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Three Main Genes in the MAPK Cascade Involved in the Chinese Jujube-Phytoplasma Interaction

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Abstract: Chinese jujube (*Ziziphus jujuba* Mill.) is an important economic forest species and multipurpose fruit tree in the family of Rhamnaceae. Phytoplasmas are significant prokaryotic pathogens, associated with more than 1000 plant diseases. Jujube witches' broom disease (JWB) is a typical phytoplasma disease, caused by '*Candidatus* Phytoplasma ziziphi'. Mitogen-activated protein kinase (MAPK) cascades are highly universal signal transduction modules and play crucial roles in regulating innate immune responses in plants. Thus, in the current study, systematical expression profiles of 10 *ZjMPK* and 4 *ZjMPKK* genes were conducted in plantlets with JWB disease, plantlets recovered from JWB disease, the tissues showing different disease symptoms, and resistant/susceptible cultivars infected by JWB phytoplasma. We found that most *ZjMPK* and *ZjMKK* genes exhibited significant up- or down-regulation expression under phytoplasma infection, but the top three differentially expressed genes (DEGs) were *ZjMPK2*, *ZjMKK2* and *ZjMKK4*, which showed the biggest times of gene's significant difference expression in all materials. Based on STRING database analysis, *ZjMKK2* and *ZjMPK2* were involved in the same plant-pathogen interaction pathway, and Yeast two-hybrid screening showed that *ZjMKK2* could interact with *ZjMPK2*. Finally, we deduced a pathway of jujube MAPK cascades which response to '*Candidatus* Phytoplasma ziziphi' infection. Our study presents the first gene-family-wide investigation on the systematical expression analysis of *MAPK* and *MAPKK* genes in Chinese jujube under phytoplasma infection. These results provide valuable information for the further research on the signaling pathway of phytoplasma infection in Chinese jujube.

Keywords: Chinese jujube; Jujube witches' broom; *Candidatus* Phytoplasma ziziphi; Phytoplasma infection; mitogen-activated protein kinase; differential gene expression; plant-pathogen interaction

1. Introduction

Plant pathogenic phytoplasmas, members of the class Mollicutes, are non-helical, cell wall-less bacterial organisms that are confined in the phloem sieve elements of infected plants [1,2]. Phytoplasmas are important prokaryotic pathogens that are associated with more than 1000 plant species, including many important economically plants [3,4]. Recently, phytoplasma effectors (such as SAP54, SAP11 and

TENGU) have been shown to modulate plant development, including indeterminate leaf-like flower development, morphology alternation and sterility in Arabidopsis plants [5–7]. These results indicate that phytoplasma effectors play an important role in phytoplasma pathogenesis.

Chinese jujube (*Ziziphus jujuba* Mill.), also known as Chinese date, is an important economic forest species and multipurpose fruit tree in the family of Rhamnaceae. It has been cultivated in China for up to 7000 years and has been introduced to 47 countries throughout Americas, southern and eastern Asia, Europe, and Australia [8]. Jujube witches' broom (JWB) caused by '*Candidatus* Phytoplasma ziziphi' [9], was a typical phytoplasma disease. Since 1980s, JWB has become one of the most serious and destructive disease in jujube cultivation and the initial mechanism of phytoplasma infection on Chinese jujube has been revealed [10].

Phytoplasmas duplicate exclusively inside phloem sieve elements of host plants and within insect vectors [11]. They are transmitted by phloem-sap-feeding insects in a circulative and persistent manner and by vegetative propagation of plants, in particular, by grafting [12]. Although axenic cultivation of grapevine yellows phytoplasma under microaerophilic growing conditions was reported [13], most phytoplasma strains are not successfully cultivated in vitro due to the complex media and strict anaerobic conditions [14]. So, interactions between phytoplasmas pathogen and host plants are hindered by the fact that phytoplasmas cultivation on artificial media is difficult, and they cannot be transmitted mechanically [15,16].

Mitogen-activated protein kinase (MAPK) cascades are highly universal signal transduction modules and play crucial roles in regulating innate immune responses in eukaryotes, including yeasts, animals and plants [17–20]. A typical MAPK cascade consists of three classes of hierarchically organized protein kinases, i.e., MAPK kinase kinase (MAPKKK/MEKK), MAPK kinase (MAPKK/MEK) and MAPK (MAPK/MPK) which are linked in various ways with upstream receptors and downstream targets [21]. Increasingly studies have clearly identified some complete MAPK cascades particularly in immune response [19,20]. For example, AtMEKK1-AtMKK4/5- AtMPK3/6-WRKY22/29 has been implicated in flagellin-mediated innate immune response [22]. Another MAPK cascade pathway AtMEKK1-AtMKK1/2-AtMPK4-MKS1/WRKY33 has been shown to negatively regulate plant immune signaling [23,24]. Orthologs of the MAPK cascades in *Arabidopsis* have the same function in other plant species, such as tobacco [25,26], rice [27], and cotton [28].

In a previous study, our group found that the expression level of most *ZjMPK* and *ZjMPKK* genes were down-regulated in jujube plantlets within phytoplasma infection [29]. Ye et al. [30] found that differentially expressed proteins of Chinese jujube under phytoplasma infection through iTRAQ proteomics method, which was related to MAPK signaling pathway. However, there is still uncertainty on the role of MAPK cascade pathways in the Chinese jujube-phytoplasma interaction.

In Chinese jujube, we have systematical and typical materials which could be used for investigating the molecular interaction mechanism between jujube and phytoplasma. The materials include plantlets with JWB disease, plantlets recovered from JWB disease, tissues showing different disease symptoms, and JWB-resistant/susceptible jujube cultivars [31]. Furthermore, our research group has identified the *ZjMPK* and *ZjMPKK* gene families at genome level [29]. Thus, in this study, expression analysis of *ZjMPK* and *ZjMPKK* genes were comprehensively investigated in diseased plantlets, recovered plantlets from JWB diseased and healthy plantlets. In the meanwhile, the temporal and spatial expression profiles of *ZjMPK* and *ZjMPKK* genes were carried out in tissues showing different disease symptoms and in JWB-resistant/susceptible cultivars [10] under phytoplasma infection. These results will provide valuable information for functional dissection of the important candidate genes and facilitate the further study on the mechanism of plant-phytoplasma interaction.

2. Results

2.1. Expression Patterns of ZjMPK and ZjMKK Genes in Healthy, JWB-diseased and Recovered Plantlets

To detect the JWB disease of the different jujube plantlets (Figure S1), the phytoplasma concentration was measured at the tissue levels in the healthy plantlets, diseased plantlets and recovered plantlets. DAPI staining showed no fluorescent spots in the sieve element (SE) of healthy plantlets and recovered plantlets, but the fluorescent spots formed a large bright circle in the SE of the diseased plantlets (Figure S2).

Furthermore, gene expression profiles under biotic stresses usually act as indicators of gene function. Therefore, according to above results, we investigated the expression patterns of 10 *ZjMPK* and 4 *ZjMKK* genes in healthy, JWB-diseased and recovered plantlets by qRT-PCR and try to find out the potential genes responding to phytoplasma infection. As shown in Figure 1, the expression level of 5 *ZjMPKs* (1, 5, 7, 8 and 9) and the *ZjMKK2* genes in diseased plantlets were down regulated compared to healthy plantlets, especially *ZjMPK1*, *ZjMPK5*, *ZjMPK7* and *ZjMKK2* with moderate-high fold change values of 1.87, 1.62, 1.74 and 1.80, respectively. In the meanwhile, none of the 14 genes were up regulated in diseased plants when compared to healthy plants. In addition, the expression level of *ZjMPK3*, *4*, *6*, *10* and *ZjMKK3* show none significant changes in all three materials which demonstrate these genes might not be involved in jujube-phytoplasma interaction. Moreover, when comparing the expression level of these 14 genes in recovered plantlets to the diseased plantlets, only *ZjMPK5*, *ZjMPK8*, *ZjMPK9* and *ZjMKK2* were up-regulated and these genes are down regulated in diseased plantlets (Figure 1) which could suggest that *ZjMPK5*, *ZjMPK9* and *ZjMKK2* were the potential genes involved in phytoplasma infection.

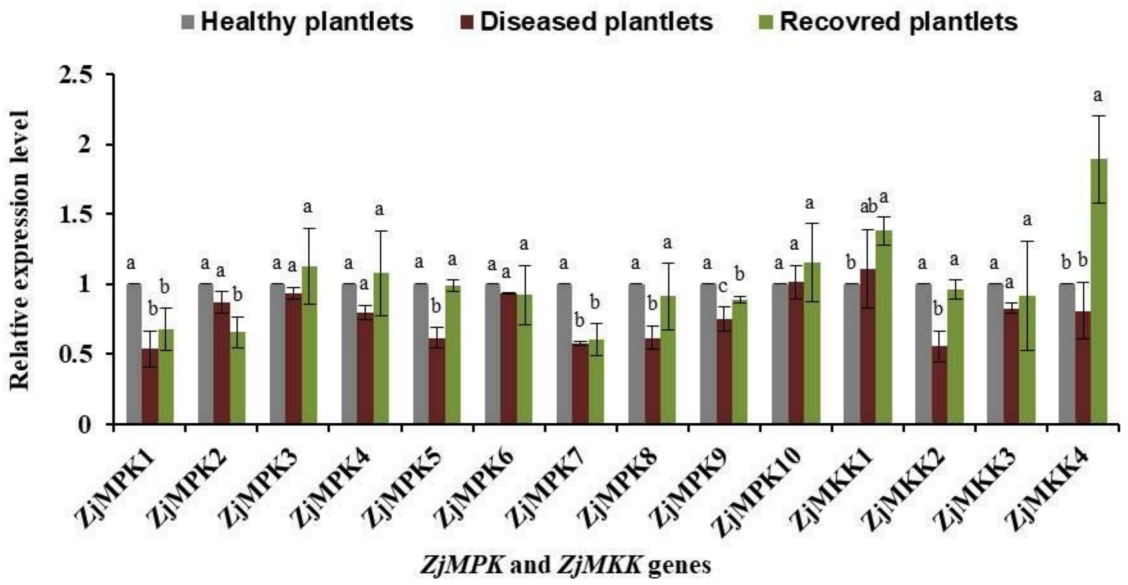

Figure 1. The expression modules of *ZjMPK* and *ZjMKK* genes in diseased plantlets and recovered plantlets. *ZjACT* was used as the internal standard. The healthy plantlets were used as control. The mean expression value was calculated from 3 independent replicates. The vertical bars indicate the standard deviation. Different letters indicate significant difference at the level of $p < 0.05$.

2.2. Expression Modules of ZjMPK and ZjMKK Genes in Tissues Showing Different JWB Disease Symptoms

Based on above results, most *ZjMPK* and *ZjMKK* genes have strong response in diseased and recovered plantlets, but whether these genes have the similar expression trend in different JWB disease symptoms tissues remains unclear. Therefore, the *ZjMPK* and *ZjMKK* genes expression modules were investigated in small leaves, phyllody and non-symptomatic leaves of diseased jujube at five stages. As shown in Table 1, the significant difference times of gene expression amount to 45 which include

21 times of up-regulation and 24 times of down-regulation, the differentially expressed genes (DEGs, the gene's relative expression change more than two-fold) distribute to 9 *ZjMPK*s and 2 *ZjMKK*s. Among them, the top 2 DEGs of *ZjMPK* gene family were *ZjMPK2* (10 times, up-regulated 9 times and down-regulated 1 time) and *ZjMPK9* (7 times up-regulation). In *ZjMKK* gene family, *ZjMKK2* was the most extinctive DEG with the 9 significant difference times include 6 times down-regulation and 3 times up-regulation.

Table 1. The number of *ZjMPK* and *ZjMKK* genes with significant difference expression in non-symptomatic leaves, phyllody and small leaves at five stages.

Gene Name	Up-Regulated Expression Times	Down-Regulated Expression Times	Total Times
ZjMPK1	0	1	1
ZjMPK2	9	1	10
ZjMPK3	0	4	4
ZjMPK4	0	2	2
ZjMPK5	2	2	4
ZjMPK6	0	2	2
ZjMPK7	0	4	4
ZjMPK8	0	0	0
ZjMPK9	7	0	7
ZjMPK10	0	1	1
ZjMKK1	0	0	0
ZjMKK2	3	6	9
ZjMKK3	0	0	0
ZjMKK4	0	1	1
In all	21	24	45

Note: The gene's relative expression changes more than two-fold were considered as significant differences. The significant difference times of one gene is the sum of that gene with significant difference expression in non-symptomatic leaves, phyllody and small leaves at five stages, which include Up-regulated expression times and Down-regulated expression times. Up-regulated expression (Down-regulated expression) means that the gene's relative expression level higher (lower) than 2 fold-changes.

Moreover, the differentially expressed genes (DEGs) had similar expression profiles in different JWB disease symptoms tissues of their own except *ZjMPK2* (Figures 2 and 3). Because diseased jujube sprout at the late April, witches' broom disease could only be shown in young branches limited that in May we could not collect the samples of phyllody and small leaves, thus we did the expression level of corresponding gene analysis from June to October. In non-symptomatic leaves, *ZjMPK2* showed a slight decrease in transcript abundance from June to August, but sharply increased at September, then strictly decreased. In phyllody, *ZjMPK2* showed a significant down-regulation from June to July, and then markedly increased at August, but strictly decreased from September to October. In small leaves, the expression level of *ZjMPK2* slightly increased from June to July, then sharply increased at August, but significantly decreased at following stages.

Base on the significant difference times of gene expression in three JWB diseased tissues at five stages, *ZjMPK2* and *ZjMKK2* were screened out as candidate genes in response to phytoplasma infection. As shown in Figure 3, it can be observed that *ZjMKK2* expression was mainly down-regulated in diseased tissues from June to October, while *ZjMPK2* expression was up-regulated.

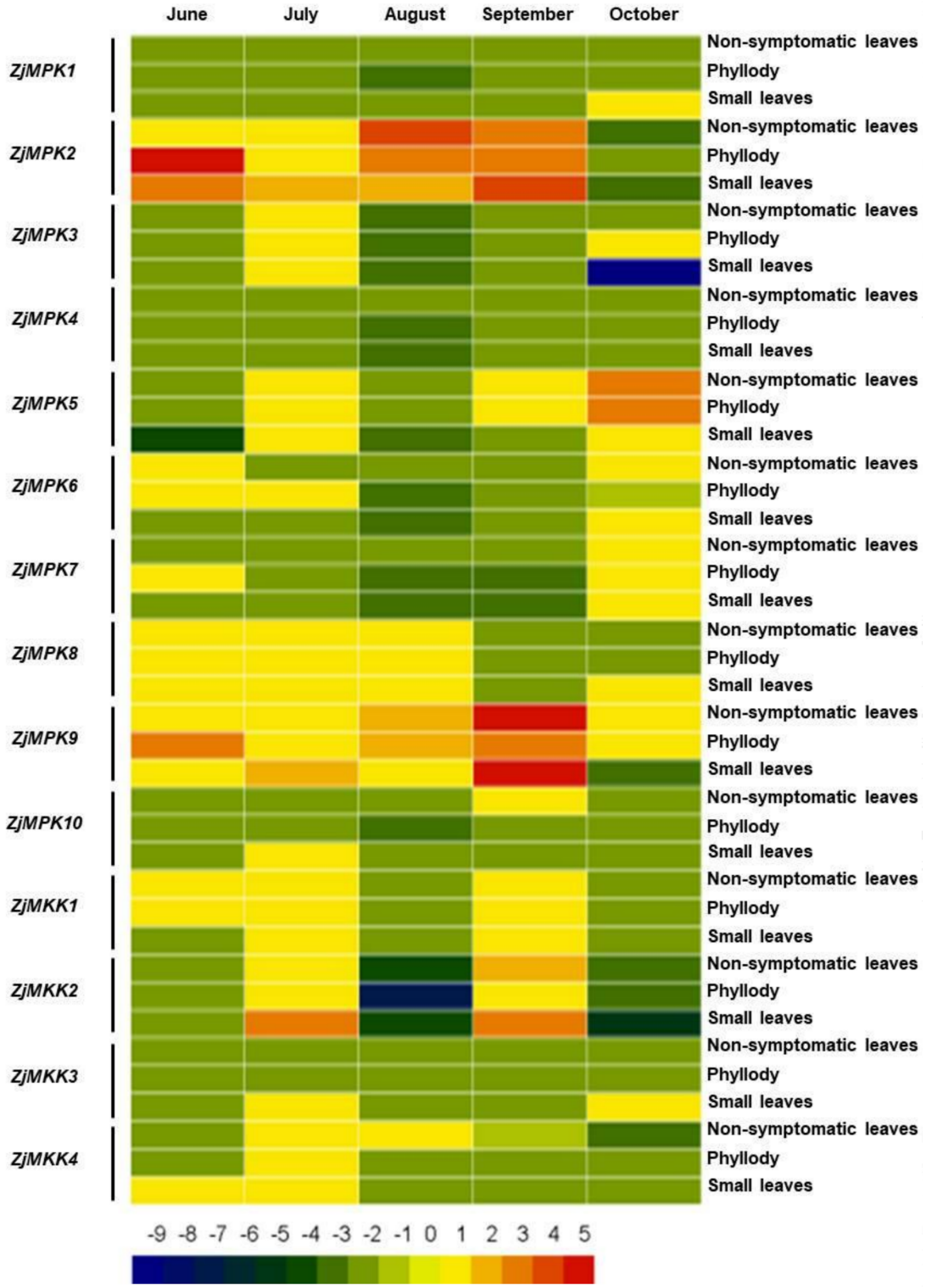

Figure 2. The expression profiles of *ZjMPK* and *ZjMKK* genes in tissues showing different JWB disease symptoms. The heatmap was generated by Heml 1.0 using expression fold-change. Up-regulated genes were colored in red, down-regulated genes were colored in blue (color code in the bottom).

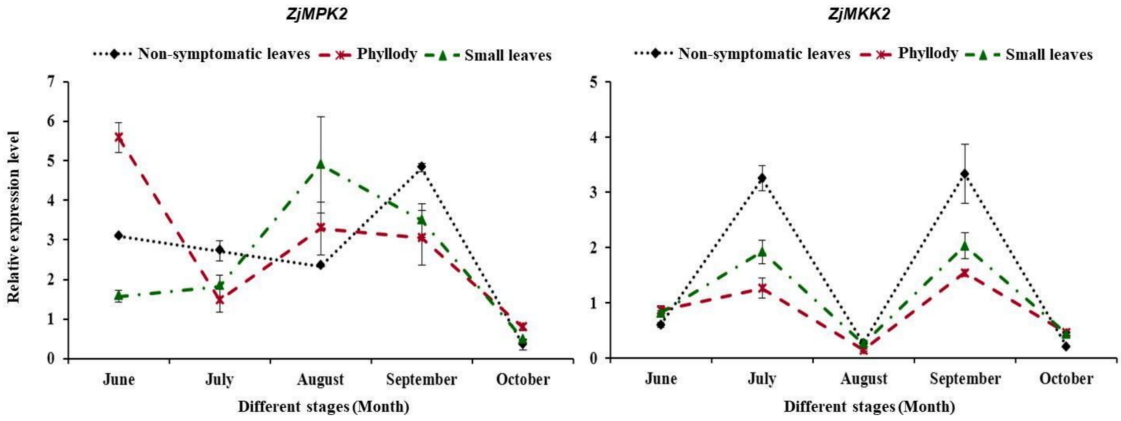

Figure 3. The expression trends of *ZjMPK2* and *ZjMKK2* genes in tissues showing different JWB disease symptoms. *ZjACT* was used as the internal standard. The leaves from healthy trees were used as control. The mean expression value was calculated from 3 independent replicates. The vertical bars indicate the standard deviation.

2.3. Expression Profiles of ZjMPK and ZjMKK Genes in Resistant and Susceptible Cultivars

Furthermore, in order to understand the expression divergence of *ZjMPK* and *ZjMKK* genes in resistant and susceptible cultivars after infected by phytoplasma, the expression profiles of *ZjMPK* and *ZjMKK* genes were conducted. As shown in Figure 4 and Table S1, 8 of 10 *ZjMPK* genes and 3 of 4 *ZiMKK* genes shown significant difference expression in both resistant and susceptible cultivars. Among them, *ZjMPK2* and *ZjMKK4* have the biggest times of gene's significant difference expression, such as *ZjMPK2* (8 times, up-regulated 7 times and down-regulated 1 time) and *ZjMKK4* (8 times, up-regulated 2 times and down-regulated 6 times). *ZjMPK2* transcript abundance significantly increased (expression changes > 2-fold) in susceptible cultivar after phytoplasma infection. Meanwhile, *ZjMKK4* showed a decreased transcript level of more than 2-fold in susceptible cultivar after phytoplasma infection. In contrast, *ZjMKK4* was significantly up-regulated in resistant cultivar.

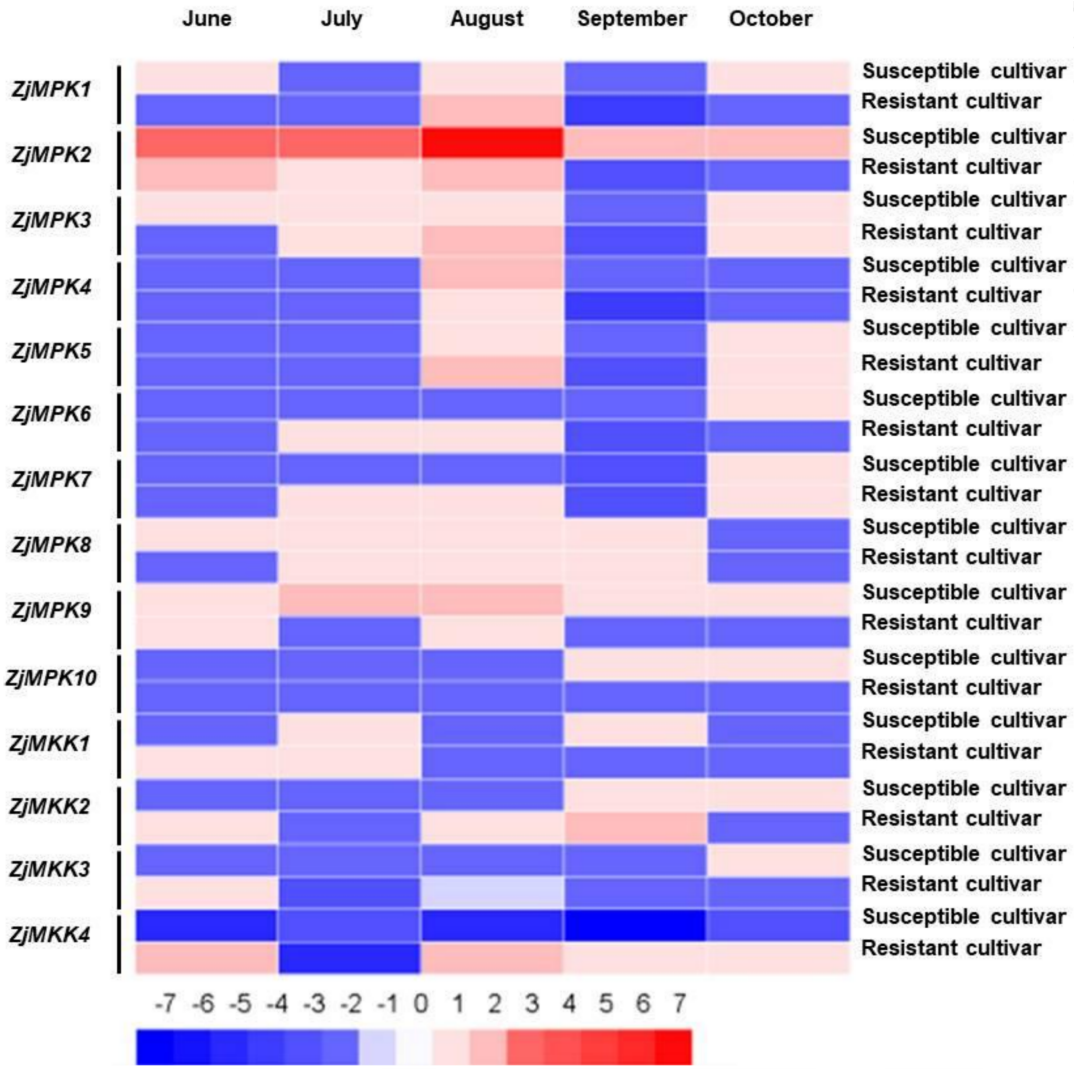

Figure 4. The expression profiles of *ZjMPK* and *ZjMKK* genes in JWB-resistant/susceptible cultivars after phytoplasma infection. The heatmap was generated by Heml 1.0 using expression fold-change. Up-regulated genes were colored in red, down-regulated genes were colored in blue (color code in the bottom).

2.4. Candidate Genes Identification and Protein-protein Interaction Analysis

According to the significant difference times of gene expression in all materials, *ZjMPK2*, *ZjMKK2* and *ZjMKK4* were selected as candidate genes which might play crucial roles in response to phytoplasma infection.

Protein-protein interaction analysis showed that *ZjMPK2* and *ZjMKK2* were involved in the same plant-pathogen interaction pathway (Figure S3), and our present study showed that *ZjMKK2* could interact with *ZjMPK2* by yeast two hybrid analysis (Figure S4). The homologous proteins of *ZjMPK2* and *ZjMKK2* were AtMPK3 and AtMKK6, respectively. AtMPK3 have been demonstrated to be the significant regulators in response to pathogen infections in *Arabidopsis*. ZjMKK4 was homologous with AtMKK10 which involved in the regulation in biological process.

2.5. The Transcriptional Difference of Candidate Genes between Susceptible and Resistant Cultivars

In order to clarify the transcriptional difference of candidate genes between susceptible and resistant cultivars, the expression profiles of *ZjMPK2*, *ZjMKK2* and *ZjMKK4* were analyzed after phytoplasma infection, as it shown in Figure 5. We found that *ZjMPK2* expression level in susceptible

cultivar was generally higher than that in resistant cultivar after phytoplasma infection. However, *ZjMKK2* and *ZjMKK4* transcripts showed a reverse trend compared to *ZjMPK2*, in particular, regarding the expression level of *ZjMKK4*. The relative expression level of *ZjMKK4* in susceptible cultivar was significantly lower than that in resistant cultivar.

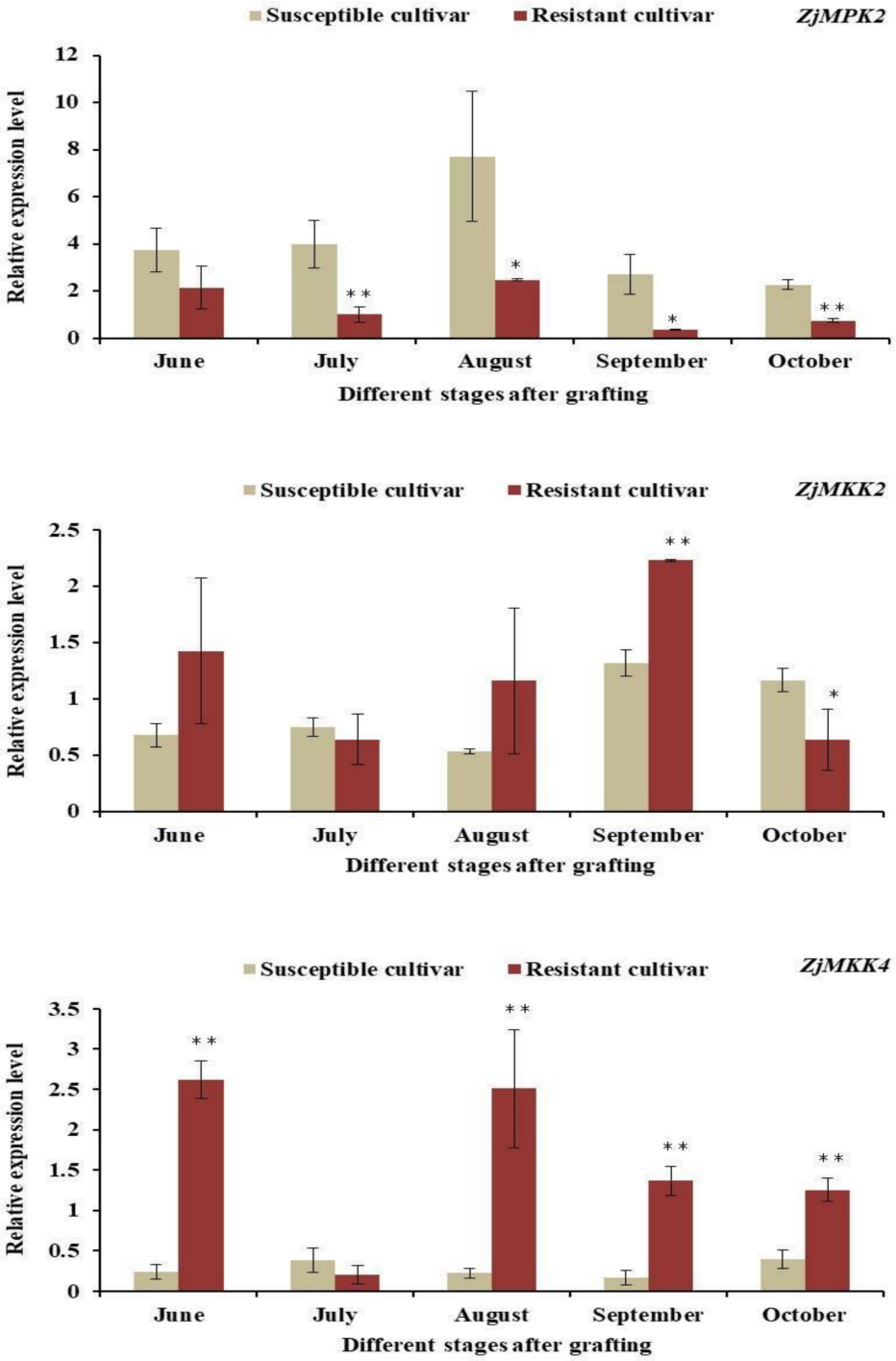

Figure 5. The expression modules of *ZjMPK2*, *ZjMKK2* and *ZjMKK4* genes in JWB-resistant/susceptible cultivars after phytoplasma infection. *ZjACT* was used as the internal standard. The 'Xingguang' and 'Junzao' scions which grafted on healthy rootstocks were used as control. The mean expression value was calculated from 3 independent replicates. The vertical bars indicate the standard deviation. $p < 0.05$ and $p < 0.01$ were considered as significant (shown as *) and highly significant difference (shown as **), respectively.

3. Discussion

As the core pathway of signal transduction networks, MAPK cascades play vital roles in defense response in plants. Previously, we have identified the *ZjMPK* and *ZjMKK* genes family from jujube genomic database, a systematic bioinformatics analysis of all *ZjMPK* and *ZjMKK* genes has been studied, and the expression profiles of all *ZjMPK* and *ZjMKK* genes under biotic and abiotic stresses were also conducted [29]. All of these results demonstrate all *ZjMPK* and *ZjMKK* genes function importantly in the signaling transduction under biotic stress. In this study, the transcription level of *ZjMPK* and *ZjMKK* genes was systematically analyzed under phytoplasma stress. Finally, *ZjMPK2*, *ZjMKK2* and *ZjMKK4* were identified as candidate genes which response to JWB phytoplasma infection.

MAPK regulatory pathway genes play crucial roles in plant developmental processes. For example, *MAPKK*s such as *AtMKK6* in *Arabidopsis* and *NtMEK1* in tobacco were found directly regulating cytokinesis and mitosis [32–35]. Although the biological functions of the *MAPKs* are not fully understood, the *MAPKs* of same sub-groups are likely to be involved in similar physiological responses [19]. Our previous research has proved that *ZjMKK2* was closely related to *AtMKK6*, and they belong to the same sub-group in the phylogenetic tree [29], so *ZjMKK2* might play critical role in the regulation of the physiological processes as *AtMKK6* and *NtMEK1* did. Meanwhile, our previous study also found that the cytokinin content of Chinese jujube significantly increased after phytoplasma infection; it resulted in the symptom of small leaves and witches' broom [10]. All of these bioinformatics analyses, physicochemical indexes and apparent symptoms proved that phytoplasma might regulate the transcription of *ZjMKK2* gene after inoculation, then cytokinin metabolic pathways of host was modulated, and then morphology of Chinese jujube was altered, such as phyllody, small leaves and witches' broom. So, *ZjMKK2* was selected as the key candidate gene which might play a significant role in jujube-phytolasma interaction.

MAPK cascade genes have been shown to play pivotal roles in regulation of plant defense responses. So far, the best characterized MAPK cascade genes are *AtMPK3/6* in *Arabidopsis* and their orthologs in other plant species. In *A. thaliana*, *AtMPK3/AtMPK6* which belong to Group A have been described to be positive regulators of innate immunity [36–38]. In addition, *AtMKK7* (belong to Group D) played a critical role in activation of plant systemic acquired resistance in *A. thaliana* [39]. Our previous study found that *ZjMPK2* was homologous to *AtMPK3*/*AtMPK6*, which belongs to Group A [29]. Furthermore, *ZjMKK4* has close phylogenetic relationship with *AtMKK7*, and they were classified into Group D [29]. It has been indicated that MAPKs proteins classified in the same groups might serve similar functions in different species [40]. Hence, we can deduce the potential functions of *ZjMPK2* and *ZjMKK4* according to the known *AtMPKs* and *AtMKKs* in *Arabidopsis*, *ZjMPK2* and *ZjMKK4* serve as candidate genes might play significant roles in the process of host-phytoplasma interaction. Ye et al. found that four receptor kinase FLS2 genes were down-regulated in phytoplasma-infected jujube, which implied that FLS2/flg22 perception within the pattern-triggered immunity system may exist in the jujube-phytoplasma interaction [30]. In addition, they show the MAPK cascade was activated after phytoplasma infection which could further regulate the induction of WRKY33, this result demonstrated the downstream regulation of MAPK cascade could be WRKY transcription factors. Moreover, MAPK cascade signaling networks were induced by FLS2/flg22 perception in the plant defense to bacterial pathogens [41] and we have demonstrated the protein of *ZjMKK2* could interact with *ZjMPK2* with two hybrid yeast analysis (Figure S4). Therefore, based on our results and the study by Ye et al. [30], we deduce the following hypothesis of Chinese jujube response to JWB phytoplasma infection (Figure 6). Firstly, the phytoplasma infection could be perceived by FLS2 like receptor, then with the unknown signaling transduction, the *ZjMKK2* and *ZjMKK4* were induced in different expression pattern between susceptible and resistant cultivars of Chinese jujube, furthermore the *ZjMPK2* was activated by *ZjMKK2* or ZjMKK4 which could further activate WRKY transcription factor. However, there were maybe different MAPK cascade pathways and regulation modules in susceptible and resistant cultivars of Chinese jujube. *ZjMKK2* and *ZjMKK4* transcription levels decreased significantly in susceptible cultivar after phytoplasam infection, while

their expressions increased strictly in resistant cultivar and in recovered plantlets. The results indicated that phytoplasma infection might activate two ZjMKKs (*ZjMKK2* and *ZjMKK4*), but their functions depended on which downstream MAPK it associates with [30,42]. Subsequently, as the sole candidate MAPK gene, *ZjMPK2* expression level significantly increased after phytoplasma infection in most materials. But the transcript abundance of *ZjMPK2* in resistant cultivar was down-regulated and was significantly lower than in susceptible cultivar. Meanwhile, *ZjMPK2* expression was also significantly down-regulated in recovered plantlets compared to diseased plantlets. These perhaps could serve as evidence for that lower expression level of *ZjMPK2* might play vital role in JWB defense response. These differential expression regulations of *ZjMPK2*, *ZjMKK2* and *ZjMKK4* genes between susceptible and resistant cultivars may be a resistant mechanism in which plant-phytoplasma interaction are fine-tuned, although additional work is needed to confirm this hypothesis.

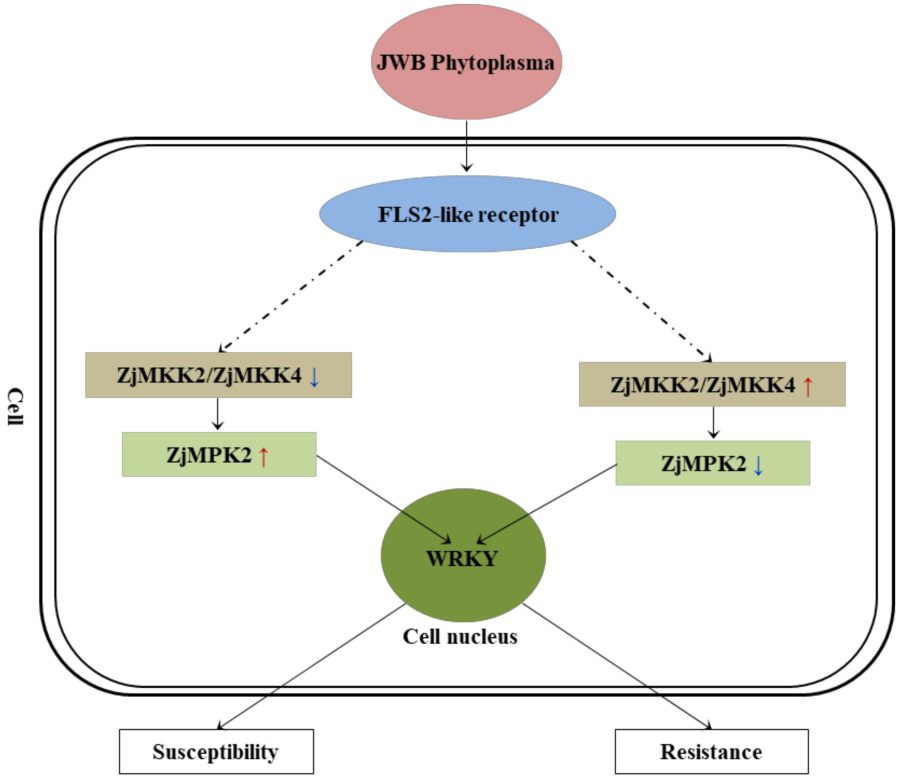

Figure 6. Possible MAPK cascades pathway of Chinese jujube under JWB phytoplasma infection. Different color arrows indicate up-regulation (Red) and down-regulation (Blue).

Because MAPK cascade kinases are post-translationally regulated by phosphorylation, and their functions depend on the amplitude and kinetics of activation, the loss of a functional gene product might not reveal the exact function of a MAPK cascade. So, a combination of biochemical and genetic studies, including both loss-of-function and gain-of-function approaches, will be required to understand the complex roles of MAPK cascade kinases in plant defense responses.

4. Conclusions

Altogether, our study presents the first gene-family-wide investigation on the systematical expression analysis of *MAPK* and *MAPKK* genes in Chinese jujube under phytoplasma infection. Most *ZjMPK* and *ZjMKK* genes were responsive to JWB phytoplasma infection. And, *ZjMPK2*, *ZjMKK2* and *ZjMKK4* genes were selected as important candidate genes for further functional studies.

5. Materials and Methods

5.1. Diseased, Recovered and Healthy Plantlets

The cultivar for this experiment was *Z. jujuba* Mill. 'Goutouzao'. The JWB diseased plantlets (the healthy plantlets were infected by phytoplasma through vitro micrografting [43]) and the recovered plantlets (the JWB diseased plantlets were cultured in antibiotic medium with 25 mg·L^{-1} tetracycline) were used as test groups, the healthy plantlets were used as control (Figure S1). The JWB phytoplasma concentration in the diseased plantlets, recovered plantlets and healthy plantlets was detected by DAPI staining [44] at histological level (Figure S2). Every ten plantlets were pooled as one sample, three independent biological replications were sampled separately, then immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

5.2. The Tissues Showing Different JWB Disease Symptoms

The cultivar for this experiment was *Z. jujuba* Mill. 'Dongzao'. Small leaves (shoot with witches-broom), phyllody (floral organs becoming leaf-like) and non-symptomatic leaves were used as test group which selected from the same branch of diseased tree, and the normal leaves from healthy trees were used as control, as shown in Figure S5. The detection of JWB phytoplasmas in the infected materials in June was conducted by DAPI staining at histological level and quantitative realtime PCR analysis (qRT-PCR) at the molecular level from June to October [44]. All samples were collected at five stages (on the 15th day of each month from June to October). Each material was sampled with three replicates from independent trees. All samples were rapidly frozen in liquid nitrogen and kept at −80 °C for RNA isolation.

5.3. Resistant and Susceptible Cultivars

A JWB-resistant cultivar (*Z. jujuba* Mill. 'Xingguang') and a JWB-susceptible cultivar (*Z. jujuba* Mill. 'Junzao') were used as scions for top grafting (as shown in Figure S6). The rootstock was JWB-susceptible cultivar *Z. jujuba* Mill. 'Dongzao'. The 'Xingguang' and 'Junzao' scions were grafted on rootstocks with JWB (Test group) and healthy ones (Control group), respectively. Three replicates were conducted of each grafting treatment. All the experimental trees were cultivated in the natural environmental conditions. The mature leaves in the middle of bearing shoot from sprouted scions were collected at five stages (on the 15th day of each month from June to October). The sampled leaves were frozen by liquid nitrogen immediately and stored at −80 °C until RNA extraction.

'Xingguang' (resistant cultivar) and 'Junzao' (susceptible cultivar) sprouted around 25 days after grafting on rootstocks (May 15th). The resistant cultivar 'Xingguang' only displayed slight symptoms at the initial stage after grafting (60 days, June 20th) and then reverted to normal growth. In infected 'Junzao', the visual JWB symptoms, such as elongated pedicel and virescent flowers, were firstly detected at 45 days after grafting (June 5th), and then small leaves and witches' broom were observed at 60 days after grafting inoculation (June 20th). The JWB phytoplasma presence of the samples was detected by quantitative real-time PCR (qRT-PCR) [44]. The expression of phytoplasma *TMK* gene in jujube samples was analyzed and *ZjACT* was used as an internal control. The detection of the phytoplasma in the two cultivars was shown in Figure S7.

5.4. Total RNA Extraction

Total RNA of the samples was isolated according to the manufacturer's instructions of TIANGEN RNA Extraction Kit. DNaseI treatment was used to remove contaminating genomic DNA. TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Dalian, China) was applied in the synthesis of double-stranded cDNA according to the instructions.

5.5. Quantitative Real-Time PCR System

The Quantitative Real-time PCR (qRT-PCR) was carried out on the Bio-Rad iQ™ 5 using TransStart Top Green qPCR SuperMix AQ131 (TransGen Biotech, Beijing, China). The 20 μL reaction system contained 10 μL of 2×SYBR Premix ExTaq™ (TransGen Biotech, Beijing, China), 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.

5.6. Expression Analysis

The expression profile analysis of the target genes was carried out by qRT-PCR, with *ZjActin* as the internal control [45]. Primer sequences for qRT-PCR analysis were shown in our previous study [29]. The relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method [46]. The gene's relative expression change more than two-fold was noted as significant difference and the gene was considered as differentially expressed genes (DEGs) [30].

5.7. Heatmap Construction

The expression profiles of all *ZjMPK* and *ZjMKK* genes in different samples were illustrated by a color gradient heatmap. The heatmap was constructed by heatmap software Heml 1.0 using expression fold-changes.

5.8. Protein-Protein Interaction Analysis

To determine the interaction network of candidate genes, STRING database [47] was used to predict protein-protein interactions. Firstly, the nucleotide sequences of candidate genes were translated into amino acid sequences by Primer Premier 5. Secondly, *Arabidopsis thaliana* was selected as reference template. Lastly, the amino acid sequences of candidate genes were used as query sequences to search interaction pathway in STRING database.

5.9. Yeast Two-Hybrid Screening (Y2H)

ZjMKK2 fused to the GAL4 activation domain (AD) was expressed in combination with *ZjMPK2* fused to the GAL4 DNA-binding domain (BD) in yeast strain AH109. Then the yeast cells were spotted on selective medium lacking leucine/tryptophan (-LW), lacking leucine/tryptophan/histidine (-LWH) and lacking tryptophan/leucine/adenine/histidine (-LWAH), respectively. The BD-fused *ZjMPK2* was co-expressed with empty AD as the negative control. The plates were incubated for 3 days at 30 °C.

5.10. Statistical Analysis

Data were shown as the means ± standard deviation (SD) of independent experiments. Statistical analysis was performed by SPSS 16.0 software (IBM, Armonk, NY, USA) using Independent-Samples T-test and One-Way ANOVA method.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/5/392/s1, Figure S1: The healthy, diseased, and recovered plantlets. A: Healthy plantlets; B: Diseased plantlets; C: Recovered plantlets. Bar = 2 cm. Figure S2: Phytoplasma determination 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 SE of the diseased plantlets. C, No fluorescent spots in the SE of recovered plantlets. The number and size of fluorescent spots represent the number of phytoplasma. Bar = 100 μm. Figure S3: The protein-protein interaction analysis of *ZjMPK2*, *ZjMKK2* and ZjMKK4 by STRING database. Figure S4: Interaction between *ZjMPK2* and ZjMPKK2 with yeast two-hybrid analysis. *ZjMKK2* fused to the GAL4 activation domain (AD) was expressed in combination with *ZjMPK2* fused to the GAL4 DNA-binding domain (BD) in yeast strain AH109. Then the yeast cells were spotted on selective medium lacking leucine/tryptophan (-LW), lacking leucine/tryptophan/histidine (-LWH) and lacking tryptophan/leucine/adenine/histidine (-LWAH), respectively. The BD-fused *ZjMPK2* was co-expressed with empty AD as the negative control. Figure S5: The tissues showing different JWB disease symptoms. A: Small leaves; B: Phyllody; C: Non-symptomatic leaves; D: Healthy leaves. Bar = 2 cm. 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. Figure S6:

The resistant and susceptible cultivars were grafted on JWB diseased rootstocks. The JWB symptoms of small leaves, phyllody and witches'-broom in susceptible cultivar were observed, but the resistant cultivar scions only showed symptom slightly at initial stage after grafting inoculation and then reversed to normal growth. Figure S7: Expression analysis of phytoplasma *TMK* in susceptible and resistant varieties which were grafted on JWB diseased rootstocks. Table S1: The number of *ZjMPK* and *ZjMKK* genes with significant difference expression in JWB-resistant/susceptible cultivars at five stages after phytoplasma infection.

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