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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

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