

A WRKY transcription factor gene isolated from *Poncirus trifoliata* shows differential responses to cold and drought stresses

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Abstract

Partial sequences of a number of cDNAs showing homology with WRKY genes were amplified from two-day cold-acclimated *Poncirus trifoliata* by reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers designed based on conserved signature sequences of the WRKY domain. The full-length sequence of one of these cDNAs designated as *PtrWRKY2* was obtained using rapid amplification of cDNA ends (RACE) method. *PtrWRKY2* cDNA was 2,070 bp in length containing an open reading frame (ORF) encoding a polypeptide of 540 amino acids. The polypeptide was homologous to WRKY transcription factors (TFs) having double WRKY domains with Cys₂His₂ signature motif. The *PtrWRKY2* gene was clustered with putative WRKY TFs having double WRKY domains and showed close phylogenetic relation with putative WRKY TFs mostly from woody perennial plants. The expression of the *PtrWRKY2* gene was analyzed by Northern blot in cold-hardy *Poncirus* and cold-sensitive *Citrus* species, *Citrus grandis* (pummelo) having the same gene with more than 95% nucleotide sequence homology. The results revealed that the expression of the *PtrWRKY2* gene was initially induced in response to cold in both cold-hardy *Poncirus* and cold-sensitive pummelo; however, the expression was decreased at 1 h of cold acclimation and then, clearly repressed in *Poncirus* and pummelo. On the other hand, drought stress had no effect on the expression of the *PtrWRKY2* gene in pummelo, but its expression was repressed by drought stress in *Poncirus* indicating a negative regulation. The results showed that *PtrWRKY2* encodes a WRKY TF and its expression showed differential responses to cold acclimation and dehydration stress in *Poncirus* and *Citrus*.

Keywords: Abiotic stress; *Citrus*; Gene expression; Northern blot; Phylogenetic analysis; RACE.

Abbreviations: ORF- Open reading frame; RACE- Rapid amplification of cDNA ends; TFs-Transcription factors; TDR- Time-domain reflectometry.

Introduction

Development of abiotic stress tolerant plants is one of the major objectives of plant breeding programs. Studies involving abiotic stress tolerance in different plants revealed that the expression of hundreds of genes was increased in response to abiotic stresses, including cold, drought and salinity (Fowler and Thomashow, 2002; Seki et al., 2002; Rabbani et al., 2003; Hwang et al., 2005; Rensink et al., 2005). Among these genes, WRKY transcription factors (TFs) regulating the expression of abiotic and biotic stress-responsive genes in plants constitute one of the major groups. Although it was originally thought that WRKY TFs are specific to plants, they were later found in other eukaryotes and it was postulated that they have originated in early eukaryotes and later expanded to plants (Zhang and Wang, 2005). WRKY genes constitute a large gene family in plants due to duplications many times during evolution (Zhang and Wang, 2005). The model plant *Arabidopsis thaliana* contains 74 WRKY genes, rice (*Oryza sativa*) has 109, and the genome of a woody plant, poplar (*Populus* spp.) has 104 WRKY genes (Eulgem and Somssich, 2007; Ross et al., 2007; Pandey and Somssich, 2009). In addition, WRKY genes have been isolated and characterized from different economically important agricultural crops, including sweet potato (Dellagi et al., 2000), pepper (Park et al., 2006), barley (Sun et al., 2003), parsley (Cormack et al., 2002) and grapevine (Marchive et al., 2007). WRKY TFs contain one or two DNA-binding domains with approximately 60 amino acids in length and a conserved WRKYGQK sequence followed by

CX₄₋₇CX₂₂₋₂₃HXH zinc finger motif. They bind specifically W-box regulatory elements with the conserved sequence of (T)TGCA(C/T) (Eulgem et al., 2000; Yamasaki et al., 2008; Pandey and Somssich, 2009). WRKY proteins have roles in various physiological processes, including abiotic and biotic stress response (Oh et al., 2006; Ryu et al., 2006; Miller et al., 2008; Jiang and Deyholos, 2006, 2009), senescence (Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2001), seed coat and trichome development (Johnson et al., 2002), hormon signaling (Zhang et al., 2004; Zhang et al., 2008), root development (Devaiah et al., 2007) and regulation of biosynthetic pathways (Xu et al., 2004). *Citrus* is an economically important fruit crop commercially grown in tropical and subtropical regions of the world. Abiotic stresses limit the production of citrus outside of these regions and cause significant losses in production. Commercial *Citrus* species are sensitive to cold, drought and salt stresses. *Poncirus trifoliata*, a close relative of *Citrus*, is cold tolerant and used in citrus production as a rootstock in some citrus growing regions. For this reason, citrus breeders have attempted to integrate the cold hardiness and drought tolerance into commercial *Citrus* varieties and rootstocks. Since these traits are controlled by many genes, the development of *Citrus* cultivars resistant to abiotic stresses has not been achieved yet. Many genes induced in response to cold have been isolated from cDNA libraries of cold-tolerant *P. trifoliata* by Northern blot, differential display and subtractive hybridization (Cai et al., 1995; Robbins and

Louzada, 2005; Zang et al., 2005; Şahin-Çevik and Moore, 2006a). Homologs of some of these genes were also identified in different *Citrus* species (Hara et al., 1999, 2001; Porat et al., 2002; Robbins and Louzada, 2005). A number of genes induced in response to drought stress were also identified in mandarin using microarray technology (Gimeno et al., 2009). Additionally, some drought induced genes, including dehydrins, a 9-cis-epoxycarotenoid dioxygenase gene and a RING zinc finger protein have also been isolated from different *Citrus* types (Sanchez-Ballesta et al., 2004; Rodrigo et al., 2006; Şahin-Çevik and Moore, 2006b; Tadeo et al. 2008). It was shown that the expression of some cold-induced genes, such as dehydrins and a RING zinc finger protein were also induced by drought stress in *Citrus* and *Poncirus* (Sanchez-Ballesta et al., 2004; Şahin-Çevik and Moore, 2006b). Isolation and characterization of a number of WRKY proteins from *Arabidopsis thaliana*, rice (*Oryza sativa*), poplar (*Populus* spp.), papaya (*Carica papaya*) and sorghum (*Sorghum bicolor*) showed that they are conserved in various types of plants and involved in a wide variety of cellular functions (Eulgem et al., 2000; Zhang et al., 2004; Xie et al., 2005; Eulgem and Somssich, 2007; Ross et al., 2007; Pandey and Somssich, 2009). A partial sequence of a WRKY gene was previously identified in a cDNA library from cold-acclimated *P. trifoliata* and the full-length cDNA sequence of this WRKY gene (*PtrWRKY1*) was induced in response to cold and drought (unpublished data). In this study, isolation and cloning of one additional WRKY gene from *P. trifoliata* and its expression in response to cold and drought stresses in cold-hardy *P. trifoliata* and a cold-sensitive *Citrus* species, *Citrus grandis* (pummelo) is reported.

Results and discussion

Cloning of the *PtrWRKY2* gene from *Poncirus*

Fragments ranging from 400 to 2000 bp were amplified from cDNAs synthesized using total RNA of two-day cold-acclimated *Poncirus* seedlings by RT-PCR using degenerate primers. The amplified cDNAs were directly cloned into pGEM-T Easy vector and a number of clones were selected and sequenced. The BLAST analysis of the sequences obtained from these clones showed that some of these cDNAs had homology with WRKY TFs from other plants (Şahin-Çevik and Çevik, 2007). One of them was selected for isolation of the full-length cDNA using the 3' and 5' RACE PCR method. No distinct cDNA bands were amplified by RACE PCR with gene specific primers, but nested gene specific primers enabled the amplification of the 3' and 5' ends of the gene. As a result of the nested RACE PCR using MSC47 and MSC48 primers two fragments of about 700 bp and 950 bp corresponding respectively to the 3' and 5' ends which overlap with the original partial cDNA sequence were obtained. The partial sequence of the cDNA isolated from two-day cold-acclimated *Poncirus* by RT-PCR using degenerate primers and the 3' and 5' RACE PCR with the gene specific nested primers were assembled to obtain the full-length sequence of a WRKY gene, designated as *PtrWRKY2* (Accession Number JX236033). *PtrWRKY2* cDNA was 2,070 bp in length, consisting of a 71-bp 5'-untranslated region (UTR), a complete open reading frame (ORF) of 1,623 bp encoding a polypeptide of 540 amino acids, followed by a 3'-UTR of 379 bp (Fig 1.). Comparison of the *PtrWRKY2* with other WRKY proteins from different plants revealed that the *PtrWRKY2* had 46 to 64% amino acid

sequence identity with WRKY proteins from other plants. While the sequence identity of the *PtrWRKY2* with WRKY proteins from woody perennial plants, such as *Vitis* spp. and *Populus* spp. was more than 60%, it shared less than 50% sequence identity with herbaceous plants, such as *Cucumis sativus*, *Glycine max* and *Arabidopsis*. The comparison also showed that the most conserved regions of the *PtrWRKY2* protein were located between amino acids 170 and 230 and 375 and 475, corresponding respectively to the N- and C-terminal WRKY DNA-binding domains (Fig 2.). Sequences of the WRKY DNA-binding domains of the *PtrWRKY2* gene were compared with that of other WRKY TFs from various plants species by conserved domain search (data not shown). The sequence alignment showed that both WRKY domains of the *PtrWRKY2* were identical to the conserved consensus sequence WRKYGQK of the WRKY domain. In addition, a Cys₂His₂ zinc finger motif was present in the downstream of both WRKY domains of the *PtrWRKY2* (Fig 2.). Since this protein contains two WRKY domains with Cys₂His₂ zinc finger motif, it is classified in the group I type WRKY proteins in the superfamily (Eulgem et al., 2000). Phylogenetic analysis of the *PtrWRKY2* with other WRKY TFs from different plants showed that the putative *Poncirus* WRKY protein was clustered with different WRKY TFs having double WRKY domains from various plants. The *PtrWRKY2* was in the same phylogenetic group with putative WRKY TFs mostly from woody perennial plants, including *Vitis* spp. and *Ricus comminus*, *Populus* spp. and their hybrids. In addition, WRKY proteins from a few herbaceous plants, such as *Cucumis sativus* was in the same phylogenetic group and the *PtrWRKY2* was also closely related with a cluster containing WRKY genes from *Glycine max* (Fig 3.). The phylogenetic groups were supported by high bootstrap values. The phylogenetic group containing the *PtrWRKY2* did not include any well-characterized and functionally known WRKY proteins from *Arabidopsis* or other plants (Fig 3.). Significant homologies and close phylogenetic relations with putative WRKY proteins mostly from woody perennial plants indicated that the *PtrWRKY2* isolated from *Poncirus* is possibly structurally and functionally similar to WRKY proteins from other perennial plants.

The expression analysis of the *PtrWRKY2* gene in response to cold stress

To understand the role of the *PtrWRKY2* gene in abiotic stress response, Its expression was studied by Northern blot analysis in response to cold and drought stresses in two species, cold-hardy *Poncirus* and cold-sensitive pummelo having the same gene with more than 95% nucleotide sequence homology. Northern blot analysis and quantification of the expression data revealed that the *PtrWRKY2* gene was induced about 50% at 0.5 h cold acclimation, but the expression was decreased and repressed about 10-25% thereafter in cold-hardy *Poncirus* (Fig 4.). A similar expression pattern was detected in response to cold acclimation in cold-sensitive pummelo plants. Quantification of the *PtrWRKY2* gene expression showed that the expression was initially slightly increased about 10% at 0.5 h of the cold acclimation in pummelo and then, gradually decreased at 1 and 2 h of cold acclimation. While the expression was induced again at 1 d of cold acclimation, it was almost completely (87-90%) repressed thereafter in cold-sensitive pummelo (Fig 4.). The results indicated that the expression of the *PtrWRKY2* gene was initially induced in response to cold

Table 1. Primers used for isolation and expression analysis of the *PtrWRKY2* gene.

Primer code	Sequence* (5'-3')	Purpose of use	Orientation	Location	Gene
MSC45	GTAGCTTTGCTGGGGATGGCGAAGTCTCGG	3' RACE	Sense	943-973	<i>PtrWRKY2</i>
MSC46	CCGAGACTTCGCCATCCCCAGCAAAGCTAC	5' RACE	antisense	943-973	<i>PtrWRKY2</i>
MSC47	GAAGAAAGACAACCTAGCAAATTCAAGCCC	3' Nested RACE	Sense	1121-1151	<i>PtrWRKY2</i>
MSC48	CAACAATCAGAATATGTGCACTTGTAGTAGC	5' Nested RACE	antisense	655-686	<i>PtrWRKY2</i>
MSC54	<i>TAATACGACTCACTATAGGGTGC</i> GGCCAGAACATCTAAG	Probe synthesis	antisense	1423-1443	18S rRNA
MSC58	TTAACGAGGATCCATTGGAGGGC	Probe synthesis	Sense	511-534	18S rRNA
MSC59	TACTAGGAATTCCTCGTTGAAGACC	Probe synthesis	antisense	1545-1570	18S rRNA
MSC62	<i>TAATACGACTCACTATAGGGAGGCTCACTAATAATCTTC</i>	Probe synthesis	antisense	1665-1695	<i>PtrWRKY2</i>
MSC63	GTGCACATATTCTGATTGTTG	Probe synthesis	Sense	665-686	<i>PtrWRKY2</i>

* Italicized letters indicate T7 promoter sequence

in both cold-hardy *Poncirus* and cold-sensitive pummelo plants; however, it was clearly repressed after 1 h and 1 d of cold acclimation in *Poncirus* and pummelo, respectively. These data indicate that the *PtrWRKY2* gene may play a role in cold response in *Poncirus* and pummelo plants. It was shown that expression of a number of *WRKY* genes was changed in response to abiotic stress in different plants (Rizhsky et al., 2002; Seki et al., 2002; Hwang et al., 2005). It was demonstrated that 41 of 103 *WRKY* genes from rice showed higher or lower expression in response to cold, drought or salinity stresses (Ramamoorthy et al., 2008). In soybean, 25 *WRKY* genes showed differential expression when subjected to cold, drought or salinity stress (Zhou et al., 2008). The expression of the *PtrWRKY2* gene was induced at the beginning of the cold acclimation, but it was repressed at the end of the experiment. Similarly, Mare et al. (2004) showed that expression of the *Hv-WRKY38* gene was first increased during plants exposure to +2 °C then, the level of expression was dropped. However, the expression of this gene was strongly and constantly induced after plants were exposed to temperatures below the freezing point. The results indicated that the *HvWRKY38* gene was induced in response to low freezing temperatures and may have role in freezing tolerance rather than low temperature stresses. Considering the *PtrWRKY2* gene has a similar expression pattern with the *HvWRKY38* gene, it may also be induced more stably in response to freezing temperatures in *Poncirus* and/or pummelo plants as the *HvWRKY38* gene. In addition, since induction of the *Hv-WRKY38* gene was observed at early stages of cold acclimation, it might have a role in early cold-responsive gene expression. Similarly, the expression of the *PtrWRKY2* gene was also induced at 0.5 h cold treatment and the expression was declined thereafter implying that this gene may also have a role in early cold-responsive gene expression in *Poncirus* and pummelo.

The expression of the *PtrWRKY2* gene in response to drought stress

Since a number of abiotic stress responsive genes, including *WRKY* genes were induced in response to more than one stresses (Fowler and Thomashow, 2002; Seki et al., 2002; Ramamoorthy et al., 2008; Zhou et al., 2008; Jiang and Deyholos, 2009), the expression of the *PtrWRKY2* gene was also studied in response to drought. When the expression of the *PtrWRKY2* gene was studied in response to drought in dehydrated *Poncirus* and pummelo seedlings, no increase was observed in the expression of the *PtrWRKY2* gene in *Poncirus* and pummelo indicating that it is only induced in response to cold, but not to drought in both species analyzed (Fig 4.). On the other hand, while the expression of the *PtrWRKY2* was not changed in pummelo, a gradual decrease was observed in its expression starting at 7 d of dehydration only in *Poncirus*. Quantitative analysis of expression data

obtained from the Northern blot indicated that drought stress has no effect on the expression of the *PtrWRKY2* gene in pummelo, but the expression was repressed 27-50% by drought stress in *Poncirus*. The low or no signal detected in the Northern blot at 13 d in *Poncirus* and 13 and 15 d in pummelo was not due to changes in the expression, but it was rather due to low amount of RNA loaded into the gel. Over dehydration of leaves towards the end of drought stress treatment generally affected the yield and quality of the total RNA recovered from these samples. Although the spectrophotometer readings were similar to other samples, gel analysis showed that the yield and quality of total RNA from both species especially pummelo with broader leaf were adversely affected by prolonged dehydration. The expression of the *PtrWRKY2* gene was repressed in *Poncirus* during dehydration indicating that this gene may be negatively regulated during drought stress in *Poncirus*. Some *WRKY* TFs function as negative regulators of stress-responsive gene expression in plants. Although the expression of the *TcWRKY53*, isolated from *Thlaspi caerulescens* a heavy metal hyperaccumulator growing in soils contaminated with heavy metals, was up-regulated in response to NaCl, drought and cold stresses, it was negatively regulated by the osmotic stress in tobacco (Wei et al., 2008). A similar result was obtained from the *WRKY48* gene isolated from *Arabidopsis*. The expression of the *WRKY48* gene was induced after 2 h and 4 h post infiltration (hpi) with virulent *Pseudomonas syringae* and started to decrease until reaching to the basal levels in 24 hpi (Xing et al., 2008). On the other hand, the expression of the *PtrWRKY2* was not changed in response to drought in pummelo and similar results were obtained from other *WRKY* TFs from different plants. Although expression of the *GhWRKY3* gene from cotton was up-regulated by infection fungal pathogens, treatments with phytohormones and wounding, its expression was not affected by exposure to abiotic stresses, such as drought, cold or salinity (Guo et al., 2011). In another study, Zhou et al. (2008) showed that 39 out of 64 *WRKY* TFs from soybean did not show any changes in their expression in response to drought, cold and salt stresses.

Materials and methods

Plant materials

Seeds of *P. trifoliata* cv. Rubideaux (*Poncirus*) and *Citrus grandis* cv. Rein King (pummelo) were obtained from the National Clonal Germplasm Repository for Citrus & Dates in Riverside, CA. The seeds were planted in a soilless medium with 70% peat, 20% perlite and 10% vermiculite in pots and seedlings were grown and maintained in a controlled environmental growth chamber at 28 °C and 16-h photoperiod provided by cool white fluorescent light (100 μmol m⁻² s⁻¹).

Abiotic stress treatments

Cold acclimation

To isolate more *WRKY* genes from *Poncirus* and determine the expression of the *WRKY* gene in response to cold, ten potted seedlings of about two-year-old *Poncirus* and pummelo plants with two-month-old flushes grown in controlled environment growth chamber at 28 °C were acclimated for 2 d or 7 d at 4 °C with a 16-h photoperiod provided by cool white fluorescent light (100 μmol m⁻² s⁻¹). Leaf tissue samples were collected and bulked for each species just before the cold acclimation (0 h) and at 0.5, 1, 2, 4, 8, 24 h and 2, 4 and 7 d of cold acclimation. All tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until used for total RNA isolation.

Drought treatment

To determine the expression of the *WRKY* gene in response to drought stress, 10 seedlings from each *Poncirus* and pummelo plants were maintained in the controlled environment growth chamber at 28 °C with a 16-h photoperiod provided by cool white fluorescent light (100 μmol m⁻² s⁻¹) for one week without irrigation. Then, water continued to be withheld from the seedlings for additional one week and the water content of the soil in each pot was measured with a TrimeEasy time-domain reflectometry (TDR) probe (Innotech, Germany). Leaf tissue samples were collected from at least five different seedlings with the same or very close water content and leaf samples from different plants were bulked just before the drought treatment at specific time points of 7, 9, 11, 13 and 15 d of dehydration. All tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until used for total RNA isolation.

Total RNA Isolation

To isolate total RNA from cold and drought treated plants, leaf samples from these plants were ground to powder using liquid nitrogen and total RNA was isolated from about 100-150 mg leaf samples using Trizol Reagents (Invitrogen, USA) according to a previously reported protocol (Simms et al., 1993). Total RNA concentration was measured by a UV visible spectrophotometer (BioRad, USA) and the samples were stored at -80 °C until used.

Degenerate primers

To amplify more *WRKY* genes from *Poncirus*, a pair of degenerate primers was designed based on the consensus sequence of WRKY domain using the multiple sequence alignment of *Poncirus* PtrWRKY1 (accession number CXO65984) and WRKY proteins from other plant species (Eulgem et al. 2000; Zhang and Wang 2005) in the GenBank database. To amplify the region between two WRKY domains without annealing primers to each other, a forward degenerate primer (MSC 29 5'-GAY GGN TAY AAY TGG MGN AAR-3') was designed based on the conserved DGYNWRK amino acids and a reverse degenerate primer (MSC 30 5'-NAC YTG YTT YTG NCC RTA-3') was designed based on the conserved YGQKQV amino acids sequence of WRKY DNA-binding domains. These primers were used to amplify genes encoding WRKY TFs with two

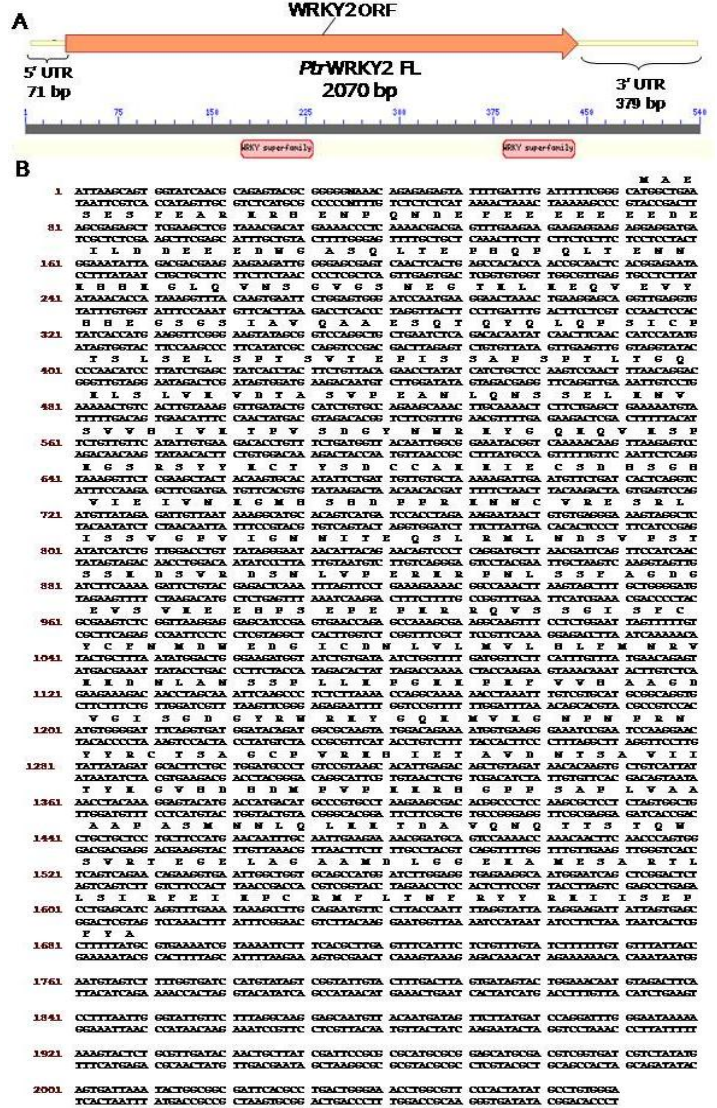


Fig 1. The full-length sequence of the *PtrWRKY2* cDNA (Accession Number JX236033). (A) The map of the full-length cDNA sequence showing open reading frame and the 5' and 3' untranslated regions. (B) The full-length cDNA sequence and the predicted translation of the *WRKY* gene open reading frame. The amino acid sequence is shown on the top of the cDNA sequence.

DNA-binding domains as previously described (Şahin-Çevik and Çevik, 2007).

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription-polymerase chain reaction (RT-PCR) with degenerate primers was used to amplify interdomain regions of WRKY TFs with two DNA-binding domains and the full-length cDNA sequences of the *PtrWRKY2* gene. First, cDNA was synthesized from total RNA isolated from two-day cold-acclimated *Poncirus* by the oligo dT primer using PrimeScript reverse transcriptase (Takara, Japan). For the cDNA synthesis, 1 μg total RNA was denatured at 65 °C for 5 min and quickly chilled on ice for a few minutes. A reaction mixture containing 1X cDNA synthesis buffer, 10 pmole oligo-dT primer, 0.2 mM dNTPs, 10 U RNAsin and 10 U PrimeScript was added to the denatured RNA and incubated at 42 °C for 90 min. Then, a portion of synthesized



Fig 2. Multiple sequence alignment of the full-length amino acid sequences of the *PtrWRKY2* with *WRKY* proteins from different plants in the GenBank. The alignment was generated by the AlignX module of Vector NTI suite. The codes preceding the scientific names of plants are the GenBank accession numbers of protein sequences. The conserved *WRKY* DNA-binding domain is indicated by the line above the sequence alignment. The C_2H_2 zinc finger motifs conserved in all *WRKY* domains, are indicated by arrowheads below the sequence alignment.

cDNA (5 μ l) were mixed with 1X PCR buffer containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U Ex Taq DNA polymerase (Takara, Japan) and 100 pmole forward and reverse degenerate or 20 pmole of gene specific primers for amplification of targeted sequences. PCR amplification was conducted in a 50 μ l reaction mixture in PTC1148 MJ Mini Thermocycler (Bio-Rad, USA). The thermocycler was programmed at 94 $^{\circ}C$ for 30 s for initial denaturation, 40 cycles of 94 $^{\circ}C$ for 30 s for denaturation, 45-55 $^{\circ}C$ for 30 s for primer annealing and 72 $^{\circ}C$ for 1 min for primer extension followed by one final cycle at 72 $^{\circ}C$ for 10 min for final primer extension. PCR products were separated in 1.5% agarose gel by electrophoresis in TAE buffer and visualized and photographed under UV light with the Doc-It (UVP, England) imaging system.

Rapid amplification of cDNA ends (RACE)

Sequences showing homology with *WRKY* genes were used for designing gene-specific and nested reverse and forward primers to obtain the 5' and 3' ends of the full-length cDNAs by rapid amplification of cDNA ends (RACE) method. The sequences and other information about the primers used for the 5' and 3' RACE are listed in Table 1. All steps of RACE were performed using the Smart RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's

instructions. Total RNA isolated from two-day cold-acclimated *Poncirus* seedlings was used as template for generating the 5' and 3' RACE ready cDNAs by RT-PCR using PowerScript reverse transcriptase and Smart II oligo and the 5' CDS primer A or with the 3' CDS primer for the 5' and 3' RACE ready cDNAs, respectively. The 3' and 5' ends of the gene were amplified by RACE PCR using the Advantage 2 DNA polymerase with the universal primer (UPM) in combination with gene specific primers MSC45 and MSC46. The RACE PCR was diluted and used as a template for amplification of the 3' and 5' ends of the *WRKY* gene by the nested RACE PCR using the Advantage 2 DNA polymerase with the universal nested primer and gene specific nested primers MSC47 and MSC48. The amplified cDNA fragments were purified from the gel using the Qiaquick gel extraction kit (Qiagen, Germany) and cloned into the pGEM-T Easy plasmid vector by T-A cloning method and plasmids carrying the 3' and 5' sequences were purified as described below.

Cloning of RT-PCR and RACE PCR products

PCR products containing the partial or the full-length sequences of the *WRKY* gene were directly cloned into the pGEM-T Easy plasmid vector using a T-A cloning kit (Promega, USA). For this purpose, 5 μ l of PCR products of

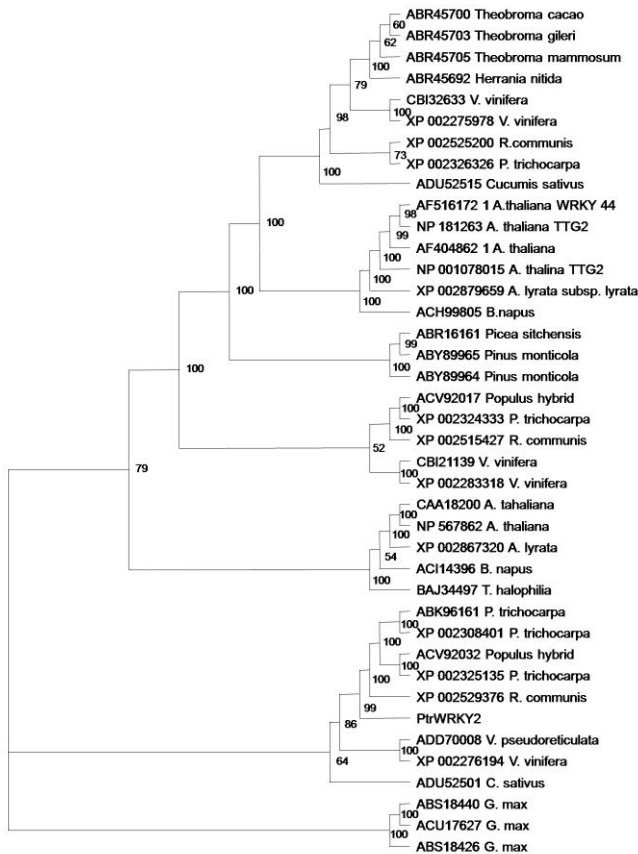


Fig 3. The phylogenetic analysis of amino acid sequences of the PtrWRKY2 transcription factor with other plant WRKY proteins in the GenBank databases. The phylogenetic tree was constructed from multiple sequence alignment of WRKY proteins from the GenBank generated by the AlignX module of Vector NTI suite using the neighbor joining clustering algorithm in Clustal X2 program. The GenBank accession numbers of the WRKY proteins and the sources and bootstrap values of the clusters are indicated in phylogram.

degenerate primers were ligated into the pGEM-T Easy plasmid vector, transformed into *Escherichia coli* JM109 competent cells and recombinant colonies were selected by blue/white screening on LB medium supplemented with ampicillin, 5-bromo-4-chloro-Indolyl- β -D-galactoside (X-Gal) and isopropyl-beta-thiogalactopyranoside (IPTG) according to the manufacturer's instructions. White colonies were screened by a colony PCR with *Taq* DNA polymerase using the forward and reverse M13 primers. Colonies containing the pGEM-Teasy plasmid with potential inserts of the WRKY gene were grown overnight and plasmids were isolated using the plasmid miniprep kit (Qiagen, Germany) and the presence of insert DNA was confirmed by *EcoRI* digest.

Sequencing and sequence analyses

To determine sequences of cloned partial and full-length cDNAs obtained from RACE and RT-PCR, the inserts were sequenced in both directions by automated cycle sequencing using the forward and reverse M13 universal primers. Sequences were analyzed using Vector NTI Suite (Invitrogen, USA) program and compared with other WRKY genes in GenBank. The partial sequences of cloned WRKY cDNA obtained from the RT-PCR and the 3' and 5' RACE

PCR were assembled and aligned to determine the full-length cDNA sequence of the WRKY gene. The full-length cDNA sequence and the deduced amino acid sequence were compared to nucleotide and amino acid sequences of previously identified WRKY TFs from different plant species in the GenBank database. The conserved domains within the protein sequences encoded by WRKY cDNAs were detected by a conserved domain search and compared with proteins containing the same or similar domain(s). Multiple alignments of the full-length amino acid sequences were generated by the AlignX module of Vector NTI suite. The phylogenetic analysis was performed by ClustalX2 using the neighbor joining algorithm and the phylogenetic tree was visualized by TreeView program.

Probe labeling and Northern blot hybridizations

Northern blot hybridization was conducted to determine the expression of the *PtrWRKY2* gene in response to cold and drought stresses in cold-hardy *Poncirus* and cold-sensitive *Citrus* species, *Citrus grandis* (pummelo) having the same gene with more than 95% nucleotide sequence homology in the coding region. The region for gene specific probes for *PtrWRKY2* gene and the 18S ribosomal RNA (rRNA) were first amplified from cDNA clone by PCR with the Advantage 2 Polymerase using a gene specific forward primer and a reverse primer with T7 promoter (MSC63 and MSC62; MSC58 and MSC54), respectively. The sequences and other information about the primers used for probe synthesis and labeling are listed in Table 1. The PCR amplified gene fragments with T7 promoter sequence at the 3' end of the antisense strand were purified by Qiaquick PCR purification kit (Qiagen, Germany), denatured and used as the template for synthesis of DIG labeled antisense riboprobes specific to WRKY gene and 18S rRNA using a DIG RNA labeling kit (Roche, Germany) according to the manufacturer's instructions. About 3 μ g of total RNA isolated from stress-treated and untreated control *Poncirus* and pummelo plants were separated on denaturing agarose gels with formaldehyde and formamide. The separated RNAs were transferred to a nylon membrane (Roche, Germany) by capillarity and fixed onto the membrane by UV cross linking (Stratagene, USA). Northern blot hybridization was performed as previously described (Şahin-Çevik and Moore, 2006b). After hybridizations with the gene specific probe, membranes were also hybridized with an 18S ribosomal RNA (rRNA) probe for loading and transfer control. The hybridization reactions were visualized by the ChemiDoc-It (UVP, England) chemiluminescent imaging system. The expressions of the *PtrWRKY2* gene and the 18S rRNA were quantified and analyzed by the LabWorks (UVP, England) image analysis software. The level of the expression at each time point was determined quantitatively by dividing the optical density values of the *PtrWRKY2* gene after background subtraction by that of the 18S rRNA used as reference. Then, changes in the expression of the *PtrWRKY2* gene defined as percent induction or repression were calculated by subtraction of the determined expression value of the control (0 h) from the expression values of the different time points of stress treatment followed by multiplication by 100. The results were graphically presented with Northern blot hybridization data.

Conclusion

The *PtrWRKY2* gene isolated and characterized in this study encodes WRKY TFs and showed differential expression in

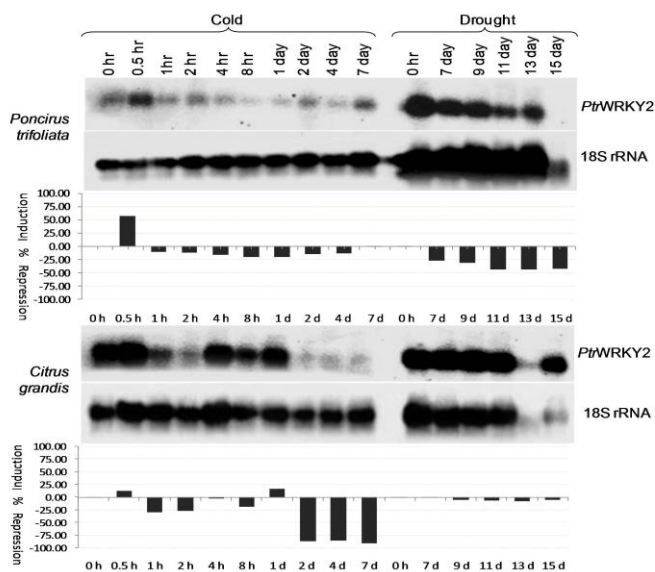


Fig 4. The expression analyses of the *PtrWRKY2* cDNA in response to cold and drought in *Poncirus trifoliata* (*Poncirus*) and *Citrus grandis* (pummelo) detected by antisense DIG-labeled riboprobe. The type of the environmental stress treatment and the duration of the treatment are indicated on the top and stress treated plant species were indicated on the left. The expression of the 18S rRNA was used as a loading and transfer control and is shown below the expression of the specific gene. Quantification of the expression data was presented under the RNA gel blot.

response to cold in both cold-hardy *Poncirus* and the most cold-sensitive *Citrus* species, *Citrus grandis* (pummelo) and in response to drought stress only in *Poncirus*. Further studies are needed to find its actual function of this gene in cold hardiness and drought tolerance in *Poncirus* and *Citrus*. The involvement of the *PtrWRKY2* gene should also be characterized in response to different abiotic and biotic stresses. In addition, over expression of the *PtrWRKY2* gene can also be studied to explore its function in cold and drought response in *Poncirus* and improvement of cold and drought tolerance in commercial *Citrus* species.

Acknowledgments

This study was financially supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) project number 104O158. The author thank to National Clonal Germplasm Repository for Citrus & Dates in Riverside, CA for providing seeds for plant material.

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