Identification and expression analyses of WRKY genes reveal their involvement in growth and abiotic stress response in watermelon (Citrullus lanatus)

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Abstract

Despite identification of WRKY family genes in numerous plant species, a little is known about WRKY genes in watermelon, one of the most economically important fruit crops around the world. Here, we identified a total of 63 putative WRKY genes in watermelon and classified them into three major groups (I-III) and five subgroups (IIa-IIe) in group II. The structure analysis indicated that ClWRKYs with different WRKY domains or motifs may play different roles by regulating respective target genes. The expressions of ClWRKYs in different tissues indicate that they are involved in various tissue growth and development. Furthermore, the diverse responses of ClWRKYs to drought, salt, or cold stress suggest that they positively or negatively affect plant tolerance to various abiotic stresses. In addition, the altered expression patterns of ClWRKYs in response to phytohormones such as, ABA, SA, MeJA, and ETH, imply the occurrence of complex cross-talks between ClWRKYs and plant hormone signals in regulating plant physiological and biological processes. Taken together, our findings provide valuable clues to further explore the function and regulatory mechanisms of ClWRKY genes in watermelon growth, development, and adaption to environmental stresses.

Introduction

Transcription factors (TFs) play an essential role in regulating the transcription of specific target genes by binding to their promoters. The WRKY gene family is one of the 10 largest transcription factor families in plants [1]. Typically, proteins in this family possess one or two highly conserved WRKY domains which include a conserved WRKYGGQK heptapeptide at N-terminus and a distinctive zinc-finger like motif C$_2$H$_2$ or C$_2$HC at C-terminus [2, 3]. To regulate gene expression, the WRKY domain binds to the cis-acting element W box (TTGACC/T)
in the promoter of the target gene [4]. In addition to W box, WRKY proteins can also bind to other elements, such as a sugar-responsive (SURE) cis-element (TAAGATTACTAATA GGAA) and a pathogen-responsive element PRE4 (TGCCTT), indicating the multiplicity in the mechanism of their functions [3].

Since the first cloning and characterization of the WRKY cDNA, SPF1 from sweet potato [5], numerous WRKY gene families have been analyzed from more than 100 other plant species [6]. In Arabidopsis, 72 WRKY genes were classified into three groups I-III based on the number of WRKY domains and the features of their zinc-finger structure [2]. Group I proteins contain two WRKY domains, one at the C- and the other at the N-terminus, whereas group II and group III proteins only contain one single WRKY domain at N-terminus. Both group I and group II proteins have the same pattern of potential zinc ligands (C-X4,5-C-X12,23-H-X-H), while, Group III proteins contain one C2-H-C zinc-finger motif. Furthermore, group II proteins can be accurately divided into five subgroups (IIa+b, IIc, IId+e) based on the phylogenetic data of the WRKY domains [2, 7]. Phylogenetic analysis has proven that the groups II and III originated from the oldest group I proteins [7, 8], and the group II genes are not monophyletic [3]. In addition, genomic comparison from a model species to a less-studied species can provide important information on the expansion and the evolution of the WRKY gene families in plants [9].

Numerous studies have established the important roles of WRKY proteins in various physiological processes such as seed germination [10], lateral root formation [11], flowering time [12], fruit ripening [13], leaf senescence [14], and metabolic processes as well [15, 16]. WRKY proteins also play important regulatory roles in plant defense against various biotic and abiotic stresses, such as pathogens, nutrient deficiency, UV-B, heavy metals, salinity, drought and cold stress [17–19]. For instance, overexpression of BnWRKY33 enhances resistance to Sclerotinia sclerotiorum in transgenic oilseed rape [20], while, double mutants of AtWRKY54 and AtWRKY70 in Arabidopsis clearly show enhanced tolerance to osmotic stress due to improved water retention and stomatal closure [21]. Notably, the regulatory roles of WRKY proteins are closely associated with multiple plant hormone-mediated signal pathways. In rice, various phytohormone treatments significantly alter expression patterns of 54 WRKY genes [22]. AtWRKY50 and AtWRKY51 work as positive regulators in the salicylic acid (SA) signaling pathway but as negative regulators in jasmonic acid (JA) signaling [23]. Increasing number of studies show that abscisic acid (ABA) signaling pathway is involved in the WRKY proteins-mediated responses of plants to various abiotic stresses [24–26].

The watermelon (Citrullus lanatus (thunb.) Matsum. & Nakai) is one of the most economically important fruit crops in the world. According to FAOSTAT2014, watermelon is cultivated all over the world with total area of 3.48 million hectares and annual production of 11.10 million tons, making it among the top five most consumed fresh fruits (http://www.fao.org/). Since a high-quality draft genome sequence of the East Asia watermelon cultivar 97103 has been reported [27], many transcription factors including mitogen-activated protein kinase (MAPK), no apical meristem-ATAF1/2-cup shaped cotyledon (NAC), and nuclear factor Y (NF-Y) have been subsequently identified and analyzed in watermelon [28–30]. However, there is still little information about WRKY genes in watermelon and their responses to environmental stresses and plant hormones. In this study, we performed a genome-wide identification of CIWRKYS in watermelon and analyzed their classification, chromosome distribution, phylogeny, structure, duplication, conserved motifs, and expression patterns in different tissues. Moreover, we further investigated the expression profiling of CIWRKY genes in response to abiotic stresses and plant hormones to exploit their potential functions in abiotic stress tolerances. Our study identified a subset of potential candidate CIWRKYs which can be utilized.
for enhancement of stress tolerance in Cucurbitaceae through genetic manipulation and rational breeding.

Materials and methods

Identification and annotation of WRKY genes in watermelon

To genome-wide identify WRKY genes in watermelon genome, both BLAST and Hidden Markov Model (HMM) methods were used in this study. Firstly, 22 and 102 WRKY proteins identified from Arabidopsis (https://www.arabidopsis.org/) and rice (http://rice.plantbiology.msu.edu/) were used as query sequences to search against the watermelon protein database (Version1, http://www.icugi.org) using BLASTP program with default settings. In addition, the HMM profile of the WRKY DNA-binding domain (Pfam: PF03106) downloaded from Pfam database (http://pfam.xfam.org/) was also exploited for identification of WRKY genes from watermelon using HMMER3.0 with E-value setting to 1e-2 [31]. Then, all putative non-redundant candidates were further subjected to identify partial or intact WRKY homologs in watermelon genome using TBLASTN methods (E-value setting to 1e-10). After parsing the BLAST files with in-house perl scripts, the new homologs were validated using non-redundant protein database from NCBI, and only sequences with best hit of WRKY protein were considered as candidate genes. Finally, all non-redundant putative WRKY genes were examined by the presence of WRKY domains using Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd/), Pfam and ScanProsite (http://prosite.expasy.org/scanprosite/).

Using the software JoinMap 4.0 (https://www.kyazma.nl/index.php/mc.JoinMap/), the distribution of watermelon WRKY genes were constructed based on their chromosomal locations. Additionally, the molecular weight (MW), Theoretical isoelectric point (pI), instability index, aliphatic index, Grand average of hydropathicity (GRAVY) of watermelon WRKY proteins were predicted via the ProtParam tool from ExPASy (http://web.expasy.org/protparam/). An advanced protein subcellular localization prediction tool WoLF PSORT (https://wolfsort.hgc.jp/) was used to predict the subcellular localization.

Multiple sequence alignment, classification, and phylogenetic analysis

The protein sequences of ClWRKY genes obtained from watermelon, as well as 7 AtWRKY genes (AtWRKY6, AtWRKY11, AtWRKY22, AtWRKY25, AtWRKY56, AtWRKY60 and AtWRKY66), were aligned using software MUSCLE [32], and visually edited by Genedoc to analyze the conserved WRKY core domain (60 amino acid). A further multiple sequence alignment of 184 complete protein sequences (listed in S1 Table) including 57 ClWRKY genes from watermelon, 72 AtWRKY from Arabidopsis and 55 CsWRKY from cucumber, was performed using MUSCLE. Based on the alignment, a neighbor-joining phylogenetic tree was constructed using MEGA 7.0 with 1000 bootstrap value and Jones-Taylor-Thornton (+ G) method [33]. An online software iTOL was applied to beautify the phylogenetic tree (http://itol.embl.de/).

Gene structure analysis and identification of motifs

The exon-intron organization of the watermelon WRKY genes were generated by comparing their coding sequences (CDS) with their respective full-length sequences (http://cucurbitgenomics.org/) using the online program Gene Structure Display Server (GSDS: http://gds.cbi.pku.edu.cn) [34]. The conserved motifs of CIWRKY proteins were analyzed online using Multiple Expectation Maximization for Motif Elicitation (MEME) with default parameters (http://meme-suite.org/tools/meme).
Gene duplication and synteny analysis

Duplication pattern and synteny analysis were performed following the procedures described previously [35]. All watermelon WRKY protein sequences were searched against themselves and proteins of *Arabidopsis* respectively, using BLASTp program with E-value setting to $1 \times 10^{-10}$ and output format as tabular (-m 8). Then, the destination tabular file, as well as the GFF files of watermelon and *Arabidopsis* genomes, were inputted into software MCScanX to analyze duplication types and syntenic relationship [36], and visualized using CIRCOS (http://circos.ca/).

Plant material and treatments

The seeds of watermelon inbred line ‘Y34’, a typical East Asia ecotype were provided by the Cucurbits Germplasm Resource Research Group at Northwest A&F University, Yangling, Shaanxi, China. For tissue-specific analysis, germinated seeds were directly sown in the experimental base at Northwest A&F University, Yangling, Shaanxi, China (34˚20’N, 108˚24’E), and the roots, stems, leaves, tendrils, fruit, male and female flowers were sampled separately during the fruit maturation period. For other treatments, germinated seeds were sown in plastic pots (8 cm × 7 cm × 7 cm) filled with commercial peat-based compost (Shaanxi Yufeng Seed Industry Co., Ltd., Yangling, China). The seedlings were grown under spring time natural light in a greenhouse at Northwest A&F University, where the temperature was 28–35˚C /16–20˚C (day/night). All plants were uniformly watered daily and nourished weekly with 1/2 strength Hoagland’s solution. Seedlings at the four-week stage were used for the following treatments.

Hormone treatments were performed by spraying leaves with 100 μM ABA [28], 1 mM SA [37], 100 μM methyljasmonate (MeJA), and 10 mM ethephon (ETH) [38], while leaves sprayed with distilled water served as control. Leaves were collected at 0.5, 1, 6, 12, 24, and 48 hours after treatments.

The salinity stress treatment was carried out by irrigating plants with 300 mM NaCl solution (80 mL per plant) in the pots [39], followed by sampling leaves at 6, 24, 48, 72, 96, and 120 hours after treatment. Similarly, plants irrigated with distilled water were used as control. For cold treatment, plants were kept in a growth chamber at 4˚C under a light intensity of 300 mmol·m⁻²·s⁻¹ PPFD [40], then leaves were sampled at 1, 3, 6, 12, 24, and 48 hours after the commencement of cold treatment, whereas seedlings kept at 27±1˚C under the same light condition were used as control. The drought stress treatment was accomplished creating a natural drought condition [41], and the control plants were well-watered to 70 ± 5% field capacity based on weighing. Then the drought-stressed and control leaves were sampled at 24, 48, 96, and 192 hours after treatment. In our study, at each time point of each treatment, the topmost second fully expanded leaves from four plants were pooled together in each biological sample, and three biological replicates were used in all treatments. Harvested samples were rapidly frozen in liquid nitrogen and stored at -80˚C for further analysis.

RNA isolation and semi-quantitative reverse-transcription PCR analysis

Total RNA of sampled leaves was extracted using the RNA Simple Total RNA Kit (DP432, TIANGEN, China). The integrity and quality of RNA were analyzed via 1.0% agar gel electrophoresis and the NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific, USA). Approximately 1 μg of total RNA was used for the synthesis of first strand of cDNA by the FastKing RT Kit with gDNase (KR116, TIANGEN, China). Gene-specific primers for the *ClWRKY* genes were designed using Primer Premier 6.0 (S2 Table). Semi-quantitative reverse-transcription (RT) PCR (LifeECO-TC96, BIOER, China) was done following the PCR procedures: initial denaturation at 94˚C for 90 s, followed by 40 cycles of denaturation at 94˚C for
30 s, annealing at 58 ± 5°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The amplification was done in a 20 μl reaction volume, which contained 10.0 μl 2×Taq Master Mix (E005, novoprotein, China), 0.6 μl of each primer (10 μM), 1.0 μl cDNA template, and 7.8 μl ddH2O. Meanwhile, a housekeeping gene β-actin gene (Cla007792) was used as internal control [42]. All PCR products were measured on a 2.0% agarose gels and imaged under UV light (ChampGel 6000, SAGECREATION, China) for gene expression analysis.

Real-time quantitative PCR
The quantitative RT-PCR was conducted on a LightCycler® 96 real time-PCR machine (Roche, Switzerland) using SYBR® Green Premix (TaKaRa Biotechnology, Dalian, China). The amplification was done in a 20 μl reaction volume, which contained 10.0 μl SYBR Green Premix, 0.8 μl of each primer (10 μM), 1.0 μl cDNA template (80 ng/μl), and 7.4 μl ddH2O. The PCR parameters were pre-denaturing at 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s. Melt-curve analyses were carried out using a program with 95°C for 15 s and then a constant growth from 60°C to 95°C with temperature increasing steps of 0.3°C/s. The gene-specific primers were same as those used for semi-quantitative RT-PCR (S2 Table). The watermelon β-actin gene (Cla007792) was used as an internal reference gene [42]. Each treatment was repeated using three technical replicates. All data from real-time quantitative PCR was calculated for relative expressions following the 2−ΔΔCt method as described by Livak and Schmittgen [43]. The relative expressions values were log2 transformed and were displayed in heat map using MeV 4.8.1(http://www.mybiosoftware.com).

Results
Identification and characterization of WRKY genes
As shown in Table 1, a total of 63 putative CIWRKY genes, including 57 full-length WRKY genes and 6 WRKY partial homologs, were finally identified in watermelon by HMM (PF03106) and TBLASTN search methods. All of these CIWRKY genes could be mapped on the chromosomes from chromosome 1 to 11 and were renamed from CIWRKY1 to CIWRKY63 based on their order on the chromosomes (Table 1; S1 Fig). The coding sequences (CDS) of 57 full-length CIWRKYs ranges from 366 to 2295 bp and the molecular weight (WM) of them varies from 14049.65 to 82969.81 Da. According to the isoelectric point (pI), 32 CIWRKYs were acidic proteins with pI value less than 7.0, and the remaining 25 proteins were basic proteins. According to the instability index, most of CIWRKY proteins, with the value of instability index higher than 40.0, were instability, except CIWRKY13 and CIWRKY29. Additionally, the WoLF PSORT prediction showed that 53 CIWRKY proteins were localized in nucleus, suggesting CIWRKY proteins play regulatory roles mainly in cell nucleus.

Multiple sequence alignment and phylogenetic analysis
The conserved heptapeptide of WRKYGQK is the most obvious structural feature of WRKY proteins, which can interact with the W box to activate target genes [2]. The WRKY domains spanning approximate 60 amino acids were analyzed using multiple sequence alignment (Fig 1). Heptapeptide WRKYGQK in most CIWRKY proteins was highly conserved. However, that in CIWRKY1 and CIWRKY47 mutated into KRQVEVQ and WRKYGKK, respectively, and that in CIWRKY2, CIWRKY5, CIWRKY11 and CIWRKY46 was missed. Additionally, identified zinc-finger motifs in some proteins encoded by CIWRKY3, CIWRKY15, CIWRKY24, and CIWRKY44 were missed.
| Group | Subgroup | Gene name | Gene locus ID | Chromosome | Start site | End site | Genomic (bp) | CDS (bp) | ORF (aa) | MW (Da) | pI | Instability index | Aliphatic index | GRAVY | Subcellular Localization |
|-------|----------|-----------|---------------|------------|------------|----------|--------------|----------|---------|---------|----|------------------|---------------|--------|-------------------------|
| I     |          | CIWRKY13  | Cha015673     | 2          | 2511354    | 2513937  | 2584        | 1308     | 435     | 4724.69 | 5.81 | 38.39           | 64.41         | -0.861 | Nuc                    |
|       |          | CIWRKY16  | Cha013402     | 2          | 2971764    | 4976     | 1365        | 454      | 49833.11 | 8.35  | 65.88         | 56.48         | -0.760 | Nuc                    |
|       |          | CIWRKY17  | Cha008104     | 3          | 162031     | 162530   | 2300        | 1765     | 584     | 63832.83 | 7.71 | 59.59           | 45.75         | -0.911 | Nuc                    |
|       |          | CIWRKY20  | Cha009557     | 3          | 1597067    | 1597906  | 5237        | 1944     | 647     | 70986.48 | 4.80 | 45.09           | 66.43         | -0.647 | Nuc                    |
|       |          | CIWRKY32  | Cha010216     | 5          | 3116426    | 3116869  | 2637        | 1482     | 493     | 53734.45 | 6.90 | 58.42           | 57.93         | -0.865 | Nuc                    |
|       |          | CIWRKY35  | Cha018733     | 6          | 2151065    | 2151842  | 2587        | 1542     | 513     | 55676.55 | 6.73 | 57.67           | 55.28         | -0.965 | Nuc                    |
| II    |          | CIWRKY13  | Cha004492     | 10         | 4866664    | 4871803  | 2435        | 1365     | 49833.11 | 8.35 | 65.88           | 56.48         | -0.760 | Nuc                    |
|       |          | CIWRKY16  | Cha009557     | 10         | 4866664    | 4871803  | 2435        | 1365     | 49833.11 | 8.35 | 65.88           | 56.48         | -0.760 | Nuc                    |
|       |          | CIWRKY17  | Cha008104     | 10         | 4866664    | 4871803  | 2435        | 1365     | 49833.11 | 8.35 | 65.88           | 56.48         | -0.760 | Nuc                    |
|       |          | CIWRKY20  | Cha009557     | 10         | 4866664    | 4871803  | 2435        | 1365     | 49833.11 | 8.35 | 65.88           | 56.48         | -0.760 | Nuc                    |
|       |          | CIWRKY32  | Cha010216     | 5          | 2151065    | 2151842  | 2587        | 1542     | 513     | 55676.55 | 6.73 | 57.67           | 55.28         | -0.965 | Nuc                    |
|       |          | CIWRKY35  | Cha018733     | 6          | 2151065    | 2151842  | 2587        | 1542     | 513     | 55676.55 | 6.73 | 57.67           | 55.28         | -0.965 | Nuc                    |
|       |          | CIWRKY13  | Cha021021     | 5          | 2432874    | 2433174  | 2910        | 909      | 302     | 33770.91 | 6.34 | 41.19           | 71.36         | -0.663 | Nuc                    |
|       |          | CIWRKY16  | Cha022362     | 5          | 2432874    | 2433174  | 2910        | 909      | 302     | 33770.91 | 6.34 | 41.19           | 71.36         | -0.663 | Nuc                    |
|       |          | CIWRKY17  | Cha022362     | 5          | 2432874    | 2433174  | 2910        | 909      | 302     | 33770.91 | 6.34 | 41.19           | 71.36         | -0.663 | Nuc                    |
|       |          | CIWRKY18  | Cha022362     | 5          | 2432874    | 2433174  | 2910        | 909      | 302     | 33770.91 | 6.34 | 41.19           | 71.36         | -0.663 | Nuc                    |

(Continued)
| Group | Subgroup | Gene name | Gene locus ID | Chromosome | Start site (bp) | End site (bp) | Genomic CDS ORF (bp) | MW (Da) | pI | Instability index | Aliphatic index | GRAVY | Subcellular Localization |
|-------|----------|-----------|---------------|------------|----------------|--------------|---------------------|---------|----|------------------|----------------|-------|------------------------|
| II d  | CIWRKY6  | Cla013967 | 1             | 2695865    | 26959762      | 1109         | 894 297             | 32337.68 | 9.64 | 48.87            | 74.24          | -0.528 | Nucl                   |
| II d  | CIWRKY14 | Cla006772 | 2             | 9026189    | 9027547       | 1359         | 870 289             | 31279.29 | 9.58 | 46.61            | 72.63          | -0.495 | Nucl                   |
| II d  | CIWRKY31 | Cla020642 | 5             | 2806432    | 28065905      | 1584         | 1143 380            | 41065.28 | 9.60 | 50.51            | 62.63          | -0.592 | Nucl                   |
| II d  | CIWRKY33 | Cla009969 | 5             | 3315463    | 33156285      | 1647         | 1056 351            | 37729.73 | 9.60 | 53.38            | 60.40          | -0.503 | Nucl                   |
| II d  | CIWRKY36 | Cla018870 | 6             | 23022662   | 23024071      | 1410         | 837 278             | 30229.44 | 9.75 | 42.23            | 66.98          | -0.560 | Nucl                   |
| II d  | CIWRKY39 | Cla006015 | 7             | 2332176    | 2333889       | 1714         | 756 251             | 27970.95 | 9.67 | 48.00            | 63.71          | -0.707 | Nucl                   |
| II d  | CIWRKY51 | Cla014818 | 9             | 6327518    | 6329225       | 1708         | 1071 356            | 39557.25 | 9.54 | 56.93            | 79.97          | -0.478 | Nucl                   |
| II e  | CIWRKY9  | Cla009853 | 1             | 33474006   | 33477082      | 3077         | 1455 484            | 54152.82 | 5.95 | 59.70            | 50.79          | -0.998 | Nucl                   |
| II e  | CIWRKY18 | Cla019646 | 3             | 8018421    | 8019422       | 1002         | 831 276             | 30132.24 | 5.15 | 45.40            | 55.83          | -0.752 | Nucl                   |
| II e  | CIWRKY19 | Cla019756 | 3             | 9691619    | 9695338       | 3720         | 1203 400            | 43272.40 | 5.96 | 42.58            | 55.90          | -0.732 | Nucl                   |
| II e  | CIWRKY25 | Cla021207 | 5             | 1219248    | 1220421       | 1174         | 996 331             | 36795.95 | 5.78 | 65.63            | 55.98          | -0.779 | Nucl                   |
| II e  | CIWRKY38 | Cla002243 | 7             | 1054123    | 1056739       | 2617         | 903 300             | 31750.79 | 5.32 | 59.85            | 54.37          | -0.700 | Nucl                   |
| II e  | CIWRKY56 | Cla017355 | 10            | 1685484    | 16856049      | 1207         | 954 317             | 35609.63 | 4.67 | 60.56            | 71.36          | -0.733 | Extr                   |
| III   | CIWRKY23 | Cla021170 | 5             | 925058     | 926889        | 1832         | 1017 338            | 37697.76 | 5.09 | 49.63            | 68.67          | -0.574 | Nucl                   |
| III   | CIWRKY27 | Cla004233 | 5             | 8987121    | 898806        | 1386         | 1065 354            | 39061.06 | 5.35 | 49.07            | 62.57          | -0.620 | Nucl                   |
| III   | CIWRKY40 | Cla007306 | 7             | 6705089    | 6711611       | 6523         | 978 325             | 35540.94 | 6.86 | 59.33            | 66.37          | -0.478 | Nucl                   |
| III   | CIWRKY41 | Cla007307 | 7             | 6714426    | 6716677       | 2252         | 858 285             | 31505.05 | 6.36 | 44.77            | 64.14          | -0.518 | Nucl                   |
| III   | CIWRKY45 | Cla010918 | 5             | 71145037   | 71145784      | 1748         | 1086 361            | 40510.40 | 5.69 | 55.65            | 53.77          | -0.897 | Nucl                   |
| III   | CIWRKY52 | Cla015003 | 9             | 8487793    | 8489885       | 2093         | 921 306             | 33831.35 | 5.46 | 60.15            | 58.59          | -0.625 | Nucl                   |
| III   | CIWRKY61 | Cla018059 | 10            | 28283762   | 28284680      | 919          | 831 276             | 30809.43 | 5.90 | 54.42            | 61.88          | -0.734 | Nucl                   |
| NG    | CIWRKY2  |           |               |            |               |             |                      |         |     |                  |                |       |                        |
| NG    | CIWRKY5  |           |               |            |               |             |                      |         |     |                  |                |       |                        |
| NG    | CIWRKY11 |           |               |            |               |             |                      |         |     |                  |                |       |                        |
| NG    | CIWRKY43 |           |               |            |               |             |                      |         |     |                  |                |       |                        |
| NG    | CIWRKY46 |           |               |            |               |             |                      |         |     |                  |                |       |                        |
| NG    | CIWRKY48 |           |               |            |               |             |                      |         |     |                  |                |       |                        |

NG, no group; CDS, coding sequence; ORF, open reading frame; MW, molecular weight; pI, theoretical isoelectric point; GRAVY, grand average of hydropathicity; Nucl, nuclear; Mito, mitochondrial matrix; Extr, extracellular; Cyto, cytoplasmic.

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**Fig 1. Alignment of 63 ClWRKY and 7 AtWRKY domain amino acid sequences.** Alignment was accomplished using MUSCLE. ‘N’ and ‘C’ indicate the N-terminal and C-terminal WRKY domain of a specific WRKY protein, respectively. The amino acids forming the zinc-finger motif are highlighted in blue, the conserved WRKY amino acid domains is highlighted in green. 

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To analyze the evolutionary relationships, a total of 184 WRKY genes, including 57 from watermelon, 55 from cucumber, and 72 from *Arabidopsis*, were used to generate a phylogenetic tree (Fig 2). Based on the WRKY domains and the specific zinc-finger motifs, 57 ClWRKYs were classified into three groups (I-III) with 11 ClWRKYs in group I, 39 in group II, and 7 in group III. The ClWRKYs in Group II were further divided into five subgroups (IIa-IIe) with the most genes in Group IIc. Obviously, proteins in group I contained two WRKY
domains located at both the N-terminus and C-terminus and the zinc finger motif of C2H2 type at N-terminus and C-terminus was C-X4-C22-H-X-H and C-X4-C23-H-X-H, respectively (Fig 1). By contrast group I, group II and group III genes had only one WRKY domain. Proteins in group IIa, IIb, IId and Ile contained a zinc finger motif of C-X5-C23-H-X-H, whereas those in group Iic contained a zinc finger motif of C-X4-C23-H-X-H at C-terminus, except CIWRKY1. Group III proteins contained a C2HC zinc-finger motif of C-X7-C-X23-H-X-C at C-terminus. In addition, sequence comparisons and phylogenetic analyses showed that WRKY proteins from three species appeared scattered across the branches of the evolutionary tree, implying that they experienced duplications after the lineages diverged. Meanwhile, a total of 42 WRKY genes from cucumber and watermelon were clustered as 21 pairs (Fig 2), indicating that they were the orthologous WRKY domains from the same lineage.

Gene structure and conserved motifs analysis

The exon-intron analysis was performed to obtain a better insight into the structure of CIWRKY genes, which exhibited relatively smaller variation in numbers of exons and introns. As shown in Fig 3, 57 CIWRKY genes had two to six exons. Among them, 27 CIWRKYs had three exons, followed by eleven CIWRKYs with four exons, eight CIWRKYs with two exons, eight CIWRKYs with five exons, and three CIWRKYs with six exons. These divergences suggested that both exon gain and loss was occurred during the evolution of the WRKY gene family. CIWRKY genes in the same group usually seemed to have similar exon-intron structures. For instance, six genes from seven members in Group III had three exons. In comparison to CIWRKYs in group II and group III, those in group I had more exons, ranging from four to six. These findings provided an additional foundation to support the classification of CIWRKYs.

We further searched for the conserved motifs in 57 CIWRKY proteins using MEME program (Fig 4). In total, 24 conserved motifs named as motif 1 to motif 24 were identified and the details of the 24 putative motifs were listed in S3 Table. Motif 1 and 2 were found in most of CIWRKY genes. Motifs 1, 3, and 15 contained a WRKYGQK sequence. As expected, most CIWRKY proteins in the same group or subgroup possessed similar motifs, suggesting their functional equivalency. For example, motifs 1, 2, 3, 4, and 9 were present in group I proteins, which contained two WRKY domains, whereas group III proteins possessed motifs 1, 2, 8, and 17.

Gene duplication and synteny analysis

Currently, we evaluated the gene duplication events of CIWRKY genes using MCScanX program. Ninety-four syntenic relations of CIWRKYs were identified as duplication events in watermelon genome (Fig 5; S4 Table), and 45 CIWRKY genes were located within syntenic blocks on all watermelon chromosomes. Chromosome 1, 5 and 10 had more duplication regions and that could partly explain the larger numbers of CIWRKY genes located on these three chromosomes. Gene duplication events are defined as either tandem duplications, with two or more genes located on the same chromosome, or segmental duplications, with duplicated genes present on different chromosomes [44]. There were 11 CIWRKY genes clustered into 6 tandem duplication event regions on watermelon chromosome 1 (CIWRKY4/CIWRKY6 and CIWRKY7/CIWRKY8), 5 (CIWRKY23/CIWRKY27 and CIWRKY27/CIWRKY30), 7 (CIWRKY40/CIWRKY42), and 10 (CIWRKY59/CIWRKY60). The other 88 syntenic relations of CIWRKY genes were confirmed as segmental duplications, suggesting that most CIWRKY genes were possibly generated by gene segmental duplication.
Identification and expression analysis of watermelon *WRKY* genes

**Fig 3. Illustration of the gene structure of 57 CWRKY transcription factors.** Genes were separated into their respective groups with different colors. Exons were shown using green round-corner rectangle while introns were shown using black solid lines (5'→3').

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Fig 4. Schematic representation of 24 conserved motifs in watermelon WRKY proteins. Conserved motifs were named as motif 1 to motif 24, and different motif was shown as colored boxes with their names in the center of the boxes. The colored boxes were ordered manually according to the results of the MEME analysis. The length of each box in the figure does not represent the actual motif size.

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Arabidopsis was widely used as a model system for plant WRKY TFs research [45]. In order to further explore evolutionary and functionality connections between watermelon and Arabidopsis WRKY genes, a synteny analysis was performed. A total of 103 pairs of syntenic relations were identified, including 51 AtWRKY genes and 45 ClWRKY genes (Fig 6; S5 Table). Out of

Fig 5. Synteny analysis of watermelon WRKY genes. Chromosomes 1–11 were shown with different colors and in a circular form. Colored curves indicated the details of syntenic regions in watermelon genome.

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these genes, 13 AtWRKY genes and 18 CiWRKY genes were found to be associated with at least three synteny events, and three CiWRKY genes (CiWRKY8, CiWRKY18 and CiWRKY23) were involved in six synteny events. A lot of synteny events indicate that numerous WRKY genes existed before the divergence of the Arabidopsis and watermelon.
Expression profiles of the CIWRKY genes in different tissues

By means of semi-quantitative observation, we analyzed the expression profiles of 57 full-length CIWRKY genes in seven different tissues including roots, stems, leaves, tendrils, fruit, male flowers, and female flowers under normal growth conditions. Then, the gene-specific primers of 52 CIWRKYs were successfully found in our study (S2 Table). The results showed that 52 CIWRKYs genes were detected in at least one of the seven tested tissues (Fig 7). Among them, 17 (33%) CIWRKY genes were expressed in all tested tissues. The other genes were detected in only one or several tissues. For instance, CIWRKY37 and CIWRKY42 were found only in roots, whereas CIWRKY15 was preferential accumulation in male flower. Some genes such as CIWRKY61 and CIWRKY56 were not observed in any tested tissue possibly due to their expression in other tissues or too low expression level (below detection limit via semi-quantitative observation).

Expression patterns of the CIWRKY genes under various abiotic stresses

To investigate the potential roles of CIWRKY genes in response to abiotic stresses, we analyzed their dynamic response after exposure to drought, cold, and salinity stresses using qRT-PCR. CIWRKY genes exhibited different expression patterns in response to different stresses (Fig 8). Drought treatment continuously induced the expression of about half of the detected CIWRKY genes but reduced the expression of four CIWRKY genes including CIWRKY18, CIWRKY23, CIWRKY27 and CIWRKY58. The expression of CIWRKY15, CIWRKY25 and CIWRKY61 transiently increased at 24 h but decreased at 192 h after drought treatment. Additionally, 11 CIWRKYs such as CIWRKY12, CIWRKY34, and CIWRKY41 slightly induced or reduced at the earlier or later period of drought stress. Similarly, cold stress (4˚C) continuously up-regulated and down-regulated about half of detected CIWRKYs and four CIWRKY genes (CIWRKY13, CIWRKY32, CIWRKY51 and CIWRKY56), respectively. Several CIWRKYs such as CIWRKY27 showed a transient down-regulation at 1 h but then continuously up-regulated with the advancement of cold stress. Most of CIWRKY genes were up-regulated but only CIWRKY18 were down-regulated by the NaCl treatment. Some CIWRKYs such as CIWRKY58 showed a transient down-regulation at the median or later period of salt stress. Notably, the expressions...
of CIWRKY14 and CIWRKY60 were up-regulated by all stresses, suggesting that these two genes may play a core role in plant tolerance to diverse abiotic stresses.

Expression patterns of the CIWRKY genes to hormone treatments

Numerous evidences have indicated that WRKY TFs are involved in signal pathways of various plant hormones such as ABA, MeJA, SA, and ETH [46]. As shown in Fig 9, ABA treatment induced both up- and down-regulation of almost all CIWRKYs at different time points. Most of CIWRKYs were down-regulated or remained unchanged at 0.5 and 1 h but ABA application up-regulated their expression at 12 h and 24 h. Similarly, many of CIWRKY genes were both up-regulated and down-regulated at different periods by MeJA, as well as ABA treatment. Most of CIWRKYs were induced or remained unchanged at 0.5, 6, 12, and 48 h but MeJA treatment reduced their expression at 24 h. Being different with ABA and MeJA, SA application induced continuous up-regulation of 12 CIWRKYs and down-regulation of three CIWRKYs. The other CIWRKYs showed different expression patterns at different time-points after SA treatment. In response to ETH treatment, about a half of CIWRKY genes showed continuous up-expression from 1 to 48 h, with some exceptions showing down-expression at 24 h and 48 h. ETH treatment down-regulated expression of the other CIWRKYs. These results indicate

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that ClWRKY genes are positively or negatively involved in regulatory pathways of plant hormones and thus play important roles in plant growth, development, and defense against environmental stresses.

### Discussion

#### Annotation and characterization of WRKY genes in watermelon genome

Due to the important roles of WRKY transcriptional factors in plant growth, development, and response to stresses, a large number of WRKY families have been identified in diverse plant species (S6 Table). However, there is dearth of information about WRKY genes in watermelon. Here, we identified a total of 63 putative ClWRKY genes in watermelon. In addition to 57 full-length ClWRKY genes, 6 ClWRKY partial homologs (ClWRKY2, ClWRKY5, ClWRKY11, ClWRKY43, ClWRKY46 and ClWRKY48) were found using TBLASTN method with in-house Perl scripts. This operation ensured that WRKY genes were complete in watermelon genome, although no particular annotation information was available for these 6 WRKY partial homologs. Moreover, our further analysis revealed the properties (i.e. amino acid length, molecular weight, isoelectric point, and instability) and subcellular location of ClWRKY proteins.

According to the classification of WRKY genes in Arabidopsis [2], 57 full-length ClWRKY genes were also classified into three groups (I-III) and five subgroups (IIa-IIe) of group II based on their conserved WRKY domain and zinc-finger motif. The size of ClWRKYs in each group is similar to that of CsWRKYs in cucumber [47], indicating a similar evolutionary pattern between watermelon and cucumber (Fig 2). However, the size of the ClWRKY gene family (63) is small compared to that of model plants such as Arabidopsis (72) and rice (102). The differences in the number of WRKY genes in group III are the primary cause of the diverse sizes of WRKY gene families (S6 Table). Group III WRKY genes have been described as a newly

![Fig 9. Expression patterns of ClWRKY genes under exogenous hormone treatments.](https://doi.org/10.1371/journal.pone.0191308.g009)
defined and the most dynamic group with a large number of duplications events [7]. Therefore, group-III WRKY genes may play important roles in plant evolution.

The number of exons in CIWRKY genes varied from two to six (Fig 3), and the exon-intron structural diversification might be caused by the rearrangement and fusions of different chromosome fragments [44, 48]. This finding provided an additional foundation to support the classification of CIWRKYs and a way to find out which group WRKY genes might be of a more ancient origin [8]. Moreover, we should pay attention to the roles of special motif in only one group, such as motif 16 in group IId proteins and motif 17 in group III proteins (Fig 4), which may possess some hitherto uncharacterized roles.

Multiple sequence alignments revealed that WRKYGQK sequence was highly conserved in most of CIWRKY TFs. The WRKYGQK sequence was considered to be important for recognizing and binding to W box elements in the promoter of target genes [3]. Previous studies have reported a number of variants of the WRKYGQK sequence in diverse plant species and proteins with these variants may recognize the other binding elements other than the W box element [7, 49]. In watermelon WRKY genes, we found two variants of the WRKY domain: WRKYGKK in CIWRKY1 and KRQYEVQ in CIWRKY47 (Fig 2). WRKYGKK sequence was the most common variant in many plants and proteins with this sequence instead of WRKYGQK could bind specifically to WK box (TTTTCCAC) [50, 51]. Strikingly, variant KRQYEVQ sequence was found only in watermelon (CIWRKY1). Variations of WRKYGQK motif might change DNA binding specificities of downstream target genes, and thus it would be interesting to validate the biological functions of CIWRKY1 and CIWRKY47. Moreover, deletion events of the zinc-finger motif occurred in four CIWRKY genes (CIWRKY3, CIWRKY15, CIWRKY24 and CIWRKY44) in group IIC. The replacement or deletion of the zinc-finger motif might lead to the evolution and classification of WRKY genes [44, 52]. However, it remains largely unknown whether the deletions of zinc-finger motif influence the function and the expression patterns of WRKY genes.

**Expansion and synteny of CIWRKY genes**

Gene duplications played a crucial role in genomic rapid expansions and evolution of gene families [53]. A number of evidence pointed that duplication and expansion events happened in plant WRKY genes [44, 54, 55]. Here, 6 tandem duplication event regions and 88 segmental duplications event regions of CIWRKY genes were confirmed in watermelon chromosome (Fig 5; S4 Table), suggesting that low-tandem and high-segmental duplications events existed in CIWRKY genes family. This finding is in agreement with that in Arabidopsis and Populus trichocarpa [52, 54], but incongruent with that in rice [21], which could be due to a large scale artificial selection and the differences of life cycle between rice and watermelon. Interestingly, three CIWRKY genes (CIWRKY23, CIWRKY27 and CIWRKY40) with tandem duplication were belonged to group III genes, implying that a different pattern of duplication may cause smaller size of group III WRKY genes in watermelon.

Moreover, most of CIWRKY genes were found in syntenic genomic regions of Arabidopsis (Fig 6; S5 Table). The large numbers of gene duplication events between watermelon and Arabidopsis will help us to understand the functions of CIWRKY genes. For example, over-expression of AtWRKY57 in rice improves drought and salt tolerance [56]. Moreover, AtWRKY70 is involved in brassinosteroids (BRs)-regulated growth and negatively affects drought responses [57]. Meanwhile, CIWRKY60 and CIWRKY61 were in the synteny region with these well-known AtWRKY genes, predicting a similar functional mechanism of CIWRKY60 and CIWRKY61 in drought resistance in watermelon. Nevertheless, gene functional study is required to better predict their roles.
Possible roles of ClWRKY genes in normal and stress conditions

Results from numerous studies demonstrate that WRKY transcription factors play very critical role in different tissues to regulate plant growth and developmental processes [3]. For instance, virus-induced silencing of GmWRKY58 and GmWRKY76 in soybean causes severe stunted growth with reduced leaf size and plant stature [58], and overexpression of the OsWRKY31 could reduce lateral root formation and elongation [10]. In our study, CIWRKY13, CIWRKY31, CIWRKY51 and CIWRKY63 showed higher expression levels in all tested tissues (Fig 7). Among them, CIWRKY13 and CIWRKY63 were orthologous genes of CsWRKY49 and CsWRKY37, respectively, implying that these genes play key roles in the whole-plant growth and development [21, 47]. Furthermore, some CIWRKY genes were specifically expressed in a certain tested tissue. As shown in Fig 7, CIWRKY15 were preferentially expressed on male flower, suggesting that CIWRKY15 might play important roles during development of male reproductive organs. In addition, CIWRKY genes with low expression level in all tested tissues might be expressed specifically in other tissues such as seeds, or could be induced under environment stimuli. For example, CIWRKY56 and CIWRKY61 were not detected in leaves under normal condition, but they were induced by abiotic stresses (Fig 7), suggesting the involvement of these genes in stress signaling.

Increasing evidence show that WRKY proteins in various plant species are involved in the response plants to various abiotic stresses such as drought, cold, and salt [16, 59]. In Arabidopsis, rice, and cucumber, at least 20, 54, and 23 WRKY genes were identified in response to diverse abiotic stress, respectively [17, 21, 47]. The responses of CIWRKYs to abiotic stress and plant hormones treatments can provide useful clues for dissecting the potential roles of WRKY genes in watermelon. In this study, we found that most of CIWRKYs positively or negatively responded to drought, cold, and salt stresses, and their responses altered with the degree of stresses. These results provided a useful reference for functional verification of CIWRKYs under environmental stresses. Additionally, the expression patterns of CIWRKYs in watermelon responses to abiotic stresses differed greatly from those in other plant species such as cucumber and pear [47, 60], suggesting that there were different gene duplication and evolution ways among different plant species.

Orthologous genes are generally supposed to retain equivalent functions in different species and to share other key properties [60]. The comparative analysis of CIWRKY genes with their homologous genes in other plant species helped to predict the potential functions of WRKY proteins in watermelon. In cucumber, CsWRKY46 can be induced by various stresses and its overexpression conferred cold tolerance to transgenic plants by positively regulating signaling pathway [47, 61]. As its orthologous genes, CIWRKY8, CIWRKY34 and CIWRKY53 also showed significant up-regulation under salt, cold, and drought stress, respectively (Fig 8), these genes may play similar roles with CsWRKY46 in stress response. Ding et al. [62] demonstrated that AtWRKY46 played dual roles in regulating plant responses to drought and salt stresses. Its orthologous gene CIWRKY23 also was found to be up-regulated under drought and salt stresses. AtWRKY25 in Arabidopsis has been known to respond to both heat and salt treatments [63]. Similarly, its orthologous gene CIWRKY60 is obviously and continuously induced by multiple abiotic stresses. These results imply that a single WRKY gene may play various regulatory roles in response to stresses. In addition, AtWRKY70 and AtWRKY54 cooperate as negative regulators for stomatal closure and thus regulate osmotic stress tolerance in Arabidopsis [20]. The negative responses of their orthologous genes CIWRKY41 and CIWRKY61 at the end of drought treatment imply they can play negative regulatory roles at the later period of drought.
Given that plant hormones such as ABA, SA, JA, and ETH, play critical roles in regulating plant growth, development, and defense against various abiotic and biotic stresses [17, 64, 65], a number of WRKY genes have been demonstrated to be involved in diverse plant hormone signal pathways [20, 23]. Ding et al. [66] revealed that the expression of AtWRKY46 was repressed by ABA signal, but induced by an ABA-independent signal under osmotic and salt stress, as well as AtWRKY54, AtWRKY70, and CsWRKY46 [20, 61]. As homologous of these well-known WRKY genes, ClWRKY60, ClWRKY23, ClWRKY41 and ClWRKY61 expressions in watermelon were up-regulated by drought stress (Fig 8), but were down-regulated by ABA at 0.5 or 1 h after treatment (Fig 9). Thus, these four CIWRKY genes may regulate watermelon response to drought stress via interacting with ABA signaling. In addition, Cho et al. [67] and Yang et al. [68] found that CIWRKY70 (same as CIWRKY41 in this study) and CIWRKY1 (same as CIWRKY17 in this study) play a positive regulatory role in plant resistance against pathogen attack. In our study, CIWRKY41 and CIWRKY17 were significantly up-regulated by SA treatment, implying that these two genes were involved in SA-mediated signaling pathways in plant defense responses. These results indicate that the regulatory mechanism of WRKY proteins under abiotic stresses is complex and the functional dissection of WRKY genes in signaling pathways and stress responses will be an important research topic for the future.

Conclusions

In this study, we identified watermelon WRKY family genes and analyzed their expression patterns for the first time. A total of 63 putative CIWRKY genes in watermelon were obtained using comprehensive computational approaches. All 63 CIWRKY genes located on 11 chromosomes were classified into three major groups (I-III) and five subgroups (IIa-IIe) in group II. CIWRKYs may play multiple regulatory roles based on their varied structures with conserved or varied WRKY domain and different motifs. The expression of CIWRKYs in different tissues indicated that they were involved in various tissue growth and development. Furthermore, the diverse responses of CIWRKYs to abiotic stresses suggested that they positively or negatively participated in plant tolerance against drought, salt, or cold stress. Altered expression patterns of CIWRKYs by phytohormones such as, ABA, SA, MeJA, and ETH, implied the existence of complex crosstalks between CIWRKYs and plant hormone signals in regulating plant physiological and biological processes. Therefore, our findings provide valuable clues for further research on the function and regulatory mechanisms of CIWRKY TFs in watermelon growth, development, and adaption to environmental stresses.

Supporting information

S1 Table. Full-length protein sequence of WRKY genes from watermelon, Arabidopsis, and cucumber. (XLSX)

S2 Table. The primers of WRKY genes in watermelon. (XLSX)

S3 Table. The details of the 24 putative motifs of watermelon WRKY genes. (DOCX)

S4 Table. The synteny regions between watermelon WRKY genes. (XLSX)

S5 Table. The synteny regions between watermelon and Arabidopsis WRKY genes. (XLSX)
S6 Table. Number of WRKY TF gene family members present in major crop plants and horticultural plants.

S7 Table. The relative expression of watermelon WRKY genes via qRT-PCR in response to various abiotic stresses and hormone treatments.

S1 Fig. Genetic map position on watermelon chromosomes of WRKY gene family. The numbers indicate the start site of WRKY genes located on chromosomes.

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References
1. Wei KF, Chen J, Chen YF, Wu LJ, Xie DX. Molecular phylogenetic and expression analysis of the complete WRKY transcription factor family in maize. DNA Res. 2012; 19(2):153–164. https://doi.org/10.1093/dnares/dsr048 PMID: 22279089.
2. Eulgem T, Rushton PJ, Robatzek S, Somssich IE. The WRKY superfamily of plant transcription factors. Trends Plant Sci. 2000; 5(5):199–206. PMID: 10785665.
3. Rushton PJ, Somssich IE, Ringler P, Shen QJ. WRKY transcription factors. Trends Plant Sci. 2010; 15(5):247–258. https://doi.org/10.1016/j.tplants.2010.02.006 PMID: 20304701.
4. Ingo Ciolkowski DW, Birkenbihl Rainer P., Somssich Imre E.. Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function. Plant Molecular Biology. 2008; 68(1–2):81–92. https://doi.org/10.1007/s11103-008-9353-1 PMID: 18523729.
5. Ishiguro S, Nakamura K. Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the S' upstream regions of genes coding for sporamin and beta-amyrase from sweet potato. Molecular & General Genetics Mgg. 1994; 244(6):563–571. PMID: 7969025.
6. Jin J, Tian F, Yang D-C, Meng Y-Q, Kong L, Luo J, et al. PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. Nucleic Acids Res. 2017; 45(D1):D1040–D1045. https://doi.org/10.1093/nar/gkw982 PMID: 27924042.
1. Zhang Y, Wang L. The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. BMC Evol Biol. 2005; 5:1–12. https://doi.org/10.1186/1471-2148-5-1 PMID: 15629062.

2. Wu KL, Guo ZJ, Wang HH, Li J. The WRKY family of transcription factors in rice and Arabidopsis and their origins. Dna Res. 2005; 12(1):9–26. PMID: 16106749.

3. Flagel LE, Wendel JF. Gene duplication and evolutionary novelty in plants. New Phytol. 2009; 183(3):557–564. https://doi.org/10.1111/j.1469-8137.2009.02923.x PMID: 19554345.

4. Gu Y, Li W, Jiang H, Wang Y, Gao H, Liu M, et al. Differential expression of a WRKY gene between wild and cultivated soybeans correlates to seed size. J Exp Bot. 2017; 68(11):2717–2729. https://doi.org/10.1093/jxb/erx246 PMID: 28472462.

5. Zhang J, Peng Y, Guo Z. Constitutive expression of pathogen-inducible OsWRKY31 enhances disease resistance and affects root growth and auxin response in transgenic rice plants. Cell Res. 2008; 18(4):508–521. https://doi.org/10.1038/cr.2007.104 PMID: 18071364.

6. Wei L, Wang H, Yu D. Arabidopsis WRKY transcription factors WRKY12 and WRKY13 oppositely regulate flowering under short-day conditions. Molecular Plant. 2016; 9(11):1492–1503. https://doi.org/10.1016/j.molp.2016.08.003 PMID: 27592586.

7. Cheng Y, JalalAhamed G, Yu J, Yao Z, Ruan M, Ye Q, et al. Putative WRKYs associated with regulation of fruit ripening revealed by detailed expression analysis of the WRKY gene family in pepper. Sci Rep. 2016; 6:39000. https://doi.org/10.1038/srep39000 PMID: 27991526.

8. Balazadeh S, Riano-Pachon DM, Mueller-Roeber B. Transcription factors regulating leaf senescence in Arabidopsis thaliana. Plant Biol (Stuttg). 2008; 10 Suppl 1:63–75. https://doi.org/10.1111/j.1438-8677.2008.00988.x PMID: 18721312.

9. Amato A, Cavallini E, Zenoni S, Finezzo L, Begheldo M, Ruperti B, et al. A grapevine TTG2-Like WRKY transcription factor is involved in regulating vacuolar transport and flavonoid biosynthesis. Front Plant Sci. 2016; 7:1979. https://doi.org/10.3389/fpls.2016.01979 PMID: 28105033.

10. Singh AK, Kumar SR, Dwivedi V, Rai A, Pal S, Shasany AK, et al. A WRKY transcription factor from Withania somnifera regulates triterpenoid withanolide accumulation and biotic stress tolerance through modulation of phytosterol and defense pathways. New Phytol. 2017; 215(3):1115–1131. https://doi.org/10.1111/nph.14663 PMID: 28649699.

11. Phukan UJ, Jeena GS, Shukla RK. WRKY transcription factors: molecular regulation and stress responses in plants. Front Plant Sci. 2016; 7:760. https://doi.org/10.3389/fpls.2016.00760 PMID: 27376564.

12. Ji J, Ma S, Ye N, Jiang M, Cao J, Zhang J. WRKY transcription factors in plant responses to stresses. J Integr Plant Biol. 2017; 59(2):86–101. https://doi.org/10.1111/jipb.12513 PMID: 27995748.

13. Karanja BK, Fan L, Xu L, Wang Y, Zhu X, Tang M, et al. Genome-wide characterization of the WRKY gene family in radish (Raphanus sativus L.) reveals its critical functions under different abiotic stresses. Plant Cell Rep. 2017. https://doi.org/10.1007/s00204-017-2190-4 PMID: 28819820.

14. Wang Z, Fang H, Chen Y, Chen K, Li G, Gu S, et al. Overexpression of BnWRKY33 in oilseed rape enhances resistance to Sclerotinia sclerotiorum. Mol Plant Pathol. 2014; 15(7):677–689. https://doi.org/10.1111/mpp.12123 PMID: 24521393.

15. Li J, Besseau S, Toronen P, Sipari N, Kollist H, Holm L, et al. Defense-related transcription factors WRKY70 and WRKY54 modulate osmotic stress tolerance by regulating stomatal aperture in Arabidopsis. New Phytol. 2013; 200(2):457–472. https://doi.org/10.1111/nph.12378 PMID: 23815736.

16. Ramamoorthy R, Jiang SY, Kumar N, Venkatesh PN, Ramachandran S. A comprehensive transcriptional profiling of the WRKY gene family in rice under various abiotic and phytohormone treatments. Plant Cell Physiol. 2008; 49(6):865–879. https://doi.org/10.1093/pcp/pcn061 PMID: 18413358.

17. Gao QM, Venugopal S, Navarre D, Kachroo A. Low oleic acid-derived repression of jasmonic acid-inducible defense responses requires the WRKY50 and WRKY51 proteins. Plant Physiol. 2011; 155(1):464–476. https://doi.org/10.1104/pp.110.166876 PMID: 21030507.

18. Yan H, Jia H, Chen X, Hao L, An H, Guo X. The cotton WRKY transcription factor GhWRKY17 functions in drought and salt stress in transgenic Nicotiana benthamiana through ABA signaling and the modulation of reactive oxygen species production. Plant Cell Physiol. 2014; 55(12):2060–2076. https://doi.org/10.1093/pcp/pcu133 PMID: 25261532.

19. Jaffar MA, Song A, Faeem M, Chen S, Jiang J, Liu C, et al. Involvement of CmWRKY10 in Drought Tolerance of Chrysanthemum through the ABA-Signaling Pathway. Int J Mol Sci. 2016; 17(5):393. https://doi.org/10.3390/ijms17050393 PMID: 27187353.

20. Luo DL, Ba LJ, Shan W, Kuang JF, Lu WJ, Chen JY. Involvement of WRKY transcription factors in abscisic-acid-induced cold tolerance of banana fruit. J Agric Food Chem. 2017; 65(18):3627–3635. https://doi.org/10.1021/acs.jafc.7b00915 PMID: 28445050.
28. Song Q, Li D, Dai Y, Liu S, Huang L, Hong Y, et al. Characterization, expression patterns and functional analysis of the MAPK and MAPKK genes in watermelon (*Citrullus lanatus*). BMC Plant Biol. 2015; 15:298. https://doi.org/10.1186/s12870-015-0681-4 PMID: 26700161.

29. Lv X, Lan S, Guy KM, Yang J, Zhang M, Hu Z. Global expression landscape of NAC transcription factor family and their responses to abiotic stresses in *Citrullus lanatus*. Sci Rep. 2016; 6:30574. https://doi.org/10.1038/srep30574 PMID: 27491393.

30. Yang J, Zhu J, Yang Y. Genome-wide identification and expression analysis of NF-Y transcription factor families in watermelon (*Citrullus lanatus*). J Plant Growth Regul. 2017; 1–18.

31. Finn RD, John T, Jain M, Coggill PC, John SS, Hans-Rudolf H, et al. The Pfam protein families database. Nucleic Acids Res. 2010; 38(Database issue):211–222. https://doi.org/10.1093/nar/gkm960 PMID: 18307903.

32. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32(5):1792–1797. https://doi.org/10.1093/nar/gkh340 PMID: 15034147.

33. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016; 33(7):1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904.

34. Hu B, Jin J, Guo JY, Zhang H, Luo J, Gao G. GSDS 2.0: an upgraded gene feature visualization server. Bioinformatics. 2015; 31(8):1296–1297. https://doi.org/10.1093/bioinformatics/btv285 PMID: 25504850.

35. Zhang H, Wei C, Yang X, Chen H, Yang Y, Mo Y, et al. Genome-wide identification and expression analysis of calcium-dependent protein kinase and its related kinase gene families in melon (*Cucumis melo* L.). Plos One. 2017; 12(4):e0176352. https://doi.org/10.1371/journal.pone.0176352 PMID: 28437432.

36. Wang Y, Tang H, Debarry JD, Tan X, Li J, Wang X, et al. MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. Nucleic Acids Res. 2012; 40(7):e49–e49. https://doi.org/10.1093/nar/gkr1293 PMID: 2217600.

37. Yang JH, Yuan G, Li YM, Qi XH, Zhang MF. Salicylic acid-induced enhancement of cold tolerance through activation of antioxidative capacity in watermelon. Sci Hortic. 2008; 118(3):200–205.

38. Zhang M, Yang XP, Xu JH, Liu G, Yao XF, Li PF, et al. Cloning and differential expression analysis of defensin gene Cldef2.2 from watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai). Acta Horticulturae. 2016; (1110):49–56.

39. Li H, Chang J, Chen H, Wang Z, Gu X, Wei C, et al. Exogenous melatonin confers salt stress tolerance to watermelon by improving photosynthesis and redox homeostasis. Front Plant Sci. 2017; 8:295. https://doi.org/10.3389/fpls.2017.00295 PMID: 28298921.

40. Kozik EU, Wehner TC. Tolerance of Watermelon seedlings to low-temperature chilling injury. Hortscience. 2014; 49(3):240–243.

41. Mo Y, Yang R, Liu L, Gu X, Yang X, Wang Y, et al. Growth, photosynthesis and adaptive responses of wild and domesticated watermelon genotypes to drought stress and subsequent re-watering. Plant Growth Regul. 2016; 79(2):229–41. https://doi.org/10.1007/s10725-015-0128-9.

42. Kong Q, Yuan J, Gao L, Zhao S, Jiang W, Huang Y, et al. Identification of suitable reference genes for gene expression normalization in qRT-PCR analysis in watermelon. PLoS One. 2014; 9(2):e90612. https://doi.org/10.1371/journal.pone.0090612 PMID: 24587403.

43. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4):402–408. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609.

44. Guo C, Guo R, Xu X, Gao M, Li X, Song J, et al. Evolution and expression analysis of the grape (*Vitis vinifera* L.) WRKY gene family. J Exp Bot. 2014; 65(6):1513–1528. https://doi.org/10.1093/jxb/eru007 PMID: 24510937.

45. Dong J, Chen C, Chen Z. Expression profiles of the *Arabidopsis* WRKY gene superfAMILY during plant defense response. Plant Mol Biol. 2003; 51(1):21–37. PMID: 12602888.

46. Peleg Z, Blumwald E. Hormone balance and abiotic stress tolerance in crop plants. Curr Opin Plant Biol. 2011; 14(3):290–295. https://doi.org/10.1016/j.pbi.2011.02.001 PMID: 21377404.

47. Ling J, Jiang W, Yang Y, Yu H, Mao Z, Gu X, et al. Genome-wide analysis of WRKY gene family in *Cucumis sativus*. BMC Genomics. 2011; 12:471. https://doi.org/10.1186/1471-2164-12-471 PMID: 21956985.

48. Xu G, Guo C, Shan H, Kong H. Divergence of duplicate genes in exon-intron structure. Proc Natl Acad Sci. 2012; 109(4):1187–1192. https://doi.org/10.1073/pnas.1109047108 PMID: 22232673.
49. Xie Z, Zhang ZL, Zou X, Huang J, Ruas P, Thompson D, et al. Annotations and functional analyses of the rice WRKY gene superfamly reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiol. 2005; 137(1):176–189. https://doi.org/10.1104/pp.104.054312 PMID: 15618416.

50. Van Verk MC, Pappaioannou D, Neeleman L, Bol JF, Linthorst HJ. A Novel WRKY transcription factor is required for induction of PR-1a gene expression by salicylic acid and bacterial elicitors. Plant Physiol. 2008; 146(4):1983–1995. https://doi.org/10.1104/pp.107.112789 PMID: 18263781.

51. Zhou QY, Tian AG, Zou HF, Xie ZM, Huang J, et al. Soybean WRKY-type transcription factors GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant Biotechnol J. 2008; 6(5):486–503. https://doi.org/10.1111/j.1476-7565.2008.00336.x PMID: 18384508.

52. He H, Dong Q, Shao Y, Jiang H, Zhu S, Cheng B, et al. Genome-wide survey and characterization of the WRKY gene family in Populus trichocarpa. Plant Cell Reports. 2012; 31(7):1199. https://doi.org/10.1007/s00299-012-1241-0 PMID: 22371255.

53. Vision TJ, Brown DG, Tanksley SD. The origins of genomic duplications in Arabidopsis. Science. 2000; 290(5499):2114–2117. https://doi.org/10.1126/science.290.5499.2114 PMID: 11118139.

54. 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 Biol. 2004; 4:10. https://doi.org/10.1186/1471-2229-4-10 PMID: 15171794.

55. Song H, Wang P, Lin JY, Zhao C, Bi Y, Wang X. Genome-wide identification and characterization of WRKY gene family in peanut. Front Plant Sci. 2016; 7:534. https://doi.org/10.3389/fpls.2016.00534 PMID: 27200012.

56. Jiang Y, Qiu Y, Hu Y, Yu D. Heterologous expression of AtWRKY57 confers drought tolerance in oryza sativa. Front Plant Sci. 2016; 7:145. https://doi.org/10.3389/fpls.2016.00145 PMID: 26904091.

57. Chen J, Nolan TM, Ye H, Zhang M, Tong H, Xin P, et al. Arabidopsis WRKY46, WRKY54, and WRKY70 transcription factors are involved in brassinosteroid-regulated plant growth and drought responses. Plant Cell. 2017; 29(6):1425–1439. https://doi.org/10.1105/tpc.17.00364 PMID: 28576847.

58. Yang Y, Chi Y, Wang Z, Zhou Y, Fan B, Chen Z. Functional analysis of structurally related soybean GmWRKY58 and GmWRKY76 in plant growth and development. J Exp Bot. 2016; 67(15):4727–4742. https://doi.org/10.1093/jxb/erw252 PMID: 27335454.

59. Chen L, Song Y, Li S, Zhang L, Zou C, Yu D. The role of WRKY transcription factors in plant abiotic stresses. Biochim Biophys Acta. 2012; 1819(2):120–128. https://doi.org/10.1016/j.bbabio.2011.09.002 PMID: 21963428.

60. Huang X, Li K, Xu X, Yao Z, Jin C, Zhang S. Genome-wide analysis of WRKY transcription factors in white pear (Pyrus bretschneideri) reveals evolution and patterns under drought stress. BMC Genomics. 2015; 16:104. https://doi.org/10.1186/s12864-015-2233-6 PMID: 26704366.

61. Zhang Y, Yu H, Yang X, Li Q, Ting J, Wang H, et al. CsWRKY46, a WRKY transcription factor from cucumber, confers cold resistance in transgenic-plant by regulating a set of cold-stress responsive genes in an ABA-dependent manner. Plant Physiol Biochem. 2016; 108:478–487. https://doi.org/10.1016/j.plaphy.2016.08.013 PMID: 27592172.

62. Ding ZJ, Yan JY, Xu XY, Yu DQ, Li GX, Zhang SQ, et al. Transcription factor WRKY46 regulates osmotic stress responses and stomatal movement independently in Arabidopsis. Plant J. 2014; 79(1):13–27. https://doi.org/10.1111/tpj.12538 PMID: 24773321.

63. Li S, Fu Q, Chen L, Huang W, Yu D. Arabidopsis thaliana WRKY25, WRKY26, and WRKY33 coordinate induction of plant thermotolerance. Planta. 2011; 233(6):1237–1252. https://doi.org/10.1007/s00425-011-1375-2 PMID: 21336597.

64. Kazan K. Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Sci. 2015; 20(4):219–229. https://doi.org/10.1016/j.tplants.2015.02.001 PMID: 25731753.

65. Khan MI, Fatta M, Per TS, Anjum NA, Khan NA. Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. Front Plant Sci. 2015; 6:462. https://doi.org/10.3389/fpls.2015.00462 PMID: 26175738.

66. Ding ZJ, Yan JY, Li CX, Li GX, Wu YR, Zheng SJ. Transcripti on factor WRKY46 modulates the development of Arabidopsis lateral roots in osmotic/salt stress conditions via regulation of ABA signaling and auxin homeostasis. Plant J. 2015; 84(1):56–69. https://doi.org/10.1111/tpj.12958 PMID: 26252246.

67. Cho S, Kang E, Min K, Lee Y, Kim Y, Yang K, et al. A positive regulatory role of the watermelon CWRKY70 gene for disease resistance in transgenic Arabidopsis thaliana. Biologia plantarum. 2012; 56(3):565–567. https://doi.org/10.1007/s10535-012-0070-x

68. Yang BY, Huo XA, Li PF, Wang CX, Duan HJ. Construction of cDNA expression library of watermelon for isolation of CWRKY1 transcription factors gene involved in resistance to Fusarium wilt. Indian J Biochem Bio. 2014; 51(4), 302. PMID: 25296501.