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Ectopic expression of a grapevine transcription factor *VvWRKY11* contributes to osmotic stress tolerance in *Arabidopsis*

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Abstract Plant WRKY transcriptional factors play an important role in response to biotic and abiotic stresses. In this study, a WRKY transcription factor was isolated from grapevine. This transcription factor showed 66% and 58% identity at the DNA and amino acid sequence levels, respectively, with *Arabidopsis AtWRKY11* genes, and was therefore designated *VvWRKY11*. Phylogenetic analysis and structure comparison indicated that VvWRKY11 protein belongs to group IIc. The VvWRKY11 protein was shown to be located in the nucleus based on green fluorescent protein analysis. Yeast one-hybrid analysis further indicated that VvWRKY11 protein binds specifically to the W-box element. The expression profile of *VvWRKY11* in response to treatment with phytohormone salicylic acid or pathogen *Plasmopara viticola* is rapid and transient. Transgenic *Arabidopsis* seedlings overexpressing *VvWRKY11* showed higher tolerance to water stress induced by mannitol than wild-type plants. These results clearly demonstrated that the *VvWRKY11* gene is involved in the response to dehydration stress. In addition, the role of VvWRKY11 protein in regulating the expression of two stress response genes, *AtRD29A* and *AtRD29B*, is also discussed.

Keywords WRKY transcription factor · Grapevine · Osmotic stress · Defense response

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Introduction

Biotic and abiotic stresses negatively influence plant growth and crop productivity. Plants have evolved diverse defense mechanisms that enable them to adapt to environmental stresses [1]. A common feature of plant defense responses is the transcriptional activation of numerous genes upon external stimuli, including biotic and abiotic stresses [2]. Genes induced under stress conditions can be divided into two groups [3]. The first code for proteins that protect plants against environmental stresses, and the second are involved in signal transduction. For example, antifreeze proteins, manganese superoxide dismutases and FtsH proteins can enhance plant tolerance to cold, salt and drought stresses, respectively [4–6]. Protein kinases and phosphatases possess converse functions in signal transduction pathways [7, 8]. It has been reported that several kinds of transcription factor (TF) such as DREB, ERF, ZFP, QM, and WRKY are involved in response to various environmental stresses [9–13]. Therefore, it is clear that transcriptional regulation of plant defense-related genes is a vital part of plant defense responses [3].

The WRKY family represents a major group of plant-specific transcriptional regulators, and encodes a large of group of transcription factors [9]. For example, there are more than 74 and 81 WRKY TFs in *Arabidopsis* and rice, respectively [14]. The *WRKY* genes are characterized by the presence of one or two highly conserved WRKY domains. The WRKY domain is about 60 amino acids in length and consists of the absolutely conserved sequence motif WRKYGQK and a Cys(2)His(2) or Cys(2)HisCys zinc-binding motif [9]. The two motifs are necessary for the high binding affinity of WRKY proteins to W-boxes, which contain an invariant DNA sequence (T)(T)TGAC(C/T) [15–18]. The W-boxes are a major class of *cis*-acting elements and are present in the promoters of many defense-related genes. For example, the *Vitis vinifera* WRKY1 protein binds specifically to W-box elements present in the promoter regions of two pathogen-defense genes, *PR1* in parsley and *NPR1* in *Arabidopsis* [17].

WRKY proteins can be divided into three groups according to the number and type of WRKY domains [9, 19]. Group I proteins possess two WRKY domains, whereas those in groups II and III have only one WRKY domain. WRKY transcription factors are key regulators of responses to microbial infection in plants. For example, gene expression profile analyses indicated that 49 of 72 *WRKY* genes tested in *Arabidopsis* responded to bacterial infection [20]. Activation of the transcription factors *WRKY22*/*WRKY29* confers resistance to both bacterial and fungal pathogens in *Arabidopsis* [21]. The transcription factor WRKY33 regulates the antagonistic relationship between defense pathways mediating responses to *Pseudomonas syringae* and necrotrophic pathogens in *Arabidopsis* [22]. The importance of WRKY transcription factors in plant disease response is further demonstrated by the strategy of modulating gene expression. For example, overexpression of *WRKY70* significantly increases resistance to virulent pathogens [23]. Moderate expression of *WRKY18* significantly increases the expression levels of pathogenesis-related genes, leading to resistance to the bacterial pathogen *P. syringae* in *Arabidopsis* [24]. In addition to regulating the expression of defense-related genes, WRKY transcription factors are also involved in hormone responses. For example, *Arabidopsis WRKY70* has been shown to regulate cross talk between jasmonate (JA)- and salicylate (SA)-regulated disease response pathways [23]. Overexpression of *WRKY70* results in constitutive expression of SA-induced pathogenesis-related genes, whereas antisense suppression of *WRKY70* activates JA-responsive/COI1-dependent genes. The transcription factor *WRKY21* from *Larrea tridentata* encodes an activator of the abscisic acid (ABA) signaling pathway [25]. The rice *WRKY71* gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells [26]. Moreover, *WRKY* genes are also involved in plant development such as leaf senescence [27, 28] and embryogenesis [29], and abiotic stress responses such as nutrient stress [30, 31], wounding [32], cold and drought [33].

To date, there are few reports about the characterization of *WRKY* genes from grapevine. In this study, we isolated a WRKY family transcription factor, designated *VvWRKY11*, from cv. Beifeng. The subcellular localization of VvWRKY11 was examined and its ability to bind specifically to W-box DNA elements was demonstrated. The expression of *VvWRKY11* is inducible by SA treatment. The biological role of *VvWRKY11* was assessed by its overexpression in *Arabidopsis.* Transgenic lines carrying the *VvWRKY11* gene exhibited reduced susceptibility towards osmotic stress, suggesting that *VvWRKY11* is involved in response to plant drought tolerance.

Materials and methods

Plant materials

Two-node cuttings of the grapevine cv. Beifeng derived from an intercross between *V. thunbergii* and *V. vinifera* were planted in pots and grown in a greenhouse at 23 ± 2°C, 70–80% relative humidity with a 14 h light/10 h dark photoperiod. The *Arabidopsis* seedlings used in this study belong to Columbia ecotype.

Recovery of full-length *VvWRKY11* cDNA

To identify members of the grapevine WRKY family, the *Arabidopsis* transcription factor *WRKY11* was used as a query sequence to BLAST against *Vitis* EST database. An EST showing a high degree of similarity in both amino acid and DNA sequences with the *Arabidopsis WRKY11* gene was identified. Based on the EST sequence, a gene specific primer 5′-CTGCATCGCGACCTGTGAGTGAGAG-3′ was then designed to recover the 5′-end sequence using the rapid-amplification of cDNA end (RACE) method. The PCR program consisted of 30 cycles of 30 s at 95°C, 30 s at 58°C, and 2 min at 72°C. PCR products of the expected size were cloned into pGEM-T vector (Promega, Madison, WI) and then subjected to sequencing.

Analysis of VvWRKY11 binding to W-box using a yeast one-hybrid system

Yeast one-hybrid analysis was performed using a Clontech system (Clontech, Mountain View, CA). A W-box-related *cis*-acting element located between −995 and −976 bp upstream of the promoter region of the grapevine *VST1* gene and its corresponding W-box mutant (mW-box) element

were synthesized [34]. The nucleotide sequences of W-box and mW-box elements were 5′-GCCGTTGAAAAGTCAA ATGA-3′ and 5′-GCCGTTGAAAATATTAATGA-3′, respectively. These two elements were individually inserted into the *Sma*I site of pHis3 vector and the *Xho*I site of pLacZi vector, respectively (Clontech). Four constructs designated pW-box-His3, pmW-box-His3, pW-box-LacZi, and pmW-box-LacZi were generated. The whole coding region of *VvWRKY11* was cloned in-frame into the yeast expression vector pGAD424 to fuse with the GAL4 activation domain, generating the expression vector pGAD424-VvWRKY11. All the constructs were confirmed by direct sequencing, and then transferred into yeast (*Saccharomyces cerevisiae*) strain YM4271 (Clontech). The empty vector pGAD424 was also transformed as a negative control. The transformed yeast cells were grown on SD medium containing 40 mM 3-aminotrizole (3-AT; a competitive inhibitor of the *His3* gene product), and β-galactosidase activity was assessed according to the manufacturer's instruction (Clontech, USA).

Subcellular localization of the VvWRKY11 protein using green fluorescent protein (GFP) fusion proteins

The coding region of *VvWRKY11* was amplified using a pair of gene-specific primers, 5′-GTCGACGACATCTTCC TTTGTCTGGTAGT-3′ and 5′-CGCGGATCCAGTTGAC TCGAACACCAAGCC-3′, containing *Sal*I and *Bam*HI sites at the respective 5′-ends. The PCR product was digested using *Sal*I and *Bam*HI, and then inserted into the 5′-end of the green fluorescent protein (GFP) coding region of the vector pJIT163 (a gift from Prof. HongQing Ling from Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The construct and empty vector pJIT163 were then transformed into onion epidermal cells using the bombardment procedure [35]. The transformed onion cells were incubated at 25°C in darkness for 16–20 h. Images were captured using a laser scanning confocal microscope (Leica, Wetzlar, Germany) at the wavelength of 488 nm.

Construction of *VvWRKY11* expression vector and *Arabidopsis* transformation

The coding region of *VvWRKY11* was amplified using a pair of primers, 5′-CGGGGTACCGACATCTTCCTTTG TCTGGTAGT-3′/5′-TGCTCTAGAATTTACAGATCCAA CCCATTCCC-3′, containing KpnI and XbaI sites at respective 5′-ends. The PCR products were cloned into pGEM-T Easy vector (Promega) and confirmed by direct sequencing. Subsequently, the plasmid DNA of the *VvWRKY11* clone was digested with *Kpn*I and *Xba*I. DNA fragments containing the full-length cDNA of *VvWRKY11* were recovered and cloned into pCAMBIA2300. The expression vector of *VvWRKY11* was introduced into *Agrobacterium tumefaciens* GV3101, and *Arabidopsis* transformation was performed using the floral dip method [36]. Transgenic seedlings were grown in a growth chamber at 22°C and 70% relative humidity with a 16 h photoperiod.

Osmotic stress treatment of *Arabidopsis* transgenic lines expressing *VvWRKY11* gene

Wild-type and T2 seeds were surface-sterilized with 20% (v/v) commercial bleach for 10 min. The sterilized seeds were plated on 1/2 MS medium at 4°C for 2–4 days and then transferred to growth chambers. To examine the effect of osmotic stress on the growth of *VvWRKY11* transgenic and wild-type plants, 4-day-old seedlings were transferred and vertically plated onto 1/2 MS media supplemented with different concentration of mannitol. The Chlorophyll contents of leaves were measured 7 days after mannitol treatment. A total of 0.05 grams of leaves from each sample were ground in liquid nitrogen. The ground leaves were extracted with 80% acetone, and the supernatant was incubated in the dark for 2 h at room temperature. Optical density (OD) was measured at 663 and 645 nm. The contents of chlorophyll *a* and *b* were calculated according to the method described previously [37].

Pathogen challenge of grapevine and transgenic *Arabidopsis*

For grapevine, two-node cuttings of cv. Beifeng were planted in pots and grown in greenhouse at 23 ± 2°C, 70–80% relative humidity with a 14-h photoperiod. *P. viticola* inoculum was collected from sporulated field leaves, resuspended in water and adjusted to 50,000 sporangia/ml using a Fuchs–Rosenthal hemocytometer (Thoma, Freiburg, Germany). The sporangia suspension was then sprayed onto the abaxial leaf surfaces.

For transgenic *Arabidopsis*, virulent *P. syringae* pv. tomato strain DC3000 (*Pst* DC3000) was cultured at 28°C on King's B medium supplemented with 50 mg/ml rifampicin. Inoculation was performed using the injection method [38]. Briefly, 5-week-old plants were infiltrated with a bacterial suspension ($OD_{600} = 0.0001$ in 10 mM $MgCl_2$) using a needless syringe. The inoculated plants were kept in a moist chamber at 23–25°C in darkness for 24 h and then under an 8-h photoperiod. The infected leaf tissues were collected on days 0 and 3 after inoculation. To assess pathogen challenge, the leaves were homogenized in

10 mM $MgCl_2$ and tenfold dilutions were then plated onto King's B medium agar containing 50 mg/ml rifampicin. The bacterial colony forming units were counted after overnight incubation. Three replicates of each experiment were conducted.

RNA extraction and gene expression analysis

Total RNA was extracted according to a method reported previously [39], and was then treated with 10 units of RNase-free DNase I (Takara Bio Inc., Kyoto, Japan) to remove genomic DNA contamination. The first-strand cDNA was synthesized in a total volume of 25 μl containing 2 μg of total RNA template, 500 ng of M13APN, and 200 units of Promega M-MLV Reverse Transcriptase according to the manufacturer's instructions.

Expression of the *VvWRKY11* gene in *Arabidopsis* T2 transgenic lines was analyzed by RT–PCR. Total RNA was extracted from young seedlings, and RT–PCR was carried out using a two-step procedure. Two primer pairs 5′-CGCATGCTGTCTCATCAGACCAATC-3′/5′-GAGCTGGAGTACTTTCCGGAGATATC-3′ and 5′-AAGCTTGCTGATAACTGTACTGGT-3′/5′-GGTTTGGAACTCAGTGACATCA-3′ were designed to amplify *VvWRKY11* and tubulin genes, respectively. PCR was performed for 27 and 22 amplification cycles for *VvWRKY11* and tubulin, respectively.

To examine the expression profiles of *AtRD29A* and *AtRD29B* genes under osmotic stress condition, 4-week-old wild-type and transgenic *Arabidopsis* seedlings were treated with 500 mM mannitol. Leaves were harvested at different stage after treatment, and subjected to RNA extraction. Expression profiles of two ABA-mediated cell signaling genes *AtRD29A* and *AtRD29B* and the actin gene were investigated by quantitative real-time PCR, and the following pairs of primers were used: 5′-CCAGAGATGATTTTGTGGAGACGAG-3′/5′-CACTTGAGTTTGATCTCCCACCG-3′, 5′-AAGTAGAGAGTGGATTGGGAAGAGAC-3′/5′-GAAGCTAACTGCTCTGTGTAGGTGC-3′, 5′-GGTAACATTGTGCTCAGTGGTGG-3′/5′-AACGACCTTAATCTTCATGCTGC-3′, respectively. PCR amplification was performed using the 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Each reaction well contained 2.5 μl of template cDNA, 12.5 μl of 2× Quantitect SYBRR® Green I Mix (Applied Biosystems), and 12.5 pmol of a gene-specific primer. The PCR program consisted of 95°C for 10 min, followed by 40 cycles at 94°C for 15 s and 60°C for 1 min. Three replicates of each reaction were performed, and data was analyzed according to the comparative C_T method [40]. The two gene expression levels were normalized against the actin gene expression level.

Results

Isolation of the *VvWRKY11* gene in grapevine

The coding DNA sequence of *Arabidopsis* transcription factor *AtWRKY11* was BLAST against the *Vitis* EST database, and an EST sequence with a significant hit from *V. vinifera* (GenBank accession no. EC935078) was identified. The EST consists of 3′-untranslated region (UTR) and a poly(T) tail, and its deduced amino acid sequence showed 61% identity with the AtWRKY11 protein. Thus, the EST was deemed to be a fragment of the grapevine *WRKY* cDNA sequence, and the 5′-end sequence of the grapevine *WRKY* gene was sequentially recovered using 5′-RACE method. The coding sequence of the grapevine *WRKY* gene was compared with the *Arabidopsis* protein database using BLASTX program, and two top hits were found: AtWRKY11 (E values = $6e^{-104}$) and AtWRKY17 (E values = $5e^{-96}$), suggesting that the grapevine *WRKY* gene may be an orthologous gene of *AtWRKY11*. Moreover, the cDNA sequence of the grapevine *WRKY* gene was compared with the *V. vinifera* whole genome sequence database, and the result showed that it matches exactly with a contig genomic DNA sequence (GenBank accession no. AM462490). This result further indicated that the cDNA sequence was most likely derived from the *V. vinifera* genome. Therefore, the grapevine *WRKY* gene was designated *VvWRKY11*, and deposited in the NCBI database with accession no. AM886167.

The cDNA sequence of *VvWRKY11* gene is 1,255 bp in length, including 31 bp of the 5′-UTR and 207 bp of the 3′-UTR. The *VvWRKY11* gene is composed of three exons and two introns, and its genomic structure is similar to that of *AtWRKY11*, with the last two exons almost identical in size (Fig. 1). The deduced polypeptide encoded by the *VvWRKY11* gene consists of 338 amino acids with a molecular mass of 25 kDa and isoelectric point of 5.03. Like the *Arabidopsis* AtWRKY11 protein, the grapevine VvWRKY11 contains the following four domains: a conserved WRKY domain, a putative nuclear localization signal or sequence (NLS), a HARF domain and a N-terminal C domain which is unique to group II members of the WRKY family (Fig. 1). The WRKY domains of VvWRKY11 and AtWRKY11 show 88% identity at the amino acid sequence and are characterized by a single C–C–H–H zinc finger. Moreover, phylogenetic analysis clearly indicated that VvWRKY11 belongs to group IId (Supplementary Fig. 1).

VvWRKY11 binds to W-box elements

Yeast cells transformed with both pGAD-VvWRKY11 and pW-box-His3 vectors can grow on SD/His-/Leu-/Ura-

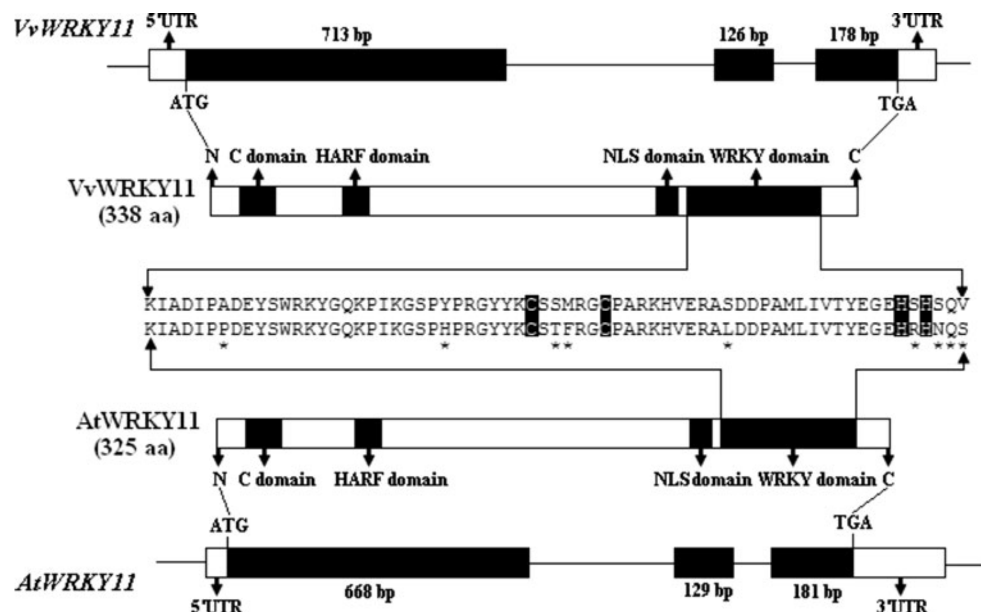

Fig. 1 Comparison of the structures of *Arabidopsis* and grapevine *WRKY11* genes and their proteins. The sequence differences between WRKY domains are marked with *stars*, and the zinc fingers are highlighted in *black boxes*

media supplemented with 40 mM 3-AT (Fig. 2c) and show β-galactosidase activity as well (Fig. 2d), suggesting that the interaction between VvWRKY11 and the W-box element activates transcription of the two reporter genes *HIS3* and *LacZ* genes. Conversely, yeast strains transformed with both pmW-box-His3 and pGAD-VvWRKY11 or pGAD424 vectors could not grow on SD/His-/Leu-/Ura-media supplemented with 40 mM 3-AT (Fig. 2c) and did not exhibit any β-galactosidase activity (Fig. 2d). This indicated there is no interaction between VvWRKY11 and the mW-box element, resulting in the failure of synthesis of both HIS and LacZ proteins. In short, the *HIS3* expression analysis and the X-gal colony-lift filter assay strongly indicated that VvWRKY11 binds specifically to TGAC, the sequence of the W-box core motif.

VvWRKY11 protein is localized in the nucleus

As a putative transcription factor, VvWRKY11 is likely to be localized in the nucleus because it consists of a putative nuclear localization signal or sequence (Fig. 1). To confirm the subcellular localization of VvWRKY11, the open reading frame of *VvWRKY11* was fused in-frame to the *hGFP* coding sequence under the control of the CaMV35S promoter. The fused expression vector was then introduced into onion epidermal cells for transient expression. The VvWRKY11-hGFP fusion protein is located exclusively in the nucleus (Fig. 3b, c), whereas the control hGFP is uniformly distributed throughout the cell (Fig. 3e, f). This result clearly indicated that VvWRKY11 is localized in nucleus.

Expression profile of grape *VvWRKY11* in response to *P. viticola* infection and SA

Downy mildew (*P. viticola*) is one of the most important diseases in grape. Since WRKY gene family is related to plant defense response, the role of *VvWRKY11* in response to biotic stress in grapevine was investigated after inoculation of *P. viticola*. The expression of *VvWRKY11* was monitored at different time intervals after treatment with the pathogen in cv. Beifeng by real-time PCR, and the results are shown in Fig. 4. The levels of the *VvWRKY11* transcript increased significantly at 2 h after pathogen inoculation (hpi) and reach a peak at 4 hpi. The transcript accumulation decreased significantly at 12 hpi, and rapidly reverted to the normal level at 24 hpi.

The plants of grapevine cv. Beifeng were also sprayed with 5 mM SA solution, and the expression of *VvWRKY11* in leaves was monitored at different time intervals after treatment. The accumulation of *VvWRKY11* transcripts increased significantly at 2 h after SA treatment and decreased rapidly 4 h after treatment (Fig. 4). This result clearly indicated that the response of *VvWRKY11* to SA is both rapid and transient.

Transgenic *Arabidopsis* overexpressing *VvWRKY11* shows tolerance to osmotic stress

The putative role of *VvWRKY11* in response to abiotic stress was investigated by ectopic expression in *Arabidopsis*. More than ten *Arabidopsis* transgenic lines were generated, and two lines, TL-1 and TL-2, showed high-level expression of the grapevine gene *VvWRKY11* based

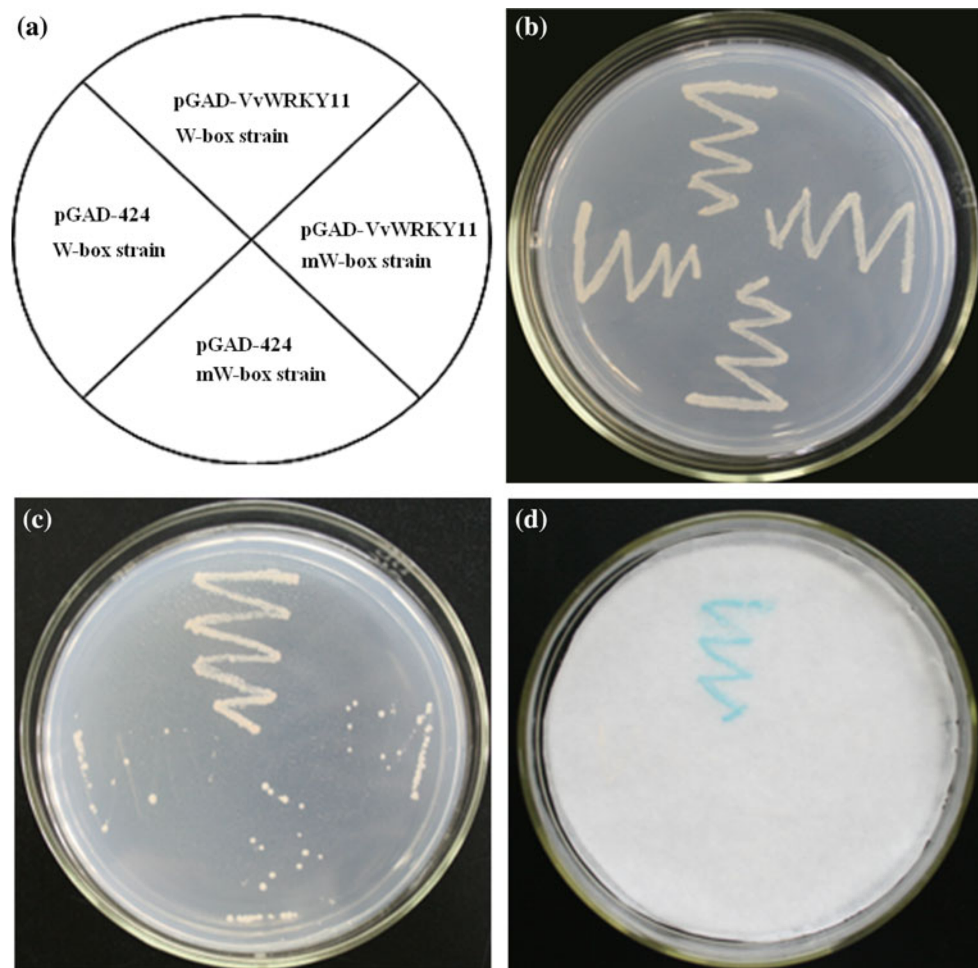

Fig. 2 Assay of VvWRKY11 binding to the W-box using a yeast one-hybrid system. **a** Schematic illustration of the positions of four kinds of yeast strains transformed with W-box-His3 or mW-box-His3 combined with pGAD-VvWRKY11 or pGAD-424; **b** Transformed yeast strains growing on SD/Leu-/His-/Ura-media; **c** Yeast strains growing on SD/Leu-/His-/Ura-media supplemented with 40 mM 3-aminotrizole (3-AT); d Analysis of β-galactosidase activity of different yeast strains using the colony-lift filter method

on the results of semiquantitative RT–PCR analysis (Fig. 5a). Thus, the two transgenic lines were selected for further analysis of resistance to osmotic stress. Four-day-old transgenic and wild-type *Arabidopsis* seedlings were transferred onto MS media plates containing different concentration of mannitol and placed in a vertical orientation for a further 7 days. Both the wide-type and transgenic seedlings grew well on MS plates without mannitol, and no obvious differences in seedling growth or root development were observed (Fig. 5d-I). However, the growth of both wild-type and transgenic seedlings was significantly inhibited under stress conditions in the presence of both 400 and 500 mM mannitol (Fig. 5d-II, III). The leaves of wild-type plants began to turn yellow on MS medium supplemented with 400 mM mannitol (Fig. 5d-II) and were completely yellow on MS medium supplemented with 500 mM mannitol (Fig. 5d-III). However, the leaves of transgenic lines were green on MS media supplemented with both 400 and 500 mM mannitol (Fig. 5d-II, III). Moreover, the contents of chlorophyll in transgenic lines were much higher than those in wild-type seedlings in the presence of 500 mM mannitol (Fig. 5c). These results unambiguously suggest that ectopic expression of *VvWRKY11* in *Arabidopsis* contributes to tolerance to osmotic stress.

The expression profiles of two stress response genes *AtRD29A* and *AtRD29B* in wild-type and transgenic *Arabidopsis* seedlings under osmotic stress condition were also assessed (Fig. 5e). The transcript accumulations of both *AtRD29*A and *AtRD29*B in *Arabidopsis* transgenic seedlings were significantly higher than those in wild-type seedlings at 5 h after treatment (Fig. 5e), suggesting that *VvWRKY11* may be involved in the regulation of the two stress response genes.

Ectopic expression of *VvWRKY11* in *Arabidopsis* contributes little to *Pseudomonas* resistance

The leaves of 4-week-old wild-type and transgenic *Arabidopsis* plants were syringe-infiltrated with a bacterial suspension of *Pst* DC3000. The bacterial densities were calculated 0 and 3 days after inoculation, and the results are shown in Fig. 5b. There was no significant difference in disease index between the transgenic and wild-type plants,

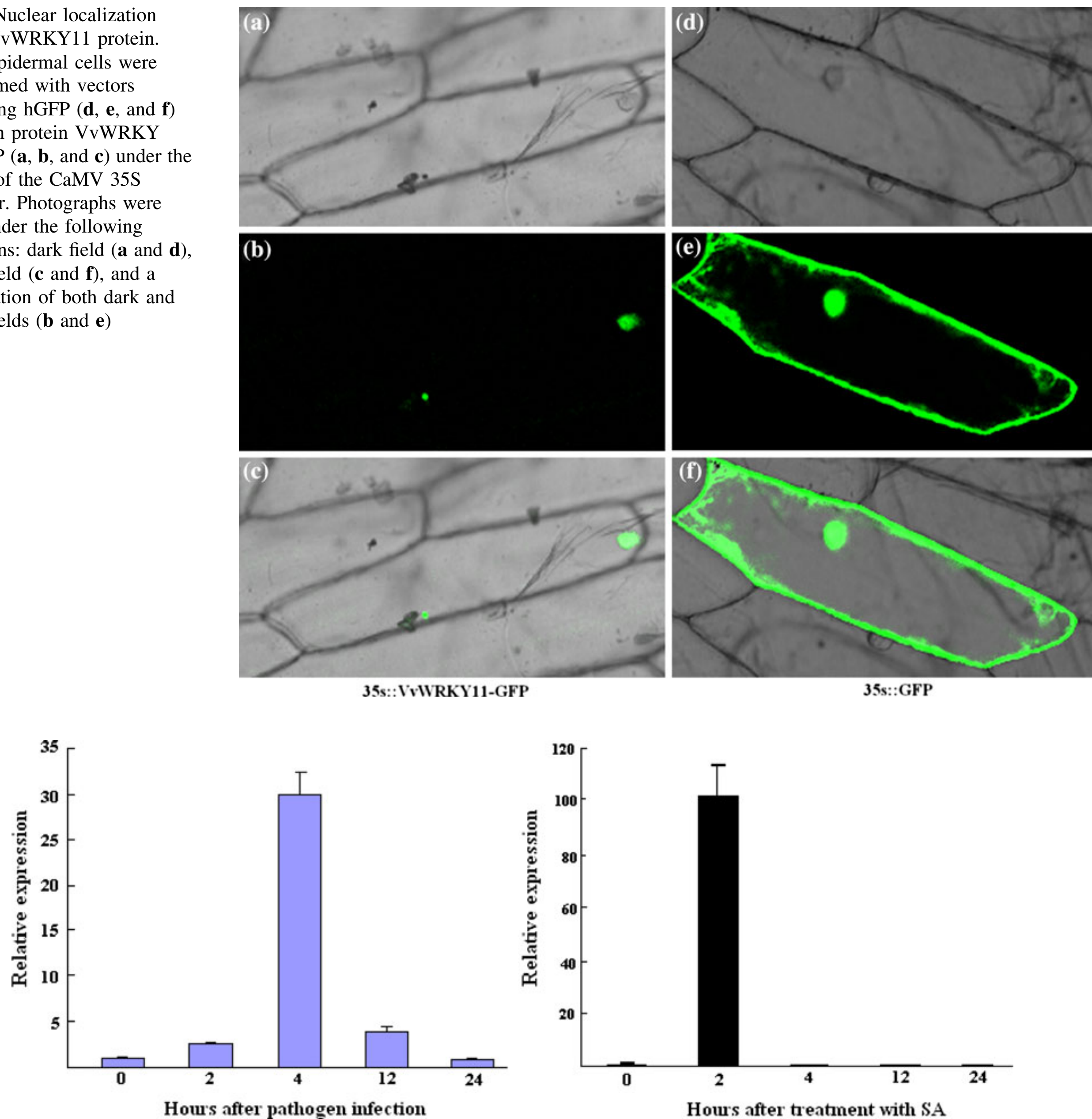

Fig. 3 Nuclear localization of the VvWRKY11 protein. Onion epidermal cells were transformed with vectors expressing hGFP (**d**, **e**, and **f**) or fusion protein VvWRKY 11-hGFP (**a**, **b**, and **c**) under the control of the CaMV 35S promoter. Photographs were taken under the following conditions: dark field (**a** and **d**), bright field (**c** and **f**), and a combination of both dark and bright fields (**b** and **e**)

Fig. 4 Expression of grapevine *VvWRKY11* in response to treatment with *P. viticola* and SA

suggesting that the expression of *VvWRKY11* has no effect on the improvement of resistance to *P. syringae* in *Arabidopsis*.

Discussion

WRKY transcription factors have been isolated in a variety of plants [32, 33, 41, 42], and their functions have been widely investigated in *Arabidopsis*. However, there are few reports on biological roles of *WRKY* genes in grapevine. To date, only two *WRKY* genes, *VvWRKY1* and *VvWRKY2*, have been identified in grapevine, and they have been shown to be involved in the response to biotic stress [17, 43]. Recently, an extensive EST collection was conducted in *Vitis vinifera* (357,849 sequences in the NCBI database as of Sep. 2009), which provides the opportunity to isolate *WRKY* genes in grapevine. Here, we reported the isolation and characterization of a new transcription factor *VvWRKY11* from the grapevine genome.

WRKY proteins can be divided into three groups. Group I proteins contain two WRKY domains at N- and C-terminal regions, respectively, whereas the other two groups possess only one WRKY domain at C-terminal region [9].

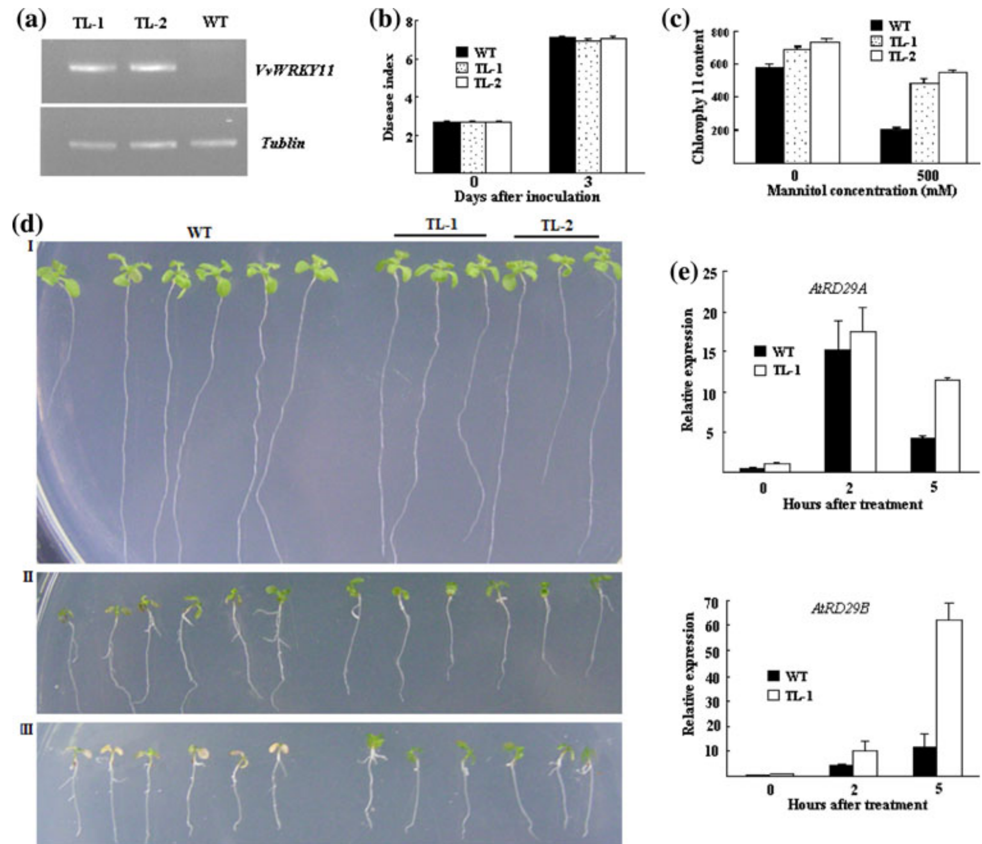

Fig. 5 Comparison between wild type and transgenic *Arabidopsis* lines expressing *VvWRKY11*. **a** Expression of *VvWRKY11* testified by RT–PCR analysis. **b** Resistance to *Pseudomonas syringae*. **c** Chlorophyll contents under mannitol stress. The values were measured 7 days after treatment, and the analysis was repeated three times for each sample. **d** Resistance to osmotic stress. Four-day-old seedlings were transferred from the growth chamber and placed vertically on MS plates supplemented with 0 mM mannitol (I), 400 mM mannitol (II), and 500 mM mannitol (III). Photographs were taken 11 days after treatment. **e** Expression profiles of *AtRD29A* and *AtRD29B* in 4-week-old seedlings under conditions of osmotic stress. WT and TL represented wild-type and transgenic line, respectively

It has been reported that the C-terminal domain of the two-WRKY-domain proteins appears to be ancestor to those with only a single WRKY domain [44]. Thus, the N-terminal WRKY domains of group I members were not selected and used for phylogenetic analysis in this study. The phylogenetic tree derived from the amino acid sequences of the C-terminal WRKY domains indicated that group I and group IIc are clustered in the same clade with a bootstrap value of 45 (supplementary Fig. 1). Recently, a total of 46 *WRKY* genes were isolated from canola, and phylogenetic analysis was conducted based on the amino acid sequences of WRKY domains [42]. Interestingly, the results also showed that groups I and IIc are clustered into the same clade. Thus, group I seems to have a higher degree of similarity with group IIc than with the other groups, suggesting that groups I and IIc are most likely derived from a common ancestor. In addition, the N-terminal WRKY domain of *AtWRKY10* genes in *Arabidopsis* has been found to be lost after the divergence of dicots from monocots [9]. Thus, it is reasonable to speculate that the loss of the N-terminal domain may have occurred during the evolution of group IIc members.

It has been reported that *WRKY* genes appear to have originated in early eukaryotes, and have been duplicated many times during plant evolution, resulting in a large gene family for WRKY proteins in higher plants [44]. Gene duplication has played an important role in generating functional diversity within the plant *WRKY* gene family. For example, two closely related homologs exist in *Arabidopsis*, *AtWRKY11* and *AtWRKY17*, both of which consist of three exons with the second exon identical in length (supplementary Table 1). This suggests that *AtWRKY11* and *AtWRKY17* must be derived from a common ancestor. Functional analysis further demonstrated that the *Atwrky11* mutant rather than *Atwrky*17 mutant shows enhanced resistance to *Pst*, suggesting the duplicated genes have diverged in function [45]. Intriguingly, we compared the cDNA sequence of *VvWRKY11* against the database of the whole-genome shotgun sequence of *V. vinifera*, and a homologous sequence, designated *VvWRKY17*, was identified. *VvWRKY11* and *VvWRKY17* have a similar genomic structure with the second exon identical in size (supplementary Table 1), and overall 69% amino acid sequence identity. Thus, *VvWRKY11* and *VvWRKY17* must also have a common ancestor. Likewise, rice *OsWRKY51* and *OsWRKY68* are homologous to *Arabidopsis AtWRKY11* or *AtWRKY17*. *OsWRKY51* and *OsWRKY68* share an overall 53% identity in amino acid sequence and are identical in the size of the second exon (supplementary Table 1), indicating they are derived from a common ancestor. Moreover, *VvWRKY11*, *VvWRKY17*, *OsWRKY51*, and *OsWRKY68* all share similar genomic structure and more

than 50% of overall identity in amino acid sequence with both *AtWRKY11* and *AtWRKY17*. Taken together, these results suggest that the three gene pairs *AtWRKY11*/*AtWRKY17*, *VvWRKY11*/*VvWRKY17*, and *OsWRKY51*/*OsWRKY68* must be derived from a common ancestor, which is most likely duplicated before the divergence of monocots and dicots, resulting in homologous *WRKY* gene pairs in different higher plants.

WRKY transcription factors have been implicated in the regulation of various biological processes, including pathogen response and hormone signaling [46]. Our study presented here demonstrated that the expression of grapevine *VvWRKY11* is strongly induced at 4 h after *P. viticola* challenge and then returns to normal at 24 h after inoculation. The grapevine gene *VvWRKY11* in response to pathogen challenge is quite similar to its ortholog of *Arabidopsis AtWRKY11*, which shows rapid and transient induction with a peak at 2 h after *Pst* infection [45]. However, expression of the canola *BnWRKY11* gene, an ortholog of *Arabidopsis AtWRKY11*, is unaffected by pathogen treatment [42]. This discrepancy may have been due to the difference in time points selected for monitoring gene expression. Both *AtWRKY11* and *VvWRKY11* expression levels were examined within 12 or 24 h after inoculation, whereas the measures of *BnWRKY11* in response to challenge with the two pathogens *Sclerotinia sclerotiorum* and *Alternaria brassicae* were conducted more than 12 h post-inoculation. The induction of *AtWRKY11* and *VvWRKY11* is transient and their expression levels decreased significantly after 12 h postinoculation, suggesting that the response of *WRKY11* genes to pathogen infection should be measured in the early stage. Therefore, the observation that *BnWRKY11* is not induced by pathogens may have been due to missing the ideal stage for monitoring gene expression in response to pathogen stress. It is worth noting that the accumulation of JA occurs within the 1st hour after pathogen infection [47]. The pathogen-induced accumulation of JA may repress the expression of *WRKY11* genes, leading to the transient induction of the *VvWRKY11* gene in response to pathogen *P. viticola* infection. Moreover, *AtWRKY11* does not respond to JA [47, 48], suggesting that it acts upstream in the JA signal transduction pathway [45]. Here, we further demonstrated that *VvWRKY11* is involved in the response to SA, and shows rapid and transient induction with a peak at 2 h after SA treatment. More recently, it has also been found that the expression of *VvWRKY1* is induced by SA treatment [17]. *VvWRKY1* and *VvWRKY11* belong to groups I and IIc, respectively. WRKY proteins belonging to different groups share common biological roles in the response to SA treatment, suggesting the functional redundancy of *WRKY* genes in plants.

Most defense-related genes contain W-box elements in their promoters that are specifically recognized by WRKY proteins [49–51]. Therefore, the rapid increases in the expression of *WRKY* genes is generally believed to play important roles in activating or attenuating expression of downstream defense-related genes, such as *PR* and *NPR1* genes[45, 52–55]. As mentioned above, *VvWRKY11* is inducible by pathogen. However, the transgenic *Arabidopsis* seedlings overexpressing *VvWRKY11* did not show any enhanced resistance to *Pst*. The *AtWRKY11* gene acts as negative regulator of basal resistance to *Pst* in *Arabidopsis*. The grapevine *VvWRKY11* is the ortholog of *AtWRKY11*. It is thus appears that *VvWRKY11* is also a negative regulator of basal resistance in grapevine. Therefore, ectopic expression of *VvWRKY11* in *Arabidopsis* contributes little to *Pseudomonas* resistance.

WRKY transcription factors are well known to be involved in response to a wide range of abiotic stresses. For example, microarray analysis has revealed that the expressions of many WRKY transcription factors can be induced by abiotic stresses such as drought, salinity, and cold [56, 57]. Barley *HvWRKY38* is involved in cold and drought stress responses [32]. Overexpression of *TcWRKY53* in tobacco under drought stress strongly inhibited the expression of stress response genes such as *ERF5* and *EREBP-1* [58]. However, the functional roles of the grapevine *WRKY* genes in tolerance to abiotic stress have not been fully investigated. In this study, we investigated the biological role of *VvWRKY11* in response to dehydration stress induced by mannitol, which has been widely used to evaluate plant tolerance to water stress [58, 59]. Transgenic *Arabidopsis* seedlings overexpressing *VvWRKY11* show higher tolerance to dehydration stress than wild-type plants, suggesting that *VvWRKY11* is a positive regulator of resistance to dehydration stress. Moreover, the *Arabidopsis* gene *AtWRKY11* acts as a negative regulator of basal resistance [45]. In addition, Chlorophyll content is an important factor in determining plant growth capacity [60]. Our study indicated that *Arabidopsis* plants overexpressing *VvWRKY11* have higher chlorophyll level than wild-type plants (Fig. 5c). Taken together, these results suggest that *WRKY11* genes may be associated with both biotic and abiotic stress response signaling pathways and therefore play multiple roles in plants.

Stress response genes, such as *RD29A* and *RD29B*, contain at least two types of *cis*-acting element, the dehydration-responsive element (DRE) and the ABA-response element (ABRE), in their promoter regions [61, 62]. It is thus clear that higher internal ABA levels are closely related to plant tolerance to osmotic stress [63]. Here, we showed that the expression levels of *AtRD29A* and *AtRD29B* in *VvWRKY11* transgenic *Arabidopsis* seedlings were higher than those in wild-type plants under dehydration stress, suggesting that *VvWRKY11* gene product affects the expression of *AtRD29A* and *AtRD29B* in *Arabidopsis*. At least two possible

explanations may explain this observation. First, interaction between DRE binding protein (DREB) and ABRE binding protein (ABEB) has been proposed to be involved in the regulation of *AtRD29A* expression [61]. Meanwhile, it has been reported that *DREB2A* gene requires posttranslational modification before it can activate downstream genes under normal growth conditions [62]. Thus, the VvWRKY11 protein may interact with DREB and/or ABEB, resulting in the increased expression of *AtRD29A* and *AtRD29B*. Second, we checked the genomic DNA sequence of *AtRD29A* and found several TGAC-like sequences in the promoter region. The TGAC-like sequences are the core of W-box. Thus, it seems that the VvWRKY11 protein may regulate the expression of *AtRD29A* and *AtRD29B* at transcriptional level. In short, the *WRKY11* gene plays an important role in improving plant tolerance to water stress. This result will be helpful toward obtaining a comprehensive understanding regarding the roles of the WRKY11 protein in the regulation of biological responses to biotic and abiotic stresses.

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