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Genome-wide Analysis of the *WRKY* Family Genes and their Responses to Cold Stress in Watermelon

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Abstract

Li Y.-M., Zhu L., Zhu H.-Y., Song P.-Y., Guo L.-Q., Yang L.-M. (2018): Genome-wide analysis of the *WRKY* family genes and their responses to cold stress in watermelon. Czech J. Genet Plant. Breed., 54: 168–176.

The WRKY transcription factors play important roles in various physiological processes, especially in regulating plant resistance to environmental stresses. Watermelon (*Citrullus lanatus*) plants suffer from various stressful climate conditions during their growth, especially cold stress. However, little information about the exact role of WRKYs in watermelon responses to cold was available. In this study, a total of 57 candidate *ClWRKY* genes from watermelon genome were identified and they were distributed unevenly on 11 chromosomes. After excluding five *ClWRKY* genes with incomplete WRKY domains, phylogenetic analysis showed that the remaining 52 *ClWRKY* genes could be divided into three groups with 11 members in Group 1, 34 in Group 2, and seven in Group 3. The *ClWRKY* genes in group 2 could be further classified into five subgroups with three members in 2a, five in 2b, 13 in 2c, six in 2d, and seven in 2e, respectively. The expression profiles of *ClWRKY* genes in response to cold stress could be classified into four types: four *ClWRKY* genes had little or no change in transcript levels, seven *ClWRKY* genes had irregular expression patters, 17 *ClWRKY* genes were upregulated, and 25 *ClWRKY* genes were downregulated. The different regulation patterns of *ClWRKY* genes in response to low-temperature treatment revealed that the *WRKY* gene family was crucial for cold stress tolerance and there were multiple regulatory pathways involved in cold resistance.

Keywords: *Citrullus lanatus*; cold stress; expression profile; phylogenetic analysis; WRKY transcription factor

The WRKY transcription factor family is one of the gene families that have the largest number of members in plants. There are one or two conserved WRKY domains with about 60 amino acids in all WRKYs, a conserved sequence WRKYGQK at the N-terminal and a C_2H_2 or C_2HC type zinc finger motif at the C-terminal region (Eulgem *et al.* 2000). The WRKY domain usually modulates gene transcription via facilitating the binding of WRKY proteins to W-box motif (TTGACC/T) in the promoter regions of target genes (Sun *et al.* 2003). The WRKYs can be classified into three groups by WRKY domain numbers and the type of zinc finger motifs (Eulgem *et al.* 2000). Two WRKY domains were found in WRKY proteins of Group I and the zinc finger motif was a C_2H_2 type. The WRKY proteins in Group II and III have a single WRKY domain followed by a C_2H_2 and a C_2HC type zinc finger motif, respectively, and the members in Group II can be further divided into five subgroups (II-a, b, c, d, and e, respectively) based on the phylogeny of the WRKY domains (Eulgem *et al.* 2000).

It has been revealed that members of the WRKY transcription factor family play important roles in

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plant resistance to stresses. Real-time PCR (RT-PCR) analysis found that 23 *CsWRKY* genes were responsive to at least one abiotic stress (cold, drought or salinity) in *Cucumis sativus*, and transcripts of 36 *VvWRKY* genes were changed when suffering from cold in *Vitis vinifera* (Ling *et al.* 2011; Wang *et al.* 2014a). Overexpression of *AtWRKY34* in *Arabidopsis thaliana* led to an increased sensitivity to cold stress (Zou *et al.* 2010). The study in tobacco indicated that constitutive expression of *BcWRKY46* could reduce the susceptibility of transgenic tobacco to freezing, abscisic acid (ABA), salt, and dehydration (Wang *et al.* 2012).

Watermelon (*Citrullus lanatus* /Thunb./ Matsum & Nakai) is a very important economic vegetable crop worldwide, which is favoured by consumers all over the world. Watermelon plants usually suffer from various stressful climate conditions during their growth, especially for cold stress, which will greatly influence its yield and quality. In this study, the genome-wide identification of the *WRKY* genes in watermelon was performed and their chromosome distribution, gene classification, phylogenetic relationship and expression patterns in response to cold stress were analysed. The results will provide an important basis for selecting the candidate *WRKY* genes for genetic improvements of cold resistance in watermelon.

MATERIAL AND METHODS

Identification of WRKYs in watermelon. The annotated watermelon genome assembly and protein sequences of the East Asia watermelon cultivar 97 103 were downloaded from Cucurbit Genomics Database (http://www.icugi.org/cgi-bin/ICuGI/index.cgi). The genome-wide identification of candidate genes encoding WRKY proteins in watermelon was completed by HMMER V.3 using the raw Hidden Markov Model (HMM) (Finn *et al.* 2014). The WRKY domain (ID: PF03106) sequence was downloaded from the Pfam database (http://pfam.xfam.org/) and then used as the seed to extract all WRKY proteins in watermelon genome. We also retrieved the information on chromosome locations and CDS (Coding Sequence) of each gene from the Cucurbit Genomics Database (http://cucurbitgenomics.org/).

Multiple sequence alignment, and phylogenetic analysis. The AtWRKY sequences were obtained from the Arabidopsis genome database (http://www.Arabidopsis.org/). The conserved WRKY domains in WRKY proteins were extracted using the SMART database (http://smart.embl-heidelberg.de/). Then the multiple alignment analysis of WRKY domains was performed using Clustal X 2.1 (Larkin *et al.* 2007). The phylogenetic tree was constructed using the neighbour-joining method of the MEGA 6.0 software based on the amino acid sequences of WRKY domains with the following parameters: Poisson model, pairwise deletion, and bootstrap values calculated with 1000 iterations (Tamura *et al.* 2013).

Treatment of watermelon plants with low-temperature stress. A cold-resistant watermelon cultivar ZXG01016 introduced from Zhengzhou Fruit Research Institute was grown at the Research Station of College of Horticulture at Henan Agricultural University, Zhengzhou, China. When growing to three leaves, seedlings were moved to a light growth incubator under a 16 h photoperiod at a photosynthetic photon flux density of 600 μmol/m^2/s and a temperature cycle of 8°C/8°C (day/night). Then leaf samples were collected after 0, 3, 6, 9, 12, 24, and 48 h of cold treatment. Three technical replicates and three biological replicates were used for each sampling time point.

Real time reverse transcription quantitative PCR (RT-qPCR). Primers used for RT-qPCR analysis are shown in Table 1. Total RNA was extracted using TRIzol reagent (Invitrogen, USA). First-strand cDNA synthesis and qRT-PCR were processed using the methods according to our previous study (Li *et al.* 2015). The watermelon *elongation factor 1-a* gene (*EF1a*) was used as the internal reference gene (Kong *et al.* 2014). Comparative analysis of gene expressions was calculated using the $2^{-\Delta\Delta c(T)}$ method (Livak & Schmittgen 2001). Changes (more than two-fold) after cold treatment were regarded as significantly different.

RESULTS

Genome-wide identification of WRKYs in watermelon genome. In this study, a total of 57 candidates were identified. They were designated as *ClWRKY1* to *ClWRKY57* according to their physical positions on watermelon chromosomes. As shown in Figure 1, 57 *ClWRKY* genes were unevenly distributed across eleven watermelon chromosomes. Twelve *ClWRKY* genes were clustered on chromosome 5, which was the largest in number, while there was only one *ClWRKY* located on chromosome 4. Among the 57 ClWRKYs, ClWRKY17 was the longest protein with 836 amino

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Table 1. Sequences of primers designed for qRT-PCR for the *WRKY* genes in watermelon

Genes	Description	Sequences(5'→3')	Genes	Description	Sequences(5'→3')
ClWRKY1	forward reverse	---	*ClWRKY30*	forward reverse	CGATCTGATGAGCTTTCCAA CTTGAGGGTTGCTTGTGAGA
ClWRKY2	forward reverse	AGGGTGTCGACGATGAAGAT ACCATCGTCAAGAACATCCA	*ClWRKY31*	forward reverse	TTATTCCGAAACCACCATCA CCATCAATACGACGACGTTC
ClWRKY3	forward reverse	AATCCTTCTTCCTCTTCCTC GTTATTCCACGACAACACAT	*ClWRKY32*	forward reverse	GCAACCGACTGAGTTGAGAA AATTGACAGGCAAACCATCA
ClWRKY4	forward reverse	ACCACCTCTTTCTACTTCTT CTTCTTGGAACAGTGACATC	*ClWRKY33*	forward reverse	GGTTTACAGAGCATGGAGCA TGAATTTGGAGACGGTGAAA
ClWRKY5	forward reverse	AAGTTGGAAGGGTAATGGTA GGTGGTATTGTTGTTGTTCA	*ClWRKY34*	forward reverse	---
ClWRKY6	forward reverse	TCTACCTACAGAGTTGTTGAG GAAGAACGATGAAGGAGTTG	*ClWRKY35*	forward reverse	TGCAGAAGAGAGTGGTGTCC TGGGATAAGGAGAGCCTTTG
ClWRKY7	forward reverse	---	*ClWRKY36*	forward reverse	ATGGGCAGAAGCCAATTAAG ATAAGCATCGACGGGTCTTC
ClWRKY8	forward reverse	TACGAAGGAAACCACAACCA CATGGATCCTGAGAGAAGCA	*ClWRKY37*	forward reverse	CAGGTTATTGCATCAATGGG GGAGCCATTGCACTTCTTCT
ClWRKY9	forward reverse	CAATTGGGAGTGTCAACAATG CGCCATTTGTAACCATCATC	*ClWRKY38*	forward reverse	GGCTCGATCTTGTGATGAGA ATCTCAGCCACTCCTCCAAC
ClWRKY10	forward reverse	AAATTGGTGAAAGGCAATCC GTCATGCTGACCCTCATACG	*ClWRKY39*	forward reverse	AAAGAATGGAGGGTATGGAT GAGGTTGTAAGAATCGGAAG
ClWRKY11	forward reverse	---	*ClWRKY40*	forward reverse	GTCTGCTAGGAATGGAGTAG GGCTTCTCATCTTCTTTCTC
ClWRKY12	forward reverse	CACAACTTCCATCATCACAA CCACACTAGAAGGAACAATATC	*ClWRKY41*	forward reverse	TACCAAGCGATTGAGAGCAG CATTGGTCCATTCCATTTCA
ClWRKY13	forward reverse	TTGACCGGAACCAGTAACAA GCACTGTTGAAGAGGGTTGA	*ClWRKY42*	forward reverse	GGAAGAAGATGGTGAAGAAC ACCCTCGTATGTTGTTATCA
ClWRKY14	forward reverse	GAATATGGTGAAGCCGGAGT TGAGGGTAATTGTTGCCGTA	*ClWRKY43*	forward reverse	CTGTAGCGACGATAATGATTC TATCCATCCTTCACAACCAA
ClWRKY15	forward reverse	AAGAAGGGCAGAAGAGGGAT GTACTTTCTCCAGGCCCAAG	*ClWRKY44*	forward reverse	CGATGATGACGAGAATAAGAG GGAGTTTACGACAGGATTTG
ClWRKY16	forward reverse	TCCGTTGTTGTTTCCGACTA AAATTAGACGGCTGGTCCAC	*ClWRKY45*	forward reverse	CATCATCATCACTTGCTTCA TGCCATTATTGCTCCTAAGA
ClWRKY17	forward reverse	ACACAGATAGATTCCTCACA AGACTGATGACGATGAAGAT	*ClWRKY46*	forward reverse	AATCCGAGAACTTAGACGAT TCACTACAGAATCCGAACAT
ClWRKY18	forward reverse	TCAACCAATCACAACGCTCT AAGCAGGAAGCAATGAAGGT	*ClWRKY47*	forward reverse	TCGATCTTCCATCAACAAGC CTGTCGTCCACTGCAGAAAT
ClWRKY19	forward reverse	CAACTGCCGAGTGAAGAAGA TTCGTGCTCTGAAGAATTGG	*ClWRKY48*	forward reverse	ATATTCTGGACGATGGCTAT TATGTGGTTATCACGGACTT
ClWRKY20	forward reverse	TAAGGTTTGCAATGGGACTG CCACCGATATCCATCATCAA	*ClWRKY49*	forward reverse	GGAACTACAGAACCATCACT TATGTCATCGTCATCAACCT
ClWRKY21	forward reverse	ACGAGAAGGGACGAAGAGAA CCACCGATATCCATCATCAA	*ClWRKY50*	forward reverse	CCAAACCCAGATGATGAACA CCCTATCCCTATTGCAGCTT
ClWRKY22	forward reverse	CATCACAAGCAACATCAGAG CCCAAATATCCGAAGACAGA	*ClWRKY51*	forward reverse	AGCTACAACCGAAACAACCC ACTCCTGTTTCAGCATGTGG
ClWRKY23	forward reverse	GGTGCGATGTGAAGAAGAGA CTGTTGTTGGTGGATTCTGG	*ClWRKY52*	forward reverse	CAATACTCAACCTCTCAGATTC CATCAAATGCCCTCTCAAAT
ClWRKY24	forward reverse	GAAAGCATAGTTGCAGCCAA CCCATGAGTTCAAGAGAGCA	*ClWRKY53*	forward reverse	GCTCGAGGTTCCACTGGTAT CGTACTTTCGTGCTTTGCAT

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Table 1 to be continued

Genes	Description	Sequences(5'→3')	Genes	Description	Sequences(5'→3')
ClWRKY25	forward reverse	AGACCTCGAACTCGGAGAAA TGGAGCCCTAATGATAACCC	*ClWRKY54*	forward reverse	CATCGGTAGTCATTCTTTCC TATGTCATCTCTGGCTTCTT
ClWRKY26	forward reverse	---	*ClWRKY55*	forward reverse	CCAATTTCCTCAAAGCTTCC TGCAACTTCCTTCTTGACCA
ClWRKY27	forward reverse	AGATTACGCTTCGTCGGAGT AACAATGCCAGCAAATTCAG	*ClWRKY56*	forward reverse	TGGATCTATGAAATCGGA-GAAG CCTGGGAAACTTGTCGTTCT
ClWRKY28	forward reverse	AACAACTCCCATCAGCCTTC AATTGATGGTGGTAGTCGCA	*ClWRKY57*	forward reverse	TAATAGTGAACCGAGTGGAT CATCTCTGTCATCTGTTATCTC
ClWRKY29	forward reverse	AAGATGAAGACGAACCTGAT GCACAATGATTCTGGATTCT	*EF1a-F*	forward reverse	AGCACGCTCTTCTTGCTTTC ACGATTTCGTCGTACCTTGC

Dashes in the case of *ClWRKY1*, *7*, 11, *26* and *34* mean invalid amplification products

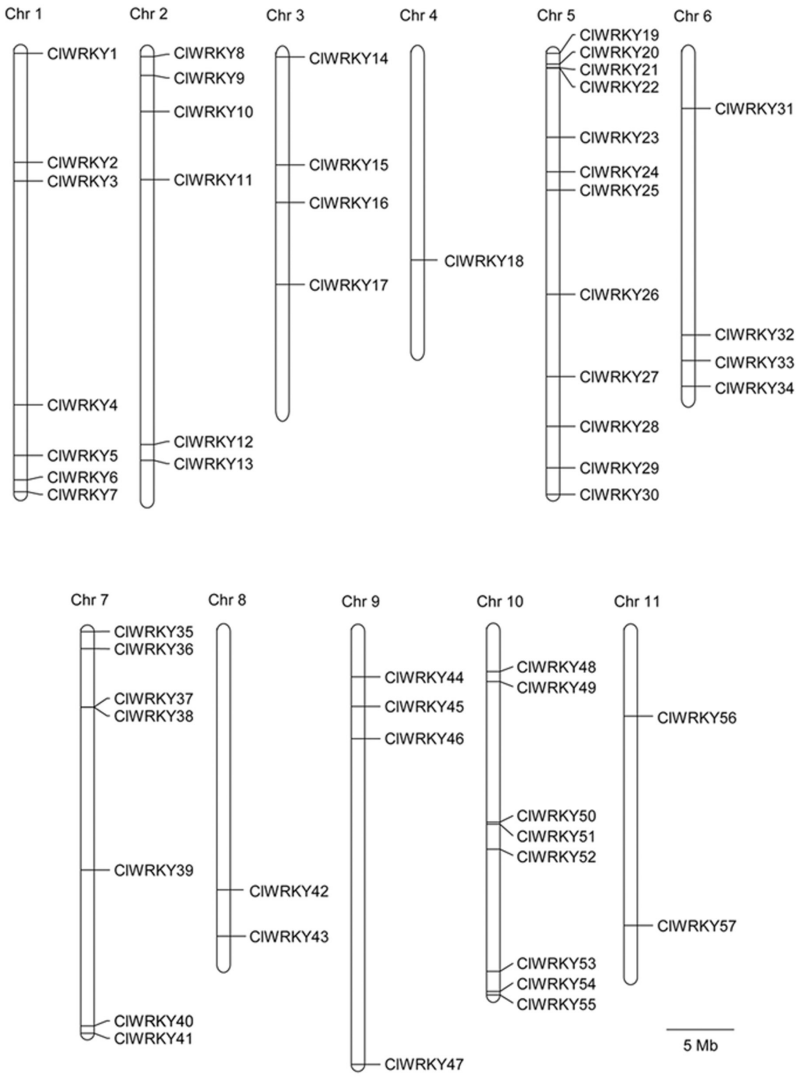

Figure 1. Distribution of 57 *ClWRKY* genes on watermelon chromosomes; the chromosome numbers are indicated at the top of each chromosome; to simplify the presentation, all the *WRKY* genes were renamed as *ClWKRY1* to *ClWRKY57* according to their locations on the chromosomes from top to bottom

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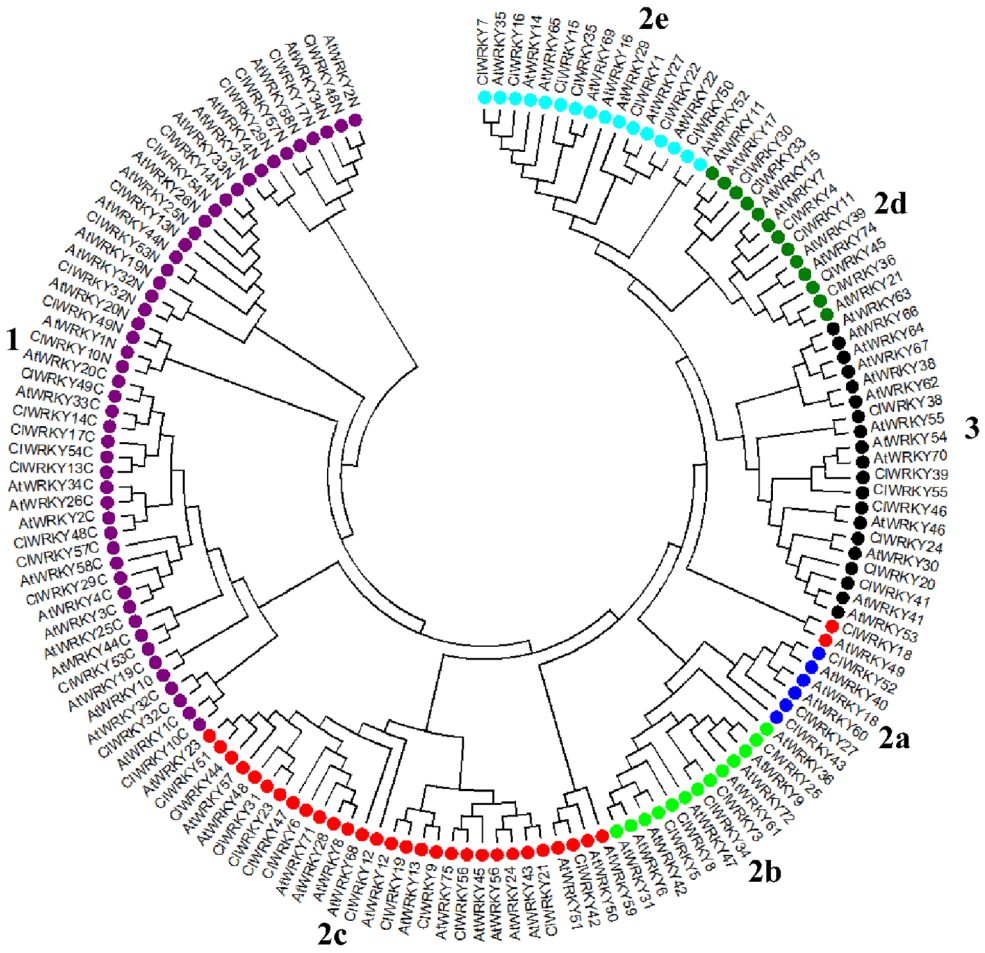

Figure 2. Unrooted phylogenetic tree of WRKY domains in watermelon and *Arabidopsis*; the groups were indicated by different colour, and their names (1, 2a, 2b, 2c, 2d, 2e, and 3) are shown on the outside of the circle; Group 1 WRKY proteins named with the suffix -N or -C indicate the N- or the C-terminal WRKY domain, respectively

acids (aa), while the shortest was ClWRKY56 with only 121 aa. The average length of ClWRKY proteins was about 361 aa. The nucleotide and protein sequences of 57 *ClWRKY*s are listed in Figure S1 in Electronic Supplementary Material (ESM).

The phylogenetic analysis and classification of WRKYs in watermelon. A total of 63 WRKY domains exist in 57 ClWRKYs (Figure S1 in ESM). For the two WRKY domains in the same protein sequence, ClWRKY with the suffix "N" or "C" represented the WRKY domain located at the N- and C-terminus, respectively. ClWRKY2, 26, 28, 37, and 40 were excluded from the phylogenetic analysis and classification due to the loss of the zinc finger domain.

As shown in Figure S2 in ESM, according to results of sequence alignments of conserved WRKY domains in ClWRKYs and classification rules employed for AtWRKYs, all 52 ClWRKYs were divided into three groups. In Group 1, there were 11 ClWRKYs containing two WRKY domains and a C_2H_2-type zinc finger motif. 34 ClWRKYs with a single WRKY domain and a C_2H_2-type zinc finger motif were assigned to Group 2, which was further divided into five subgroups with three in subgroup 2a, five in 2b, 13 in 2c, six in 2d, and seven in 2e. There were seven ClWRKYs with a single WRKY domain and a C_2HC-type zinc finger motif in Group 3. Although the WRKYGQK motif was highly conserved, one amino acid variation was found in ClWRKY42 in subgroup 2c, which contained a WRKYGKK motif. The detailed information on ClWRKYs is listed in Table 2.

To investigate the phylogenetic relationships among the ClWRKYs, an unrooted phylogenetic tree was constructed based on sequence alignments of WRKY domains of ClWRKYs and AtWRKYs (Figure 2). As can be seen in the phylogenetic tree, different from

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Table 2. Identified *WRKY* genes in watermelon

Gene name	Gene ID	Chr	Physical position	WRKY domain Group Conserved heptapeptide	Zinc-finger type	Domain number	Group
ClWRKY1	Cla004938	1	623905-625501	WRKYGQK	C2H2	1	2e
ClWRKY2	Cla008480	1	8796142-8797069	WRKYGQK	no conserved stretch	1	NG
ClWRKY3	Cla008346	1	10188973-10192981	WRKYGK	C2H2	1	2b
ClWRKY4	Cla013967	l1	26958654-26959762	WRKYGQK	C2H2	1	2d
ClWRKY5	Cla014433	1	30737723-30740093	WRKYGQK	C2H2	1	2b
ClWRKY6	Cla009748	1	32574758-32576313	WRKYGQK	C2H2	1	2c
ClWRKY7	Cla009853	1	33474006-33477082	WRKYGQK	C2H2	1	2e
ClWRKY8	Cla007656	2	46121-49115	WRKYGQK	C2H2	1	2b
ClWRKY9	Cla007761	2	997830-999818	WRKYGQK	C2H2	1	2c
ClWRKY10	Cla015673	2	2511354-2513937	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY11	Cla006772	2	9026189-9027547	WRKYGQK	C2H2	1	2c
ClWRKY12	Cla013485	2	28883241-28885653	WRKYGQK	C2H2	1	2c
ClWRKY13	Cla013402	2	29711771-29716746	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY14	Cla008104	3	160231-162530	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY15	Cla019646	3	8018421-8019422	WRKYGQK	C2H2	1	2e
ClWRKY16	Cla019756	3	9691619-9695338	WRKYGQK	C2H2	1	2e
ClWRKY17	Cla009557	3	15970670-15975906	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY18	Cla018197	4	19676671-19679194	WRKYGQK	C2H2	1	2a
ClWRKY19	Cla021067	5	117031-118615	WRKYGQK	C2H2	1	2c
ClWRKY20	Cla021170	5	925058-926889	WRKYGQK	C2HC	1	3
ClWRKY21	Cla021203	5	1196965-1198094	WRKYGQK	C2H2	1	2c
ClWRKY22	Cla021207	5	1219248-1220421	WRKYGQK	C2H2	1	2e
ClWRKY23	Cla021806	5	6402958-6404527	WRKYGQK	C2H2	1	2c
ClWRKY24	Cla004233	5	8987121-8988506	WRKYGQK	C2HC	1	3
ClWRKY25	Cla013052	5	10364374-10369111	WRKYGQK	C2H2	1	2b
ClWRKY26	Cla002084	5	18166261-18168850	WRKYGQK	No conserved stretch	1	NG
ClWRKY27	Cla021021	5	24328741-24331742	WRKYGQK	C2H2	1	2a
ClWRKY28	Cla020642	5	28064322-28065905	WRKYGQK	No conserved stretch	1	NG
ClWRKY29	Cla010216	5	31164260-31166896	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY30	Cla009969	5	33154639-33156285	WRKYGQK	C2H2	1	2d
ClWRKY31	Cla009235	6	4823071-4824445	WRKYGQK	C2H2	1	2c
ClWRKY32	Cla018733	6	21510656-21513842	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY33	Cla018870	6	23022662-23024071	WRKYGQK	C2H2	1	2d
ClWRKY34	Cla019127	6	25287085-25291877	WRKYGQK	C2H2	1	2b
ClWRKY35	Cla002243	7	1054123-1056739	WRKYGQK	C2H2	1	2e
ClWRKY36	Cla006015	7	2332176-2333889	WRKYGQK	C2H2	1	2d
ClWRKY37	Cla014665	7	18910904-18913128	WRKYGQK	No conserved stretch	1	NG
ClWRKY38	Cla007306	7	6705089-6711611	WRKYGQK	C2HC	1	3
ClWRKY39	Cla007307	7	6714426-6716677	WRKYGQK	C2HC	1	3
ClWRKY40	Cla010867	7	30588853-30589646	WRKYGQK	No conserved stretch	1	NG

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Table 2 to be continued

Gene name	Gene ID	Chr	Physical position	WRKY domain Group Conserved heptapeptide	Zinc-finger type	Domain number	Group
ClWRKY41	Cla010918	7	31145037-31146784	WRKYGQK	C2HC	1	3
ClWRKY42	Cla021984	8	19308051-19308824	WRKYGQK	C2H2	1	2b
ClWRKY43	Cla022362	8	22951835-22953972	WRKYGQK	C2H2	1	2a
ClWRKY44	Cla015154	9	3914241-3917158	WRKYGQK	C2H2	1	2c
ClWRKY45	Cla014818	9	6327518-6329225	WRKYGQK	C2H2	1	2d
ClWRKY46	Cla015003	9	8487793-8489885	WRKYGQK	C2HC	1	3
ClWRKY47	Cla005515	9	34426506-34428311	WRKYGQK	C2H2	1	2c
ClWRKY48	Cla004431	10	4866664-4871403	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY49	Cla004492	10	4199747-4205383	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY50	Cla017355	10	16854843-16856049	WRKYGQK	C2H2	1	2e
ClWRKY51	Cla017345	10	17001468-17004493	WRKYGQK	C2H2	1	2c
ClWRKY52	Cla017213	10	18648384-18649816	WRKYGQK	C2HC	1	2a
ClWRKY53	Cla017851	10	26731256-26733477	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY54	Cla018026	10	28064812-28067485	WRKYGQK/WRKYGQK	C2H2	2	1
ClWRKY55	Cla018059	10	28283762-28284680	WRKYGQK	C2HC	1	3
ClWRKY56	Cla003370	11	7658790-7659877	WRKYGQK	C2H2	1	2c
ClWRKY57	Cla016540	11	22331642-22336203	WRKYGQK/WRKYGQK	C2H2	2	1

Chr – chromosome

Group 2, which was divided into three distinct clades: subgroup 2c, 2b+2a, and 2d+2e, Group 1 and Group 3 were monophyletic. Subgroup 2c showed a closer relationship with the C-terminal domains of Group 1 than with the N-terminal domains. The phylogenies of ClWRKYs in the tree were in accordance with the ClWRKYs' classification results.

Expression analysis of ClWRKYs under cold treatment in watermelon. To study the function of *ClWRKY*s, the expressions of 57 *ClWRKY*s in response to cold were investigated using RT-qPCR (Figure 3). Since the Ct values of the amplification curve of *ClWRKY1*, *7*, *26*, and *34* were higher than 35 cycles in the cDNA templates of treated leaves, their expression results were excluded. The expressions of four *ClWRKY*s (*ClWRKY2*, *28*, *37*, and *40*) encoding proteins with incomplete WRKY domains were also analysed and they are shown as Group 4 in Figure 3.

Four *ClWRKY*s (*ClWRKY3*, *4*, *23,* and *55*) showed little or no change in transcript levels under cold stress. Among the differentially regulated genes, 17 *ClWRKY*s were significantly induced including three *ClWRKY* genes in Group 1 (*ClWRKY13*, *14*, and *54*), nine in Group 2 (*ClWRKY43*, *5*, *25*, *12*, *31*, *56*, *11*, *30,* and *33*), four in Group 3 (*ClWRKY20*, *38*, *41*, and *46*), and one in Group 4 (*ClWRKY37*). The upregulated genes exhibited three different expression patterns: transcripts of 11 genes (*ClWRKY12*, *20*, *25*, *30*, *33*, *37*, *41*, *46*, *54*, and *56*) accumulated more quickly after cold treatment with the highest expression occurring in the first 12 hours, and then tended to recover during the next two days; six genes (*ClWRKY13* and *14* in Group 1, *43* in 2a, *5* in 2b, *31* in 2c, and *38* in 3) had the highest expressions at 24 or 48 h. Among the induced genes, *ClWRKY11*, *12*, *13*, 20, *31*, and *54* showed the highest induced expression after cold treatment. The highest expressions of *ClWRKY20* and *54* after cold treatment were about 21 and 16 times higher than the level at 0 h, respectively. The transcripts of eight *ClWRKY* genes in Group 1 (*ClWRKY10*, *17*, *29*, *32*, *48*, *49*, *53*, and *57*), 13 in Group 2 (*ClWRKY8*, *6*, *9*, *19*, *44*, *47*, *51*, *45*, *15*, *16*, *22*, *35*, and *50*), one in Group 3 (*ClWRKY39*), and three in Group 4 (*ClWRKY2*, *28*, and *40*) were remarkably suppressed after cold treatment. Though the expression levels of *ClWRKY57*, *6*, *8*, *9*, *16*, *39*, and *35* were recovered at 24 or 48 h of cold treatment, the change of downregulated genes was inhibited more intensively under cold stress than that of the upregulated ones. Some genes were inhibited more than 10 times after cold, especially for *ClWRKY10* (16-fold), *53* (16-fold), *44* (20-fold), *50* (35-fold), *2*(30), and *40* (16). The other seven genes (*ClWRKY27*, *52*, *18*, *21*, *42, 36* in Group 2, and *ClWRKY24* in Group 3) exhibited irregular expression patterns.

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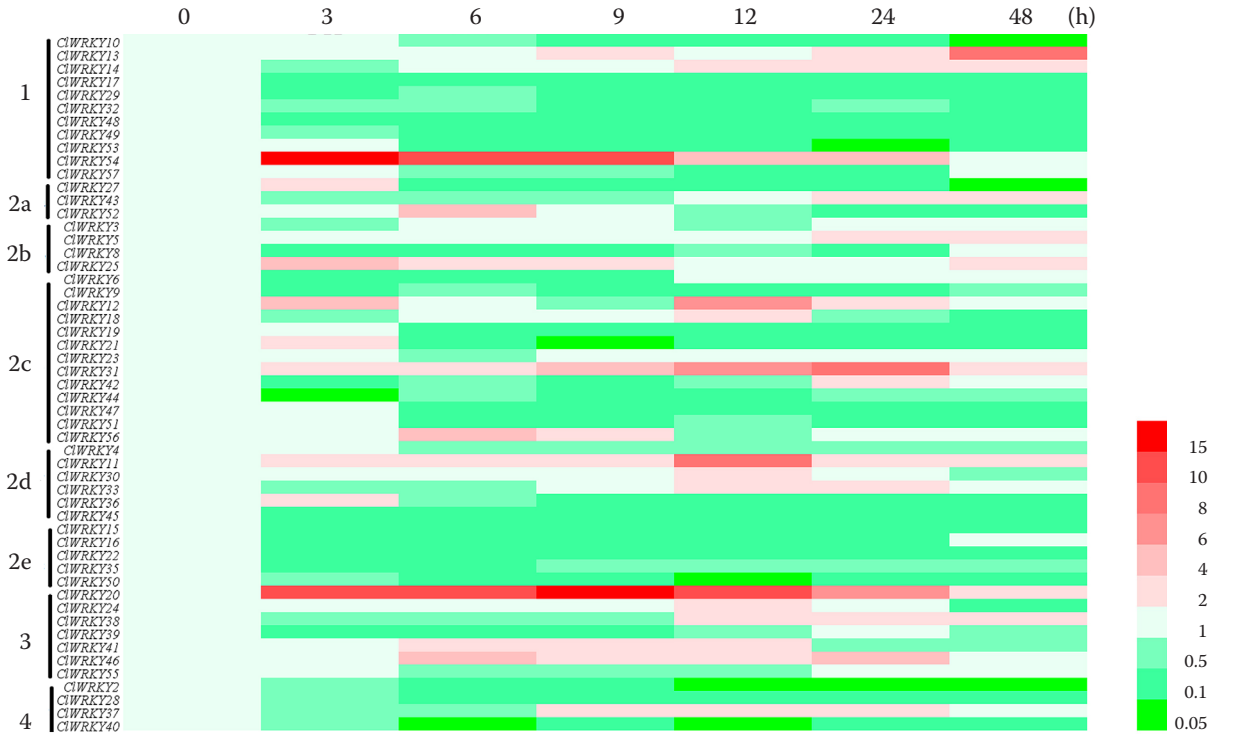

Figure 3. Expression patterns of 53 *ClWRKY* genes in watermelon leaves under cold (8°C) by RT-qPCR analysis; the colour scale is shown on the right of the heat map indicating expression values with blue indicating low transcript abundance and red indicates high levels; four *ClWRKY*s encoding proteins with incomplete WRKY domains were assigned to Group 4; values represent means ± SD of three replicates

DISCUSSION

WRKY proteins are members of a transcription factor family in higher plants. Here we found 57 candidate WRKYs proteins in watermelon, and 52 of them with a complete WRKY domain were categorized into three previously described groups (Eulgem *et al.* 2000). Online Resource 2 showed that the WRKYGQK motif was replaced by WRKYGKK in the sequence of ClWRKY42 in subgroup 2c. The study in tobacco found that when the conserved WRKY domain in *NtWRKY12* was changed from WRKYGQK to WRKYGKK, its binding sequence was also changed (Van Verk *et al.* 2008).Whether the function of ClWRKY 42 is also altered in watermelon after a variation in the sequence needs further study.

The conserved WRKY domains were regarded as a crucial element for binding with the W-box and for the activation of defence-related genes (Sun *et al.* 2003). In addition, the C-terminal WRKY domains in Group 1 play the main role for sequence-specific DNA binding (Eulgem *et al.* 2000). In our study, the subgroup 2c (Figure 2) showed a close relationship with C-terminal WRKY domains of Group 1. All this evidence further supported that WRKYs in Group 2 and 3 may be evolved from Group 1 by a domain structure loss of the Group 1 N-terminal WRKY domains (Zhang & Wang 2005). The genes in Group 3 were considered to be evolutionarily the youngest and the most adaptable (Zhang & Wang 2005). It has been deduced that a mutation in the zinc finger motif of Group 2 proteins from the conserved His residue into a Cys residue may lead to the evolution of Group 3 WRKYs (Xie *et al.* 2005). In our study, the ClWRKYs in Group 3 were sister to subgroup 2d + 2e in the phylogenetic tree. The result supports the speculation that Group 3 members have evolved from Group 2 during the process of plant adaption to stressful conditions (Wang *et al.* 2014b).

Previous studies have established that the cold response pathway of the WRKY family is an important component of the stress acclimation response (Chen *et al.* 2012). In our study, as much as 93% of the *ClWRKYs* was found to be changed significantly in transcript levels after cold treatment (Figure 3). It was observed that the majority of 13 selected *WRKYs* in poplar were early response genes displaying a pattern of "induced amplification recovering" after stress treatment (Wang *et al.* 2014b). In our study, most of the upregulated genes increased rapidly after

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cold treatment, reached a peak at 3, 6, 9 or 12 h, and after that began to recover to the normal level. This may be because that *ClWRKY*s would be induced and contribute to cold tolerance by activating their downstream genes, then started to decline when their task was completed as has been indicated in another study (Wang *et al.* 2014b). Similar patterns were also observed in some of downregulated genes, which were repressed firstly, and then recovered later. Most studies focus on the functions of upregulated genes in cold resistance. However, extensive downregulations in gene expressions occur at the same time during cold acclimation. The downregulations of gene expressions may also be an important component of the adaptation to cold stress (Skinner 2009), and their expressions may not be compatible with the enhancement of cold tolerance. Though we found that five ClWRKYs (ClWRKY2, 26, 28, 37, and 40) did not have the complete WRKY domain, the transcript levels of *ClWRKY2*, *28*, *37*, and *40* could be significantly changed, which could also be found in other plants (Xie *et al.* 2005). Therefore, their exact biological functions in plants are still inconclusive.

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