Genome-wide identification of MAPKKK genes and their response to phytoplasma stress in Chinese jujube (Ziziphus jujuba Mill.)

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Research article

Keywords: Chinese jujube, MAPKKKs, Jujube witches' broom, Phytoplasma, Expression profiles

Posted Date: August 13th, 2019

DOI: https://doi.org/10.21203/rs.2.9872/v2

Version of Record: A version of this preprint was published on February 10th, 2020. See the published version at https://doi.org/10.1186/s12864-020-6548-6.

Abstract

Backgrounds Mitogen activated protein kinase (MAPK) cascades play vital roles in signal transduction in response to various biotic and abiotic stresses. In the previous study we have identified 10 ZjMAPKs and 5 ZjMAPKKs in Chinese jujube genome and found some crucial members of ZjMAPKs and ZjMAPKKs might function importantly in the process of phytoplasma infection. But how these ZjMAPKKs were modulated by ZjMAPKKKs during this process is still elusive and little information is known about the MAPKKKs in Chinese jujube. Results In the current study, 56 ZjMAPKKKs were identified in the jujube genome and all of them contain the key S-TKc (serine/threonine protein kinase) domain which distributed in all 12 chromosomes. Phylogenetic analysis showed that these ZjMAPKKKs could be classified into two subfamilies, of which 41 belonged to Raf, and 15 to MEKK subfamily. In addition, the ZjMAPKKKs in each subfamily share the same conserved motifs and gene structures, one pair of ZjMAPKKKs (15/16) was the only tandem duplication event on Chromosome 5. Furthermore, the expression profiles of these MAPKKKs in response to phytoplasma disease were investigated by qPCR. In the three main infected tissues (witches' broom leaves, phyllody leaves, apparent normal leaves), the ZjMAPKKK26 and 45 were significantly up regulated and the ZjMAPKKK3, 43 and 50 were down regulated. While the ZjMAPKKK4, 10, 25 and 44 were significant highly induced in the sterile cultivated tissues infected by phytoplasma, and the ZjMAPKKK7, 30, 35, 37, 40, 41, 43 and 46 were significantly down regulated. Conclusions The identification and classification analysis of ZjMAPKKKs was firstly reported and some key individual ZjMAPKKKs genes might play essential roles in response to phytoplasma infection. This could provide initial understanding for the mechanism that how the ZjMAPKKKs were involved in jujube - phytoplasma infection.

Background

Mitogen activated protein kinase (MAPK) cascades, consisting of three specific kinase families: MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MAPKKs) and MAP kinases (MAPKs), are essential middle signal modules from signal sensing to the activation of related transcript factors in response to biotic and abiotic stresses such as drought, salt, cold and pathogen attack [1-3]. Among the signalling transduction, the conserved serine/threonine MAPKKKs could be activated by the plasma membrane receptors, then phosphorylate the MAPKKs which further activate the MAPKs by sequentially phosphorylation. Finally, the MAPKs could regulate other kinases or related transcription factors in response to various stresses [4, 5]. Furthermore, each MAPK cascade family consists of a series of members and the number of them is significantly different. For instance, , the MAPKKKs family composes greater members and shows more complex sequence diversity comparing to the other two families. The members belong to this family could be classified into Raf, ZIK and MEKK subfamilies, according to characteristic sequence motifs [6]. The structural disparity of MAPKKKs in each subfamily could be observed, the Raf subfamily has a C-terminal kinase domain and a long N-terminal regulatory domain, while ZIK proteins only have the N- terminal kinase domains and MEKK subfamily has less conserved kinase domain. In addition, thethe long N- terminal regulatory domain is the backbone for Raf and ZIK subfamilies [1, 6].

MAPK cascades have been implicated in the signal transduction in distinct plants innate immunity [7, 8]. In *Arabidopsis*, the *MEKK1-MKK4/5-MPK3/6-WRKY22/WRKY29/FRK1* cascade was involved in the innate

immunity signalling transduction and the *MEKK1-MKK1/MKK2-MPK4* kinase cascade could negatively activate *MEKK2* which further leads to SUMM2-mediated immune responses [9, 10]. In tobacco, the *NPK1-MEK1-Ntf6* could regulate *WRKY/MYB* transcription factors to participate in the tobacco mosaic virus infection pathway [11]. In addition, the *MAPKKKα-MKK2/MKK4-MPK2/MPK3* cascades took part in the Pto-mediated effect or triggered immunity (ETI) pathway by regulating the transcription factor *TGA* in tomato [12]. Hence, the MAPKs pathway is indeed involved in the pathogen attack and might also play essential roles in response to phytoplasma infection in Chinese jujube.

Hitherto, the *MAPKKKs* family has been characterized in plant kingdom. 80 *MAPKKKs* was firstly identified in *Arabidopsis* in 2002 [6, 13]. From then on, an array of *MAPKKKs* has been identified from different plants, including rice (75 members), *Zea mays* (71 members), *Vitis vinifera* (45 members), *Malus* domestic (72 members), *Musa nana* (77 members), and so on [14-17]. However, little is known about the biological information and function of *MAPKKK* gene family in Chinese jujube, even though the detailed information of *ZjMAPKKs* and *ZjMAPKs* has been reported by our group [18].

Jujube witches' broom disease (JWB) caused by '*Candidatus Phytoplasma* ziziphi' has become a devastating disease in Asia [19]. This disease shows three typical symptoms, including witches' broom, phyllody and yellowing [20]. The physiological and biochemical behaviors of jujube infected by phytoplasma has been widely studied [21], but the molecular mechanism behind is still elusive. Recently, the MAPKs in response to phytoplasma infection have been reported with analysis of RNA-seq transcriptomics in Chinese jujube [22], this study provides the insight that MAPKs must play important roles during this process. In addition, *ZjMPK2*, *ZjMKK2* and *ZjMKK4* have been demonstrated to be the main and key genes involved in Chinese jujube-phytoplasma interaction and ZjMKK2 could interact with ZjMPK2 with the yeast two hybrid analysis [18, 23]. All these results demonstrate the important function of MAPK cascades in response to phytoplasma infection and it is necessary to do the identification and initial functional analysis of *ZjMAPKKKs* to build the complete MAPK cascade signalling transduction pathway. Thus, in this study, the *ZjMAPKKKs* were identified by the genome wide analysis and the phylogenetic analysis, gene structure and conserved motifs of *ZjMAPKKKs* were also predicted. Furthermore, the expression profiles of these *ZjMAPKKKs* in response to phytoplasma infection were investigated by qPCR. These investigation and identification of the *ZjMAPKKKs* and the detection of their initial functions to phytoplasma infection could facilitate the understanding of the mechanism how the *ZjMAPKs* cascades involved in JWB defense response.

Method

Identification of *ZjMAPKKKs* in Chinese jujube

The *ZjMAPKKKs* gene family was identified according to our previous study on the identification of *ZjMAPKKs* and *ZjMAPKs* with some modifications [18]. Firstly, the whole protein sequences of *MAPKKKs* in *Arabidopsis* were retrieved from TAIR databases **(Additional file 1)**. These sequences were as queries to search against in the whole jujube genome database (accession JREP00000000) [24]. In addition, the alignments of all *Arabidopsis MAPKKK* sequences were used to construct a HMM profile (http://hmmer.org/download.html) to search the other potential *MAPKKKs* members in jujube genome

database. All the potential *ZjMAPKKKs* genes were confirmed by HMMER tools which contain protein kinase domain (PF00069) [25], while the redundancy was removed, and the remaining sequences were identified as *ZjMAPKKKs* family. Furthermore, the ExPASy Proteomics Server (http://expasy.org/) was used to calculate the theoretical pI (isoelectricpoint) and Mw (molecular weight) of the putative *ZjMAPKKKs* [26].

Gene structure, protein domain and motif analysis of *ZjMAPKKKs* genes

The open reading frames (ORF) of 56 *ZjMAPKKKS* were analyzed through the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) ORF finder and the main protein domains of *ZjMAPKKKs* were recorded by blastp of NCBI as well **(Additional file 2)**. GSDS (Gene Structure Display Server, http://gsds.cbi.pku.edu.cn/) was exploited to determine the exon/intron structures of individual *ZjMAPKKKs* by aligning the cDNA sequences to their corresponding genomic DNA sequences [27]. MEME database was exploited to identify the conserved motifs of *ZjMAPKKKs* [28]. The ideal motif widths were set to be between six and fifty, and each protein sequence of the *ZjMAPKKKs* subfamily was used to find the higher number of conserved domains [29].

Sequence alignment, phylogenetic and gene duplication analysis

All the protein sequences of *ZjMAPKKKs* were aligned by ClusterX software with default parameters. Then the alignment of the protein sequences of 56 *ZjMAPKKKs* and 80 *AtMAPKKKs* were performed to do the phylogenetic analysis using MEGA 6.06 alignment explorer. The parameters of alignment as follows: gap opening penalty, 10.00; gap extension penalty, 0.50 (both in pairwise alignment and multiple alignment); protein weight matrix, gonnet; residue-specific penalties, on; hydrophilic penalties, on; gap separation distance, 0; end-gap separation, on; use negative matrix, off; and delay divergent cutoff (%), 30. Phylogenetic trees were constructed by the neighbor-joining (NJ) method. The parameters of the constructed trees were: statistical method, neighbor-joining; scope, all selected taxa; test of phylogeny, bootstrap method; number of bootstrap replications, 1000; substitutions type, amino acid; model/method, poisson model; rates among sites, uniform rates; pattern among lineages, same (homogeneous); and gaps/missing data treatment: complete deletion. Multiple Collinearity Scan toolkit (MCScanX) was adopted to analyze the gene duplication events, with the default parameters and the homologous relationships were drawn using the Circos software [30, 31].

Plant materials and treatments

Ziziphus jujuba Mill. 'Dongzao' which was cultivated in the Experimental Station of Chinese Jujube, Hebei Agricultural University, were used as the experimental materials. The leaf samples were collected from at least three healthy jujube trees and three trees infected with JWB for each time point. All these experimental trees were cultivated in the naturally environmental conditions [21]. The visual JWB symptoms, such as phyllody leaves (floral organs becoming leaf-like) and apparent normal leaves (infected but none symptom), were firstly observed earlier in June, and then witches' broom leaves (shoot with small leaves) were observed at the middle of Junein each year. Finally, the mature leaves of witches' broom leaves (shoot with small leaves), phyllody leaves (floral organs becoming leaf-like) and apparent normal leaves (infected but none symptom), were collected from the same branch of diseased trees in June, July, August, and September (on

the 15th day of each month). The leaves from healthy trees were used as control for each time point. The different type materials were shown in **Additional file 3**. The detection of JWB phytoplasmas in the infected materials in June was detected by DAPI staining at histological level and quantitative real-time PCR analysis (qRT-PCR) at the molecular level from June to September [21]. Each material was sampled with three replicates from independent trees. In addition, the sterile cultivated JWB diseased plantlets of *Ziziphus jujuba* Mill. 'Goutouzao' were used as another parallel testing group and the healthy plantlets were used as control (As shown in **Additional file 4**). The detection of JWB phytoplasmas in the diseased and healthy sterile cultivated plantlets was detected by DAPI staining at histological level (**Additional file 5**) [23]. Every ten plantlets were pooled as one sample, three independent biological replications were sampled separately. All samples were rapidly frozen in liquid nitrogen and kept at -80 °C for RNA isolation and qPCR analysis.

RNA extraction and qRT-PCR analysis

Total RNA from the leaves was extracted by the TIANGEN RNA Extraction Kit. The genomic DNA contamination from the samples was removed by digesting with DNase. The cDNA synthesis was performed by TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa) according to the protocol of the manufacturer protocol using 1 μg of RNA template.

The qRT-PCR was carried out on the Bio-Rad iQ™ 5 using TransStart Top Green qPCR SuperMix AQ131 (TransGen Biotech, China). The 20 μL reaction system contained 10 μL of 2×SYBR Premix ExTaq™, 0.4μL each of 10 μM primers, 1μL diluted cDNA and 8.2 μL ddH_2O. The thermal profile was pre-incubation for 3 min at 94 °C, followed by 40 cycles of 5 s at 94 °C, 15 s at 55~63 °C and 15 s at 72°C.Relative expression levels of *ZjMAPKKKs* were calculated with the $2^{-\Delta\Delta Ct}$ method [30] using *ZjActin* as endogenous control for normalization[31]. The primer sequences of *ZjMAPKKKs* for qPCR were shown in **additional file 6**. The gene expression with more than or less than two-fold changes was considered as significant difference [23].

Heatmap construction

The expression profiles of all *ZjMAPKKKs* in different samples were illustrated by a color gradient heatmap. The heatmap was constructed by heatmap software Heml 1.0 using Log2 based expression fold-changes.

Results

Genome-wide identification of *ZjMAPKKKs*

A total of 56 *ZjMAPKKKs* were defined and all of them have the key S-TKc (serine/threonine protein kinase) domain and other conserved protein kinase domains (**Additional file. 2**). With the purpose of clearly understanding and discrimination of the *MAPKKK* genes, the Locus of *MAPKKKs* to *ZjMAPKKKs* according to the nomenclature suggestions of *Arabidopsis* was designated as*,* of which *Zj* is short for *Ziziphus jujuba* and the series number of *ZjMAPKKK1-56* was coded in term of their locations on chromosomes (**Table 1**). The *ZjMAPKKKs* distributed all over the 12 pseudo-chromosomes, excepted *ZjMAPKKK44-56* could not match to corresponding chromosome.

Specific information of each CDS and amino acid sequences of *ZjMAPKKKs* were listed in the **Additional file 1 and 7**. In addition, according to the specific conserved signature motif, all the *ZjMAPKKKs* could be divided into two subfamilies (Raf and MEKK); no ZIK subfamily members could be identified. Furthermore, as shown in **Table 1**, the length of the CDS sequence ranged from 762 bp (*ZjMAPKKK36*) to 4455 bp (*ZjMAPKKK7*), with an average length of 1804 bp. The amino acid sequence length of ZjMAPKKKs varied from 253 (*ZjMAPKKK36*) to 1484 (*ZjMAPKKK7*) amino acids (aa); average length was 600 aa. The predicted molecular weight (Mw) of these proteins ranged from 28.29 (*ZjMAPKKK36*) to 160.66 (*ZjMAPKKK7*) and the theoretical isoelectric point (pI) ranged from 4.78 to 9.34, respectively.

Phylogenetic analysis of *ZjMAPKKKs* genes

In order to assess the phylogenetic relationships between Chinese jujube and *Arobidopsis*, the phylogenetic tree was constructed with the total 136 protein sequences (56 ZjMAPKKKs and 80 AtMAPKKKs). As illustrated in **Fig. 1**, the members of AtMAPKKKs could be clustered into three categories, Raf, ZIK and MEKK, indicating that the method for the phylogenetic tree was reliable. However, the 56 members of ZjMAPKKKs could only be clustered into two subfamilies, Raf and MEKK. In addition, the largest Raf subfamily consisted of 41 members and the other 15 members of ZjMAPKKKs belonged to MEKK subfamily, and none of them could be defined into ZIK subfamily. Morever, some *ZjMAPKKKs* located on the same chromosome, shown little divergence, but clustered into the same group, such as *ZjMAPKKK36, 37* and *40*, *ZjMAPKKK15* and *16*, *ZjMAPKKK38* and *39*. These results indicated that some duplication of *ZjMAPKKKs* took place during the evolutionary process of jujube.

Conserved domains and gene structure analysis of *ZjMAPKKKs*

Within the analysis of MEME software, five main conserved motifs were identified in all 56 *ZjMAPKKKs* (**Fig.2**). The motif 1, 3 and 4 were founded in all the *ZjMAPKKKs*, while the other two motifs were observed in all the members of Raf subfamily. Additionally, the members of MEKK subfamily could be divided into two groups, one group contains motifs 1-4, including *ZjMAPKKK21, 56, 6, 31, 10* and *25.* The remaining members only consisted of motifs 1, 3 and 4. These results illustrated that the ZjMAPKKKs share the same conserved motifs which further indicate that the protein structures for each subfamily were highly conserved.

For the analysis of the contents of exon/introns, the difference among *ZjMAPKKKs* was significant. As shown in **Fig. 3** and **Additional file 8**, the number of exons in *ZjMAPKKKs* ranged from 1 (*ZjMAPKKK9, 12, 29, 35, 36, 44* and *54*) to 19 (*ZjMAPKKK42*). Interestingly, the members of *ZjMAPKKKs* which only contained 1 exon, all belong to MEKK subfamily (47%), but the highest number of exons in this subfamily could arrive to 17 (*ZjMAPKKK25* and *10*), the average number was 5.6. This demonstrated that more significant loss and gain of exon took place in this subfamily during the process of evolution. For the subfamily of Raf, the number of exon varied from 2 (*ZjMAPKKK16* and *28*) to 19 (*ZjMAPKKK42*), with the average number of 9.56. Even the significant variation of the number of exons existed in the Raf and MEKK subfamilies, some exon structure patterns were clearly conserved in close paralogs. For instance, the *ZjMAPKKK24* and *49* have 12 exons, *ZjMAPKKK37* and *40* have 2 exons, and they were all closely clustered in the same phylogenetic tree. Collectively, the evolutionary different organization of *ZjMAPKKKs* gene structures between Raf and MEKK

subfamilies indicated that the tandem and segmental duplication events might occur in the ancient time and the diverse exon structures might function differently in the jujube genome.

Furthermore, with the multiple protein alignment of *ZjMAPKKKs*, the Raf-specific signature motif: GTXX(W/Y)MAPE was founded in the Raf subfamily and the kinase domain located at the N terminal or C terminal. In contrast, the less conserved MEKK-specific signature motif: G(T/S)PX(W/F)MAPEV was observed in MEKK subfamily, while the kinase domain located at three positions: N- or C-terminal or the central part of the proteins (**Fig.4**). The features of the signature motifs of ZjMAPKKKs were consistent with other orthologues in other plants that fulfill them the important role in diverse signal transduction in plants.

Synteny analysis of *ZjMAPKKK* genes

Furthermore, a tandem duplication event was firstly analyzed according to the principle that two or more genes located on a chromosomal region within 200 kb [34]. As shown in **fig.5**, One pair of *ZjMAPKKKs* (15/16) was the only tandem duplication event on LG5. In addition, 13 segmental duplication events with 22 *ZjMAPKKKs* were also identified. These results indicated that some *ZjMAPKKKs* were possibly generated by gene duplication and the segmental duplication events played a major driving force for *ZjMAPKKKs* evolution.

Phytoplasma detection in different phytoplasma infected tissues

In order to get insight to understand the function of *ZjMAPKKKs* involved in phytoplasma infection, the expression level of individual *ZjMAPKKKs* was detected by qPCR in two kind of plant materials one material was infected by phytoplasma with three different symptoms, including witches' broom leaves, phyllody leaves and apparent normal leaves from diseased plants (*in vivo*) and the other plant material was sterile cultivated tissues of JWB plantlets (*in vitro*). Firstly, the phytoplasma concentration in in the first plant material with three sympotems has been detected by Xue *et al.* (2018) [21], and the phytoplasma determination in the sterile cultivated tissues of JWB plantlets shown that the fluorescent spots formed a large bright circle in the petiole phloem (**Additional file 5**). These results guaranteed the following test on the function of *ZjMAPKKKs* in response to phytoplasma infection.

Expression analysis of *ZjMAPKKKs* in witches' broom leaves

As shown in **Additional file 9 and Fig. 6 (A)**, the heat map showed the expression levels of *ZjMAPKKKs* with significantly different patterns in witches' broom leaves from June to September. 42 candidates expressing level could be detectable, but the expression levels of the other 14 *ZjMAPKKKs* were very low and could not be detectable. Finally these undetectable expressing *ZjMAPKKKs* genes were considered as the redundant candidates in our research and were not selected for further calculation and analysis. Among them, the most significant transcript induction took place at the early stage (June or July) when the witches' broom began to grow. For instance, the *ZjMAPKKK13, 14, 15, 23, 34,42, 44, 47* and *56* were highly induced in June or July (above 2-folds), afterwards, began to decrease from August to September as shown in **Fig. 6 (B)**. However, the *ZjMAPKKK3, 43* and *50* were down-expressed from June to September. In addition, two members of *ZjMAPKKKs* (*26* and *45*) remained high level expression constantly from June to September, indicating these two candidates might be the potentially key *MAPKKKs* in response to phytoplasma infection. However, it

should be kept in mind that the clustering of the expression profiles of *ZjMAPKKKs* was not consistent with the gene similarities, illustrating the gene function did not only rely on the gene structure.

Expression analysis of *ZjMAPKKKs* in phyllody leaves

As depicted above, the transcript abundance of *ZjMAPKKKs* were as well as investigated in the tissue of phyllody leaves. The heat map of the expressing *ZjMAPKKKs* was indicated in **Fig. 7 (A)**. Several of the *ZjMAPKKKs* were highly expressed in June or July and then the expression levels of them decreased from August to September, while most of the *ZjMAPKKKs* showed no significant changes or down regulated. For the details of each single *ZjMAPKKKs* expressing level could be seen in the **Fig. 7 (B)**, the *ZjMAPKKK10, 14, 15 34, 44* and *56* were significantly up regulated at the early stage (June or July). Howeverten of the *ZjMAPKKKs* showed significantly down regulated, including *ZjMAPKKK3, 16, 18, 41, 43, 50, 51, 52, 53* and *55*. As same as the expressing pattern of *ZjMAPKKK26* and *45* in the tissue of witches' broom leaves, these two members were also highly up regulated from June to September in the tissue of phyllody leaves.

Expression analysis of *ZjMAPKKKs* in apparent normal leaves

Furthermore, the apparent normal leaves that were infected by phytoplasma but shown no apparent symptoms was used to test which *ZjMAPKKKs* really play roles in response to phytoplasma infection in the non-strong disease leaves . Interestingly, the heat map figure showed different expressing pattern of *ZjMAPKKKs* in the tissue of apparent normal leaves (**Fig. 8B**). Seldom genes were highly up regulated and most of them showed down regulated pattern. For example, the *ZjMAPKKK1, 3, 7, 16, 17, 18, 19, 41, 43, 50, 51* and *53* were down regulated from June to September, while the *ZjMAPKKK28, 34* and *47* were significantly up regulated in June or July, and the *ZjMAPKKK*27 and 54 were up regulated from August or September. However, *ZjMAPKKK*26 and 45 showed the same high expressed pattern in the tissue of apparent normal leaves from June to September (**Fig. 8 A**).

To sum up, in the above-mentioned three phytoplasma infected tissues from four different time series, *ZjMAPKKK26* was significantly up regulated (8 times) and the *ZjMAPKKK45* were highly induced 10 times. Moreover, with the infecting development of phytoplasmas, the symptoms of which observed on jujube leaves were gradually stronger from the apparent normal leaves, phyllody leaves to withches' broom leaves [21]. For the analysis of this aspect, the expression level of *ZjMAPKKK26* was highly induced in phyllody leaves (~6 fold) in June, but not within other two symptom leaves. Then with time and infecting development, *ZjMAPKKK26* was up regulated in witches' broom leaves (~3 fold) in July and down regulated in phyllody leaves (~2 fold) in the meantime. However, *ZjMAPKKK45* was highly induced in apparent normal leaves and phyllody leaves (~3 and ~6 fold, respectively) in June and began to be down regulated from July to September, but it was also induced in witches' broom leaves constantly (~2 fold) (**Fig. 6, 7 and 8**). These results demonstrated within the infection of phytoplasmas, the *ZjMAPKKK26* was quickly response in phyllody leaves and then highly induced in witches' broom leaves, however, *ZjMAPKKK45* response faster than *ZjMAPKKK26* because of its high expression in apparent leaves in June. In contrast to these two up regulated *ZjMAPKKK* genes, the*ZjMAPKKK3, 43* and *50* were down regulated 9, 9, 10 times, respectively.

Expression analysis of *ZjMAPKKKs* in the sterile cultivated tissues of JWB plantlets

After investigating the expression profiles of *ZjMAPKKKs* in field tissues, we further detected their expression levels in the sterile cultivated (*in vitro*) tissues of JWB plantlets and the healthy plantlets were used as control. As shown in **Fig. 9**, the expressing profiles of *ZjMAPKKKs* indicated significant disparities compared with the above results. Only four members of *ZjMAPKKKs* were significantly induced in the disease plants, i.e., *4, 10, 25* and *44*. While the *ZjMAPKKK7, 30, 35, 37, 40, 41, 43* and *46* were significantly down regulated. The others showed none significantly changes.

Discussion

MAPK cascades, as conserved signal transducing modules in eukaryotes, are widely studied in the plant kingdom, including *Arabidopsis*, rice, maize, apple and so on [6, 10, 13, 14, 16]. In Chinese jujube, 10 *MAPKs* and 5 *MAPKKs* have been identified according to our previous study [18]. The structures of these genes were mostly as similar as to other plants, such *Arabidopsis*, poplar or apple [18]. In the current study, it is the first time to report the *MAPKKKs* in Chinese jujube genome, 56 *ZjMAPKKKs* were identified and the number of *ZjMAPKKKs* was approximately larger than that of *VviMAPKKKs* [15], but significantly smaller (nearly 45%) compared with the number of *MdMAPKKKs* [16]. Moreover, all the ZjMAPKKKs could be classified into two main subfamilies (Raf and MEKK), and interestingly none of them belong to ZIK subfamily. The reason for the disappearance of ZIK subfamily may due to the loss function of them during the evolutionary process in Chinese jujube, because the Raf subfamily was considered as the original members of *MAPKKKs* family [32]. In addition, the rate of intron loss is faster than the rate of intron gain after segmental duplication [33] and in our study the number of the conserved motifs and exons were all higher in Raf subfamily compared with that in MEKK subfamily, and 13 segmental duplication events including 22 *ZjMAPKKKs* were also identified, these data may further illustrate that the Raf subfamily contains original genes and the segmental duplication occurred during the long evolutionary history. This is consistent with what have been found in maize [14]. Furthermore, only 43 members of *ZjMAPKKKs* were sparsely located on the 12 chromosomes and the *ZjMAPKKK44-56* could not find the corresponding position in chromosomes (**Table 1**). All these findings may demonstrate that the evolutionary duplication process of *ZjMAPKKKs* took place and the unknown location of *ZjMAPKKKs* may confer a number of paralogous genes and play critical roles during the biological process, such as *ZjMAPKKK44, 45, 46,* and *50*, which might involve in the process of phytoplasma infection. This is different with our previous study on *ZjMAPKs* and *ZjMAPKKs* that they did not experience genome duplication in the evolutionary process [18].

MAPK cascades have been demonstrated as key important signalling modules in response to biotic and abiotic stresses, particularly the apparently wide implication in pathogen attack [34]. Among the *MAPKKKs*, the MEKK subfamily has been widely studied, while the biological function of Raf subfamily is still elusive at present. In *Arabidopsis*, the MAPK cascades signaling module *MEKK1-MKK4/MKK5-MPK3/MPK6* was proposed to be activated in the interaction with *flg22* treatment [35]. *EDR1*, as member of Raf subfamily, was indicated to be responsible for the salicylic acid induced powdery mildew attack [36]. In tomato, *MAPKKKε* and *MAPKKKα* play important roles in the cell death signaling associated with plant immunity [37, 38]. In wheat, a *MAPKKK* named *TaFLR* could be activated by leaf rust pathogen *Puccinia triticina* [39]. While in grapevine, *VqMAPKKK38* could be highly induced by powdery mildew infection [40]. All the evidence suggested that the member of MAPKKKs is essential in the pathogen attack signal transduction. In this study,

we demonstrate that the *ZjMAPKKK26* and *45* were significantly up regulated and the *ZjMAPKKK3, 43* and *50* were down regulated in the three main infected leaves. While in the sterile cultivated (*in vitro*) tissues of JWB, four members of *ZjMAPKKKs* (*4, 10, 25* and *44*) were significantly induced in the JWB disease plants. And the *ZjMAPKKK7, 30, 35, 37, 40, 41, 43* and *46* were significantly down regulated. The potential candidate genes responses to phytoplasmas were different *in vivo* and *in vitro.* This was consistent with our previous work that *ZjMPK1* was considered as the potential genes related with phytoplasmas infection *in vitro* [18] which was also different with our late work that *ZjMPK2* was the main genes which could be regulate with *ZjMEK2 in vivo* [23]. The reason behind this might be that more environmental factors (light, temperature, etc.) affect the phytoplasma growth, evolution and infection in vivo. Even though, all these candidates which have been depicted above might be the potential genes which involved in phytoplasma infected signal transduction by recruiting the *MAPKKs* and *MAPK* families. Moreover, the *ZjMAPKKK43* which is homologous to *AtRaf1* in *Arabidopsis* was down regulated in all the phytoplasma infected tissues, and the *ZjMAPKKK10* was highly induced in the sterile cultivated tissues of JWB plantlets, this gene is homologous to *AtMEKK1* in *Arabidopsis*. Thus, these two genes might be the key gene in response to phytoplasma infection, because the *AtMEKK1* has already been demonstrated function importantly in the innate immunity in *Arabidopsis* by activation of *MKK4/MKK5-MPK3/MPK6* [35]. Moreover, the *ZjMKK2* could activate the *ZjMPK2* to play essential roles in JWB defense response [23], Ye *et al.* [22] have illustrated that after the phytoplasma infection, the MAPKs could also be activated and furthermore, the transcription factor *WRKY33* was regulated. Taking all together, the potential *ZjMAPKKKs* mediates *ZjMKK2-ZjMPK2* to *WRKY* transcription factors in response to phytoplasma infection need to be illustrated in the future study. In addition, the scaffold *RACK 1* (Receptor for Activated C Kinase 1) was identified in *Arabidopsis* which tethers the *MAPKKKs* to the plasma membrane and associates with Gb subunit to involve in immune responses [41]. Therefore, it is worth noting to demonstrate the function of *MAPKKKs* candidates and the relationship to *RACK1*in response to phytoplasma infection in Chinese jujube in the further study.

Conclusions

With various informatics analyses of *MAPKKKs* in Chinese jujube genome, 56 members of *ZjMAPKKKs* were identified and named according to their location in the chromosomes. The phylogeny, conserved motifs and intron/exon analysis confirmed their identity as members of each family. Moreover, the expression profiles of *ZjMAPKKKs* were detected by qPCR in four materials with different level of JWB (jujube witches' broom) symptom, including the tissues of witches’ broom leaves, phyllody leaves, apparent normal leaves and the sterile cultivated tissues of JWB, respectively. The *ZjMAPKKK26* and *45* were significant up regulated and the *ZjMAPKKK3, 43* and *50* were down regulated in the three main infected tissues. While in the sterile cultivated tissues of JWB plantlets, four members of *ZjMAPKKKs* were significantly induced in the disease plants, such as *4, 10, 25* and *44*. And the *ZjMAPKKK7, 30, 35, 37, 40, 41, 43* and *46* were significantly down regulated. In conclusion, our results provide the initial insight of *ZjMAPKKKs* that they could be potentially involved in phytoplasma infection.

Abbreviations

JWB: Jujube witches' broom; **S-TKc:** Serine/threonine protein kinase; **MAPK:** Mitogen-activated protein kinase; **MAPKK:** MAPK kinase; **MAPKKK:** MAPK kinase kinase; **ORF:** Open reading frames; **qPCR:** Quantitative real-time PCR; ***Zj*:** *Ziziphus jujuba*□**Chr:** Chromosome; **PI:** The theoretical isoelectric point of proteins; **MW:** The theoretical molecular weight of proteins; **RACK 1:** Receptor for Activated C Kinase 1

Declarations

Ethics approval and consent to participate

The healthy and diseased jujube trees used in this study were from the Experimental Station of Chinese Jujube, Hebei Agricultural University, in Baoding, Hebei. Chinese jujube is one of traditional and widespread fruit trees in China, and it is not an endangered species. No specific permits are required for sample collection on Chinese jujube.

Consent for publication

Not applicable.

Ethical standards

This research does not contain any studies with human participants or animals.

Availability of data and materials

All data and materials are presented in the main paper and additional file. In addition, the whole protein sequences of MAPKKKs in *Arabidopsis* were retrieved from TAIR databases. The CDS and genome sequences of MAPKKKs in jujube were retrieved from the whole jujube genome database (accession JREP00000000) in NCBI.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Funds for Hebei Distinguished Young Scholar (C2016204145), the Youth Fund of Hebei Province Natural Science Foundation (C2016204157), Green Channel Fund of Hebei Province Natural Science Foundation (C2019204308), the Significance Fund of Hebei Province Natural Science Foundation (C2017204114), and the Agricultural University of Hebei Foundation for Leaders of Disciplines in Science Technology, Funds for Young Talent Project of Hebei Agricultural University Foundation (grant number YJ201853). Funds were used for the design of the study and collection, analysis, and interpretation of data and in writing the manuscript, as well as in the open access payment.

Authors' contributions

JZ and ML designed the research and wrote the paper. ZL and LW performed the experiments, analyzed the data and wrote the paper. CX, YC, WG and YZ performed the experiments and participated in the data analysis. All authors read and approved the final the manuscript.

Acknowledgement

Not applicable.

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Tables

Table 1 Characteristic of MAPK Kinase Kinases from *Ziziphus Jujuba* Mill. (*ZjMAPKKKs*)

Group	Name	Locus ID	Chr	Location	CDS (bp)	Amino acid length (AA)	PI	MW (KD)
Raf	ZjMAPKKK1	LOC107412947	Chr1	512097-518184	3351	1116	5.56	123.87
	ZjMAPKKK2	LOC101223021	Chr1	588730..592155	1062	353	7.16	39.83
	ZjMAPKKK3	LOC107413171	Chr1	6034745..6041047	1380	459	8.99	52.10
	ZjMAPKKK4	LOC107414729	Chr1	7211380..7216386	1119	372	9.01	42.35
	ZjMAPKKK5	LOC107422643	Chr1	17322629..17330021	1707	568	6.55	64.31
	ZjMAPKKK7	LOC107427772	Chr1	28684428..28693993	4455	1484	5.27	160.66
	ZjMAPKKK8	LOC107429777	Chr1	31236415..31242279	3432	1143	7.67	126.67
	ZjMAPKKK11	LOC107412267	Chr2	23790620..23798118	2913	970	5.83	107.05
	ZjMAPKKK13	LOC107415263	Chr4	1873602..1877368	1059	352	7.66	39.46
	ZjMAPKKK14	LOC107417160	Chr4	23620629..23633413	2208	735	6.1	83.02
	ZjMAPKKK15	LOC107417666	Chr5	2301847..2306994	1248	415	8.14	46.31
	ZjMAPKKK16	LOC107417677	Chr5	2314642..2318435	1257	418	7.62	46.80
	ZjMAPKKK17	LOC107417907	Chr5	5814146..5820584	3789	1262	5.18	139.68
	ZjMAPKKK18	LOC107417903	Chr5	5822068..5828809	3825	1274	5.43	140.42
	ZjMAPKKK19	LOC107418405	Chr5	10268020..10272429	1074	357	8.97	40.45
	ZjMAPKKK20	LOC107418996	Chr5	18139031..18145136	2343	780	6.38	87.04
	ZjMAPKKK22	LOC107421353	Chr6	17987031..17997512	2832	943	8.15	105.39
	ZjMAPKKK23	LOC107420099	Chr6	2889952..2898479	2856	951	5.53	104.94
	ZjMAPKKK24	LOC107422567	Chr7	14102760..14109942	3444	1147	5.87	127.99
	ZjMAPKKK26	LOC107423093	Chr7	21216345..21219607	1173	390	7.92	43.53
	ZjMAPKKK27	LOC107423594	Chr7	27636786..27641226	1251	416	6.11	46.81
	ZjMAPKKK28	LOC107424157	Chr8	4344698..4348053	2049	682	8.81	79.10
	ZjMAPKKK30	LOC107424832	Chr8	9154812..9160628	1179	392	9.11	44.23
	ZjMAPKKK32	LOC107426395	Chr9	4943575..4946720	1203	400	6.29	44.66
	ZjMAPKKK33	LOC107426719	Chr9	5733599..5742140	3945	1314	5.32	144.52
	ZjMAPKKK34	LOC107427400	Chr9	18534579..18538786	1032	343	5.84	38.71
	ZjMAPKKK38	LOC107428906	Chr10	10418263..10423580	1299	432	8.15	48.84
	ZjMAPKKK39	LOC107428931	Chr10	11520310..11525561	1299	432	7.74	48.89
	ZjMAPKKK41	LOC107430036	Chr11	2650983..2657196	2181	726	6.97	81.01
	ZjMAPKKK42	LOC107431473	Chr11	19821853..19829329	1707	568	5.51	64.02
	ZjMAPKKK43	LOC107432147	Chr12	5159272..5168476	2556	851	5.98	93.67
	ZjMAPKKK45	LOC107408109	Unplaced Scaffold	688..6689	1425	474	9.12	53.23
	ZjMAPKKK46	LOC107405634	Unplaced Scaffold	5820..12905	1341	446	5.58	50.42
	ZjMAPKKK47	LOC107407393	Unplaced Scaffold	6471..11148	1125	374	7.13	42.29
	ZjMAPKKK48	LOC107406964	Unplaced Scaffold	13195..15977	1005	334	7.68	37.91
	ZjMAPKKK49	LOC107404883	Unplaced Scaffold	14404..17983	1251	416	6.24	46.77
	ZjMAPKKK50	LOC107406505	Unplaced Scaffold	16102..20361	1059	352	7.17	39.84
	ZjMAPKKK51	LOC107405705	Unplaced Scaffold	33936..39585	1158	385	7.51	42.93
	ZjMAPKKK52	LOC107403422	Unplaced Scaffold	48143..64604	1647	548	5.13	61.65
Raf	ZjMAPKKK53	LOC107435406	Unplaced Scaffold	61008..63977	1479	492	9.34	56.56
	ZjMAPKKK55	LOC107435407	Unplaced Scaffold	134848..138183	1482	493	9.22	56.35
MEKK	ZjMAPKKK6	LOC107423632	Chr1	18688160..18695042	2700	899	9.29	96.87

ZjMAPKKK9	LOC107432528	Chr1	34451996..34453515	1428	475	4.78	53.09
ZjMAPKKK10	LOC107411974	Chr2	21809712..21815908	2046	681	5.64	74.86
ZjMAPKKK12	LOC107414154	Chr3	19912625..19913971	1266	421	4.95	46.85
ZjMAPKKK21	LOC107420999	Chr6	10913503..10919747	1839	612	5.53	67.87
ZjMAPKKK25	LOC107423026	Chr7	20770505..20775489	2058	685	6.78	75.80
ZjMAPKKK29	LOC107424505	Chr8	6765530..6767083	1071	356	4.93	39.97
ZjMAPKKK31	LOC107425633	Chr8	20944047..20949800	1563	520	9.11	56.65
ZjMAPKKK35	LOC107427543	Chr9	20198543..20199481	939	312	6.52	35.52
ZjMAPKKK36	LOC107428154	Chr10	1469136..1470695	762	253	7.01	28.29
ZjMAPKKK37	LOC107428813	Chr10	8441149..8442948	825	274	4.89	30.43
ZjMAPKKK40	LOC107429056	Chr10	13522021..13523553	1035	344	5.36	37.67
ZjMAPKKK44	LOC107409320	Unplaced Scaffold	335..2009	1335	444	4.8	50.18
ZjMAPKKK54	LOC107434197	Unplaced Scaffold	108777..110183	1119	372	5.46	41.39
ZjMAPKKK56	LOC107435014	Unplaced Scaffold	155489..160603	1836	611	5.64	67.84

Note: Chr: chromosome; PI: the theoretical isoelectric point of proteins; MW: The theoretical molecular weight of proteins.

Additional Files

Additional file 1: Fig. S1 The protein sequences of MAPKKKs from *Ziziphus jujuba* Mill. and *Arabidopsis thaliana*.

Additional file 2: Table S1 The number of main protein domains of ZjMAPKKKs.

Additional file 3: Fig. S2 The healthy and diseased plantlets. A: Healthy plantlets; B: Diseased plantlets.

Additional file 4: Fig. S3 The tissues showing different JWB disease symptoms. A: Witches' broom leave; B: Phyllody leaves; C: Apparent normal leaves; D: Healthy leaves. A, B and C were used as test group which collected from the diseased trees. D was used as control which collected from the healthy trees.

Additional file 5: Fig. S4: Determination of phytoplasma in the sieve element in jujube petiole phloem by using 4',6-diamidino-2-phenylindole (DAPI). A, No fluorescent spots in the sieve element (SE) of healthy plantlets. B, The fluorescent spots formed a large bright circle in the sieve element (SE) of the diseased plantlets. The number and size of fluorescent spots represented the number of phytoplasma. Bar = 100 μm.

Additional file 6: Table S2 The primer sequences of *ZjMAPKKKs* for qRT-PCR.

Additional file 7: Fig. S5 The CDS sequences of *ZjMAPKKKs.*

Additional file 8: Table S3 The number of introns and exons of *ZjMAPKKK* genes

Additional file 9: Table S4 The fold change values of *ZjMAPKKKs* in the leaves of witches' broom, phyllody and apparent normal symptoms, respectively.

Figures

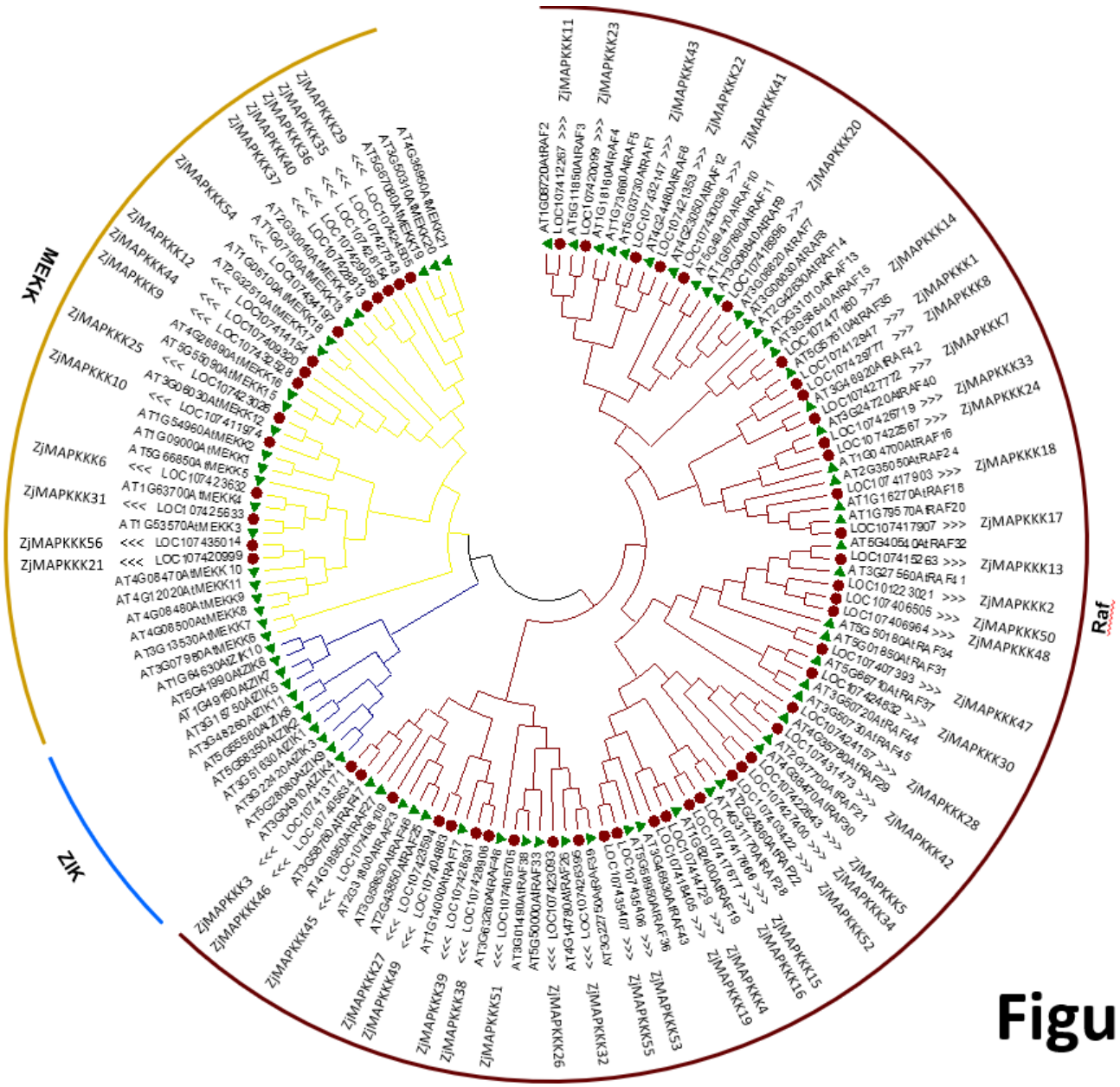

Figure.1

Figure 1

The phylogenetic analysis of ZjMAPKKKs (Ziziphus jujuba Mill.) and AtMAPKKKs (Arabidopsis thaliana) with total 136 protein sequences. The MEGA 6.0 was used to construct the phylogenetic tree with the neighbor-joining (NJ) method, and 1000 bootstrap replications were performed to indicate the reliability. All the ZjMAPKKKs were clustered into two groups, called Raf and MEKK subfamilies.

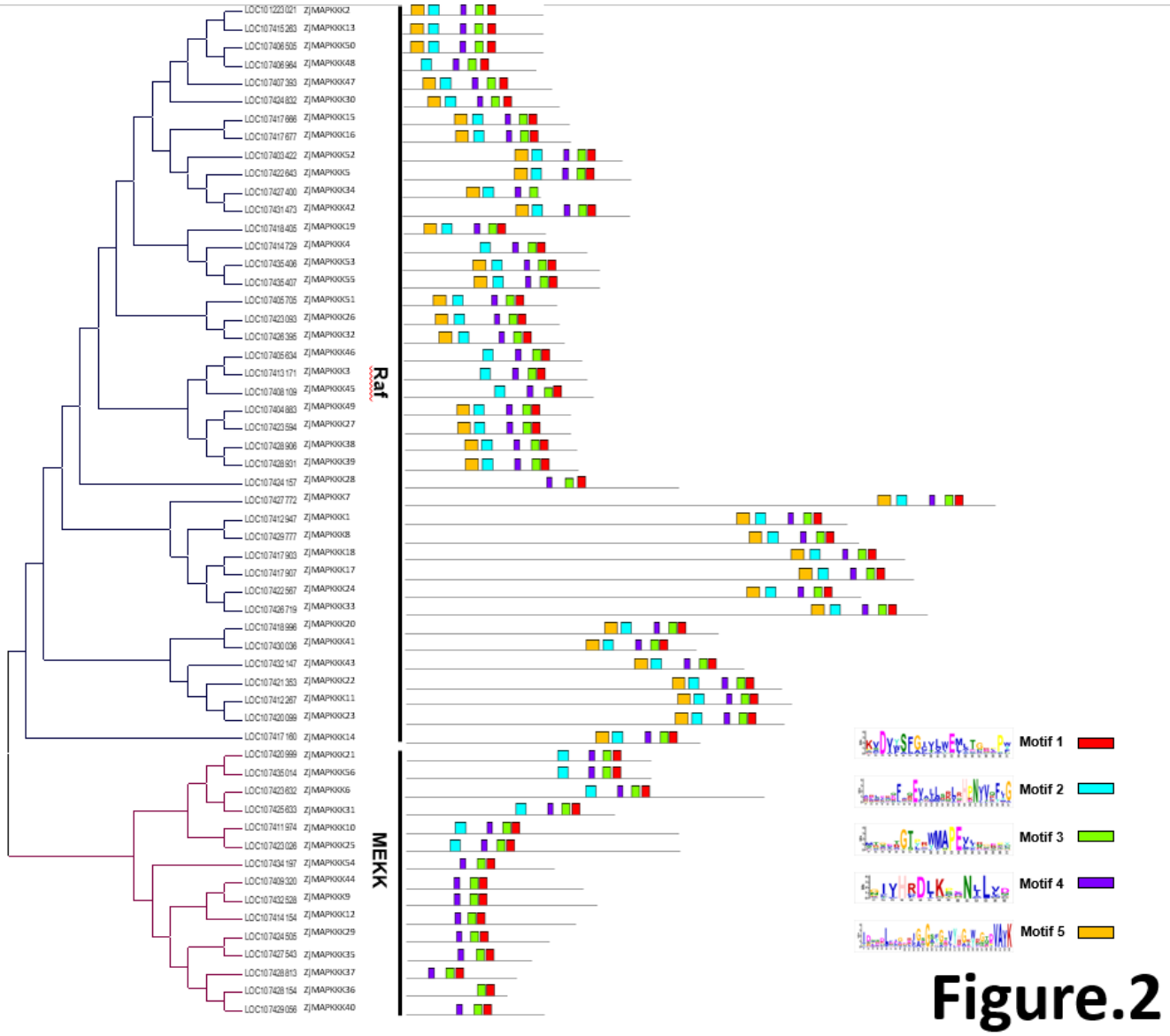

Figure 2

Identifing the conserved motifs of ZjMAPKKKs in corresponding to the phylogenetic tree. The MEME database was used to do the motifs' identification in according to the protein sequences.

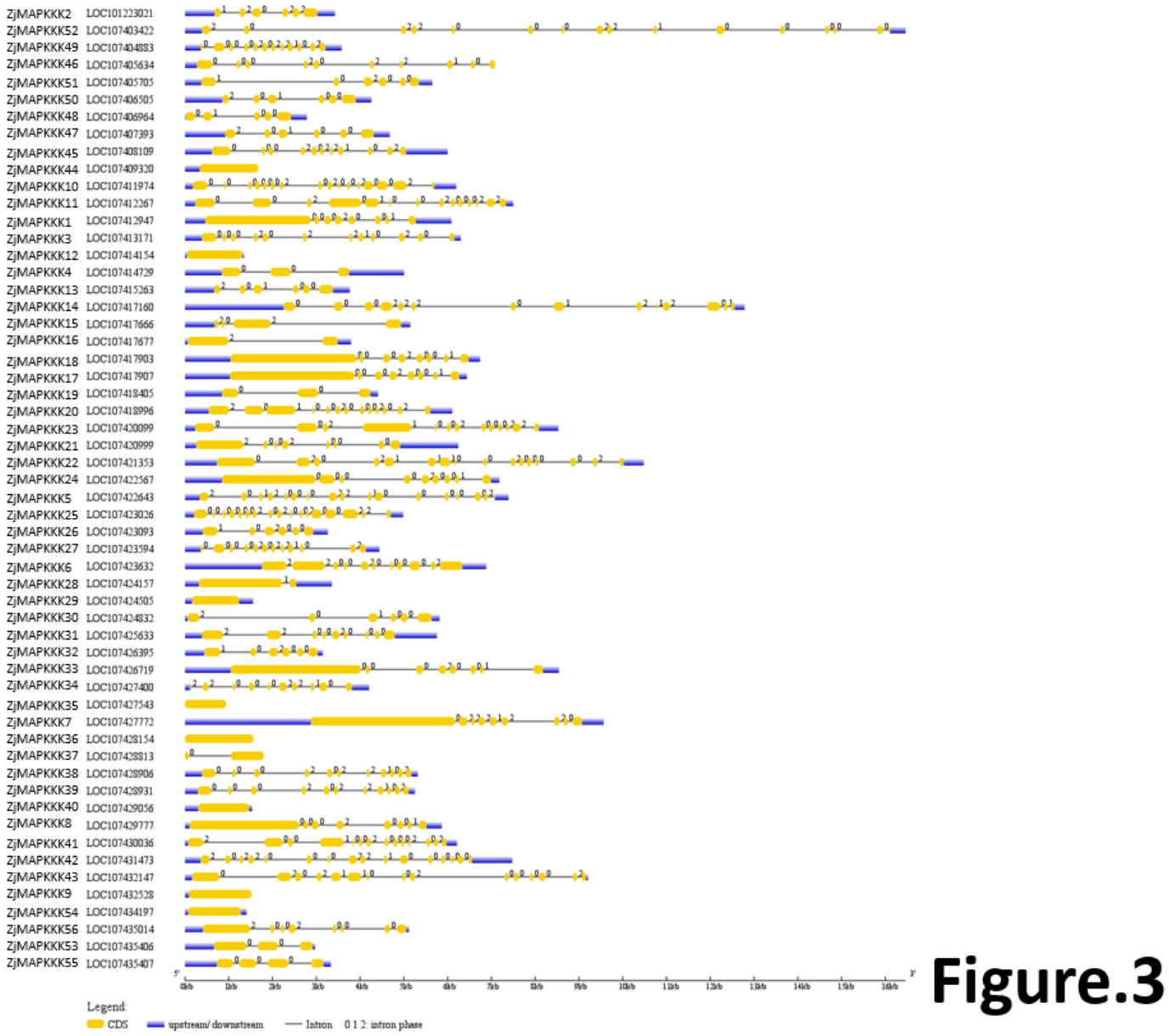

Figure.3

Figure 3

Schematic diagrams of ZjMAPKKKs structures The yellow, blue boxes and the black lines indicated the exons, UTRs and introns, respectively. 0, 1 and 2 illustrated different intron phases.

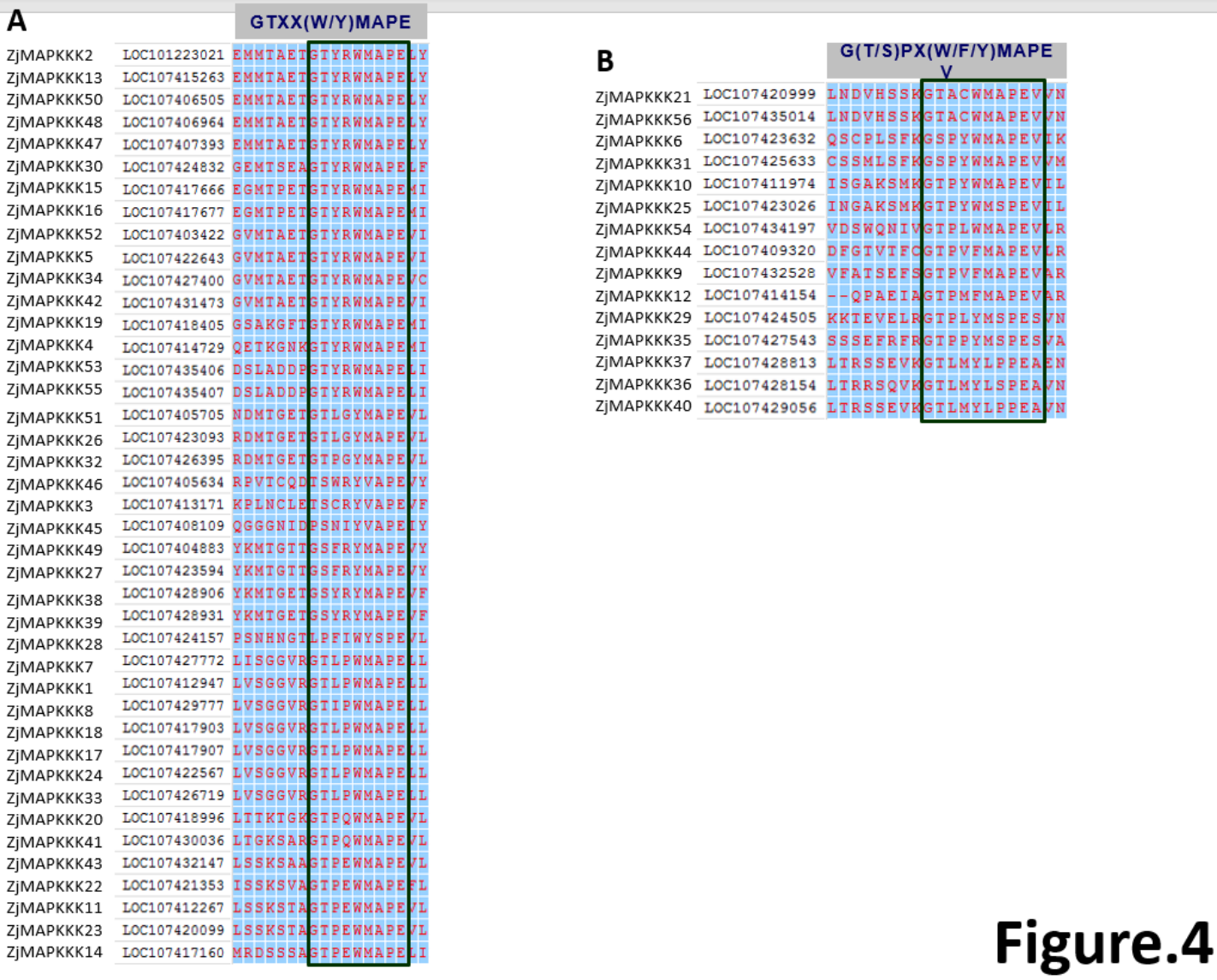

Figure.4

Figure 4

Protein sequence alignment of Raf subfamily (A) and MEKK subfamily (B) from Ziziphus jujuba Mill. The conserved signature motif GTXX(W/Y)MAPE and GTPEFMAPE(L/V)(Y/F) were found in the Raf and MEKK subfamily, respectively.

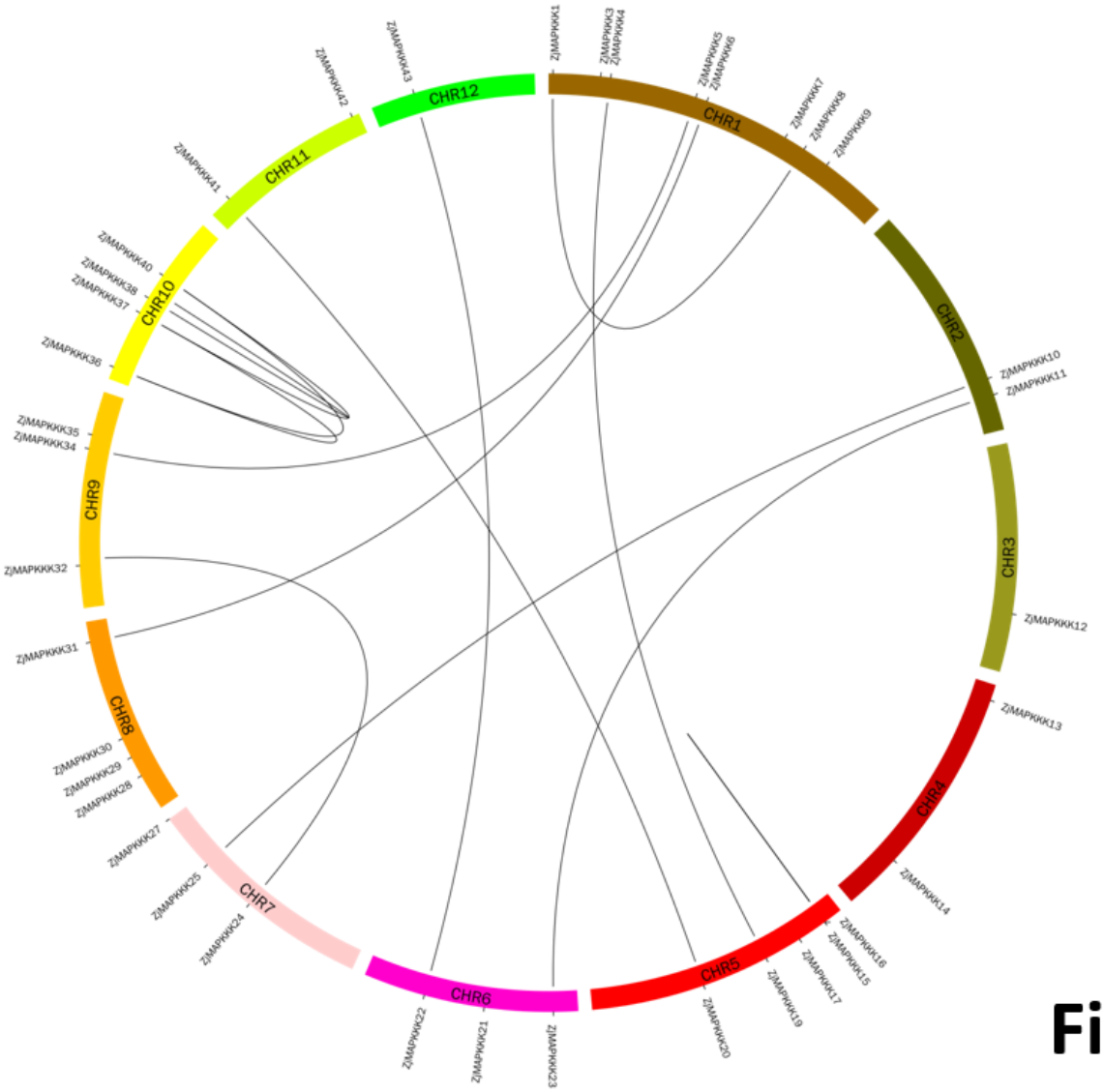

Figure.5

Figure 5

The synteny analysis of ZjMAPKKKs gene in the jujube genome. The black lines indicated duplicated ZjMAPKKKs gene pairs.

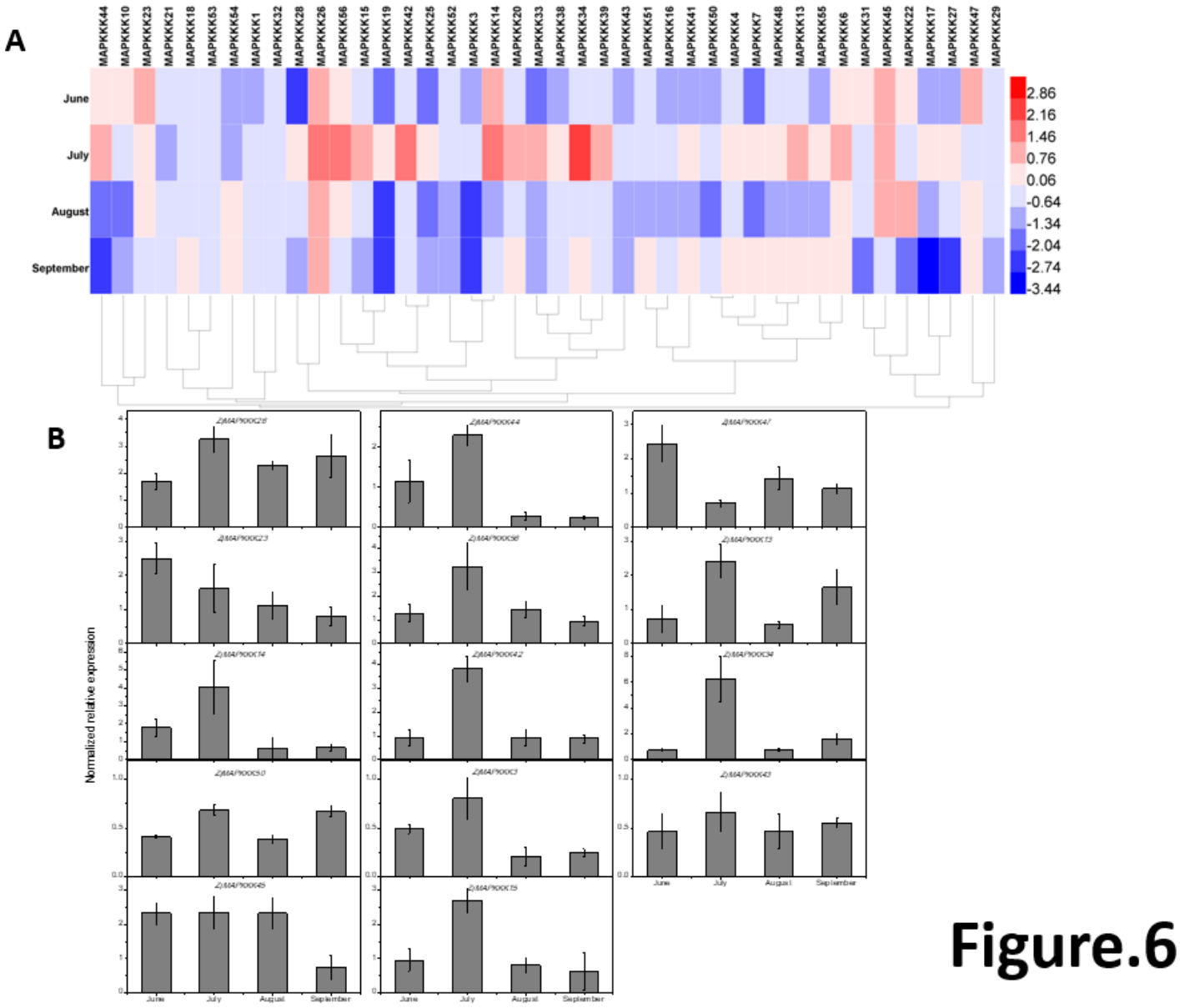

Figure.6

Figure 6

Relative expression profiles of ZjMAPKKKs in jujube witches' broom leaves from June to September. The expression levels of ZjMAPKKKs in healthy trees' leaves were used as control. (A) Heat map analysis of the ZjMAPKKKs based on the Log2 based fold change values. (B) The relative expression level of the representative members of ZjMAPKKKs in three independent replications and the error bar represents standard deviation (SD).

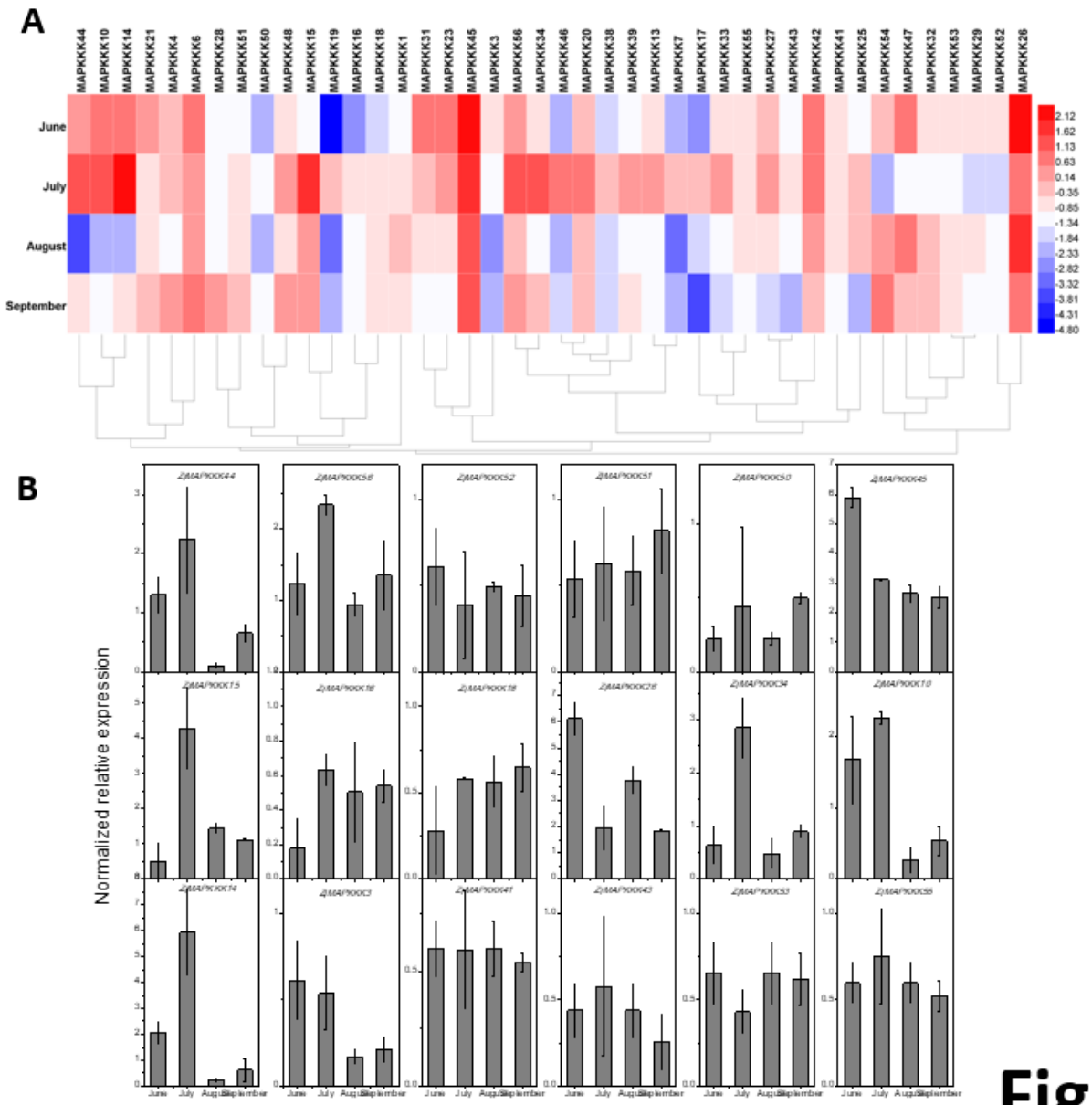

Figure.7

Figure 7

Relative expression profiles of ZjMAPKKKs in phyllody leaves from June to September. The expression levels of ZjMAPKKKs in healthy trees' leaves were used as control. (A) Heat map analysis of the ZjMAPKKKs based on the Log2 based fold change values. (B) The relative expression level of the representative members of ZjMAPKKKs in three independent replications and the error bar represents standard deviation (SD).

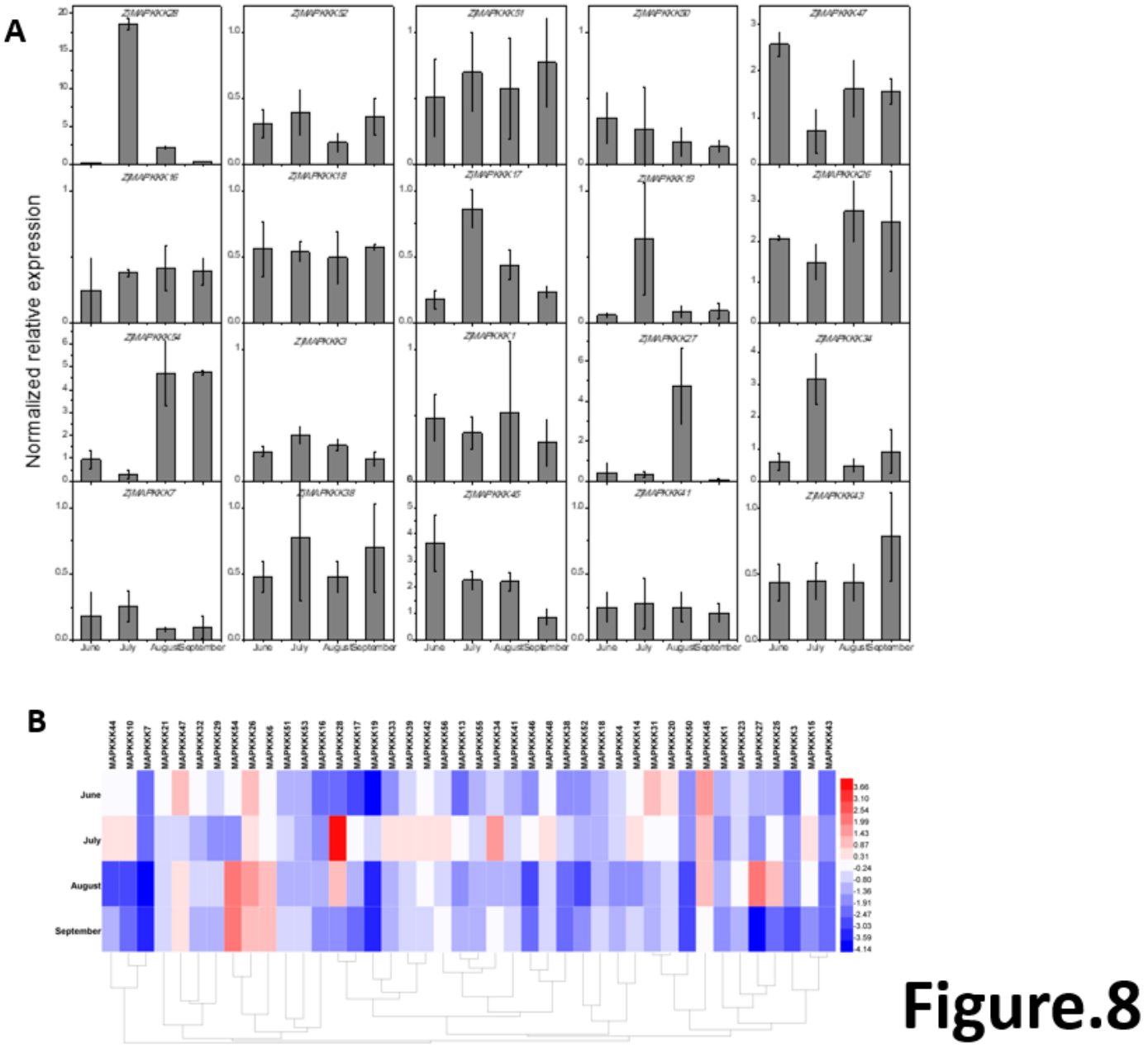

Figure.8

Figure 8

Relative expression profiles of ZjMAPKKKs in apparent normal leaves from June to September. The expression levels of ZjMAPKKKs in healthy trees' leaves were used as control. (A) The relative expression level of the representative members of ZjMAPKKKs in three independent replications and the error bar represents standard deviation (SD). (B) Heat map analysis of the ZjMAPKKKs based on the Log2 based fold change values.

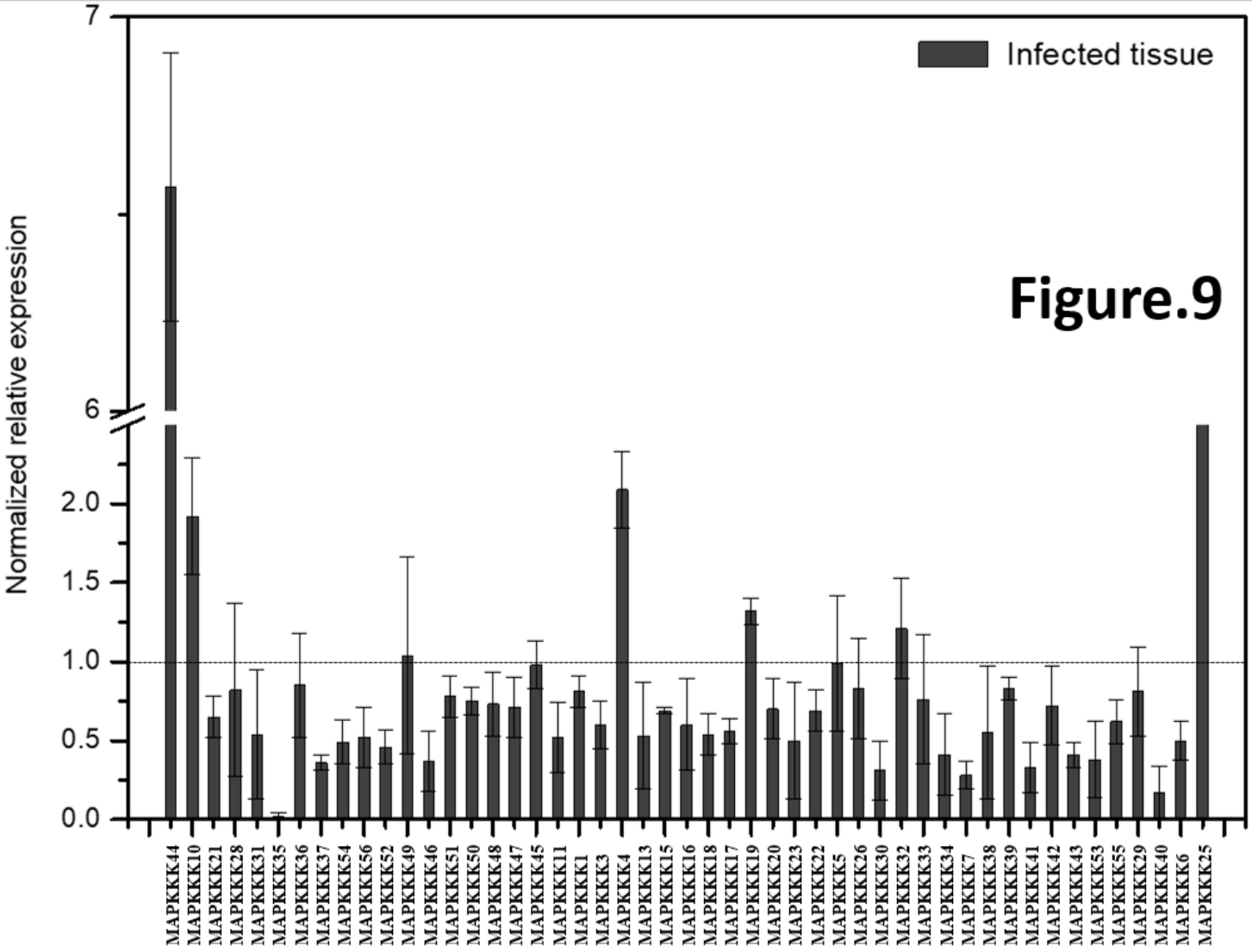

Figure 9

The expression profiles of ZjMAPKKKs in phytoplasma diseased plantlets and healthy tissue cultivated plantlets. The healthy plantlets were used as control. Three independent replications were performed and the error bar represents standard deviation (SD).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.xls
- supplement2.xls
- supplement3.xls
- supplement4.xls
- supplement5.pdf
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