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Genome-wide analysis of the *WRKY* gene family in the cucumber genome and transcriptome-wide identification of WRKY transcription factors that respond to biotic and abiotic stresses

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Abstract

Background: Cucumber (*Cucumis sativus* L.) is an economically important vegetable crop species. However, it is susceptible to various abiotic and biotic stresses. WRKY transcription factors play important roles in plant growth and development, particularly in the plant response to biotic and abiotic stresses. However, little is known about the expression pattern of *WRKY* genes under different stresses in cucumber.

Results: In the present study, an analysis of the new assembly of the cucumber genome (v3.0) allowed the identification of 61 cucumber *WRKY* genes. Phylogenetic and synteny analyses were performed using related species to investigate the evolution of the cucumber *WRKY* genes. The 61 *CsWRKYs* were classified into three main groups, within which the gene structure and motif compositions were conserved. Tissue expression profiles of the *WRKY* genes demonstrated that 24 *CsWRKY* genes showed constitutive expression (FPKM > 1 in all samples), and some *WRKY* genes showed organ-specific expression, suggesting that these *WRKYs* might be important for plant growth and organ development in cucumber. Importantly, analysis of the *CsWRKY* gene expression patterns revealed that five *CsWRKY* genes strongly responded to both salt and heat stresses, 12 genes were observed to be expressed in response to infection from downy mildew and powdery mildew, and three *CsWRKY* genes simultaneously responded to all treatments analysed. Some *CsWRKY* genes were observed to be induced/repressed at different times after abiotic or biotic stress treatment, demonstrating that cucumber *WRKY* genes might play different roles during different stress responses and that their expression patterns vary in response to stresses.

Conclusions: Sixty-one *WRKY* genes were identified in cucumber, and insight into their classification, evolution, and expression patterns was gained in this study. Responses to different abiotic and biotic stresses in cucumber were also investigated. Our results provide a better understanding of the function of *CsWRKY* genes in improving abiotic and biotic stress resistance in cucumber.

Keywords: Cucumber, WRKY, Abiotic stress, Biotic stress, Expression profiling

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Background

Throughout their lifecycle, plants frequently encounter many different types of stresses that severely prevent them from reaching optimal growth and that may have a great impact on yield production [1–4]. Some of these stresses are abiotic stress factors such as temperature, drought, salt, and heavy metal stress. By contrast, biotic stress factors involve the interaction of plants with insect, nematode, viral, bacterial, fungal, or oomycete origins that use the plant as a food source [2, 5]. To withstand or cope with these different stresses, plants have evolved a series of adjustment mechanisms including a broad regulation of numerous genes to mediate plant physiological and biochemical processes [6, 7]. Therefore, the study of genes involved in these mechanisms is important for the development of biotechnological tools to enhance desirable agronomic traits, such as plant growth and productivity. Transcription factors (TFs), including members of the AP2/ERF, NAC, MYB, and WRKY families, participate in plant tolerance against abiotic and biotic stresses by modulating the expression of defence-related genes [8–14].

The *WRKY* gene family is one of the largest and most extensively studied TF families in higher plants [15]. Since the first *WRKY* gene was cloned in sweet potato, the identification of *WRKY* genes has been performed in various plant species, including *Arabidopsis thaliana* (72) [16], *Oryza sativa* (103) [17], *Zea mays* (120) [18], and *Solanum lycopersicum* (81) [19]. WRKY TFs share a conserved DNA-binding domain that contains a highly conserved WRKYGQK heptapeptide followed by a C2H2- or C2HC-type zinc finger motif [15, 20]. WRKY TFs function by recognizing and binding W-box *cis*-elements (TTGACC/T) of target genes, and both the heptapetide sequence and zinc finger motif are required for this high binding activity [15, 21, 22]. Based on the number of WRKY domains and the type of zinc fingers, WRKY TFs can be classified into three phylogenetically distinct groups: Group I WRKYs, which have two WRKY domains; Group II WRKYs, which have one WRKY domain, while both group I and II WRKYs contain one C2H2-type zinc finger motif (C-X4–5-C-X22–23-H-X1-H); and Group III members, which feature one WRKY domain and a C2HC-type motif (C-X7-C-X23-H-X1-C). Moreover, Group II is further divided into five subgroups (IIa-IIe) based on phylogenetic analyses [23–25].

WRKY TFs have been reported to be involved in many aspects of plant development [25, 26], including senescence [27, 28], trichome development [29], biosynthesis of secondary metabolites [21, 30–32], flowering [33, 34], and seed development and germination [35–37]. Substantial evidence has demonstrated that many *WRKY* genes also participate in various stress responses. For example, the expression of 18 *WRKY* genes was shown to

be induced by exposure to salt stress in the roots of Arabidopsis [38]. *WRKY6* and *WRKY42* were identified to participate in the response to low Pi stress by regulating *PHO1* expression [39]. WRKY TFs from Arabidopsis were also shown to regulate the defence response positively and/or negatively against bacterial pathogens [16, 40], fungal pathogens [41–43] and nematodes [44, 45]. The expression levels of 13 *OsWRKY* genes from rice were examined in response to different treatments, including salt, polyethylene glycol (PEG), and cold or heat stresses, and 10 *WRKY* genes were down- or upregulated in response to these abiotic stresses. Moreover, WRKY proteins from tomato (*S. lycopersicum*) [19, 46], *Brassica napus* [47], soybean (*Glycine max*) [48], rice (*O. sativa*) [49, 50], wheat (*Triticum aestivum* L.) [51] and other plant species were shown to play critical roles in the response to various biotic and abiotic stresses.

According to the above mentioned discussion, WRKY TFs may participate in multiple pathways, leading to an array of physiological responses. The elucidation of the evolution and duplicative expansion of *WRKY* genes seems to be related to the diversity of their functions [20, 24]. The evolutionary studies of the *WRKY* gene family and large-scale genome-wide analyses of *WRKY* genes indicated that Group IIa genes, which compose the group with the fewest number of members, were the last to evolve and appear to have originated from Group IIb genes. Furthermore, Group IIa TFs play many important roles in the regulation of biotic and abiotic stress responses [20].

Cucumber (*Cucumis sativus* L.), one of the most economically important vegetable crop species, produces tender fruits that are edible organs [52, 53]. In addition, cucumber is extensively used as a model system in the study of sex determination, vascular biology, and induced defence responses [54]. In cucumber cultivation, yield and quality are frequently affected by different types of biotic and abiotic stresses, leading to a decline in cucumber output. Therefore, the identification of new functional genes for resistance to stresses is gaining considerable interest. Based on the cucumber genome (v1.0), 57 *WRKY* genes were identified, and 23 of them had been shown to be differentially expressed in response to at least one abiotic stress [55]. Low-coverage Sanger sequences and short high-coverage Illumina sequences were used to assemble draft cucumber genomes (v1.0 and v2.0); thus, these genomes are incomplete and of low quality. A high-quality and complete cucumber genome assembly (v3.0) is currently available for use in comparative genomics and genetic research [56]. Here, a new genome-wide identification of cucumber WRKYs was performed by the use of the cucumber (Chinese Long, 9930) genome (v3.0). We identified 61 *WRKY* genes and classified them into three groups.

Comprehensive analyses including the gene structures, chromosomal locations, conserved protein domains, and phylogenetic analysis were further performed. The expression profiles of genome-wide *CsWRKY* genes in cucumber plants under different stresses were investigated. Our results will provide valuable clues for future work on the function of WRKYs in cucumber.

Results

The cucumber genome contains 61 *WRKY* genes
In a previous study, 57 *WRKY* genes were identified in the cucumber (Chinese long, 9930) genome (v1.0) [55]. Recently, an updated version (v3.0) was released in CuGenDB (http://cucurbitgenomics.org/), and the v1.0 was eliminated. Therefore, we identified cucumber *WRKY* genes in the cucumber genome (v3.0), and 61 *WRKY* genes were identified by a hidden Markov model (HMM) search using the WRKY domain (PF03106). These genes were proven to contain WRKY domains according to Pfam and SMART analysis. Among the previous 57 *WRKY* genes, five (*CsWRKY53-CsWRKY57*) were not conclusively mapped to any chromosome on the basis of the cucumber genome (v1.0) [55]; however, all 61 of the *WRKY* genes identified in this study could be mapped onto the chromosomes on the basis of the current version of the cucumber genome (v3.0) (Additional file 1: Fig. S1). There are seven chromosomes in the cucumber genome; the *WRKY* genes were not evenly dispersed across all chromosomes. Chromosome 3 harboured the highest number of *CsWRKY* genes (15, 24.59%), while only five (8.20%) were found on chromosome 5. Except for chromosomes 1 and 4, the number of *WRKY* genes we identified mapped onto every chromosome was at least one more than that in a previous study (Additional file 2: Fig. S2). Based on their order on the chromosomes, the *WRKY* genes identified in this study were renamed *CsWRKY1* to *CsWRKY61* (Additional file 1: Fig. S1), and this nomenclature approach was identical to that used in the previous study. A comparison of the currently known WRKY TFs in the cucumber genomes (Gy14, v1.0; 9930, v2.0 and v3.0) is listed in Additional file 3: Table S1.

For these 61 *WRKY* genes, the length of the coding DNA sequence (CDS) and the protein sequence, the protein molecular weight (MW), and the isoelectric point (pI) were analysed (Table 1 and Additional file 3: Table S1). The largest protein was CsWRKY8, comprising 1118 amino acids (aa), whereas the smallest one was CsWRKY47 (119 aa), corresponding to MWs ranging from 13.95 (CsWRKY47) to 124.59 (CsWRKY8) kDa. The pIs of the WKRYs ranged from 5.11 (CsWRKY10) to 10.08 (CsWRKY54). According to the predicted results of subcellular localization, all these CsWRKY proteins might be localized to the nucleus. The subcellular

localization of CsWRKY50 (in this paper named CsWRKY47) [53] could support this claim.

Multiple sequence alignment, phylogenetic relationship, and classification of CsWRKY proteins
The WRKY domains, which comprise approximately 60 aa, of the newly identified CsWRKYs were first aligned, and seven AtWRKY domains (AtWRKY58, 56, 21, 35, 46, 40, and 6) from each group or subgroup were randomly selected as representatives for analysis. The highly conserved sequence WRKYGQK was found within a total of 58 CsWRKY proteins, while the others (CsWRKY10, CsWRKY47, and CsWRKY50) had a single amino acid substitution: K for Q (Fig. 1 and Table 1).

A phylogenetic tree was constructed using the neighbour-joining (NJ) method by MEGA 5.0 software with 1000 bootstrap tests and based on multiple alignments of cucumber and Arabidopsis [16] WRKY domain aa sequences (Additional file 4: Table S2). As shown in Fig. 2, cucumber WRKY proteins could be categorized into three large groups (Group I-III) on the basis of the classifications of WRKYs in Arabidopsis [25]. Among the sequences of the 61 CsWRKY proteins, 11 sequences were assigned to Group I, 43 sequences belonged to Group II, and seven were assigned to Group III. In Group I, 10 members contained two WRKY domains (an N-terminal and a C-terminal WRKY domain), whereas CsWRKY42 had lost its C-terminal WRKY GQK-like stretch; these 11 members all harboured C2H2-type zinc finger motifs (C-X4-C-X22–24-H-X-H). The members of Group II contained a WRKY domain and could be further classified into five subgroups (IIa-IIe). Moreover, three members were classed in IIa, which was the group with the smallest number of members; 5, IIb; 20, IIc; 7, IId; and 8, IIe. Although most of the members of Group II had integral C2H2-type zinc finger motifs, partial absence of the zinc finger motif sequence was present in CsWRKY14 and CsWRKY47. Except for CsWRKY40, whose zinc finger motif was almost entirely absent, the CsWRKYs classed in Group III harboured a WRKY domain and contained a C2HC-type zinc finger motif. The 'leucine-rich repeat' (LRR) motif, which is a typical domain of resistance (R) proteins and is found in WRKY proteins of some species, such as Arabidopsis and rice, was not observed in the WRKY proteins of cucumber.

The Group IIa *WRKY* genes were found to be the last to evolve, as these genes compose the only group absent from the spike moss *Selaginella moellendorffii* [20]. The *WRKY* gene family members in many species have been identified, and their detailed numbers were listed in Table 2. Thus, we investigated the duplication and diversification of Group IIa *WRKYs* during evolution based on the available *WRKY* IIa genes in different species,

Table 1 Features of *WRKY* genes identified in cucumber

| Name | Gene ID (V3) | ORF | Intron number | AA | WRKY domain | | | Group |
					Conserved heptapeptide	Zinc-finger type	Domain number	
CsWRKY1	CsaV3_1G002180	1773	4	590	WRKYGQK	C2H2	1	II b
CsWRKY2	CsaV3_1G004720	1731	4	576	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY3	CsaV3_1G007870	1845	3	614	WRKYGQK	C2H2	1	II b
CsWRKY4	CsaV3_1G028960	1521	4	506	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY5	CsaV3_1G032000	849	2	282	WRKYGQK	C2H2	1	II d
CsWRKY6	CsaV3_1G033110	858	2	285	WRKYGQK	C2H2	1	II e
CsWRKY7	CsaV3_1G037680	1242	2	413	WRKYGQK	C2H2	1	II e
CsWRKY8	CsaV3_1G044520	3357	6	1,118	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY9	CsaV3_2G013650	1047	2	348	WRKYGQK	C2H2	1	II d
CsWRKY10	CsaV3_2G017720	618	2	205	WRKYGKK	C2H2	1	II c
CsWRKY11	CsaV3_2G017760	741	3	246	WRKYGQK	C2H2	1	II c
CsWRKY12	CsaV3_2G032460	939	3	312	WRKYGQK	C2H2	1	II a
CsWRKY13	CsaV3_2G032470	612	3	203	WRKYGQK	C2H2	1	II a
CsWRKY14	CsaV3_2G034030	681	2	226	WRKYGQK	C2	1	II c
CsWRKY15	CsaV3_2G035630	882	2	293	WRKYGQK	C2H2	1	II d
CsWRKY16	CsaV3_3G003840	2244	4	747	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY17	CsaV3_3G004410	1602	5	533	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY18	CsaV3_3G007160	660	3	219	WRKYGQK	C2H2	1	II c
CsWRKY19	CsaV3_3G008170	1014	2	337	WRKYGQK	C2HC	1	III
CsWRKY20	CsaV3_3G008580	546	1	181	WRKYGQK	C2H2	1	II c
CsWRKY21	CsaV3_3G008610	1011	2	336	WRKYGQK	C2H2	1	II e
CsWRKY22	CsaV3_3G015290	972	2	323	WRKYGQK	C2H2	1	II c
CsWRKY23	CsaV3_3G018790	1521	4	506	WRKYGQK	C2H2	1	II b
CsWRKY24	CsaV3_3G021980	1101	2	366	WRKYGQK	C2HC	1	III
CsWRKY25	CsaV3_3G026600	999	3	332	WRKYGQK	C2H2	1	II c
CsWRKY26	CsaV3_3G026920	513	1	170	WRKYGQK	C2H2	1	II c
CsWRKY27	CsaV3_3G033000	990	1	329	WRKYGQK	C2HC	1	III
CsWRKY28	CsaV3_3G033350	1431	4	476	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY29	CsaV3_3G035430	1419	4	472	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY30	CsaV3_3G047140	1053	2	350	WRKYGQK	C2H2	1	II d
CsWRKY31	CsaV3_4G001260	873	2	290	WRKYGQK	C2H2	1	II e
CsWRKY32	CsaV3_4G003030	648	1	215	WRKYGQK	C2H2	1	II c
CsWRKY33	CsaV3_4G006110	810	1	269	WRKYGQK	C2H2	1	II e
CsWRKY34	CsaV3_4G006120	603	1	200	WRKYGQK	C2H2	1	II c
CsWRKY35	CsaV3_4G006480	1068	2	355	WRKYGQK	C2HC	1	III
CsWRKY36	CsaV3_4G025110	840	2	279	WRKYGQK	C2H2	1	II c
CsWRKY37	CsaV3_4G034570	1176	2	391	WRKYGQK	C2H2	1	II d
CsWRKY38	CsaV3_4G036610	981	4	326	WRKYGQK	C2H2	1	II a
CsWRKY39	CsaV3_5G001010	1035	2	344	WRKYGQK	C2H2	1	II e
CsWRKY40	CsaV3_5G011060	639	1	212	WRKYGQK	-	1	III
CsWRKY41	CsaV3_5G011080	849	2	282	WRKYGQK	C2HC	1	III
CsWRKY42	CsaV3_5G033090	1173	3	390	WRKYGQK	C2H2	1	I

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Table 1 Features of *WRKY* genes identified in cucumber *(Continued)*

Name	Gene ID (V3)	ORF	Intron number	AA	WRKY domain			Group
					Conserved heptapeptide	Zinc-finger type	Domain number	
CsWRKY43	CsaV3_5G038330	1521	3	506	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY44	CsaV3_6G013820	921	2	306	WRKYGQK	C2H2	1	II c
CsWRKY45	CsaV3_6G018960	1287	4	428	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY46	CsaV3_6G028510	1359	4	452	WRKYGQK	C2H2	1	II c
CsWRKY47	CsaV3_6G032100	360	3	119	WRKYGKK	C2	1	II c
CsWRKY48	CsaV3_6G032480	921	2	306	WRKYGQK	C2H2	1	II c
CsWRKY49	CsaV3_6G042200	735	2	244	WRKYGQK	C2H2	1	II c
CsWRKY50	CsaV3_6G042280	600	2	199	WRKYGKK	C2H2	1	II c
CsWRKY51	CsaV3_6G043450	885	2	294	WRKYGQK	C2H2	1	II d
CsWRKY52	CsaV3_6G048830	1872	5	623	WRKYGQK	C2H2	1	II b
CsWRKY53	CsaV3_6G051490	786	2	261	WRKYGQK	C2H2	1	II c
CsWRKY54	CsaV3_6G052610	1566	2	521	WRKYGQK	C2H2	1	II e
CsWRKY55	CsaV3_7G002670	1302	4	433	WRKYGQK/WRKYGQK	C2H2	2	I
CsWRKY56	CsaV3_7G003470	1608	4	535	WRKYGQK	C2H2	1	II-b
CsWRKY57	CsaV3_7G005750	897	2	298	WRKYGQK	C2H2	1	II e
CsWRKY58	CsaV3_7G022650	894	2	297	WRKYGQK	C2H2	1	II c
CsWRKY59	CsaV3_7G025370	891	2	296	WRKYGQK	C2HC	1	III
CsWRKY60	CsaV3_7G027490	768	3	255	WRKYGQK	C2H2	1	II d
CsWRKY61	CsaV3_7G030110	729	2	242	WRKYGQK	C2H2	1	II c

including eight dicots (Arabidopsis, castor bean, cucumber, grape, tomato, pear, potato, and poplar) and six monocots (barley, rice, maize, bread wheat, *Brachypodium* and millet). The WRKY domain sequence of these *WRKY* IIa genes was used to construct a phylogenetic tree via MEGA 5.0 (Additional file 5: Table S3).

As shown in the phylogenetic tree we constructed, the WRKY IIa proteins were categorized into seven clades (Fig. 3). WRKYs from the phylogenetically closer species clustered together in the same clade. For example, the members of clades 1 and 2 were all from dicots, whereas clades 4–7 contained proteins only from monocots; clade 3 was further divided into two different subclades (clades 3a and 3b) based on members from dicots or monocots, implying that the different evolutionary patterns of Group IIa WRKYs in dicots and monocots may have occurred after their divergence. WRKY members from one species clustered together at most within the three clades, and all the WRKY proteins that divided into three clades were from monocots. For the *WRKYs* from dicots, each of the four species (cucumber, grape, pear, and poplar) contributed at least one gene to clade 1 and clade 2; however, the three species (castor bean, tomato, and potato) clustered specifically within clade 1 or clade 2. These results suggested that numerous

evolutionary splits and diversifications of *WRKYs* have occurred among different species.

Gene structure and motif composition of *CsWRKYs*

Gene structural diversity can reflect the evolution of multigene families [68]. Therefore, we analysed the exon-intron organization within the ORF (open reading fame) sequences of each *CsWRKY* gene (*CsWRKY40*, which lacked a zinc finger motif, was removed) to acquire more insight into the evolution of the WRKY family in cucumber. Previous studies showed that the majority of *Populus* and soybean WRKY members harboured two to four introns [48, 62]. Consistently, more than 80% of the members of the *CsWRKY* genes contain two to four introns (seven with one intron, 29 with two introns, 10 with three introns, 12 with four introns, two with five introns, and one with six introns) (Fig. 4 and Table 1). As shown in Fig. 4, a greater number of introns were observed in Group I, which varied from three to six. All WRKY domains typically contain an intron, and the position of this intron is extremely highly conserved [57]. We found that all *CsWRKYs* contained an intron in their WRKY domains. This intron within the Groups I (the C-terminal WRKY domain), IIc, IId, IIe, and III *WRKY* genes had the same
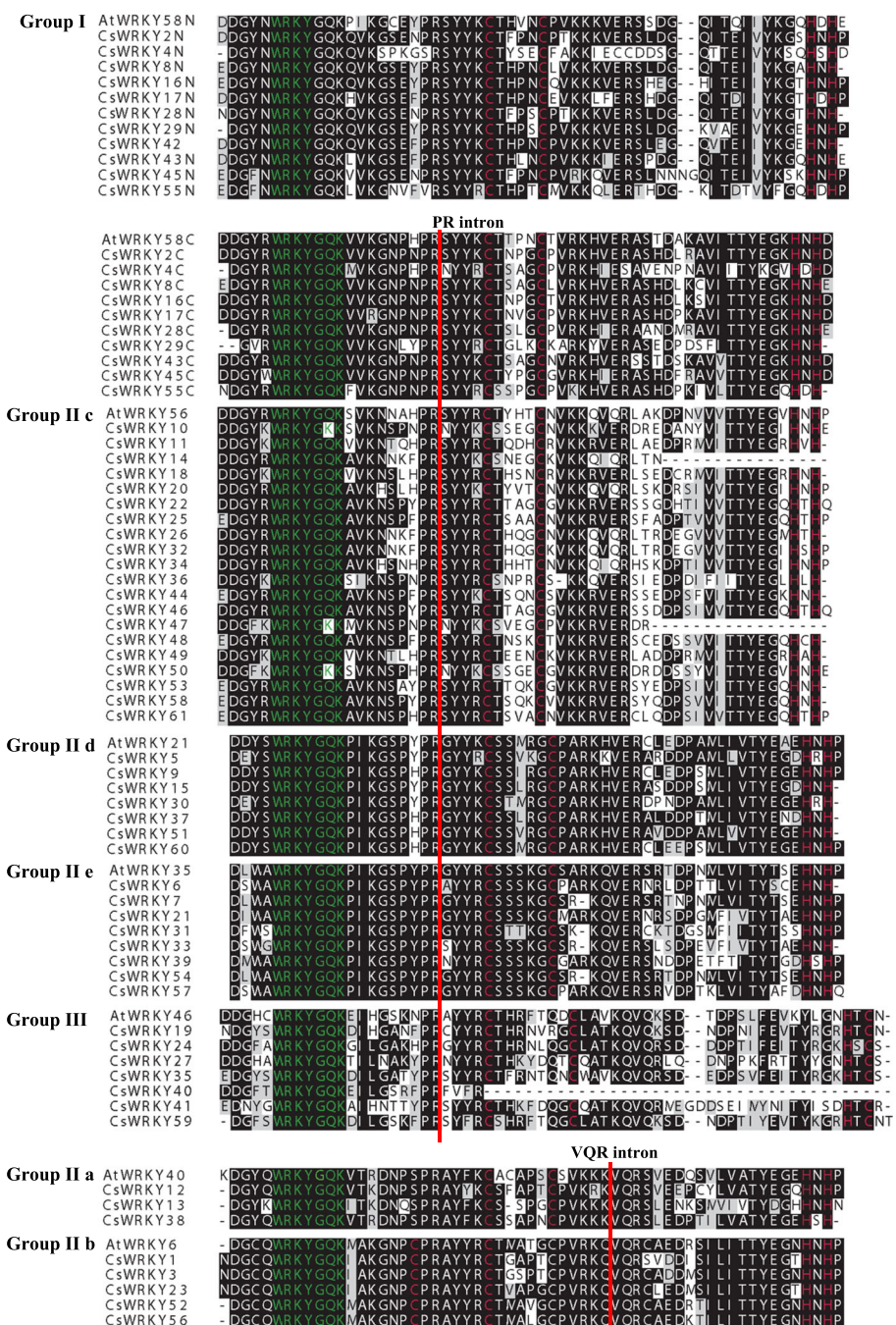
Fig. 1 Alignment of 61 cucumber (CsWRKY) and 8 Arabidopsis (AtWRKY) WRKY domain sequences. For Group I WRKY proteins, N-terminal and C-terminal WRKY domains are represented by 'N' and 'C', respectively. The typical amino acid residues within WRKY domain and zinc-finger motif are in green and red color, respectively. The position of the intron in the genome is indicated by a red line for each WRKY subfamily

location, which was after the codons for the invariant PR amino acid sequence (PR intron) (Fig. 1). The VQR intron, which occurs before the invariant VQR amino acid sequence, was observed in the Group IIa and IIb genes.

To better understand the conservation and diversification of *CsWRKYs*, the putative motifs of all CsWRKY proteins were predicted by MEME motif analysis. As expected, the CsWRKYs that were categorized into the same group shared highly similar motif compositions (Fig. 4 and Additional file 6: Table S4). For instance, motif 9 was found to be specific to Groups IId and IIe, whereas motif 10 was unique to Groups IIb and IIc; Groups IIe and IIc contained only two or three motifs,
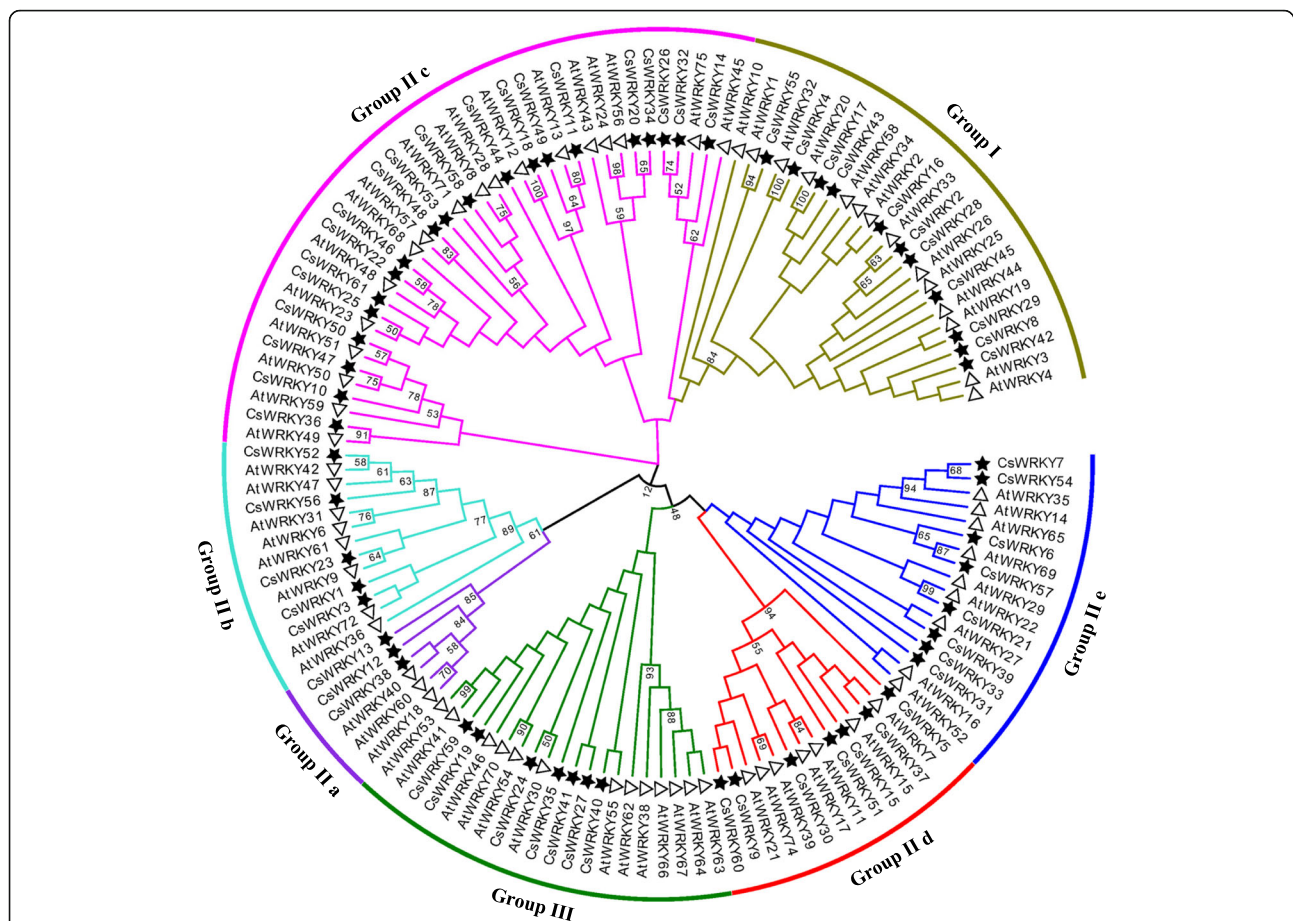
Fig. 2 The phylogenetic tree of the total WRKY proteins from cucumber and Arabidopsis. The WRKY domains were used for phylogenetic analysis by MEGA5 software with bootstrap test of 1000 times. The arcs with different color represent 7 subgroups of WRKY proteins. The black solid star and hollow triangle represent WRKY domain from cucumber and Arabidopsis, respectively

while Group IIb harboured 5 motifs. The functions of most of these motifs remain to be elucidated.

Overall, the closely related CsWRKYs in the phylogenetic tree shared similar gene structural and common motif compositions, suggesting that the CsWRKYs within the same group may play similar functional roles.

Synteny analysis of *CsWRKY* genes
The segmental duplication events occurring in the cucumber WRKY family were investigated by conducting a synteny analysis of the *CsWRKY* genes using BLASTP and MCScanX. As shown in Fig. 5, 14 segmental duplication events involving 25 *WRKY* genes were observed (Additional file 7: Table S5). In contrast, tandem duplication events, which were defined by a chromosomal region within 200 kb containing two or more genes, were not identified for cucumber *WRKY* genes. These results suggested that some *CsWRKYs* were possibly generated by segmental duplication events and that the evolution of *CsWRKY* genes may have been driven, at least in part, by segmental duplication events.

The phylogenetic mechanisms of the cucumber WRKY family were further explored by constructing comparative syntenic maps of cucumber associated with five representative species, including three dicots (Arabidopsis, tomato and watermelon) and two monocots (rice and maize) (Fig. 6). Fifty-two, 29, 27, 9, and 5 *CsWRKY* genes showed syntenic relationships with those in the other five species: watermelon, tomato, Arabidopsis, rice and maize, respectively. A total of 52 *WRKY* collinear gene pairs between cucumber and watermelon were identified, followed by cucumber and Arabidopsis (41), cucumber and tomato (37), cucumber and rice (9), and cucumber and maize (7) (Additional file 8: Table S6). Both cucumber and watermelon belong to the gourd family, and more than 85% of the *CsWRKY* genes showed a syntenic relationship with *WRKYs* in watermelon, and one *CsWRKY* gene was associated with only one syntenic gene pair, indicating that *WRKY* genes in cucumber and watermelon evolved from the same ancient *WRKY* genes. *CsWRKY21* and *CsWRKY28* were found to be associated with two syntenic gene pairs

Table 2 Summary of the number of WRKY proteins in diverse plant species

Species	Name	Total	Group							NG	References
			I	IIa	IIb	IIc	IId	IIe	III		
Ananas comosus	AcWRKY	54	12	2	7	13	7	5	8	0	[22]
Arabidopsis thaliana	AtWRKY	72	13	4	7	18	7	9	14	0	[16]
Brachypodium distachyon	BdWRKY	86	15	3	6	21	6	10	23	2	[57]
Brassica napus	BnWRKY	300	78	11	34	55	28	30	51	13	[47]
Cucumis sative	CsWRKY	61	14	3	5	17	7	8	7	0	This study
Glycine max	GmWRKY	188	32	14	33	42	21	20	26	0	[48]
Gossypium raimondii	GrWRKY	112	20	7	16	26	16	13	14	0	[58]
Gossypium arboreum	GaWRKY	109	19	7	16	26	14	13	14	0	[58]
Hordeum vulgare	HvWRKY	45	8	4	1	11	5	3	13	0	[59]
Manihot esculenta	MeWRKY	85	17	5	14	20	8	9	12	0	[60]
Musa acuminate	MusaWRKY	153	14	7	11	15	15	13	6	72	[61]
Oryza sativa	OsWRKY	103a	15	4	8	15	7	11	36	0	[17]
Populus trichocarpa	PtrWRKY	100	22	5	9	27	13	13	10	1	[62]
Pyrus bretschneideri	PbrWRKY	103	17	6	10	24	15	16	15	0	[63]
Ricinus communis	RcWRKY	47	9	3	10	12	3	5	5	0	[64]
Sesamum indicum	SiWRKY	71	12	4	11	18	7	8	7	4	[65]
Solanum lycopersicum	SlWRKY	81	15	5	8	16	6	17	11	3	[19]
Solanum tuberosum	StWRKY	82	14	6	5	16	15	12	14	0	[66]
Triticum aestivum	TaWRKY	160	20	16	3	34	17	11	57	2	[51]
Vitis vinifera	VvWRKY	72	15	5	7	22	8	8	7	0	[67]
Zea mays	ZmWRKY	120b	15	6	8	22	13	16	30	0	[18]

a98 WRKY genes in japonica and 102 in indica rice. bother 10 *ZmWRKY* genes were classied in Group II. *NG no group*

between cucumber and tomato/rice/maize; some *CsWRKY* genes were associated with three collinear gene pairs (between cucumber and tomato/Arabidopsis *WRKY* genes), speculating that these *CsWRKYs* may play an important role in the evolution of the *WRKY* gene family. Importantly, collinear *CsWRKY21* gene pairs were observed between cucumber and all of the other five species, suggesting that this orthologous pair may have formed before the divergence of dicotyledonous and monocotyledonous plants.
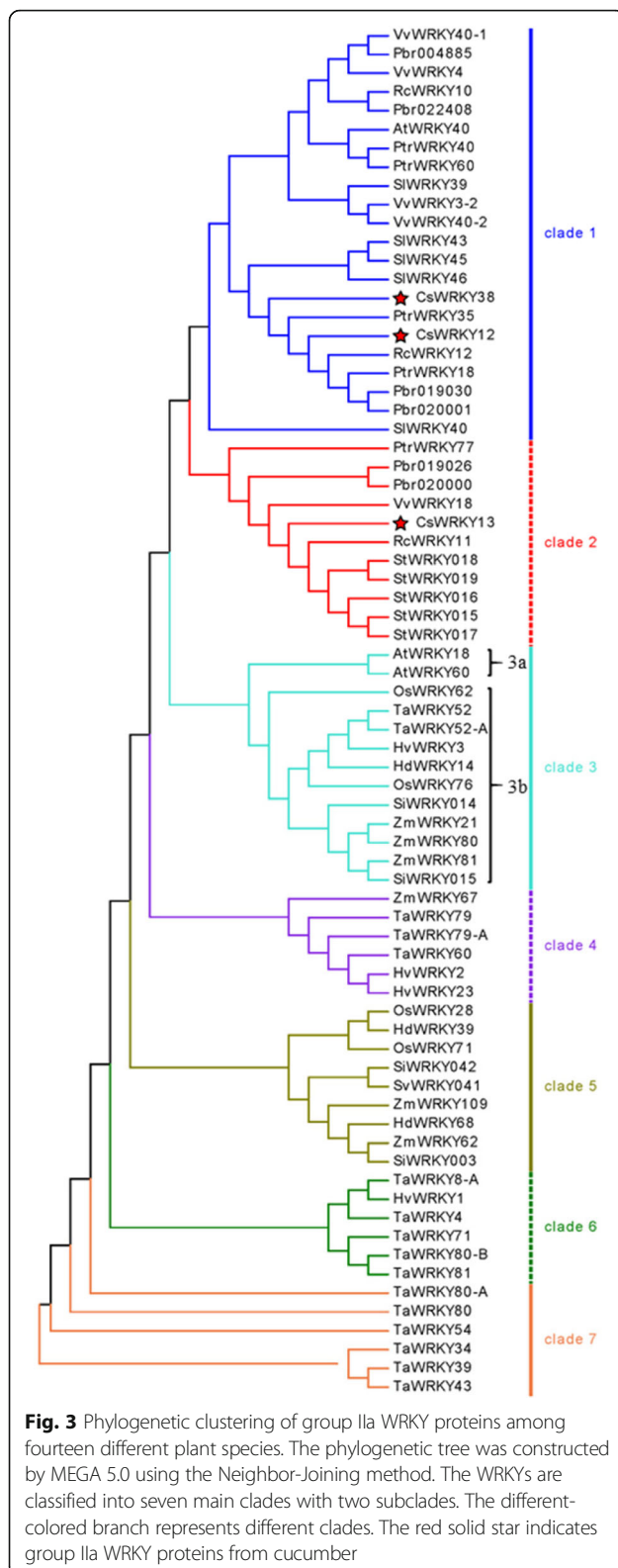
CsWRKYs expression profiles in different organs
The expression patterns of all 61 *CsWRKYs* were investigated using a standard transcriptome analysis procedure based on public transcriptomic data of different tissues of cucumber, including roots, stems, leaves, female flowers, male flowers, ovaries, expanded unfertilized ovaries, expanded fertilized ovaries, and tendrils [69]. Among the 61 *CsWRKY* genes, 41 *CsWRKYs* were expressed in all detected samples (FPKM> 0), and 24 genes showed constitutive expression (FPKM> 1 in all samples) (Additional file 9: Table S7). Some *CsWRKY* genes showed preferential expression across all tissues tested. Nineteen genes in the roots, two genes in the

tendrils (*CsWRKY50/59*), and two genes in the female flowers (*CsWRKY48/12*) exhibited the highest transcript levels. The expression analysis of the different fruit developmental stages showed that several genes (*CsWRKY9/40/54*) had higher expression in the ovaries than in the expanded ovaries (fertilized and unfertilized). In addition, the transcript levels of some *CsWRKYs* (such as *CsWRKY19/27/41/57*) decreased in the fertilized expanded ovaries (Fig. 7). These results indicated that these genes may play roles in many aspects of cucumber plant development, including ovary development and fruit fertilization.

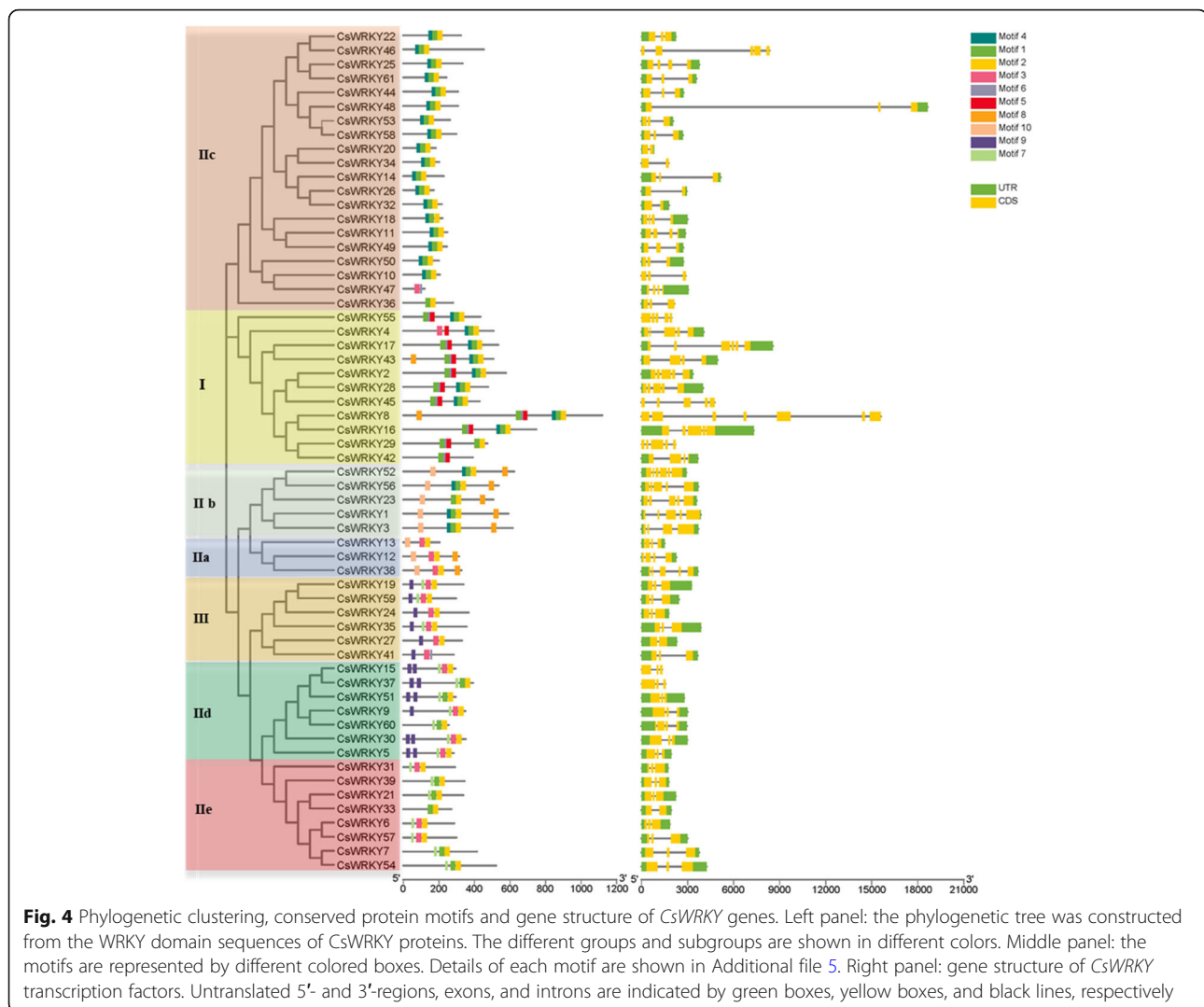### Expression patterns of CsWRKYs in response to abiotic and biotic stresses
To confirm whether *CsWRKYs* are involved in the response to various stresses, we analysed the comprehensive expression patterns of *CsWRKY* genes under different abiotic and biotic stresses, including salt, heat, downy mildew (DM, *Pseudoperonospora cubensis*) and powdery mildew (PM, *Podosphaera fusca*), based on public transcriptome information [54, 70, 71] and transcriptomic data that we generated.

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Fig. 3 Phylogenetic clustering of group IIa WRKY proteins among fourteen different plant species. The phylogenetic tree was constructed by MEGA 5.0 using the Neighbor-Joining method. The WRKYs are classified into seven main clades with two subclades. The different-colored branch represents different clades. The red solid star indicates group IIa WRKY proteins from cucumber

transcriptomic data [70] (Additional file 10: Table S8). We observed that the expression levels of *CsWRKY27*, *CsWRKY41* and *CsWRKY50* considerably increased in response to salt stress. Moreover, seven genes exhibited the opposite trend with exposure to salt stress (Fig. 8a). Previous studies have shown that Silicon (Si) application can improve plant growth under salt stress [72]. Among the seven genes that were downregulated under salt stress, the expression levels of six genes reverted to normal expression levels, and the DEGs (differentially expressed genes) were all upregulated in response to exogenous Si treatment, implying a potential role of these *WRKY* genes in the Si-based alleviation of salt stress (Fig. 8a). Furthermore, the expression patterns of all 61 *CsWRKY* genes in the transcriptomic data, which were derived from leaves subjected to different heat treatment durations, were investigated in this study (Additional file 10: Table S8). Correlation and cluster analyses were used to explore the similarity among the transcriptomes. Two samples (HT3h_2 and HT6h_2) were removed due to their poor uniformity, and the remaining seven samples were used for the following analysis (Additional file 11: Fig. S3). As shown in Fig. 8b, 21 *CsWRKY* genes were significantly induced/repressed by heat stress. The variation trend of the expression of most *WRKY* genes in response to heat stress for 3 h (hours) was consistent with that for 6 h. Overall, the transcript levels of five *CsWRKY* genes (*CsWRKY27/41/50/52/57*) were significantly affected by both salt and heat stress treatments.

To explore the potential functions of *CsWRKYs* in resistance to biotic stresses, we performed a *CsWRKYs* expression analysis of the susceptible and resistant cucumber lines inoculated with PM for 48 h based on published RNA-seq data [71] (Fig. 9a and Additional file 10: Table S8). Eleven and 12 *CsWRKY* genes that were differentially expressed were identified in the susceptible and resistant cucumber lines, respectively, compared with the controls. These results indicated that these *WRKYs* may be influenced by PM stress. The expression patterns of *CsWRKY10* and *CsWRKY50* were opposite in the susceptible and resistant cucumber lines under inoculation with PM, implicating the important role of these two *WRKY* genes in the response to PM infection. The expression of *CsWRKY* genes in response to DM infection was obtained by transcriptome analysis based on RNA-seq data published by Adhikari et al. [54] (Additional file 10: Table S8). Twenty-five *CsWRKY* genes in cucumber were involved in responses to DM infection, indicating that they were induced to play a role in response to DM infection (Fig. 9b). We identified 12 *CsWRKY* genes (*CsWRKY10/14/19/27/28/32/35/46/50/52/59/61*) that were differentially expressed in response to the inoculation of PM and DM (Fig. 9),
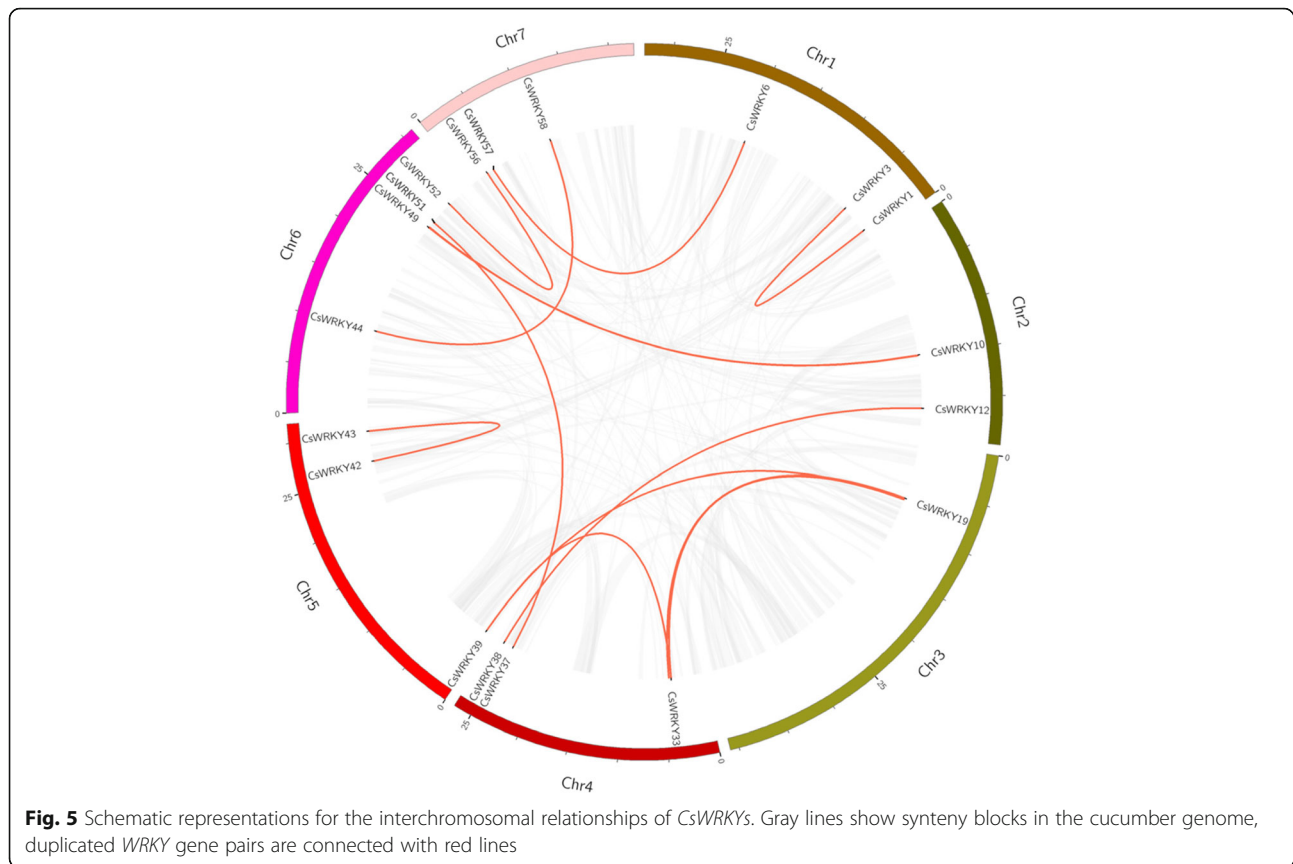
To investigate the potential functions of *CsWRKYs* in resistance to salt stress, we performed a *CsWRKYs* expression analysis after salt treatment based on public

Fig. 4 Phylogenetic clustering, conserved protein motifs and gene structure of *CsWRKY* genes. Left panel: the phylogenetic tree was constructed from the WRKY domain sequences of CsWRKY proteins. The different groups and subgroups are shown in different colors. Middle panel: the motifs are represented by different colored boxes. Details of each motif are shown in Additional file 5. Right panel: gene structure of *CsWRKY* transcription factors. Untranslated 5′- and 3′-regions, exons, and introns are indicated by green boxes, yellow boxes, and black lines, respectively

indicating that these genes may play key roles in responses to biotic stresses. Some *CsWRKY* genes were affected only by inoculation of PM and/or DM and not by abiotic (heat and salt) stresses (Figs. 8 and 9). For instance, *CsWRKY46* was expressed significantly in response to inoculation of PM and DM but not to salt and heat stresses; moreover, *CsWRKY15* was not induced/repressed by any of the tested treatments except inoculation of PM. In addition, the expression levels of several *CsWRKY* genes were significantly affected by both abiotic stresses and biotic stresses (Figs. 8 and 9). For instance, *CsWRKY27*, *CsWRKY50* and *CsWRKY52* simultaneously responded to all treatments analysed, and the expression of *CsWRKY59* was affected by all tested treatments except salt treatment.

Discussion
Although *WRKY* genes had been identified in the cucumber (9930) genome (v1.0) [55], it is essential to re-identify them. Because the information of the *WRKY* genes identified in v1.0 were no longer available, due to the elimination of v1.0 from CuGenDB (http://cucurbit-genomics.org/), whereas the assemblies (v2.0 and v3.0) were available. Therefore, we identified and characterized the WRKY family in the cucumber (9930) genome (v3.0). It is composed of 61 members, which were designated CsWRKY1 to CsWRKY61 on the basis of their chromosomal location; this number is higher than that identified in a previous study (57 *WRKY* genes) [55]. Compared with these previously reported *CsWRKY* genes, nine new *CsWRKY* genes were mapped onto the chromosomes, and five previous *CsWRKY* genes that could not be conclusively mapped to any chromosome were considered obsolete according to the current version of the cucumber genome (v3.0) (Additional file 1: Fig. S1 and Additional file 2: Fig. S2).

Based on the gene structures, amino acid sequences, conserved structural domains and phylogenetic

Fig. 5 Schematic representations for the interchromosomal relationships of *CsWRKYs*. Gray lines show synteny blocks in the cucumber genome, duplicated *WRKY* gene pairs are connected with red lines

relationships with *A. thaliana*, the 61 CsWRKY proteins were similar to the typical WRKY family proteins in other species with classifications into Groups I, IIa, IIb, IIc, IId, IIe and III (Table 1, Figs. 1 and 2). Rinerson et al. [20] proposed that there were four major WRKY TF lineages in flowering plants, Groups I + IIc, Groups IIa + IIb, Groups IId + IIe, and Group III, accurately reflecting the evolution of the WRKY family. This was also verified in cucumber; for example, the members of Groups IIa and IIb (or Groups IId and IIe) were divided into two subclades, which involved the same clade; some WRKY TFs from Group IIc were classified into one subclade together with the members of Group I (Fig. 2). Three CsWRKY proteins (CsWRKY10/47/50) in Group IIc showed sequence variation in their WRKY domains. Domain loss, which seems to be common in monocotyledons, is one of the divergent forces for expansion of the *WRKY* gene family [73, 74]; however, these loss-of-domain events occur less for dicotyledons than for monocotyledons. For example, Group I contains one protein (AtWRKY10) having only one WRKY domain in Arabidopsis [74]. In cucumber, except CsWRKY42, all WRKY proteins of Group I have two WRKY domains, and the event by which the zinc finger motif was lost was also found in three WRKY TFs (CsWRKY14/40/47) (Fig. 1 and Table 1). According to previous studies, both

the heptapeptide motif WRKYGQK and the zinc finger motif are required for the high binding affinity of WRKY TFs to their cognate *cis*-acting W-box element (TTGA CC/T). Therefore, variations in the heptapeptide motif and loss of the zinc finger motif might influence normal interactions of CsWRKYs with target genes, and it might be worth further studying the binding specificities and functions of these five CsWRKY proteins.

Both tandem duplication and chromosomal/segmental duplications contributed to the expansion of the *WRKY* gene family [24]. Comparison of the number of *WRKY* genes in the cucumber genome with other sequenced dicotyledon genomes showed that cucumber has fewer genes (Table 2). Fourteen segmental duplication events within 25 *WRKY* genes were observed (Fig. 5 and Additional file 7: Table S5), while tandem duplication events were lacking. Therefore, the lack of tandem duplication might be a possible reason for the smaller number of *CsWRKY* genes, and segmental duplication was a major driver of *WRKY* gene expansion during the cucumber evolutionary process. Moreover, we identified that more than 85% (52 of 61) of *CsWRKY* genes showed orthologous relationships with *ClWRKY* genes (Additional file 8: Table S6), indicating that the segmental duplication of *WRKY* genes might have occurred in diploid progenitors before the divergence of the cucumber and watermelon.
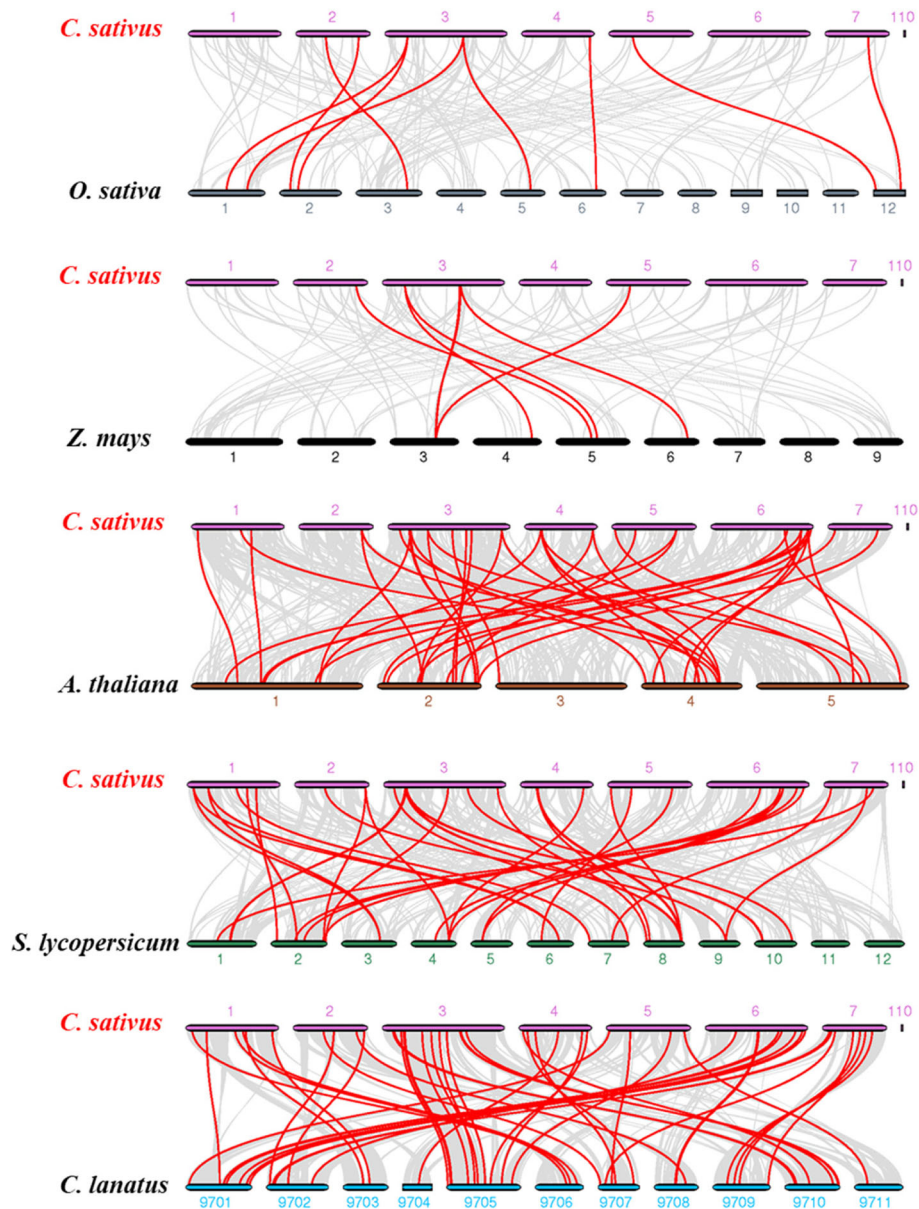
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Fig. 6 Synteny analysis of *WRKYs* between cucumber and other plant species. The collinear blocks are marked by gray lines, while the collinear gene pairs with *WRKY* genes are highlighted in the red lines. '*C. sativus*', '*O. sativa*', '*Z. mays*', '*A. thaliana*', '*S. lycopersicum*' and '*C. lanatus*' indicate *Cucumis sativus, Oryza sativa, Zea mays, Arabidopsis thaliana, Solanum lycopersicum,* and *Citrullus lanatus,* respectively

In 2015, Rinerson et al. used the genome sequences of a moss to propose the hypothesis that Group III genes were not the last to evolve; rather, Group IIa genes were [20]. Among the *WRKY* gene family, Group IIa genes compose the subclade with the smallest number of members and appear to play many important roles in the response to different stresses [20]. The availability of increased numbers of Group IIa members of sequenced plant genomes could provide additional clues about the evolution of the WRKY TF family. In this study, we found that members of the plant WRKY Group IIa from

closely related species tended to be clustered together, and there were monocot (clades 1, 2 and 3a)- and dicot (clades 3b and 4–7)-specific clades (Fig. 3). These results suggested that *WRKY* IIa genes might have evolved independently after the divergence of monocots and dicots.

It is well known that gene expression is correlated with gene function [75]. In this study, the expression pattern of all 61 *CsWRKY* genes was analysed in nine different tissues of cucumber, including the roots, stems, leaves, flowers, ovaries, and tendrils. We found that 19 *CsWRKY* genes were expressed specifically in the roots
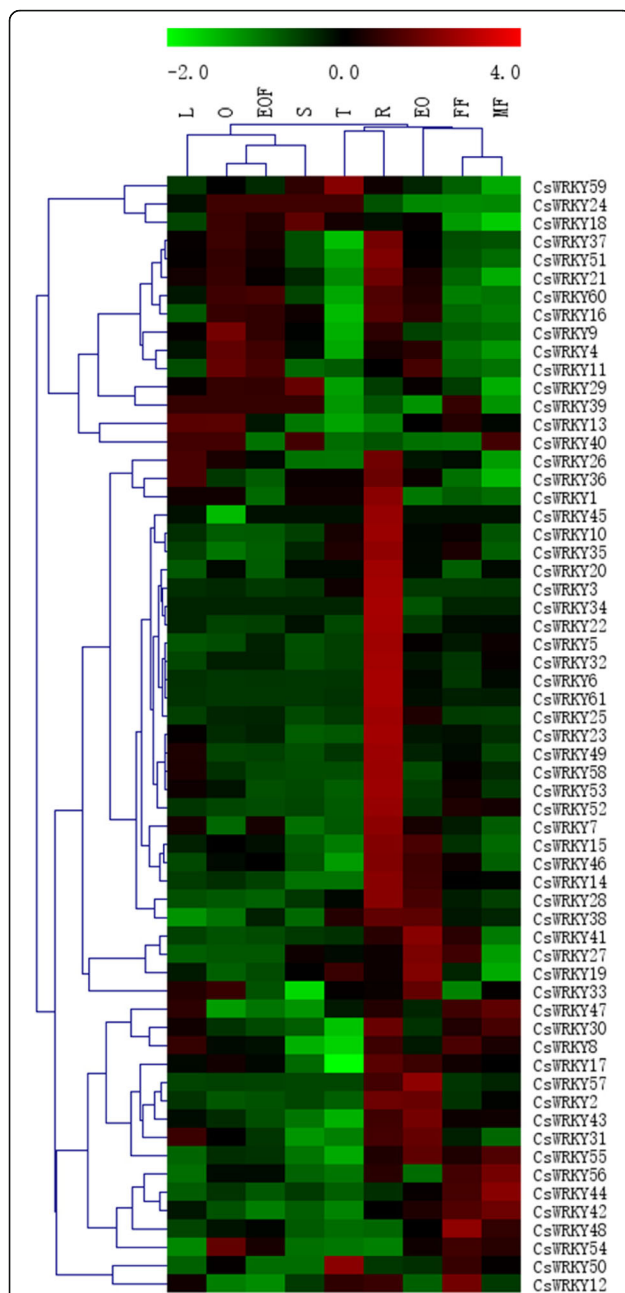
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Fig. 7 Tissue-specific expression of *WRKY* genes in cucumber. The transcriptional levels of *CsWRKY* genes in nine tissues of cucumber 9930 were investigated based on a public transcriptome data, and only one biological replication was used for each tissue sample [69]. The genome-wide expression of *CsWRKY* genes was shown on a heatmap using FPKM value, and the expression data were gene-wisely normalized by MeV (Multiple Experiment Viewer) software. -2.0 to 4.0 was artificially set with the color scale limits according to the normalized value. The color scale shows increasing expression levels from green to red. L, leaves; O, ovary; EOF, expanded fertilized ovary; S, stem; T, tendril; R, root; EO, expanded unfertilized ovary; FF, female flower; MF, male flower

(Fig. 7). As previously reported, *AtWRKY23*, *AtWRKY75* and *AtWRKY6* were found to regulate root development [76, 77], and their close genetic homologous genes in cucumber, *CsWRKY25*, *CsWRKY32* and *CsWRKY52*, respectively, were expressed specifically in the roots. According to these results, all genes expressed specifically in the roots were assumed to be key regulators of root development and may play roles in response to various stresses that first affect plants below ground. Additionally, *CsWRKY50* and *CsWRKY59* were highly expressed in the tendrils, which are considered abnormal leaves in cucumber, implying that they may regulate leaf morphogenesis in cucumber. Noticeably, the expression profiles of cucumber *WRKY* genes in this study were not consistent with the results in the study of Ling et al. [55]. For example, *WRKY18* and *WRKY56* have extremely different patterns of expression profiles between these two studies (Fig. 7) [55]. The reason might be that although they have the same name, they actually are different genes. This prediction could be supported by that *WRKY56* could not be conclusively mapped to any chromosome in the previous study [55], but to chromosome 7 in our study (Additional file 1: Fig. S1), and that *WRKY18* was localized in different position on chromosome 3 (Additional file 1: Fig. S1) [55]. However, now it is very difficult to clarify the reason because that the v1.0 of the cucumber genome sequences is not available yet, and Ling et al. did not provide the gene or protein sequences of WRKYs in their paper [55].

WRKY proteins constitute one of the most important TF families and are involved in responses to biotic and abiotic stresses [25]. At least 26 and 54 *WRKY* genes were identified to respond to abiotic stress in Arabidopsis and rice, respectively [38, 78]. In this study, we further explored the expression of 61 *CsWRKY* genes under multiple stresses. Most of them were induced/repressed by at least one of the stresses that we tested (heat, salinity, and inoculation of DM and PM), indicating that the *CsWRKYs* play crucial roles in cucumber stress responses. Four *CsWRKY* genes, *CsWRKY9*, *CsWRKY18*, *CsWRKY48* and *CsWRKY57*, were responsive to heat and/or salinity stress but not to the inoculation of DM and PM (Figs. 8 and 9). Previous studies have revealed that one *WRKY* gene can function in response to several stresses. For example, overexpressing *AtWRKY30* improved tolerance to oxidative and salinity stresses during seed germination [79]. Among these four genes, the transcript level of *CsWRKY57* was significantly affected by both salt and heat stress treatments (Fig. 8), suggesting that this gene acts as the most important gene to regulate susceptibility to abiotic stresses in cucumber. Correspondingly, 10 *CsWRKY* genes (*CsWRKY1/3/4/15/ 21/22/30/46/53/58*) were affected only by inoculation of PM and/or DM and not by abiotic (heat and salt)
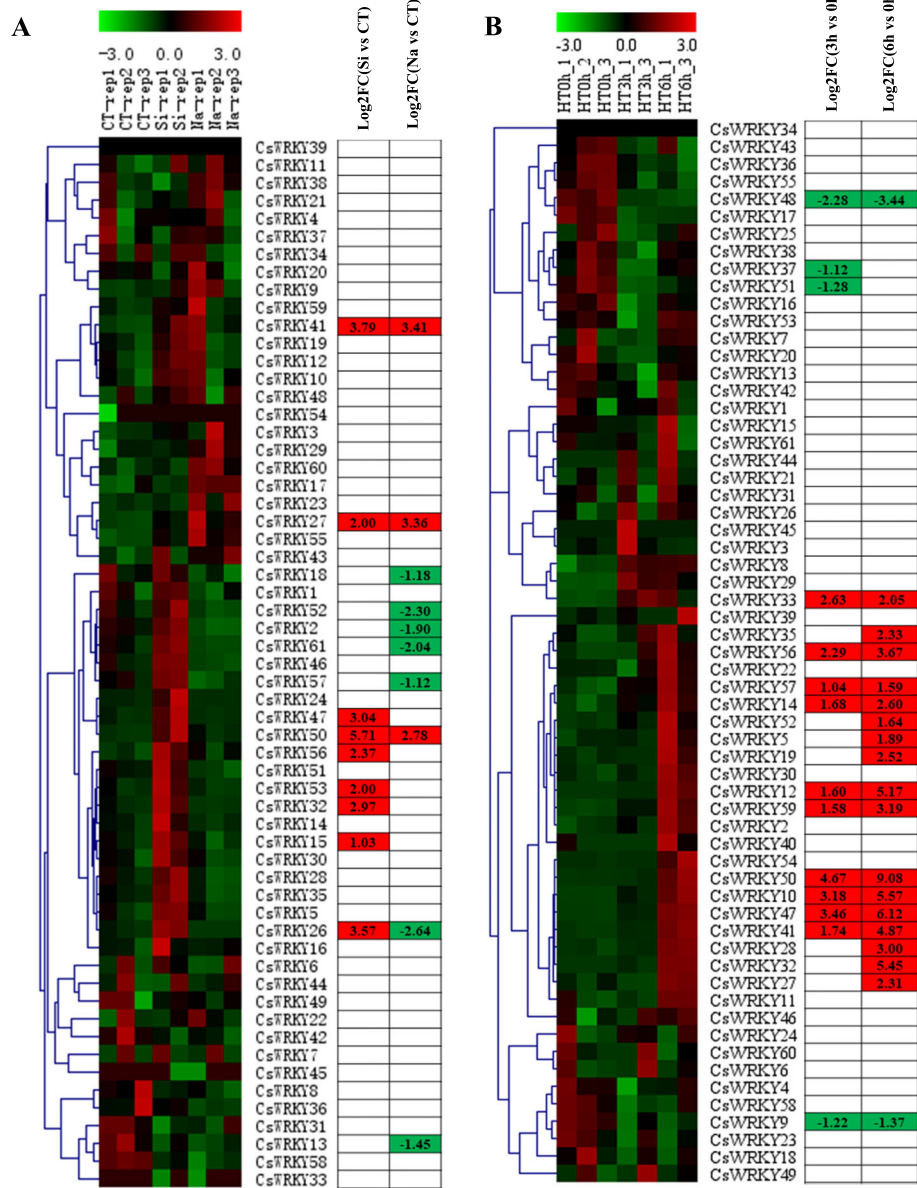
Fig. 8 Expression profiles of *CsWRKY* genes in response to various abiotic stress treatments. The transcriptional levels of *CsWRKY* genes in response to salt (**a**) and heat (**b**) stresses were investigated based on a public transcriptome data [70] and transcriptome data that we performed, respectively. The genome-wide expression of *CsWRKY* genes under different treatments was shown on a heatmap using FPKM values, and the expression data were gene-wisely normalized by MeV software. -3.0 to 3.0 was artificially set with the color scale limits according to the normalized value. The color scale shows increasing expression levels from green to red. In the table, differentially expressed genes (DEGs) were highlighted by red (up-regulation) and green (down-regulation). FC, fold-change; CT, control; HT, heat treatment; HT0h = heat treatment for 0 h (hours); HT3h = heat treatment for 3 h; HT6h = heat treatment for 6 h

stresses (Figs. 8 and 9), and only *CsWRKY46* was observed to be differentially expressed in response to inoculation of PM and DM. These results revealed significant differences in the stress-induced expression of *WRKYs* in response to abiotic and biotic stresses. In addition, 22 *CsWRKY* genes were significantly affected by both abiotic stresses and biotic stresses (Figs. 8 and 9), indicating that some *CsWRKY* genes have similar functions in response to both abiotic

and biotic stresses. For instance, the expression of *CsWRKY59* was affected by all tested treatments except salt treatment; *CsWRKY27*, *CsWRKY50* and *CsWRKY52* simultaneously responded to all treatments that we analysed. The expression of *OsWRKY67* was activated by rice blast inoculation; overexpression of *OsWRKY67* in rice plants enhanced resistance to leaf blast, panicle blast and bacterial blight [80]; and its orthologue in cucumber,
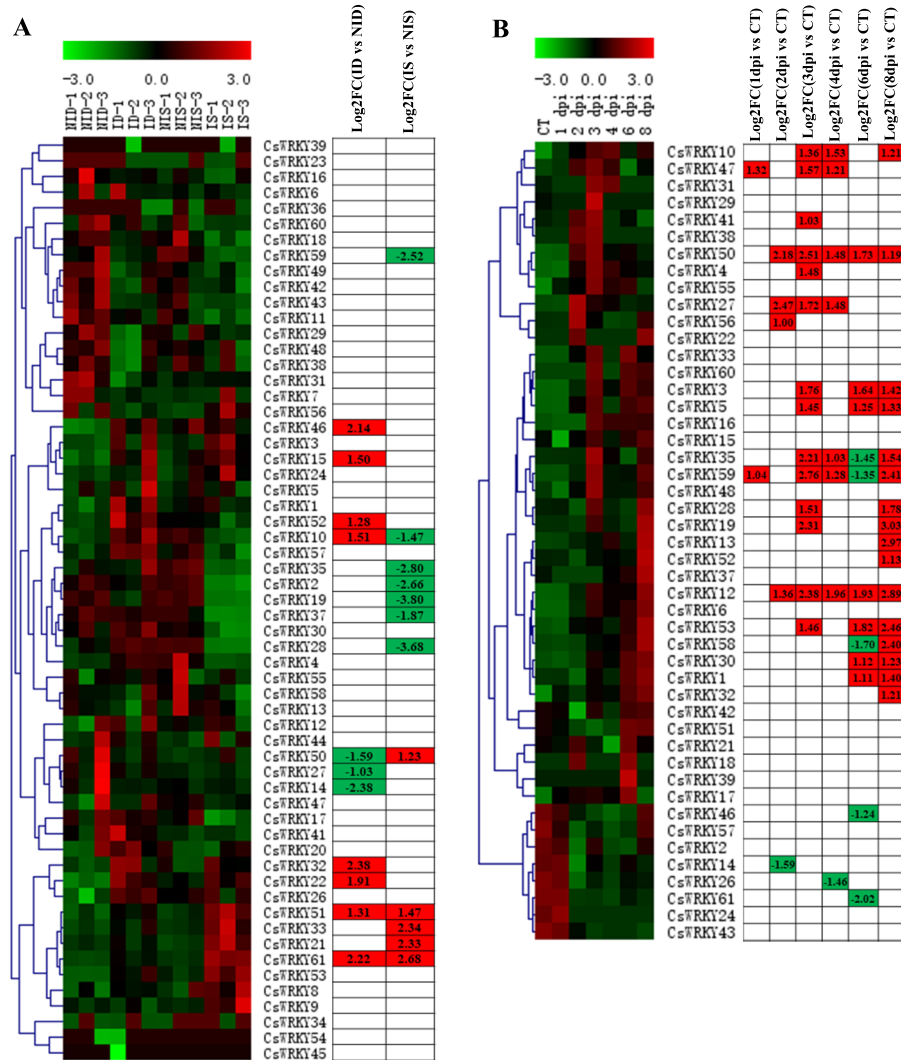
Fig. 9 Expression analysis of *CsWRKYs* under biotic stresses. The *CsWRKY* genes transcripts were determined after the infection of powdery mildew (PM) for 48 h (**a**) [71] and of downy mildew (DM) for 1 to 8 days post inoculation (**b**) [54], respectively. Without inoculation as the control (CT). The FPKM values of *CsWRKY* genes under different treatments were gene-wisely normalized and shown on a heatmap by MeV software. -3.0 to 3.0 was artificially set with the color scale limits according to the normalized value. The color scale shows increasing expression levels from green to red. In the tables, red shadings indicated up-regulated genes, green shading indicated down-regulated genes. FC, fold-change; ID, DM inoculated susceptible cucumber line D8 leaves; NID, non-inoculated D8 leaves; IS, DM inoculated resistant cucumber line SSL508–28 leaves; NIS, non-inoculated SSL508–28 leaves; DPI, day post inoculation

CsWRKY50, was also induced by biotic stresses, suggesting the potential value of *CsWRKY27, CsWRKY50* and *CsWRKY52* in improvements to cucumber abiotic and biotic stress tolerance. Moreover, the expression of 12 *CsWRKY* genes (*CsWRKY6/16/17/24/29/31/38/ 39/42/43/55/60*) was not observed in response to either the biotic stresses or abiotic stresses we analysed in this study.

As shown in Fig. 9, the expression of *CsWRKY19* was downregulated by PM infection but upregulated by DM. The results indicated that *WRKY* genes might play

different roles under different stress responses. Further analysis showed that responses to stresses occurred at different timepoints. *CsWRKY10* and *CsWRKY47* responded to heat stress at 3 h, whereas the expression of *CsWRKY28* and *CsWRKY35* was affected at 6 h after heat stress began; *CsWRKY56* was highly expressed only at 2 dpi, while infection by DM upregulated the expression of *CsWRKY12* and *CsWRKY50* between 2 to 8 dpi, suggesting that *CsWRKY* genes might play important regulatory roles at different stages in cucumber abiotic and biotic stress tolerance.

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Overall, these above findings provide insights into the potential functions of cucumber *WRKY* genes. The differential expression in response to different stresses indicated their functional diversification. Some *CsWRKY* genes might specifically respond to biotic or abiotic stress, while several genes may respond to both biotic and abiotic stress. In addition, some *CsWRKY* genes might not be involved in stress responses. These results are helpful for future functional characterization of *CsWRKY* genes and for the genetic improvement of the abiotic and biotic stress resistance of cucumber.

Conclusions

In the present study, 61 cucumber *WRKY* genes were identified, and a comprehensive analysis of those *CsWRKY* genes was carried out. First, the chromosomal location, conserved motifs, evolutional relationships and gene structure of the cucumber *WRKY* genes were examined. The expression patterns of the *CsWRKY* genes in nine different tissues of cucumber cultivar 9930 and in response to various stresses then showed that these genes may play important roles in cucumber growth and development. Furthermore, our results revealed differences and similarities in the stress-induced expression of *CsWRKYs* in response to abiotic and biotic stresses. In conclusion, our study provided a foundation for future studies into the functions of *WRKY* genes important in responses to abiotic and biotic stresses and the identification of new sources of resistance for breeding programmes.

Methods

Gene identification and chromosomal locations

The hidden Markov model (HMM) file of the WRKY domain (PF03106), downloaded from the Pfam protein family database (http://pfam.sanger.ac.uk/), was used for the identification of *WRKY* genes from the cucumber genomic database (v3.0) by HMMER 3.0. The default parameters were employed, and the cutoff value was 0.01. All *CsWRKY* genes that were queried from the cucumber genomic data based on the HMMER results were further examined to confirm the existence of the WRKY domain sequences through the Pfam (http://pfam.xfam.org/search#tab-view=tab1) and SMART (http://smart.embl-heidelberg.de/) databases. We then manually examined each candidate gene to ensure the conserved heptapeptide sequence within the predicted WRKY domain and used PCR amplification and sequencing to further validate select *CsWRKY* genes. Sixty-one *WRKY* genes were ultimately identified and mapped to cucumber chromosomes according to their physical location information from the cucumber genomic database. The

subcellular localization of CsWRKY proteins was predicted using CELLO (http://cello.life.nctu.edu.tw/).

CsWRKY genes structure analysis, classification and phylogenetic analysis

The gene structures of all identified cucumber WRKY genes were identified by the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/). The cucumber *WRKY* genes were classified into different groups according to the classification scheme of Arabidopsis *WRKY* genes and the WRKY domain alignments of CsWRKY and AtWRKY proteins. Alignments of the amino acid sequences of the following were performed using ClustalX with default settings: WRKY domains from cucumber and Arabidopsis (excluding the C-terminal domains of Group I); 61 full-length CsWRKYs; and the Group IIa WRKY domains from Arabidopsis [16], castor bean [64], cucumber, grape [67], tomato [19], pear [63], potato [66], poplar [62], barley [59], rice [17], maize [18], bread wheat [51], *Brachypodium* [57] and millet [81]. Phylogenetic trees were then constructed based on the alignments using the neighbour-joining (NJ) method of MEGA 5.0. The trees were visualized and optimized via Evolview (http://www.evolgenius.info/evolview).

Motif composition analysis of CsWRKY proteins

The motifs within the 61 cucumber WRKY protein sequences were identified using the MEME online program (http://meme.nbcr.net/meme/intro.html) with the following parameters: number of repetitions, any; maximum number of motifs, 10; and optimum width of each motif, between 6 and 300 residues.

Analysis of gene duplication

The Multiple Collinearity Scan toolkit (MCScanX) was used to examine the gene duplication events, with the default parameters [82]. To explore the syntenic relationships of the *WRKY* genes obtained from cucumber and other selected species, syntenic analysis maps were constructed using MCScanX.

Regulatory elements in the promoter regions of *CsWRKY* genes

The elements in the 1.5 kb promoter fragments (upstream sequences of the CsWRKY-encoding sequences) of the *CsWRKY* genes were analysed using the online PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Transcriptome analysis of *WRKY* genes in cucumber

The expression patterns of the *CsWRKYs* were analysed based on published RNA-seq data (SRA046916) [69]. Clean tags were remapped to the cucumber genome

sequence (http://cucurbitgenomics.org/, v3.0) by Biomarker Technologies (Beijing, China), and the FPKM values were recalculated. These analyses were performed on 9 different cucumber tissues: roots, stems, leaves, female flowers, male flowers, ovaries, expanded unfertilized ovaries, expanded fertilized ovaries, and tendrils. Only one biological replication was used for each tissue sample [69]. The genome-wide expression of the *CsWRKY* genes was shown on a heatmap using MeV (Multiple Experiment Viewer) software, and the expression levels are shown by a colour bar that changes from green to red.

Transcriptome analysis of *CsWRKYs* in response to abiotic and biotic stresses

The expression regulation of *CsWRKY* genes responsive to different stresses was obtained from publicly available transcriptomic data, which were downloaded from Gene Expression Omnibus and analysed to reveal the genome-wide differentially expressed genes after treatment with salt (GSE116265) [70] and inoculation with DM (SRP009350) [54] and PM (GSE81234) [71]. Every treatment had three or two biological replicates. The FDR (or *P* value) and absolute value of log2 (fold-change) that were published in the original literature were used for the identification of DEGs [54, 70, 71]. Because the gene ID shown was according to the cucumber genome v2.0, we cross-referenced the gene IDs of the *CsWRKYs* with those of the cucumber genome v3.0. The expression of the *CsWRKY* genes was then shown by a heatmap using MeV software.

The seedlings of the 'Chinese long' inbred line 9930, which was obtained from X. Gu Lab of Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, and used for cucumber genome sequencing, were treated at 42 °C, and the leaves of the seedlings were taken at 0, 3 and 6 h after treatment for transcriptome sequencing in Novogene (Beijing, China). Three biological replicates were performed. The transcript abundance of *CsWRKY* genes was calculated as fragments per kilobase of exon model per million mapped reads (FPKM). The sequencing reads data were submitted to the National Center for Biotechnology Information (NCBI) GEO Sequence Read Archive with accession number of GSE151055.

For the transcriptome analysis of *CsWRKYs* in response to abiotic and biotic stresses, a threshold of FDR (or *P* value) ≤ 0.05 and an absolute value of log2 (fold-change) ≥ 1 were used to define DEGs.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-020-02625-8.

> **Additional file 1: Figure S1.** The *WRKY* genes we identified mapped on every chromosome according to the current version of cucumber genome (v3.0).

> **Additional file 2: Figure S2.** The number of *WRKY* genes mapped on every chromosome according to cucumber genome v1.0 (previous study) and v3.0 (this study).
>
> **Additional file 3: Table S1.** List of the 61 *CsWRKY* genes identified in this study.
>
> **Additional file 4: Table S2.** List of WRKY domains sequence derived from cucumber and Arabidopsis.
>
> **Additional file 5: Table S3.** List of WRKY domain sequences of WRKY IIa proteins.
>
> **Additional file 6: Table S4.** Analysis of conserved motifs in cucumber WRKY proteins.
>
> **Additional file 7: Table S5.** Segmentally duplicated *CsWRKY* gene pairs.
>
> **Additional file 8: Table S6.** Orthologous relationships between cucumber and Arabidopsis/tomato/watermelon/rice/maize.
>
> **Additional file 9: Table S7.** List FPKM of *WRKY* genes in cucumber different tissues.
>
> **Additional file 10: Table S8.** RNA-seq data (FPKM values) of *CsWRKY* genes under salt/heat treatment/powdery mildew /downy mildew infection.
>
> **Additional file 11: Figure S3.** Hierarchical clustering of cucumber gene expression profiles under heat treatment. A, Hierarchical cluster of expressed cucumber genes in 9 samples. In the color panels, each transverse line represents a single gene and the color of the line indicates the expression level of the gene relative to the mean center in a specific sample: red, high expression; green, low expression level. B, The hierarchical clustering on the gene expression matrix, using the Pearson correlation coefficient as a proxy of similarity between transcriptomes. HT0h = heat treatment for 0 h (hours), HT3h = heat treatment for 3 h, HT6h = heat treatment for 6 h.

Abbreviations
TFs: Transcription factors; HMM: Hidden Markov Model; CDS: Coding DNA sequence; MW: Molecular weight; pl: Isoelectric point; aa: amino acid; NJ: Neighbor-joining; R protein: Resistance protein; ORF: Open reading frame; DEGs: Differential expression genes; h: Hours; Si: Silicon; DM: Downy mildew; PM: Powdery mildew

Authors' contributions
ZR and CC conceived and designed the experiments. XC and JH provided transcriptome data. CC analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and its additional files; Cucumber sequences in this article can be found from the CuGenDB (http://cucurbitgenomics.org/); The *Arabidopsis thaliana* sequences in this article were downloaded from TAIR (https://www.arabidopsis.org/index.jsp). The public transcriptome data are available at Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/). The sequencing reads data of cucumber in response to high temperature stress are deposited at GEO with accession number of GSE151055 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE151055). All plant materials were selected from cucumber provided by the Z. Ren lab, Shandong Agricultural University, Taian, China.

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