattern is conserved from yeast to humans. The MAPKKK activates MAPKK, which, in turn, activates MAPK. The activation of MAPK, in turn, transmits upstream signals to downstream response molecules, which jointly regulate cell growth, differentiation, stress adaptation to the environment, and other important physiological or pathological processes[4, 5].

MAPK is a group of serine-threonine protein kinases that can be activated by different extracellular stimuli. MAPK is one of the most important protein kinases in signal transduction and is crucial in plant growth and environmental stress response[6]. The regulation of MAPK activity is reversible[7]. MAPK phosphatase can be inactivated by the dephosphorylation of MAPK. MAPK can also enter the nucleus to activate transcription factors and regulate gene expression[5]. MAPK was first found in Alfalfa (*Medicago sativa* Linn). So far, more than 50 kinds of MAPK genes have been isolated from plants, including Arabidopsis[8], poplar[9], and rice[10]. According to the amino acid sequence comparison of the conserved TXY gene sequence in MAPK, it can be divided into two subtypes, TEY and TDY, in which the former includes three groups A, B, and C, and the latter includes group D[11]. There is no unique and specific docking domain of MAPK in each group of plants, and the consensus of docking domain is group specific[12]. MAPK plays a key role in plant response, growth, and development. For instance, *AtMAPK3* is crucial for oxidative stress in *Arabidopsis thaliana*[13], and PsMAPK4 is involved in mechanical injury[14].

MAPKK is the pathway intermediate of the other two kinases. When plants are stimulated by external stress factors, they can form various signaling pathways with the other two kinases. It connects various MAPK cascade reaction pathways in plants to form a network of aggregation points[4]. Compared to the plant MAPK, MAPKK is a dual kinase that can directly act on T and Y of the T-X-Y motif of MAPK (T is Thr, Y is Tyr). According to the amino acid sequence analysis of tomato[15], poplar[9], rice[11], and Arabidopsis[8], the MAPKK family can be divided into four groups: A, B, C, and D.

In plant cells, the MAPK signaling is involved in almost all kinds of stress responses and development processes, forming complex networks[17–20] to cope with various abiotic stresses (trauma, cold, high salt, ultraviolet (UV) radiation, ozone, reactive oxygen species, drought, high and low osmotic pressure, heavy metals) and pathogens[21–24]. Different elicitors can evoke a common MAPK response, and multiple functional pathways usually share the same MAPK cascade[25].

Holley et al.[26] studied the induction of three MAPK analogs and *LeMAPK1–3* in the suspension cells of *Lycopersicon peruvianum* under the action of the peptide signal molecule systemin, oligosaccharides, and UVB. *LeMAPK1/2* can be independently induced by the above three elicitors, while *LeMAPK3* can only be induced by UVB. Asa et al.[27] found that flg22-MEKK1-MKK4/5-MPK3/6-WRKY22/29 could promote disease resistance gene expression using the *Arabidopsis* protoplast transient expression system. Mao et al.[28] found two MAPK cascades involved in the disease resistance response of *Arabidopsis thaliana*. Infection with AvrPto/AvrPtoB or overexpression of *LeMAPKKKa* and *LeMKK2* in tomatoes can activate *LeMAPK1*, *LeMAPK2*, and *LeMAPK3*, and in vivo and in vitro kinase tests show an upstream and downstream relationship among them[29]. Stulemeijer et al.[30] found that the abundance of the pathogenic bacteria *Xanthomonas campestris* in *LeMKK2* and *LeMPK2*-silenced tomato plants were significantly higher than that in the control. These results indicate that *LeMAPKKKa-LeMKK2*-

LeMPK1/LeMPK2/LeMPK3 pathway is involved in disease resistance in tomatoes. Gang[31] showed that the overexpression of the *VvMKK2/VvMKK4/VvMPK6* gene in *Arabidopsis* improved the plant's ability to resist salt and drought stress. Yang et al.[32] reported that the binding of Ca^{2+} /CaM with CRLK1 upregulates *CRLK1* activity, thereby activating the downstream *AtMEKK1* pathway to regulate chilling stress response. *CRLK1-MEKK1-MKK2-MPK4/MPK6* signaling pathway exists in *A. thaliana* to transmit chilling and salt stress signals and regulate gene expression. Ichimura et al.[33] identified the first complete MAPK signaling pathway AtMEKK1-AtMKK1-AtMPK4 in *A. thaliana* by the yeast two-hybrid test, and this pathway can transmit drought and mechanical damage signals. Hadiarto et al.[34] showed that AtMEKK1 was quickly induced after injury treatment in *A. thaliana* and interacted with the downstream MKK1 and MKK2. These results indicate that the MEKK1-MKK1/2-MPK4 signaling pathway exists in *A. thaliana* and is involved in drought and mechanical injury response. Luo et al.[35] showed that the homologous gene *GhMPK6* of *AtMPK6* was also involved in abscisic acid-induced CAT1 activation and H_2O_2 production in cotton. In *A. thaliana*, the YODA-MKK4/5 (MKK7/9)-MPK3/MPK6 cascade pathway negatively regulates stomatal development[36]. Other studies have also shown that MKK7/9, the upstream kinase of MPK3/6, participates in stomatal development[37].

Walnut (*Juglans regia* L.), a thermophilic tree belonging to the genus *Walnut* of the Walnut family, is an important wood, ecological, economic, and bioenergy tree [38]. Chilling stress affects the normal growth and development of walnut. As discussed earlier, the MAPK and MAPKK family are extensively involved in plants' response to abiotic stress [8, 13, 25], especially in regulating plant cold tolerance [32–33]. However, reports on the relationship between the MAPK-MAPKK family members and walnut cold tolerance are scarce. Hence, in this study, we aimed to obtain the MAPK and MAPKK family members in the walnut genome through bioinformatics. Subsequently, we analyzed the physical and chemical properties, gene structure, conserved motif, evolutionary relationship, and promoter region binding sites of the family members. We also analyzed the transcriptional expression of the family members in different tissues and under chilling stress, and the gene analysis of the MAPK-MAPKK members in response to cold stress will lay a foundation for understanding this regulatory network and improve walnut stress resistance through efficient breeding and molecular genetic strategies.

2. Materials and Methods

2.1. RNA-sequencing and quantitative reverse transcription PCR (qRT-PCR) of walnut tissues

We collected the leaves of 'Qingxiang' and 'Liaoning No.8' grafted on the same rootstock potted seedlings treated with chilling stress 0.5 °C for 0, 4, 8, 12, 24, 36, and 48 h. The leaves collected from the 0 and 48 h treatment groups were subjected to RNA-sequencing. The total RNA was extracted and reverse transcribed. The gene-specific primers used for qRT-PCR were designed using NCBI (Table 1). The sequencing data were stored in the National Genome Science Data Center (<https://bigd.big.ac.cn/?lang=zh>) with the project ID PRJCA006073.

The tissues of the roots, bud, and young stems, young leaves, and male flowers, female flowers, and mature leaves of 'Qingxiang' walnut were collected from the herbarium of the Hebei Agricultural University. After subjecting the tissues to chilling stress 0.5 °C for 0, 4, 8, 12, 24, 36, and 48 h, they were sent to the Beijing Nuohe Zhiyuan Science and Technology Co., Ltd. (Beijing, China) for RNA sequencing. The kit used for RNA extraction was from Tiangen, China (product number: DP441), and the kits for reverse transcription and real-time PCR were from Takara Bio, Japan (product numbers: RR047A and RR403A, respectively).

The qRT-PCR experiments were performed on an FX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using a TB Green® Fast qPCR Mix (Takara Bio). *JrActin* was used as the reference gene to calculate the relative fold differences based on the comparative cycle threshold ($2^{-\Delta\Delta\text{Ct}}$). The PCR solution (20 µL) contained 10 µL of SYBR-Green PCR Master Mix, 0.2 µM of each primer, 100 ng of cDNA template, and nuclease-free water. The PCR conditions were as follows: 95°C for 5 min; 94°C for 10 s, 60°C for 30 s, and 72°C for 30 s (35 cycles); followed by a final extension at 72°C for 3 min.

Table 1
The primers of qRT-PCR.

Primer Name	Forward Primer	Reverse Primer	Gene ID
JrMAPK2	ACAATCATTCGTTAGGGCTT	TTGTA CT TGGCTGTAACTC	Table 2 – 1
JrMAPK3	TTCGACTTTGAGCAAAAACC	GCAGACACACCAAAGTAATG	Table 2 – 1
JrMAPK10	ACAGTTACAGGGTACAACAG	CCAAGATGTTGCCGAATATG	Table 2 – 1
JrMAPK15	CTGTGGAAGAGGTAGAGAGA	GCTATTTTCTTCATGGCGAC	Table 2 – 1
JrCOR1	TGTACAAACTGACTCTGTGG	TTAACACATCCATGCAGACA	108984723
JrCOR4	GGTCCAAAATAATCAAGCG	CAGACCTTATCAGCTCACTC	109010700
JrCOR5	AGCTGGTAACATTTCTCCTG	ACGTTGTCCACAAAAGAAG	108998923
JrCOR7	GGATATCGGACTTCCATCAC	TAGATGATCTCTGGCCTGTT	108995330
JrCOR9	ATCCTAACCTAAGCGAATGG	CACGTGTCAGTTATATCGGT	108984239

2.2. Statistical Analysis

The data were analyzed using the SPSS 17.0 software.

3. Results

3.1. Identification and analysis of the MAPK and MAPKK families in *Juglans regia* genome

The MAPK and MAPKK families subfamily were identified using the protein sequence information from *Arabidopsis*, and a hidden Markov model was constructed and the protein data were searched in the Pfam database. We obtained 16 MAPKs, 8 MAPKKs, which were numbered according to their annotation information in walnut (Tables 2 – 1, 2–2).

The shortest and longest lengths among the MAPKs were 3132 bp and 20057 bp, respectively. The ORF lengths of these genes ranged between 993–1872 bp, and their protein lengths ranged between 330–623 amino acids. Further, the molecular weight (MW) of the proteins ranged between 37.52 kDa (JrMAPK1) and 70.76 kDa (JrMAPK5), and the isoelectric point (pI) ranged between pH 5.04 (JrMAPK16) and 9.19 (JrMAPK12), with 50% of proteins being weakly acidic (pI < 7) and 50% partially basic.

Table 2
– 1. Information of MAPKs in *Juglans regia*.

Gene Name	Transcript ID	Locus ID	Gene length (bp)	Protein length (AA)	ORF length (bp)	PI
<i>JrMAPK1</i>	XM_018952042.1	LOC108980971	3957	330	993	8.56
<i>JrMAPK2</i>	XM_018956622.1	LOC108984609	5546	400	1203	5.44
<i>JrMAPK3</i>	XM_018963328.1	LOC108989647	5189	375	1128	5.59
<i>JrMAPK4</i>	XM_018965593.1	LOC108991370	3380	372	1119	6.67
<i>JrMAPK5</i>	XM_018968431.1	LOC108993499	8395	623	1872	9.17
<i>JrMAPK6</i>	XM_018968507.1	LOC108993546	3170	368	1107	8.51
<i>JrMAPK7</i>	XM_018970449.1	LOC108994997	7786	556	1671	8.57
<i>JrMAPK8</i>	XM_018980337.1	LOC109002546	8489	603	1812	6.47
<i>JrMAPK9</i>	XM_018982649.1	LOC109004185	4534	379	1140	6.50
<i>JrMAPK10</i>	XM_018983827.1	LOC109005059	5826	390	1173	5.33
<i>JrMAPK11</i>	XM_018984996.1	LOC109005905	7113	562	1689	8.65
<i>JrMAPK12</i>	XM_018993027.1	LOC109011723	6008	603	1812	9.19
<i>JrMAPK13</i>	XM_018995980.1	LOC109013779	20057	520	1563	7.27
<i>JrMAPK14</i>	XM_019002985.1	LOC109020511	3132	368	1107	7.66
<i>JrMAPK15</i>	XM_019004886.1	LOC109022081	4479	374	1125	5.38
<i>JrMAPK16</i>	XM_019005092.1	LOC109022242	3897	371	1116	5.04

Among the MAPKKs, the shortest and longest lengths were 1074 bp and 11789 bp, respectively. The ORF lengths of these genes ranged from 948 to 1569 bp, the protein lengths from 315 to 522 amino acids, and their MW from 35.5 (JrMAPK1) to 58.02 kDa (JrMAPK5). Further, their pI ranged from 5.32 (JrMAPK16) to 9.26 (JrMAPK12), with 50% of proteins being weakly acidic (pI < 7) and 50% partially basic.

Table 2
2. Information of MAPKKs in *Juglans regia*.

Gene Name	Transcript ID	Locus ID	Gene length (bp)	Protein length (AA)	ORF length (bp)	PI
<i>JrMAPKK1</i>	XM_018954584.1	LOC108983062	1676	352	1059	9.26
<i>JrMAPKK2</i>	XM_018968108.1	LOC108993253	1176	315	948	7.63
<i>JrMAPKK3</i>	XM_018980752.1	LOC109002837	9865	354	1065	5.84
<i>JrMAPKK4</i>	XM_018986827.1	LOC109007236	6286	522	1569	5.32
<i>JrMAPKK5</i>	XM_018989318.1	LOC109008993	11789	354	1065	5.74
<i>JrMAPKK6</i>	XM_018997755.1	LOC109015276	1074	328	987	7.25
<i>JrMAPKK7</i>	XM_019000840.1	LOC109018692	6880	354	1065	5.90
<i>JrMAPKK8</i>	XM_019001287.1	LOC109019062	1314	378	1137	8.51

The SignalP 3.0 Server and TMHMM method (a membrane protein topology prediction method based on a hidden Markov model) was used to analyze protein signal peptide prediction and transmembrane structure (Tables 3 – 1, 3 – 2). Among the screened proteins, while JrMAPK1 and JrMAPKK11 had a signal peptide. ExPasy was used to predict the physicochemical properties of a protein, such as the gene instability index and hydrophobicity. The instability coefficient of members of the signaling cascade with only JrMAPK14, JrMAPK6, JrMAPK12, JrMAPK5, JrMAPK7, JrMAPK11, and JrMAPK4 being stable. All screened proteins were hydrophilic, and the average hydrophobicity of the proteins ranged from approximately for 0.101 (JrMAPKK5). The ngLOC and Nuc-PLoc online software prediction results showed that these proteins were located in the nucleus, chloroplast, cytoplasm, mitochondria, and PLA.

We also analyzed the differences in the post-translational modifications of the MAPK-MAPKK members, which might be helpful in the subsequent study of protein function. Using the Net NGlyc 1.0 and NetPhos 3.1 Server, we found that the member proteins contained multiple phosphorylations, N-glycosylation, and O-glycosylation sites. However, there were differences in the number of serine, threonine, and tyrosine phosphorylation sites among the proteins.

Table 3
– 1. Information on the physical and chemical properties of MAPKs in *Juglans regia*.

Protein Name	Molecular weight(KDa)	Transmembrane structure	Signal peptide	Instability index	Hydropathicity	Subcellular localization	Number of Phosphorylation sites			Number of Glycosylation sites	
							Ser	Thr	Tyr	N-Gly	O-Gly
JrMAPK1	37.52	No	NO	40.66	-0.435	Nucleus.	16	9	8	1	18
JrMAPK2	45.62	No	NO	40.8	-0.348	Nucleus.	14	8	9	2	4
JrMAPK3	43.49	No	NO	41.23	-0.266	Nucleus.	7	10	5	2	6
JrMAPK4	42.57	No	NO	39.83	-0.197	Nucleus.	14	8	7	0	6
JrMAPK5	70.76	No	YES	35.33	-0.458	Nucleus.	29	20	9	3	20
JrMAPK6	42.16	No	NO	33.38	-0.171	Nucleus.	7	12	6	0	2
JrMAPK7	63.23	No	NO	36.57	-0.478	Nucleus.	26	11	13	2	24
JrMAPK8	68.77	No	NO	46.53	-0.531	Nucleus.	34	17	11	0	45
JrMAPK9	43.11	No	NO	43.77	-0.308	Nucleus.	17	12	7	1	11
JrMAPK10	44.59	No	NO	44.04	-0.265	Nucleus.	9	7	5	3	5
JrMAPK11	64.08	No	NO	37.68	-0.483	Nucleus.	27	11	12	1	23
JrMAPK12	68.39	No	NO	33.51	-0.410	Nucleus.	24	16	9	2	26
JrMAPK13	59.00	No	NO	40.02	-0.541	Nucleus.	32	13	10	1	33
JrMAPK14	42.28	No	NO	32.51	-0.284	Nucleus.	11	11	4	0	5
JrMAPK15	43.16	No	NO	47.72	-0.278	Nucleus.	7	8	5	2	4
JrMAPK16	42.74	No	NO	46.83	-0.323	Nucleus.	17	9	7	2	5

Table 3
– 2. Information on the physical and chemical properties of MAPKKs in *Juglans regia*.

Protein Name	Molecular weight(KDa)	Transmembrane structure	Signal peptide	Instability index	Hydropathicity	Subcellular localization	Number of Phosphorylation sites			Number of Glycosylation sites	
							Ser	Thr	Tyr	N-Gly	O-Gly
JrMAPKK1	39.30	No	NO	63.71	-0.438	Nucleus.	22	8	2	1	15
JrMAPKK2	35.50	No	NO	49.37	-0.271	Nucleus.	16	11	3	0	9
JrMAPKK3	40.02	No	NO	47.58	-0.128	Nucleus.	22	8	3	0	14
JrMAPKK4	58.02	No	NO	44.61	-0.180	Nucleus.	32	14	9	1	29
JrMAPKK5	39.32	No	NO	41.44	-0.101	Nucleus.	21	10	2	1	9
JrMAPKK6	37.05	No	NO	57.30	-0.283	Nucleus.	9	12	2	0	7
JrMAPKK7	39.82	No	NO	46.24	-0.169	Nucleus.	24	7	3	0	13
JrMAPKK8	42.33	No	NO	52.83	-0.243	Nucleus.	20	14	4	2	25

3.2. Phylogenetic tree construction

The amino acid sequences of MAPK and MAPKK family members of *A. thaliana* and walnut were downloaded from the TAIR and NCBI websites, respectively. Based on the amino acid sequences of the member proteins, the phylogenetic tree was constructed by the neighbor-joining

method using MEGA7.0 software. As shown in Fig. 1, the evolutionary relationship among the members of the MAPK and MAPKK families were analyzed. Based on the classification of *A. thaliana*, the MAPK-MAPKK pathway in *Juglans regia* was divided into four subgroups—A, B, C, and D. In the MAPK family, subgroups C and D had the highest number of members (six), while subgroup A had the least (three). In the MAPKK family, subgroup D had the highest number of members (four) while subgroup B had the lowest (one).

3.3. Multiple sequence alignment, gene structure, and conserved motif analysis

Next, we performed the sequence alignment and motif analysis of the MAPK and MAPKK families in *Juglans regia*. We observed that in the MAPK family (Fig. 2), the P-loop, C-loop, CD-domain, and activation-loop were highly conserved. Similarly, the S/TxxxxS/T and the G(T/S)Px(W/Y/F)MAPEV motifs were highly conserved in the MAPKK family and the MEKKK subfamily, respectively. These findings were consistent with previous findings from other species, such as cucumber [38].

The conserved motif distribution of the sequence and structure of these proteins was analyzed using the MEME software (Fig. 3). A common conserved motif distribution was found among the MAPK and MAPKK families and MEKKK subfamily in *Juglans regia*. However, the Ham subfamily lacked the corresponding conserved motif compared with the other members of the subfamily.

The results showed that some members did not contain introns but had similar gene structures in the same subgroup.

3.4. Analysis of promoter elements

The online analysis software PlantCare was used to predict the distribution of stress tolerance and hormone-related elements in the promoter region of the MAPK and MAPKK families in *Juglans regia*. As shown in Table 4, the promoter region of the member genes was rich in hormone signaling and stress response regulatory elements. Further, several members contained light response elements, which might be due to the abiotic stress response.

Table 4
Part of stress-related and hormone in the promoter region of MAPK, MAPKK families in *Juglans regia*.

Gene name	Abscisic acid responsive element	SA responsive element	MeJ responsive element	Auxin responsive element	Gibberellin responsive element	Light responsive element	Low-temperature responsive element	Drought-Inducibility element
<i>JrMAPK2</i>					✓			
<i>JrMAPK3</i>								
<i>JrMAPK4</i>						✓	✓	
<i>JrMAPK5</i>					✓	✓		
<i>JrMAPK6</i>		✓		✓	✓	✓		
<i>JrMAPK7</i>					✓	✓	✓	
<i>JrMAPK8</i>		✓				✓		
<i>JrMAPK9</i>								
<i>JrMAPK10</i>					✓			
<i>JrMAPK11</i>	✓				✓	✓	✓	
<i>JrMAPK12</i>						✓		
<i>JrMAPK13</i>	✓					✓		
<i>JrMAPK14</i>			✓		✓	✓		
<i>JrMAPK15</i>			✓			✓		
<i>JrMAPK16</i>								✓
<i>JrMAPKK1</i>		✓				✓		
<i>JrMAPKK2</i>						✓		
<i>JrMAPKK3</i>								
<i>JrMAPKK4</i>								
<i>JrMAPKK5</i>						✓		
<i>JrMAPKK6</i>						✓		
<i>JrMAPKK7</i>								
<i>JrMAPKK8</i>								

3.5. Analysis of expression patterns and response patterns of the MAPK-MAPKK genes in different tissues and organs

The expression of 16 *JrMAPK*, 8 *JrMAPKK* genes in seven tissues was analyzed using the transcription data of 'Qingxiang.' The expression of different genes in the same tissue was different, and the expression of the same gene in different tissues was also significantly different (Fig. 5A). We observed the highest expression of *JrMAPK9* in female flowers, *JrMAPK3* in roots, *JrMAPK7* in old leaves, *JrMAPK11* in tender stems, *JrMAPKK6* in young leaves, *JrMAPKK1* in male flowers, and *JrMAPKK9* in buds. Notably, *JrMAPK9* had a high expression level in female flowers; *JrMAPK3*, *JrMAPK15*, *JrMAPKK7*, and *JrMAPKK8* in roots; and *JrMAPK7* in old leaves. Further, *JrMAPKK1* had high expression in male flowers but low expression in other tissues.

Further, we analyzed the expression patterns of the members of the MAPK and MAPKK families by RNA sequencing. The RPKM values were converted into Log2P values, and the expression heat map of the MAPK and MAPKK families were drawn (Fig. 5B). The sequencing result demonstrated that the expression of some *JrMAPK*, *JrMAPKK* genes responded to chilling stress. For instance, chilling stress of 4 h significantly decreased the expression of *JrMAPK10*, *JrMAPK12*, and *JrMAPK14*. Under chilling stress of 8 h, *JrMAPKK12* expression significantly increased, while *JrMAPKK9* expression significantly decreased. The expression of *JrMAPK2* and *JrMAPKK15* significantly increased, and that of *JrMAPK2* significantly decreased under a 12 h chilling stress. Under 24 h chilling stress, the expression of *JrMAPK1* and *JrMAPKK1* significantly increased, while *JrMAPK16* expression decreased. Under chilling stress of 36 h, *JrMAPK9* expression was significantly downregulated. Finally, a 48 h chilling stress significantly increased the expression of *JrMAPK5*, *JrMAPK3*, *JrMAPKK7*, *JrMAPKK8*, and

JrMAPKK6 and significantly decreased *JrMAPKK5* and *JrMAPKK11* expression (Figure B). Further, as shown in Fig. 4B, the expression of *JrMAPK3* was the highest in roots. Thus, *JrMAPK*, *JrMAPKK* may be crucial in chilling stress responses.

3.6 Correlation. of expression profile of MAPK-MAPKK gene families in *Juglans regia* under chilling stress

The expression profiles of *JrMAPK*, *JrMAPKK* were analyzed by the SPSS software under chilling stress. Correlation analysis of the MAPK-MAPKK expression profile (Table 5) showed that *JrMAPK1* negatively correlated with *JrMAPKK2*, *JrMAPK2* expression negatively correlated with *JrMAPKK1*, *JrMAPKK8*, and *JrMAPKK2*. Further, the expression of *JrMAPK3* positively correlated with that of *JrMAPKK1*, *JrMAPKK2*, and *JrMAPKK8* and negatively correlated with *JrMAPKK4*. *JrMAPK4* expression negatively correlated with *JrMAPKK3* expression and positively correlated with *JrMAPKK4* and *JrMAPKK5*. *JrMAPK5* negatively correlated with *JrMAPKK1*, *JrMAPKK2*, and *JrMAPKK4* expression and positively correlated with *JrMAPKK3* expression. The above correlations were all statistically significant.

Table 5
of expression profile of MAPK-MAPKK gene families in chilling stress.

	<i>JrMapkk1</i>	<i>JrMapkk2</i>	<i>JrMapkk3</i>	<i>JrMapkk4</i>	<i>JrMapkk5</i>	<i>JrMapkk7</i>	<i>JrMapkk8</i>
<i>JrMapk1</i>	-0.374	-0.624*	0.053	0.026	0.028	-0.178	-0.485
<i>JrMapk2</i>	-0.748**	-0.666*	0.123	0.267	0.082	-0.038	-0.824**
<i>JrMapk3</i>	0.794**	0.777**	0.097	-0.614*	-0.236	0.361	0.881**
<i>JrMapk4</i>	0.051	0.163	-0.813**	0.738**	0.838**	-0.577*	0.033
<i>JrMapk5</i>	-0.619*	-0.599*	0.657*	-0.254	-0.612*	0.172	-0.573
<i>JrMapk6</i>	-0.152	0.331	-0.788**	0.829**	0.816**	-0.718**	-0.137
<i>JrMapk7</i>	-0.099	0.536	-0.289	0.290	0.437	-0.564	0.043
<i>JrMapk8</i>	-0.779**	-0.414	-0.417	0.849**	0.461	-0.632*	-0.745**
<i>JrMapk9</i>	-0.188	0.347	-0.607*	0.713**	0.525	-0.798**	-0.047
<i>JrMapk10</i>	0.721**	0.691*	-0.212	-0.214	0.199	0.120	0.822**
<i>JrMapk11</i>	-0.496	-0.559	-0.226	0.492	0.376	-0.471	-0.576*
<i>JrMapk12</i>	-0.484	-0.428	0.678*	-0.464	-0.561	0.119	-0.318
<i>JrMapk13</i>	-0.356	-0.357	0.787**	-0.581*	-0.763**	0.379	-0.245
<i>JrMapk14</i>	0.013	-0.109	0.242	-0.224	-0.133	-0.087	0.047
<i>JrMapk15</i>	-0.186	-0.223	0.003	-0.090	0.020	-0.260	-0.241
<i>JrMapk16</i>	-0.514	-0.676*	0.231	-0.034	0.000	0.182	-0.697*

3.7 Correlation of the relative expression of four MAPK genes with five cold-related (COR) genes in *Juglans regia* under chilling stress by qRT-PCR

Next, we analyzed the correlation of the relative expression of the four MAPK genes with the five COR genes of the two *Juglans regia* varieties under different chilling stress treatment times by qRT-PCR. The expression of *JrMAPK2*, *JrMAPK3*, *JrMAPK15*, *JrCOR1*, *JrCOR4*, and *JrCOR9* were the highest in the 'Liaoning No. 8' variety treated with chilling stress for 12 h (Fig. 6). The expression of *JrMAPK2*, *JrMAPK3*, *JrMAPK15*, *JrCOR1*, and *JrCOR4* initially decreased and then increased under chilling stress. *JrMAPK3*, *JrCOR4*, *JrCOR5*, and *JrCOR7* expression was the highest in the 'Qingxiang' variety treated with chilling stress for 48 h (Fig. 7).

The MAPK and COR genes in 'Liaoning No. 8' were more active than those in 'Qingxiang,' which might indirectly explain why 'Liaoning No. 8' is more resistant to cold stress than 'Qingxiang.' In 'Liaoning No. 8,' As shown in Table 7 and Table 8, *JrMAPK2* expression significantly positively correlated with *JrCOR1* expression and *JrMAPK3* and *JrMAPK15* expression significantly positively correlated with *JrCOR4*, *JrCOR5*, *JrCOR7*, and *JrCOR9* expression. In 'Qingxiang,' the *JrMAPK2* expression significantly positively correlated with *JrCOR1*, *JrCOR4*, and *JrCOR7* expression. *JrMAPK3* expression significantly positively correlated with *JrCOR4* and *JrCOR1* expression. Further, *JrMAPK10* expression

significantly positively correlated with that of *JrCOR9*, and *JrMAPK15* expression significantly positively correlated with *JrCOR4*, *JrCOR7*, and *JrCOR1* expression. Hence, these findings indicate that these four MAPK genes are involved in chilling stress responses.

Table 7
Correlation of the relative expression of four MAPK and five COR gene in 'Qingxiang' under different chilling stress time.

	<i>JrMAPK2</i>	<i>JrMAPK3</i>	<i>JrMAPK10</i>	<i>JrMAPK15</i>	<i>JrCOR1</i>	<i>JrCOR4</i>	<i>JrCOR5</i>	<i>JrCOR7</i>	<i>JrCOR9</i>
<i>JrMAPK2</i>	1	-0.168	-0.285	-.191	0.886**	-0.193	-0.168	-0.192	-0.189
<i>JrMAPK3</i>	-0.168	1	-0.378	0.989**	-0.314	0.989**	0.993**	0.980**	0.986**
<i>JrMAPK10</i>	-0.285	-0.378	1	-0.265	-0.130	-0.326	-0.294	-0.332	-0.255
<i>JrMAPK15</i>	-0.191	0.989**	-0.265	1	-0.339	0.988**	0.995**	0.974**	0.992**
<i>JrCOR1</i>	0.886**	-0.314	-0.130	-0.339	1	-0.286	-0.284	-0.254	-0.285
<i>JrCOR4</i>	-0.193	0.989**	-0.326	0.988**	-0.286	1	0.996**	0.997**	0.996**
<i>JrCOR5</i>	-0.168	0.993**	-0.294	0.995**	-0.284	0.996**	1	0.989**	0.998**
<i>JrCOR7</i>	-0.192	0.980**	-0.332	0.974**	-0.254	0.997**	0.989**	1	0.991**
<i>JrCOR9</i>	-0.189	0.986**	-0.255	0.992**	-0.285	0.996**	0.998**	0.991**	1

Table 8
Correlation of the relative expression of four MAPK and five COR gene in 'Liaoning 8' under different chilling stress time.

	<i>JrMAPK2</i>	<i>JrMAPK3</i>	<i>JrMAPK10</i>	<i>JrMAPK15</i>	<i>JrCOR1</i>	<i>JrCOR4</i>	<i>JrCOR5</i>	<i>JrCOR7</i>	<i>JrCOR9</i>
<i>JrMAPK2</i>	1	0.886**	-0.254	0.910**	0.992**	0.951**	-0.120	0.778*	-0.319
<i>JrMAPK3</i>	0.886**	1	-0.043	0.822*	0.874*	0.896**	-0.017	0.689	-0.038
<i>JrMAPK10</i>	-0.254	-0.043	1	-0.459	-0.269	-0.044	0.100	-0.253	0.903**
<i>JrMAPK15</i>	0.910**	0.822*	-0.459	1	0.868*	0.893**	0.078	0.910**	-0.464
<i>JrCOR1</i>	0.992**	0.874*	-0.269	0.868*	1	0.912**	-0.170	0.706	-0.338
<i>JrCOR4</i>	0.951**	0.896**	-0.044	0.893**	0.912**	1	0.024	0.863*	-0.125
<i>JrCOR5</i>	-0.120	-0.017	.100	0.078	-0.170	0.024	1	0.357	0.146
<i>JrCOR7</i>	0.778*	0.689	-0.253	0.910**	0.706	0.863*	0.357	1	-0.341
<i>JrCOR9</i>	-0.319	-0.038	0.903**	-0.464	-0.338	-0.125	0.146	-0.341	1

4. Discussion

The MAPK pathway is one of the most important pathways in the plant signaling network and is associated with plant growth, development, and stress response. In recent years, research on the MAPK signal transduction pathway in plants has mainly focused on tobacco, Arabidopsis, and other model plants and field crops. There are 21 MAPKs reported in Arabidopsis[8], 17 in rice[10], and 21 in poplar[9]. The group A MAPK is involved in various biotic and abiotic stress and hormone responses. For instance, *AtSIPK* can be induced by injury, salt, osmotic pressure, and pathogen-derived elicitors[11]. Studies on the group B MAPK pathway are scarce, but few studies have shown its involvement in environmental stress response and cell division. For example, *AtMPK4* of *A. thaliana* can participate in biotic and abiotic stress responses. Information on groups C and D is limited, with only *AtMPK7* expression known to control a 24 h circadian rhythm[5].

MAPKK is intermediate between MAPKKK and MAPK. MAPKK is not only the concentration point of the upstream signal but also the bifurcation point of the downstream signal and thus plays a crucial regulatory function. Compared to MAPK and MAPK, the number of MAPKK genes identified in plants is relatively small, such as 10 in Arabidopsis, 11 in rice, and 11 in poplar[38–41].

Research on MAPK genes in fruit trees is limited. To date, the published reports on MAPK in fruit trees mainly focus on apple[44], pear[42], jujube[43], and grape[31]. Fruit trees are not evolutionary close to tobacco, Arabidopsis, and other model plants, and there are differences even in their abiotic stress response. Therefore, studying the MAPK signaling pathway in fruit trees is necessary.

Genetic evidence suggests that cold stress activates *MPK3* and *MPK6* genes, which, in turn, activate the upstream of ICE1 to regulate the CBF-dependent cold signaling pathway [45]. *OsMAPK3* slows down the degradation of *OsBHLH002/OsICE1* mediated by *OsHOS1* during low-

temperature stress and enhances chilling tolerance in rice[46]. MYB15, an R2R3 type MYB transcription factor, directly binds to the MYB-binding site in the CBF promoters, inhibiting the cold-induced expression of CBF[47].

In this study, we selected four JrMAPKs according to their expression level in the transcriptome, which belonged to the homologous genes of AtMAPK3/6. Through qRT-PCR, we found that for different exposure periods of chilling stress, JrMAPK expression positively correlated with several CORs to various degrees. Our results were similar to rice[46] but opposite to those in Arabidopsis[47]. It may be because walnut is a highly heterozygous genomic tree species and its response to cold stress is more complicated than that of Arabidopsis. However, the specific mechanism of action needs to be further elucidated.

5. Conclusion

Members of 16 MAPKs and 8 MAPKKs in walnut(*Juglans regia*) were identified, and JrMAPK2, JrMAPK3, JrMAPK10, and JrMAPK15 may play an important role in walnut response to chilling stress. We believe that the expression characteristics of the MAPK-MAPKK gene families under chilling stress will provide a useful reference for the research of the MAPK family in fruit trees.

Abbreviations

qRT-PCR	Quantitative real-time PCR
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MKP	Mitogen activated protein kinase phosphatase
ZIK	ZR1-interacting kinase
ORF	The open reading frame
MW	Molecular weights
PI	Isoelectric point
NJ	Neighbor-joining
DEGs	Differentially expressed genes
FPKM	The fragments per kilobase of transcript per million mapped reads
SA	Salicylic acid
MeJ	Methyl jasmonate
CBF	C-repeat binding transcription factor
COR	Cold-relate

Declarations

Author Contributions: K.L. and Y.L. wrote the main manuscript text and prepared all figures; L.J. and G.Z. formal Analysis; H.W. improved resources; Q.L., R.Z. curation Data; all author improved funding acquisition. All authors reviewed the manuscript. AND to have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work.

Funding Declaration: This study was supported by National Key Research and Development Program of the 14th Five-Year Plan (2022YFD16000400), Binzhou Polytechnic Doctoral Fund (2022bzbs01), Regular Natural Science Projects of Binzhou Polytechnic (2024yjkt31, 2024yjkt32, 2023yjsx13), Shandong Provincial Department of Education 2025 Vocational College Teacher Visiting and Training Project Funds were used for the design of the study, sample collection, analysis or interpretation of data, and writing the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available in the NCBI data portal (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_001411555.2/).

Conflicts of Interest: The authors declare no conflict of interest.

Ethics declaration: not applicable.

Consent for publication: All authors agree to publish.

Clinical trial number: not applicable.

References

1. Zhang Z, Li Cui. Research progress on plant MAPK cascade pathway and its function [J]. J Northwest. 2014;42(04):207–14. University (NATURAL SCIENCE EDITION).
2. Pan jiaowen. Isolation and functional analysis of D group mitogen activated protein kinase MAPK gene *zmmpk17* in maize [D]. Shandong Agricultural University; 2013.
3. Jiang Yong L, Shenqiu. Molecular basis and functional regulation of cell signal transduction [M]. Sci Press, 2005:141–6.
4. Liu Xue. Identification of MAPKK gene and screening of drought resistance related functional genes in potato [D]. Gansu Agricultural University; 2017.
5. Chen Yafei F. bin, Zhao Xiaoming, Research progress of MAPK cascade pathway in plant signal transduction [J]. Bull Bot, 2005 (03): 357–65.
6. Davies WJ, Zhang J. Root signals and the regulation of growth and development of plants in drying soil [J]. Annu Rev Plant Biol. 1991;42(1):55–76.
7. Chang L, Karin M, Mammalian. MAP kinase signaling cascades [J]. Nature. 2001;410:37–40.
8. Hamel LP, Nicole MC, Sritubtim S, et al. Ancient signals: comparative genomics of plant MAPK and MAPKK gene families [J]. Trends Plant Sci. 2006;11(4):192–8.
9. Nicole MC, Hamel LP, Morency MJ, et al. MAP-ping genomic organization and organ-specific expression profiles of poplar MAP kinases and MAP kinase kinases [J]. BMC Genomics. 2006;7(1):223–46.
10. Reyna NS, Yang Y. Molecular analysis of the rice MAP kinase gene family in relation to *Magnaporthe grisea* infection [J]. Mol Plant Microbe Interact. 2006;19(5):530–40.
11. Jonak C, Okresz L, Bgre L, et al. Complexity, cross talk and integration of plant MAP kinase signaling [J]. Curr Opin Plant Biol. 2002;5:415–24.
12. Mohanta TK, Arora PK, Mohanta N, et al. Identification of new members of the MAPK gene family in plants shows diverse conserved domains and novel activation loop variants [J]. BMC Genomics. 2015;16(1):58–77.
13. Kovtun Y, Chiu WL, Tena G. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants [J]. Proceedings of the National Academy of Sciences, 2000, 97(6): 2940–2945.
14. Ortiz-Masia D, Perez-Amado Kovtun Y, Chiu WL et al. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants [J]. Proceedings of the National Academy of Sciences, 2000, 97(6): 2940–2945.
15. Kong Fuling. Isolation and expression analysis of tomato mitogen activated protein kinase (*slMAPK*) family genes [D]. Zhejiang University; 2012.
16. Ichimura K, Shinozaki K, Tena G. Mitogen-activated protein kinase cascades in plants: a new nomenclature [J]. Trends Plant Sci. 2002;7:301–8.
17. Asai T, Tena G, Plotnikova J, et al. MAP kinase signalling cascade in *Arabidopsis* innate immunity [J]. Nature. 2002;415:977–83.
18. Yang KY, Liu YD, Zhang S. Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco [J]. Proceedings of the National Academy of Sciences, 2001, 98: 741–746.
19. Ekengren SK, Liu Y, Schiff M, et al. Two MAPK cascades, *NPR1*, and *TGA* transcription factors play a role in Pto-mediated disease resistance in tomato [J]. Plant J. 2003;36:905–17.
20. Jin HL, Liu YD, Yang KY, et al. Function of a mitogen-activated protein kinase pathway in N gene-mediated resistance in tobacco [J]. Plant J. 2003;33:719–31.
21. Jonak C, Okresz L, Bogrw L, et al. Complexity, cross talk and integration of plant MAP kinase signaling [J]. Curr Opin Plant Biol. 2002;5:415–24.
22. Liu XM, Kyung Eun Kima, Kim KC, et al. Cadmium activates *Arabidopsis* *MPK3* and *MPK6* via accumulation of reactive oxygen species [J]. Phytochemistry. 2010;71:614–8.
23. Hirt H. Connecting oxidative stress, auxin and cell cycle regulation through a plant MAPK pathway [J]. Proc Natl Acad Sci USA. 2000;97:2405–24057.

24. Yeh CM, Hsiao LJ, Huang HJ. Cadmium activates a mitogen activated protein kinase gene and MBP kinases in rice [J]. *Plant Cell Physiol.* 2004;45:1306–3283.
25. Zhang M, Su J, Zhang Y, et al. Conveying endogenous and exogenous signals: MAPK cascades in plant growth and defense [J]. *Curr Opin Plant Biol.* 2018;45:1–10.
26. Holley Susan R, Yalamanchili Roopa D, Moura Daniel S, et al. Convergence of signaling pathways induced by systemin, oligosaccharide elicitors, and ultraviolet-B radiation at the level of mitogen-activated protein kinases in *Lycopersicon peruvianum* suspension-cultured cells [J]. *Plant Physiol.* 2003;132(4):1728–38.
27. Rasmussen Magnus W, Milena R, Morten P, John M. MAP Kinase Cascades in Arabidopsis Innate Immunity [J]. *Front Plant Sci.* 2012;3:169–74.
28. Mao G, Meng X, Liu Y. Phosphorylation of a WRKY transcription factor by two pathogenresponsive MAPKs drives phytoalexin biosynthesis in *Arabidopsis* [J]. *Plant Cell.* 2011;23(4):1639–53.
29. Pozo Olga P, Kerry F, Martin Gregory B. MAPKKK α is a positive regulator of cell death associated with both plant immunity and disease [J]. *EMBO J.* 2004;23(15):3072–82.
30. Stulemeijer Iris JE, Stratmann Johannes W, Joosten Matthieu. H A J. Tomato mitogen- activated protein kinases *LeMPK1*, *LeMPK2*, and *LeMPK3* are activated during the Cf-4/Avr4-induced hypersensitive response and have distinct phosphorylation specificities [J]. *Plant physiology*, 2007, 144(3): 1481–1494.
31. Wang G. Identification and expression analysis of grape MAPK gene family and functional verification of some genes [D]. Nanjing Agricultural University; 2014.
32. Yang Tianbao C, Shubho Y, Lihua, et al. A calcium/calmodulin-regulated member of the receptor-like kinase family confers cold tolerance in plants [J]. *J Biol Chem.* 2010;285(10):7119–24.
33. Ichimura K, Mizoguchi T, Irie K, Morris P, et al. Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in *Arabidopsis* [J]. *Biochem Biophys Res Commun.* 1998;253(2):532–43.
34. Hadiarto Toto N, Takashi M, Daisuke, et al. Activation of Arabidopsis MAPK kinase kinase (*AtMEKK1*) and induction of AtMEKK1-AtMEK1 pathway by wounding [J]. *Planta.* 2006;223(4):708–14.
35. Juan Luo L-L, Zhao S-Y, Gong, et al. A cotton mitogen-activated protein kinase (*GhMPK6*) is involved in ABA-induced *CAT1* expression and H₂O₂ production [J]. *J Genet.* 2011;38(11):557–65.
36. Wang Huachun N, Njabulo L, Yidong, et al. Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in *Arabidopsis* [J]. *Plant Cell.* 2007;19(1):63–73.
37. Takahashi Fuminori Y, Riichiro I, Kazuya, et al. The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in *Arabidopsis* [J]. *Plant Cell.* 2007;19(3):805–18.
38. Wang J, Pan C, Wang Y, et al. Genome-wide identification of MAPK, MAPKK, and MAPKKK gene families and transcriptional profiling analysis during development and stress response in cucumber[J]. *BMC Genomics.* 2015;16(1):386–407.
39. Colcombet J, Hirt H. *Arabidopsis* MAPKs: a complex signaling network involved in multiple biological processes [J]. *Biochem J.* 2008;413:217–26.
40. Hamel LP, Nicole MC, Sritubtim S, et al. Ancient signals: comparative genomics of plant MAPK and MAPKK gene families [J]. *Trends Plant Sci.* 2006;11:192–8.
41. Zhang Mengling W, Lixin X, Chaoling et al. Identification of MAPKKK family genes in jujube and analysis of their expression in response to Jujube Witches' broom [A]. Chinese society of horticulture. Proceedings of the annual academic meeting and 90th Anniversary Conference of Chinese society of Horticulture in 2019 [C]. Chinese society of Horticulture: Chinese society of horticulture, 2019: 1.
42. Jiao Jin. Bioinformatics analysis of MAPK family genes and functional verification of *pbrmapk15* in pear [D]. Nanjing Agricultural University; 2017.
43. Zhao Yanlin P, Jin LZ, et al. Cloning and analysis of MAPK gene fragment of *Zizyphus jujuba* [J]. *J Hebei Agricultural Univ.* 2010;33(06):43–6.
44. Peng Lixin. Cloning, expression characteristics and signal transduction of MAPK gene in apple under water stress [D]. Shandong Agricultural University; 2003.
45. Li H, Ding Y, Shi Y, Zhang X, Zhang S, Gong Z, Yang S. MPK3- and MPK6-mediated ICE1 phosphorylation negatively regulates ICE1 stability and freezing tolerance in *Arabidopsis* [J]. *Dev Cell.* 2017;43:630–42.
46. Li H, Ding Y, Shi Y, Zhang X, Zhang S, Gong Z, Yang S. MPK3- and MPK6-Mediated ICE1 phosphorylation negatively regulates ICE1 stability and freezing tolerance in *Arabidopsis* [J]. *Developmental Cell*, 2017, 43(5):630–642.
47. Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, Zhu JK. A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance [J]. *J Biol Chem.* 2006;281:37636–45.

Figures

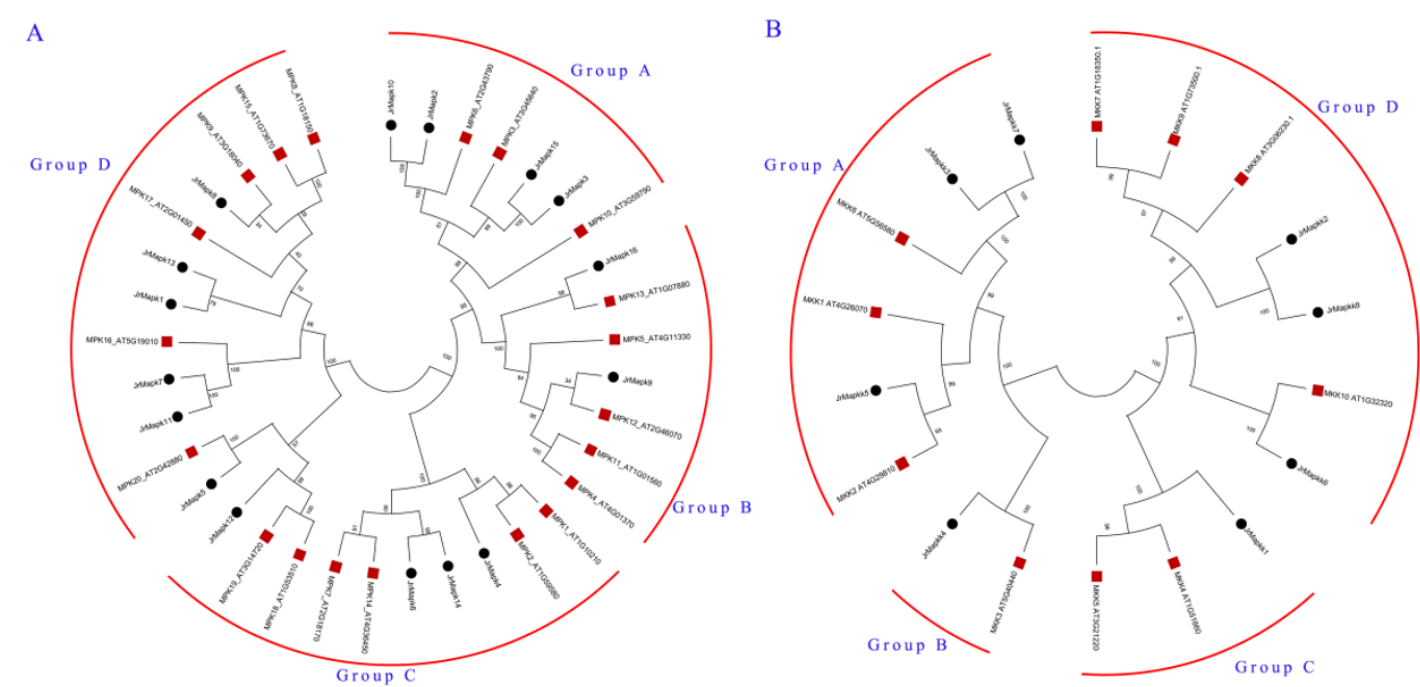


Figure 1

The phylogenetic tree of the MAPK, MAPKK families in *Juglans regia* and *Arabidopsis thaliana*. A: The phylogenetic tree of the MAPK gene family in *Juglans regia* and *Arabidopsis thaliana*; B: The phylogenetic tree of the MAPKK gene family in *Juglans regia* and *Arabidopsis thaliana*;

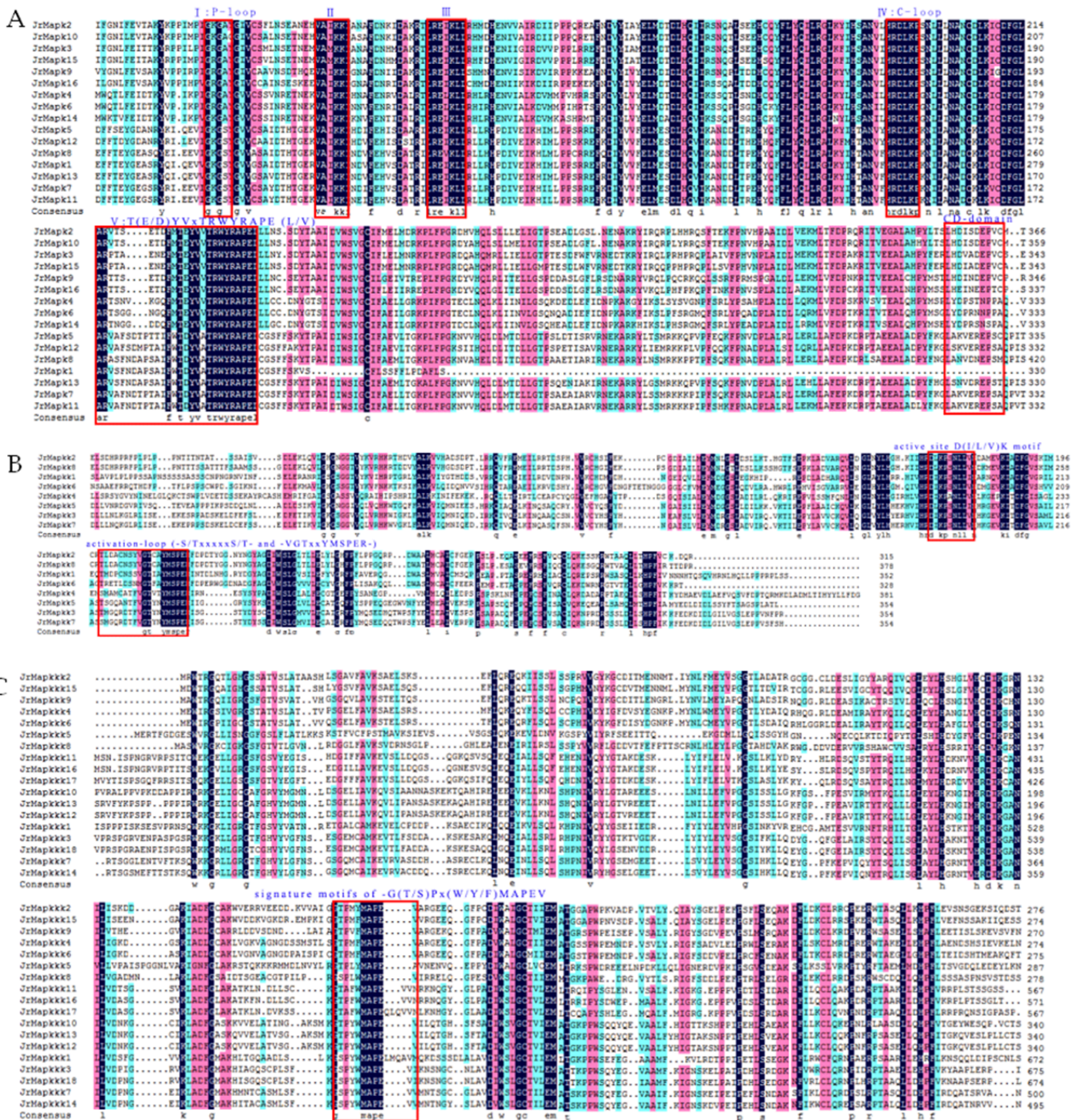


Figure 2

Sequence alignment and motif analysis of MAPK, MAPKK families and MEK family in *Juglans regia*. The key region is marked by a red line. A: Sequence alignment and motif analysis of MAPK family in *Juglans regia*, P-loop, C-loop, CD-domain and activation-loop are marked by red line; B: Sequence alignment and motif analysis of MAPK family in *Juglans regia*, S/TxxxxS/T motif are marked by red line; C: Sequence alignment and motif analysis of MAPK family in *Juglans regia*, signature motifs are marked by red line.

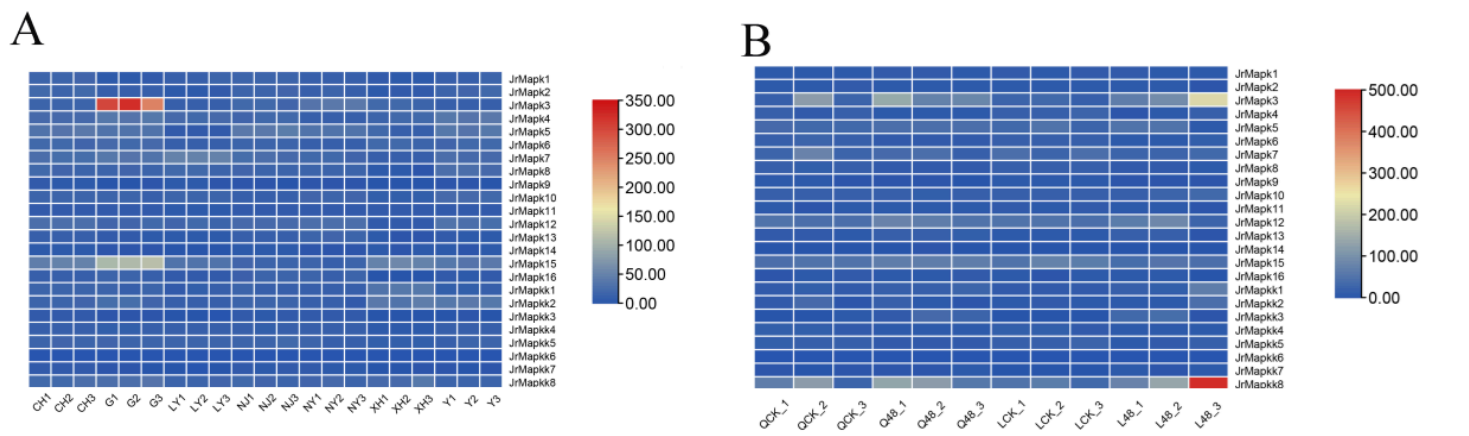


Figure 5

A: The expression of *JrMAPK*, *JrMAPKK* and *JrMAPKKK* in different tissues. B: The expression of *JrMAPK*, *JrMAPKK* in chilling stress .

Note: CH: female flower; G: root; LY: mature leaves; NJ: young stem; NY: young leaves; XH: male flower; Y: bud.

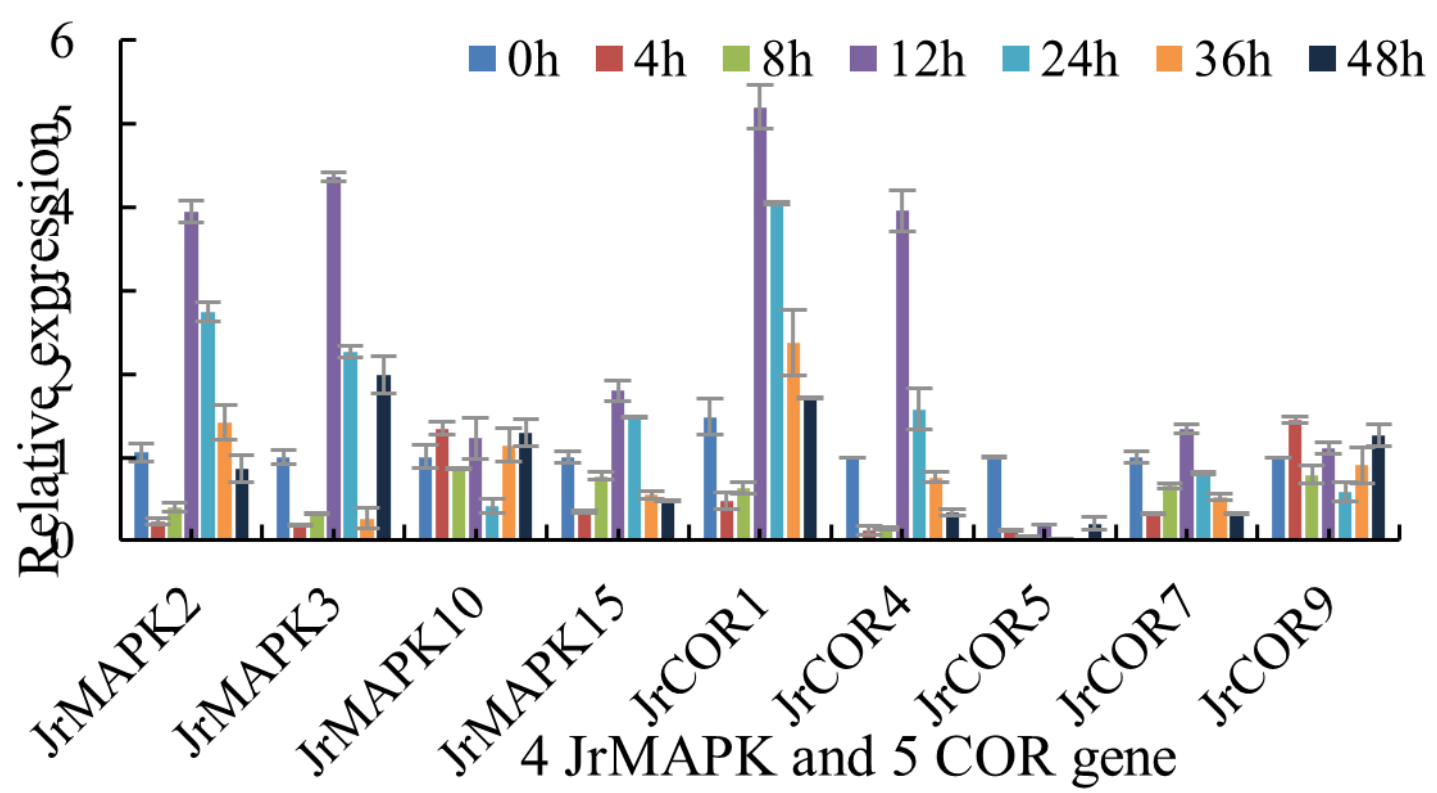


Figure 6

The relative expression of four MAPK and five COR gene in 'Liaoning 8' under different chilling stress time.

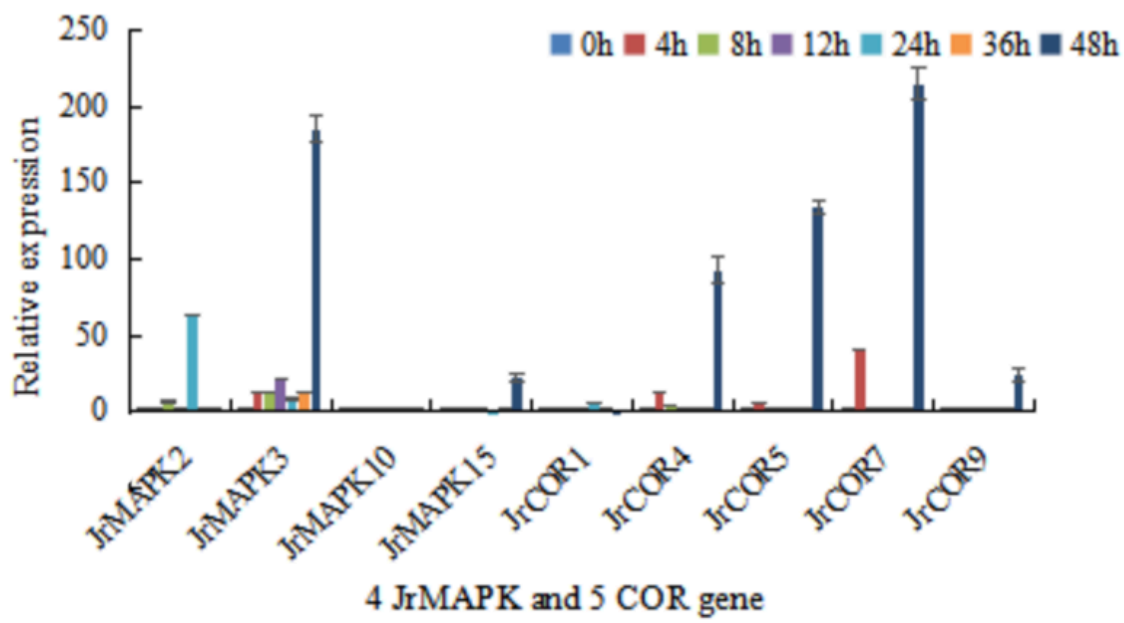


Figure 7

The relative expression of four MAPK and five COR gene in 'Qingxiang' under different chilling stress time.