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Transcription factors WRKY11 and WRKY17 are involved in abiotic stress responses in *Arabidopsis*

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ABSTRACT

Plant WRKY transcription factors play a vital role in abiotic stress tolerance and regulation of plant defense responses. This study examined *AtWRKY11* and *AtWRKY17* expression under ABA, salt, and osmotic stress at different developmental stages in *Arabidopsis*. We used reverse transcriptase PCR, quantitative real-time PCR, and promoter:GUS lines to analyze expression. Both genes were upregulated in response to abiotic stress. Next, we applied the same stressors to seedlings of T-DNA insertion *wrky11* and *17* knock-out mutants (single and double). Under stress, the mutants exhibited slower germination and compromised root growth compared with the wild type. In most cases, double-mutant seedlings were more affected than single mutants. These results suggest that *wrky11* and *wrky17* are not strictly limited to plant defense responses but are also involved in conferring stress tolerance.

1. Introduction

WRKY transcription factors (TFs) are members of the WRKY-GCM1 superfamily of zinc finger TFs (Babu et al., 2006), currently found only in plants (Ulker and Somssich, 2004). These proteins have a characteristic N-terminal WRKYGQK motif with slight modifications and a C-terminal zinc-finger-like motif. The motifs are vital to WRKY TFs binding with W-boxes (TTGACT/C; cis-acting elements) (Ciolkowski et al., 2008). *Arabidopsis* has 74 WRKY genes divided into three groups, each with subgroups based on WRKY domains and inferred phylogeny (Eulgem et al., 2000).

Abiotic stress limits crop productivity and influences gene expression (Xiong et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Hirayama and Shinozaki, 2010); common stressors include salinity, drought, osmotic stress, heat, and cold. Stress can increase abscisic acid (ABA) biosynthesis and accumulation, as part of plant defense responses (Koornneef et al., 1998). The multiple morphological, physiological, biochemical, and molecular strategies that plants have evolved to cope with stress are under genetic modulation (Joshi et al., 2016). Notably, WRKY TFs are crucial in regulating plant reactions to biotic and abiotic stress (Rushton et al., 2010; Phukan et al., 2016). Currently, we still do not fully understand how the numerous WRKY TFs function under diverse environmental stressors (Ulker and Somssich, 2004; Chen et al., 2012), despite research on multiple plant species (Gadjev et al., 2006; Miller et al., 2008; Pnueli et al., 2002; Zhou et al., 2016; Jiang and Deyholos, 2006, 2009Jiang and Deyholos, 2009). These studies have shown us, however, that WRKYs form interconnecting networks to regulate multiple stress responses synchronously (Banerjee and Roychoudhury, 2015). Additionally, examples from rice, *Arabidopsis*, tobacco, ginseng, and sunflowers have shown that a single WRKY TF can mediate multiple abiotic stress responses. For example, salt-inducible WRKY25 and WRKY33 influenced salt stress response in *Arabidopsis* (Jiang and Deyholos, 2009). Similarly, *GhWRKY41/SpWRKY1* in transgenic tobacco improved drought and

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salt tolerance through regulating stomatal conductance and reactive-oxygen-species levels (Chu et al., 2015). Moreover, *AtWRKY46* in *Arabidopsis* conferred tolerance to both osmotic and salt stress tolerance through effects on ABA signaling and auxin homeostasis, which improved lateral root formation (Ding et al., 2015). In *Papaver somniferum*, ABA, wounding, salt, cold, methyl jasmonic acid (MeJA), and drought stress all induced *PsWRKY* expression; this gene also played a role in regulating the benzylisoquinoline pathway (Phukan et al., 2016; Mishra et al., 2013). Overall, WRKY TFs appear to be strictly regulated in a complex signaling network that allows plants to perform under a variety of abiotic stress conditions (Chen et al., 2012). Based on their known function, WRKY TFs are excellent candidates for engineering plants with better stress tolerance.

Arabidopsis WRKY11 and *WRKY17* are members of the WRKY IId subfamily. Both genes are involved in regulating plant defense. For instance, they are negative regulators of basal resistance against *Pseudomonas syringae* (Journot-Catalino et al., 2006). In a previous study, we challenged *wrky11* and *wrky17* loss-of-function *Arabidopsis* mutants using beet cyst nematodes (Ali et al., 2014), and demonstrated that both single and double mutants were susceptible to nematodes, suggesting that both genes function in resistance against this pathogen. However, we still have not analyzed gene expression or characterized the two TFs in response to different abiotic stressors. Therefore, in this study, we used phylogenetic analysis, reverse transcriptase (RT)-PCR, quantitative real-time (qRT)-PCR, histochemical GUS staining, and quantitative GUS assays to investigate *WRKY11* and *WRKY17* function under abiotic stress. We also used single and double knock-out mutants (*wrky-11*, *wrky17*, and *wrky-11/17*) to demonstrate the importance of both genes in conferring tolerance against environmental stress.

2. Materials and methods

2.1. In silico analysis of WRKY IId subfamily

Eight *Arabidopsis* WRKY genes (WRKY07, 11, 15, 17, 21, 22, 39, and 74) were aligned using ClustalW in MEGA 7.0 (Kumar et al., 2016). A member of the WRKY IIe subgroup (WRKY22) was included as an outgroup. All conserved residues were used to construct a neighbor-joining (NJ) phylogenetic tree (1000 replicates). Potential WRKY domains—*WRKY domain* (IPR003657/PF03106) and *Calmodulin binding domain* (IPR004178/PF02888)—were identified using InterPro v. 60.0 (https://www.ebi.ac.uk/interpro/). Protein sequences were scanned in MEME suite 4.11.2 (http://alternate.meme-suite.org/) to validate the presence of WRKY IId-specific motifs. Amino acid substitutions within WRKY domains of WRKY11 and 17 were checked in MEGA 7.0 using default parameters.

2.2. Plant materials and cultivation

Single and double mutants of *wrky11* and *wrky17* were obtained from previous research (Journot-Catalino et al., 2006). The promoterWRLY11:GUS line was provided by Dr. Thomas Kroj from the Laboratory of Plant-Microorganism Interactions (Castanet Tolosan, France), while we had previously developed the promoter WRLY17:GUS line using pMAA-Red plasmid vectors (Ali et al., 2012, 2014). *Arabidopsis* seeds (Columbia-0; Promoter:GUS lines; T-DNA insertion WKRY11, 17 knock-out mutants) were grown in 9-cm diameter Petri dishes on a modified MS medium (Epple et al., 1997) under sterile conditions. Dishes were kept in a growth chamber at 25 °C under a long-day photoperiod (16 h/8 h light/dark cycle) (Ali et al., 2013). A subset of seedlings grown on MS medium were harvested 5 d and 14 d after germination, washed, frozen in liquid nitrogen, and immediately stored at −80 °C until RNA extraction (roots were stored separately).

Soil for growing 14 d-old seedlings was first incubated at −80 °C for 3 d to kill any insect pupae. Plants were cultivated in growth chambers at 25 °C under a long-day photoperiod (Ali et al., 2013). Subsequently, 5-week-old leaves, cauline leaves, stems, flowers, and siliques were harvested, washed with sterile distilled water, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.3. Abiotic stress assays

Germination and growth assays were performed with age-matched (planted and harvested at the same time) populations of wild type and mutants. Seeds were sterilized and plated on 0.8% agar plates containing half-strength MS medium supplemented with various ABA concentrations (0, 1, 1.5, and 2 μM), NaCl (0, 50, 100, and 150 mM), or D-mannitol (0 mM and 200 mM) (all from Sigma-Aldrich, St. Luis, Mo, USA) (Ali et al., 2013). Germination was defined as radical protrusion from the seed coat. Three plates with 20 seeds per line were scored in germination assays, while three plates with six plants each per line were scored for root growth.

2.4. Reverse transcriptase (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Reverse transcriptase PCR was performed with the RT-PCR Master Mix (USB) following manufacturer's protocol. Resultant RNA was used in cDNA synthesis with Superscript III reverse transcriptase (Invitrogen) and random primers (oligo(dN)6), following manufacturer's protocol. Next, qRT-PCR was performed on an ABI PRISM 7300 Sequence Detector (Applied BioSystems). The reaction mixture (25 μL) contained 12.5 μL Platinum SYBR Green qPCR SuperMix with UDG and ROX (Invitrogen), 2 mM $MgCl_2$, 0.5 μL of each forward and reverse primer (10 μM), 2 μL cDNA, and water. Forward and reverse primers for both PCRs were the same as those used in previous work (Ali et al., 2014). Primer efficiencies were determined using different cDNA concentrations from the wild-type seedlings. Control reactions with no cDNA template ruled out false positives. Dissociation runs ensured the absence of primer dimers. The 18S gene was used as an internal reference for all qRT-PCR reactions. Results were calculated in SDS (Sequence Detection Software) version 2.0 (Applied BioSystems). Relative expression (fold change) was calculated with the ΔΔCt method (Livak and Schmittgen, 2001).

2.5. Histochemical GUS staining and quantitative GUS assay

Promoter:GUS plant tissues were stained in a mix of X-gluc (Biomol, Hamburg, Germany), 0.1 M sodium phosphate buffer (pH 7.0), 0.1% Triton-X 100, 0.5 mM K3[Fe(CN)6], 0.5 mM K4[Fe(CN)6], and 10 mM Na2EDTA at 37 °C, across multiple time points. After staining, chlorophyll was removed from photosynthetic tissues with 70% (v/v) ethanol. Seedlings (including the plant parts of interest) were stained before and after flowering. Seeds were imbibed at 4 °C on filter paper soaked in sterile water under continuous white light for 4 d before GUS staining for 24 h.

Measurement of quantitative GUS activity followed previous protocols (Jefferson, 1989; Shah et al., 2013). A 150-μL reaction mixture of 1 mM 4-methylumbelliferyl-β-D-glucuronide (4-MUG, GUS substrate) was generated by adding 50 μL of 3 mM 4-GUS to 100 μL of protein extract in a black 96-well microtiter plate. The reaction occurred through incubation at 37 °C for 5 min and was stopped with the addition of 50 μL Na2CO3 (0.5 M). Fluorescence was measured at 355-nm excitation and 460-nm emission in the FLUOstar Omega micro-plate reader (BMG Labtech) using 4-MU standards (10 mM stock in ethanol, diluted in GUS extraction buffer) ranging from 1 to 100 μM.

2.6. Co-expression network

Gene modules of *AtWRKY11* and *AtWRKY17* were compared using the NetworkComparer server built in PlaNet (http://aranet.mpimp-golm.mpg.de/network-comparer.html, Mutwil et al., 2009, 2011;