The tomato SlWRKY gene plays an important role in the regulation of defense responses in tobacco

Jing-bin Li, Yu-shi Luan*, Hui Jin

School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116024, China

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WRKY-type transcription factors are involved in multiple aspects of plant growth, development and stress responses. SlWRKY, a cDNA clone encoding a polypeptide of 552 amino acids and exhibiting the structural features of group I of WRKY protein family, was isolated from tomato (Solanum lycopersicum L. cv Zhongshu No. 4) using the homologous cloning method. Semi-quantitative RT-PCR analysis indicated that SlWRKY was up-regulated by salt and drought treatment in tomato seedlings. To investigate the biological roles of SlWRKY, we generated transgenic tobaccos overexpressing the SlWRKY and analyzed their responses to salt and drought stresses. Transgenic tobacco plants exhibited more vigorous growth than wild-type plants and display high tolerance to salt and drought stresses. In order to minimize oxidative damage, the activities of antioxidant enzymes were increased but EC and the MDA content were decreased in the transgenic tobacco leaves. Furthermore, it was observed that the SlWRKY proteins regulate the downstream genes and increased the expression of defense-related PR1 and PR2 genes. These results demonstrate that SlWRKY plays an important role in responding to abiotic stress.

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1. Introduction

Plants are constantly challenged with various types of abiotic stresses. To survive these challenges, plants not only have developed elaborate mechanisms to perceive external signals, but also implemented adaptive responses with proper physiological and morphological changes [1]. In recent years, researches concerning the mechanisms of protection against tolerance to environmental stress in plants have become more systematic. More and more studies that examine the plants’ responses to the environment have now focused on the level of gene regulation. Research on transcription factors has become a focus of current plant gene research. Once an external stimulus/signal makes contact with a plant, it usually passes through a series of signals and eventually induces the expression of many stress-responsive genes [2,3]. Among the numerous transcription factors, WRKY transcription factors were a new type of transcription factor which has been discovered in more recent years.

WRKY transcription factor super-family is a large family of transcription factors that are characterized by the presence of one or two 60-amino acid WRKY domains, including a very highly conserved WRKYGQK heptapeptide at its N-terminus with a zinc-finger-like motif at its C-terminus [4]. Most of identified WRKY proteins can bind W box (TTGAC[C/T]) to regulate gene expression. This family of genes is widely distributed among terrestrial plants. They have rapid, transient and tissue-specific expression features that could be induced under abiotic (cold, drought, and salinity) and biotic (pathogen) stresses, and could also participate in biotic and abiotic stress responses [5]. The factors involved in plant defense have been widely investigated in the recent years [6]. So far, there are 137 and 89 WRKY genes identified in rice and Arabidopsis, respectively [7]. In rice, different factors have been shown to confer resistance toward bacteria, fungi and environment stress, such as OsWRKY11 [8], OsWRKY13 [9] and OsWRKY89 [10]. Likewise, over-expression of OsWRKY23 [11] or OsWRKY45 [12] resulted in enhanced disease resistance and drought tolerance in Arabidopsis. In Arabidopsis, WRKY genes are differentially regulated following treatment with an avirulent strain of a bacterial pathogen or SA [13]. Over-expression of either AtWRKY25 or AtWRKY33 in Arabidopsis increases salt tolerance [14]. Over-expression GmWRKY13, GmWRKY21 and GmWRKY54 in Arabidopsis resulted in the plants being more tolerant to cold stress, drought and salt stress [15]. These examples illustrate that WRKY factors form part of the signaling processes associated with transcriptional reprogramming when plants encounter high salt, heat, cold, drought or pathogen. Although there is much information implicating WRKY proteins of many plants in plant defense responses, few experiments have reported the use of tomato transcription factor
WRKY for gene expression analysis under salt-related or drought-related stresses.

In this study, we isolated and characterized SlWRKY and examined the changes in responses toward various abiotic stresses by tobacco plants overexpressing SlWRKY. Physiological and biochemical changes of the transgenic plants during stresses and putative downstream genes were also analysed. Our results provide a useful reference for understanding the molecular mechanism of transcriptional regulation of the SlWRKY gene in tomato.

2. Materials and methods

2.1. Plant materials and growth conditions

Tomato (Solanum lycopersicum L. cv Zhongshu No. 4) or Tobacco (Nicotiana tabacum L. cv 89) seeds were surface sterilized and placed in conical flask containing Murashige and Skoog medium supplemented with 30 g/L sucrose, adjusted to pH 5.7, and solidified with 8 g/L agar. Seedlings were grown for 3–4 weeks under fluorescent light for 16 h at 25 ± 3 °C.

2.2. Nucleic acid extraction and cDNAs synthesis

Total RNA was extracted from 6-week-old tomato leaves using TRIZOL reagent according to the manufacturer's instructions (Takara). All RNA samples were subjected to reverse-transcription for the synthesis of the first cDNA strand using a Takara Reverse Transcription Kit.

2.3. Isolation of the WRKY gene and sequence analysis

The full-length of WRKY was cloned from tomato using homology-based cloning and RT-PCR methods [16]. Degenerate PCR primers were designed based on the WRKY conserved coding region from Solanum tuberosum (GenBank Accession No. ABU49723.1) and Capsicum annuum (GenBank Accession No. AA086886.1). Primers sequences were: WRKY-F: 5'-TTGGMGAAARYGGCMARAR-3' and WRKY-R: 5'-GGGCTGTYTTNCTCCTGCTANTG-3'. One of the ESTs has significant similarity to the Micro-Tom, database named Solanum lycopersicum cDNA, HTC in fruit (GenBank Accession No. AK326880). Used a pair of primer: WRKY-F: 5'-TTTATTCTTACTTCTCAGCAG-3' and WRKY-R: 5'-TTTTTTATATCAAATTAT-3' to find the full-length of SlWRKY as described previously [17]. The DNAMAN software and BLAST software online (http://www.ncbi.nlm.nih.gov) were used to analyze the DNA and protein sequences. A conserved WRKY superfamilly domain was found via searching Conserved Domains Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Multiple alignments were prepared using ClustalX 1.8. The online tool Compute pl/MW program (http://www.expasy.org/ proteomics/) was used to predcict the theoretical pl (isoelectric point) and Mw (molecular weight) of SlWRKY protein.

2.4. Stress treatment and semi-quantitative RT-PCR analysis

Aseptic seedlings of tomato were grown in MS medium containing 200 mM NaCl or 2% polyethylene glycol. Plant samples were harvested at various times after stress treatments and frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.5. Generation of SlWRKY-overexpressing tobacco plants

The coding region of SlWRKY was amplified with using forward primer 5'-TCCGCCCGGGATGACTCCTGCTATGCTTT-3' (Sma I sequence is underlined) and reverse primer 5'-GCCAGCTACGGGCCAGAAGTATC-3' (Sac I sequence is underlined), and inserted into the pBI121 vector with a modified cauliflower mosaic virus (CaMV) 35S promoter. The resultant recombinant vector pBI121-SlWRKY was transformed into Agrobacterium tumefaciens strain EHA105 by a freeze–thaw method. Single colonies of the bacteria were picked and grown at 28 °C in 50 mL of liquid YEB medium containing 100 mg/L carbenicillin and 50 mg/L kanamycin. Transformation of tobacco was performed using A. tumefaciens leaf disc method.

The putative transfromants were screened using genomic PCR analyses. Genomic DNA was extracted from tobacco leaves using CTAB extraction buffer as described previously [18]. For RT-PCR analysis, total RNA was extracted as described above. PCR products were detected by electrophoresis in 1% agarose gels.

2.6. Stress tolerance assay of the transgenic plants

Aseptic seedlings of transgenic tobacco and wild-type (WT) were submerged into 1/2 MS medium containing 200 mM NaCl or 2% polyethylene glycol. To make a deep analysis of the contribution of SlWRKY to the salt and drought tolerance, the 4-week-old seedlings of transgenic-tobacco and WT were transferred to the pots. The pot was filled till 3/4 full with soil mixture (vermiculite/soil = 1:1). Seedlings were cultured in a greenhouse (25 °C, 16 h light) and irrigated with 200 mM NaCl solution or holding water for two weeks. Then after 2 weeks, the plants of drought treatment were rehydrated and their recoveries were assessed. Plants were classified as alive or dead based on their color. Plants exhibiting green color in >50% of their tissue were counted as surviving plants.

2.7. Measurements of EC, MDA content, antioxidant enzyme activities and chlorophyll content

The electrical conductivity (EC) was calculated by the method of Liu et al. [19]. Lipid peroxidation of leaves was measured in terms of malondialdehyde (MDA) content, as described by Xu et al. [20] and expressed as mmol/g fresh weight (FW). The superoxide dismutase (SOD) activity was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). One unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of NBT by 50%. Peroxidase (POD) activity was measured using guaiacol and H2O2 as substrates and increase in absorbance at 470 nm due to oxidation of guaiacol was recorded as described previously [21]. The content of chlorophyll was extracted from a known fresh weight of leaves in 80% (v/v) aqueous acetone and spectrophotometrically determined and expressed as mg/g fresh weight [22].

2.8. Analysis of downstream genes regulated by SlWRKY genes

In transgenic and WT tobaccos, RT-PCR was used to check for the presence of expressions of the two defense marker genes, PR1 and PR2. The gene-specific primers were as follows: PR1 (forward primer: 5'-CCCAAAATTCTCACAAG-3' and reverse primer: 5'-TTAGTATGGACTTTCGCCTCT-3'); PR2 (forward primer: 5'-ATGGCTTCTTGCAGCTGCCCCTG-3' and reverse primer: 5'-GACTCAAAGTGTTCCTCCTGATA-3'). Primers sequences of the housekeeping gene actin were: forward: 5'-GTAGGTTGTGGTGACACT-3' and reverse: 5'-GGGAGCCAGGCGGTAT-3'.

2.9. Statistical analysis

The data of root lengths, fresh biomasses, EC, MDA contents, activities of antioxidant enzymes and chlorophyll content were analyzed. Values on columns are means of experiments conducted in triplicate and standard errors of means is indicated in bars. The experiment was independently repeated three times. Duncan
multiple range tests were performed by using one-way analysis of variance (ANOVA) on SPSS 17.0 version for Windows software and a p value <0.05 was considered statistically significant.

3. Results

3.1. Identification of a WRKY cDNA from tomato

The full-length cDNA of SlWRKY was 2152-bp, which contained an open reading frame (ORF) of 1659-bp encoding 552 amino acids, an untranslated region of 130-bp at the 5'-end and 363-bp of the noncoding region at the 3'-end. The estimated molecular mass of the predicted protein product was 6047.3 and an isoelectric point of 6.99. Two WRKY DNA-binding domains were found via searching Conserved Domains Database (Fig. 1A). For investigating the relationship among the SlWRKY and other WRKY proteins, ClustalX 1.8 was used to analyze the multiple alignments, and the results showed that these available WRKY proteins were found to possess two WRKY domains (Fig. 1B). Based on the number of WRKY domains and the features of the zinc finger motifs, WRKY family are divided into three groups [23]. All of the above data strongly indicated that SlWRKY belonged to group I WRKY family.

3.2. Constitutive expression of SlWRKY in tomato

In order to address the biological function of SlWRKY, semi-quantitative RT-PCR was used to examine its expression pattern in tomato. As shown in Fig. 2A, under salt treatment, rapid accumulation of SlWRKY transcript was observed after 2 h, with a maximum accumulation after 4 h, followed by a gradual decline after 8 h. Under drought treatment (Fig. 2B), SlWRKY transcript level increased only after 12 h but declined to background level beyond with longer treatment. Increases in SlWRKY expression level in response to salt and drought stresses shown here were similar to the response to similar stresses reported for OsWRKY45 [12]. These results strongly suggest that SlWRKY is involved in basal tolerance of salt and drought stress.

3.3. Confers salt and drought tolerance in transgenic tobacco

In order to evaluate the functional significance, the SlWRKY cDNA was cloned into the binary vector pBI121 under the transcriptional control of the CaMV 35S promoter and the resulting plasmid was introduced into tobacco using the Agrobacterium-mediated leaf disc transformation. Transformants were screened for resistance to kanamycin and then confirmed by PCR analysis. The PCR results of three representative positive transformants are shown in Fig. 2C and D. All three lines showed the presence of a 1659-bp band corresponding to size of the SlWRKY cDNA while no band was detected in the case of WT tobaccos.

Both transgenic lines and WT tobaccos (control) were placed in the 1/2 MS medium, containing 200 mM NaCl or 2% polyethylene glycol. After 45 days of growth in the salt and drought stress conditions, significant difference was observed in root lengths and fresh biomass. The transgenic lines showed more developed root systems and higher fresh biomass compared with WT. Different transgenic lines showed discrepant salt resistance (Fig. 3A and B) and drought resistance (Fig. 3C and D), and the best one was Line 17.

MDA is one of the final decomposition products in membrane lipid peroxidation, which is also taken as an indicator of the extent of damage caused by membrane lipid peroxidation. POD and SOD are ubiquitous among aerobic organisms and it plays a role in reducing intracellular reactive oxygen species and thereby

Fig. 1. Structure and sequence analysis of SlWRKY. (A) CDD analysis showing the two conserved domains of WRKY proteins. (B) ClustalX analysis, comparison of deduced amino acid sequences of WRKY family proteins that have high sequence similarity with SlWRKY. Two black boxes above the sequence represented the highly conserved DNA-binding domain (WRKY domain).
providing a protective effect against cell damage. Under the same stress conditions, a high reactivity means an increase in the ability to clear free radicals and better protection for the plants. Our results showed that EC and MDA content were significantly reduced whereas POD and SOD levels were significantly upregulated in the three transgenic lines compared with the WT after salt treatments (Fig. 3E–H) and drought treatments (Fig. 3I–L), suggesting that overexpression of SlWRKY in tobacco plants helped to protect the plants from lipid peroxidation incurred by stresses. EC, MDA content, POD and SOD activity are an important factor in determining plant growth and defense capacity [24]. Expression of SlWRKY gene in tobacco increased salt and drought stress tolerance. These studies indicated transgenic plants could stay the normal metabolism by accumulated osmotic substance under stress condition and SlWRKY may be associated with abiotic stress response signaling pathways and play multiple roles in plants.

3.4. Response of tobaccos to salt and drought stress after transplanting

After 2 weeks, seedlings of WT plants were wilting or turning yellowish, whereas the transgenic seedlings grew nearly normal and their leaf size was doubled in comparison with the size before the treatment (Fig. 4A). The results indicated that the overexpression of SlWRKY enhanced the salt tolerance. After withholding of water for 2 weeks, all transgenic tobaccos and WT plants wilted. However, recovery on normal watering
was seen, the transgenic lines were able to recover whereas WT plants remained in the wilted stage (Fig. 4B). These results suggested that overexpression of SIWRKY conferred a beneficial trait to the transgenic plants that enabled the plants to better withstand the condition of drought and helped them to recover once the condition was reversed.

The possible effect of SIWRKY on the control of downstream gene expressions through transcriptional regulation was also investigated. In the case of WT tobacco, expressions of the PR1 and PR2 genes were at very low levels compared to transgenic tobacco (Fig. 4C). This again showed the contribution of SIWRKY in regulating the expression levels of downstream genes.

WRKY proteins can specifically recognize some defense-related genes containing W-box elements in their promoters [25]. Therefore, the rapid increases in the expressions of WRKY genes are generally considered to play important roles in activating the expressions of downstream defense-related genes, such as PR1 and PR2 genes [26]. Overexpression of SIWRKY in transgenic tobacco resulted in increased expressions of downstream PR genes, which are likely to be involved in regulating resistance to salt, drought and pathogen stresses, and this was also consistent with those mentioned previously [27]. As mentioned above, due to the potential activity against biotic and abiotic stresses, tomato SIWRKY gene can be used to enhance resistance against biotic stress. Therefore, the functions are valuable for further investigations.

Salt and drought stresses can reduce the photosynthetic pigments of plants and decline in photosynthesis was seen as well as hindering of normal development of the plants. From the data shown in Fig. 4D and E, the chlorophyll content of WT leaves was significantly reduced in response to salt and drought stresses compared to transgenic lines.

4. Discussion

WRKY genes were among several families of transcription factor genes that are well evidenced to have important regulatory roles in plants subjected to various high-salinity or drought stresses [28,29]. Recently, it was also reported that overexpression of TaWRKY2 and TaWRKY19 could improve the salt and drought stress tolerance in Arabidopsis [30]. In this study, the up-regulated expression of SIWRKY in tobacco gave direct evidence.

SIWRKY, is the WRKY transcription factor isolated form tomato. It has two WRKY DNA-binding domains and belonged to group I WRKY family (Fig. 1). Its expression could be rapidly accumulated by not only salt treatments but also drought treatments (Fig. 2A and B). To further study the functions of SIWRKY, we transformed this gene into tobacco plants and used it in transgenic studies for stress-tolerance. Overexpression of SIWRKY improved stress tolerance in transgenic tobacco plants as revealed from changes in physiological parameters including root lengths and fresh biomass. MDA concentration has widely been utilized to differentiate salt-tolerant and salt-sensitive cultivars [31]. It was also previously reported to be directly related to drought [32]. Some antioxidant enzymes are altered when plants are subjected to stresses. These antioxidants have been touted as beneficial for mitigating the effects of biotic and abiotic stresses [33]. It is interesting to note that the SIWRKY promotes tolerance through the regulation of other physiological parameters, such as EC, MDA, POD and SOD (Fig. 3). The antioxidant enzymes were significantly higher whereas both the MDA and EC levels were lower in stressed transgenic plants than those in stressed control plant.

After transplanting, three independent SIWRKY-transgenic lines (2, 4 and 17) with higher transgene expressions showed better growth than the WT plants (Fig. 4A and B). We also analyzed for their chlorophyll produced under stresses (Fig. 4D and E), and the higher level of chlorophyll produced by the transgenic plants may provide some sort of protection against damage sustained by the onset of adverse environmental conditions. These results indicate that SIWRKY-overexpressing tobaccos exhibited salt and drought stress tolerance.

Otherwise, the differential tolerance of these transgenic plants to different stresses may reflect specificities of SIWRKY protein in DNA binding and regulation of downstream genes. PR genes are defense marker genes and it has been found to increase stress tolerance [34]. In SIWRKY-overexpressing tobaccos, the PR1 and PR2 expression was up-regulated compared with that in WT plants (Fig. 4C). Expression profiles of downstream genes suggested that SIWRKY had a specific moderate affinity with the promoter of PR1 and PR2, and it may improve stress tolerance in transgenic plants by direct binding and activating the PR genes.

Transgenic tobacco over-expressing SIWRKY displayed significant improvement in survival following salt and drought tolerance. Further study is needed to elucidate the mechanisms by which SIWRKY operates to enhance resistance against environmental stresses. Salt and drought is one of the major factors to limit the tomato yield in China. It will be very interesting and meaningful to further investigate the important roles of SIWRKY in response
to salt and drought and the effects of over-expressing SiWRKY on stress tolerance in tomato.

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