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Genome-Wide Identification, Evolution, and Expression Analysis of the MAPK Gene Family in Rosaceae Plants

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Abstract: Mitogen-activated protein kinases (MAPKs) are crucial regulators in coping with abiotic and biotic stresses, including drought, salinity, fungi, and pathogens. However, little is known about the characteristics, evolution process, and functional divergence of the MAPK gene family in Rosaceae plants. A total of 97 MAPK members were identified in six Rosaceae species, including 12 genes in *Fragaria vesca*, 22 genes in *Malus domestica*, 23 genes in *Pyrus bretschneideri*, 12 genes in *Prunus mume*, 14 genes in *Prunus persica*, and 14 genes in *Rosa chinensis*. All MAPK members of six Rosaceae plants were categorized into four clusters by the phylogenetic relationship analysis. Collinearity analysis discovered that both segmental duplication and tandem duplication contributed to the expansion of MAPK family genes in Rosaceae plants. And the analysis of motifs and gene structures indicated that the evolution of the MAPK gene family was highly conserved among phylogenetic clusters in Rosaceae species. In addition, the d_N/d_S rates of MAPK paralogous gene pairs were below one, suggesting the MAPK gene family in Rosaceae was driven by purifying selective pressure. Furthermore, functional divergence analysis discovered that 14 amino acid residues were detected as potentially key sites for functional divergence of MAPK family genes between different cluster pairs, specifically Type I functional divergence. The analysis of functional distance indicated that cluster C retained more of the original functional features, while cluster B exhibited functional specialization. Moreover, the expression profiles revealed that *PmMAPK8*, *PmMAPK9*, and *PmMAPK10* were both highly expressed under drought stress and low temperature conditions. This study aims to comprehensively analyze the evolutionary process and functional analyses of the MAPK gene family in Rosaceae plants, which will lay the foundation for future studies into MAPK genes of Rosaceae in response to drought and cold stress.

Keywords: mitogen-activated protein kinase (MAPK); gene family; evolution analysis; functional divergence; gene expression

Citation: Yang, Y.; Tang, H.; Huang, Y.; Zheng, Y.; Sun, Y.; Wang, Q. Genome-Wide Identification, Evolution, and Expression Analysis of the MAPK Gene Family in Rosaceae Plants. *Horticulturae* **2023**, *9*, 1328. https://doi.org/10.3390/horticulturae9121328

Academic Editor: Jiafu Jiang

Received: 9 November 2023
Revised: 1 December 2023
Accepted: 7 December 2023
Published: 11 December 2023

1. Introduction

Plants simultaneously suffer from multiple adverse stimuli, including abiotic and biotic stress, during growth and development. To adapt to adverse environmental conditions, plants have evolved effective and sophisticated defense mechanisms, among which signal perception, signal transmission, signal amplification, and signal transduction are the vital response pathways in stress tolerance [1,2]. The mitogen-activated protein kinase (MAPK) cascade is a signal transduction pathway that is evolutionarily conserved. This pathway plays a crucial role in various biological processes such as cell growth, differentiation, plant immunity, and stress response [3–5]. The typical MAPK cascade pathway comprises three kinds of kinases, namely MAPK, MAPKK (MAPK kinase), and MAPKKK (MAPKK kinase) [4–6]. When plants encounter a stimulus signal, serine/threonine residues of S/TXXXXXS/T (X represents any amino acid) located in the activation loop of MAPKK

are phosphorylated by the activated MAPKKKs. Subsequently, the activation of MAPKKs leads to the phosphorylation of threonine and tyrosine residues located in the activation loop of downstream MAPKs, thus initiating the activation of these MAPKs [7].

Mitogen-activated protein kinases (MAPKs), as the terminal member of the MAPKKK-MAPKK-MPAK cascade, play critical roles in regulating the stress-responsive genes to express and improve plant stress tolerance by phosphorylating multiple substrates downstream, such as transcription factors, enzymes, protein kinases, and structural proteins [8,9]. In recent years, the detection and characterization of the MAPKs gene family have made significant progress in plants, such as *Arabidopsis thaliana* [6], *Oryza sativa* [10–12], *Populus trichocarpa* [13], *Malus domestica* [14], *Vitis vinifera* [15], *Triticum aestivum* [16], *Ziziphus jujuba* [17], *Helianthus annuus* [18], *Cucumis sativus* [19], *Gossypium hirsutum* [20], *Brassica napus* [21], *Camellia sinensis* [22], *Fagopyrum tataricum* [23], *Fragaria* × *ananassa* [24], *Prunus mume* [25]. In plants, MAPK family members are divided into two subtypes, including the TEY subtype and the TDY subtype, based on the amino acid residues in the middle site of the TXY motif. Furthermore, the TEY subtype of MAPKs in plants was classified into three groups. The TEY subtype of MAPKs contains the conserved docking domain (CD domain) in the C-terminus, serving as the binding domain for upstream MAPKKs. In contrast, the TDY subtype of MAPKs lacks the CD domain [26,27].

The evolutionary history of MAPKs provides insights into their functional characteristics. Gene duplication events and functional divergence are both associated with the evolutionary process of the MAPK gene family [28]. During its evolutionary history, the MAPK family expanded through whole-genome duplications (WGD) in the angiosperms genome. These gene duplications provided opportunities for functional divergence and specialization [29]. As a result, distinct MAPK genes acquired subfunctionalization, neofunctionalization, or nonfunctionalization in various cellular processes [30,31]. Available studies have reported that MAPKs participated in plant signal transduction to withstand various stresses, including drought, cold, salinity, pathogens, and physical damage [32]. For example, *AtMPK3*, *AtMPK6*, and *AtMPK4* have been widely reported to confer resistance to osmotic stress and pathogen infection through signal transduction in Arabidopsis [33–35]. In rice, *OsMPK5*, as an abscisic acid-inducible kinase, was reported to enhance rice tolerance to abiotic stresses but decrease resistance to pathogen infection [36]. A recent investigation revealed that *OsMPK1* plays a vital role in responding to drought and salt stress by regulating the expression *OsABA2* [37]. Overexpression of *BnMAPK1* and *BnMAPK4* in *Brassica napus* could enhance the drought stress and fungi infection tolerances, respectively [38,39]. In addition, the *GhMAP3K14-GhMKK11-GhMPK31* signal pathway has been proven to play a vital role in enhancing the drought tolerance of cotton [40].

Rosaceae plants, such as *Fragaria vesca*, *Malus domestica*, *Prunus persica*, *Pyrus bretschneideri*, *Prunus mume*, and *Rosa chinensis* are widely cultivated on a global scale because of their fruit production and landscape application. Although the MAPK family has been extensively explored in several Rosaceae plants, little is known about the evolution history and functional analyses of the MAPK family in Rosaceae plants. In our research, all MAPK gene family members were identified from wild strawberry, apple, Chinese pear, peach, Chinese rose, and *P. mume* genomes. Subsequently, gene structure, protein motif, phylogenetic evolution, collinear and synteny relationships, selective force, gene functional divergence, and gene expression were conducted to obtain an extensive and comprehensive understanding of the MAPK family in Rosaceae.

2. Materials and Methods

2.1. MAPK Gene Family Identification in Rosaceae Plants

The genome data of *Fragaria vesca* (v4.0) [41], *Malus domestica* (v1.1) [42], *Pyrus bretschneideri* (v1.1) [43], *Rosa chinensis* (v1.1) [44], and *Prunus persica* (v1.0) [45] were derived from the Genome Database for Rosaceae, which is available at https://www.rosaceae.org/ (accessed on 8 June 2023). And the genome information of *Prunus mume* was acquired from the genome project, which is available at http://prunusmumegenome.bjfu.edu.cn/index.

jsp (accessed on 8 June 2023) [46]. To exactly identify the members of the MAPK family, 20 protein sequences of MAPK in *Arabidopsis thaliana* from The Arabidopsis Information Resource (https://www.arabidopsis.org/, accessed on 8 June 2023) were exacted as queries. The local BLASTP was conducted in BioEdit 7.0.9.0 software for searching the MAPK members of six Rosaceae plants by setting expectation value $<e^{-10}$. Furthermore, the protein sequences of all obtained MAPKs were submitted in SMART (http://smart.embl.de/, accessed on 8 June 2023) to check their conserved domain MAPK-specific S_TKc.

For exploring the evolutionary relationships of MAPKs among six Rosaceae plants, DNAMAN was used for multiple sequence alignment of all MAPK genes in six Rosaceae plants. Moreover, protein physicochemical properties analysis was conducted in ExPASy (https://www.expasy.org, accessed on 8 June 2023).

2.2. Gene Structure and Protein Motifs Analyses

The visualization of Gene Structures was performed as described by Yang [47]. To identify the conserved motifs, the protein sequences were submitted to the MEME Version 5.4.1 (accessible via https://meme-suite.org/meme/index.html, accessed on 8 June 2023). Furthermore, TBtools was utilized to present all the results of the sequence analyses.

2.3. Phylogenetic Tree Construction and Conservative Analysis

Cluster X was utilized to align all MAPK full-length protein sequences of six Rosaceae plants after resetting all gaps. Subsequently, the Mega software (version 7.0) was employed to construct the system evolution tree through the neighbor-joining (NJ) method with 1000 bootstrap replications [48]. Phylogenetic trees of MAPK in six Rosaceae plants were further embellished by Evolview, which is available at http://www.evolgenius.info/evolview (accessed on 8 June 2023).

2.4. Gene Duplications and Collinearity Analysis

To identify the chromosomal location of all MAPKs in six Rosaceae plants, the gff3 file of apple, Chinese pear, wild strawberry, peach, rose, and *P. mume* genomes were acquired from the Genome Database. The Protein BLAST (available at https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 8 June 2023) platform was utilized for collinearity analysis by submitting the MAPK protein sequences of six Rosaceae plant species online. The BLASTP ($e < 10^{-5}$) output and gff3-files of the genome were employed to identify different types of gene duplications, including whole-genome, segmental, and tandem duplications, among apple, Chinese pear, wild strawberry, peach, rose, and *P. mume* by the McscanX tool kit. The TBtools-II v1.120 software was used to visualize the gene duplications and collinearity relationship.

2.5. Selective Pressure Estimation

The yn00 algorithm in Phylogenetic Analysis Maximum Likelihood (PAML 4.9j) was utilized to determine the synonymous (d_S) substitution rates, the non-synonymous (d_N) substitution rates, and the d_N/d_S (ω) ratio of MAPKs gene pairs after MAPK sequence alignment. The MAPK gene duplication and divergence time were calculated by the formula: $T = d_S/2\lambda$ Mya based on the constant λ ($\lambda = 1.5 \times 10^{-8}$ for dicots) [47].

2.6. Functional Divergence Detection

For functional divergence analysis, the DIVERGE 2.0 software was utilized to calculate the coefficients of function divergence with Type I and Type II after constructing the phylogenetic tree. Type I functional divergence resulted in alterations of functional constraints, which were associated with shifts of evolutionary rates between two replicated genes following gene duplication events. Additionally, Type II functional divergence could potentially affect the physicochemical properties of amino acid residues. Furthermore, the type I functional branch length was calculated by the formula: $bF_{(i)} = -\ln(1 - \theta_i)$ by the DIVERGE 2.0.

2.7. Gene Expression Profile of MAPK Genes

To further explore the MAPK functions, we obtained raw data of all PmMAPK genes from an RNA-seq dataset (GEO: GSE40162) that covered various tissues, including leaves, stems, roots, buds, and fruits in *P. mume* [46]. We also collected transcriptome data (NCBI: PRJNA1046642) of stems in *P. mume* during three seasons (Autumn, Winter, And Spring) from three different regions: Beijing (9°54′ N, 116°28′ E); Chifeng (42°17′ N, 118°58′ E); and Gongzhuling (ZGL, 43°42′ N, 124°47′ E) [49]. Additionally, transcriptome data (NCBI: PRJNA1046397) of leaves in *P. mume* were acquired during the drought stress stage, including drought for 3 days (DL1), drought for 7 days (DL2), drought for 15 days (DL3), rehydration for 5 days (RW), and a fully watered group as the control group [50]. We used the row-based Z-Scores algorithms to normalize the expression levels and then employed the Heatmap Illustrator 1.0 software to generate the heatmap [18,51].

3. Results

3.1. Identification and Sequence Analyses of the MAPK Gene Family in Rosaceae

After removing redundant sequences, a total of 97 MAPK family members were examined from the genomes of six Rosaceae species, with 22, 23, 14, 12, 14, and 12 MAPK genes being identified in *Malus domestica* (*MdMAPK1-MdMAPK22*), *Pyrus bretschneideri* (*PbMAPK1-PbMAPK23*), *Prunus persica* (*PpMAPK1-PpMAPK14*), *Prunus mume* (*PmMAPK1-PmMAPK12*), *Rosa chinensis* (*RcMAPK1-RcMAPK14*), and *Fragaria vesca* (*FvMAPK1-FvMAPK12*), respectively. The length of 97 MAPK proteins ranged from 352 to 691 amino acids (aa) (Table S1).

To better understand the protein sequence characteristics of the MAPK family, multiple sequence alignment was employed in the DNAMAN 7.0 software. The alignment results displayed that the 97 identified MAPK proteins contained I-XI characterized multiple protein domains and conserved docking domain (named CD domain) (Figure S1). Moreover, TXY motifs with phosphorylation activation in the Activation-loop were identified in MAPK family members of Rosaceae (Figure 1).

3.2. Phylogenetic Tree Construction of MAPK Genes

To deeply explore the evolution history of MAPK genes in Rosaceae, the phylogenetic tree of 97 MAPK protein sequences was constructed by MEGA7 using the maximum likelihood (ML) and neighbor-joining (NJ) methods (Figure 2). According to the evolutionary relationships, 97 MAPK proteins were divided into four distinct subgroups, namely cluster A, cluster B, cluster C, and cluster D. Based on the phosphorylation motif presenting in the activation loop, cluster A, cluster B, and cluster C belonged to TDY subtype while cluster D falls under TEY subtype. Statistical analyses discovered that the largest cluster (cluster D) contained 38 members, whereas the least cluster (cluster C) contained 13 members (Table S2). Moreover, the MAPK members of six Rosaceae were presented in every cluster (Figure 2), suggesting that highly homologous and conserved processes occurred in MAPK family.

3.3. Sequence Analyses of the MAPK Gene Family in Rosaceae

In total, 15 conserved motifs ranging from 6 to 50 amino acid residues of MAPK proteins were analyzed by the MEME suite to better understand the differentiation and evolution of MAPK proteins in six Rosaceae plants. Analyses of the motif distribution displayed that the motif number of the MAPK protein varied from 10 to 14. And motif 1, motif 2, motif 3, motif 4, motif 5, motif 7, motif 9, and motif 14 were shared by all MAPK proteins (Figure 3B). Among these shared motifs, motif 7 contained the core sites of TEY/TDY conserved domain (Figure S2). Specifically, motif 6, motif 8, motif 12, motif 13, and motif 15 were only presented in cluster A and cluster B. In addition, motif 10 was located at the N-terminal region of cluster D but located at the C-terminal region of partial members in cluster A.

Figure 1. Multiple sequence alignment of the *MAPK* gene family in Rosaceae. Dark blue-highlighted amino acid sites are identical, and other colored residues represent amino acid sites that are similar in protein sequences. The activated loop and conserved docking (CD) domain are marked by the red rectangle.

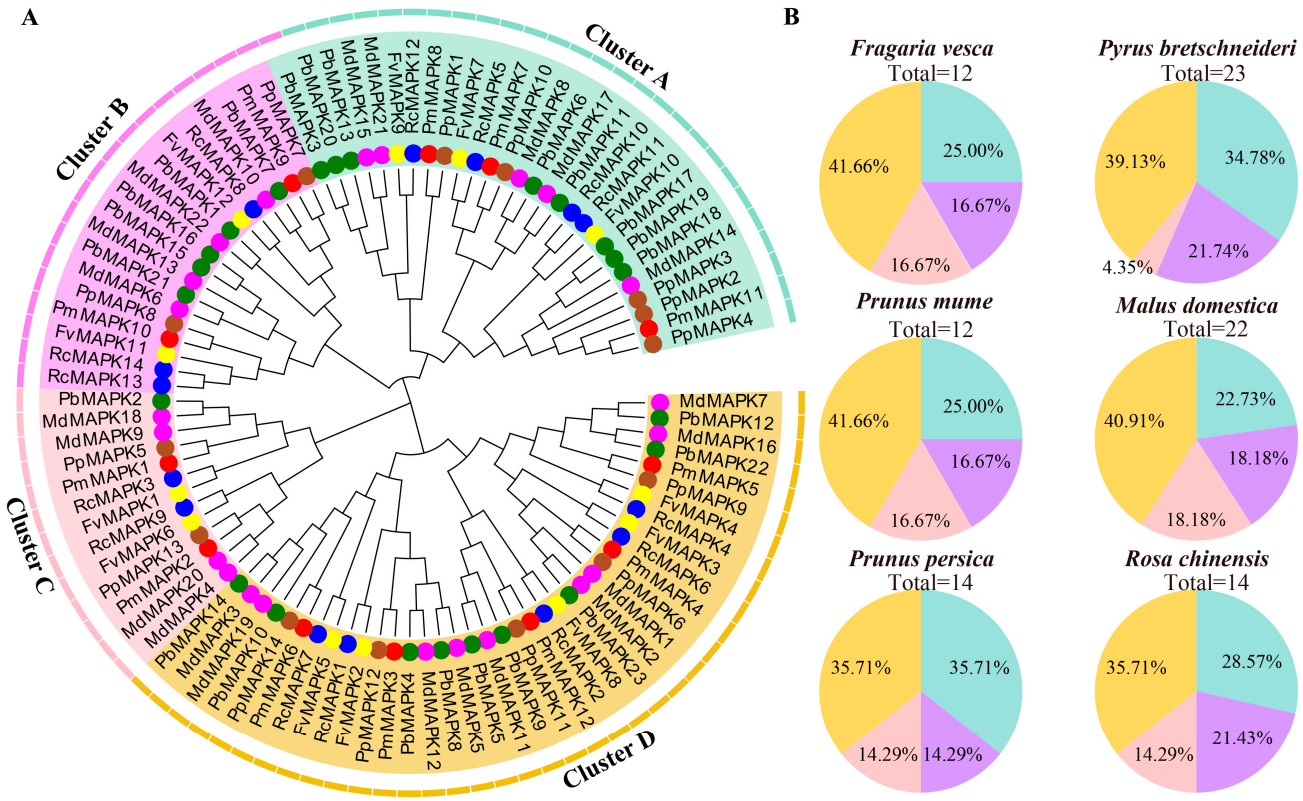

Figure 2. Phylogenetic tree of MAPK family genes from *Malus domestica*, *Pyrus bretschneideri*, *Prunus persica*, *Prunus mume*, *Rosa chinensis*, and *Fragaria vesca*. (**A**) The 97 MAPK genes were divided into four clusters: Cluster A; Cluster B; Cluster C; and Cluster D. Cyan, violet, pink, and gold represent clusters A, B, C, D, respectively. (**B**) The proportion of MAPK genes of four groups in six Rosaceae species.

Gene structure differentiation is of utmost importance in the adaptive evolution of the gene family. Previous studies have highlighted that gene structure diversity provided an essential basis for evaluating the phylogenetic relationship among gene family members [52]. The gene structure analyses displayed that the exons of MAPK ranged from 2 to 13 (Figure 3C). Combined with the phylogenetic tree, the exon-intron structures were relatively conservative in the same cluster, but diverse in different groups. In cluster A, 85.71% of genes contained 10 exons, *PbMAPK18* and PbMAPK19 consisted of 13 exons, whereas *PmMAPK9* and *PmMAPK7* contained 9 and 13 exons, respectively. In cluster B, 83.33% of genes contained 10 exons, while *RcMAPK14*, *PmMAPK9*, and *PmMAPK10* comprised 9 exons. In cluster C, all genes contained 2 exons. In cluster D, 78.95% of genes contained 6 exons, and other genes consisted of 5 exons.

3.4. Collinearity Analysis

To explore the expansion of MAPK family genes in evolutionary history, gene duplication, including tandem duplication and segmental duplication, was identified in apple, Chinese pear, wild strawberry, peach, rose, and *Prunus mume*. Based on the chromosomal localization of 97 MAPK genes (Table S3), 12 gene pairs of tandem duplication were identified in six Rosaceae plants. The collinearity relationship of paralogous homologous pairs was analyzed by MCScanX and Advanced Circos and visualized in TBtools (Figure 4), where 3, 12, 2, 2, 8, 3 paralogous homologous pairs were detected in peach, apple, wild strawberry, rose, Chinese pear, and *Prunus mume*, respectively.

Furthermore, the synteny relationships among MAPK genes were examined in whole genomes of six Rosaceae plants (Figure 5). The analysis revealed 326 orthologous gene pairs among six Rosaceae species genomes. Specifically, there were 34 orthologous gene

pairs between apple and Chinese pear, followed by apple and *P. mume* (29 orthologous gene pairs), apple and wild strawberry (26 gene pairs), apple and peach (26 gene pairs), as well as apple and rose (24 gene pairs). In addition, 22 orthologous gene pairs were discovered between Chinese pear and *P. mume*, Chinese pear and wild strawberry, Chinese pear and peach, respectively, while 20 and 18 gene pairs were examined between Chinese pear and rose, peach, and *P. mume*. Moreover, there were 17 gene pairs shared between *P. mume* and two other species, namely wild strawberry and rose. Additionally, 16 gene pairs were detected between peach and rose, as well as between wild strawberry and rose (Table S4). These findings highlight the genetic similarities and potential relationships among six Rosaceae plants.

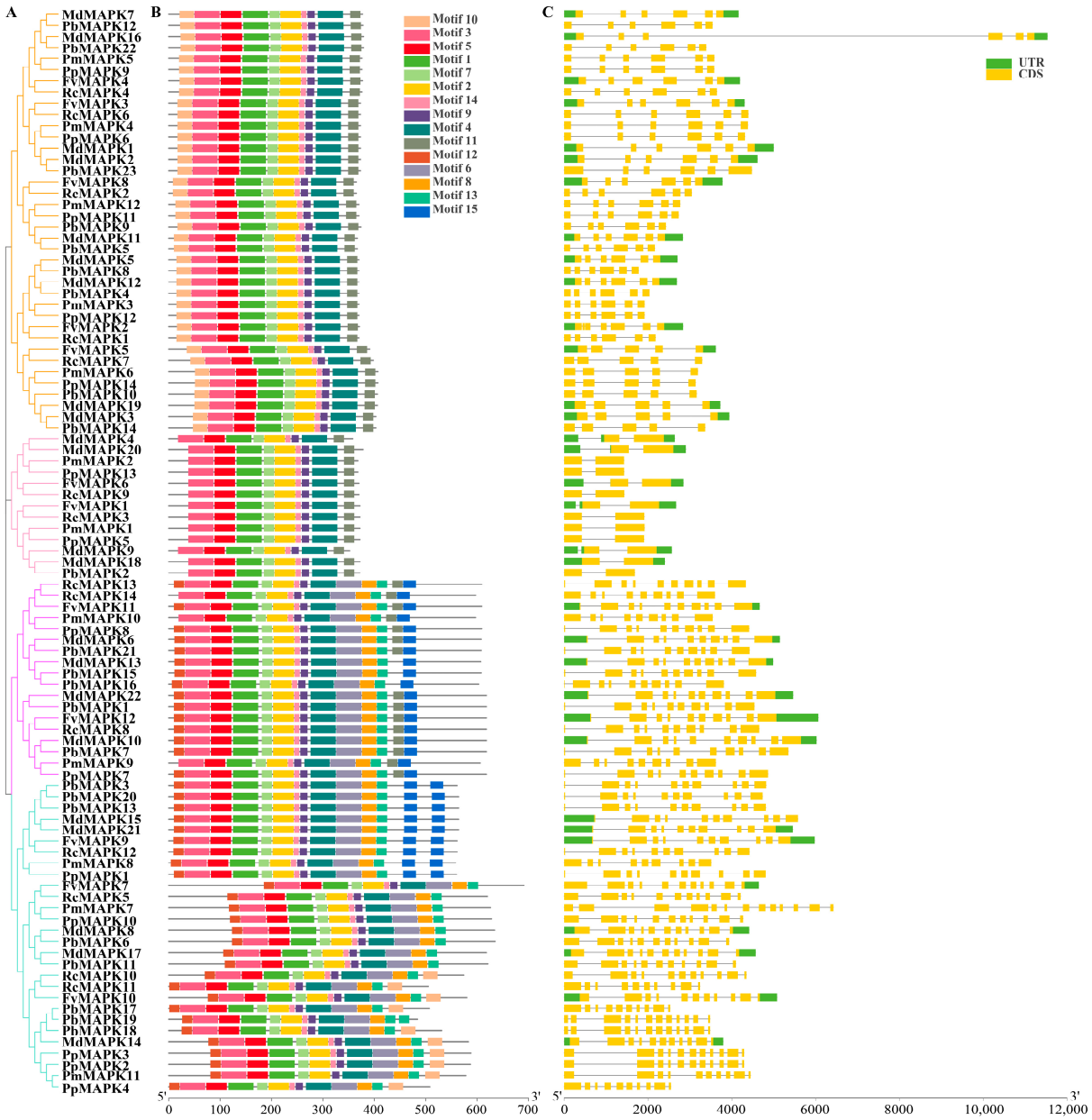

Figure 3. Motif and gene structure analyses of the *MdMAPK*, *PbMAPK*, *PmMAPK*, *PpMAPK*, *RcMAPK*, and *FvMAPK* gene families. (**A**) Phylogenetic tree of MAPK family genes from apple, Chinese pear, wild strawberry, peach, Chinese rose, and *Prunus mume*. The MAPK genes were classified into 4 clusters, and cyan, violet, pink, gold represent clusters A, B, C, D, respectively. (**B**) Motif analyses of MAPK family genes in six Rosaceae species. (**C**) Gene structure analyses of MAPK family genes in six Rosaceae species.

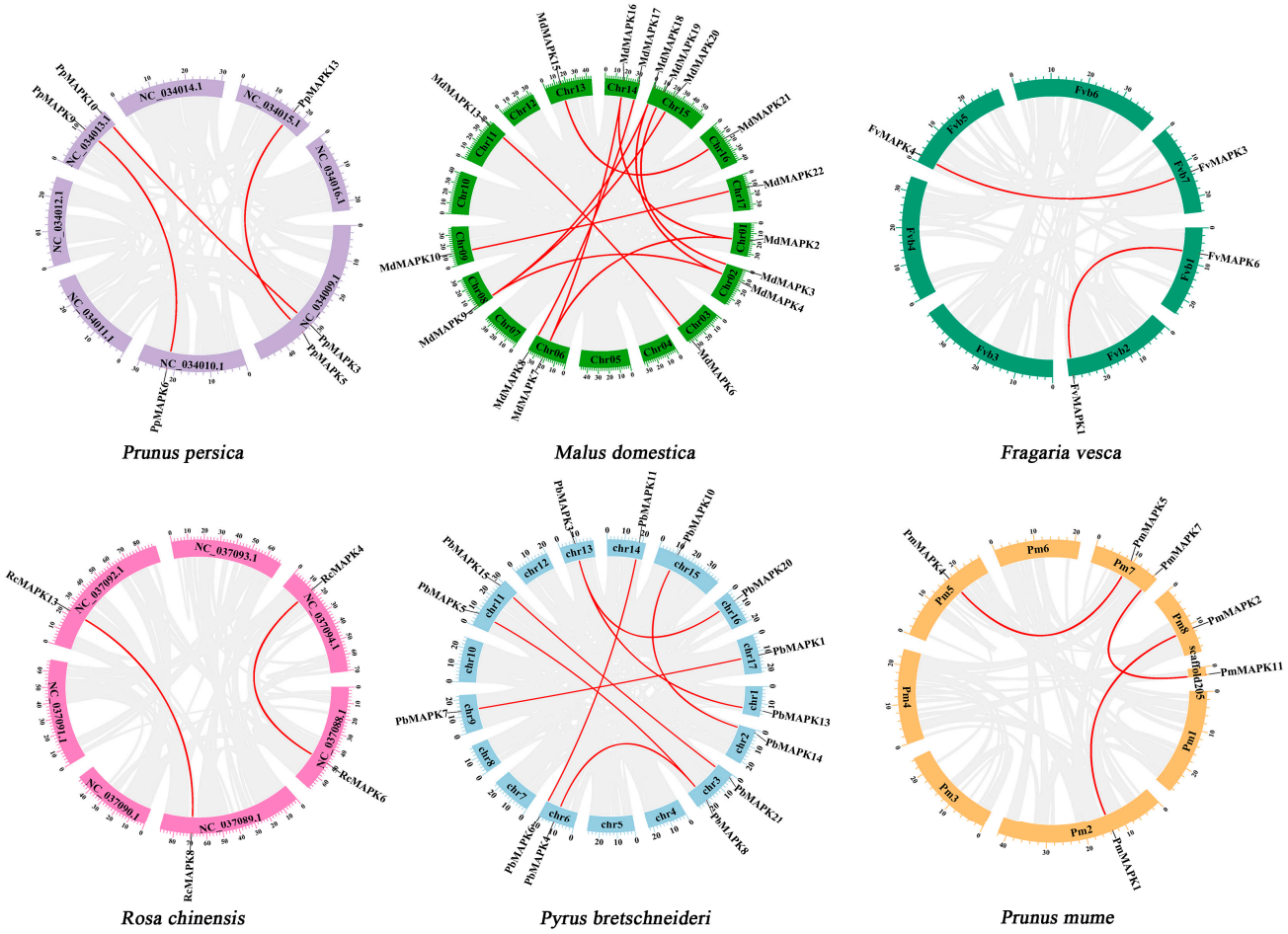

Figure 4. Segmental duplication events and collinearity analyses of MAPKs family in six Rosaceae plants. Red links highlight segmental duplication of MAPK gene pairs from *Fragaria vesca*, *Malus domestica*, *Pyrus bretschneideri*, *Prunus mume*, *Prunus persica*, and *Rosa chinensis*.

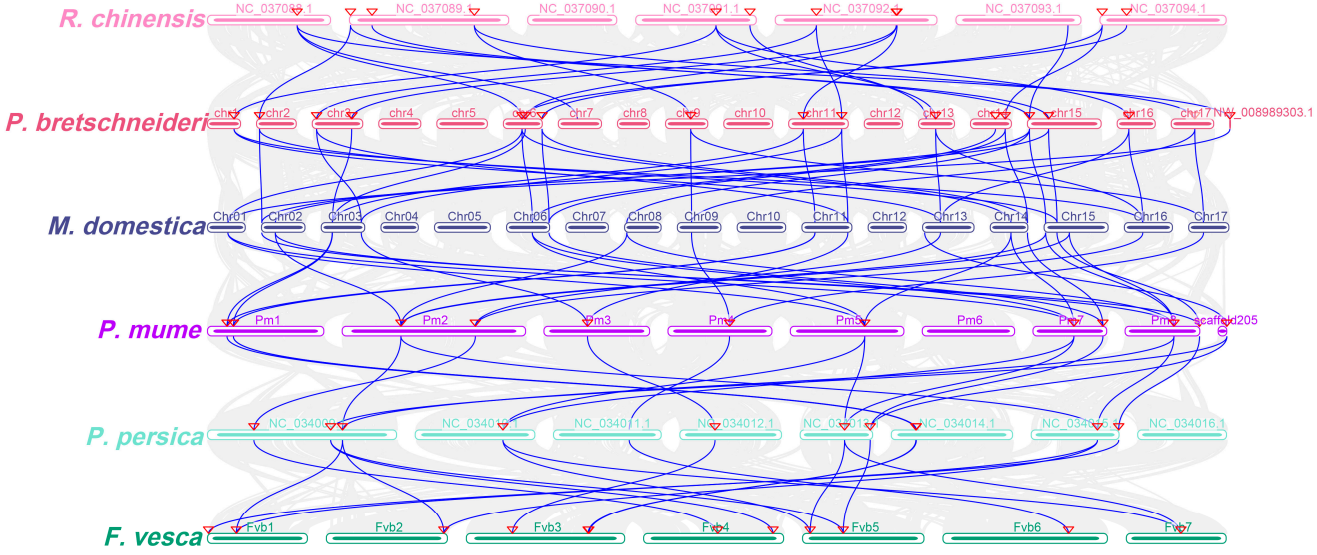

Figure 5. The synteny analysis of MAPK family genes between *Fragaria vesca*, *Malus domestica*, *Pyrus bretschneideri*, *Prunus mume*, *Prunus persica*, and *Rosa chinensis*. Blue links highlight collinear gene pairs with MAPKs between six Rosaceae plants.

3.5. Selective Force Analyses of MAPK Family

The d_N/d_S (ω) rates were calculated to explore the evolutionary history and selection pressure on the MAPK family of six Rosaceae species. In general, $d_N/d_S < 1$, $d_N/d_S = 1$, and $d_N/d_S > 1$ represent purity selections, neutral selections, and positive selections, respectively. In Figure 6, it can be observed that all the d_N/d_S values of MAPK duplicated pairs in apple, Chinese pear, wild strawberry, peach, rose, and *Prunus mume* were far below 1. This suggested that there was a strong purifying selection acting on amino acid substitution within the MAPK family in six Rosaceae plants after the gene duplication. The d_S values mainly ranged from 0.8 to 4.0 in apple, Chinese pear, wild strawberry, peach, rose, and *P. mume* (Figure 7). In wild strawberry and rose, the d_S values peaked between 2.0 to 2.4, which showed that a large-scale evolution event of the MAPK family happened about 67–80 million years ago. While the d_S values of *P. mume*, apple, and Chinese pear peaked between 1.6 and 2.0, indicating the large-scale evolution event of MAPK family was estimated to have taken place approximately 53–67 million years ago. In peach, the d_S values peaked between 1.6 and 2.4 suggesting that the large-scale evolution event of the MAPK family probably occurred about 53–80 million years ago.

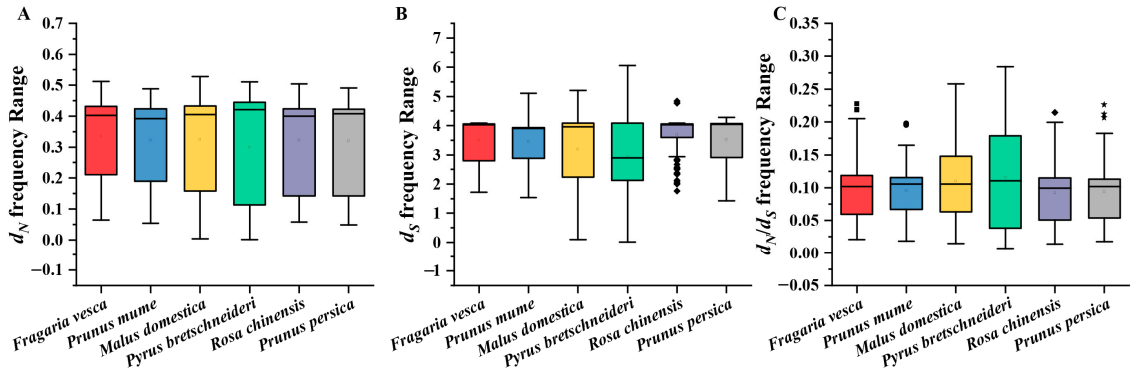

Figure 6. The selective pressure analyses of MAPKs family in six Rosaceae species. (**A**) d_N frequency analysis for six Rosaceae plants. (**B**) d_S frequency analysis for six Rosaceae plants. (**C**) d_N/d_S frequency analysis for six Rosaceae plants.

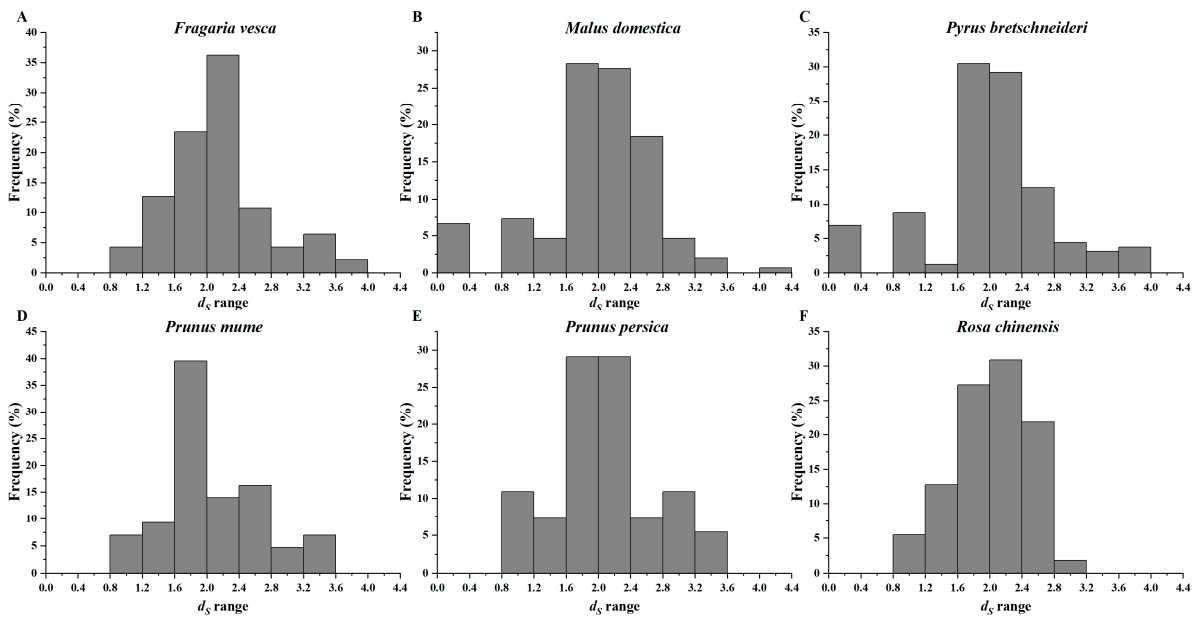

Figure 7. The d_S values distribution in six Rosaceae species. The d_S values ranged in *Fragaria vesca* (**A**), *Malus domestica* (**B**), *Pyrus bretschneideri* (**C**), *Prunus mume* (**D**), *Prunus persica* (**E**), and *Rosa chinensis* (**F**), respectively.

3.6. Gene Functional Divergence Analysis

To analyze the impact of amino acid substitutions on the diversity of gene function within the MAPK family genes, the Diverge II software was employed to calculate the Type I and Type II Divergence for six pairs of clusters. The coefficient θ value=0 represents no function divergence while the coefficient θ value = 1 represents determinant function divergence [53]. The Type I coefficient values of six cluster groups ranged from 0.130 to 0.814 ($p < 0.01$), indicating significant gene functional divergence happened among different clusters. Among them, the θ_I value of B/C pairs was the largest and the θ_I value of A/C pairs was the smallest. The cut-off value $Q_k \geq 0.90$ was set for searching the vital amino acid site substitution. In total, 14 key amino acid sites were identified among different clusters, among which the cluster B/C group was detected in 6 amino acid sites, while the cluster A/B, A/D, B/D, and C/D pairs were checked in 3, 2, 2, 1 amino acid site substitutions (Figure 8), with 6 amino acid residues located in a conserved domain of the MAPK family while others were located in C terminal domain. The Type II coefficient values of six pairs were relatively less than the Type I coefficient values but all of them were greater than 0. Based on MFE z score = θ_{II}/SE of Type II, we found the *p*-value of clusters A/C, A/D, B/C, B/D, and C/D were less than 0.05, which indicated that A/C, A/D, B/C, B/D, and C/D underwent significant type II functional divergences (Table 1).

Table 1. Type I and type II functional divergences of MAPK gene family in Rosaceae.

Pairs	Type I					Type II	
	$\theta_I \pm$ SE [a]	LRT	MFE z Score	*p*-Value	$Q_k \geq 0.90$ [b]	$\theta_{II} \pm$ SE [c]	*p*-Value
A/B	0.636 ± 0.133	22.965	−6.711	0.000	330, 497, 528	0.032 ± 0.040	0.425
A/C	0.240 ± 0.110	4.802	−2.894	0.004	-	0.381 ± 0.043	0.000
A/D	0.436 ± 0.093	22.199	−6.444	0.000	507, 530	0.273 ± 0.061	0.000
B/C	0.687 ± 0.127	29.222	−7.328	0.000	269, 330, 384, 523, 524, 560	0.362 ± 0.042	0.000
B/D	0.451 ± 0.099	20.673	−7.160	0.000	269, 528	0.258 ± 0.060	0.000
C/D	0.238 ± 0.071	11.335	−5.758	0.000	384	0.118 ± 0.062	0.058

[a] The coefficient value of Type I function divergence with its error. [b] The number of amino acid sites under posterior probability (Qk ≥ 0.90). [c] The coefficient value of Type II function divergence with its error.

To investigate the function constraints of gene clusters after gene duplication, the functional distance of four clusters was analyzed. The b_F values were varied among four clusters, among which cluster C exhibited the lowest bF value (0.138) compared to other clusters and cluster B was observed the largest b_F value (Figure S3). These results discovered that cluster C preserved more original function features, while cluster B was involved in functional specialization.

3.7. Gene Expression Patterns Analysis of PmMAPKs

To investigate the function of the MAPK gene family, the expression patterns of *PmMAPK*s were analyzed in various tissues, as well as under drought stress and under different temperature conditions, by using three transcriptome datasets. In Figure 9A, we observed that 12 *PmMAPK*s were categorized into three groups based on gene expression patterns. Specifically, *PmMAPK12* and *PmMAPK2* showed high expression levels in buds, while *PmMAPK10*, *PmMAPK11*, and *PmMAPK7* exhibited high expression levels in fruits. *PmMAPK6*, *PmMAPK1*, and *PmMAPK9* demonstrated high expression in stems. Additionally, *PmMAPK4* exhibited high expression in leaves, while *PmMAPK3*, *PmMAPK8*, and *PmMAPK5* showed high expression in roots. We also analyzed the expression profiles of *PmMAPK*s under drought stress. *PmMAPK8*, *PmMAPK10*, *PmMAPK6*, and *PmMAPK9* were found to be upregulated after drought treatment, whereas *PmMAPK4* and *PmMAPK12* were downregulated. Furthermore, *PmMAPK11*, *PmMAPK3*, and *PmMAPK7* showed a pattern of downregulation followed by upregulation (Figure 9B). The cluster analysis of *PmMAPK*s expression under different temperature conditions resulted in the classification

of the 12 *PmMAPK*s into four groups (Figure 9C). Notably, *PmMAPK3*, *PmMAPK8*, and *PmMAPK9* were highly expressed during the winter in Beijing, Chifeng, and Gongzhuling. Additionally, *PmMAPK4* and *PmMAPK12* were highly expressed in Autumn. *PmMAPK2* and *PmMAPK10* showed relatively high expression levels in Autumn and Winter.

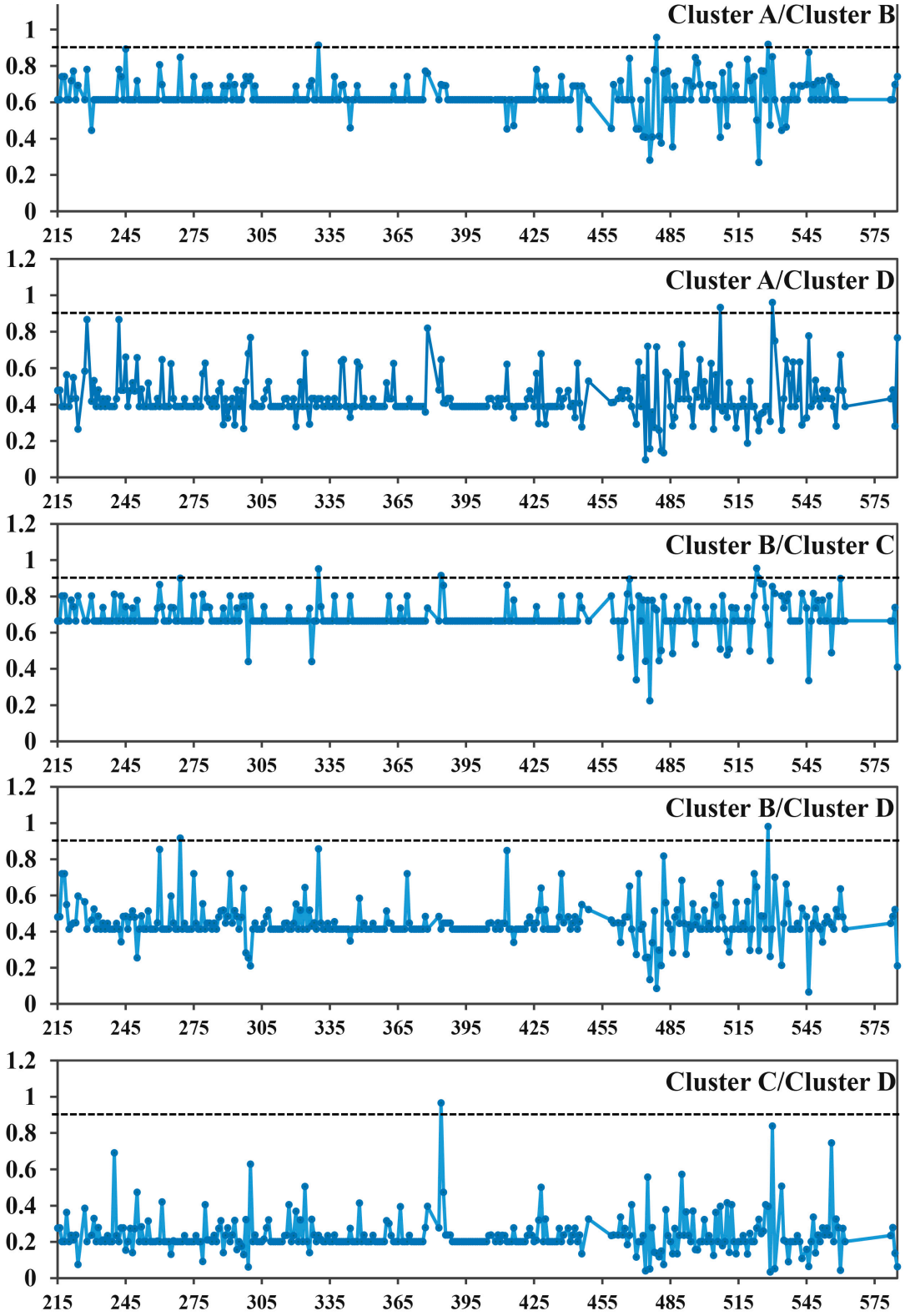

Figure 8. Type I functional divergence among the MAPK family genes of six Rosaceae plants. The cutoff = 0.90 is shown by the dotted line.

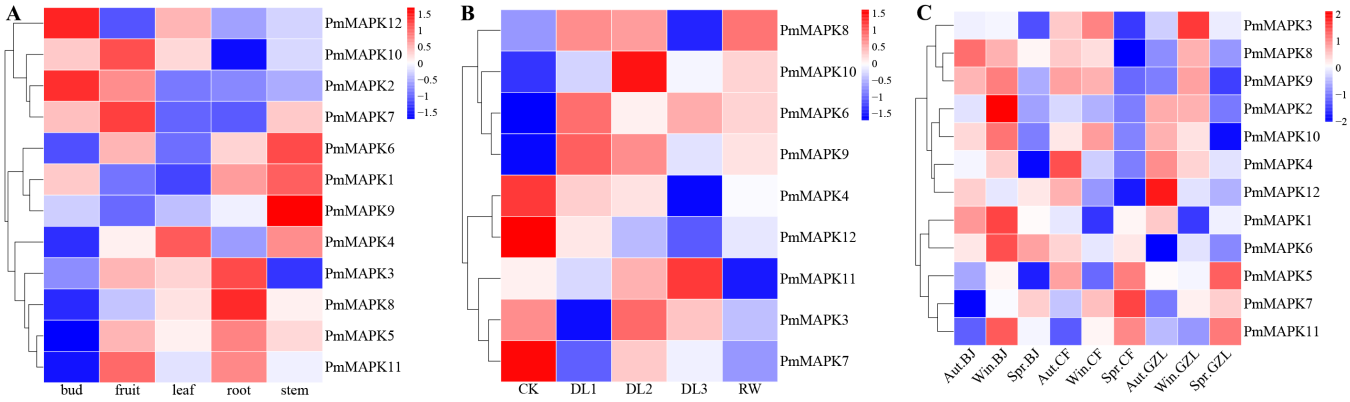

Figure 9. Expression profiles of *PmMAPK* genes in *Prunus mume*. (**A**) Gene expression of *PmMAPK*s in buds, fruits, leaves, roots, and stems. (**B**) Gene expression of *PmMAPK*s at different stages (DL1 represents drought for 3 days, DL2 represents drought for 7 days, DL3 represents drought for 15 days, and RW represents rehydration for 5 days) in *P. mume*'s response to drought stress. (**C**) Gene expression of *PmMAPK*s in stems during different seasons (Autumn, Winter, And Spring) from three regions (Beijing, Chifeng, and Gongzhuling). Aut, Autumn; Win, Winter; Spr, Spring. BJ, Beijing; CF, Chifeng; GZL, Gongzhuling.

4. Discussion

The MAPK cascade plays an essential role in plant growth, development, and environmental stress responses, such as drought, salt, cold, fungi, and pathogens. It serves as a signal pathway that allows plants to respond and adapt to complicated conditions. As the final member of the MAPKKK-MAPKK-MPAK cascade, MAPKs transfer upstream signals to downstream stress-responsive genes for promoting and enhancing plant tolerance to abiotic and biotic stress. In recent years, studies have systematically identified and examined the MAPK gene family members in Rosaceae plants, including apple [14], pear [54], wild strawberry [55], and *Prunus mume* [25]. However, the evolutionary relationship and gene function divergence of MAPK in Rosaceae plants remained unclear. In this study, 97 MAPK genes were detected in six Rosaceae plants, including *Fragaria vesca* (12 genes), *Malus domestica* (22 genes), *Pyrus bretschneideri* (23 genes), *Prunus mume* (12 genes), *Prunus persica* (14 genes), and *Rosa chinensis* (14 genes). The MAPK gene number is closely related to genome size, gene duplication, and ploidy [56]. The genome sizes of 649.7 Mb for apple, 224.6 Mb for peach, 219 Mb for wild strawberry, 400.57 Mb for *Pyrus bretschneideri*, 280 Mb for *Prunus mume*, and 512 Mb for *Rosa chinensis* were determined through genome assembly, respectively. This suggested no apparent connection was observed between the genome size and the MAPK family gene number in Rosaceae plants. The gene number of apple and pear was higher than others due to the whole genome replication event. Moreover, the number of identified MAPKs in this study was different from the gene number of MAPK in previous studies. For example, 22 MAPK genes of apple were detected in this study, in accordance with the report in 2023 [25], while 26 MAPK members of apple were reported in 2013 [14]. In *P. mume*, 12 MAPK genes were identified in this study, whereas Wen et al. discovered 11 *PmMAPK*s [25]. The variation in MAPK family gene numbers within the same species in different reports may be attributed to the utilization of different visions of assembly genomes and the strict parameter settings during HMM (Hidden Markov Model) searching and local BLAST analysis. Therefore, it is essential to take into account these factors to gain an extensive analysis of the MAPK family in a particular species.

To explore the phylogenetic relationship and molecular evolution of MAPKs in six Rosaceae plants, 97 genes were divided into four clusters through a phylogenetic tree construction, which was consistent with most previous studies, such as in Arabidopsis [6,33], *Brassica rapa* [28], and lettuce [57]. However, MAPK family genes in rice were classified into six groups [12,56], and MAPKs in kiwifruit were divided into five clades [58], which may result from the methods used to construct the evolutionary tree and the phylo-

genetic tree distance. Among four clusters, clusters A, B, and C contained the TDY motif, while cluster D contained the TEY motif in six Rosaceae plants, indicating that MAPKs in Rosaceae are an evolutionarily conserved family. Conserved motifs analysis discovered that the composition and order of conserved motifs within the same group presented similarity, while motifs in different groups exhibited diversity. For example, it was observed that only cluster D members contained a specific motif 10 at the N-terminal region. On the other hand, MAPK members of cluster A specifically had motif 10 located at the C-terminal region. This distinction suggests that the presence of motif 10 is not uniform across all clusters of the MAPK family, with different clusters exhibiting distinct patterns and locations for this specific motif. This variation in motif location within the different clusters of the MAPK family may be involved in the function diversity of genes in different biological processes among different clusters, while the gene function diversity may enhance plant tolerance to environmental stress [23,59].

The expansion of the gene family is associated with gene duplication, especially tandem duplication and segmental duplication [29,60]. Collinearity relationship analyses and chromosomal localization of MAPK family genes in six Rosaceae species discovered that 12 tandem duplication gene pairs were found in the MAPK family. Meanwhile, 30 paralogous homologous gene pairs were identified in six Rosaceae species, which resulted from segmental duplication. These findings revealed that the expansion and evolution of MAPK family genes in Rosaceae were influenced significantly by segmental and tandem duplication, which is consistent with findings in poplar [61], sugarcane [62], and maize [63]. Furthermore, synonymous and nonsynonymous substitution analyses discovered that all d_N/d_S rates of MAPK paralogous genes were far lower than 1, showing the adaptive evolution of the MAPK gene family was driven by purifying selection. And the MAPK family proteins underwent a rather conservative evolution process in Rosaceae species. Moreover, the d_S values peaked much earlier in apple, Chinese pear, *P. mume*, and peach than in wild strawberry and rose. These results indicated the relatively recent duplication of the MAPK gene family in apple, Chinese pear, *P. mume*, and peach.

The functional divergences of the gene family were primarily driven by gene duplication events and the subsequent potential substitution of amino acid sites. These events play a significant role in the evolution and diversification of gene functions [53]. Therefore, to identify the essential amino acid sites, DIVERGE 2.0 was employed to analyze the gene functional divergence of the MAPK family in Rosaceae. The coefficient estimation of divergence types showed there were significant differences in Type I functional divergences among four clusters of the MAPK family in six Rosaceae plants, which suggested Type I functional divergences played an important role in MAPK gene functional divergence. In addition, the potential key residues for functional divergence of the MAPK proteins were detected in six cluster pairs, involving 14 amino acid sites. Among them, eight amino acid residues were located in the C terminal of the MAPK protein sequence and six amino acid residues were found in the conserved domain of the MAPK gene family. These results discovered that the functional divergence of MAPK genes in Rosaceae plants was driven by diverse evolution rates after gene duplication [64].

The MAPK cascade pathway is the vital signal module, which transfers environmental stimuli into cellular responses [32,65]. In recent years, numerous genes in the MAPK cascade pathway have been reported on for their functions in response to drought and cold stress. For instance, studies have shown that *AtMAPK3*, *AtMAPK4*, and *AtMAPK6* participate in osmotic or cold stress responses in Arabidopsis [33,65,66]. Furthermore, *OsMPK1* has been reported to function in drought tolerance in rice [37]. In addition, *GhMPK31* was involved in regulating drought tolerance in cotton via the MAPK cascade [40]. Moreover, *PbrMAPK13* could respond to chilling stress by changing the expression profile [54]. This research focused on the analysis of expression patterns of *PmMAPK*s under drought stress and different temperature conditions. Four genes (*PmMAPK8*, *PmMAPK10*, *PmMAPK6*, and *PmMAPK9*) were found to be upregulated in leaves when *P. mume* was exposed to drought stress. These results revealed that these genes played a positive role in *P. mume's*

response to drought stress. Moreover, 12 MAPK genes showed complex expression patterns in Autumn, Winter, and Spring in Beijing, Chifeng, and Gongzhuling. Three genes (*PmMAPK3*, *PmMAPK8*, and *PmMAPK9*) were highly expressed in Winter (−5~−22 °C), meanwhile *PmMAPK4* and *PmMAPK12* were highly expressed in Autumn (3~5 °C). These results indicated that *PmMAPK4* and *PmMAPK12* functioned when withstanding cold stress, while the high expression levels of *PmMAPK3*, *PmMAPK8*, and *PmMAPK9* could help *P. mume* survive in extremely low-temperature conditions. Among the 12 *PmMAPK* genes, *PmMAPK2* and *PmMAPK10* showed relatively high expression levels in Autumn and Winter, indicating that *PmMAPK2* and *PmMAPK10* may work constantly under low-temperature conditions. These results will lay the foundation for future function studies of MAPK genes in Rosaceae's response to drought and cold stress.

5. Conclusions

In this study, 97 MAPK family members were identified in six Rosaceae plants, including *Fragaria vesca*, *Malus domestica*, *Pyrus bretschneideri*, *Prunus mume*, *Prunus persica*, and *Rosa chinensis*. In addition, comprehensive analyses for chromosomal localization, protein motif, gene structure, phylogenetic relationship, collinear, and synteny relationships, selective force, gene functional divergence, and gene expression patterns of MAPK genes were conducted. Based on the analysis of collinear and synteny relationships, it was found that segmental and tandem duplication promoted the MAPK gene expansion of Rosaceae. Synonymous and nonsynonymous substitution analyses discovered that the purifying selection force primarily drove the evolution of the MAPK gene family in Rosaceae plants. Moreover, the gene functional divergence of the MAPK family in Rosaceae plants was facilitated by Type I functional divergence. *PmMAPK8*, *PmMAPK9*, and *PmMAPK10* exhibited high expression levels under both drought stress and low-temperature conditions. In summary, this study provides valuable insights into the evolutionary process and functional analyses of the MAPK gene family in Rosaceae plants, and it will contribute to future investigations into MAPK genes of Rosaceae in response to abiotic stress, especially drought and cold stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9121328/s1, Table S1: Features of MAPK family genes from the *Fragaria vesca*, *Malus domestica*, *Pyrus bretschneideri*, *Prunus mume*, *Prunus persica*, and *Rosa chinensis* genome; Table S2: Number distribution of MAPK genes in Clusters; Table S3: Chromosomal location of the MAPK family genes in *Fragaria vesca*, *Malus domestica*, *Pyrus bretschneideri*, *Prunus mume*, *Prunus persica*, and *Rosa chinensis*; Table S4: Duplication events between *Fragaria vesca*, *Malus domestica*, *Pyrus bretschneideri*, *Prunus mume*, *Prunus persica*, and *Rosa chinensis*. Figure S1: Multiple sequence alignment of 97 MAPK genes from *Malus domestica*, *Pyrus bretschneideri*, *Prunus persica*, *Prunus mume*, *Rosa chinensis*, and *Fragaria vesca*. The ninety-seven identified MAPK proteins contained I-XI characterized multiple protein domains; Figure S2: Amino acid residues of 15 conserved motifs within MAPK protein sequences in six Rosaceae species; Figure S3: Functional distances of Clusters A, B, C, D.

Author Contributions: Conceptualization, Y.Y., Y.S. and Q.W.; methodology, Q.W.; software, Y.Y. and Y.H.; validation, Y.Y. and Y.H.; formal analysis, Y.Y. and Y.Z.; investigation, Y.Z.; resources, Y.Y.; data curation, Y.Y. and H.T.; writing—original draft preparation, Y.Y.; writing—review and editing, H.T., Y.S. and Q.W.; visualization, Y.H.; supervision, Y.S.; project administration, Y.S. and Q.W.; funding acquisition, H.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Science and Technology Plan Project of Tianjin, grant number: 21KPHDRC00020.

Data Availability Statement: The RNA-seq data were deposited in NCBI database (Accession ID: GSE40162, PRJNA1046642, and PRJNA1046397).

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

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