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Genome-wide analysis of the WRKY gene family and their positive responses to phytoplasma invasion in Chinese jujube

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Abstract

Background: The WRKY gene family is one of the most important families in higher plants. As transcription factors, they actively respond to biotic and abiotic stress and are also involved in plant development. Chinese jujube (*Ziziphus jujuba* Mill.) is the largest type of dried fruit tree in China in terms of production, but its production is largely limited by phytoplasma infection, and the information about the role of WRKY genes under phytoplasma stress was still limited.

Results: We identified 54 *ZjWRKYs* in the jujube genome and classified them into three subgroups according to conserved WRKY domains and zinc-finger structure. 41 *ZjWRKYs* were distributed on 11 of 12 pseudo chromosomes in Chinese jujube. The majority of *ZjWRKYs* were highly expressed in the seven examined tissues, indicating that they play multiple roles in these vegetative and reproductive organs. Transcriptome data showed that most of the characterised *ZjWRKYs* were highly expressed at later stages of fruit development. RT-qPCR demonstrated that the expression of 23 *ZjWRKYs* changed following phytoplasma infection, suggesting that they are involved in signalling pathways that respond to phytoplasma stress. Then, STRING analysis and yeast two-hybrid screening proved that some *ZjWRKY* proteins were interacting with *ZjMAPKK* proteins, which were also involved in phytoplasma invasion. Moreover, their differential expressions were further confirmed in resistant and susceptible jujube varieties under phytoplasma stress. These results suggest that *ZjWRKYs* play significant roles in phytoplasma tolerance and should be crucial candidate genes for jujube-phytoplasma interaction.

Conclusions: 54 *ZjWRKYs* in Chinese jujube were identified and classified into three subgroups. 41 *ZjWRKYs* were unevenly distributed along the chromosomes. The majority of *ZjWRKYs* were highly expressed in various tissues. Most of the *ZjWRKYs* were positive responses to phytoplasma invasion, and that provided candidate genes for the future studies of jujube-phytoplasma interaction.

Keywords: WRKYs, Chinese jujube, Bioinformatics analysis, Gene expression, Phytoplasma invasion

Background

The WRKY transcription factors (TFs) bind to a specific promoter sequence in the target gene, known as a W-box, and can positively or negatively regulate target gene expression. The WRKY proteins have one or two DNA binding domains that are 60 amino acids long and contain

the conserved heptapeptide WRKYGQK followed by a zinc-finger motif C₂H₂ (CX₄₋₅CX₂₂₋₂₃HXH) or C₂HC (CX₇CX₂₃₋₂₄HXC) [1]. The WRKY family contains important transcription factors that have multiple functions in processes such as embryogenesis [2], trichome and seed development [3], leaf senescence [4], flowering [5], fruit and pollen development [6], biomass accumulation [7], secondary metabolite biosynthesis [8] and hormone signalling [9]. WRKY transcription factors are also crucial regulatory components of plant responses to pathogen infection. In Arabidopsis, several WRKY genes have been

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experimentally characterised and associated with responses to fungal or bacterial pathogens [10–12]. AtWRKY70 is required for R gene-mediated pathogen resistance, determining the balance between the SA- and JA-dependent defence systems [13, 14]. Many WRKY genes also act in defence signalling; for example, AtWRKY38 and AtWRKY62 act as negative regulators of basal resistance towards bacterial pathogens [15]. In rice, overexpression of OsWRKY30 enhanced resistance to the rice sheath blight fungus *Rhizoctonia solani* and the blast fungus *Magnaporthe grisea* [16, 17]. Owing to their important roles, the WRKY family has been widely studied in many plant species, such as Arabidopsis, rice, grape, apple, pear, and peach [18–22]. However, the information of this gene family in Chinese jujube and their roles under phytoplasma stress was still limited.

Chinese jujube is the largest type of dried fruit tree in China in terms of production [23] and the most important species of family Rhamnaceae. It is cultivated mainly for its fruits, which can be eaten fresh or dried or as raw materials for making Chinese herbal medicine. However, jujube production is threatened by several devastating diseases, such as jujube witches' broom disease (JWB). The genome of Chinese jujube was recently published [24, 25], paving the way for further investigations. Our transcriptome data indicated that some WRKY genes respond to JWB phytoplasma infection. Since the WRKY family plays a crucial role in biotic stress response, identifying WRKY genes in Chinese jujube and determining their possible functions in response to phytoplasma stress have important significance.

Here, we report on the genome-wide analysis of the WRKY family in Chinese jujube. A non-redundant set of WRKY genes was identified in this species. Subsequently, chromosomal location was determined, phylogenetic and motif analyses were also performed as a base for further comparative genomics studies. Moreover, expression patterns of ZjWRKYs in various tissues and under phytoplasma stress were also investigated. The interacting proteins of ZjWRKYs were also screened. The ZjWRKYs involved in phytoplasma invasion were considered good candidates for subsequent studies of the jujube-phytoplasma interaction.

Results

Identification of ZjWRKYs in Chinese jujube

A total of 54 non-redundant putative WRKY coding sequences (Table 1) were identified in the jujube genome sequence. The sequences were named from ZjWRKY1 to ZjWRKY54 according to their gene structure and motifs. The ORF length for ZjWRKY genes ranged from 522 bp (ZjWRKY26) to 2205 bp (ZjWRKY8), and they encoded proteins ranging from 173 to 734 amino acids (aa) in length, with predicted pIs ranging from 4.65 (ZjWRKY32) to 9.09 (ZjWRKY1) (Table 1).

Previous genome evolution studies showed that Chinese jujube is closely related to species of the family Rosaceae [24, 26], so the WRKY genes of three Rosaceae species (apple, pear and peach) and Arabidopsis were compared with that of Chinese jujube (Additional file 1). Compared with Arabidopsis, apple and pear [18, 19, 21], there are fewer WRKY genes in jujube, but the number was similar to that of peach [22]. The smaller number of WRKY genes in Chinese jujube and peach may be due to the occurrence of only one genome duplication event during the evolution of the two species [24, 27]. Based on the above comparison, it was suggested that most of the expected WRKY genes in jujube were identified.

Conserved motifs and phylogenetic tree construction of ZjWRKYs

The phylogenetic tree of the ZjWRKY proteins was constructed by aligning multiple domain sequences (Fig. 1). The ZjWRKY proteins were classified into three groups (Group I, II and III) (Table 1) according to their WRKY and zinc-finger motifs. The domain sequences in the ZjWRKY gene family were highly conserved. There were 8 motifs among ZjWRKYs and proteins in the same group had similar numbers and types of motifs (Fig. 2, Additional file 2). The WRKY domain (WRKYGQK, Motif 1) was highly conserved among the 54 proteins (Additional file 2) and only two of them contained variations. The group II proteins ZjWRKY25 and ZjWRKY26 showed a WRKY motif with one amino acid modifications (WRKYGKK) (Table 1, Fig. 2). Motif 2 was also highly conserved except in the two Group III proteins ZjWRKY52 and ZjWRKY53. Motif 5 and Motif 8 were specific to groups I and III respectively.

Group I had 8 proteins (Table 1), that contained two WRKY motifs, and two C₂H₂ zinc-finger motifs. Group II was the biggest group and included 34 proteins that contained a WRKY motif and a C₂H₂ zinc-finger motif. According to the phylogenetic analysis, the 34 genes could be further divided into five subgroups (IIa to e) that included 3, 10, 11, 3 and 7 genes, respectively (Table 1). The members of subgroups IIa, IIb, IIc and IIe had a CX₅CX₂₃HX₁H zinc-finger motif, while that of subgroup IIc had a CX₄CX₂₃HX₁H structure (Table 1). Group III contained 12 proteins, and they had one WRKY motif and a C₂HC zinc-finger motif (CX₇CX₂₃HX₁C, Table 1).

The chromosomal location and gene structure of ZjWRKYs

Of the 54 ZjWRKY genes, 41 were mapped to 11 of 12 pseudo chromosomes in the jujube genome (Fig. 3), and 13 genes were located on 12 scaffolds (Table 1, Additional file 3). ZjWRKYs were not evenly distributed across the 11 pseudo chromosomes (Fig. 3). Ten ZjWRKYs (18.5%) were located on Chr. 11, whereas only one ZjWRKY gene was on Chr. 5 and 8 each. No

Table 1 The information of WRKY gene family in Chinese jujube

Gene Name	NCBI Reference	ORF (bp)	Size (aa)	MW(D)	PI	Conserved motif	Domain pattern	Zinc finger	Group	Exon number
ZjWRKY1	XM_016044069.1	1434	477	52053.75	9.09	2 × [WRKYGQK]	C-X ₄ -C-X ₂₂₋₂₃ -HXH	C ₂ H ₂	I	5
ZjWRKY2	XM_016042179.1	1629	542	60186.57	7.09	2 × [WRKYGQK]	C-X ₄ -C-X ₂₂₋₂₃ -HXH	C ₂ H ₂	I	5
ZjWRKY3	XM_016047139.1	1904	583	63795.60	6	2 × [WRKYGQK]	C-X ₄ -C-X ₂₂₋₂₃ -HXH	C ₂ H ₂	I	6
ZjWRKY4	XM_016025559.1	1506	501	55188.92	6.52	2 × [WRKYGQK]	C-X ₄ -C-X ₂₂₋₂₃ -HXH	C ₂ H ₂	I	4
ZjWRKY5	XM_016037165.1	1566	521	57263.14	5.12	2 × [WRKYGQK]	C-X ₄ -C-X ₂₂₋₂₃ -HXH	C ₂ H ₂	I	5
ZjWRKY6	XM_016019228.1	1077	358	39860.32	8.82	2 × [WRKYGQK]	C-X ₄ -C-X ₂₂₋₂₃ -HXH	C ₂ H ₂	I	4
ZjWRKY7	XM_016024358.1	1629	542	59088.72	8.91	2 × [WRKYGQK]	C-X ₄ -C-X ₂₂₋₂₃ -HXH	C ₂ H ₂	I	5
ZjWRKY8	XM_016020284.1	2205	734	80415.39	5.9	2 × [WRKYGQK]	C-X ₄ -C-X ₂₂₋₂₃ -HXH	C ₂ H ₂	I	1
ZjWRKY9	XM_016029235.1	951	316	34758.07	8.44	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ila	4
ZjWRKY10	XM_016014490.1	951	316	35076.43	8.68	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ila	5
ZjWRKY11	XM_016028547.1	801	266	29844.33	8.99	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ila	4
ZjWRKY12	XM_016022282.1	1863	620	67027.91	6.26	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	5
ZjWRKY13	XM_016029844.1	1902	633	67897.98	6.12	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	6
ZjWRKY14	XM_016043879.1	1611	536	58692.78	6.48	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	6
ZjWRKY15	XM_016036346.1	1125	374	40424.14	8.09	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	3
ZjWRKY16	XM_016036345.1	1659	552	60686	7.71	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	7
ZjWRKY17	XM_016037515.1	1512	503	56033.17	5.48	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	5
ZjWRKY18	XM_016039435.1	1251	416	44528.06	9.13	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	3
ZjWRKY19	XM_016014870.1	1878	625	67519.49	7.97	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	5
ZjWRKY20	XM_016014977.1	1764	587	63426.84	8.8	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	4
ZjWRKY21	XM_016014513.1	1878	625	67533.52	7.97	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ilb	5
ZjWRKY22	XM_016038792.1	579	192	21894.40	9.43	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	2
ZjWRKY23	XM_016040974.1	636	211	24030.21	8.47	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	3
ZjWRKY24	XM_016026686.1	597	198	22899.66	9.23	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	2
ZjWRKY25	XM_016014637.1	588	195	21527.72	6.73	WRKYGKK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	3
ZjWRKY26	XM_016028953.1	522	173	19750.96	5.59	WRKYGKK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	3
ZjWRKY27	XM_016011550.1	1008	335	37240.76	6.34	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	4
ZjWRKY28	XM_016024581.1	1038	345	39158.92	6.76	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	3
ZjWRKY29	XM_016039492.1	933	310	34331.69	5.65	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	3
ZjWRKY30	XM_016011683.1	1110	369	40614.00	5.16	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	3
ZjWRKY31	XM_016036211.1	1008	335	37135.37	6.43	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	3
ZjWRKY32	XM_016041473.1	756	251	28026.78	4.65	WRKYGQK	C-X ₄ -C-X ₂₃ -HXH	C ₂ H ₂	Ilc	2
ZjWRKY33	XM_016028867.1	1116	371	40189.57	9.57	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ild	3
ZjWRKY34	XM_016011768.1	1101	366	39383.33	9.64	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ild	3
ZjWRKY35	XM_016036017.1	1083	360	40498.69	9.65	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ild	4
ZjWRKY36	XM_016045200.1	1497	498	53218.21	5.81	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ile	3
ZjWRKY37	XM_016020139.1	858	285	31393.44	5.62	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ile	3
ZjWRKY38	XM_016025812.1	849	282	30475.83	5.46	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ile	3
ZjWRKY39	XM_016022820.1	1422	473	51502.28	5.19	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ile	3
ZjWRKY40	XM_016044078.1	969	322	36328.33	8.98	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ile	4
ZjWRKY41	XM_016044080.1	1068	355	38920.61	5.92	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ile	3
ZjWRKY42	XM_016036213.1	870	289	32200.49	5.27	WRKYGQK	C-X ₅ -C-X ₂₃ -HXH	C ₂ H ₂	Ile	3
ZjWRKY43	XM_016013400.1	1215	404	45202.30	6.64	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	4
ZjWRKY44	XM_016040779.1	1113	370	41825.52	5.26	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3

Table 1 The information of WRKY gene family in Chinese jujube (Continued)

Gene Name	NCBI Reference	ORF (bp)	Size (aa)	MW(D)	PI	Conserved motif	Domain pattern	Zinc finger	Group	Exon number
ZjWRKY45	XM_016022705.1	1176	391	43607.65	5.9	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3
ZjWRKY46	XM_016033893.1	1065	354	40462.32	5.16	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	4
ZjWRKY47	XM_016041850.1	960	319	36005.79	5.25	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3
ZjWRKY48	XM_016013401.1	1035	344	38711.93	5.39	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3
ZjWRKY49	XM_016041861.1	981	326	37477.17	8.15	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3
ZjWRKY50	XM_016041806.1	930	309	35609.87	5.91	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3
ZjWRKY51	XM_016047371.1	924	307	35268.77	6.71	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3
ZjWRKY52	XM_016041801.1	948	315	35731.33	6.46	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3
ZjWRKY53	XM_016041802.1	972	323	37104.55	8.78	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3
ZjWRKY54	XM_016041803.1	951	316	36421.82	5.76	WRKYGQK	C-X ₇ -C-X ₂₃ -HXC	C ₂ HC	III	3

ZjWRKY gene was found on Chr. 7. Additionally, the gene structure was highly conserved within each group, especially in groups II d, II e, and III. We found that Group I genes contained more introns and were more complicated than genes in the other two groups (Fig. 4). Tandem duplications were present in 40.7% of ZjWRKY genes (ZjWRKY1, 15, 16, 18, 26, 29, 31, 33, 35, 39, 40, 41, 42, 45, 47, 49, 50, 52, 53, and 54), which contributed to the expansion of the ZjWRKY gene family. This dynamic was particularly evident in Group III in which 8 out of the 12 genes (66.7%) mapped to duplicated chromosome or scaffold regions.

Expression profiles of ZjWRKYs in various tissues/organs

To investigate the tissue-specific expression of the jujube WRKY genes, RT-PCR was used to determine their expression patterns in seven tissues. The expression patterns of 26 ZjWRKY genes were analysed and are shown in Fig. 5a. Of the 26 ZjWRKY genes, six genes were actively expressed in at least five tissues, including ZjWRKY6, 9, 10, 13, 29, and 42. A total of 8 ZjWRKY genes (ZjWRKY2, 22, 25, 33, 36, 44, 45 and 48) were found to be upregulated in only one or two tissues, indicative of the tissue-specific expression of these genes. ZjWRKY24 expression could only be detected in roots and old branches. The expression of the remaining genes was comparatively low in the different organs, suggesting that genes in the same group might have different functions. These results showed that most of the ZjWRKY genes had diverse tissue-specific expression patterns, indicating that ZjWRKYs play multiple roles in various organs.

Moreover, a heat map of our RNA-Seq data highlighted differential expression of ZjWRKYs during jujube fruit development (Fig. 5b), and most of the genes were expressed at different levels. The genes of group II e were mainly expressed at before white mature period (BWM) and white mature period (WM), and the expression of group II b genes was lower at young fruit period (Y) except for

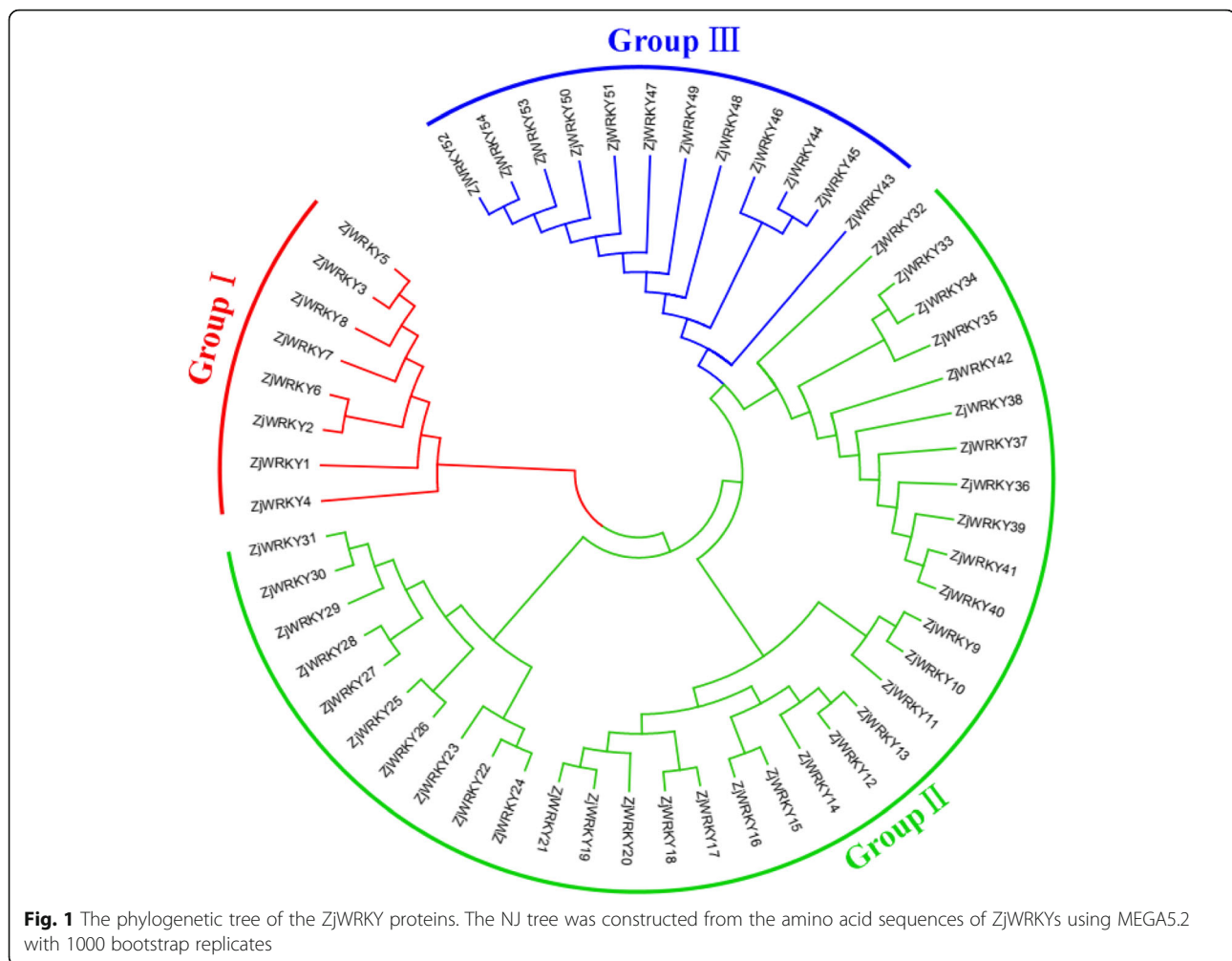
ZjWRKY13. ZjWRKY8, 26, 47, and 48 were only involved in the development of young fruit, suggesting the role for these WRKY genes in jujube fruit development.

ZjWRKYs involved in the jujube-phytoplasma interaction

The expression of the phytoplasma *TMK* gene was not detected in the healthy leaves, however its highly expression was found in other diseased tissues, including the apparently normal leaves (Additional file 4). Among the 30 ZjWRKY genes detected, 17 ZjWRKYs (ZjWRKY2, 3, 6, 9, 10, 15, 18, 22, 24, 26, 34, 36, 37, 38, 42, 44, and 45) were significantly upregulated under phytoplasma stress, while 5 ZjWRKYs (ZjWRKY5, 8, 33, 47, and 49) were downregulated (Fig. 6a). The expression of ZjWRKY32 first increased and then decreased in diseased jujube. Most Group II genes were upregulated under phytoplasma stress. ZjWRKY37, ZjWRKY38, and ZjWRKY44 were significantly upregulated in phyllody leaves. ZjWRKY5 and ZjWRKY49 were significantly downregulated in diseased leaves. These ZjWRKY genes displayed noticeable changes in expression and should play vital roles in jujube-phytoplasma interactions.

STRING analysis displayed that WRKY proteins could function by interacting with each other, as well as with MPK3 (Additional file 7A). Furtherly, yeast two-hybrid screening proved that ZjWRKY9 and ZjWRKY37 were interacting with ZjMAPKK6 (Fig. 6b), and ZjWRKY26 was interacting with ZjMAPKK2 (Fig. 6c). In previous study, it was found that ZjMAPKs and ZjMAPKKs were also involved in phytoplasma infection [28].

To confirm the identities of these ZjWRKY genes for phytoplasma tolerance, we analysed the transcript profiles of ZjWRKY genes in a JWB-resistant variety and a susceptible variety (Fig. 7). The detection of the phytoplasma in the two varieties was shown in Additional file 4. After phytoplasma infection, the expression of 9 ZjWRKYs (ZjWRKY2, 9, 22, 24, 29, 34, 36, 42, and 45) in the susceptible variety were higher than in the resistant variety, and they were also upregulated in above diseased tissues (Fig. 6). In contrast,



the expression of *ZjWRKY5* and *ZjWRKY49* in the resistant variety was higher than in the susceptible one during the early stages of infection. These two genes were significantly downregulated in diseased tissues. *ZjWRKY4* expression in the resistant variety was also higher than in the susceptible one. The above results indicated that some ZjWRKYs might play a role in phytoplasma tolerance.

Discussion

In this study, a total of 54 WRKY-encoding genes were identified in the jujube genome. These genes can be divided into three groups (Group I to III), but this number may increase in the future once problems with the assembly and annotation of the jujube genome are addressed. As in other plants, almost all of the *ZjWRKY* genes share the WRKYGQK signature motif. However, the WRKYGKK variant was found in two jujube genes (Table 1, Fig. 2). Such slight variations in this region have also been reported in other plants such as Arabidopsis and apple [29].

Gene duplication events are the biggest contributors to the rapid expansion and evolution of gene families. Previous research has demonstrated that the Arabidopsis Group III WRKY gene family expanded rapidly as a result of recent segmental and tandem duplication events [30], and we found that this was also the case in the jujube genome. There are 6 tandemly duplicated *ZjWRKY* genes (*ZjWRKY47*, *ZjWRKY49*, *ZjWRKY50*, *ZjWRKY52*, *ZjWRKY53*, and *ZjWRKY54*) in Group III. The phylogenetic analysis (Additional file 5) indicated that 6 Group III *ZjWRKYs* were grouped and then clustered with 6 other genes from Arabidopsis; this also occurred in other subgroups from apple and pear. This finding suggests that the duplications in Group III WRKY genes and tandem duplication events are the main contributors to the expansion of the Group III genes.

Previous research has demonstrated that Group I WRKY genes are the ancestors of the other WRKY genes in plants and are more likely to be constitutively expressed in different tissues [30]. In our study, the

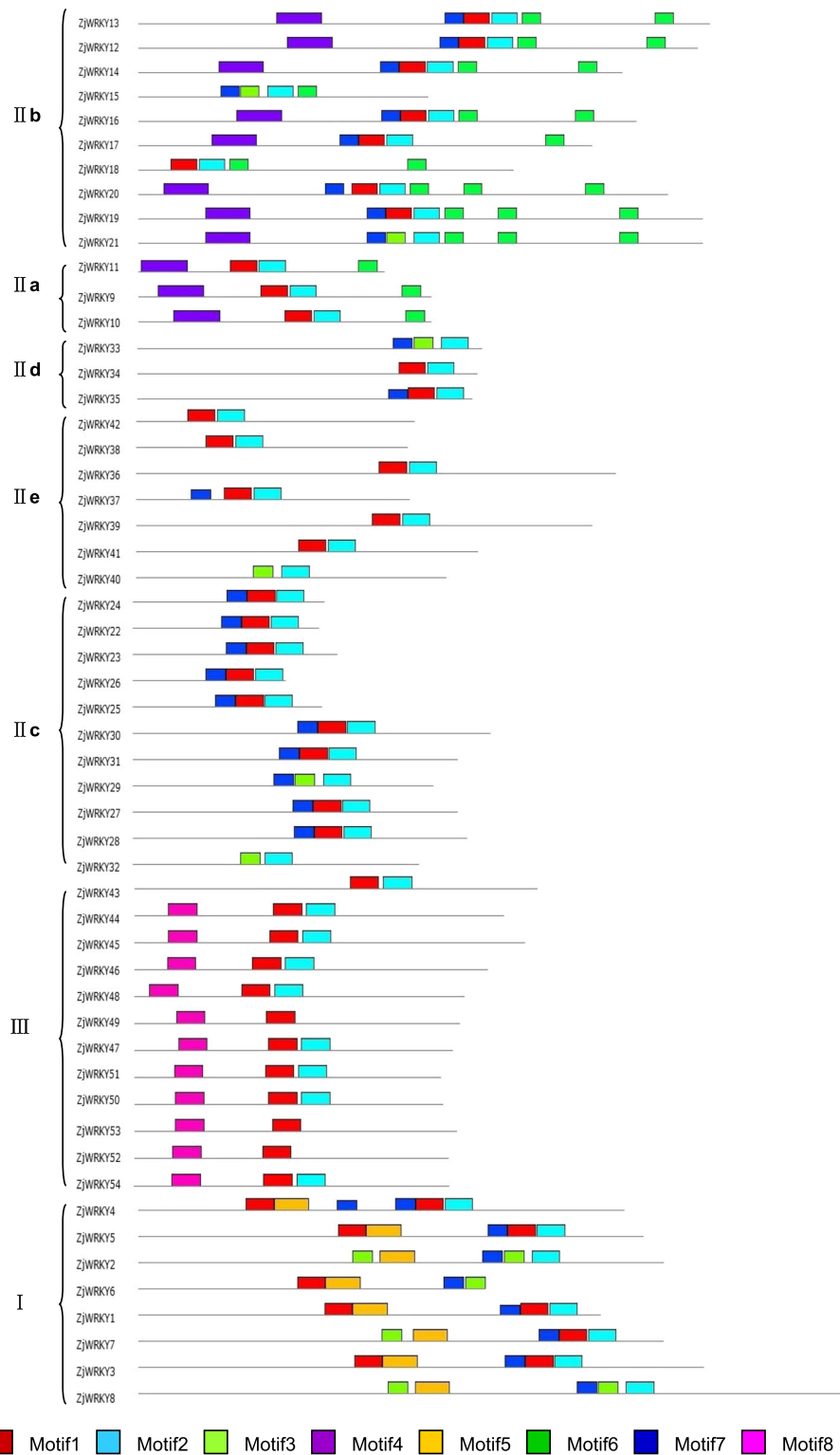
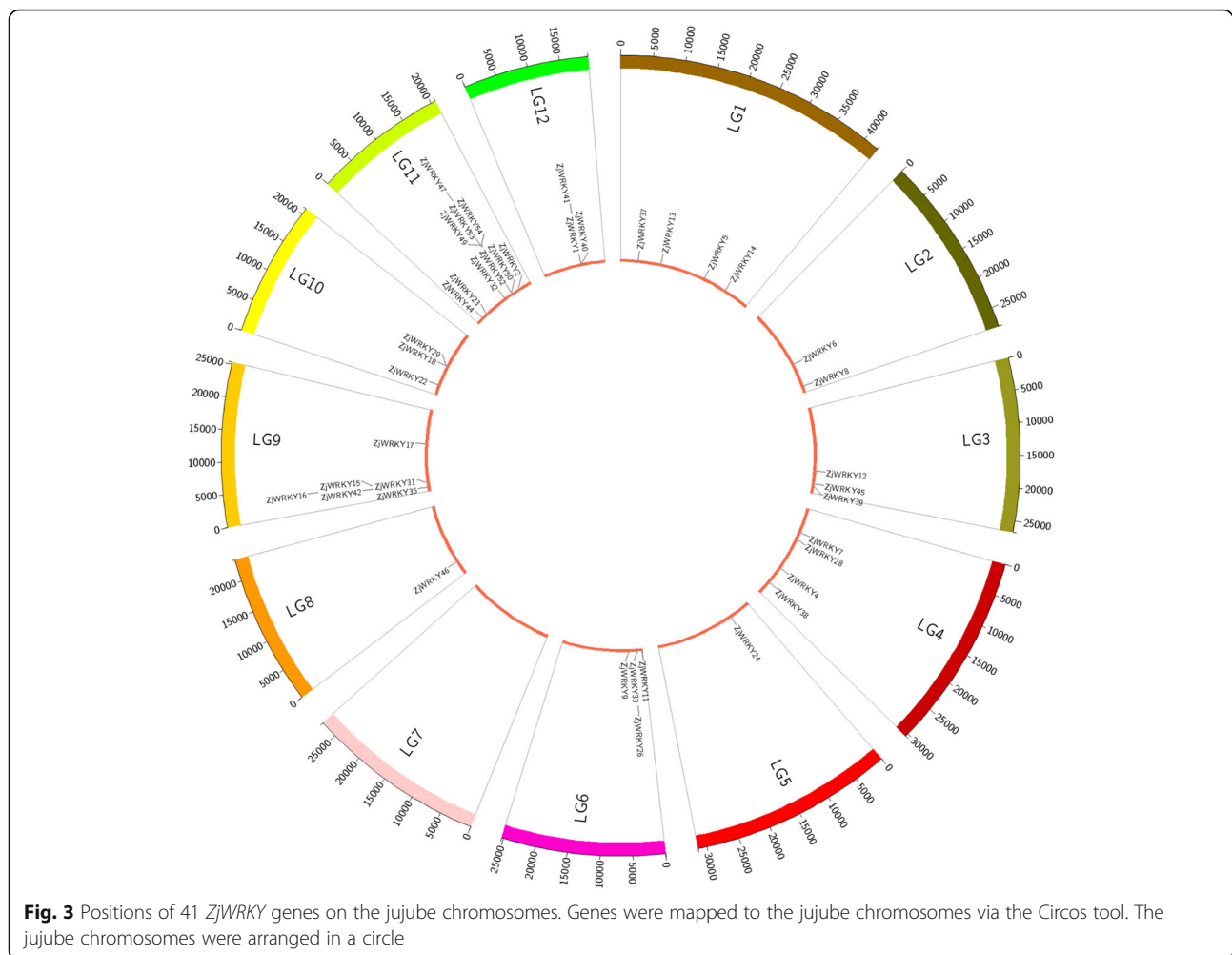


Fig. 2 Conserved motifs of the ZjWRKY proteins arranged according to their phylogenetic relationships. The motifs in the ZjWRKYs were identified using Multiple Em for Motif Elicitation (MEME). In ZjWRKY proteins, 8 conserved motifs were identified and shown in different colors

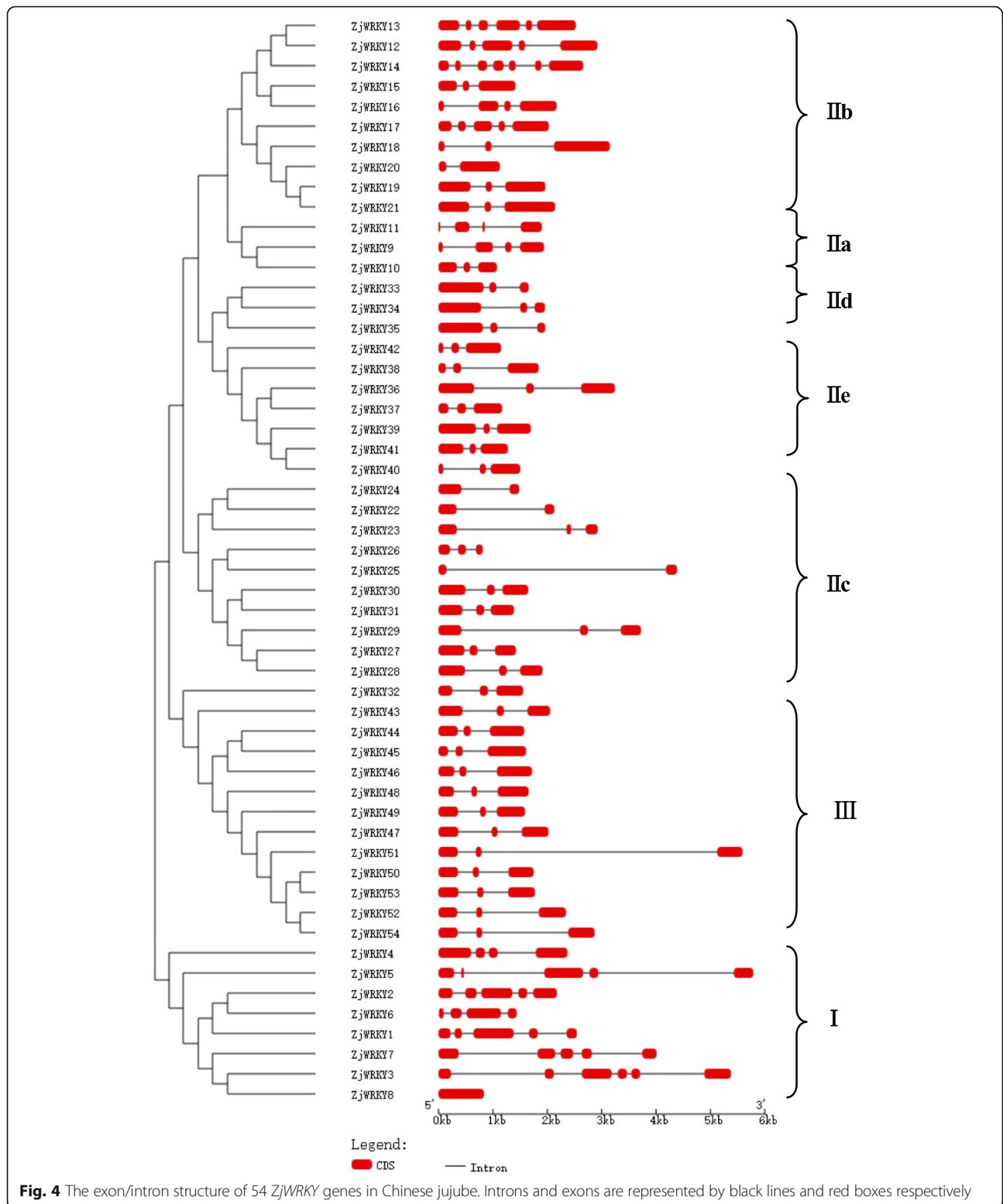


Group I genes and many genes from the other two groups were expressed in various tissues (Fig. 5), indicative of their diverse functions. These results provide some useful clues for additional investigations into the biological functions of these *WRKY* genes in jujube growth and development.

Transgenic apple lines overexpressing *MdWRKY9* were significantly shorter and had significantly lower internode lengths than control plants [31], and its two orthologues in Arabidopsis (*AtWRKY11* and *AtWRKY17*) are negative regulators of basal resistance to a bacterial pathogen [32–34]. The Group II phylogenetic tree (Fig. 8) indicates that *AtWRKY11*, 17, and *ZjWRKY34* are closely related. In this study, we found that *ZjWRKY34* was expressed at a noticeably higher level in infected jujube (Fig. 6) and in the JWB-resistant variety than in the susceptible variety at later stages of infection. STRING analysis showed that *ZjWRKY34* (the orthologous of *AtWRKY17*) can interact with MSK1 and calmodulin (CAM, Fig. 9b). MSK1 is a regulator of plant defense response and it may contribute to MPK4-regulated defense activation by coupling the kinase to specific WRKY

transcription factors. It also indicated that *ZjWRKY34* might interact with the calmodulin- Ca^{2+} complex. Inferring the potential functions of *ZjWRKY34* from the known *AtWRKYs* suggests that *ZjWRKY34* might also act as a negative regulator in the defence process during jujube-phytoplasma interactions.

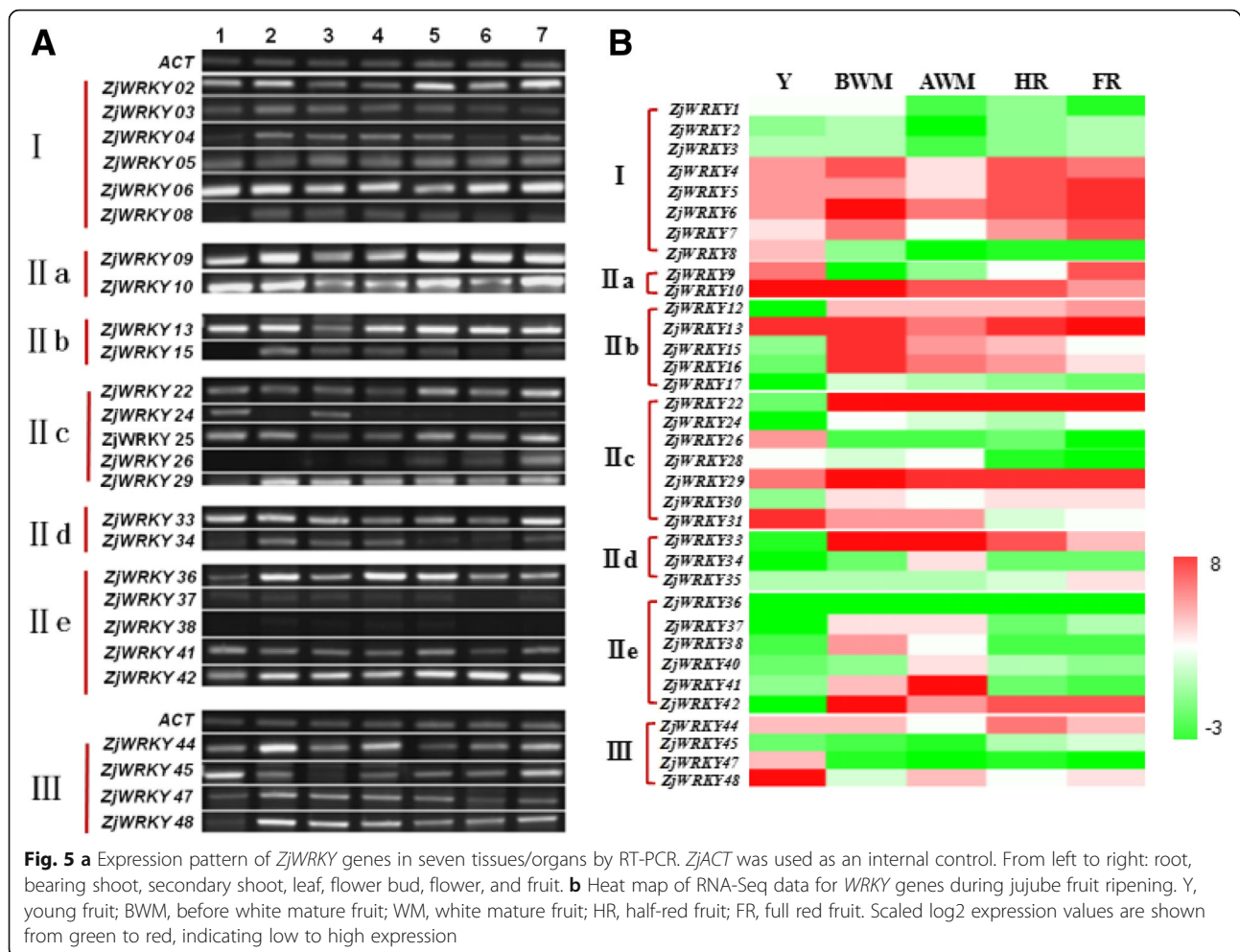
ZjWRKY9 was actively expressed in JWB-diseased tissues. The phylogenetic tree (Fig. 8) and sequence alignment showed that *ZjWRKY9* was the orthologous gene of *AtWRKY18*. Transgenic *AtWRKY18* plants had increased expression of pathogenesis-related genes and resistance to the bacterial pathogen *Pseudomonas syringae*, indicating that *AtWRKY18* can positively modulate defence-related gene expression and disease resistance [12]. *AtWRKY18/40* act in a feedback repression system controlling basal defences [10]. In the other side, high *AtWRKY18* expression can cause severely abnormal plant growth [12]. These results suggest that proper expression of *ZjWRKY9* is critical for enhancing jujube's defence response without negatively impacting plant growth. *ZjWRKY9*'s higher expression might be related



to the abnormal growth of diseased jujube trees, such as witches' broom and tiny leaves.

Overexpression of *AtWRKY28* and *AtWRKY75* induced an oxidative burst in host plants, which

suppressed the hyphal growth of *Sclerotinia sclerotiorum* and consequently inhibited fungal infection [35]. STRING analysis predicted that *AtWRKY75* could interact with *GSTU10* (Fig. 9), which can



eliminate the toxicity of oxygen bursts on plant cells and increase plant tolerance [36]. In this study, *ZjWRKY22* and *ZjWRKY24* are orthologous of *AtWRKY75* (Fig. 8), which behaved higher expression in the susceptible variety than in the resistant one (Fig. 7). That means that the two genes might eliminate the toxicity of oxygen bursts caused by phytoplasma infection. Previous study also indicated that the expression of *WRKY* gene was responsive to phytoplasma infection [37]. A divergent behaviour was previously observed for *OsWRKY28*. In rice, overexpression of *OsWRKY28* enhanced susceptibility to the rice blast fungus *Magnaporthe oryzae* and decreased accumulation of PR5 [38]. The knock-out of *OsWRKY28* led to a two-fold increase in resistance to a compatible rice blast fungus and this phenotype is accompanied by the increased expression of several defence-related genes [39]. Hence, *OsWRKY28* acts as a negative regulator of basal defence responses. Similarly, some *WRKY* genes might act as negative regulators of the basal resistance of jujube under phytoplasma stress, but further study is necessary to verify their specific functions.

Conclusions

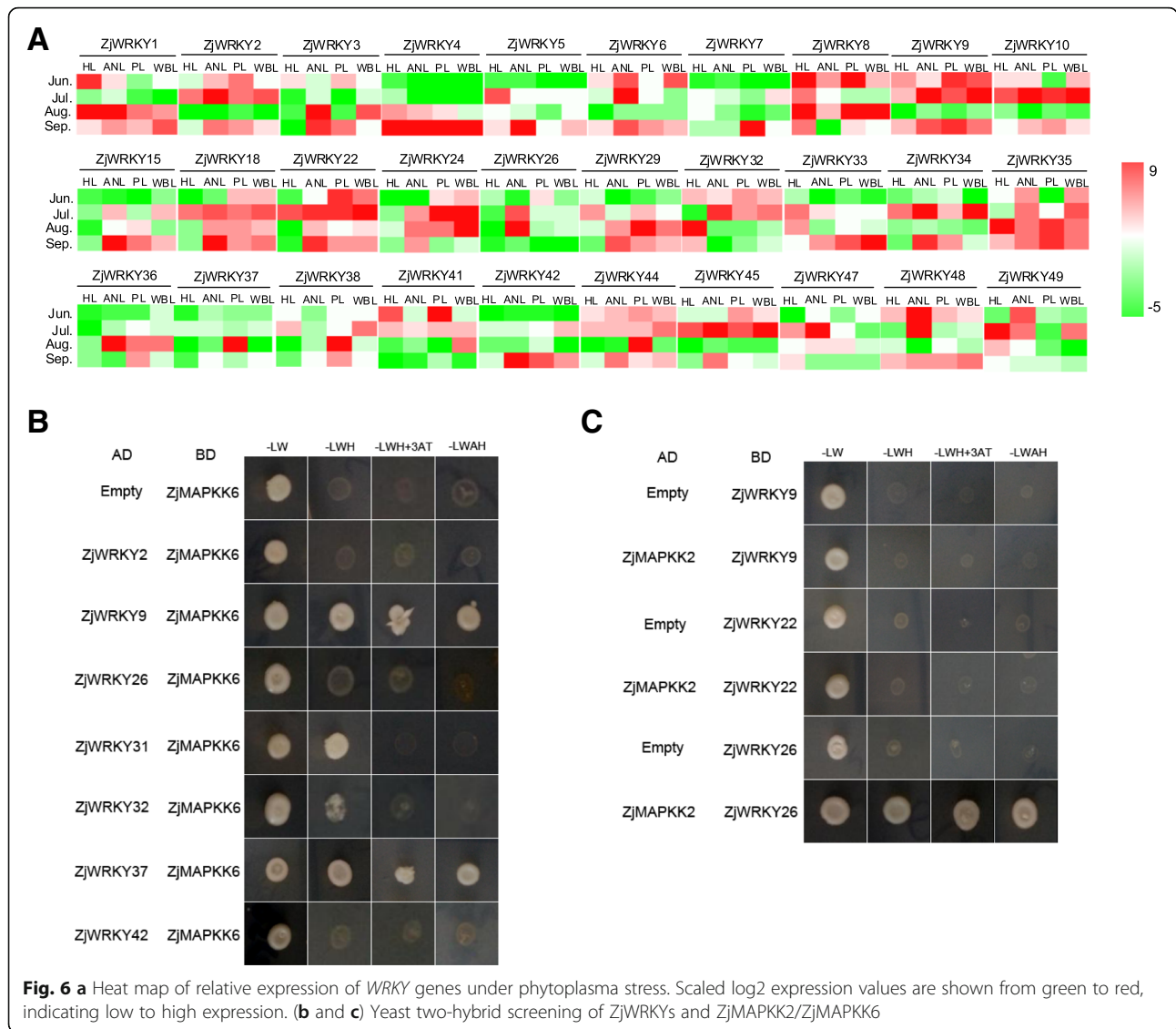
This paper described the *WRKY* gene family of Chinese jujube at the genome level. Their gene structure, chromosomal distribution, phylogenetic relationship, and tissue-specific expression patterns were presented in this study. Most of the *ZjWRKY*s were positive responses to phytoplasma invasion, and that provided meaningful candidates for the future studies of *ZjWRKY*s involved in jujube-phytoplasma interaction.

Methods

Plant material

The seven tissues including roots, young branches, old branches, leaves, flower buds, flowers and young fruits were collected from three jujube trees and used for organ-specific expression analysis.

Four kinds of tissues representing different degrees of JWB disease (apparently normal leaves (ANL), phyllody leaves (PL), and witches'-broom leaves (WBL)) from diseased trees, and healthy leaves (HL) from healthy trees were collected at four growth periods (June, July,



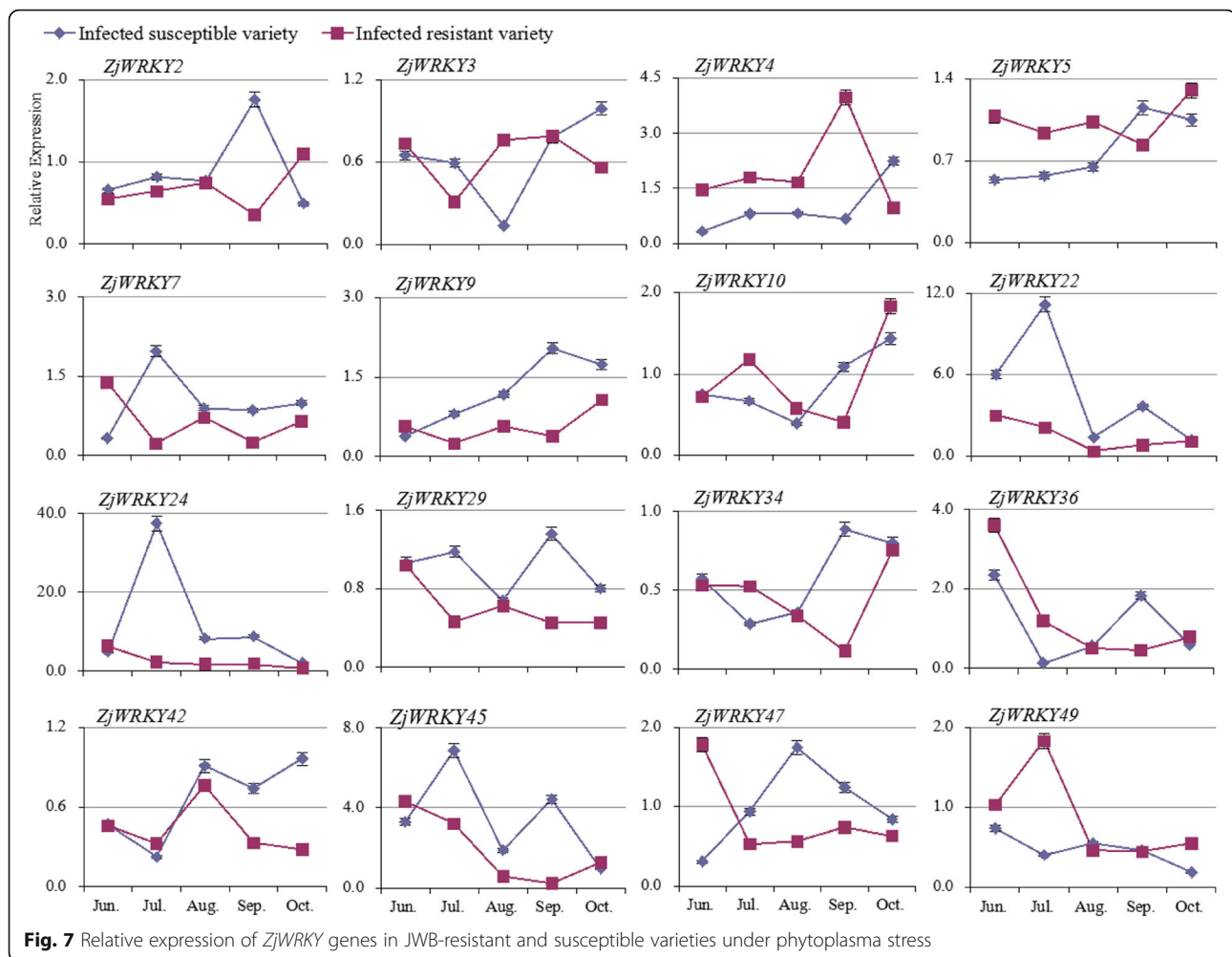
August, and September). The All treatments were conducted with three biological replicates.

Phytoplasma cannot be cultured in vitro, and thus, JWB phytoplasma infection was transmitted by grafting. A JWB-resistant variety and a susceptible variety were used as scions for grafting onto JWB-diseased and healthy trees. All grafting treatments were conducted with three replicates. The samples were collected from sprouted scions at five growth periods (June, July, August, September and October). The samples were stored at -80 °C until RNA extraction and expression analysis.

The JWB phytoplasma presence of the samples was detected by quantitative real-time PCR (qRT-PCR) [40]. The expression of phytoplasma *TMK* gene in jujube samples was analysed and *ZjACT* was used as an internal control.

Identification and protein structure analysis of *ZjWRKY*s in Chinese jujube

First, *WRKY* genes from Arabidopsis were used as queries to search the jujube genome database. Next, the Pfam (<http://pfam.xfam.org/>) and SMART (<http://smart.embl-heidelberg.de/>) databases were used to confirm the predicted jujube *WRKY* proteins. To further confirm that the amino acid sequences in our data set were *WRKY*s, we manually examined the conserved *WRKYGQK* amino acid motif at the N-terminus and the zinc-finger-like motif at the C-terminus of the predicted *WRKY* domain. Truncated and false genes were excluded from our analysis. The number of amino acids, molecular weight, and theoretical pI of *ZjWRKY* genes were predicted by ProtParam (https://web.expasy.org/compute_pi/). The conserved motifs of *ZjWRKY* proteins were detected by MEME (<http://meme-suite.org/>),



using the following parameters: number of repetitions, any; maximum number of motifs, 20; and the optimum motif widths, 6–60 amino acid residues [41].

The chromosomal location and gene structure of *ZjWRKYs*

To determine the chromosomal location of the *ZjWRKY* genes, their gene sequences were used as query sequences in BLASTN searches against the jujube genome. Each *ZjWRKY* gene was mapped to the jujube genome according to their genome coordinates. Tandem duplications were identified as previously described [42].

The website GSDB (<http://gsds.cbi.pku.edu.cn/>) was used to predict the number of exons from the coding domain sequences (CDS) and DNA sequences of the *WRKY* genes [43].

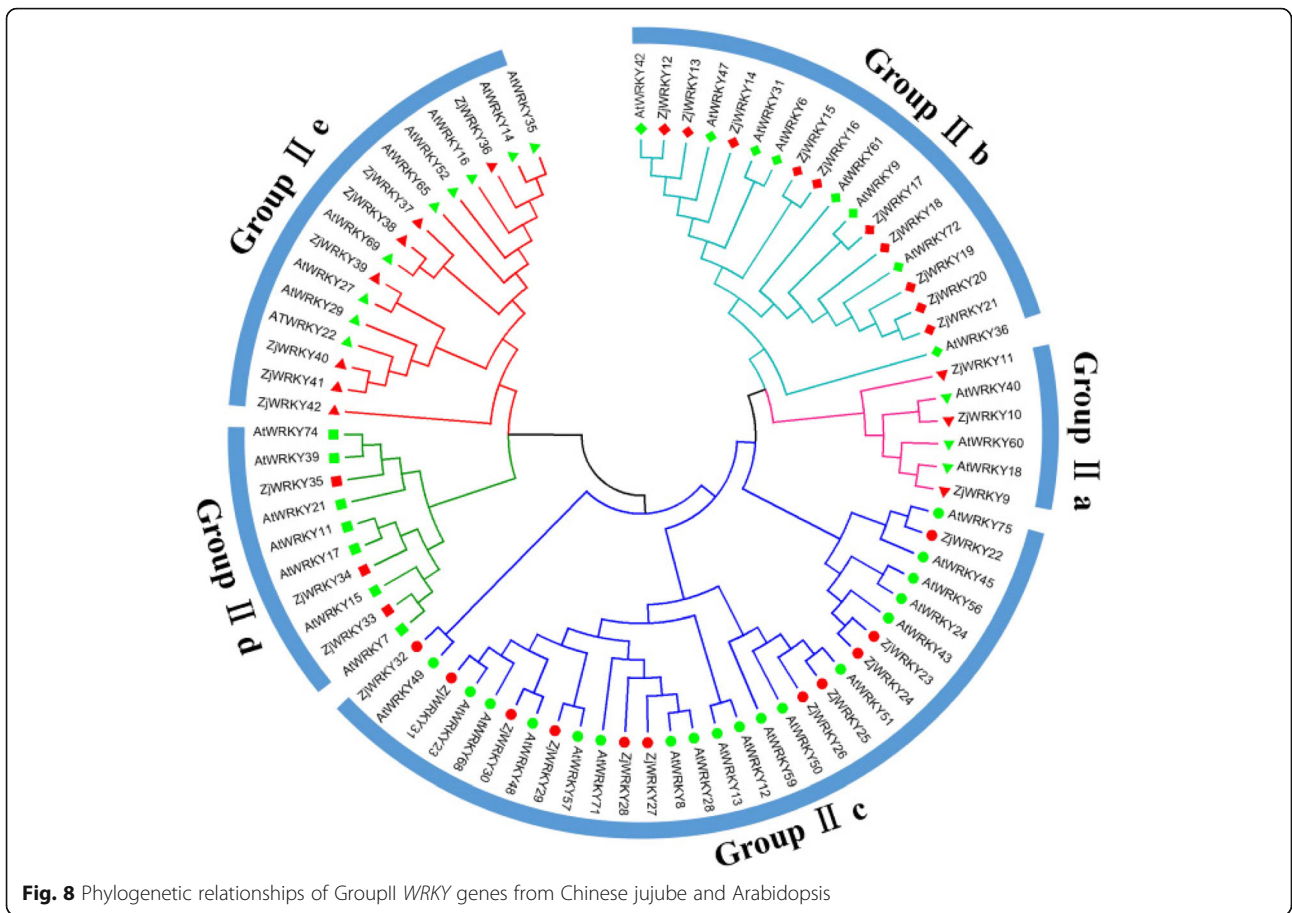
Multiple sequence alignment and phylogenetic tree construction

The jujube *WRKY* proteins were classified into different groups based on their conserved domains. A phylogenetic tree was constructed from the amino acid sequences of

WRKY conserved domains from jujube (54 sequences). The *Arabidopsis thaliana* *WRKY* proteins were retrieved from the TAIR database (<http://www.arabidopsis.org/>) as reported previously. Additionally, *WRKY* proteins of three other species (*Persica prunus* [22], *Pyres bretschneideri* [21], and *Malus domestica* [29]) were downloaded from NCBI. The classification of jujube Group II *WRKY* genes using the phylogenetic tree was dependent on the putative *Arabidopsis thaliana* orthologs. The MEGA 5.2 software and the neighbour-joining statistical method were used to construct a rooted phylogenetic tree [44–46]. The evolutionary distances were obtained using the p-distances method, and these distances were used to estimate the number of amino acid substitutions per site. The reliability of each phylogenetic tree was established by conducting 1000 bootstrap sampling iterations.

RNA isolation and expression and statistical analysis

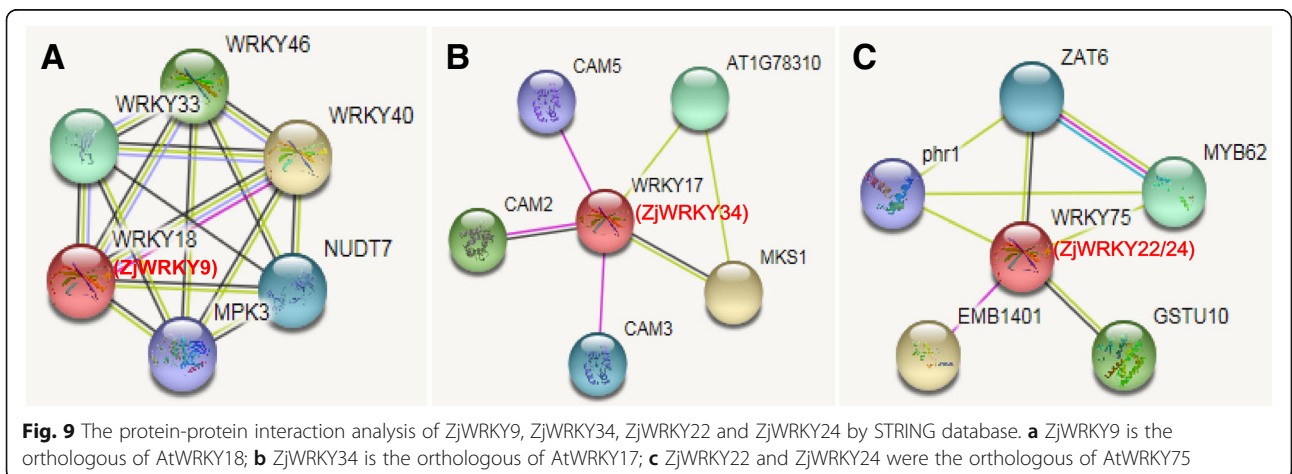
Total RNA was extracted using an RNAPrep Pure Plant Kit (TIANGEN) according to the manufacturer's protocol.



After genomic DNA was removed by RNase-free DNase I (TIANGEN), RNA concentration and purity were checked on a NanoDrop2000 spectrophotometer. First-strand cDNA was synthesized by reverse transcribing 500 ng of total RNA with FastQuant RT Super Mix Kit (TIANGEN). The cDNA was used as the template for qRT-PCR.

Gene expression was detected by qRT-PCR. The primers used in this study are listed in Additional file 6.

PCR products were amplified in triplicate using the Bio-Rad iQ™5 with TransStart Top Green qPCR Super-Mix AQ131 (TransGen Biotech, China) in 20 μL reactions. Each reaction contained 10 μL of 2 × TransStart® Top Green qPCR SuperMix, 0.4 μL each of 10 μM primers, 8.2 μL of ddH₂O and 1 μL of cDNA. The thermal profile for RT-qPCR was as follows: preincubation for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C,



10 s at 53–58 °C, and 10 s at 72 °C. Three biological replicates were performed for each treatment. Threshold cycle values were calculated using iCycler software, and *ZjACT* was used as an internal control [47]. Relative transcript levels were calculated according to the $2^{-\Delta\Delta CT}$ method [48].

Yeast two-hybrid screening (Y2H)

ZjWRKY protein is fused to the Gal4 DNA-binding domain (BD) and the screening proteins are fused to the Gal4 activation domain (AD). The AD-fused *ZjWRKY* and BD-fused *ZjMAPK* were amplified using the primers shown in Supplementary Table S1, and cloned into the pGADT7 vector and pGBKT7 respectively. *ZjWRKYs* were digested by *Sma*I and the *ZjMAPKs* were digested by *Eco*RI and co-transformed AH109 strain with pairs of appropriate pGADT7 and pGBKT7 vectors. Successful co-transformants were selected on synthetically defined medium lacking tryptophan and leucine (SD/–Trp/–Leu). To examine protein–protein interactions, freshly transformed yeast colonies were resuspended in 10 μ L sterile deionized water, and 0.5 μ L aliquots were spotted upon medium lacking leucine and tryptophan (–LW) and medium lacking leucine, tryptophan, histidine (–LWH), supplemented with 7 mM 3-Amino-1,2,4-triazole (3-AT; Sigma Aldrich) (–LWH + 3AT) and medium lacking leucine, tryptophan, histidine, adenine (–LWAH). Growth was scored after 3 d of incubation at 28 °C.

Additional files

Additional file 1: Number of WRKY gene family from Chinese jujube and other species (DOC 28 kb)

Additional file 2: The amino acid sequences of 8 motifs among *ZjWRKY* proteins. (DOC 160 kb)

Additional file 3: Positions of 13 *ZjWRKY* genes on the jujube scaffolds. The jujube scaffolds were arranged in a circle. (DOC 319 kb)

Additional file 4: Expression analysis of phytoplasma TMK in four kinds of leaves (A) and in susceptible and resistant varieties (B). (DOC 31 kb)

Additional file 5: The phylogenetic analysis of Group III WRKYs of *Ziziphus jujuba*, *Arabidopsis thaliana*, *Pyres Bretschneideri*, *Persica Prunus* and *Malus domestica*. (DOC 89 kb)

Additional file 6: The primers of *ZjWRKY* genes used in this study. (DOC 36 kb)

Abbreviations

ANL: Apparently normal leaves; CDS: Coding domain sequences; HL: Healthy leaves; JWB: Jujube witches' broom disease; PL: Phyllody leaves; qRT-PCR: Quantitative real-time PCR; TFs: Transcription factors; WBL: Witches'-broom leaves

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funding bodies had no role in the design of the study, sample collection, analysis or interpretation of data and in writing the manuscript.

Availability of data and materials

All data and materials are presented in the main paper and additional supporting file.

Authors' contributions

JZ and ML designed the research; CX, HL and JZ performed the experiments, analyzed the data and wrote the paper. ZL, LW and HF participated in the data analysis. YZ and XW performed RT-PCR, RT-qPCR experiments. All authors read and approved the final the manuscript.

Ethics approval and consent to participate

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

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

- Eulgem T, Rushton P, Robatzek S, Somssich I. The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* 2000;5:199–206.
- Lagace M, Matton DP. Characterization of a WRKY transcription factor expressed in late torpedo-stage embryos of *Solanum chacoense*. *Planta.* 2004;219(1):185–9.
- Johnson CS, Kolevski B, Smyth DR. Transparent TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. *Plant Cell.* 2002;14:1359–75.
- Miao Y, Laun T, Zimmermann P, Zentgraf U. Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. *Plant Mol Biol.* 2004;55:853–67.
- Li W, Wang H, Yu D. Arabidopsis WRKY transcription factors WRKY12 and WRKY13 oppositely regulate flowering under short-day conditions. *Mol Plant.* 2016;9:1492–503.
- Guan Y, Meng X, Khanna R, LaMontagne E, Liu Y, Zhang S. Phosphorylation of a WRKY transcription factor by MAPKs is required for pollen development and function in Arabidopsis. *PLoS Genet.* 2014;10(5):e1004384.
- Rinerson CI, Scully ED, Palmer NA, Donze-Reiner T, Rabara RC, Tripathi P, et al. The WRKY transcription factor family and senescence in switchgrass. *BMC Genomics.* 2015;16:1–17.
- Suttipanta N, Pattanaik S, Kulshrestha M, Patra B, Singh SK, Yuan L. The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *Plant Physiol.* 2011;157(4):2081–93.
- Zhang ZL. A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells. *Plant Physiol.* 2004;134(4):1500–13.
- Pandey SP, Roccaro M, Schon M, Logemann E, Somssich IE. Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. *Plant J.* 2010;64(6):912–23.

11. Mukhtar MS, Deslandes L, Auriac MC, Marco Y, Somssich IE. The Arabidopsis transcription factor WRKY27 influences wilt disease symptom development caused by *Ralstonia solanacearum*. *Plant J*. 2008;56(6):935–47.
12. Chen C, Chen Z. Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor. *Plant Physiol*. 2002;129(2):706–16.
13. Li J, Brader G, Kariola T, Tapio Palva E. WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J*. 2006;46:477–91.
14. Knoth C, Ringle J, Dangl JL, Eulgem T. Arabidopsis WRKY70 is required for full RPP4-mediated disease resistance and basal defense against *Hyaloperonospora parasitica*. *Mol Plant-Microbe Interact*. 2007;20:120–8.
15. Kim KC, Lai Z, Fan B, Chen Z. Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. *Plant Cell*. 2008;20:2357–71.
16. Peng X, Hu Y, Tang X, Zhou P, Deng X, Wang H, Guo Z. Constitutive expression of rice WRKY30 gene increases the endogenous jasmonic acid accumulation, PR gene expression and resistance to fungal pathogens in rice. *Planta*. 2012;236:1485–98.
17. Viana VE, Busanello C, da Maia LC, Pegoraro C, Costa de Oliveira A. Activation of rice WRKY transcription factors: an army of stress fighting soldiers? *Curr Opin Plant Biol*. 2018;45:268–75.
18. Cannon SB, Mitra A, Baumgarten A, Young ND, May G. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biol*. 2004;4(1):10.
19. Ross CA, Liu Y, Shen QJ. The WRKY gene family in rice (*Oryza sativa*). *J Integr Plant Biol*. 2007;49(6):827–42.
20. Wang L, Zhu W, Fang L, Sun X, Su L, Liang Z, Wang N, Londo JP, Li S, Xin H. Genome-wide identification of WRKY family genes and their response to cold stress in *Vitis vinifera*. *BMC Plant Biol*. 2014;14(1):103.
21. Huang X, Li K, Xu X, et al. Genome-wide analysis of WRKY transcription factors in white pear (*Pyrus bretschneideri*) reveals evolution and patterns under drought stress. *BMC Genomics*. 2015;16(1):1104.
22. Chen M, Tan Q, Sun M, Li D, Fu X, Chen X, Xiao W, Li L, Gao D. Genome-wide identification of WRKY family genes in peach and analysis of WRKY expression during bud dormancy. *Mol Gen Genomics*. 2016;291:1319–32.
23. Liu MJ. Chinese jujube: botany and horticulture. In: *Horticultural reviews*, vol. 32; 2010. p. 229.
24. Liu MJ, Zhao J, Cai QL, et al. The complex jujube genome provides insights into fruit tree biology. *Nat Commun*. 2014;5:5315.
25. Huang J, Zhang C, Zhao X, Fei Z, Wan K, Zhang Z, Pang X, Yin X, Bai Y, Sun X, Gao L, Li R, Zhang J, Li X. The jujube genome provides insights into genome evolution and the domestication of sweetness/acidicity taste in fruit trees. *PLoS Genet*. 2016;12(12):e1006433.
26. Liu MJ, Zhao J, Wang JR, Liu ZG, Liu GC. Phylogenetic analysis of 25 plant species representing 19 angiosperm families and one gymnosperm family based on 390 orthologous genes. *Plant Syst Evol*. 2017;303(3):413–7.
27. Verde I, Abbott AG, Scalabrin S, et al. The high-quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. *Nat Genet*. 2013;45(5):487–94.
28. Liu Z, Zhang L, Xue C, Fang H, Zhao J, Liu M. Genome-wide identification and analysis of MAPK and MAPKK gene family in Chinese jujube (*Ziziphus jujuba* mill.). *BMC Genomics*. 2017;18:855.
29. Meng D, Li Y, Bai Y, et al. Genome-wide identification and characterization of WRKY transcriptional factor family in apple and analysis of their responses to waterlogging and drought stress. *Plant Physiol Biochem*. 2016;103:71–83.
30. Zhang Y, Wang L. The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. *BMC Evol Biol*. 2005;5(1):1.
31. Zheng X, Zhao Y, Shan D, et al. MdWRKY9 overexpression confers intensive dwarfing in the M26 rootstock of apple by directly inhibiting brassinosteroid synthetase MdDWF4 expression. *The New Phytologist*. 2017;217(3):1086–98.
32. Journot-Catalino N, Somssich IE, Roby D, Kroj T. The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in *Arabidopsis thaliana*. *Plant Cell*. 2006;18:3289–302.
33. Ali MA, Wicczorek K, Kreil DP, Bohlmann H. The beet cyst nematode *Heterodera schachtii* modulates the expression of WRKY transcription factors in syncytia to favour its development in *Arabidopsis* roots. *PLoS One*. 2014;9:e102360.
34. Jiang CH, Huang ZY, Xie P, Gu C, Li K, Wang DC, Yu YY, Fan ZH, Wang CJ, Wang YP, et al. Transcription factors WRKY70 and WRKY11 served as regulators in rhizobacterium *Bacillus cereus* AR156-induced systemic resistance to *Pseudomonas syringae* pv. *Tomato* DC3000 in *Arabidopsis*. *J Exp Bot*. 2016;67:157–74.
35. Chen X, Liu J, Lin G, et al. Overexpression of AtWRKY28 and AtWRKY75 in *Arabidopsis* enhances resistance to oxalic acid and *Sclerotinia sclerotiorum*. *Plant Cell Rep*. 2013;32(10):1589–99.
36. Edwards R, Dixon DP. Plant glutathione transferases. *Method Enzymol*. 2005;401:169–86.
37. Ye X, Wang H, Chen P, Fu B, Zhang M, Li J, Zheng X, Tan B, Feng J. Combination of iTRAQ proteomics and RNA-seq transcriptomics reveals multiple levels of regulation in phytoplasma-infected *Ziziphus jujuba* mill. *Hortic Res*. 2017;4:17080.
38. Chujo T, Miyamoto K, Shimogawa T, Shimizu T, Otake Y, Yokotani N. OsWRKY28, a PAMP responsive transrepressor, negatively regulates innate immune responses in rice against rice blast fungus. *Plant Mol Biol*. 2013;82(1):23–37.
39. Delteil A, Blein M, Faivre-Rampant O, Guellim A, Estevan J, Hirsch J. Building a mutant resource for the study of disease resistance in rice reveals the pivotal role of several genes involved in defence. *Mol Plant Pathol*. 2012;13(1):72–82.
40. Xue C, Liu Z, Dai L, Bu J, Liu M, Zhao Z, Jiang Z, Gao W, Zhao J. Changing host photosynthetic, carbohydrate and energy metabolisms play important roles in phytoplasma infection. *Phytopathology*. 2018;108(9):1067–77.
41. Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res*. 2009;37(Web Server):W202–8.
42. Paterson AH, Bowers JE, Chapman BA. Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. *Proc Natl Acad Sci*. 2004;101(26):9903–8.
43. Guo A, Zhu Q, Chen X, Luo JGSDS. A gene structure display server. *Yi Chuan*. 2007;29:1023–6.
44. Lui S, Luo C, Zhu L, Sha R, Qu S, Cai B, Wang S. Identification and expression analysis of WRKY transcription factor genes in response to fungal pathogen and hormone treatments in apple (*Malus domestica*). *Journal of Plant Biology*. 2017;60(2):215–30.
45. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 2011;28(10):2731–9.
46. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987;4(4):406–25.
47. Bu J, Zhao J, Liu M. Expression stabilities of candidate reference genes for RT-qPCR in Chinese jujube (*Ziziphus jujuba* Mill.) under a variety of conditions. *PLoS One*. 2016;11(4):e0154212.
48. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*. 2001;25(4):402–8.

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