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MAPK Gene Family in *Lactuca sativa*: Genome-Wide Identification, Regulatory Network, and Expression Patterns in Stem Development and Stress Responses

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Abstract: Mitogen-activated protein kinases (MAPKs) play essential roles in the process of stress response and plant growth and development. MAPK family genes have been identified in many plant species. In this study, 18 *LsMAPK* genes were identified in lettuce (*Lactuca sativa*). The *LsMAPK* members were divided into Group A, B, C, and D by phylogenetic tree analysis among Arabidopsis, rice, and lettuce. *Cis*-elements, which relate to abiotic stress, phytohormone response, and transcription factor binding site, were identified to exist in the promoter region of *LsMAPK* genes. Chromosomal location analysis showed the *LsMAPK* genes were distributed on eight chromosomes except chromosome 6. Interaction network analysis showed that *LsMAPK*s could interact with MAPK kinase (MAPKK), protein-tyrosine-phosphatase (PTP), and transcription factors (WRKY, bZIP). Quantitative reverse transcription PCR (qRT-PCR) showed that *LsMAPK* genes were induced by different abiotic stresses, hormone response, and stem enlargement. The comprehensive identification and characterization of *LsMAPK* genes in stem lettuce will lay a theoretical foundation for the functional analysis of *LsMAPK* genes and advance our knowledge of the regulatory mechanism of MAPK genes in plants.

Keywords: stem lettuce; MAPK gene; genome-wide identification; abiotic stress; stem enlargement; expression profile

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1. Introduction

External stimuli, including abiotic stress (such as drought, salt, or extreme temperatures) and biotic stress (such as insect or pathogen infection), affect plant growth and development. Plants have developed certain defense mechanisms to counteract negative effects of extracellular stimuli [1,2]. The mitogen-activated protein kinase (MAPK) cascade pathway serves as the key pathway in eukaryotic signal transduction. It can regulate various cellular signaling cascades and participate in various fundamental biological processes. The MAPK cascade pathway consists of MAPK (MPK), MAPK kinase (MAPKK/MKK), and MAPKK kinase (MAPKKK/MEKK/MAP3K). The MAPK cascade pathway can be activated after stimulation of plant cells by adverse environmental factors [3,4].

Although at the bottom of the MAPK signaling cascade, MAPK genes are considered to be just some of the principal and highly conserved signaling molecular in eukaryotes [5]. As an important signal transduction mode, MAPK genes can be phosphorylated by activated MAPKKs and then modulate cellular response for normal growth and development by phosphorylating downstream target genes (transcription factor or other key proteins) [5–7]. Subsequently, the phosphorylated transcription factor could regulate target genes by binding the *cis*-elements existing in their promoter regions. Therefore, it is important to understand the process of signal transduction and regulation pathways in plants under different stresses. Thus, we focus on the analysis of the molecular mechanism of MAPKs involved in various biological processes.

MAPK, a kind of serine/threonine protein kinase, regulates eukaryotic cell signal transduction [8,9]. Eleven conserved motifs (I-XI) have been identified to exist in MAPKs. TXY motif, containing the phosphorylation sites, has been identified in subdomains VII and VIII. TXY motif is a key determinant of MAPK activity. The X residues in TXY motifs vary among different MAPKs. MAPKs are divided into A, B, C, and D subfamilies based on phylogenetic analysis and phosphorylation motifs. The TEY motif is the activation site of A, B, and C subfamily members, while the TDY phosphorylation motif is the activation site for members of the D subfamily [10,11]. Numerous MAPK genes have been identified and reported in different plants. Up to now, there are 20, 43, 16, 92, 56, 28, and 20 in Arabidopsis [12], strawberry [8], tartary buckwheat [13], *Brassica napus* [14], cotton [15], sunflower [16], and barley [17].

Large numbers of studies have shown that MAPK genes play critical roles in response to different stimuli. For example, Arabidopsis *AtMPK3* and *ZmMAPK1* genes positively regulated drought stress response [18,19]. Sorghum *SbMPK14* gene improved drought hypersensitivity by promoting water loss [20]. Similar results were shown in *Zea mays* MAPK genes. *ZmMPK3* and *ZmSIMK1* enhanced plant growth by increasing tolerance to high salinity [21,22]. MAPK genes are also involved in plant development and physiological processes. *AtMPK4* participated in photosynthesis regulation, plant growth, and immune defense [23]. *AtMAPK3* and *AtMAPK6* were required for another development [24]. MAPK proteins can participate in different biological processes through multiple regulation mechanisms. Numerous studies have shown that MAPK proteins can interact with other proteins such as transcription factors. For example, AtMAPK8 promoted seed germination by interaction with the TCP14 transcription factor [25]. *Nicotiana tabacum* WRKY transcription factors (WRKY4, WRKY6, and WRKY10) were able to interact with MAPK proteins to modulate plant defense against whiteflies [26]. *Magnaporthe oryzae* MAPK protein MoMps1 showed interaction with an APSES family transcription factor, and the interaction was required for hyphal and conidial morphogenesis, appressorial function and pathogenicity of *M. oryzae* [27].

Lettuce (*Lactuca sativa* L.) is a popular vegetable with several cultivars such as oil lettuce, leaf lettuce, and stem lettuce. Stem lettuce is a vegetable with low fat and high nutritional value. Studies on stem lettuce have mainly focused on its cultivation techniques and the effect of different fertilizers on yield and quality. The molecular mechanisms of stem lettuce growth and development remain unclear. Previous research has found that lettuce *LsMAPK4* may be involved in high-temperature bolting in lettuce crops [28]. Here, we identified and analyzed the most important and highly conserved signaling molecular MAPK genes in lettuce. The exon–intron structure, phylogenetic relationships, motif compositions, collinearity analysis, and chromosome distribution of *LsMAPK* genes were identified. To investigate the possible function of *LsMAPKs* in different biological processes, the expression profiles of *LsMAPK* genes at different stages of stem expansion, abiotic stresses, and plant hormones were also conducted. Our results provide the basis for further research on the function of *LsMAPK* genes in stem expansion and stress response.

2. Materials and Methods

2.1. LsMAPK Genes Identification in Lettuce

The lettuce genome sequence used in this study was obtained from the Lettuce Genome Resource database (https://lgr.genomecenter.ucdavis.edu/, URL (accessed on 1 December 2021)). MAPK genes from Arabidopsis were used as the query sequence to identify the homologous genes of lettuce. The conserved domains within MAPK family genes were determined using the Pfam (http://pfam.xfam.org/, URL (accessed on 1 December 2021)) and NCBI Conserved Domain Database (CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, URL (accessed on 1 December 2021)). All gene sequences encoding complete amino acid sequences with conserved domains were considered *LsMAPK* genes. Subsequently, the molecular weight (Mw) and isoelectric point (pI) of *LsMAPK*s were analyzed using the ExPASY online tool (https://web.expasy.org/protparam/, URL (accessed on

10 December 2021)). The subcellular localization analysis of LsMAPs was conducted using an online WOLF PSORT platform (https://www.genscript.com/wolf-psort.html/; URL (accessed on 10 December 2021)).

2.2. Characterization and Correlation Analysis of LsMAPKs in Lettuce

A phylogenetic tree among Arabidopsis, rice, and lettuce was constructed using the MEGA 7.0 software by the Neighbor-Joining method with 1000 bootstrap replicates. The MAPK amino acids of Arabidopsis and rice were obtained from the Arabidopsis Information Resource (https://www.arabidopsis.org/, URL (accessed on 10 December 2021)) and the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/, URL (accessed on 10 December 2021)), respectively. Conserved motif and chromosomal distribution of *LsMAPKs* were analyzed using the MEME online program (https://meme-suite.org/meme/, URL (accessed on 10 December 2021)) and MapChart software (version 2.32), respectively. The interaction network of *LsMAPK* proteins was predicted using STRING software (https://cn.string-db.org/, URL (accessed on 10 December 2021)) and visualized by Cytoscape software (version 3.9.1). The transcription start site (TSS) of *LsMAPK* genes was predicated on the website: http://www.fruitflfly.org/seq_tools/promoter.html, URL (accessed on 28 December 2021) [29]. The promoter region with 2000 bp of *LsMAPK* genes was extracted from the upstream of the TSS. Then, cis-element analysis of the promoter region was conducted by using the PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace, URL (accessed on 28 December 2021)), PlantPAN 3.0 (http://plantpan.itps.ncku.edu.tw/, URL (accessed on 28 December 2021)), and PlanCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, URL (accessed on 28 December 2021)) online databases, respectively. Gene pair collinearity analysis among lettuce, Arabidopsis, and rice was determined by MCScanX software (http://chibba.pgml.uga.edu/mcscan2; URL (accessed on 28 December 2021)).

2.3. Plant Growth and Treatments

The seeds of stem lettuce cultivar 'Yonganhong' were sown in a controlled environment chamber for 12 h photoperiod at 22 and 18 °C (day vs. night) with a light intensity of 20,000 μmol/m^2/s (lux) at Linyi University (Linyi, China). For plant hormone treatment, seedlings at the four-leaf stage were sprayed with 75 μmol/L abscisic acid (ABA), 50 μmol/L gibberellin (GA), 0.5 mmol/L salicylic acid (SA). Seedlings in the control group were sprayed with distilled water. The seedlings were collected and frozen under liquid nitrogen after spraying for 0 h and 12 h. For abiotic stress treatment, four-leaf stage seedlings were treated with 200 mmol/L NaCl (salt), 20% PEG6000 (drought), 4 °C (low temperature), and 37 °C (high temperature). The treated and untreated leaves were collected at 0 h and 12 h. Each treatment (plant hormone, abiotic stress) contained 15 seedlings and all the treatments were replicated three times and harvested after the treatments. The stem tissue of stem lettuce was also collected with three biological replicates at different stages of stem enlargement: S1 (transverse diameter length is 1 cm), S2 (transverse diameter length is 2 cm), S3 (transverse diameter length is 3 cm), and S4 (transverse diameter length is 4 cm).

2.4. Quantitative Reverse Transcription PCR (qRT-PCR) of LsMAPK Genes

To identify the differentially expressed genes, the four stem enlargement stages (S1, S2, S3, and S4) of 'Yonganhong' were chosen to conduct the RNA sequencing. The transcript abundance of *LsMAPK* genes at different stages of stem enlargement was obtained according to the RNA-Seq, which has been submitted to public transcriptome data (NCBI: PRJNA844256). The transcript abundance of *LsMAPK* genes at different developmental stages was counted by FPKM (fragments per kilobase exon per million fragments mapped). Total RNA was isolated from the samples using a plant total RNA isolation kit (Vazyme, Nanjing, China). For qRT-PCR, 6 *LsMAPK* genes involved in the process of stem enlargement were chosen to conduct expression pattern analysis. For qRT-PCR, 20 μL reaction system containing 10 μL SYBR qPCR master mix (Vazyme, Nanjing, China), 0.4 μL of each

primer, 2 µL diluted cDNA and 7.2 µL deionized water was performed. The qRT-PCR was conducted using Roche LightCycler 96 with the following procedure: 95 °C for 30 s initially, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and melting curve analysis at 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s. The calculation of relative expression levels of *LsMAPK* genes used the $2^{-\Delta\Delta CT}$ methods based on the mean value of three technical repeats referred to previous research methods [30]. The expression levels of each *LsMAPK* gene were standardized and calculated by *LsTIP41* (Lsat_1_v5_gn_5_116421) [31]. The experiments were repeated in three independent bio-replicates and tech-replicates. Primer Premier 6.0 was used to design the primer pairs used in the study (Supplemental Table S1). SPSS 17.0 software was used to analyze significant difference at 0.05 levels.

3. Results

3.1. The LsMAPK Genes in Lettuce

A total of 18 putative *LsMAPK* genes (denoted *LsMAPK01-LsMAPK18*) were identified in lettuce after homologous alignment and conservative domain verification with Arabidopsis MAPK genes. The nucleotide and amino acid sequences of *LsMAPKs* are shown in Supplemental Table S2. Sequence alignment showed the presence of TEY or TDY phosphorylation sites in the 18 *LsMAPK* genes (Supplemental Figure S1). As shown in Table 1, the amino acid length of 18 *LsMAPKs* ranged from 369 (*LsMAPK8*) to 761 (*LsMAPK11*). The *pI* and Mw of *LsMAPK* proteins varied from 4.99 (*LsMAPK14*) to 9.30 (*LsMAPK10*), and 42.416 kD (*LsMAPK1*) to 85.362 kD (*LsMAPK11*), respectively. According to the grand average of hydropathicity (GRAVY) values, which ranged from −0.174 (*LsMAPK1*) to −0.549 (*LsMAPK15*), all the *LsMAPKs* are hydrophilic proteins. Subcellular localization analysis showed that 12 *LsMAPK* proteins were located only in the cytoplasm, while two *LsMAPK* (*LsMAPK13* and *LsMAPK14*) proteins were located in the cytoplasm and the cytoskeleton. *LsMAPK9* was predicted to be located in the cytoskeleton. *LsMAPK11* and *LsMAPK18* were located in the chloroplast. *LsMAPK15* was located both in the cytoplasm and the chloroplast.

Table 1. The characteristic of *LsMAPK* genes.

Gene Name	Amino Acid/aa	ORF/bp	Molecular Weight/kD	*pI*	Instability Index	GRAVY	Subcellular Localization
LsMAPK1	371	1116	42.416	5.83	40.87	−0.174	Cytoplasm
LsMAPK2	382	1149	43.937	5.65	41.96	−0.296	Cytoplasm
LsMAPK3	571	1716	65.308	6.97	80.79	−0.541	Cytoplasm
LsMAPK4	410	1233	47.512	5.89	95.39	−0.229	Cytoplasm
LsMAPK5	378	1137	43.461	6.32	90.26	−0.388	Cytoplasm
LsMAPK6	501	1506	57.520	6.93	85.47	−0.456	Cytoplasm
LsMAPK7	373	1122	43.077	6.50	97.21	−0.236	Cytoplasm
LsMAPK8	369	1110	42.706	6.94	95.64	−0.239	Cytoplasm
LsMAPK9	370	1113	42.559	5.62	93.08	−0.272	Cytoskeleton
LsMAPK10	584	1755	66.322	9.30	79.95	−0.447	Cytoplasm
LsMAPK11	761	2286	85.362	9.08	90.46	−0.220	Chloroplast
LsMAPK12	738	2217	84.028	7.15	85.35	−0.355	Cytoplasm
LsMAPK13	453	1362	51.788	6.01	84.77	−0.430	Cytoplasm, Cytoskeleton
LsMAPK14	381	1146	43.284	4.99	93.39	−0.283	Cytoplasm, Cytoskeleton
LsMAPK15	600	1803	67.890	7.08	76.57	−0.549	Chloroplast, Cytoplasm
LsMAPK16	598	1797	67.857	9.15	77.29	−0.505	Cytoplasm
LsMAPK17	373	1119	42.879	5.38	89.38	−0.328	Cytoplasm
LsMAPK18	372	1119	42.749	5.86	95.86	−0.299	Chloroplast

Note: aa: amino acid; bp: base pair; kD: kilodalton; *pI*: isoelectric point; GRAVY: grand average of hydropathicity.

3.2. Phylogenetic Analysis of LsMAPKs in Lettuce

The amino acid sequences of 18 lettuce *LsMAPKs*, 20 Arabidopsis AtMPKs, and 16 rice OsMPKs were used to analyze the evolutionary relationships (Figure 1). As shown in Figure 1, 18 *LsMAPKs* were divided into A, B, C, and D subfamilies. The TEY motif existed in the A, B, and C subfamily *LsMAPKs*, while *LsMAPKs* in the D subfamily contained the TDY motif (Supplemental Figure S1). The D subfamily had the most *LsMAPK* members (eight), followed by the C subfamily, which had four members. The A and B subfamilies

contained an equal number of *LsMAPK* members. *LsMAPK2*, *LsMAPK9*, and *LsMAPK17* were classified into the A subfamily, which contained well-characterized AtMPK3, AtMPK6, AtMPK10, OsMPK3, and OsMPK6. *LsMAPK5*, *LsMAPK14*, and *LsMAPK18* were classified into the B subfamily, which included AtMPK4, AtMPK11, AtMPK13, AtMPK12, AtMPK5, and OsMPK16.

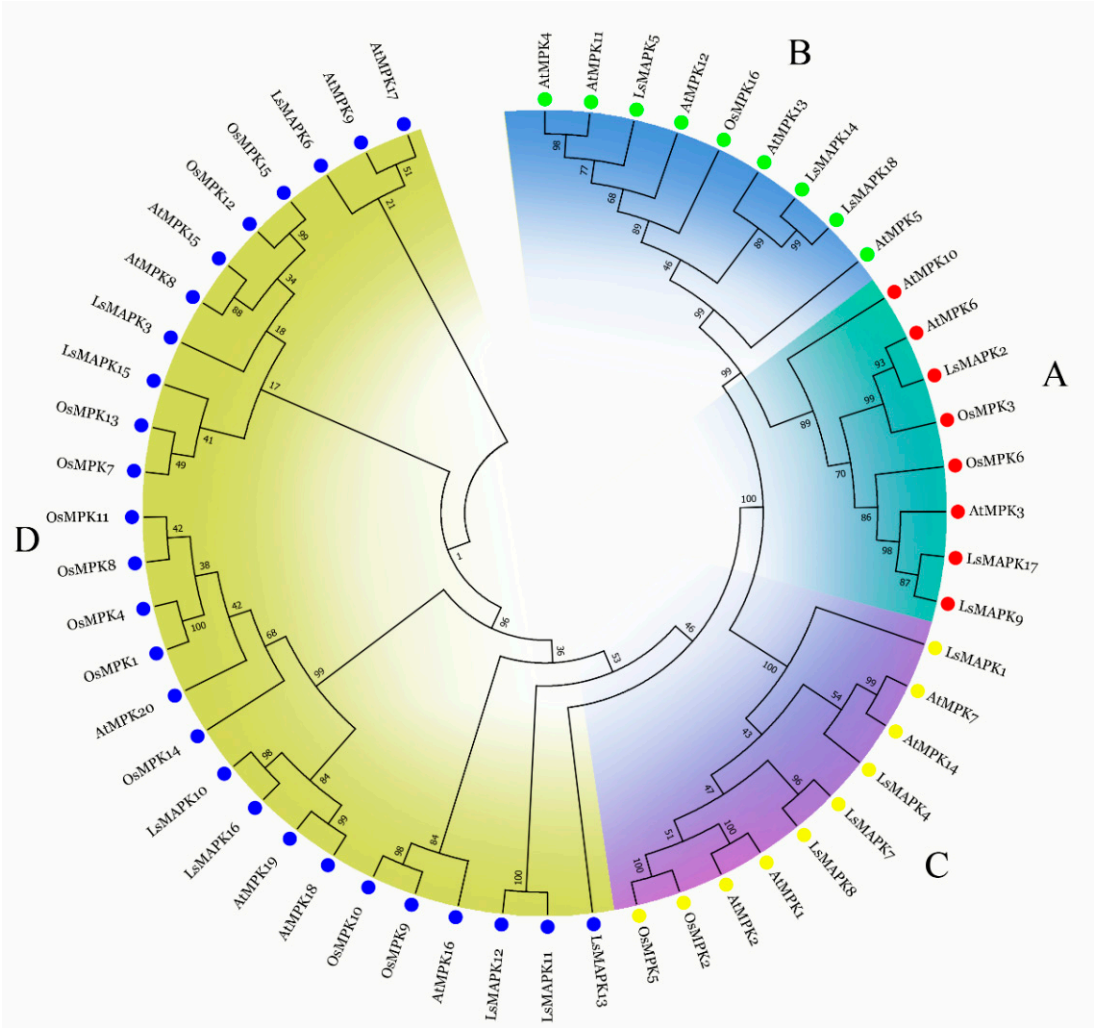

Figure 1. Phylogenetic tree of MAPK proteins among lettuce, Arabidopsis, and rice. MAPK proteins were divided into four subfamilies (A, B, C, and D), with each color group representing a subfamily.

3.3. Analysis of Gene Structure and Motif of LsMAPK Genes

The *LsMAPK* protein structure was examined using the MEME online program. As shown in Figure 2B, ten motifs were identified. Motifs 1, 2, 7, and 3 existed in all *LsMAPK* proteins, while most *LsMAPK* proteins contained motifs 4, 5, 6, and 8. *LsMAPKs* in the same subfamily had similar motifs. For example, most *LsMAPKs* belonging to Group D except *LsMAPK13* had specific motif 9. *LsMAPK* proteins belonging to Groups A and B contained motif 10.

The exon–intron structures of the identified *LsMAPK* genes were analyzed (Figure 2C). *LsMAPK* genes belonging to the same subfamily had conserved exon–intron structure. For instance, the *LsMAPKs* identified in Groups A and B, except *LsMAPK9,* had six exons, while Group C *LsMAPK* genes had two to three exons. The *LsMAPK4* in Group C had three exons, while *LsMAPK1*, *LsMAPK7*, and *LsMAPK8* each had two exons. Group D *LsMAPK* genes had eight to eleven exons. *LsMAPK3* and *LsMAPK15* had the highest number of exons (eleven).

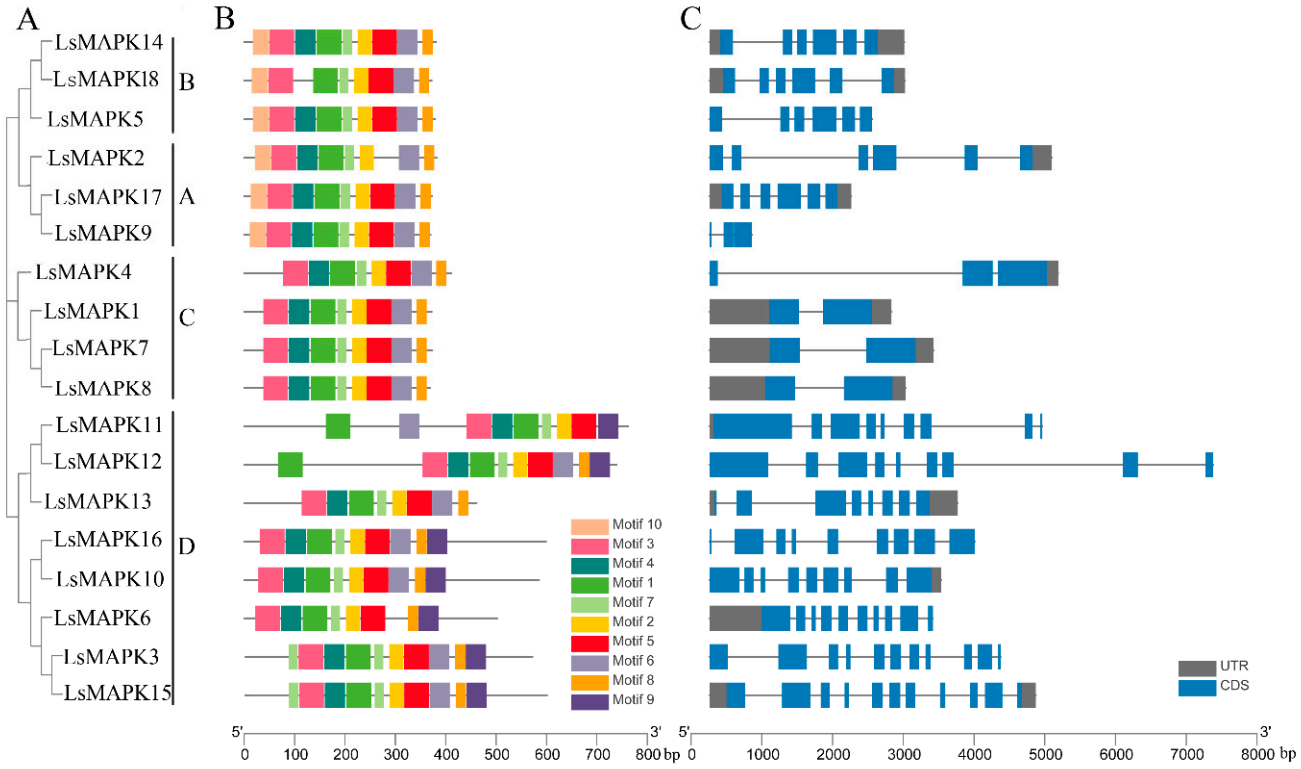

Figure 2. Gene structure analysis of *LsMAPK* genes in lettuce. (**A**) Phylogenetic tree analysis created by MEGA 7.0. (**B**) Conserved motifs analysis. Different motifs were shown by different numbers of colored boxes. (**C**) Exon–intron structures analysis. Blue boxes and grey lines represent exons and introns, respectively; grey boxes represent the UTR; UTR: untranslated region, CDS: coding sequence.

3.4. Cis-Element Analysis of LsMAPK Genes

To better understand the function of *LsMAPKs* involved in different biological processes, we analyzed the *cis*-elements existing in the promoter regions of *LsMAPK* genes by using PLCAE (Figure 3), PlantCARE database (Supplement Table S3), and PlantPAN 3.0 (Supplement Table S4), respectively. As shown in Figure 3, the identified *cis*-elements were related to abiotic stress, phytohormone responses, and transcription factors. Abiotic- related elements contained five *cis*-elements such as low-temperature responsiveness element (LTR), drought inducibility element, salt induced element, CBF, and W-box. The promoter region of nine *LsMAPK* genes contained the LTR element, which was essential for the low temperature responsive. W-box, CBFHV, and GT1GMSCAM4 motifs appeared 71, 24, and 46 times, respectively. All *LsMAPK* genes contained the wound response element W-box. According to the analysis by the PLACE and PlantCARE database, *cis*-elements related to phytohormone responses also existed in *LsMAPK* genes, including GA, auxin, ABRE, ERE, TCA-element, and CGTCA/TGACG motif (Figure 3 and Supplemental Table S3). Six *LsMAPK* genes contained the auxin-responsive element. Twelve out of eighteen *LsMAPK* genes contained a GA responsive element (GAREAT) in their promoter region. Apart from stress-related and plant hormone-related *cis*-elements, some *cis*-elements, belonging to the binding sites of transcription factors (Dof, MYB, RAV, and bZIP), were also identified by the analysis of the PLACE and PlantPAN database (Figure 3 and Supplemental Table S4). Dof transcription factor binding sites existed in the promoter region of all the *LsMAPK* genes. All *LsMAPK* genes except *LsMAPK7* contained the RAV transcription factor binding site. *Cis*-elements of the MYB transcription factor binding site were identified to exist in the promoter region of 16 *LsMAPKs*, except *LsMAPK3* and *LsMAPK6*.

	Abiotic stress					Plant hormone		Transcription factor			
	CBFHV	MYC	LTR	GT1GMSCAM4	W-box	CATATGGMSAUF	GAREA1	DOF	bZIP	MYB	RAV
LsMAPK1	1	22	2	2	3	4	3	30	5	8	8
LsMAPK2	0	6	0	3	3	0	0	21	1	10	8
LsMAPK3	0	14	0	3	1	2	2	20	5	0	6
LsMAPK4	1	4	4	0	5	0	0	10	4	3	4
LsMAPK5	1	10	0	3	4	0	1	18	0	3	9
LsMAPK6	2	4	5	4	9	2	0	23	0	0	4
LsMAPK7	0	4	0	0	1	0	2	15	0	3	0
LsMAPK8	1	22	2	1	1	2	0	11	1	1	2
LsMAPK9	3	6	1	3	4	0	1	23	5	6	6
LsMAPK10	0	2	0	1	2	0	1	43	2	2	4
LsMAPK11	0	4	0	5	7	0	0	7	0	4	7
LsMAPK12	0	8	1	2	3	2	3	16	4	3	3
LsMAPK13	1	8	1	3	4	0	1	23	3	4	2
LsMAPK14	3	8	0	2	4	0	0	16	0	1	3
LsMAPK15	2	24	1	5	2	6	1	31	3	2	4
LsMAPK16	3	6	0	5	1	0	4	13	0	5	8
LsMAPK17	2	10	1	0	11	0	1	13	4	11	13
LsMAPK18	4	16	0	4	6	0	1	28	2	2	2
Total	24	178	18	46	71	18	21	361	39	68	93

MYC: MYC recognition site found in the promoters of the dehydration-responsive gene Atrd22; MYB: binding site for plant MYB proteins;
LTR: core of low temperature responsive element (LTRE); RAV: binding consensus sequence of Arabidopsis transcription factor RAV1;
W-box: exist in promoter of Arabidopsis NPR1 gene; GAREAT: GA-responsive element; DOF: core site required for binding of Dof proteins
CATATGGMSAUR: multiple auxin response modules in the soybean SAUR15A promoter;
GT1GMSCAM4: GT-1 motif found in the promoter of pathogen- and salt-induced SCaM-4 gene;
CBFHV: CBF C-repeat binding factors; bZIP: a novel class of bZIP transcription factors binding core sequence.

Figure 3. *Cis*-element analysis of *LsMAPK* genes in lettuce by PLACE database. Boxes with different numbers represent each *cis*-element identified by the PLACE database. Different colors represent different number ranges of identified *cis*-element.

3.5. Chromosomal Location of LsMAPK Genes

As shown in Figure 4, the chromosomal distribution of 18 *LsMAPK* genes was investigated. The 18 *LsMAPK* genes were mapped on eight chromosomes except chromosome 6, which contained zero MAPK genes. Chromosomes 2, 4, and 5 had only one *LsMAPK*, i.e., *LsMAPK3*, *LsMAPK7*, and *LsMAPK8*, respectively. Chromosome 1 contained two *LsMAPK* genes. *LsMAPK1* and *LsMAPK2* were mapped on chromosomes 1. In addition, three *LsMAPK* genes were found on chromosomes 3, 8, and 9. Chromosome 7 had the largest number of *LsMAPK* genes, including *LsMAPK10*, *LsMAPK11*, *LsMAPK12*, and *LsMAPK13*. Interesting, *LsMAPK* genes belonging to the same subfamily were not distributed on the same chromosomes. For example, *LsMAPK5*, *LsMAPK14*, and *LsMAPK18*, which both belong to the Group B subfamily, were mapped on chromosomes 3, 8, and 9, respectively. *LsMAPK* genes (*LsMAPK2*, *LsMAPK9*, and *LsMAPK17*) belonging to Group A were divided into three different chromosomes (1, 8, and 9).

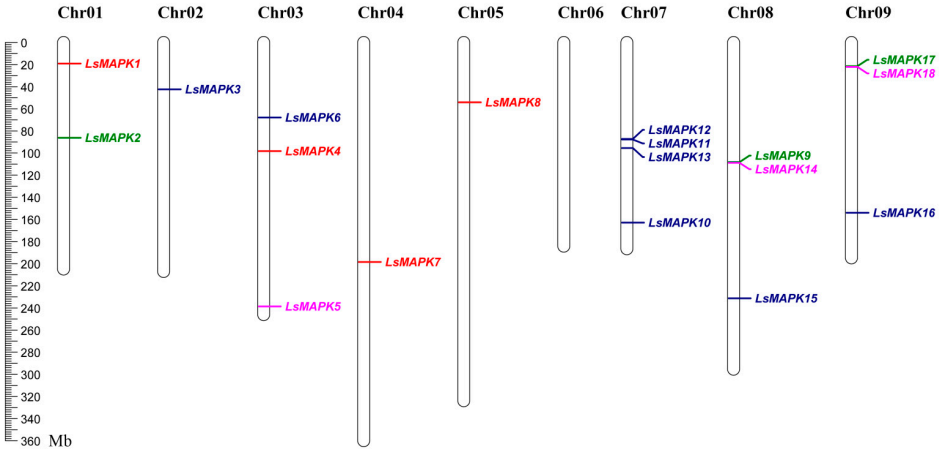

Figure 4. Chromosomal distribution of *LsMAPK* genes in lettuce chromosomes. Different colors represent different subfamily *LsMAPK* genes.

3.6. Synteny Analysis of MAPK Genes

As shown in Figure 5, seven *LsMAPK* gene pairs in lettuce chromosome were identified as collinear pairs by collinearity analysis. The collinear pairs of *LsMAPK* genes in lettuce belonged to the same subfamily, for example, *LsMAPK1* and *LsMAPK7*, *LsMAPK10* and *LsMAPK16*. To further determine the evolutionary relationship, MAPK genes of Arabidopsis and rice were chosen for synteny analysis with lettuce. Five pairs of orthologous MAPK genes were identified between Arabidopsis and lettuce. The collinear pairs between Arabidopsis and lettuce were clustered on the same branch, such as *LsMAPK4* and *AtMPK7*, *LsMAPK6* and *AtMPK17*, *LsMAPK10* and *AtMPK19* (Figure 6, Supplemental Table S5). However, no collinear pairs of MAPK genes were identified between rice and lettuce, indicating the genetic relationship between lettuce and Arabidopsis was more advanced than that of rice, and the MAPK genes were conserved in the evolution of dicotyledons, not in the evolution of monocotyledon.

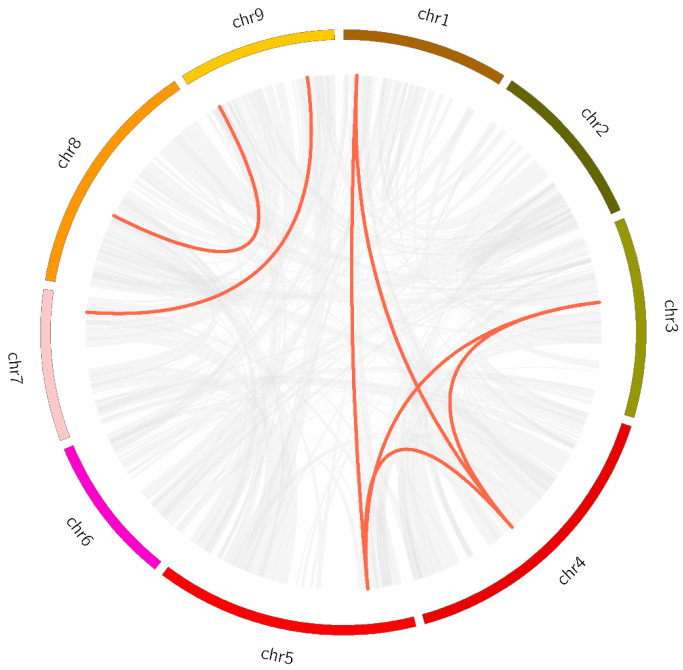

Figure 5. Synteny analysis of *LsMAPK* genes in lettuce. Colored circular rectangles represent the chromosomes (1–9) of lettuce. Grey and red curves represent the identified collinear blocks with the genomes and the collinear with *LsMAPK* genes, respectively.

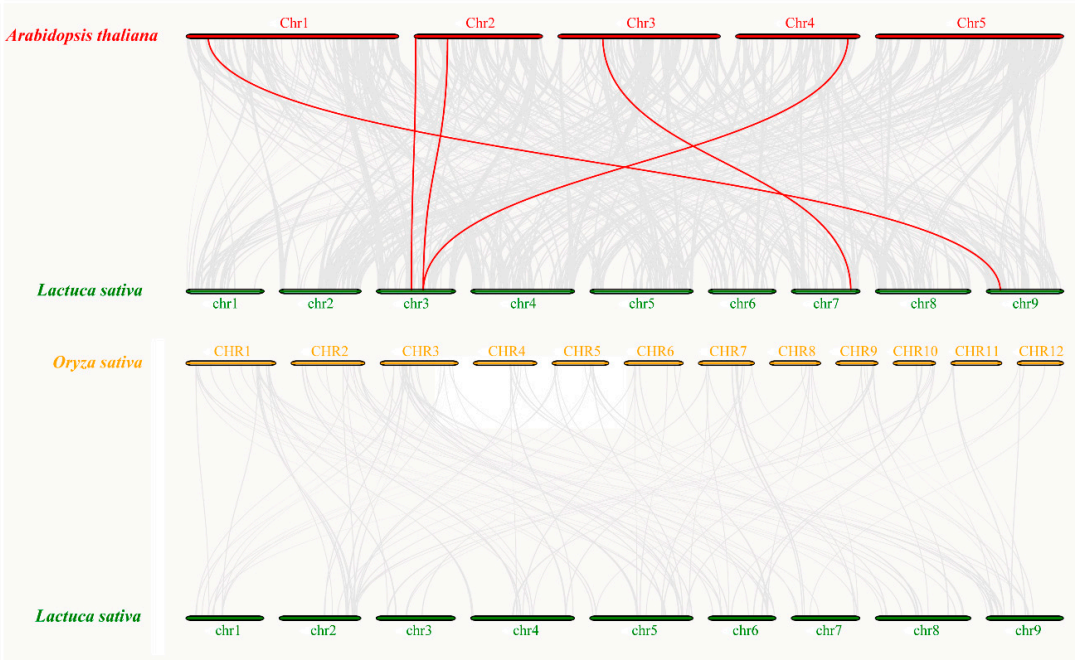

Figure 6. Comparative analysis of synteny among lettuce, Arabidopsis, and rice. The chromosomes of three plants are shown by colored circular rectangles. Grey and red curves represent the identified collinear blocks with the genomes and the collinear with MAPK genes, respectively.

3.7. Interaction Network Analysis of LsMAPK Proteins

In order to identify the interaction relationship of *LsMAPK* proteins with other proteins in lettuce, an interaction network analysis was conducted based on orthologous genes in *A. thaliana*, using STRING software. As shown in Figure 7, *LsMAPK* proteins in Groups A, B, C, and D showed complex interaction relationships with other proteins. For Group A MAPK proteins *LsMAPK2* (a homolog of Arabidopsis MPK6) and *LsMAPK9/17* (a homolog of Arabidopsis MPK3), both showed interaction with MKK2/4/5, WRKY33, PP2C (protein phosphatase 2C family protein), and protein-tyrosine-phosphatase (PTP1). For Group B MAPK proteins, MPK4 (*LsMAPK5*) and ATMAPK13 (*LsMAPK14*/18) were able to interact with MKK proteins (MKK2, MKK6), PP2C, and PTP1. Furthermore, *LsMAPK5* also showed an interaction relationship with WRKY transcription factors (WRKY33 and WRKY25). For Group C MAPK proteins, *LsMAPK1/4/7/8* proteins, the Arabidopsis MPK7 homologs, showed the same interaction networks. They both showed complex interaction with PP2C, MKK, and bZIP transcription factor. For Group D MAPK proteins, MPK9 (*LsMAPK3/6/15*), MPK16 (*LsMAPK11/12/13*), MPK18 (*LsMAPK16*), and MPK19 (*LsMAPK10*) showed ten interaction relationships with other proteins, including PTP1, PP2C, and bZIP transcription factor (HY5, HYH) (Supplemental Table S6).

3.8. Expression Patterns of LsMAPK Genes in Response to Stem Enlargement

Numerous studies have shown that MAPK genes can participate in the process of plant growth and development. In this study, the roles of *LsMAPK*s involved in the stem enlargement process of stem lettuce were evaluated by RNA-Seq (Supplemental Table S7). As shown in Figure 8, seven *LsMAPK* genes, including *LsMAPK1*, *LsMAPK8*, *LsMAPK11*, *LsMAPK12*, *LsMAPK15*, *LsMAPK17*, and *LsMAPK18*, showed different expression during four stem enlargement stages (S1, S2, S3, and S4). *LsMAPK8*, *LsMAPK11*, *LsMAPK17*, and *LsMAPK18* showed increased expression levels during the process of stem enlargement, while *LsMAPK1* and *LsMAPK12* showed decreased expression levels at S2-S4 stages compared with the S1 stage. Expression of *LsMAPK15* showed no significant change during the process of stem enlargement.

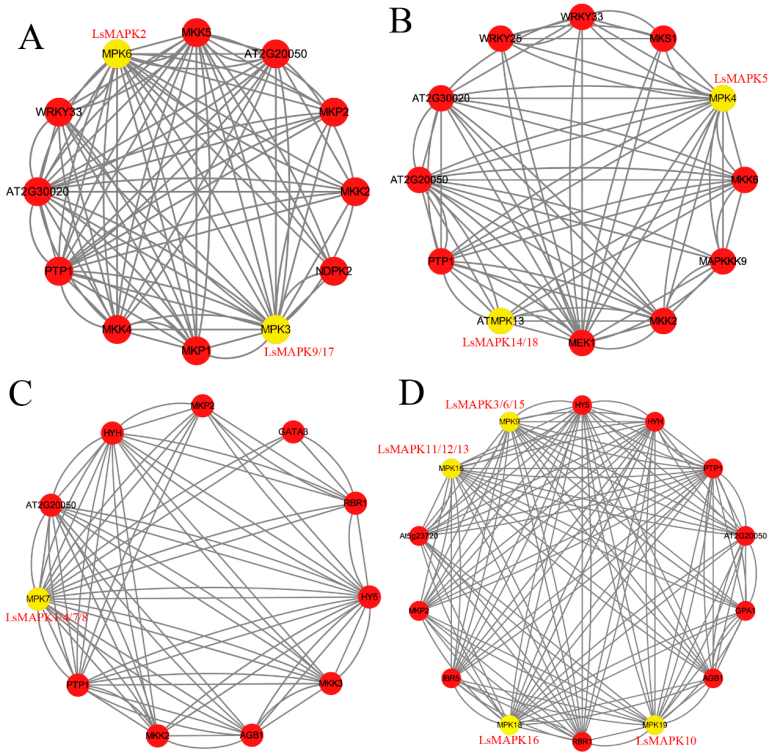

Figure 7. An interaction network analysis of *LsMAPK* proteins. (**A**) Interaction proteins of *LsMAPK* proteins belonging to the Group A subfamily. (**B**) Interaction proteins of *LsMAPK* proteins belonging to the Group B subfamily. (**C**) Interaction proteins of *LsMAPK* proteins belonging to the Group C subfamily. (**D**) Interaction proteins of *LsMAPK* proteins belonging to the Group D subfamily.

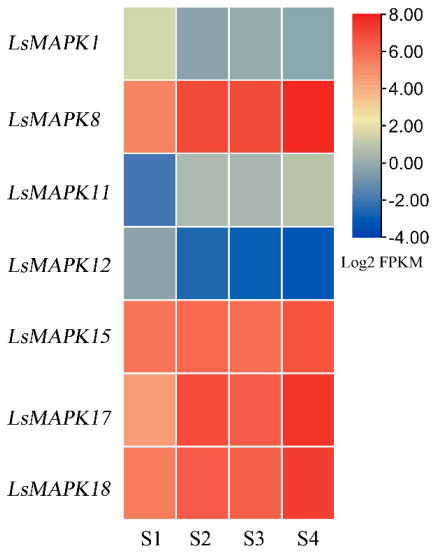

Figure 8. Expression profiles of *LsMAPK* genes by transcriptome data analysis at different lettuce stem enlargement periods. S1: diameter length is 1 cm; S2: diameter length is 2 cm; S3: diameter length is 3 cm; S4: diameter length is 4 cm. Different color represented different expression levels of *LsMAPK* genes identified by RNA-Seq.

The accuracy of the transcriptome profiles was validated by qRT-PCR analysis. The expression profiles of six *LsMAPK* genes (*LsMAPK1*, *LsMAPK8*, *LsMAPK11*, *LsMAPK15*, *LsMAPK17*, and *LsMAPK18*) were quantified at different stem enlargement stages (S1, S2, S3, and S4). As shown in Figure 9, these six genes showed differential expression patterns. For instance, *LsMAPK1* peaked at the S4 stage. The expression levels of *LsMAPK17* increased

significantly during the process of stem enlargement and peaked at the S3 stage. Compared with the S1 stage, the expression profiles of *LsMAPK11* and *LsMAPK15* decreased at the S2 and S3 stages but increased at the S4 stage. *LsMAPK18* showed decreased expression levels. Overall, the RNA-Seq and qRT-PCR analysis results of most *LsMAPK* genes were consistent, suggesting that the *LsMAPK* genes may be involved in the stem enlargement process.

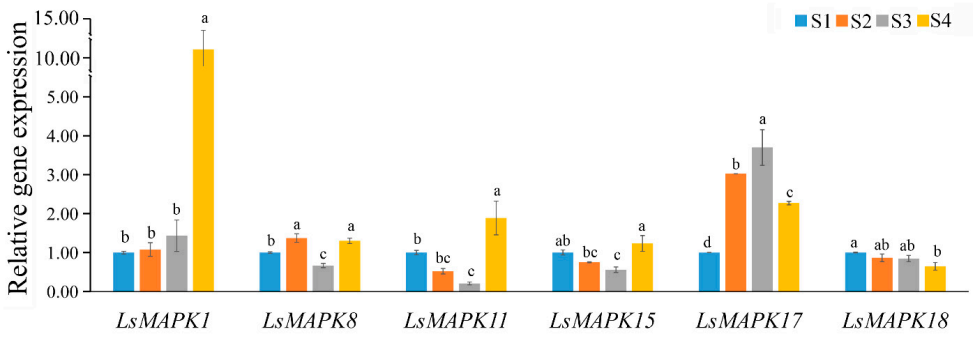

Figure 9. Expression profiles of *LsMAPK* genes at different lettuce stem enlargement periods. S1: diameter length is 1 cm; S2: diameter length is 2 cm; S3: diameter length is 3 cm; S4: diameter length is 4 cm. Bars with different lowercase letters (a, ab, bc, b, c) represented significantly different by Duncan's multiple range tests at the 0.05 levels.

3.9. Expression Levels of LsMAPK Genes Involved in Abiotic Stresses

MAPK genes not only participated in plant growth and development, but were also involved in various abiotic stresses such as drought, salt, and extreme temperature. Analysis of *cis*-elements showed that *LsMAPK* genes contained various abiotic stresses including low-temperature-responsive element LTR, and drought-induced MYB binding site (Figure 3). For a preliminary investigation of the potential role of *LsMAPK* genes under abiotic stresses, six *LsMAPK* genes (*LsMAPK1*, *LsMAPK8*, *LsMAPK11*, *LsMAPK15*, *LsMAPK17*, and *LsMAPK18*) which responded to stem enlargement periods were selected to determine the roles of *LsMAPK* genes in abiotic stress response (drought, salt, low temperature, and high temperature) by qRT-PCR. As shown in Figure 10, the expression profiles of the six *LsMAPK* genes differed after drought treatment for 12 h. The expression of three genes, including *LsMAPK1*, *LsMAPK8*, and *LsMAPK11*, increased. The expression levels of *LsMAPK1* and *LsMAPK11* both increased twice as much as CK. However, the expression levels of *LsMAPK15* and *LsMAPK18* decreased after drought treatment. There was no significant change in the expression of *LsMAPK17* under drought treatment. For salt treatment, the expression patterns of these six *LsMAPK* genes were similar to drought treatment. Four *LsMAPK* genes showed increased expression levels, including *LsMAPK1*, *LsMAPK8*, *LsMAPK11*, and *LsMAPK17*; however, the expression levels of *LsMAPK15* and *LsMAPK18* under salt treatment decreased by 0.86-fold and 0.57-fold, respectively. Low temperature also induced the expression of four *LsMAPK* genes. The expression levels of *LsMAPK1*, *LsMAPK11*, *LsMAPK17*, and *LsMAPK18* increased by 2.5-fold, 4.0-fold, 3.21-fold, and 1.32-fold, respectively, whereas *LsMAPK8* and *LsMPAK15* were insensitive to low temperature. For high-temperature treatment, only *LsMAPK1* and *LsMAPK8*, which belong to Group C, showed significantly increased expression; the expression levels of *LsMAPK1* and *LsMAPK8* increased by about 2.5-fold and 2-fold, respectively. In contrast, the expression patterns of *LsMAPK15*, *LsMAPK17*, and *LsMAPK18* decreased under high temperatures. *LsMAPK11* showed no significant change in expression level compared to the control under high temperatures.

3.10. LsMAPK Genes Expression Patterns in Response to Hormone Stresses

MAPK genes have been identified as participating in regulating plant hormone signal transduction. To investigate whether *LsMAPK* genes participate in hormone signal trans-

duction, expression levels of *LsMAPK* genes under different plant hormone treatments were investigated using qRT-PCR. As shown in Figure 11, SA induced the expression of four genes (*LsMAPK1*, *LsMAPK8*, *LsMAPK11*, and *LsMAPK17*) except for *LsMAPK15* and *LsMAPK18*. The expression levels of *LsMAPK17*, *LsMAPK1*, and *LsMAPK11* increased by about 5.5-fold, 2.5-fold, and 2.5-fold, respectively, under SA treatment. For GA treatment, three *LsMAPK* genes—*LsMAPK15*, *LsMAPK17*, and *LsMAPK18*—showed decreased expression levels. Only the expression profile of *LsMAPK11* was up-regulated under GA treatment; the expression level of *LsMAPK11* increased 2-fold. For ABA treatment, the expression level of *LsMAPK8* increased about 2-fold, while *LsMAPK1* and *LsMAPK18* expression decreased. The expression patterns of three genes, *LsMAPK11*, *LsMAPK15*, and *LsMAPK17*, showed no significant change under ABA treatment.

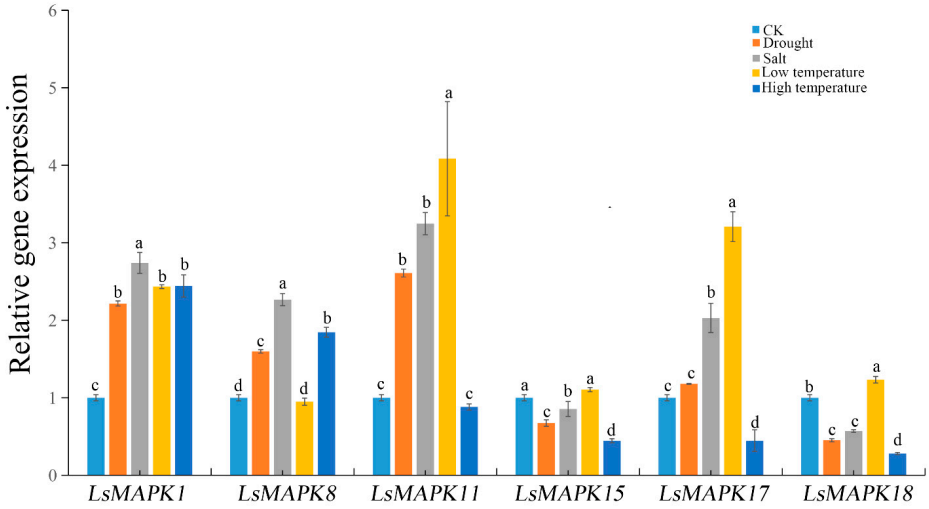

Figure 10. Relative expression of *LsMAPK* genes under salt, drought, low temperature and high temperature at 12 h. Bars with different lowercase letters (a, b, c) represented significantly different by Duncan's multiple range tests at the 0.05 levels.

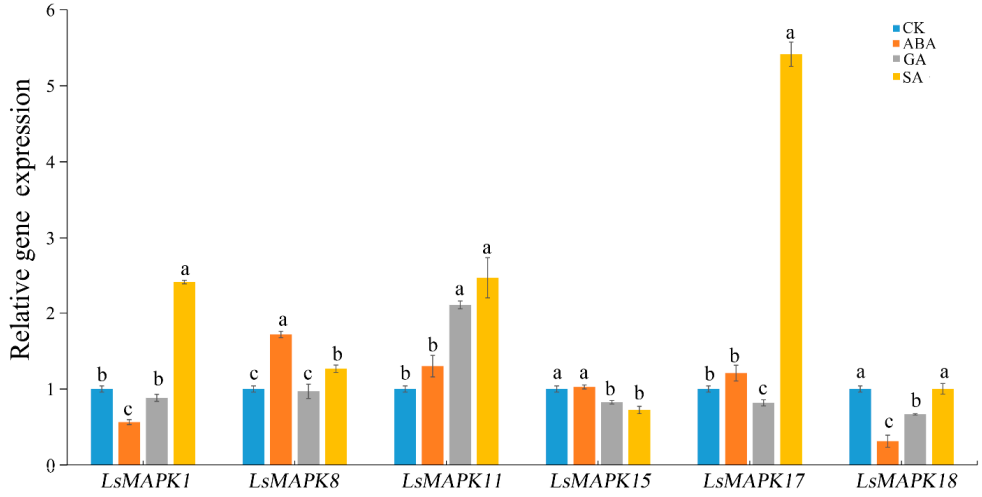

Figure 11. Relative expression of *LsMAPK* genes under SA (salicylic acid, 0.5 mmol/L), ABA (abscisic acid, 75 μmol/L), GA (gibberellin, 50 μmol/L) and untreated control (CK) at 12 h. Bars with different lowercase letters (a, b, c) represented significantly different by Duncan's multiple range tests at the 0.05 levels.

4. Discussion

During their lifetime, plants encounter various abiotic stresses which can seriously affect their growth and development, and change the distribution of plant species [32].

Higher plants adapt to various adverse environmental factors by developing complex signal transduction pathways. The MAPK cascade is one of the important signal transduction pathways in all eukaryotes and can provide developmental and environmental clues on intracellular responses [33]. Studies have shown that MAPK genes play critical roles in modulating various abiotic stress (temperature, drought, salinity, UV, heavy metal), biotic stress (pathogen infection, wounding), plant hormone response, and plant growth and development [34–36]. The possible function of gene family can be predicated by phylogenetic, evolutionary, and structural analysis. To predicate the roles of *LsMAPK* genes in lettuce, the gene structure of *LsMAPK* genes was analyzed. As shown in Figure 2, the exon–intron numbers in the same group of lettuce *LsMAPKs* were similar. The *LsMAPKs* in group C contained two or three exons, while, *LsMAPKs* in group D contained eight or eleven exons. Similar results were also shown in other plant species, such as Arabidopsis, poplar, and tomato [37–39]. The results showed that the evolution of exon–intron structure among different species was highly conserved.

Previous research has shown that there were 17 *LsMAPK* family genes in lettuce [40]. In the study, 18 *LsMAPK* genes were identified to exist in lettuce. There were some differences in the results of our study and those of Wang et al. [40]. For example, the *LsMAPK16* (Lsat_1_v5_gn_9_101600), which was not identified in 2022 by Wang et al. [40], was identified as a member of the MAPK family in our study. This difference may be related to the different *e* values used when searching the genes by BLASTP. Phylogenetic tree analysis showed the lettuce *LsMAPK*s were classified into A, B, C, and D groups based on the TE(D)Y motif. Research demonstrates that group D has the largest number of MAPK members than other groups, which indicates that group D has undergone significant expansion in the evolution of MAPKs [8]. In the study, the number of *LsMAPK* genes in different groups varied, and group D contained the largest number of members (8). Phylogenetic tree analysis also showed that 18 *LsMAPK*s in lettuce were more closely clustered with Arabidopsis than with rice, indicating the evolution relationship between lettuce and Arabidopsis was closer than with rice. The results were consistent with the results of synteny analysis. As shown in Figure 6, the collinear gene pairs among lettuce, Arabidopsis, and rice were performed. Five collinear gene pairs existed between lettuce and Arabidopsis, while no collinear gene pairs existed between lettuce and rice. The *LsMAPK* genes and their corresponding *AtMPKs* were clustered on the same branch, which indicated a similar function among these genes [41].

Plants have developed complex mechanisms to protect themselves from various abiotic stresses (extreme temperature, salinity, drought, and UV). MAPK proteins participate in plant abiotic stress response [42,43]. For instance, the *PtrMAPK*-overexpressing transgenic tobacco lines showed improved drought tolerance than wild-type (WT) varieties [44]. *BnMAPK1* from *Brassica napus* enhanced drought resistance by increasing root activity and cell water retention [45]. In our study, a large number of *cis*-elements related to abiotic stresses were found in the promoter region of *LsMAPK* genes, which indicated the possible function of *LsMAPK* genes in response to abiotic stress. The expression patterns of six *LsMAPK* genes were determined by qRT-PCR analysis. *LsMAPK1* and *LsMAPK8* were significantly induced by drought, salt, and high temperature. The function analysis of Arabidopsis *MPK3*, *MPK4*, and *MPK6* was comprehensive and thorough. *MPK3* and *MPK4*, which belong to group A, participated in plant salinity response through a complex regulation network [46]. *MPK3* and *MPK4* promoted salt tolerance by interacting with and phosphorylating key cytokinin signaling components, *ARR* genes, or heat shock factor *HSFA4A* [46,47]. In the study, the expression of *LsMAPK17*, a homolog of *AtMPK3*, was significantly induced by salt treatment and low temperature. Rice group A MAPK gene *OsMPK3* positively regulated low-temperature response by phosphorylating *OsICE1* genes, which directly targeted OsTPP1, the key enzyme in the trehalose biosynthetic pathway, to improve cold tolerance [48]. Similar, tomato *SlMPK3* enhanced low-temperature tolerance by improving antioxidant enzyme activity; however, *SlMPK1* served as a negative regulator in response to high temperature [49,50]. Arabidopsis *MPK3/MPK6* played a negative role

in response to low temperature, while the cold tolerance in mutation *MPK3*, *MPK6*, or both lines was improved compared with WT lines [51]. These results indicated that MAPK genes classified in the same group may play similar functions in response to abiotic stresses.

Although the roles of MAPK proteins in plant stress response are characterized, their functions in diverse signaling networks, including plant development such as pollen development and plant ovule development, are unclear [52,53]. In the study, the expression patterns of *LsMAPK* genes involved in stem enlargement process were detected. The expression levels of several *LsMAPK* genes, including *LsMAPK1* and *LsMAPK17*, were significantly increased in the stem enlargement process. In Arabidopsis, *AtMPK3*, *AtMPK4*, and *AtMPK6* were involved in flower development, including early pollen development, anther, and ovule integument development [54,55]. As the homolog of *AtMPK3*, the expression levels of *LsMAPK17* increased continuously in the process of stem expansion, implying they may participate in regulating this process. However, the important roles of *LsMAPK* genes in the stem enlargement process need to be explored further.

Some studies have confirmed the interconnections between MAPK signaling and plant hormones. ABA enhanced plants' resistance to various unfavorable environmental conditions and was needed for plant growth and development. Studies have shown that MAPK genes are involved in ABA signaling through different molecular biology techniques. Arabidopsis *MPK3* negatively regulated the ABA signaling pathway. The *MPK3*-overexpressed Arabidopsis seedlings were stunted even under ABA treatment [56]. Interestingly, *MPK3* also participated in ABA-inhibited stomatal opening [57]. In addition, Arabidopsis *MPK9* and *MPK12* were involved in ABA signaling [58]. Arabidopsis *MPK3* and *MPK6* were able to disrupt normal plant growth and development by inducing SA accumulation [59]. *LsMAPK17*, the homolog of *AtMPK3*, was significantly induced under SA treatment, and the expression level increased about 6-fold. The roles of MAPK genes in JA, auxin, and the ethylene signaling pathway have also been identified in plants including Arabidopsis, tomato, and tobacco [36]. Here, different expression patterns under ABA, GA and SA existed in the examined *LsMAPK* genes, indicating the potential different functions of these genes in lettuce hormone signaling pathways. In summary, when stem lettuce is threatened by different adverse environments, various stimuli (abiotic stress, plant hormone, and plant growth and development) will be transmitted to the MAPK cascades through signaling molecular on the cell membrane. MAPK cascades can phosphorylate downstream target genes such as transcription factor, thereby inducing the expression of downstream functional genes (Figure 12). These results will provide the information for the function analysis of lettuce MAPK genes.

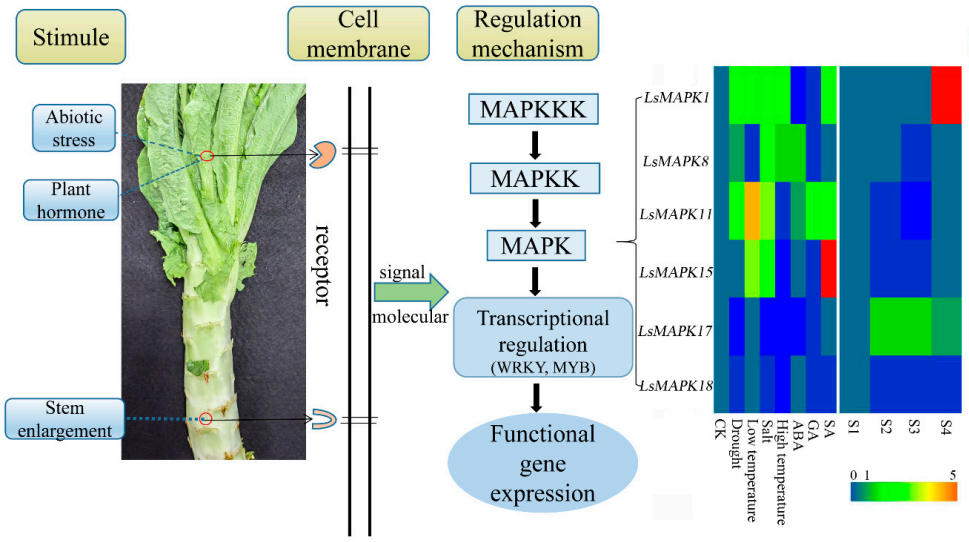

Figure 12. Possible model of stem lettuce *LsMAPK* genes involved in different stimuli.

5. Conclusions

In our study, 18 *LsMAPK* genes were identified in lettuce. The systematic analysis and identification of *LsMAPK* genes were conducted by the analysis of exon–intron structure, motif compositions, collinearity analysis, phylogenetic relationships, chromosome distribution, and expression patterns. Our study provides important information about the evolution and diversity of the MAPK gene family in lettuce. These findings can provide a basis for further analysis of the function of MAPK genes in plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8111087/s1, Supplemental Tables: Table S1. Primer sequences for qRT-PCR in the study. Table S2. The nucleotide and amino acid sequences of *LsMAPK* genes. Table S3. *Cis*-elements identified in the promoter region of *LsMAPK* genes by PlantCARE. Table S4. *Cis*-elements identified in the promoter region of *LsMAPK* genes by PlantPAN. Table S5. The paralogs and orthologs genes of MAPK genes between lettuce and Arabidopsis. Table S6. Interaction proteins between *LsMAPK* proteins and other proteins. Table S7. Expression profiles of *LsMAPK* genes by transcriptome data analysis at different lettuce stem enlargement periods. with 1, 2, and 3 representing three biological repeats. Supplemental Figure S1. The amino acid sequence alignment of *LsMAPK* proteins.

Author Contributions: Conceptualization, Y.H. and M.L.; investigation, Y.H., Y.L. and Z.L.; resources, J.D. and Y.L.; data curation, X.C. and Z.L.; writing—original draft preparation, Y.H. and J.D.; writing—review and editing, Y.H. and M.L.; funding acquisition, Y.H. and M.L. All authors have read and agreed to the published version of the manuscript.

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