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The *Reaumuria trigyna* transcription factor RtWRKY1 confers tolerance to salt stress in transgenic *Arabidopsis*

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ABSTRACT

Reaumuria trigyna (*R. trigyna*) is an endangered small shrub endemic to the Eastern Alxa–Western Ordos area in Inner Mongolia, China. Based on *R. trigyna* transcriptome data, the Group I WRKY transcription factor gene *RtWRKY1* was cloned from *R. trigyna*. The full-length *RtWRKY1* gene was 2100 bp, including a 1261-bp open reading frame (ORF) encoding 573 amino acids. *RtWRKY1* was mainly expressed in the stem and was induced by salt, cold stress, and ABA treatment. Overexpression of *RtWRKY1* in *Arabidopsis* significantly enhanced the chlorophyll content, root length, and fresh weight of the transgenic lines under salt stress. *RtWRKY1* transgenic *Arabidopsis* exhibited higher proline content, GSH-PX, POD, SOD, and CAT activities, and lower MDA content, Na^+ content, and Na^+/K^+ ratio than wild-type *Arabidopsis* under salt stress conditions. Salt stress affected the expression of ion transport, proline biosynthesis, and antioxidant related genes, including *AtAPX1*, *AtCAT1*, *AtSOD1*, *AtP5CS1*, *AtP5CS2*, *AtPRODH1*, *AtPRODH2*, and *AtSOS1* in transgenic lines. *RtWRKY1* confers tolerance to salt stress in transgenic *Arabidopsis* by regulating plant growth, osmotic balance, Na^+/K^+ homeostasis, and the antioxidant system.

1. Introduction

Soil salinity is one of the most critical environmental factors limiting germination, growth, development, and survival of plants, and it reduces yield in most major crop plants (Wang and Altman 2003; Allakhverdiev et al., 2000; Zhou et al., 2015). Salinized areas are increasing at a rate of 10% annually (Nakahara et al., 2015). High salt stress disrupts homeostasis by affecting water potential and ion distribution, and these effects occur at both cellular and entire plant levels (Zhu 2001). This disruption of homeostasis contributes to osmotic, ionic, and secondary oxidative stresses in plant cells (Liu et al., 2015; Chen et al., 2016). Because of their immobile nature, plants have evolved sophisticated mechanisms to respond to salt stress (Katsuhara et al., 2003; Verslues et al., 2006). The induction of salt stress-associated transcription factors (TFs) plays a crucial role in the response of plants to salt stress (Dang et al., 2013). Large families of TFs, such as WRKY, bZIP, AP2/ERF, GRAS, MYB, bHLH, and NAC, are integral in linking salt sensory pathways mediating plant salt stress-tolerance responses (Yu et al., 2015; Liu et al., 2015; Shukla et al., 2015; Abogadallah et al., 2011; Hartmann et al., 2015). These TFs separately or cooperatively regulate the expression of downstream salt stress-related genes, resulting in the establishment of gene networks for stress adaptation (Wang et al., 2015a; Bakshi and Oelmüller 2014; Rushton et al., 2012; Li et al., 2014b; Xu et al., 2015; Qin et al., 2013).

The WRKY family is among the largest families of TFs in plants, and it is named after the highly conserved sequence motif WRKYGQK (Eulgem et al., 2000; Li et al., 2015). Based on the number of WRKY domains and the pattern of the zinc-finger motif, the WRKY superfamily is subdivided into three groups (Eulgem et al., 2000). WRKY TFs modulate target gene expression by binding to the *cis*-acting element known as the W-box (C/T)TGAC(T/C) in the promoter region (Ulker and Somssich 2004). WRKY TFs regulate plant responses to abiotic stresses via multiple approaches, such as promoting the growth and development of plants and preventing the loss of chlorophyll under abiotic stress conditions (Cheng et al., 2012). *TaWRKY10* and *TaWRKY44* enhance drought and salt stress tolerance in transgenic tobacco, as demonstrated by the increased germination rate, root length, survival rate, and relative water content of transgenic plants exposed to these stress conditions (Wang et al., 2013, 2015b). Transgenic banana plants overexpressing *MusaWRKY71* show increased tolerance to oxidative and salt stress, as indicated by a high photosynthetic efficiency (Fv/Fm) and decreased membrane damage in assayed leaves (Shekhawat and Ganapathi 2013). WRKY TFs also confer high abiotic stress tolerance to plants by regulating homeostasis, including reactive

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oxygen species (ROS) balance, Na^+/K^+ homeostasis, and osmotic balance (Zhu 2001; Chen et al., 2012; Gong et al., 2014). *GhWRKY34*, as a positive transcription regulator, may function in plant responses to high salinity stress by maintaining Na^+/K^+ homeostasis as well as activating salt stress-related genes in cells (Zhou et al., 2015). Upon treatment with 150 mM NaCl, *wrky8* mutants in *Arabidopsis* show an increased Na^+/K^+ ratio and reduced salt stress tolerance, suggesting that WRKY8 functions in the response to salt stress by regulating Na^+/K^+ homeostasis (Hu et al., 2013). Overexpression of *GsWRKY20* in alfalfa increases drought and salt tolerance by regulating Na^+/K^+ homeostasis and osmoregulation, as indicated by a lower relative membrane permeability, MDA content, and Na^+ content, as well as a higher proline content, soluble sugar content, and K^+ content, than the controls (Tang et al., 2014). *DgWRKY3* and *DgWRKY1* overexpressing tobacco plants show high proline content and antioxidant enzyme activity, and low H_2O_2 and MDA content, and these effects are mediated by the upregulation of stress-related genes involved in osmotic adjustment, membrane protection, and oxidative stress responses (Liu et al., 2014, 2013). However, research on WRKY TFs is limited to *Arabidopsis*, rice, tobacco, and other model plants. Many wild plants in nature perform better than model plants and crops in terms of resistance to various abiotic stresses and are therefore worthy of in-depth study.

The WRKY1 transcription factor was first discovered in *Arabidopsis* and named AtWRKY1 (de Pater et al., 1996). The C-terminal WRKY domain of AtWRKY1 protein was crystallized and analyzed by X-ray (Ming-Rui et al., 2005). Meanwhile, AtWRKY1 could be induced by SA treatment, suggesting that this protein may be involved in the defense response (Ming-Rui et al., 2007). Gene functional analysis showed that *AtWRKY1* acted as a negative regulator in plant guard cell ABA signaling and in the response to drought stress. The loss-of-function mutant *atwrky1* was particularly sensitive to ABA, with respect to both ion channel regulation and stomatal movements, and less sensitive to drought than the wild type (Zhu et al., 2016). WRKY1 TFs have also been identified in other plants, such as strawberry (Encinas-Villarejo and Caballero, 2009), tobacco (Menke et al., 2005), cotton (Li et al., 2014a), and parsley (Somssich Imre et al., 1998). These WRKY1 TFs mainly function in resisting pathogens and mediating cell death and flowering. For example, strawberry FaWRKY1 and parsley WRKY1 are important elements in mediating pathogen resistance in plants (Encinas-Villarejo and Caballero, 2009; Somssich Imre et al., 1998). Overexpression of *GbWRKY1* in *Arabidopsis* resulted in a higher sensitivity to *B. cinerea* and *V. dahlia* and promoted flowering (Li et al., 2014a). Tobacco WRKY1 was phosphorylated by the MAP kinase and mediated HR-like cell death in tobacco (Menke et al., 2005; Ogata et al., 2015). Currently, although numerous WRKY TFs have been explored, the publications on WRKY1 TFs are very few and the regulatory mechanism of WRKY1 TFs involved in abiotic stress remains unclear.

The Eastern Alxa–Western Ordos area (106°27′E–111°28′E, 39°13′N–40°52′N, elevation 1500–2100 m), a salinized desert in Inner Mongolia, China, is characterized by its high soil salinity (up to 0.7% salts), hyper-drought conditions (annual average precipitation of 140.9–302.2 mm), and low temperature (annual average temperature of 6.0–9.2 °C) (Li 1990). This area is one of eight centers of biodiversity in China and is home to a group of ancient and endangered plants. *Reaumuria trigyna* (*Reaumuria* Linn genus, family *Tamaricaceae*) is an endangered dicotyledonous shrub with the features of a recretohalophyte, and it is endemic to the Eastern Alxa–Western Ordos area of China. It is regarded as a living fossil owing to its Tethys Ocean origin and is considered a key protected plant in Inner Mongolia (YQ, 1989; Yang et al., 2002; YZ, 2006; Dang et al., 2013). *R. trigyna* shows outstanding performance because of its highly efficient antioxidant system, salt glands with secretion function, and strong osmoregulatory ability (Yan, 2008; Yan et al., 2012; Dang et al., 2013; Liguo 1992). *R. trigyna* transcriptome data analysis revealed that the expression levels of 67 WRKY genes changed significantly under salt stress, such as *RtWRKY1*, a differentially expressed gene showing high transcript abundance. Of a total of 171 differentially expressed genes (DEGs), 155 DEGs were enriched in the "oxidoreductase activity" and "response to stress" GO categories, respectively. In addition, 147 genes and 74 genes were identified as DEGs regulating reactive oxygen species (ROS) scavenging and ion transport, respectively (Dang et al., 2013). WRKY TFs may perform a positive function in the response of *R. trigyna* to salt stress by regulating the expression of genes related to plant growth and homeostasis. Exploring the regulatory function of *R. trigyna* WRKY TFs via expression pattern analysis and function identification will contribute to our understanding of the molecular mechanism underlying the response of *R. trigyna* to salt stress.

In the present study, a WRKY gene named *RtWRKY1* was cloned from *R. trigyna* and shown to be induced by abiotic stresses. Overexpression of *RtWRKY1* in *Arabidopsis* conferred salt tolerance through the regulation of plant growth and development, osmoregulation, Na^+/K^+ homeostasis, and the antioxidant system.

2. Materials and methods

2.1. Plant materials and stress treatments

Seeds and seedlings of *R. trigyna* were acquired as previously described (Zhang et al., 2016). Groups of three healthy seedlings of similar size were subjected to various stress treatments as follows: seedlings were kept in Murashige and Skoog (MS) medium supplemented with 400 mM NaCl, 10 μm ABA, or 30% PEG6000 (w/v) for the salt treatment, ABA treatment, or drought treatment, respectively. Similarly, the seedlings in MS medium were exposed to 4 °C and 42 °C for cold treatment and heat treatment, respectively. The seedlings were treated for 0, 3, 6, 12, and 24 h for each condition, and samples were snap-frozen in liquid nitrogen immediately after the treatment and stored at −80 °C until analysis.

2.2. RNA extraction and first-strand cDNA synthesis

Total RNA was extracted using the Plant Plus RNA reagent (DP437, Tiangen, Beijing) according to the manufacturer's instructions. The extracted RNA was treated with RNasefree DNase I (TaKaRa Bio Inc., Otsu, Shiga, Japan) to remove residual DNA according to the manufacturer's instructions. The quality of the extracted RNA was assessed by agarose gel electrophoresis and NanoVue Plus (GE Healthcare). First-strand cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3. Cloning and sequence analysis of full-length RtWRKY1 cDNA

Based on the analysis of *R. trigyna* transcriptome data, primers for rapid amplification of cDNA ends (RACE) were designed according to the requirements of the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA), which was used to perform both 5′- and 3′- RACE according to the manufacturer's instructions. The primers for 5′- and 3′-RACE were W-1GSP1, W-1GSP2, W-1NGSP1, and W-1NGSP2 (Table 1). The PCR cycling conditions were as follows: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min for 30 cycles. Each PCR product was gel-purified, cloned into the pMD19-T vector (TaKaRa, Kyoto, Japan), and sequenced. The sequence of *RtWRKY1* was analyzed using the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/gorf/gorf.html) website to obtain its open reading frame (ORF) and putative peptide sequence. The molecular weight, pI, and motif of the putative RtWRKY1 protein were analyzed with the online tool ProtParam (http://www.expasy.org). Multialignment was performed with Clustal X, and the phylogenetic tree was drawn using the MEGA 5.0 program with the neighbor-joining method. Subcellular localization prediction analysis was performed using the online soft-