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Characterization of WRKY transcription factors in *Solanum lycopersicum* reveals collinearity and their expression patterns under cold treatment

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ABSTRACT

WRKY transcription factors play an important role in cold defense of plants. However, little information is available about the cold-responsive WRKYs in tomato (*Solanum lycopersicum*). In the present study, a complete characterization of this gene family was described. Eighty WRKY genes in the tomato genome were identified. Almost all *WRKY* genes contain putative stress-responsive *cis*-elements in their promoter regions. Segmental duplications contributed significantly to the expansion of the *SlWRKY* gene family. Transcriptional analysis revealed notable differential expression in tomato tissues and expression patterns under cold stress, which indicated wide functional divergence in this family. Ten *WRKY*s in tomato were strongly induced more than 2-fold during cold stress. These genes represented candidate genes for future functional analysis of *WRKY*s involved in the cold-related signal pathways. Our data provide valuable information about tomato WRKY proteins and form a foundation for future studies of these proteins, especially for those that play an important role in response to cold stress.

1. Introduction

Low temperature is a major environmental factor that limits the agricultural productivity and geographical distribution of many plant species [1]. Numerous studies have revealed that many *WRKY* genes are responsive to abiotic stresses including cold, heat, drought, salinity, and so on [2]. In *Arabidopsis* and *Oryza sativa*, results of microarray analyses showed that some of the *WRKY* genes were strongly regulated in response to salinity, drought, and cold stress [3]. while in *Vitis vinifera*, at least 15 *WRKY* genes showed stress-induced expression patterns due to cold [4].

As a large gene family in plants, *WRKY* expanded greatly during evolution. Gene duplication is one of the major evolutionary mechanisms for generating new genes that can diversify their functions relative to the ancestral gene [5]. Previous studies have shown that gene duplication events, which include segmental and tandem duplication, play a key role in the expansion of *WRKY* genes. For instance, in *Glycine max*, 76.7% of *WRKY* genes were segmentally duplicated and 13.5% of the genes were duplicated in tandem [6]. In addition, the divergence of expression of duplicated genes was observed widely in plants such as *G. max*, *O. sativa*, and *Brassica oleracea* [6–8], indicating that functional divergence of duplicated *WRKY* genes had occurred.

Tomato (*Solanum lycopersicum*) is one of the most important economic crops and it is cultivated worldwide. Although the function of several individual *WRKY* genes have been identified in tomato [9–11], the cold-responsive *WRKY* genes in tomato remain wholly uncharacterized. Based on a draft of the *S. lycopersicum* cv. Heinz 1706 genome sequence reported recently [12], we searched *WRKY* genes containing the WRKY domain and we analysed further their protein characterizations. Syntenic analysis was conducted to identify expansion patterns and selection pressure on *WRKY* in tomato (*SlWRKY*). We used two genotypes of tomato that differed in cold tolerance to identify cold-responsive *SlWRKY* genes; the gene expression patterns in different tissues of two tomato genotypes were detected by RT-PCR. Additionally, we used quantitative RT-PCR to compare the expression level of *SlWRKY*s between two genotypes under cold treatment.

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2. Materials and methods

2.1. Database set and sequence analysis of WRKY proteins in tomato

Candidate WRKY proteins were identified from the *S. lycopersicum* cv. Heinz 1706 genome (http://www.phytozome.net). Full-length aa sequences of all WRKY proteins in *Arabidopsis thaliana* and *Solanum tuberosum* (http://planttfdb.cbi.pku.edu.cn) were used as query sequences. A BLASTP search was performed and an E-value of 1e−10 was used as the threshold to ensure that all potential WRKY domain-encoding sequences were discovered [13]. We pooled all hits into a single data set after duplicate sequences were removed. Candidate WRKY proteins were further confirmed manually by searching for WRKY domains in the candidate aa sequences using SWISS-MODEL (http://swissmodel.expasy.org) [14].

The *SlWRKY* sequences that we obtained were submitted to GenBank database, and accession numbers were kept. Additionally, the four fields (length, molecular weight, isoelectric point and instability index) of the deduced polypeptides were calculated by ExPasy proteomics Server (http://web.expasy.org/protparam/). The proteins having an instability index <40 were considered as stable [15]. The cellular localization of each WRKY protein was carried out using the PSORT server (http://psort.hgc.jp/form.html).

2.2. In silico analysis of regulatory elements in the promoter region of SlWRKY genes

To identify *cis*-elements in the promoter regions of *SlWRKY* genes, the 1500 bp sequences upstream of the coding region of each *SlWRKY* gene were selected as promoter sequences and downloaded from Phytozome (http://www.phytozome.net). These sequences were submitted to query the PLACE website (http://www.dna.affrc.go.jp/PLACE/signalscan.html) [16].

2.3. Detection of collinear blocks and syntenic analysis of WRKY proteins

To predict collinear blocks in the tomato genome and to further classify and count duplication events, a local installation of the MCScanX toolkit was obtained from the MCScan webpage [17]. MCScanX reported blocks with at least 5 collinear gene pairs. Syntenic analysis of WRKY proteins between tomato and *Arabidopsis* was also performed by MCScanX.

2.4. Analysis of SlWRKYs expansion patterns and dating the duplication events

We analysed duplication events of *SlWRKY*s and we focused on two patterns of gene expansion: segmental duplication and tandem duplication. Segmental duplications were defined by syntenic analysis mentioned above. Two *SlWRKY*s placed on the syntenic blocks in the genome were designated as segmental duplicated pairs. Tandem duplications were identified as genes separated by five or fewer gene loci according to the tomato genome annotation ITAG 2.4 [18].

We also calculated the synonymous rate (Ks), nonsynonymous rate (Ka), and evolutionary constraint (Ka/Ks) between the duplicated pairs of *SlWRKY*s based on their coding sequence alignments, using the method of Librado and Rozas [19] as implemented in DnaSP v5. The approximate dates of the duplication events were calculated by the equation $T = Ks/2\lambda$, in which the synonymous substitution rate (λ) for tomato is 1.5×10^{-8} [20].

2.5. Plant materials and growth conditions

Two cultivars of tomato (*S. lycopersicum* Mill. cv. Lichun and *S. lycopersicum* Mill. cv. Santiam) provided by the Chinese Academy of Agricultural Sciences were used as materials. Lichun is more sensitive to cold than Santiam. Pot experiments were conducted in 7 cm × 7 cm plastic pots filled with sterilized horticultural soil (sterilized at 121 °C for 1 h). We surface-sterilized seeds of tomato with 3% sodium hypochlorite for 3 min and washed with sterile water. Two seeds were placed in each pot. Seeds of tomato were germinated and grown in a controlled environmental chamber (25 °C/20 °C during the 16/8 h light/dark photoperiod) for 40 days at a relative humidity of 60%. Different tissues including root, shoot, leaf, and cotyledon of two cultivars were collected for analysis of expression patterns of the different genes. All samples were frozen in liquid nitrogen immediately and then stored at −80 °C for the following RNA isolation.

The chilling tolerance of two cultivars was then checked. Cold treatment experiments were performed according to Li et al. [21] Briefly, seedlings were cultured in a growth chamber with the same parameters mentioned above except for temperature (4 °C). Well-developed leaves of seedlings were used and the leaf samples were collected daily for 7 days. Malondialdehyde (MDA) content, H_2O_2 content, and activities of the antioxidant enzyme system were determined as we previously reported [22]. Data were statistically analysed by analysis of variance (ANOVA), and means were compared using the least significant difference (LSD) method to assess the different properties of two cultivars under cold stress; differences with $P \leq 0.05$ were considered significant.

For analysis of relative expression of *SlWRKY*s, leaf samples of seedlings under cold treatment were collected at 0 h (used as control), 8 h, 24 h, and 48 h. Samples were frozen in liquid nitrogen immediately.

2.6. Analysis of expression patterns of SlWRKYs by RT-PCR

Total RNA was isolated from collected samples (root, shoot, leaf and cotyledon tissues of two tomato species) using the Plant Total RNA Isolation kit (Tianz Inc; Beijing, China). RNase-free DNase I (TRANSGEN Biotechnology, Beijing) was used to degrade DNA from total RNA. RNA concentration was determined by NanoDrop ND-2000 UV–Vis spectrophotometer (NanoDrop Technologies, Inc.) and the integrity of the RNA was assessed on a 1% (w/v) agarose gel. cDNA was synthesized by the AMW Reverse Transcriptase (Promega) with Oligo $(dT)_{18}$ (Promega) according to the manufacturer's instructions. A maximum of 1 μg RNA was used for each reverse–transcription reaction. Primer pairs (Supplementary material 1) for *SlWRKY*s were designed by Primer (version 5.0) software and their specificities were tested by NCBI Primer BLAST. The *UBi3* gene of tomato (GenBank accession: X58253; sense primer: 5′-TCCATCTCGTGCTCCGTCT-3′; antisense primer: 5′-CTGAACCTTTC-CAGTGTCATCAA-3′) was used as a positive control for RT-PCR [23]. The following protocol was used for RT-PCR: 94 °C for 2 min followed by 35 cycles at 94 °C for 10 s, 55 °C for 10 s, and 72 °C for 25 s, followed by a 2 min extension step at 72 °C. Three biological replicates for each reaction were performed. PCR products were detected by agarose gel electrophoresis with 2% (w/v) gel concentration.

2.7. Expression analysis of SlWRKYs under cold stress by quantitative RT-PCR

Total RNA was isolated from cold-treated leaves following the cDNA synthesis methods described above. The real-time PCR analysis was performed using BIO-RAD CFX96 real-time PCR

system (Bio-Rad, USA) with denaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 1 min. Three biological replicates were carried out and triplicate quantitative assays for each replicate were performed using Promega GoTaq qPCR Master Mix kit (Promega) according to the manufacturer's protocol. The 25 μL reaction mixture contained 10.5 μL of diluted template (1 μL of the generated first-strand cDNA diluted by 9.5 μL H_2O), 12.5 μL 2 × GoTaq qPCR Master Mix and 1 μL of each of the two gene-specific primers. The tomato *UBi3* gene was used as an internal control [23]. The relative expression level was calculated as $2^{-\Delta\Delta CT}$ [$\Delta C_T = C_{T,\ Target}$-$C_{T,\ UBi3}$. $\Delta\Delta CT = \Delta C_{T,\ treatment}$-$\Delta C_{T,\ control\ (0h)}$]. The relative expression level ($2^{-\Delta\Delta CT,\ control\ (0h)}$) in the control plant without treatment was normalized to 1 as described by Schmittgen et al. [24].

3. Results

3.1. Identification and characterization of WRKY TFs in tomato

A total of 84 transcripts in the tomato genome sequence were identified as possible members of the WRKY family (Supplementary material 2). Four transcripts were excluded due to a lack of the conserved WRKY domain in the predicted aa sequence. The remaining 80 transcripts were named from *SlWRKY1* to *SlWRKY80* based on their order on the chromosomes. Sixty-one (76.3%) WRKY protein sequences were previously annotated in the NCBI protein database and the accession numbers are listed in Supplementary material 3.

The particularization, including number of aa (length), isoelectric point (PI), molecular weight (MW), and stability was listed in Supplementary material 3. According to the detailed information, the length of the 80 identified WRKY TFs varied from 131 residues (*SlWRKY16*) to 739 residues (*SlWRKY57*). ExPasy analysis showed that WRKY proteins varied greatly in PI values (ranging from 4.74 to 9.75) and MW values (ranging from 15.5 to 79.9 kDa). According to their instability index values, 8 of 80 (10.0%) WRKY proteins were stable with an instability index < 40.

The subcellular localization of each *SlWRKY* was predicted by PSORT program. We predicted that *SlWRKYs* would be localized in the nucleus (28), cytoplasm (27), chloroplast stroma (12), microbody (5), and mitochondrial matrix space (5), while *SlWRKY47*, *SlWRKY19*, and *SlWRKY36* would be in the endoplasmic reticulum, outside and in the plasma membrane, respectively (Supplementary material 3).

3.2. Variety of cis-elements in the promoter regions of SlWRKY genes

We searched potential *cis*-acting regulatory elements in upstream (1500 bp) sequences of the *SlWRKY* genes; a number of *cis*-elements associated with stresses and phytohormone responses were found (Fig. 1). The promoters of 5 genes were unavailable as there were still gaps in the tomato genome (Supplementary material 4). SA-responsive elements (TCA-elements), MeJA-responsive elements (CGTCA-motif) and Ethylene-responsive elements (ERE) were found in the promoter regions of 46,42, and 30 *SlWRKY* genes, respectively (Fig. 1). All of them existed in the promoters of 9 genes together. We detected 11 promoters that contained WUN-motifs involved in wounding stress. ABA-responsive elements (ABREs) and MYB-binding sites (MBSs) were abundant in *SlWRKY* promoters and these elements were found in 35 and 39 promoters, respectively. Temperature-responsive elements were also discovered in the promoters of *SlWRKY* genes. The low-temperature elements (LTRs) existed in 16 promoters and the heat shock elements (HSEs) were found at the highest frequency in

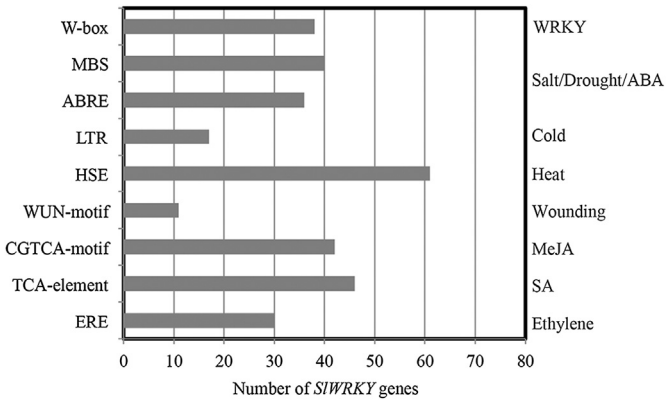

Fig. 1. The number of *WRKY* genes containing various *cis*-acting elements. W-box, WRKY binding site; MBS, MYB binding site; ABRE, ABA-responsive element; LTR, *cis*-acting element involved in low-temperature responsiveness; HSE, heat shock elements; WUN-motif, wound-responsive element; CGTCA-motif, *cis*-acting regulatory element involved in MeJA responsiveness; TCA-element, *cis*-acting element involved in SA responsiveness; ERE, ethylene-responsive elements.

61 promoters. In addition, there were different numbers of W-box elements located in 37 promoter regions (Supplementary material 4).

3.3. Collinearity and evolutionary divergence of SlWRKY genes

The collinear relationships of the duplicated gene pairs in the tomato genome were shown in Supplementary material 5. Among 34,725 genes in the tomato genome, we found 6558 collinear gene pairs distributed in 12 tomato chromosomes (18.89%; Data not shown). In *SlWRKYs*, 39 genes (48.8%) located in 10 chromosomes (except chromosome 9 and chromosome 11) had syntenic relationships. To further estimate the origin and evolutionary history of the *SlWRKY* family, we created a comparative syntenic map between the tomato and *Arabidopsis* genomes. Among 19,145 collinear gene pairs (Data not shown), large-scale syntenies containing 42 *WRKY* genes in tomato and 40 in *Arabidopsis* were identified (Supplementary material 6). Seventeen pairs of duplicated genes appeared to have an unambiguous single tomato-to-*Arabidopsis WRKY* gene correspondence (Supplementary material 7).

A summary of Ka/Ks for 38 *SlWRKY* duplicated pairs indicated that a total of 36 duplication pairs had Ka/Ks ratios <1, representing purifying selection on these genes (Supplementary material 8). However, one duplication pair (*SlWRKY39* and *SlWRKY40*) showed a Ka/Ks ratio equal to 1, suggesting that the neutral selection was also presented. Moreover, another gene pair (*SlWRKY23* and *SlWRKY50*) showed a Ka/Ks ratio > 1, indicating positive selection (Supplementary material 8).

Segmental duplications of 29 gene pairs were estimated to have occurred from 10.33 to 61.33 million years ago. Tandem duplication events for 9 pairs occurred within the last 1.33–31.67 million years.

3.4. Difference in cold tolerance between two tomato genotypes

The H_2O_2 content of Linchun was almost 15.2%–30.9% higher than that in Santiam, and a higher MDA concentration (10.9%–28.3%) was recorded for Lichun (Fig. 2). Activities of four antioxidant enzymes were fluctuant-induced during the treatment. We observed a difference in enzyme activity between the two cultivars, and all antioxidant enzyme activities were significantly higher ($P < 0.05$) in cold-tolerant Santiam than in cold-sensitive Lichun.

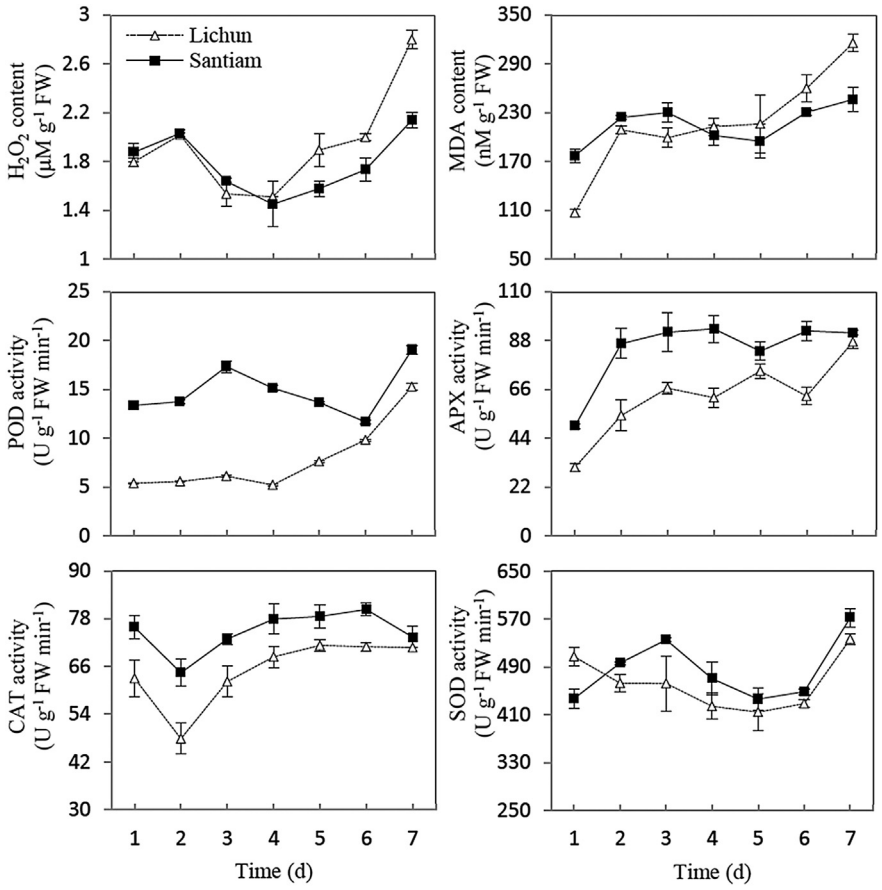

Fig. 2. Differences in H_2O_2 content, MDA content, and antioxidant enzyme activities [peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD)] in two tomato genotypes under cold stress.

3.5. RT-PCR based transcription levels detections of SlWRKYs in different tissues

Not all of the predicted genes were expressed in tomato plants (Fig. 3). Among 80 predicted genes, 76 (95.0%) genes could be detected in at least two tissues. Fifty-five *SlWRKY*s (68.8%) were found expressed in all eight tissues examined. Differential expression pattern was also observed. Seven genes (*SlWRKY25*, *35*, *36*, *37*, *40*, *41* and *45*) could not be found in root tissues and the *SlWRKY10* could only be detected in the roots of two tomato cultivars. Meanwhile, we observed different expression profiles in various tissues between two tomato cultivars: for example, *SlWRKY77* was expressed in four tissues of Linchun, but it could not be found in the cotyledon of Santiam.

3.6. Quantitative RT-PCR based expression analysis of SlWRKYs under cold treatment

Thirty-five *SlWRKY*s had the same changing tendency in two tomato genotype seedlings when exposed to low temperature. These available genes were further classified into three groups according to their expression pattern (Fig. 4; Supplementary material 9). The relative expressions of 10 genes were up regulated (Fig. 4). In contrast, 12 *SlWRKY*s were significantly ($P < 0.05$) down regulated (<0.5 fold) during the cold treatment (Supplementary material 9A). In addition, the other 13 genes presented irregular patterns (Supplementary material 9B). All the up-regulated genes were changed over 2-fold and the greatest increase in expression (nearly 114.7 fold) was found in up-regulated *SlWRKY62* after 48 h of cold treatment. Moreover, the expression levels of most *SlWRKY* genes in Santiam were significantly ($P < 0.05$) higher than that in Linchun when exposed to cold stress.

4. Discussion

This study identified and characterized 80 *SlWRKY* genes based on publicly available sequence information (Supplementary material 3). However, the physiological traits of WRKY proteins including PI and MW were extraordinarily varied, indicating a high degree of complexity within the *SlWRKY* genes. Analysis of *cis*-acting elements in promoter regions helped us to understand the biological functions of *SlWRKY*s (Fig. 1; Supplementary material 4). TCA-elements, CGTCA-motif, and ERE were distributed randomly in the promoter regions of *SlWRKY* genes, which implies that most *SlWRKY* genes are involved in SA, MeJA and the ethylene signalling response. Furthermore, MBS, ABRE, and some other stress-responsive elements such as LTR, HSE, and WUN-motif were also detected in *SlWRKY*s. The MBS and ABRE elements participated in the drought, low temperature, salt, and GA stress responses [25,26]. Thus, we concluded that *SlWRKY*s might be involved in the response to stresses such as drought, salt, and low temperature. The existence of W-box in promoters suggested that these *SlWRKY* genes might be regulated by other WRKY proteins or by themselves.

In this study, a high proportion (48.8%) of *SlWRKY* genes was distributed preferentially in duplicated blocks (Supplementary

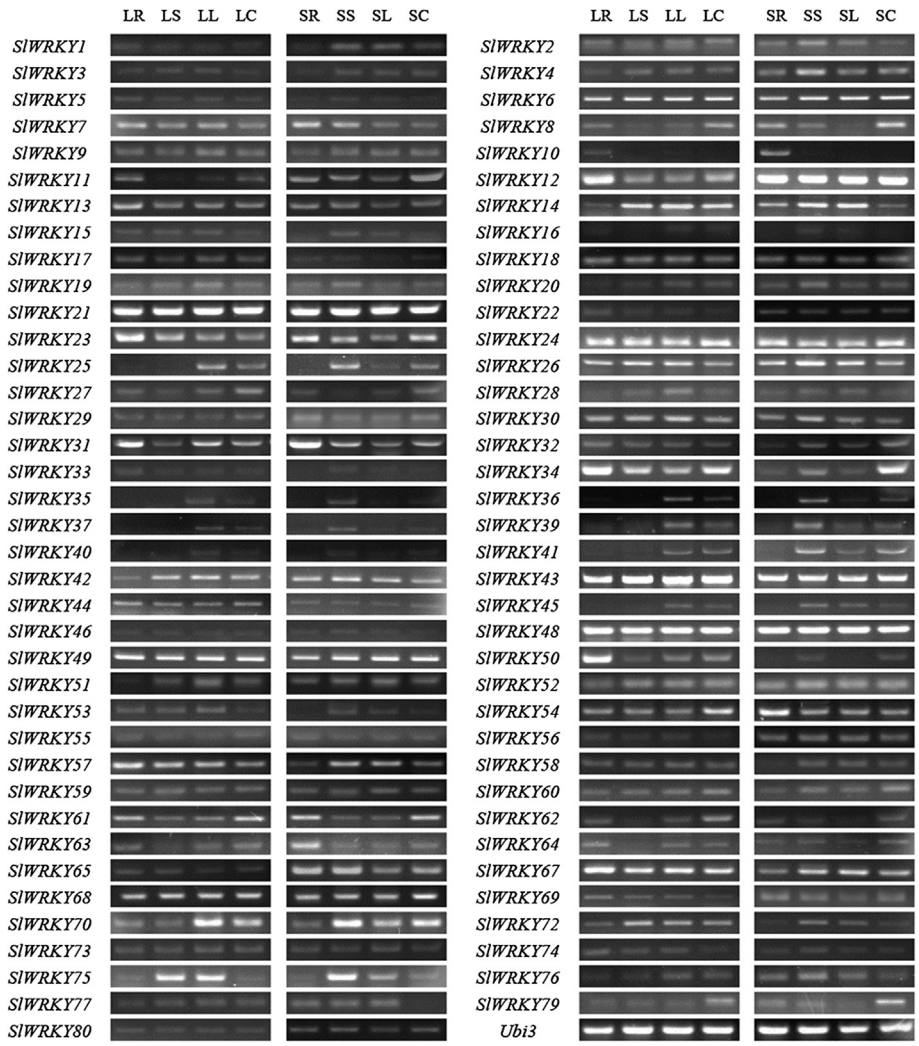

Fig. 3. RT-PCR analyses of presence of *SlWRKY* transcripts in root, shoot, leaf and cotyledon of Lichun (LR, LS, LL, and LC, respectively) and Santiam (SR, SS, SL, and SC, respectively).

material 5; Supplementary material 8), suggesting that segmental duplications contribute significantly to the expansion of the *SlWRKY* gene family. A similar evolutionary pattern for *WRKY* genes was observed in *Populus trichocarpa* [27]. In comparative syntenic analysis between tomato and *Arabidopsis*, 17 pairs of *WRKY* genes presented a single corresponding relationship, suggesting that these genes had been in the genome of the last ancestor of tomato and *Arabidopsis* (Supplementary material 7). However, complex cases like *WRKY* genes in the *Arabidopsis* had two or more counterparts in tomato with a high similarity in protein sequences, indicating that the *WRKY* genes might have undergone differential expansion in tomato and *Arabidopsis* after the divergence of the two species. New *SlWRKY* genes generated by tandem duplication were much younger than *SlWRKY* genes generated by segmental duplication. Tandem duplication in plants might have a relatively faster turnover rate, thus tandem duplicates were not retained for as long as nontandem duplicates [28]. Analysis of selection pressures analysis showed that most (94.7%) of the duplicated pairs had Ka/Ks ratios < 1, which strongly suggests that the *SlWRKY* gene family had experienced mainly strong purifying selection pressure.

Up to now, little is known about the cold-related *WRKY* genes in tomato. A comprehensive analysis of cold-responsive *WRKY* genes in two different tomato species with contrasting cold tolerance would certainly help to illustrate the function of *WRKY* genes in conveying cold hardiness in tomato. Our study demonstrated that activities of the antioxidant enzyme in Santiam were much higher than that in Lichun during cold treatment. Benefitting from the enhancement of the antioxidant system, H_2O_2 was scavenged effectively, and the accumulation of MDA was also alleviated substantially in Santiam compared to Lichun. The two tomato species could be used to identify cold-responsive *WRKY* genes, because the cold tolerance of the two genotypes was significantly different.

Genes that are highly expressed in specific tissues may be important for their development and metabolism. *GhWRKY15* was found to be abundant in the roots and stems of tobacco and displayed faster elongation at the earlier shooting stages [29]. Differential expression of *WRKY* genes in different tissues of two tomato genotypes was detected (Fig. 3). A large proportion of *SlWRKY*s could be found in all experimental tissues, indicating their fundamental roles in different cell-types in tomato.

Results from quantitative RT-PCR demonstrated 70 *SlWRKY* genes exhibited differential transcript abundance in response to cold stress, indicating that *SlWRKY* genes might play an important role in protecting tomato. Ten *SlWRKY*s were strongly induced more than 2-fold during 48 h of cold stress. Of these, *SlWRKY62* was remarkable for its highest relative expression in two tomato genotypes. This gene might be crucial in the process of cold defense in tomato.

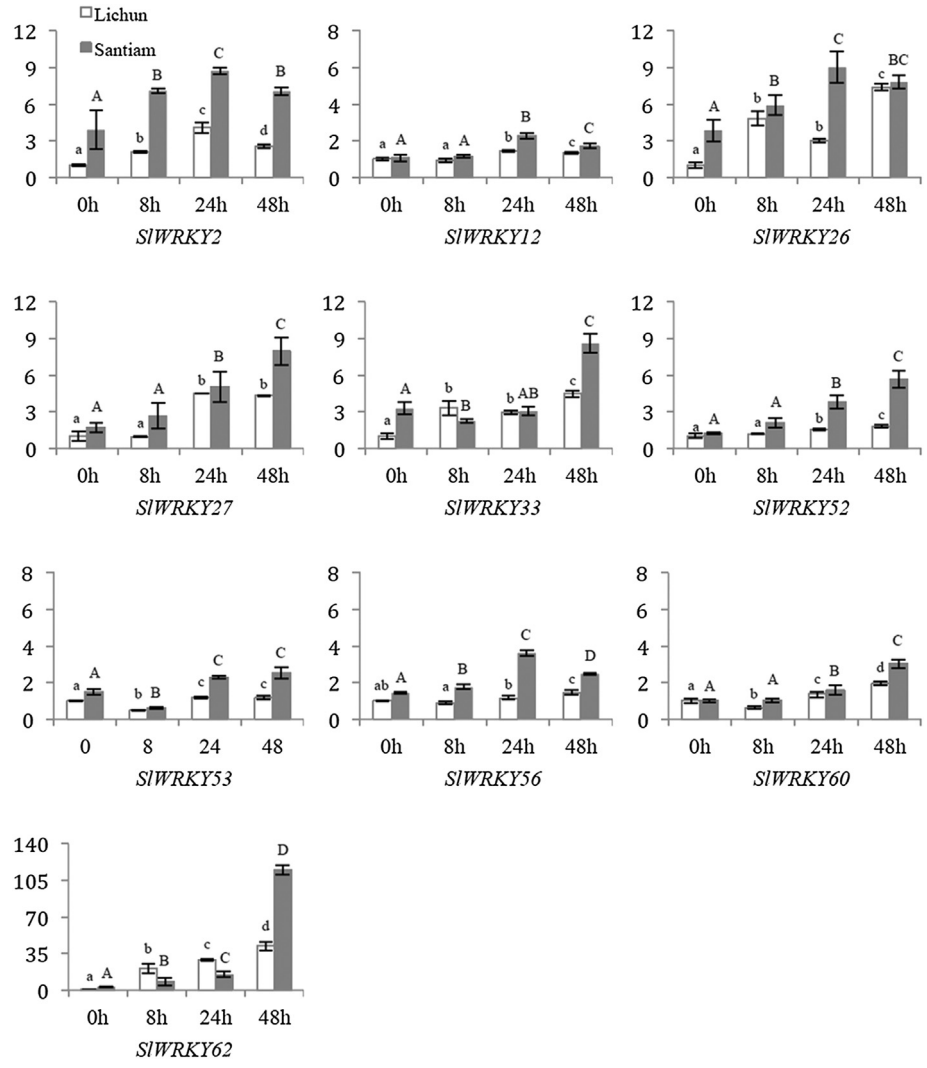

Fig. 4. Sustained up-regulated *SlWRKY* genes in two tomato genotypes under cold stress. The gene response in sensitive (Lichun) and resistant (Santiam) genotypes during cold treatment was evaluated using qRT-PCR. The values (mean ± SD) were calculated based on three biological replicates.

Although low-temperature related *WRKY*s were isolated in several species, the mechanism of how *WRKY*s respond to cold signals and regulate the expression of downstream genes is still poorly understood. Further work is needed to fully understand the function of these important genes in low-temperature signal pathways. The systematic analysis of the *SlWRKY* gene family provides an important reference for future studies on its biological functions.

Conflict of interest statement

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.085.

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