Article

Genome-Wide Identification and Expression Analysis of MAPK and MAPKK Gene Family in Pomegranate (*Punica Granatum* L.)

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Abstract: Mitogen-activated protein kinase (MAPK) cascade is involved in the regulation of a series of biological processes in organisms, which are composed of MAPKKs, MAPKKs, and MAPKs. Although genome-wide analyses of it has been well described in some species, little is known about MAPK and MAPKK genes in pomegranates. In this study, we identified 18 PgMAPKs, 9 PgMAPKKs through a genome-wide search. Chromosome localization showed that 27 genes are distributed on 7 chromosomes with different densities. Multiple sequence alignment and phylogenetic analysis revealed that PgMAPKs and PgMAPKKs could be divided into 4 subfamilies (groups A, B, C, and D), respectively. In addition, exon-introns structural analysis of each candidate gene has indicated high levels of conservation within and between phylogenetic groups. Cis-acting element analysis predicted that PgMAPKs and PgMAPKKs were widely involved in the growth, development, stress and hormone response of pomegranate. Expression profile analyses of PgMAPKs and PgMAPKKs were performed in different tissues (root, leaf, flower and fruit), and PgMAPK13 was significantly expressed in all tissues. To our knowledge, this is the first genome-wide analysis of the MAPK and MAPKK gene family in pomegranate. This study provides valuable information for understanding the classification and functions of pomegranate MAPK signal.

Keywords: *Punica granatum*; gene family; MAPK; MAPKK; gene expression

1. Introduction

In the long process of evolution, plants have evolved complex signal network regulation mechanism to adapt to the biotic and abiotic stress during growth and development [1–4]. The mitogen-activated protein kinase (MAPK) cascade is regarded as one of the typical mechanisms of signal transduction. As broad and relatively conservative evolutionary pathway, it is involved in multiple metabolic pathways such as cell division, development process and defense response in organism [5–7]. The typical MAPK cascade is composed of three specific kinases, MAP kinase (MAPK), MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK) [8]. They are activated by phosphorylation and dephosphorylation at specific sites [9]. According to sequence alignment and phylogenetic analysis of model species *Arabidopsis thaliana* [9], the MAPK and MAPKK genes can be divided into four groups (A, B, C, and D). MAPK family members all have a unique “TxY” phosphorylation motif, while the MAPKK genes are activated when serine and serine/threonine residues in the “S/TXXXXXS/T” motif [10]. Despite the highly conserved structure of plant MAPKs and MAPKKs, they have been shown to be extensively involved in plant growth, and hormonal response regulation processes, as well as diverse biological and abiotic stress [11,12]. To date, members of the MAPK cascade gene family have been identified in

several species such as *Arabidopsis thaliana* [13,14], *Oryza sativa* [15,16], *Brachypodium distachyon* [17], *Zea mays* [18], and *Hordeum vulgare* [19].

Numerous studies have shown that the MAPK cascade pathway mekk1-mkk4/5-mpk3/6 is involved in the biosynthetic pathway of phytoalexins [20]. In response to the pathogen, *Arabidopsis thaliana* induced the expression of related defense genes by activating two MAPK cascade pathways, mekk1-mkk1/2-MPK4 and mekk1-mkk4/5-mpk3/6, in which the former played a negative regulatory role [21,22], while the latter played a positive regulatory role [23]. In terms of signaling under abiotic stress, studies have shown that a variety of MAPKs, including MAPK3, MAPK4 and MAPK6, can be rapidly activated in plants under abiotic stress [23]. For example, the transcriptional level of *ZmMPK3* in maize significantly increased and rapidly increased under multiple stresses such as cold, drought, ultraviolet, salinity, heavy metal, and mechanical damage [24,25]. Overexpression of *OsMAPK5* in transgenic rice can increase its tolerance to drought, salt, and cold stress [26], and *RaMPK1*, combined with *RaMPK2* were significantly up-regulated in response to low temperature stress [27]. In the field of plant hormone signaling, MAPKs and MAPKKs are involved in the signaling of auxin, abscisic acid, and ethylene. Activity of MAPKs has been found to be directly dependent on abscisic acid stimulation in different plants such as rice [15,16], corn [18], barley [19], and tobacco [20]. *OsMAPK5* (*OsMAP1*), *OsMAPK2*, *OsMAPK44* and other MAPKs can be activated by ABA transcription [28]. MPK3 and MPK6 in *Arabidopsis* play roles in the ethylene response pathway by promoting the stability of the key transcription regulator *EIN3* [29]. It has been found that the mkk9-mpk3/MPK6 cascade controls *EIN3* transcription to regulate ethylene signaling in plant cells and links the interwoven MAPK cascade pathways to control the quantitative response and specificity in the signaling network [30]. Since the research on the MAPK and MAPKK gene families has become increasingly intensive and comprehensive, we place emphasis on these two families in this paper.

Pomegranate (*Punica granatum* L.) is an economic tree widely cultivated throughout the world [31–34]. Native to central Asia, it is known for its bright red rinds, juicy kernels, and allagenic tannins [35,36]. With the advancement of molecular biology and bioinformatics methodology, it is of great significance to study the functions about signal transduction pathways in plants under stress response, cell growth and development, and comprehensive disease resistance [37]. A new high-quality genome sequence has been published, making it possible to study the gene family of pomegranates [38]. However, no information on pomegranate MAPK and MAPKK gene family at genome-wide level is currently available.

In this study, the MAPK and MAPKK gene family members of pomegranate were systematically identified at the genome-wide level using the pomegranate “Tunisia” genomes. Physicochemical properties, chromosomal locations, gene structures, cis-acting elements, and expression patterns of PgMAPKs and PgMAPKKs were carefully analyzed, which would be conducive to further research on the application in pomegranate growth and development.

2. Materials and Methods

2.1. Genome Data Sources

Pomegranate genome-wide data (SAMN05193489) were downloaded from NCBI’s official website (<https://www.ncbi.nlm.nih.gov/biosample/SAMN05193489/>); The MAPK and MAPKK protein sequences of *Arabidopsis thaliana* were obtained from EnsemblPlants database (<http://plants.ensembl.org/index.html>) [39] (Fasta file S1, Table S1).

2.2. Identification of PgMAPKs and PgMAPKKs in Pomegranate

The protein kinase domain sequence comparison file (Pfam accession No.PF00069) was downloaded from Pfam database (<http://pfam.xfam.org/>) [40]. HMMER v3.2.1 software [41] was used to convert the comparison file into HMM model file. Selecthmm package (<https://github.com/Redpome/SelectHMM>) (E-value 10^{−10} or less) was used to get pomegranate protein

sequence containing MAPK cascade genes sequence structure domain. Moreover, the protein domains were validated by online program SMART (<http://smart.embl-heidelberg.de>) [42] and NCBI CDD (<https://www.ncbi.nlm.nih.gov/CDD>) [43]. The isoelectric points and molecular weights of *PgMAPKs* and *PgMAPKKs* were obtained by the ExPASy Proteomics Server (<http://expasy.org/>) [44].

2.3. Phylogenetic Relationship and Conserved Motif Analysis

ClustalX V2.0 [45] was used for multi-sequence alignment of identified proteins of *PgMAPKs* and *PgMAPKKs* (Fasta file S2) with parameters set as default values. The results were then submitted to GeneDoc [46] for visualization. A neighbor-joining phylogenetic tree was constructed with MEGA V7.0 [47] (selecting position model with 1000 bootstrap replicates) to explore the phylogenetic relationships of *PgMAPK* and *PgMAPKK* family members based on the full-length protein sequence. The conservative motif was obtained by online program MEME (<http://meme.nbcr.net/meme/intro.html>) [48] based on the expectation maximum (EM) algorithm. The introns and exons of *PgMAPKs* and *PgMAPKKs* genes were analyzed and visualized by TBtools V0.6696 [49]. Finally, the 2.0kb upstream DNA sequence of each candidate gene was extracted, and then submitted to Plantcare database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [50] to predict the cis-acting elements. Cis-acting elements related to growth, stress and hormones were manually retained, and then submitted to TBtools v0.6696 for drawing.

2.4. Chromosome Localization and Expression Profiles

The chromosome distribution information of *PgMAPKs* and *PgMAPKKs* were displayed by TBtools. RNA-seq data of six pomegranate cultivars were downloaded from NCBI database, including “Dabenzi”, “Tunisia”, “Baiyushizi”, “Wonderful”, “Nana”, and “Black127” (Table 1 and Table S2). All RNA-Seq data were quality-controlled by fastp [49] to obtain clean reads. The index file was constructed by Kallisto V0.44.0 [51] and then the normalized expression units (Transcripts Per Kilobase of exon model per Million mapped reads (TPM) values) of each gene were extracted by kallisto quant command. The final expression levels was from the TPM values that converted by $\log_2(\text{TPM}+1)$. Then the Heatmap package in RStudio V3.6.1 [52] was used to draw the Heatmap of *PgMAPKs* and *PgMAPKKs*.

Table 1. RNA-Seq data of pomegranate.

Accession No.	Cultivars	Sample Type	Library	Platform	Reference
SRR5279396	“Dabenzi”	Root	Paired end	Illumina HiSeq 4000	[33]
SRR5279397	“Dabenzi”	Leaf	Paired end	Illumina HiSeq 4000	[33]
SRR5279395	“Dabenzi”	Flower	Paired end	Illumina HiSeq 4000	[33]
SRR5279391	“Dabenzi”	Inner seed coat (50 days after pollination)	Paired end	Illumina HiSeq 4000	[33]
SRR5279388	“Dabenzi”	Outer seed coat (50 days after pollination)	Paired end	Illumina HiSeq 4000	[33]
SRR5279394	“Dabenzi”	Pericarp (50 days after pollination)	Paired end	Illumina HiSeq 4000	[33]
SRR5678820	“Tunisia”	Inner seed coat (50 days after pollination)	Paired end	Illumina HiSeq 4000	[33]
SRR5678819	“Baiyushizi”	Inner seed coat (50 days after pollination)	Paired end	Illumina HiSeq 4000	[33]
SRR1055290	“nana”	Mixed samples of leaves, flowers, fruit and roots	Single end	454 GS FLX Titanium	[53]
SRR1054190	“Black127”	Mixed samples of root, leaf, flower and fruit	Single end	454 GS FLX Titanium	[53]
SRR5446598	“Tunisia”	Functional male flower (3.0–5.0 mm)	Paired end	Illumina HiSeq 2500	[54]
SRR5446595	“Tunisia”	Functional male flower (5.1–13.0 mm)	Paired end	Illumina HiSeq 2500	[54]
SRR5446592	“Tunisia”	Functional male flower (13.1–25.0 mm)	Paired end	Illumina HiSeq 2500	[54]
SRR5446607	“Tunisia”	Female sterility flower (3.0–5.0 mm)	Paired end	Illumina HiSeq 2500	[54]
SRR5446604	“Tunisia”	Female sterility flower (5.1–13.0 mm)	Paired end	Illumina HiSeq 2500	[54]
SRR5446601	“Tunisia”	Female sterility flower (13.1–25.0 mm)	Paired end	Illumina HiSeq 2500	[54]
SRR080723	“Wonderful”	Pericarp	Paired end	Illumina HiSeq 2000	[55]

3. Results

3.1. Identification and Sequence Analysis of *PgMAPKs* and *PgMAPKKs*

According to the described method, 18 *PgMAPKs* and 9 *PgMAPKKs* were obtained from pomegranate whole genome, respectively (Table 2). Furthermore, the number of amino acids,

molecular weight, theoretical pI of *PgMAPKs* and *PgMAPKKs* were analyzed by ExPASy online tool. The results showed that the molecular weight was between 34910.05~70420.74 Da, the isoelectric point was between 4.94~9.38, the protein length was within the range of 314~619 amino acids, the number of amino acids varied significantly. These data were similar to those reported in economic trees such as grape [56], banana [57], and apple [58]. However, the variations in physicochemical properties among the *PgMAPKs* and *PgMAPKKs* indicated that these genes may have subfunctionalization and new functionalization [59]. Chromosomal location analyses showed that 18 *MAPKs* and 9 *MAPKKs* presented on 7 chromosomes (chr1, chr2, chr3, chr4, chr6, chr7, chr8) (Table 2 and Figure 1).

Table 2. The information of *MAPK* and *MAPKK* gene family in pomegranate.

Gene Name	Protein ID	Genomic Position	Size (aa)	MW (Da)	pIs	Types	Group
PgMAPK1	XP_031372466.1	chr8:25215947 ... 25219536	615	68,952.19	7.69	TDY	D
PgMAPK2	XP_031382378.1	chr2:26190661 ... 26195979	391	44,872.36	5.57	TEY	A
PgMAPK3	XP_031384487.1	chr3:27987357 ... 27983062	484	55,597.84	8.79	TDY	D
PgMAPK4	XP_031387033.1	chr3:6220495 ... 6221628	377	42,844.83	8.90	TGY	C
PgMAPK5	XP_031387034.1	chr3:6229201 ... 6231072	435	48,521.45	7.66	TGY	C
PgMAPK6	XP_031387035.1	chr3:6233547 ... 6234680	377	42,552.40	9.23	TGY	C
PgMAPK7	XP_031388383.1	chr3:6647361 ... 6648512	383	43,266.50	9.26	TGY	C
PgMAPK8	XP_031389841.1	chr4:494852 ... 498050	374	42,897.79	6.11	TEY	B
PgMAPK9	XP_031389944.1	chr4:8712267 ... 8715865	380	43,399.50	6.20	TEY	B
PgMAPK10	XP_031392146.1	chr4:18603577 ... 18604728	383	43,164.35	9.36	TGY	C
PgMAPK11	XP_031392360.1	chr4:34805345 ... 34809858	617	70,420.74	9.19	TDY	D
PgMAPK12	XP_031399464.1	chr6:27190634 ... 27192917	377	43,002.99	4.94	TEY	B
PgMAPK13	XP_031400536.1	chr6:27163862 ... 27162051	375	42,968.25	5.78	TEY	A
PgMAPK14	XP_031402771.1	chr1:54220111 ... 54215519	566	64,371.77	8.76	TDY	D
PgMAPK15	XP_031402779.1	chr1:54220111 ... 54215513	564	64,243.64	8.76	TDY	D
PgMAPK16	XP_031404075.1	chr7:28354650 ... 28351083	597	68,130.90	9.35	TDY	D
PgMAPK17	XP_031404901.1	chr7:21504171 ... 21503431	372	42,781.79	6.92	TEY	C
PgMAPK18	XP_031406973.1	chr7:3285226 ... 3281535	619	69,387.45	8.06	TDY	D
MAPKK1	XP_031374856.1	chr8:13616418 ... 13612292	354	39,870.90	5.96	DIK	A
MAPKK2	XP_031374857.1	chr8:13616418 ... 13612292	354	39,870.90	5.96	DIK	A
MAPKK3	XP_031381620.1	chr2:14357511 ... 14354367	352	38,956.51	5.49	DLK	A
MAPKK4	XP_031383136.1	chr2:2401332 ... 2404194	518	58,182.26	5.76	DIK	B
MAPKK5	XP_031383137.1	chr2:2401332 ... 2404194	518	58,182.26	5.76	DIK	B
MAPKK6	XP_031383138.1	chr2:2401664 ... 2404194	436	49,232.33	6.04	DIK	B
MAPKK7	XP_031388194.1	chr3:13155229 ... 13156281	350	39,123.69	9.38	DIK	C
MAPKK8	XP_031393241.1	chr4:1081579 ... 1082574	331	36,995.33	8.31	DIK	D
MAPKK9	XP_031406836.1	chr7:3593726 ... 3594670	314	34,910.05	8.35	DIK	D

Figure 1. Positions of *PgMPK* and *PgMPKK* genes on the pomegranate chromosomes. Genes were mapped to the pomegranate chromosomes by TBtools.

3.2. Conserved Motif Analysis of PgMAPK and PgMAPKK Gene Family Members

According to specific conserved motifs analysis, PgMAPKs and PgMAPKKs were found to have unique conserved motifs (Figures 1 and 2). The “VGTxxYMSPER” conserved motifs were found in 9 PgMAPKK genes, and the “T (E/D) YVxTRWYRAPE (L/V)” conserved motifs were contained in 18 PgMAPK genes (Figure 2). Through multiple sequence alignment of the protein sequences of PgMAPK family genes, “T-loop” motifs were detected in the domain (Figure 3). There was an ATP phosphorylation site catalyzed by “TxY”, which plays an important role in the function of PgMAPKs. According to previous studies of model plants, the PgMAPK gene family can also be divided into four groups (A, B, C, and D) and can be divided into subtypes of “TDY” and “TEY” according to different “TxY” phosphorylation sites (Table 1). In addition, phosphorylation domains with “S/T-XXXXX-S/T” and an anchor site with “-D (L/I/V) K-” were found in PgMAPKKs (Figure 3). PgMAPKKs were also divided into four groups (A, B, C and D) (Table 1) based on results reported in the *Arabidopsis thaliana* [60].

3.3. Phylogenetic Relationship of PgMAPKs and PgMAPKKs

In order to explore the evolutionary relationship toward PgMAPK and PgMAPKK proteins, the predicted protein sequences of 18 PgMAPKs, 9PgMAPKKs, 20 *At*MAPKs, and 10*At*MAPKKs from *Arabidopsis* were subjected to a multiple sequence alignment using the MEGA7. The N-J (Neighbor-Joining) method was then used to construct an unrooted phylogenetic tree. As shown in Figure 4, the phylogenetic tree divided PgMAPKs and PgMAPKKs into four groups (A, B, C, D). The most abundant of MAPK gene family members were in group D (7), followed by group C (6), group B (3) and group A (2). This result was consistent with previous studies on *Arabidopsis* [14], rice [15], and tomato [61]. The number of MAPKK gene family was less than that of MAPK, so there was no significant difference in the number of each group. Three (PgMAPKK1, PgMAPKK2, PgMAPKK3), three (PgMAPKK4, PgMAPKK5, PgMAPKK6), one (PgMAPKK7) and two (PgMAPKK8, PgMAPKK9) PgMAPKK genes belonged to groups A, B, C, and D respectively. The ratio of PgMAPKKs to PgMAPKs was about 1:2, which was in accordance with that in other plant (Table 3). In summary, compared with these model plants, the quantity and grouping status of MAPKs and MAPKKs were highly conserved in pomegranate (Figure 4, Table 3).

Figure 2. The specific conserved motif of MAPK and MAPKK gene in pomegranate.

Figure 3. Sequence multiple alignment and conserved motifs analysis of *PgMAPK* and *PgMAPKK* genes.

Figure 4. The phylogenetic tree of the *MAPK* and *MAPKK* gene family in pomegranate and *Arabidopsis thaliana*.

Table 3. The number of the MAPK and MAPKK gene family in Pomegranate and other plants.

Gene Family	Species	GroupA	GroupB	GroupC	GroupD	Total	Reference
MAPK	Pomegranate	2	3	6	7	18	
	Chinese jujube	2	1	2	5	11	[10]
	<i>Arabidopsis</i>	3	5	4	8	20	[14]
	Rice	2	1	2	10	15	[15]
	<i>Brachypodium distachyon</i>	2	2	3	9	16	[17]
	Maize	4	2	2	11	10	[18]
	Apple	5	6	5	10	26	[58]
	Poplar	4	4	4	9	21	[60]
	Tomato	3	4	2	7	16	[61]
	Mulberry	2	3	2	3	10	[62]
	Bread wheat	7	3	8	36	54	[3]
MAPKK	Pomegranate	3	3	1	2	9	
	Chinese jujube	2	1	0	2	5	[10]
	<i>Arabidopsis</i>	3	1	2	4	10	[14]
	Rice	2	1	2	3	8	[15]
	<i>Brachypodium distachyon</i>	2	3	2	5	12	[17]
	Apple	3	1	2	3	9	[58]
	Poplar	3	1	2	5	11	[60]
	Bread wheat	3	2	1	12	18	[3]

3.4. Gene Structure Analysis of PgMAPKs and PgMAPKKs

Gene structure analysis was supposed to provide valuable information about evolutionary and duplication events among gene families. We found that most members of the same group share similar exon/intron structures and conserved motifs. In the PgMAPKs gene family, each group showed different structural characteristics. In group A, PgMPK2 and PgMAPK13 were composed of 6 exons with the longer introns. Six exons were also found in same group of MAPKs in *Arabidopsis*, poplar and tomato. PgMPK8, PgMAPK9, and PgMAPK12 in group B had 6 exons, which was also consistent with the results of studies on tomato and poplar studies. In group C, PgMAPK5 and PgMAPK17 were only composed of two exons, and the size of each exon was strictly conservative. Compared with the highly conserved structural pattern in group C, the exon and intron distribution of PgMPKs in group D was more complex, exhibiting a diverse pattern. For example, PgMPK1, PgMAPK18 had 11 exons, while PgMAPK11, PgMAPK14, PgMAPK15, and PgMAPK16 had 10 exons, and PgMAPK3 only had 9 (Figure 5). Although there were slight differences in exon lengths, it was clear that the structural patterns of exons well conserved not only between close paralogs, but also between the PgMAPKs that apparently diverged following earlier duplication events.

The PgMAPKK gene showed two distinct structural patterns (Figure 5) which were quite similar to the MAPKK gene in *Arabidopsis* and poplar. PgMAPKK7 in group C and PgMAPKK8, PgMAPKK9 in group D both had less introns and exons, whereas the PgMAPKKs in group A and group B possessed more exon and intron junctions. In group A, PgMAPKK1 and PgMAPKK2 had 7 exons, while PgMAPKK3 had 8 exons. Consistent with other plants, PgMAPKK1 and PgMAPKK2 shared strong conservation of exonic length. As shown in Figure 5, there were highly consistency of gene structure among the three genes (PgMAPKK4, PgMAPKK5, and PgMAPKK6) in group B.

Figure 5. The exon/intron structure of *PgMAPK* and *PgMAPKK* genes. Introns and exons are represented by black lines and green boxes respectively. Note: The structure was analyzed combined phylogenetic trees and motif patterns from the perspective of evolution.

3.5. Cis-Acting Element Prediction of *PgMAPKs* and *PgMAPKKs*

We extracted upstream 2.0 KB of genome sequence for the cis-acting elements analysis. And hundreds of possible cis-acting elements downloaded from the Platcare database (Table S3). After screening, 25 cis-acting elements with clear functional information were retained (Table S4). Among them, we found that a total of 6 elements were related to stress, 9 elements were related to growth and development, and the remaining 10 elements were related to hormones (Table 4). As shown in Table 3, elements named ARE, LTR, and MBS were present in 20, 18 and 17 genes, respectively. These results indicated that *PgMAPK* and *PgMAPKK* genes were widely involved in hypoxia, low temperature and drought stress of pomegranate. Furthermore, WUN-motif was found in six genes, indicating that these genes involved in the external damage mechanism of pomegranate. The elements related to plant growth and development mainly included circadian rhythm, meristem, palisade mesophytic differentiation and endosperm. Notably, MBSI elements were found in five genes (*PgMAPK2*, *PgMAPK4*, *PgMAPK8*, *PgMAPK14*, and *PgMAPK15*), suggesting that these genes are involved in the mechanism of flavonoid synthesis in pomegranate. In addition, the cis-acting elements related to various hormone reactions (such as auxin, ethylene, salicylic acid, MeJA, GA3), indicating that *PgMAPKs* and *PgMAPKKs* were deeply involved in signal transduction of endogenous and exogenous hormone networks in pomegranate. To sum up, the various cis-acting elements in the gene promoter region suggested that the *PgMAPK* and *PgMAPKK* gene family plays a crucial role in its growth and development and stress adaptation (Table 4 and Figure 6).

Table 4. Characteristics of cis-acting regulatory elements presented in the promoter regions of *PgMAPK* and *PgMAPKK* genes.

Function	Promoter Name	Promoter Annotation	Total Number
Motifs related to stress response	ARE	cis-acting regulatory element essential for the anaerobic induction	20
	LTR	cis-acting element involved in low-temperature responsiveness	18
	MBS	MYB binding site involved in drought-inducibility	17
	TC-rich repeats	cis-acting element involved in defense and stress responsiveness	6
	WUN-motif	wound-responsive element	12
	GC-motif	enhancer-like element involved in anoxic specific inducibility	9
Motifs related to growth and development	CAT-box	cis-acting regulatory element related to meristem expression	8
	GCN4_motif	cis-regulatory element involved in endosperm expression	5
	CCGTCC-box	cis-acting regulatory element related to meristem specific activation	10
	O2-site	cis-acting regulatory element involved in zein metabolism regulation	11
	HD-Zip 1	element involved in differentiation of the palisade mesophyll cells	4
	RY-element	cis-acting element involved in seed-specific regulation	1
	MBSI	MYB binding site involved in flavonoid biosynthetic genes regulation	5
	MSA-like	cis-acting element involved in cell cycle regulation	1
Motifs related to hormone response	circadian	cis-acting regulatory element involved in circadian control	5
	ABRE	cis-acting element involved in the abscisic acid responsiveness	23
	AuxRR-core	cis-acting regulatory element involved in auxin responsiveness	1
	CGTCA-motif	cis-acting regulatory element involved in the MeJA-responsiveness	20
	TGACG-motif	cis-acting regulatory element involved in the MeJA-responsiveness	19
	ERE	ethylene-responsive element	20
	GARE-motif	gibberellin-responsive element	7
	P-box	gibberellin-responsive element	8
	TATC-box	cis-acting element involved in gibberellin-responsiveness	1
	TGA-element	auxin-responsive element	11
	TCA-element	cis-acting element involved in salicylic acid responsiveness	15

Figure 6. Promoter cis-acting element of *MAPK* and *MAPKK* genes in pomegranate.

3.6. Expression Pattern of *PgMAPKs* and *PgMAPKKs*

We used the online RNA-seq data to explore the expression patterns of *MAPK* and *MAPKK* genes in pomegranates at different developmental stages. The results showed that these 27 genes were expressed in at least one tissue organ or stage of development (Figure 6). Compared with other genes, *PgMAPK13* was highly expressed in root, flower and seed coat, while *PgMAPK4*, *PgMAPK5*, *PgMAPK6*, *PgMAPK7*, *PgMAPK8*, *PgMAPK10* had a less expression level. *PgMAPK9*, *PgMAPK12* and *PgMAPKK7* was expressed in all except pericarp as shown in Figure 7A. A high *PgMAPKK9* and

PgMAPK13 expression level was found in inner seed coat after pollination for 50 days. *PgMAPKK3* and *PgMAPKK9* expressed significantly variously during the development of functional male flower and female sterility flower as illustrated in Figure 7C. The expression patterns demonstrated that the expression level of each gene was highly variable, suggesting that these genes play an indispensable role in regulating plant growth and development.

Figure 7. Heatmap of *PgMAPK* and *PgMAPKK* gene expression in different tissues. (A) showed the expression difference of different tissues; (B) illustrated difference in inner and outer seed coat and pericarp after pollination for 50 days; (C) displayed the expression under different stages of flower development in cultivar “Tunisia”. Note: S1: Root; S2: Leaf; S3: Flower; S4: Pericarp; S5: Mixed sample of leaves, flowers, fruit, roots of “nana”; S6: Mixed sample of leaves, flowers, fruit, roots of “Black127”; S7: Inner seed coat (50 days after pollination); S8: Outer seed coat (50 days after pollination); S9: (50 days after pollination); S10: Inner seed coat of “Tunisia” (50 days after pollination); S11: Inner seed coat of “Baiyushizi” (50 days after pollination); S12: Functional male flower (13.1–25.0 mm); S13: functional male flower (5.1–13.0 mm); S14: Functional male flower (3.0–5.0 mm); S15: Female sterility flower (13.1–25.0 mm); S16: Female sterility flower (5.1–13.0 mm); S17: Female sterility flower (3.0–5.0 mm); S1–S4, S7–S9 and S12–S17 are cultivar “Dabenzi”.

4. Discussion

Generally, plants responded to various biological and abiotic stresses through physiological, biochemical and even molecular changes to resist and adapt them [59]. As an important protein kinases group, *MAPKs* and *MAPKKs* can transfer extracellular signals to intracellular signals in plant growth and development, hormone regulation and stress response [63,64]. To date, *MAPK* and *MAPKK* gene family have been identified and functionally characterized in several model plants and numerous economic trees. However, the *MAPK* and *MAPKK* gene family in pomegranate has not been reported previously.

In this study, we first identified 18 *MAPKs* and 9 *MAPKKs* in pomegranate at genome-wide level. The numbers of these family members were similar to those found in other plant species, such as *Ziziphus jujuba* (11/5) [10], *Arabidopsis thaliana* (20/10) [13,14], *Oryza sativa* (17/8) [15,16], *Brachypodium distachyon* (16/12) [17], *Malus Domestica* (26/9) [58], *Populus* (21/11) [60], *Solanum lycopersicum* (16/5) [61], *Triticum aestivum* (54/18) [3], *Capsicum annuum* (19/5) [65], *Cucumis sativus* (14/6) [66], *Citrullus lanatus* (15/6) [67], and *Jatropha curcas* (12/5) [68].

Figure 1 displayed a scattered distribution of *PgMAPKs* and *PgMAPKKs* on the 7 chromosomes of *Punica granatum*. The chromosome distribution pattern showed that the *PgMAPK* or *PgMAPKK* genes density was relatively high in certain chromosomes. Regions containing 4 or more genes in the range of 200kb or less can be regarded as gene clusters [69]. Only one gene cluster with four members in *PgMAPK* gene family and no other gene cluster was found in *PgMAPKs* (Figure 1). However, given

that our clustering criteria was relaxed to at least two genes, 7 clusters would be found (63.6% of MAPK and MAPKK family members appeared in the cluster). In addition, we also found that although the *PgMAPKK3* and the gene cluster consisting of *PgMAPKK4*, *PgMAPKK5*, and *PgMAPKK6* were all located on the same chromosome, they were physically far apart, which was consistent with the result of phylogenetic and multiple alignment analysis.

PgMAPKs were divided into three subtypes of “TDY”, “TEY”, and “TGY” according to different “TxY” phosphorylation sites by multiple sequence alignment (Table 2, Figure 3). Interestingly, all *PgMAPKs* in group C had a “TGY” motif except *PgMAPK17*. The “TGY” activation loop was similar to P38 MAPKs in mammals, rather than the T(E/D)Y motif commonly in plants [19]. As far as we know, the same results were also found in wheat, where *TaMPK25* also carries a TGY activating loop [70]. Furthermore, there were confirmed results included *MAPKs* in group B mainly involved in cell division and environmental stress response [66]. Therefore, *PgMAPK8*, *PgMAPK9*, and *PgMAPK12* may also response to various stress. *MAPKKs* in pomegranate were phosphorylated by “S/T-XXXXX-S/T” and anchored by “-D(L/I/V) K-”. Due to its wide range of functions, many *MAPKK* genes have been cloned recently, such as *AtMKK1* and *AtMKK2-5* of *Arabidopsis thaliana*, *SiMKK* of Alfalfa, *MEK1* of tomato, *NTMEK1-2* of tobacco and *ZmMEK1* of maize [7].

Since highly differentiated sequences may responsible for regulatory diversity of MAPK and MAPKK protein, we constructed a phylogenetic tree based on the predicted MAPK and MAPKK proteins of pomegranate and *Arabidopsis thaliana*. Four groups were separated in phylogenetic tree, which were named following previous studies [67]. The members in each group shared similar intron–exon structures. Consistent with previous studies, these data indicate similar origins and evolutionary patterns of *MAPK* and *MAPKK* genes in plants. It is possible that original family member had experienced replication events prior to the eudicot-monocot divergence [58,65].

The MAPK cascade genes have been studied to interact with signaling pathways mediated by hormone responsive cis-acting elements such as SA, JA, and ethylene [71]. Hormone responsive elements such as MeJA-responsive element (CGTCA-motif and TGACG-motif) [72], ethylene-responsive ERE element [73], gibberellin-responsive element (GARE-motif, P-box element, TATC-box) [74], abscisic acid-responsive ABRE [75], and salicylic acid-responsive TCA element [76] were widely found in the promoters of *PgMAPK* and *PgMAPKK* genes in *Punica granatum*. These results indicated that they were widely involved in pomegranate life cycles [77].

Previous studies have confirmed that the response of plants to the development and biological process are greatly regulated at the transcription level [66,73]. RNA-seq data showed that the expression levels of *PgMAPKs* and *PgMAPKKs* varied greatly in different tissues, which was consistent with the research of pepper and other species [65]. Obviously, some *PgMAPK* and *PgMAPKK* genes were highly expressed while others expressed at a low level or not expressed, suggesting their diverse functions in pomegranate growth and development [78]. For example, *MAPKs* in rice can regulate auxin signaling and cell cycle-related gene expression of root under cadmium stress [79].

Our results will lay the foundation for the functional characterization of *MAPK* and *MAPKK* gene family, and further understanding of the structure–function relationship among gene members. In addition, our study also provides comprehensive information and new insights into the evolution and differentiation of *PgMAPK* and *PgMAPKK* genes. These studies may provide a molecular basis for key agronomic traits, developmental, and other physiological processes in pomegranates.

5. Conclusions

In this study, a total of 18 *PgMAPK* and 9 *PgMAPKK* genes were identified in pomegranate and their phylogenetic relationships were explored. The gene structure of each group members was similar. *PgMAPKs* and *PgMAPKKs* may participate in the apical meristems, flower organ and fruit development, and the same group may have the similar expression pattern. These results lay the foundation for function studies on *PgMAPKs* and *PgMAPKKs* during their evolutionary process.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/7/1015/s1>, Fasta file S1: MAPK and MAPKK protein sequences of *Arabidopsis thaliana*, Fasta file S2: MAPK and MAPKK protein sequences of pomegranate (*Punica granatum* L.), Table S1: IDs of MAPK and MAPKK gene sequences of *Arabidopsis thaliana*. Table S2: RNA-seq origin data of 18 PgMAPKs and 9 PgMAPKKs; Table S3: All cis-acting elements of 18 PgMAPKs and 9 PgMAPKK, Table S4: 25 cis-acting elements retained 18 PgMAPKs and 9 PgMAPKKs.

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