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Two novel WRKY genes from *Juglans regia, JrWRKY6* and *JrWRKY53,* are involved in abscisic acid-dependent stress responses

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Abstract

Genes encoding plant WRKY transcription factors are important for stress response. In the current study, two WRKY transcription factor genes (*JrWRKY6* and *JrWRKY53*) were identified from walnut (*Juglans regia* L.), and their function and involvement in stress responses were characterized. Under NaCl stress, *JrWRKY6* and *JrWRKY53* were upregulated in a short time (within 6 h of seedling exposure to salt) except in roots, in which the highest induction occurred at 24 and 48 h of salt exposure. The gene expression patterns under polyethylene glycol stress were similar to those under NaCl stress. Under heat stress, both genes were induced in all tissues, except for *JrWRKY6* in leaf tissue of seedlings treated for 24 and 48 h. Both genes were also induced in all plants exposed to cold stress, except for *JrWRKY6* in root tissue of seedlings exposed for 6 h and *JrWRKY53* in root tissue exposed for 48 h. *JrWRKY6* and *JrWRKY53* also showed varied responses to abscisic acid (ABA), with the maximum expression being for *JrWRKY6* in the roots of plants treated for 1 h, and *JrWRKY53* in the leaves of plants treated for 3 h. Furthermore, under NaCl, sorbitol, heat, cold, and ABA treatments, yeast cells transformed with *JrWRKY6* and *JrWRKY53* showed an improved growth activity and density relative to the empty-vector-containing control yeast. Moreover, JrWRKY6 or JrWRKY53 could bind to the W-box motif. These results suggest that *JrWRKY6* and *JrWRKY53* can response positively to abiotic stressors and improve the plant tolerance to salinity, osmotic stress, and abnormal temperatures in a mechanism that likely involves the ABA signalling pathway and W-box binding activity.

Additional key words: cold, gene expression, heat, NaCl, polyethylene glycol, walnut.

Introduction

WRKY transcription factors (TFs) are a large family of proteins in plants that regulate plant growth, development, and biotic and abiotic stress responses (Wei *et al*. 2012, Tripathi *et al*. 2014). According to the two most prominent and defining structural characteristics of WRKY proteins [the WRKY domains and the zinc-finger motifs (C-X_{4-5}-X_{22-23}-H-X1-H and C-X_7-C-X_{23}-H-X_1-C)], WRKY TFs are classified into three groups (Eulgem *et al*. 2000). Group I TFs contain two WRKY domains and one C_2H_2 zinc-finger motif; and groups II and III TFs include one WRKY domain and a C_2H_2 or C_2-H-C zinc-finger-like motif. Furthermore, the majority of WRKY TFs belong to group II (Eulgem *et al*. 2000, Ling *et al*. 2011).

Because WRKY TFs are induced by various biotic and abiotic stressors, they have a role in regulation of

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Abbreviations: ABA - abscisic acid; CDS - coding DNA sequence; GUS - β-glucuronidase; PEG - polyethylene glycol; qPCR - quantitative polymerase chain reaction; RT - reverse transcription; SC - synthetic complete; TF - transcription factor; Y1H - yeast one-hybrid system.
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plant responses (Dong *et al*. 2003). For instance, of the selected 13 *WRKY* genes noted in *Oryza sativa*, at least 10 are induced by salt, polyethylene glycol (PEG), and temperature stresses (Ramamoorthy *et al*. 2008, Berri *et al* 2009). Sixty-four *WRKY* genes in *Glycine max* were identified under abiotic stresses, and *GmWRKY21* improves cold tolerance of a transgenic *Arabidopsis* plant. *TcWRKY53* was cloned from *Thlaspi caerulescens* and is induced by salt, cold, and osmotic stresses (Wei *et al*. 2008). The *OsWRKY11* gene in rice is induced by heat shock and drought stresses, where its overexpression improves tolerance of transgenic plants to these two stresses (Wu *et al*. 2009). Germination rate and hypocotyledon and root lengths of an *Arabidopsis wrky25* mutant are decreased in contrast to the same features in *WRKY25* transgenic *Arabidopsis*. Furthermore, expression of *WRKY25* increases transcription of heat-shock factor genes *HsfA2*, *HsfB1*, *HsfB2a*, and *Hsp101* indicating a positive role of *WRKY25* in heat stress (Li *et al*. 2009). Expression of the *CaWRKY40* gene from *Capsicum annum* is also responsive to heat stress (Dang *et al*. 2013). Therefore, WRKY proteins are important TFs in plant stress responses and worthwhile to study further.

The WRKY TFs are DNA-binding proteins that recognize W-box motifs [a core sequence: TTGAC(C/T)] found in the promoters of a large number of plant defence-related genes (Dong *et al*. 2003). For instance, the promoter of a *ThVHAc1* gene from *Tamarix hispida* contains more than one W-box motif, and the ThWRKY7 protein can bind specially to the W-box to improve the cadmium stress response of the plant (Yang *et al*. 2016). The promoter of a galactinol synthase (*GolS*) gene from *Boea hygrometrica* (*BhGolS1*) includes four W-box motifs and was shown to be bound by an early-dehydration- and abscisic acid (ABA)-inducible *WRKY* gene (*BhWRKY1*), suggesting a mechanism where *BhWRKY1* is likely to function in an ABA-dependent signalling pathway to regulate *BhGolS1* expression (Wang *et al*. 2009). ThWRKY2, ThWRKY3, and ThWRKY4, which can all bind to the W-box motif, show similar expression patterns after exposure to ABA demonstrating that these three TFs are also involved in abiotic stress responses in an ABA-dependent signalling pathway (Wang *et al*. 2015). These reports suggested that the binding activity of WRKY proteins to the W-box is important for stress response in plants; therefore, studying the binding of WRKY proteins to the W-box is an effective way to illustrate the functional mechanism of *WRKY* genes.

Whereas multiple *WRKY* genes have been found in herbal plants, such as *Arabidopsis* and rice, few studies have been conducted on *WRKY* genes in woody plants, especially in nut trees. *Juglans regia* is an important nut tree with a variety of uses (Abdallah *et al*. 2015). With the variations of the global climate, environmental factors (particularly, a frequent cold in late spring) have become limiting factors for walnut production. Therefore, knowledge about the abiotic stress response mechanism of *J. regia* is important. However, studies on *J. regia* have focused mainly on production and nut quality (Bouabdallah *et al*. 2014, Pope *et al*. 2014, Qureshi *et al*. 2014), and research of stress tolerance at the molecular level remains limited. With the aim to contribute to better understanding *WRKY* genes in woody plants, two *JrWRKY* genes were cloned from walnut cv. Xiangling, and their expression profiles as well as contribution to stress tolerance were analyzed in response to salt, drought, osmotic, cold, and heat stresses. Additionally, W-box binding activity of both WRKYs was analyzed.

Materials and methods

Plants, growth conditions, and treatments: Walnut (*Juglans regia* L. cv. Xiangling) seeds soaked in water for 8 d were sown in a mixture of turf peat and sand (2:1, v/v) in plastic pots and were watered every 2 d. The seeds were cultivated in a greenhouse at a temperature of 22 ± 2 °C, a relative humidity of 70 ± 5 %, a 14-h photoperiod, and an irradiance of 100 μmol m^{-2} s^{-1}. After 10 weeks, the seedlings were used for experiments. For NaCl and PEG stress tests, the seedlings were watered with 0.3 M NaCl or 10 % (m/v) PEG_{6000}, and samples were collected after 0.5, 1, 3, 6, 12, 24, and 48 h. For ABA treatment, the seedlings were watered with 100 μM ABA, and samples were collected after 1, 2, 3, 6, 12, 24, and 48 h. For heat and cold stresses, the seedlings were exposed to 44 °C or 10 °C for 0.5, 1, 3, 6, 12, 24, and 48 h. Non-treated seedlings were used as controls. Each treatment was conducted with at least 12 seedlings, and roots, stems, and leaves were collected separately. All experiments were repeated three times.

Identification and sequence analysis of *JrWRKY* genes: A transcriptome for leaf, stem, and root tissues under cold stress was constructed using *Solexa* technology (data not shown and not published). The *WRKY* TFs were identified according to the functional annotations of non-redundant unigenes of the transcriptome under the "WRKY transcription factor." The selected *WRKY* genes were further confirmed by *BLAST* search (http://blast.ncbi.nlm.nih.gov/) and conserved domain prediction, from which two *WRKY* TFs with complete open reading frames (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) (named as *JrWRKY6* and *JrWRKY53*) were selected for analysis. Relative molecular mass (Mr), isoelectric point (pI), and encoded

amino acid sequence were predicted by the *Compute pI/Mw* tool (http://www.expasy.org/tools/protparam.html). The JrWRKY proteins and WRKY proteins from *Vitis vinifera* (Wen *et al*. 2014) were aligned by *ClustalX*, and the JrWRKY proteins were grouped according to the alignment and *V. vinifera* WRKY classification. The probable subcellular localization of the JrWRKY proteins was predicted by *Wolfpsort* (http:// wolfpsort.org) and *SubLoc* (http:// www.bioinfo.tsinghua.edu.cn/SubLoc/cgi-bin/eu_ subloc.cgi).

Isolation of RNA, reverse-transcription, and reverse transcription quantitative PCR: The total RNA was isolated from tissue samples using the cetyltrimethyl ammonium bromide method (Yang *et al*. 2014). In brief, 0.5 μg of DNase I-treated RNA was reverse-transcribed into cDNA using a *Prime Script RT Reagent* kit (*TaKaRa*, Shiga, Japan). The synthesized cDNA was diluted to 1/10 with sterile water and used as a template for reverse transcription (RT) quantitative PCR (qPCR), which was performed using a *SYBR Green Real-Time PCR Master Mix* kit (*Toyobo*, Osaka, Japan) on an *MJ Research Opticon 2* apparatus (*Bio-Rad Laboratories*, Hercules, CA, USA). The PCR cycle parameters were 94 °C for 30 s followed by 44 cycles of 94 °C for 12 s, 60 °C for 30 s, and 72 °C for 40 s, and a final extension at 81 °C for 1 s. The *18S* rRNA (HE574850) gene was used as an internal control (Xu *et al*. 2012). The primers used are listed in Table 1 Suppl. Three independent experiments were performed to ensure the reproducibility of the RT-qPCR results. Relative expression was calculated from the threshold cycle according to the control gene or control condition (Livak and Schmittgen 2001, Yang *et al*. 2016b). All data were analyzed using the *SPSS* software package (*SPSS*, Chicago, IL, USA).

Stress tolerance analysis of *WRKY* genes in yeast: To determine a potential role of the *JrWRKY* genes in stress tolerance, *JrWRKY6* and *JrWRKY53* were analyzed in a yeast expression system. The primers used to construct the yeast expression vectors are shown in Table 1 Suppl. The recombined plasmids pYES2-JrWRKY6 and pYES2-JrWRKY53 were independently transformed into the yeast *Saccharomyces cerevisiae* strain INVSC1 (His^-, Leu^-, Trp^-, and Ura^-) by the lithium acetate method (Gao *et al*. 2012). The INVSC1 yeast colonies that harbored empty pYES2 (control), pYES2-JrWRKY6, or pYES2-JrWRKY53 were cultivated in a synthetic complete (SC)-Ura medium containing 2 % (m/v) glucose at 30 °C to an absorbance (A_{600}) value of 0.4. Then, the yeast cells were collected and diluted with the SC-Ura medium containing 2 % (m/v) galactose and incubated with shaking for another 24 h. Thereafter, the yeast cells were collected and adjusted to an A_{600} of 1.6 by SC-Ura for stress analysis. The stress conditions included 0.3 M NaCl, 0.3 M sorbitol, 10 °C, 44 °C, and 100 μM ABA. Serial dilutions (10^0, 10^1, 10^2, 10^3, and 10^4) of these yeast cells were spotted onto SC-Ura agar plates and incubated at 30 °C for 60 h and then photographed. Non-stressed yeast cells (as controls) were also spotted onto SC-Ura agar plates at the same dilutions as the treated cells and grown at 30 °C for 40 h. Meanwhile, the yeast transformants INVSC1(pYES2), INVSC1(pYES2-JrWRKY6), and INVSC1(pYES2-JrWRKY53) were treated with the above-described stressors for 0, 3, 6, 12, and 24 h, and yeast cell densities (A_{600}) were evaluated after 20 h (NaCl and sorbitol) or 12 h (10 °C, 44 °C, and ABA). The data were analyzed by the *Tukey's* test ($P = 0.05$).

Yeast one-hybrid assays and transient co-expression confirmation: To evaluate binding activity of the JrWRKY6 and JrWRKY53 proteins to the W-box (TTGACC), three tandem copies of the WRKY motif were cloned into the pHis2 vector (Yang *et al*. 2016a). The coding DNA sequences (CDSs) of *JrWRKY6* and *JrWRKY53* were individually cloned into the pGADT7-Rec2 vector (*Clontech*, Palo Alto, CA, USA) as effectors (Fig. 1*A* Suppl. and Table 1 Suppl.). Their interactions were studied using the yeast one-hybrid (Y1H) system (*Clontech*). The p53HIS2 and pGADT7-Rec2-53 vectors were used as negative and positive control vectors, respectively (Wang *et al*. 2015, Yang *et al*. 2016a). Meanwhile, triple tandem repeats of the W-box motif were fused with a *CaMV35S*-46 minimal promoter and cloned into pCAMBIA1301 to drive the β-glucuronidase (*GUS*) gene (acting as a reporter) (Fig. 1*A* Suppl. and Table 1 Suppl.). The CDSs of *JrWRKY6* and *JrWRKY53* were cloned into the pROKII vector under control of the 35S promoter, generating pROKII-*JrWRKY6* and pROKII -*JrWRKY53* (Fig. 1*C* Suppl. and Table 1 Suppl.) to act as effectors. Then, the effectors and reporters were co-transiently transformed into *Arabidopsis* using the *Agrobacterium tumefaciens* EHA105-mediated method to further confirm the binding. The EHA105(pROKII-*JrWRKY6*), EHA105(pROKII -*JrWRKY53*), and EHA105 (reporter vector) cells were grown to an A_{600} of 0.6, and then diluted to an A_{600} of 0.5 with an infection buffer (a 1/2 Murashige and Skoog liquid medium supplemented with 100 μM acetosyringone). Five-week-old *Arabidopsis* seedlings were immersed in this buffer and incubated at 25 °C with 80 rpm shaking for 8 h. Thereafter, the seedlings were rinsed three times with the 1/2 Murashige and Skoog medium and incubated in the infection buffer for another 36 h under 30 rpm shaking, during which the infection buffer was added if needed to keep the A_{600} less than 0.6. Three independent transformations were performed and every transformation included at least 12 seedlings. The co-transformed *Arabidopsis* plants were used to stain for and measure GUS activity (Liu *et al*. 2015, Yang *et al*. 2016a). A CaMV 35S-luciferase construct was also transformed together to normalize the transformation efficiency. Every experiment was repeated three times.

Results

The basic characteristics of *JrWRKY6* (*GeneBank* accession No. KP784650) and *JrWRKY53* (acc. No. KP784657) were following: the open reading frames were 1 224 and 1 083 bp in length, the deduced encoded polypeptides included 407 and 360 amino acids (the nucleic and protein sequences are shown in Fig. 3 Suppl.), the predicted molecular masses were 43.297 and 40.174 kDa, and the pI 8.50 and 5.43, respectively. Because TFs are generally nuclear proteins that play a regulatory role in nuclear responses (Forwood *et al.* 2001), we predicted that both JrWRKY6 and JrWRKY53 would be targeted to the nucleus and would play a role there, similar to that of the LbDREB protein from *Limonium bicolor* (Ban *et al.* 2011). Based on the category of VvWRKY proteins from *V. vinifera*, JrWRKY6 and JrWRKY53 were classified into the group III subfamilies (Fig. 1) according to their WRKY domain and zinc-finger motif (Wen *et al.* 2014).

To understand the responses of *JrWRKY6* and *JrWRKY53* to salt and osmotic stresses, NaCl and PEG

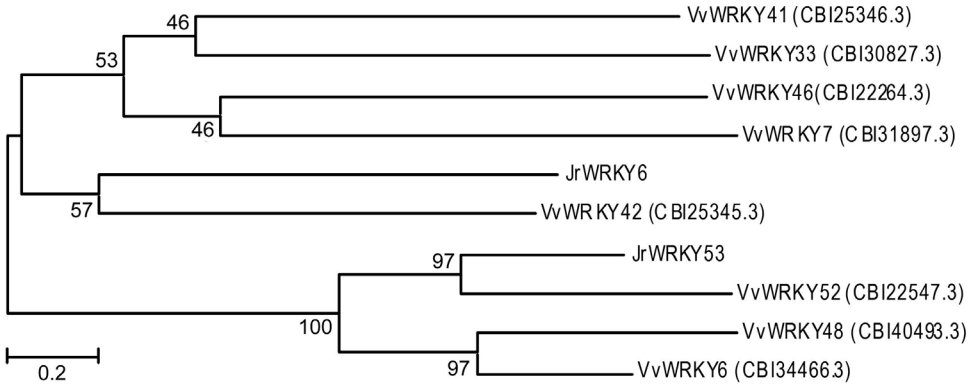

Fig. 1. A phylogenetic tree of the deduced amino acid sequences of nine JrWRKY proteins and WRKY proteins from *V. vinifera* (VvWRKY) based on sequence alignments of the encoded proteins. The alignment was constructed by *ClustalX* using the neighbour-joining method in *MEGA 4.0*.

treatments were applied. In the control, both *JrWRKY6* and *JrWRKY53* were hardly expressed in roots, stems, and leaves (Fig. 2). However, the gene expression was changed remarkably after exposure to NaCl and PEG (Fig. 3). Under the NaCl stress, *JrWRKY6* and

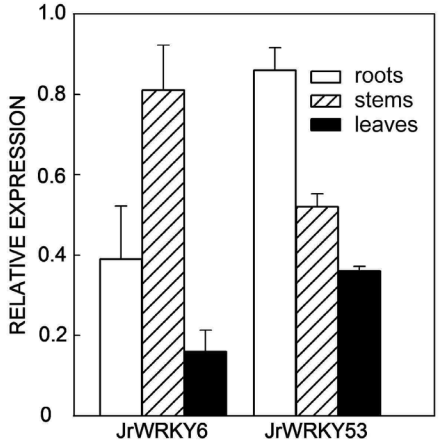

Fig. 2. The relative expressions of *JrWRKY* transcription factor genes in roots, stems, and leaves of walnut seedlings grown under normal conditions. The expression was related to the internal control gene *18S*. All of the relative expression levels were log2 transformed. The *error bars* were obtained from three replicates of reverse transcription quantitative PCR.

JrWRKY53 showed similar expression tendencies in roots, being downregulated at 3 and 6 h of the salt treatment. However, the maximum expression of *JrWRKY6* (25.46-fold of the control) was at 24 h, whereas that of *JrWRKY53* (38.32-fold of the control) was at 48 h of the treatment. In stems, *JrWRKY6* and *JrWRKY53* were induced at 0.5 - 6 h, with their most remarkable expressions (7.78-fold and 6.19-fold of the control, respectively) after the 1-h treatment. After a longer treatment (for 12 - 48 h), their expressions were mainly suppressed. In leaves, the expression profiles of the two genes were different. *JrWRKY6* increased to 1.68 - 10.93-fold of the control except at 24 h of the treatment. The expression of *JrWRKY53* tended to decline with a prolonged treatment time (12 - 24 h), whereas it was upregulated at 0.5 - 6 h (Fig. 3*A*).

Under the PEG stress, the expressions of *JrWRKY6* and *JrWRKY53* in roots were similar, where they were downregulated at 3 and 6 h but then induced to 2.53 - 18.77-fold and 1.73 - 31.78-fold of the control, respectively. In stems, the expressions of *JrWRKY6* and *JrWRKY53* were mainly induced, except at 3 and 6 h of the treatment. However, there were also some differences between the two *WRKY* genes. The expression of *JrWRKY6* was less than that of *JrWRKY53* at 3 - 12 h, whereas it was higher at 24 - 48 h of the treatment. The highest expression of *JrWRKY6* was 19.16-fold of the

control after 24 h of the treatment and the highest expression of *JrWRKY53* was 14.72-fold of the control at 12 h of the stress exposure. In leaves, the expressions of *JrWRKY6* and *JrWRKY53* increased before 6 h of the treatment and then decreased from 6 to 24 h. Both *JrWRKY6* and *JrWRKY53* achieved their maximum expressions at 6 h of the stress treatment, where they were 38.32-fold and 29.04-fold of the control, respectively. Overall, the expressions of *JrWRKY6* were higher than those of *JrWRKY53* except at 0.5, 1, and 48 h of the treatment (Fig. 3*B*).

To understand potential responses of *JrWRKY6* and *JrWRKY53* to abnormal temperatures, the *J. regia* seedlings were exposed to 44 °C or 10 °C for different

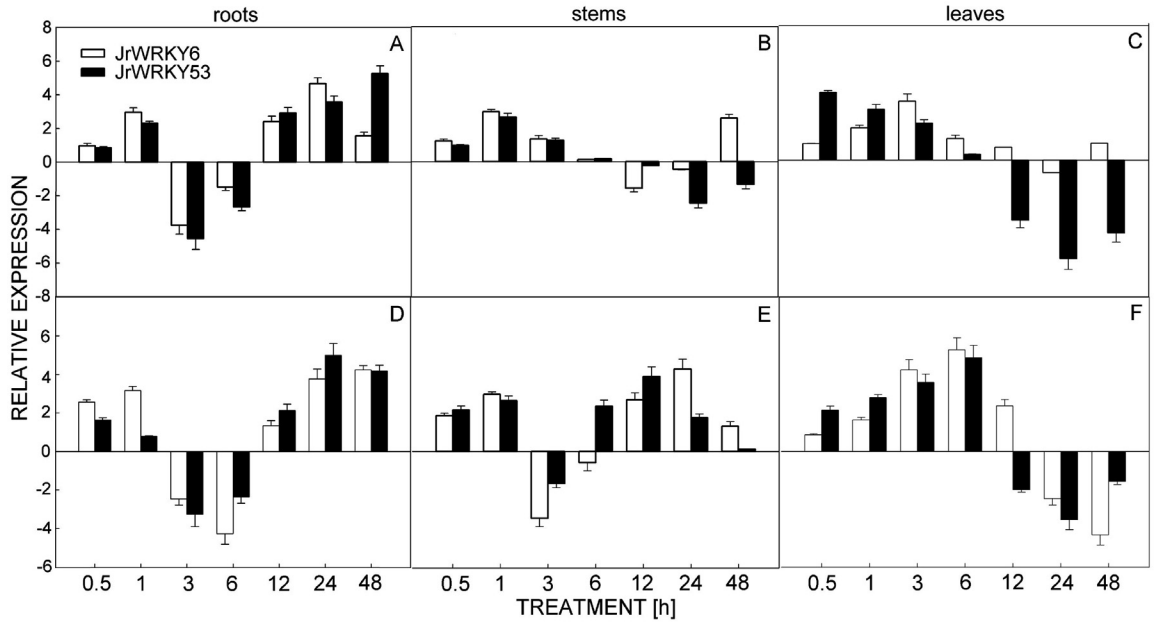

Fig. 3. The relative expressions of *JrWRKY6* and *JrWRKY53* in different walnut organs under NaCl (*A*,*B*,*C*) and PEG (*D*,*E*,*F*) stresses. The relative expression = expression under stress treatment/expression in control (0 h). All of the relative expression levels were log2 transformed. The error bars were obtained from three replicates of reverse transcription quantitative PCR.

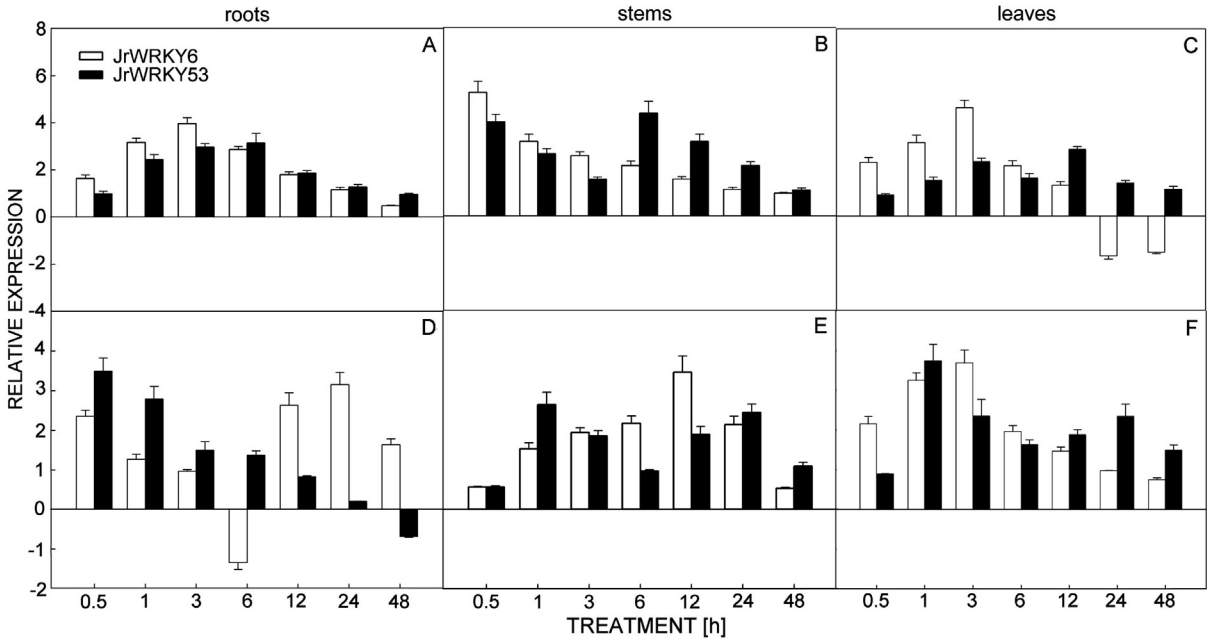

Fig. 4. The relative expressions of *JrWRKY6* and *JrWRKY53* in different walnut organs under heat (44 °C; *A*,*B*,*C*) and cold (10 °C; *D*,*E*,*F*) stresses. The relative expression = expression under stress treatment/expression in control (0 h). All of the relative expression levels were log2 transformed. The error bars were obtained from three replicates of reverse transcription quantitative PCR.

time periods. When exposed to 44 °C, *JrWRKY6* and *JrWRKY53* expressions in roots increased at all the tested time intervals with similar patterns, only the expression of *JrWRKY6* was higher than that of *JrWRKY53* at 0.5 - 3 h, whereas *JrWRKY6* was upregulated less than *JrWRKY53* at 6 - 48 h of the exposure. The maximum expression of *JrWRKY6* was 15.56-fold higher than of the control at 3 h of the treatment, whereas the highest expression of *JrWRKY53* was 8.75-fold of the control at 6 h of the exposure. In stems, the expression of *JrWRKY6* decreased from 37.53-fold to 1.95-fold of the control as the treatment time extended, whereas the expression of *JrWRKY53* declined from 15.89-fold to 2.95-fold of the control at 0.5 - 3 h of the treatment, and then increased to 20.53-fold of the control at 6 h, and then decreased again to 2.13-fold of the control at the 48 h exposure. In leaves, *JrWRKY6* was upregulated before 12 h of the stress, and its maximum expression was 24.76-fold of the control at 3 h of the treatment. *JrWRKY53* was induced at all the tested time points to 1.88 - 7.21-fold of the control (Fig. 4*A*).

Under the cold stress, the *JrWRKY6* and *JrWRKY53* expressions in roots were mainly increased, except at

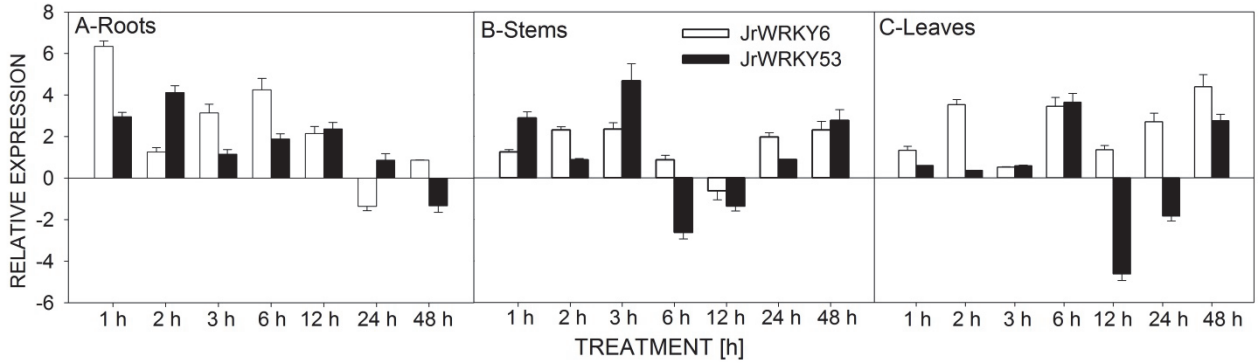

Fig. 5. The relative expressions of *JrWRKY6* and *JrWRKY53* in different walnut organs exposed to abscisic acid. The relative expression = expression under stress treatment/expression in control (0 h). The error bars were obtained from three replicates of reverse transcription quantitative PCR. All of the relative expression levels were log2 transformed.

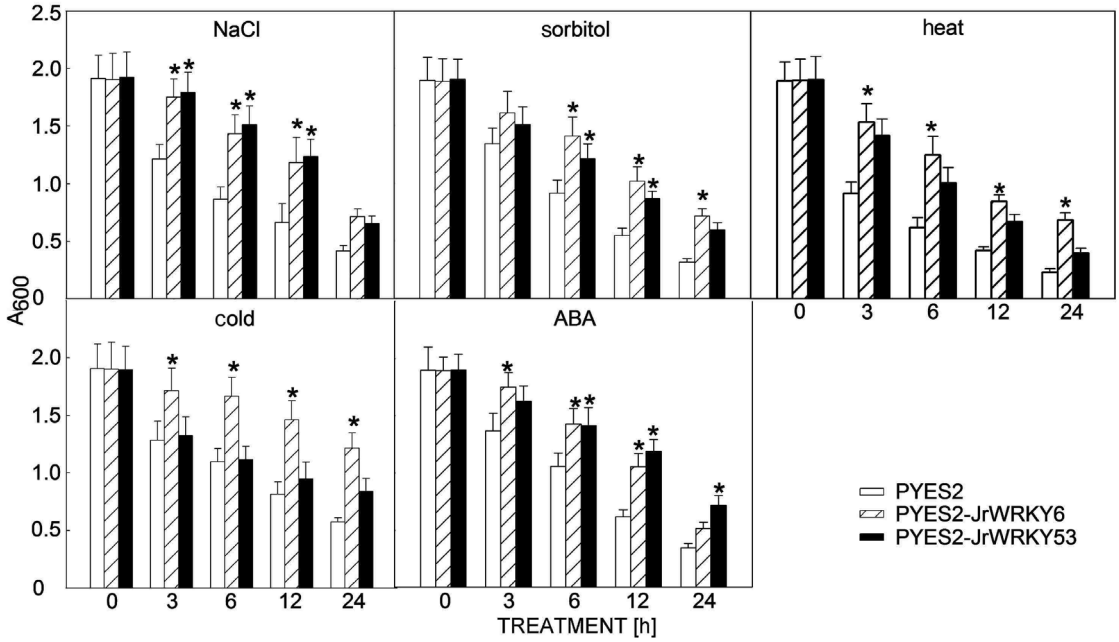

Fig. 6. The abiotic stress tolerance analysis of *JrWRKY6* and *JrWRKY53* in a yeast expression system compared with the empty pYES2 (control) yeast. The two yeast cultures were independently grown in a synthetic complete (SC)-Ura liquid medium containing 2 % (m/v) galactose at 30 °C for 20 h to A_{600} = 0.4, then collected and adjusted the yeast with SC-Ura including 2 % (m/v) galactose and cultivated to A_{600} = 1.6 for stress analysis. Yeast cell densities (A_{600}) of INVSCI (pYES2), INVSCI (pYES2-*JrWRKY6*), and INVSCI (pYES2-*JrWRKY53*) under various time intervals (0, 3, 6, 12, and 24 h) (0 h was set as a control) for NaCl, sorbitol, 10 °C, 44 °C, and abscisic acid treatments were tested. Means ± SDs, n = 3, significant differences between INVSCI (pYES2) and INVSCI (pYES2-*JrWRKY6*), and INVSCI (pYES2) and INVSCI (pYES2-*JrWRKY53*) are indicated by * ($P < 0.05$), respectively.

6 and 48 h of the treatment, respectively. The highest expression of *JrWRKY6* was 8.88-fold of the control at 24 h. The expression of *JrWRKY53* decreased with extended treatment time, and the maximum expression

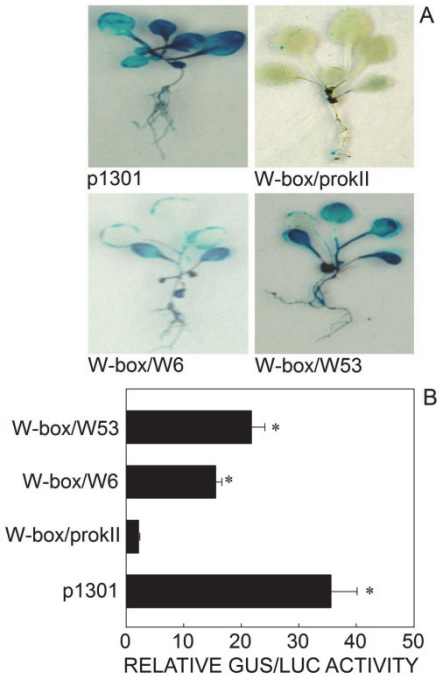

Fig. 7. The binding analysis of JrWRKY6 and JrWRKY53 to the W-box motif. A 35S::luciferase (LUC) construct was transformed together with a reporter and an effector into leaves to normalize for transformation efficiency. p1301 was used as a positive control, W-box co-transformation with the empty pROKII (W-box+ prokII) was used as a negative control. *A* - GUS staining the co-expression of the effector and reporter plasmids in *Arabidopsis* leaves. *B* - GUS activities according to *Arabidopsis*. Means ± SDs, $n = 3$; * indicates significant differences between the negative control and others ($P < 0.05$).

was 11.24-fold of the control at 0.5 h. In stems, the maximum expression of *JrWRKY6* (10.93-fold of the control) was at 12 h. The expression profile of *JrWRKY53* had two peaks at 1.5 h (6.19-fold of the control) and 24 h (5.43-fold of the control) of the treatment, respectively. In leaves, *JrWRKY6* induction was highest within 3 h (12.91-fold of the control) and lowest at 48 h (1.67-fold of the control) of the treatment. The maximum expression of *JrWRKY53* was 13.36-fold of the control at 1.5 h of the treatment, whereas the minimum was 1.85-fold of the control at 0.5 h of the stress exposure (Fig. 4*B*).

To determine whether the ABA signalling pathway is involved in stress responses, the seedlings were treated with ABA, and the expression patterns of *JrWRKY6* and *JrWRKY53* were analyzed. Both the *JrWRKY6* and *JrWRKY53* expressions were generally induced after the ABA treatment, except at a few time points. However, these genes showed completely different expression profiles in all the tissues. In roots, *JrWRKY6* increased to 1.82 - 81.57-fold of the control, except at 24 h of the ABA exposure. *JrWRKY53* was downregulated at 48 h but upregulated to the maximum value (17.51-fold of the control) at 2 h of the treatment. In stems, *JrWRKY6* was induced to 1.82 - 5.06-fold of the control, except at 12 h. *JrWRKY53* was induced at 3 h (25.63-fold of the control), whereas it was suppressed to a minimum at 6 h. In leaves, *JrWRKY6* was induced at all the time points of the treatment, and the maximum (20.92-fold of the control) was at 48 h. The expression of *JrWRKY53* was similar (1.27 - 1.51-fold of the control) at 1 - 3 h, and then it increased to a maximum (12.47-fold of the control) at 6 h of the treatment. Furthermore, the *JrWRKY53* expression was downregulated within 12 and 24 h of the ABA treatment (Fig. 5).

To understand the roles of *JrWRKY6* and *JrWRKY53* under abiotic stress, the yeast expression system was selected to analyze the two genes. The three yeast lines (pYES2-JrWRKY6, pYES2-JrWRKY53, and the control) were treated with different abiotic stressors. The results show no difference among these three yeast lines under normal culture conditions (Fig. 6 and Fig. 2 Suppl.). However, the pYES2-JrWRKY6 and pYES2-JrWRKY53 transgenic yeast exhibited increased survival rates compared with the control yeast under salt, osmotic (sorbitol), heat (44 °C), cold (10 °C), and ABA treatments (Fig. 2 Suppl.). To quantify survival differences among these yeast lines, their cell densities (A_{600}) were measured after recovery from the different treatments with the non-treated (0 h) cells used as controls. After each stress treatment, INVSC1(pYES2-JrWRKY6) and INVSC1(pYES2-JrWRKY53) displayed much higher A_{600} values than INVSC1(pYES2). Under the NaCl stress, the A_{600} values of INVSC1(pYES2-JrWRKY6) and INVSC1(pYES2-JrWRKY53) were 1.44 - 1.78-fold and 1.48 - 1.86-fold of that of INVSC1 (pYES2), respectively, and differences generated at 3 - 12 h of the stress exposure were significant. Under the sorbitol stress, the A_{600} value of INVSC1(pYES2) was 64.76 - 43.99 % of that of INVSC1(pYES2-JrWRKY6) at 6 - 24 h, and the difference was significant. The A_{600} value of INVSC1(pYES2) was 75.43 and 62.95 % of that of INVSC1(pYES2-JrWRKY53) at 6 and 12 h, respectively. Under the heat stress, the greatest difference among the three yeast strains was at 24 h, where the A_{600} values of INVSC1(pYES2-JrWRKY6) and INVSC1(pYES2-JrWRKY53) were 2.99- and 1.73-fold of that of INVSC1(pYES2), respectively. Under the cold stress, the greatest difference among the three yeast strains was also generated at 24 h, where A_{600} of INVSC1 (pYES2-JrWRKY6) and INVSC1(pYES2-JrWRKY53) were 2.12- and 1.46-fold of that of the control yeast, respectively. The A_{600} values of INVSC1(pYES2) under the ABA stress were 58.35 - 78.16 and 48.32 - 84.12 % of the A_{600} values of INVSC1(pYES2-JrWRKY6) and INVSC1(pYES2-JrWRKY53), respectively. The diffe-

rences at some time intervals were significant (Fig. 6).

Understanding whether WRKY TFs can bind to the W-box is important for comprehending the response mechanism of WRKYs in plants. To determine whether JrWRKY6 and JrWRKY53 can bind to the W-box motif, a Y1H assay was performed. All the yeast transformants grew well and had similar growth and sizes on a SD/-His/-Leu/-Trp medium containing 50 mM 3-amino-1,2,4-triazole as a positive control (Fig. 1*B* Suppl.), suggesting the binding of JrWRKY6 and JrWRKY53 to the W-box. To confirm the Y1H assay results, the CDSs of *JrWRKY6* and *JrWRKY53* were cloned into the pROKII vector as effectors, three tandem repeats of the W-box motif were inserted into the pCAMBIA1301 as reporters, and then each effector and reporter were co-transformed into *Arabidopsis* seedlings. Increased GUS staining and activity similar to that of the positive control (Fig. 7) indicates that *JrWRKY6* and *JrWRKY53* can bind specifically to the W-box motif.

Discussion

WRKY TFs are involved in plant stress responses, growth, and development. In the current study, based on their WRKY domain and zinc-finger motif, *JrWRKY6* and *JrWRKY53* were identified and classified into group III according to the WRKY proteins from *V. vinifera* (Wen *et al*. 2014). In *A. thaliana*, the binding-site preferences of *AtWRKY* TFs for the WRKYGQK motif depend on the DNA sequences adjacent to the TTGACY core motif (Ciolkowski *et al*. 2008). In *Nicotiana tabacum*, the NtWRKY12 protein contains a WRKYGKK motif and recognizes the downstream binding sequence TTTTCCAC, which is not a W-box (Van Verk *et al*. 2011). These reports indicate that the variations of the WRKYGQK motif influence the normal interaction of WRKY TFs with downstream target genes, and thus it would be interesting to investigate the functions and binding specificities of *JrWRKY* TFs in stress responses.

Under the abiotic stresses (NaCl, PEG, heat, cold) and ABA treatments, the expressions of *JrWRKY6* and *JrWRKY53* genes were generally induced in roots, stems, and leaves but with tissue-specific patterns. These results are similar to other reports on WRKY TFs. For instance in *Arabidopsis*, *AtWRKY25* and *AtWRKY33* are both significantly induced by NaCl or mannitol treatments for 24 h (Jiang and Deyholos 2009), which is similar to *JrWRKY6* and *JrWRKY53* under the NaCl or PEG stresses for 24 h. Meanwhile, *AtWRKY25* is induced by cold stress at 24 h, whereas *AtWRKY33* is upregulated by cold exposure at 6 h while suppressed by heat exposure at 24 h (Jiang and Deyholos 2009); likewise, *JrWRKY6* was induced after 24 h of exposure to the cold and 6 and 24 h of exposure to the heat, whereas the *JrWRKY53* expression increased after 6 h of the cold and 6 and 24 h of heat stresses (Figs. 3 and 4). The transcription of *OsWRKY76* in *O. sativa* increases after the cold stress for 0 - 25 h (Yokotani *et al*. 2013), which is the same as for *JrWRKY6* and *JrWRKY53*, which were mainly induced under the cold stress for 0 - 48 h (Fig. 4*B*). The expression of *BcWRKY46* from *Brassica campestris* is upregulated by the cold stress at all tested time points (0.5 - 168 h) and by the drought and NaCl treatments at 2 h (Wang *et al*. 2012). Our results also show that both *JrWRKY6* and *JrWRKY53* were induced at any cold stress time points in stems and leaves, and both the genes were upregulated after a short time (less than 3 h) of the salt stress (Figs. 3 and 4). In *V. vinifera*, most of the 59 *VvWRKY* TFs are also induced by cold stress (Wang *et al*. 2014). In *Tamarix hispida*, *ThWRKY4* is highly induced in leaves by 3 h NaCl stress (Zheng *et al*. 2013) similarly to *JrWRKY6* and *JrWRKY53* (Fig. 3). *ThWRKY4* is also upregulated by PEG stress at 3 and 6 h in both roots and leaves (Zheng *et al*. 2013). However, the expressions of *JrWRKY6* and *JrWRKY53* increased at 3 and 6 h of the PEG stress in leaves and downregulated in roots (Fig. 3). These results suggest that *WRKY* genes from different species may be induced by the same abiotic stressors but with various expression profiles, implying their potential role in abiotic stress response. Therefore, although *JrWRKY6* and *JrWRKY53* did not display similar expressions after the exposure to NaCl, PEG, heat, and cold stresses, the potential involvement of these genes in the plant tolerance to the imposed stressors was demonstrated and worth further confirmation.

In plants, ABA-independent signalling pathways are also involved in stress responses including drought, heat, and cold. They include many TFs that are master regulators of gene expression among abiotic stress responses (Yamaguchi-Shinozaki and Shinozaki 2006, Nakashima *et al*. 2014, Yoshida *et al*. 2014). Therefore, understanding whether the studied genes are involved in the ABA-dependent or ABA-independent signalling pathway is important for clarifying the regulation mechanism of stress response. Recent studies on WRKY TFs have pointed out that many *WRKY* genes are involved in the ABA-dependent signalling pathway. For example, *ThWRKY4* was induced in roots at 3, 6, 9, and 12 h of the drought or salt stress, and the overexpression of the gene improved transgenic seedling tolerance to these stressors, meanwhile, the transgenic plants increased tolerance to the ABA treatment indicating that the response of *ThWRKY4* relates to the ABA-dependant signaling pathway (Zheng *et al.* 2013). Furthermore, ThWRKY2 and ThWRKY3 were also predicted to participate in the ABA-dependant signalling pathway (Wang *et al*. 2015). CmWRKY1 TF, isolated from

transgenic *Chrysanthemum morifolium* plants, suppressed expression of genes negatively regulated by ABA [*e.g.*, ABA insensitive (*ABI*) 1 and *2*] and activated expression of genes positively regulated by ABA [*e.g.*, pyrabactin resistance 1-like 2 (*PYL2*), ABA-responsive element-binding factor 4 *(ABF4)*, and dehydration-responsive element-binding factor 1A (*DREB1A*)] indicating that *CmWRKY1* plays an important role in *Chrysanthemum* response to drought through the ABA-mediated signalling pathway (Fan *et al*. 2016). The high expression of *DREB1A*, *DREB2A*, 9-*cis*-epoxycarotenoid dioxygenase 3A (*NCED3A*), and *NCED3B* in *CmWRKY10*-overexpressing plants provided a strong evidence that the drought tolerance mechanism is associated with the ABA signalling pathway (Jaffar *et al*. 2016). The expressions of *BcWRKY46*, *OsWRKY76*, *AtWRKY25*, and *AtWRKY33* are all influenced by ABA suggesting that they are all possibly involved in the ABA signalling pathway (Li *et al*. 2011, Wang *et al*. 2012, Yokotani *et al*. 2013). In the current study, the expressions of *JrWRKY6* and *JrWRKY53* were affected by ABA, where they were mainly induced before 6 h of the stress, and thereafter suppressed at some time point in roots, stems, and leaves. These results suggest that the response mechanism of *JrWRKY6* and *JrWRKY53* may be involved in the ABA-dependant signalling pathway.

To understand the potential role of *JrWRKY6* and *JrWRKY53* in stress tolerance, the yeast expression system was used. Both the *JrWRKY6* and *JrWRKY53* transgenic yeast gained an improved tolerance to the salt, osmotic, and heat stresses. However, with regard to the cold response, the *JrWRKY6* transgenic cultures showed a significantly improved cold tolerance unlike the *JrWRKY53* transgenic line (Fig. 6) indicating the positive roles and difference of *JrWRKY* genes in abiotic stress tolerance. The expression of *JrWRKY6* was more effective at enhancing cold tolerance of the transgenic yeast compared with the control strain, whereas the yeast transformed by *JrWRKY53* did not gain the cold resistance ability (Fig. 6) suggesting that although both *JrWRKY6* and *JrWRKY53* were mainly induced by the low temperature stress, they were not necessary empowered with similar cold response abilities, which may be correlated to multiple and complex regulation pathways involved in stress response. Moreover, the transcriptional machinery has evolved from those in primitive to those in complex life forms, with more regulator genes present in plants than in yeast (Riechmann *et al*. 2000). Other studies have also analyzed TFs in yeast expression systems. For instance, overexpression of the myeloblastosis (MYB) 44 TF from *Salicornia brachiata* (*SbMYB44*) enhances the growth of yeast cells under salinity and dehydration stresses suggesting that salt and drought stress tolerance is endowed by this TF (Shukla *et al*. 2015). EsDREB2B, a novel truncated dehydration-responsive element-binding protein 2-type TF in *Eremosparton songoricum*, is induced by drought, salinity, cold, heat, and oxidative stresses and by exogenous ABA treatment. When overexpressed in yeast, the *EsDREB2B* transgenic lines have an increased tolerance to these multiple abiotic stressors indicating a possible role of *EsDREB2B* in conferring this ability (Li *et al*. 2014). Therefore, the current results suggest the positive roles of *JrWRKY6* and *JrWRKY53* in salt, osmotic, heat, and cold responses.

WRKY TFs always bind specifically to the W-box motif (core sequence: TTGACC/T) in their regulatory function against external stimuli. Understanding the special binding of WRKY proteins to the W-box in plant cells is of utmost importance to develop plants with tolerance to biotic and abiotic stressors. Our results show that JrWRKY6 and JrWRKY53 can bind to the W-box motif similarly as TFs in other reports. For instance, TFs encoded by *TaWRKY2* and *TaWRKY19*, two multiple stress-inducible genes from *Triticum aestivum*, display a specific binding to the typical W-box. Meanwhile, TaWRKY2 enhances the expression of a Cys2/His2-type zinc-finger transcriptional repressor (*STZ*) and desiccation-responsive 29B (*RD29B*) and bind to their promoters, whereas TaWRKY19 activates the expression of *DREB2A*, *RD29A*, *RD29B,* and the cold regulated gene *Cor6.6* and bind to *DREB2A* and Cor6.6 promoters (Niu *et al*. 2012). Salicylic acid-inducible OsWRKY6 also shows a physical interaction with W-box-like element 1, which positively regulates the expression of *O.sativa* pathogenesis-related 10a (*OsPR10a*) (Choi *et al*. 2015). AtWRKY30 binds with a high specificity and affinity to the W-box, and also to its own promoter (Scarpeci *et al*. 2013). These results suggest that JrWRKY6 and JrWRKY53 function by binding to the W-box.

In conclusion, the *JrWRKY6* and *JrWRKY53* genes were identified and their proteins grouped into the group III subfamily of WRKY TFs. The RT-qCR analysis shows that both *JrWRKY6* and *JrWRKY53* were mainly induced by NaCl, PEG, and low and high temperatures, but they showed varying expression profiles to these treatments, with tissue specificity. In yeast transformation tests, *JrWRKY6* and *JrWRKY53* more or less enhanced the survival rate of the recombined yeast cells indicating their positive roles in endowing the plant with abiotic stress tolerance. Furthermore, JrWRKY6 and JrWRKY53 can bind to the W-box. Taken together, the results suggest that a possible abiotic stress response of *JrWRKY6* and *JrWRKY53* involves the ABA signalling pathway and W-box binding activity. This study provides better understanding the molecular mechanisms of WRKY TFs in response to abiotic stresses in woody plants.

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