Plant Growth Regulation (2023) 99:125–135
https://doi.org/10.1007/s10725-022-00916-8

ORIGINAL PAPER

Setaria italica SiWRKY89 enhances drought tolerance in Arabidopsis

Aiying Zhang[1] · Li Zhang[2] · Erhu Guo[1] · Rui Wang[1] · Qiang Li[3] · Shihua Guo[3] · Prince Marowa[4] · Zongchang Xu[5]

Received: 13 June 2022 / Accepted: 23 September 2022 / Published online: 15 October 2022

Abstract

Foxtail millet (*Setaria italica*), a drought-tolerant plant, is grown in drylands all over the world. However, the molecular basis of drought tolerance in *S. italica* is not yet understood. Previously, we comprehensively characterised the *SiWRKY* genes and discovered that *SiWRKY89*, a homologue of *AtWRKY57*, had a noticeably higher expression level during dry conditions. In this study, a transgenic experiment was carried out in *Arabidopsis* to investigate the function of *SiWRKY89* in conferring drought tolerance. Phenotypic analysis showed that the root length of seedlings and the survival rates of mature transgenic *Arabidopsis* were greater than those of the control plants under drought conditions. Additionally, compared to the control plants, the transgenic plants had higher proline content and antioxidant activity. Furthermore, qRT-PCR investigation for abiotic stress-responsive genes revealed that *SiWRKY89*-overexpressing plants had higher expression levels than their control counterparts. Additionally, the yeast one-hybrid experiment demonstrated that *SiWRKY89* could bind to the W-box elements of *AtNCED3*. By upregulating the downstream gene *AtNCED3* and activating the reactive oxygen species scavenging mechanisms, *SiWRKY89* overexpression improved *Arabidopsis* drought tolerance. Thus, we provide a molecular and biochemical basis for drought tolerance and a candidate gene for crop breeding for drought tolerance.

Keywords *Setaria italica* · Foxtail millet · WRKY transcription factor · Drought-responsive gene · Reactive oxygen species · Transgenic *Arabidopsis*

Introduction

According to Cramer et al. (2011), drought stress affects crop output in two-thirds of the world's land and is predicted to get worse with rising temperatures brought on by climate change (Zandalinas et al. 2018). Additionally, the IPCC (2014) estimates that by 2050, the world's population would have reached over 9 billion, necessitating a 70–100% increase in crop production to meet demand for food (Godfray et al. 2010; Gupta et al. 2020). The challenges in crop breeding in this environment are to boost food production and enhance tolerance to drought stress. Foxtail millet (*Setaria italica* (L.) P. Beauv.) is mainly cultivated in dry areas, but drought stress during later growth stages (jointing and grain filling stages) can cause grain yield loss (Zhang et al. 2010; Tang et al. 2017). Some millet germplasms are drought tolerant in the seedling development stage but not in the booting stage (Zhang et al. 2010). Using conventional methods to breed drought-tolerant cultivars with higher yields is complex and time-consuming. Therefore, the key is to understand the basis of drought tolerance and identify key genes that can considerably increase plant drought tolerance throughout the growing period.

In order to identify key genes that regulate the drought response pathway in *S. italica*, several genetic and transcriptomic studies have been performed (Qie et al. 2014;

Communicated by Zhen Liang.

Aiying Zhang and Li Zhang have contributed equally to this work.

✉ Zongchang Xu
xuzc1110@163.com

[1] Millet Research Institute, Shanxi Agricultural University, Changzhi, Shanxi, China

[2] College of Agriculture, Shanxi Agricultural University, Taigu, China

[3] Faculty of Agronomy College, Inner Mongolia Agricultural University, Hohhot, China

[4] Department of Plant Production Sciences and Technologies, University of Zimbabwe, Harare, Zimbabwe

[5] Marine Agriculture Research Center, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, China

Qin et al. 2020; Wang et al. 2021; Ceasar 2022). Using high-throughput transcriptomic sequencing, whole genome expression profiling studies have revealed numerous drought response transcription factors (TFs), including WRKY, NAC, AP2/ERF, bHLH, and bZIP, which could regulate the expression of several downstream genes (Rabara et al. 2014; Qin et al. 2020).

The WRKY family is an important TF family for plant growth and development. The WRKY domain is characterised by the highly conserved WRKYGQK domain at the N-terminus and the less conserved zinc-finger domain at the C-terminus (Eulgem et al. 2000; Ulker and Somssich 2004; Rushton et al. 2010). The WRKY TFs can regulate the expression of their target genes by binding to the W-box in the target gene promoter (Ulker and Somssich 2004). The WRKY family members have been characterised in various species (Xie et al. 2005; Bencke-Malato et al. 2014; Okay et al. 2014; Zhang et al. 2017); they play important roles in plant growth, development, and biotic and abiotic stress tolerance (Chen et al. 2012; Jiang et al. 2012, 2016; Guo et al. 2022).

The functions of the *WRKY* genes in response to drought stress have been extensively established. For instance, Jiang et al. (2012) demonstrated that overexpression of *AtWRKY57* enhances *Arabidopsis'* ability to withstand drought. It was discovered that AtWRKY57 can bind to the promoters of *RD29A* and *NCED3*, increasing the expression of those genes. Additionally, it was shown that AtWRKY63 can bind to the *RD29A* promoter and regulate how plants respond to ABA and drought stress (Ren et al. 2010). In rice, research has shown that drought and heat tolerance can be enhanced by the overexpression of *OsWRKY*11 driven by an *HSP*101 promoter (Wu et al. 2009) while overexpression of *OsWRKY*45 in *Arabidopsis* enhances the tolerance to drought stress (Tao et al. 2011). Also, by altering the root architecture's length, *SbWRKY30* has been reported to make rice more tolerant to drought stress (Yang et al. 2020). Additionally, it has been suggested that the *WRKY* genes from a few other non-model crops, including soybean, sorghum, and wheat, are crucial for drought tolerance. For instance, *GsWRKY20* overexpression in soybean enhances *Arabidopsis*' ability to withstand drought by regulating the wax biosynthesis process (Luo et al. 2013), while constitutive expression of wheat *TaWRKY*2 confers salt and drought tolerance in transgenic *Arabidopsis* by directly binding and activating the drought-responsive gene *RD29B* (Niu et al. 2012).

Setaria italica has high water use efficiency, and it is considered an excellent model for investigating the WRKY regulation mechanism of drought tolerance (Lata et al. 2010). We previously performed genome-wide identification and expression profiling of *SiWRKY* genes against drought stress (Zhang et al. 2017). *SiWRKY89*, a homologous gene of *Arabidopsis* gene *AtWRKY57*, was found to be upregulated under drought stress. In this study, the role of *SiWRKY89* in drought tolerance was examined by exploring the effects of overexpressing the gene in *Arabidopsis*.

Materials and methods

Plant materials and growth conditions

Seeds of Yugu1 (a sequenced *S. italica* cultivar) were grown in a greenhouse as described in our previous study (Zhang et al. 2017). The seeds of *Nicotiana benthamiana* and *Arabidopsis thaliana* (Columbia-0) were obtained from the Tobacco Research Institute of the Chinese Academy of Agricultural Sciences, Qingdao, China. *Nicotiana benthamiana* and *Arabidopsis* seeds were sown in pots containing soil and vermiculite, and grown in a greenhouse at 22 °C under a 16 h photoperiod, with the relative humidity set to 70%.

Bioinformatic analysis of SiWRKY89

The full protein sequence of WRKY TFs from *Arabidopsis* and *S. italica* were obtained and used in a multiple sequence alignment following the method described in our previous study (Zhang et al. 2017). A phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) 5.0 program. Bootstrap values were calculated with 1000 iterations. The amino acid sequences of *SiWRKY89* and *AtWRKY57* were aligned with BioEdit software (Tom et al. 2011). To predict the regulatory mechanisms in which *SiWRKY89* is involved, potential regulatory elements within the 2 kb promoter region upstream of the *SiWRKY89* start codon were analysed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Subcellular localisation of SiWRKY89

The coding sequence of *SiWRKY*89 (without stop codon) with restriction sites *KpnI* and *BamHI* was amplified from the cDNA of Yugu1 with the specific primers *SiWRKY*89 CDS-F and *SiWRKY*89 CDS-R (Table S1). The amplified fragment was then digested with *KpnI* and *BamHI* and cloned into the corresponding sites of the pEGFP vector to generate the recombinant construct SiWRKY89-GFP driven by CaMV35S promoter. The constructed SiWRKY89-GFP vector was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The transgenic *Agrobacterium* cells were injected into tobacco leaves. After 2 to 3 days, the injected leaves were pre-stained with 4′,6-diamino-2-phenylindole (DAPI) at 5 mg/mL for 10 min. Then the fluorescence signals in the leaves were observed

under the Leica TCS SP8 laser scanning confocal microscope (Mannheim, Germany).

RNA isolation and qRT-PCR analysis

The total RNA was isolated from *S. italica* and *Arabidopsis* using a Plant Total RNA Isolation Kit (Tiangen, Beijing, China) and treated with RNase-free DNase I (RQ1, Promega, Madison, WI, USA). The cDNA was synthesised using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo(dT)18 (Promega) following the manufacturer's protocol. The cDNA samples were analysed using quantitative real-time polymerase chain reaction (qRT-PCR) on a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermocycling conditions were as follows: 95 °C for 5 min, followed by 40 cycles (95 °C for 15 s and 60 °C for 1 min). Three biological replicates were used. The primers used for qRT-PCR are listed in Table S1. A constitutive *SiACTIN* (Seita.8G043100) was used as an internal reference gene for *F. millet* (Pan et al. 2018), and *AtTUBULIN* for *Arabidopsis* (Xu et al. 2017). Relative gene expression values were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

35S:SiWRKY89 vector construction and Arabidopsis transformation

To create the *35S:SiWRKY89* construct, the coding sequence of *SiWRKY*89 with restriction sites *NcoI* and *BstEII* was amplified from the cDNA of Yugu1 with the specific primers *35SSiWRKY*89-F and *35SSiWRKY*89-R (Table S1). The amplified fragment was digested with *NcoI* and *BstEII*, and cloned into the corresponding sites of the *pCAMBIA1302* vector to generate the recombinant construct *35S:SiWRKY89* driven by the CaMV35S promoter. The *35S:SiWRKY89* vector was transformed into *A. tumefaciens* strain GV3101 by electroporation, and then transformed into *Arabidopsis* using the floral dipping method (Clough and Bent 1998). The T_0 transformants were screened on 1/2 MS medium with hygromycin (50 μg/mL). Ten T_1 transgenic lines were generated and confirmed using semi-quantitative PCR with the primers of *SiWRKY89* CDS-F and *SiWRKY89* CDS-R. The *AtACTIN2* gene was used as the reference gene (Xu et al. 2020). The seeds of T_1 generation were screened on 1/2 MS medium containing hygromycin. (50 μg/mL) to select the T_2 generation. The same process was repeated with seeds of T_2 generation to select T_3 generation. Three T_3 generation transgenic lines (*W1-3*, *W3-5*, and *W6-3*) with 100% resistance to hygromycin were considered homozygous lines and used in the subsequent experiments.

Drought treatments of transgenic Arabidopsis lines

For drought treatments in the seedling stage, the seeds of wild-type (WT), *W1-3*, *W3-5*, and *W6-3* were sown onto 1/2 MS medium. After growing for 7 days, the seedlings were transferred to square petri dishes containing 1/2 MS as controls and to some dishes containing 1/2 MS medium with 300 mM mannitol (to induce osmotic stress). After 7 days of culture, the root length of the seedlings was recorded. The seedlings were transplanted into pots containing soil and vermiculite, at four seedlings per pot, under normal watering conditions in three replicates. After 3 weeks, the plants were dehydrated by withdrawing water for 7 days. At 3 days after re-watering the plants, we evaluated the survival rates of the transgenic and WT plants.

Measurement of water loss rate, proline content, and peroxidase and superoxide dismutase activities

The water loss rate, proline content, superoxide dismutase (SOD) activity, and peroxidase (POD) activity of the drought-treated transgenic plants were measured. *Arabidopsis* leaves were collected during drought treatment. To measure the water loss rate, 20 rosette leaf from five plants were detached and weighed at four-time points over a 2 h period (He et al. 2016). The mixture of rosette leaves derived from 10 WT individuals or transgenic plants (0.1 g) under drought stress were used to evaluate proline content, and SOD and POD activities (Huang et al. 2014). The experiments were performed using appropriate kits following the manufacturer's instructions (A107-1-1, A001-3-2, and A084-3-1; Jiancheng, Nanjing, China). Three biological replicates were performed for each experiment.

Yeast one-hybrid assay

For the protein–DNA-binding experiment, an oligonucleotide sequence containing triple tandem copies of the W-box and mW-box was synthesised, annealed, and cloned into the pHIS2.1 vector forming W-box- and mW-box-specific reporter vectors. The full-length CDS sequence of *SiWRKY89* was cloned and fused with the transcription activating domain (GAL4) of the pGADT7 vector (Clontech, Santa Clara, CA, USA). To determine the interaction between DNA and protein, pGADT7 and pGADT7-SiWRKY89 were co-transformed with the reporter vectors, pHIS2.1-Wb and pHIS2.1-mWb, into the Y187 strain following the manufacturer's instructions (Clontech). All co-transformed yeast cells grown on SD/-Trp/-Leu were re-streaked on SD/-Trp/-Leu/-His with 30 mM 3-AT (3-amino-1,2,4-triazole) to examine protein–DNA interaction.

Statistical analysis

All experiments were repeated thrice and experimental data of all parameters were analysed using one-way analysis of variance in SPSS version 17.0 software (SPSS, Chicago, IL, USA). The data are shown as mean ± standard deviation (SD). Significant differences between the means were identified using Duncan's multiple range test at $P \leq 0.05$.

Results

Bioinformatic analysis of SiWRKY89

The transcript sequence of *SiWRKY89* is 1300 bp long, and *SiWRKY89* is composed of 207 amino acids. Phylogenetic analysis revealed that *SiWRKY89* was classified into the same phylogenetic clade with *AtWRKY57* (Fig. 1A). *SiWRKY89* and *AtWRKY57* showed a 22.3% identity at the amino acid level, however, they both contained the conserved WRKY domain WRKYGQK at N-terminal and the zinc-finger-like motif at C-terminal (Fig. 1B). In addition, promotor *cis*-elements analysis revealed that drought response *cis*-elements, such as ABA-responsive element, ARE, and MBS, and elements for hormone response, such

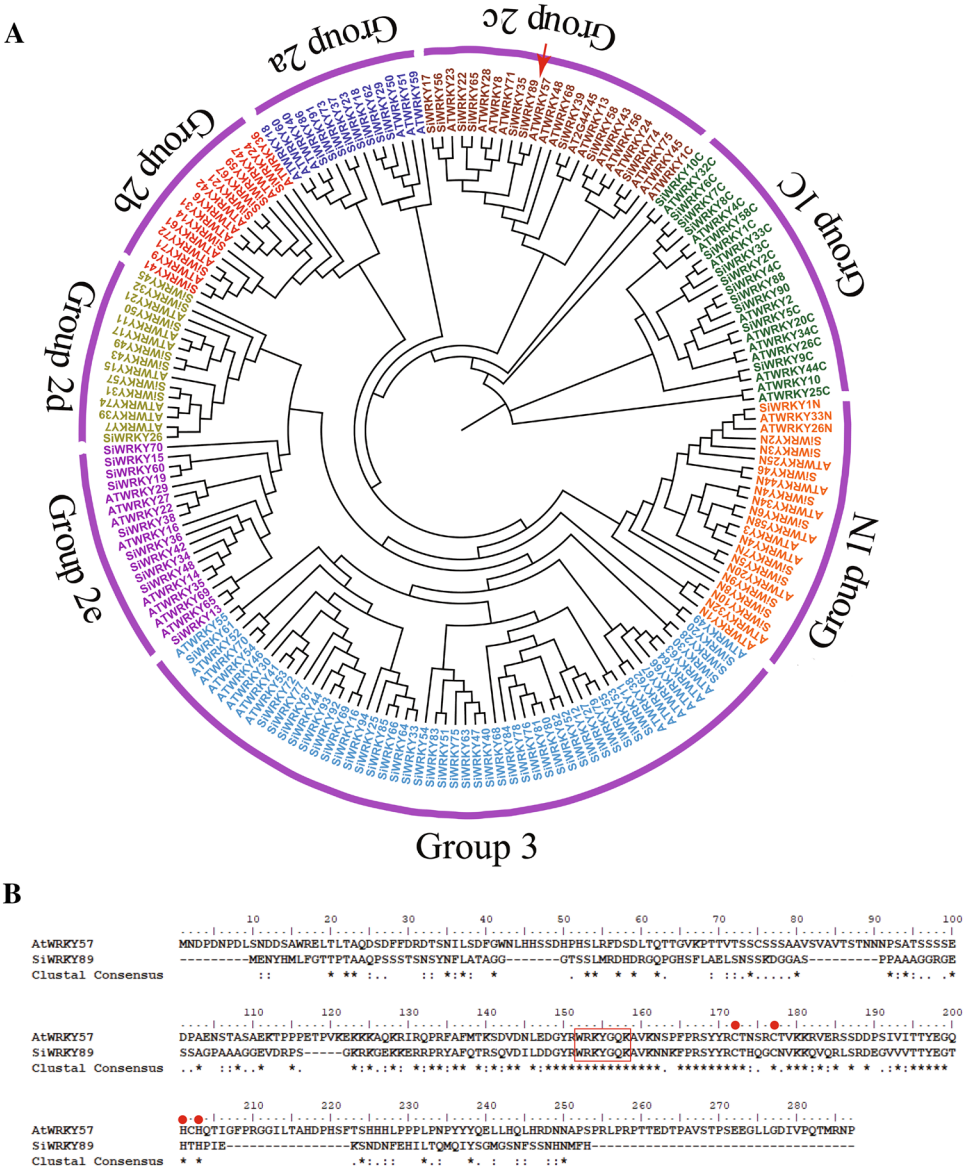

Fig. 1 *Setaria italica* and *Arabidopsis's* WRKY gene family's phylogenetic tree (**A**) and alignment study of *AtWRKY57* and *SiWRKY89* (**B**). *SiWRKY89* and *AtWRKY57* are denoted with a red arrow in the phylogenetic tree while the zinc-finger-like motif (Eulgem et al. 2000) is denoted by a red circle, and the conserved WRKY domain WRKYGQK of *SiWRKY89* and *AtWRKY57* is shown in a red box

as CGTCA-box, TGA-element, and TGACG-motif, were identified in the 2000 bp *SiWRKY89* promoter (Table 1). The results indicate that *SiWRKY89* regulates plant drought response and development possibly through hormone regulation.

SiWRKY89 subcellular localisation

The subcellular localization of SiWRKY89 was detected with a confocal microscope. The green fluorescence signal of SiWRKY89-GFP was detected in the nucleus, which was marked by DAPI staining of the nucleus-targeted control (Fig. 2), which suggests that SiWRKY89 is a nuclear protein.

Root length of 35S:SiWRKY89 transgenic lines under drought stress

The function of *SiWRKY89* was investigated by ectopically expressing the gene under the CaMV35S promoter in *Arabidopsis*. The semi-quantitative PCR results revealed that the *SiWRKY89* gene was successfully integrated into the genome of transformed *Arabidopsis* (Fig. 3C). The expression of *SiWRKY89* in three homozygous transgenic lines (*W1-3*, *W3-5*, and *W6-3*) was significantly higher than the background level in the Yugu1 leaves (Fig. 3D). The root growth of WT and transgenic *Arabidopsis* plants were comparable on 1/2 MS medium (Fig. 3A). Although the root length of W1-3 (2.47 cm) was slightly longer than that of the WT (2.43 cm) on the medium containing mannitol, W3-5 (3.15 cm) and W6-3 (2.69 cm) plants developed significantly longer roots on mannitol (Fig. 3B, E).

Survival rate and physiological indices of 35S:SiWRKY89 transgenic lines under drought

The survival rate of transgenic and WT plants was compared in order to look at the survival rate of *35S:SiWRKY89* transgenic plants under drought stress. The findings demonstrated that after 1 week of no watering, the leaves of the WT showed chlorosis and withered, whereas just a few leaves of the transgenic plants withered (Fig. 4A). Furthermore, after resuming watering, only 30% of the WT plants resumed growth, whereas over 90% of the transgenic plants resumed growth (Fig. 4B).

The physiological parameters of the WT and transgenic plants under drought stress, including the water loss rate, proline content, and SOD and POD activities, were compared in order to understand the mechanism behind the increased drought tolerance of *35S:SiWRKY89* transgenic plants. After detaching over a 2-h period, the leaves of the WT plants showed a higher water loss rate than those of the

Table 1 *Cis*-acting elements in the promoter of *SiWRKY89*

Cis-acting element	Number	Target sequence	Function
ABRE	15	ACGTG/CGCACGTGTC	*cis*-acting element involved in abscisic acid responsiveness
ARE	2	AAACCA	*cis*-acting regulatory element essential for anaerobic induction
MBS	2	CAACTG	MYB-binding site involved in drought inducibility
WUN-motif	1	AAATTTCCT	Wound-responsive element
AuxRR-core	1	GGTCCAT	*cis*-acting regulatory element involved in auxin responsiveness
W-box	5	TTGACC	WRKY transcription factor binding site

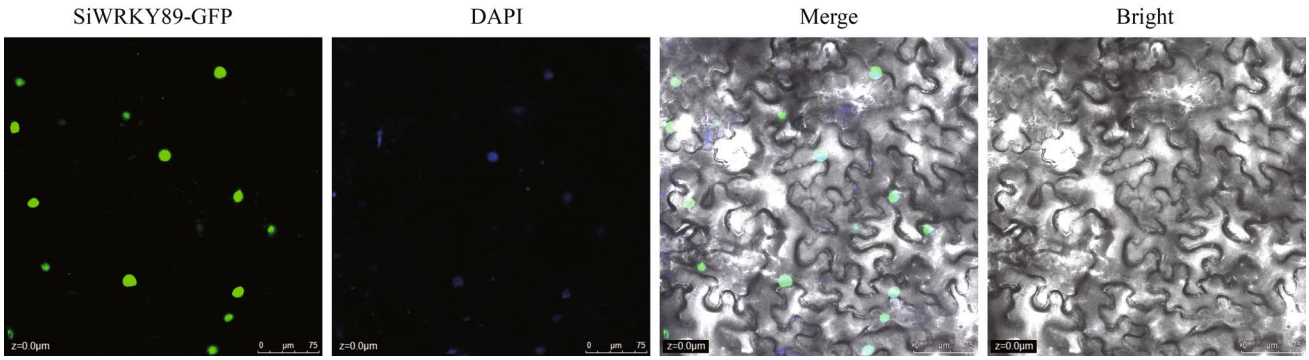

Fig. 2 Subcellular location analysis of *SiWRKY89*. The recombinant constructs of SiWRKY89-GFP and empty pEGFP were separately introduced into tobacco leaves. The fluorescence images revealed that SiWRKY89-GFP was present in the nucleus, whereas empty pEGFP was present throughout the cell

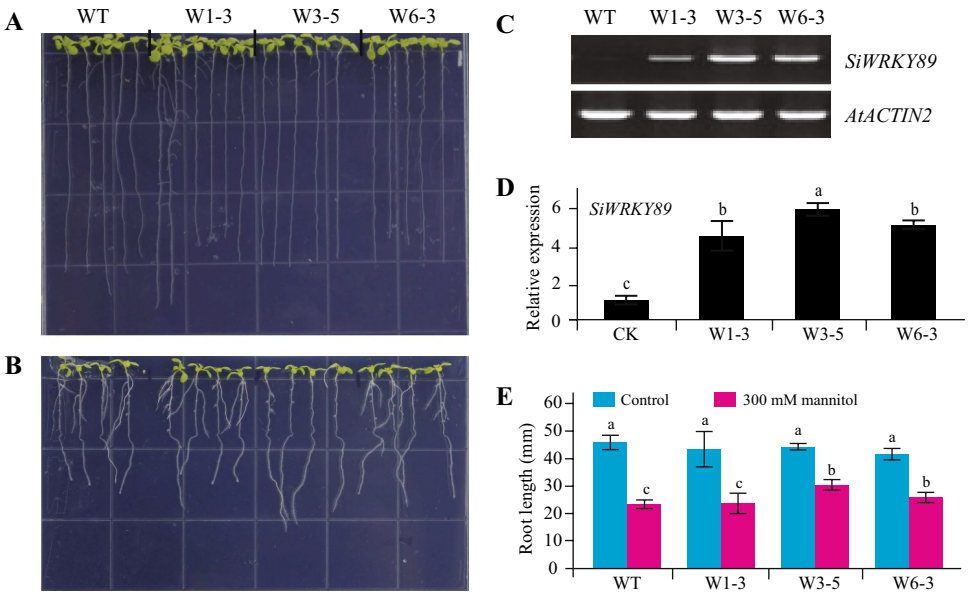

Fig. 3 Root length of 35S:*SiWRKY89* transgenic lines under drought stress. **A** 1/2 MS medium; **B** 1/2 MS medium supplemented with 300 mM mannitol; **C** semi-quantitative PCR analysis of *SiWRKY89* in WT and transgenic lines (*W1-3*, *W3-5*, and *W6-3*); **D** qRT-PCR expression analysis of *SiWRKY89* in Yugu1 leaves (CK) and transgenic lines (*W1-3*, *W3-5*, and *W6-3*). **E** Root length of 35S:*SiWRKY89* transgenic lines under drought stress. The data are shown as mean ± SD of three replicates

transgenic lines (Fig. 4C). Additionally, transgenic plants had proline content and SOD and POD activities that were 2–3fold higher than those of WT plants (Fig. 4D-F), demonstrating the critical role played by osmotic substances and the antioxidant system in the *35S:SiWRKY89* transgenic plants' responses to drought stress.

Expression of drought-responsive genes in 35S:SiWRKY89 transgenic lines under drought stress

To further understand the molecular mechanism underlying enhanced stress tolerance in 35S:*SiWRKY*89 transgenic plants, the expression of four genes, *AtSOD1*, *AtPOD*, *AtP5CS*, and *AtNCED3* was evaluated in the WT and transgenic *Arabidopsis* plants under drought stress. In transgenic lines compared to WT plants, the transcript levels of *AtSOD1, AtPOD*, and *AtP5CS* were 2–6 fold higher. In particular, the expression of *AtP5CS* was significantly increased in W1-3 (Fig. 5A–C), which is consistent with the high levels of proline and antioxidants accumulated in transgenic plants. In addition, we found that the three transgenic lines had considerably higher levels of the *AtNCED3* gene, a crucial enzyme in the ABA biosynthesis pathway (Fig. 5D), suggesting that *SiWRKY89* may be controlling this gene.

Determination of DNA-binding activity of SiWRKY89 using the yeast one-hybrid assay

To determine the DNA-binding activity of SiWRKY89, the yeast one-hybrid assay was performed. All yeast cells harbouring the two kinds of plasmids grew well on SD/-Trp/-Leu medium (Fig. 6) suggesting the success of co-transformation. On SD/-Trp/-Leu/-His medium plus 30 mM 3-AT, the cells containing pHIS2.1-Wb plus pGADT7-SiWRKY89 vectors grew well, and pHIS2.1-mWb plus pGADT7-SiWRKY89 did not grow (Fig. 6), suggesting that *SiWRKY89* could interact with W-box rather than mW-box to induce the transcription of *HIS3*. In addition, the negative control (pHIS2.1-Wb/pGADT7 and pHIS2.1-mWb/pGADT7) did not grow on SD/-Trp/-Leu/-His medium plus 30 mM 3-AT.

Discussion

Abiotic factors, especially drought stress, severely limit crop production worldwide (Godfray et al. 2010; Gupta et al. 2020). WRKY transcription factors are a large family of transcription factors in plants (Thomas et al. 2000), and the functions of WRKYs in growth and development have been extensively explored in a variety of plants (He et al. 2016). Numerous earlier investigations also reported on the various functions that WRKY transcription factors play in response to biotic and abiotic stressors (Wu et al. 2009; Ren et al. 2010; Tao et al. 2011; Chen et al. 2012; Niu et al. 2012; Luo et al. 2013; Rabara et al. 2014; Jiang et al. 2016; Yang et al. 2020). The role of the *Setaria italica* WRKY genes in the response to drought stress hasn't, however, received much thorough research. *SiWRKY89* was revealed to be up-regulated under drought stress when we previously did genome-wide identification and expression profiling of *SiWRKY* (Zhang et al. 2017).In the present study, the role of *SiWRKY*89 in drought tolerance was examined by exploring the effects of overexpressing the gene in *Arabidopsis*. The 35S:*SiWRKY*89 transgenic seedlings displayed longer roots

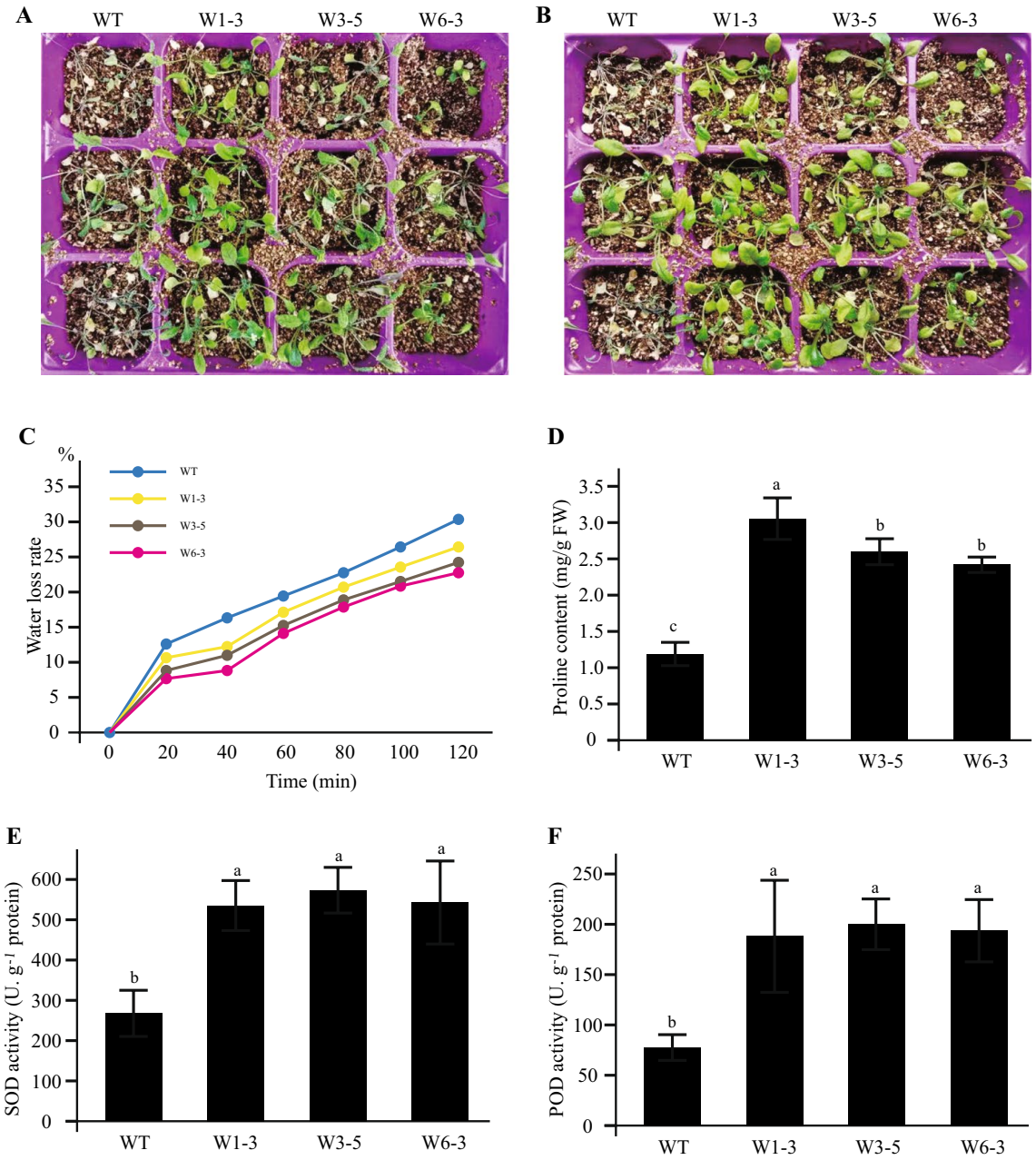

Fig. 4 Analysis of drought tolerance of *35S:SiWRKY89* transgenic lines under drought stress. WT and transgenic lines (*W1-3*, *W3-5*, and *W6-3*) were **A** under drought stress for 1 week and **B** 3 days after re-watering; **C** The water loss rate of WT and transgenic lines (*W1-3*, *W3-5*, and *W6-3*). **D** The proline content in WT and transgenic lines (*W1-3*, *W3-5*, and *W6-3*). **E** SOD activity in WT and transgenic lines (*W1-3*, *W3-5*, and *W6-3*); **F** POD activity in WT and transgenic lines (*W1-3*, *W3-5*, and *W6-3*). The data are shown as mean ± SD of three replicates

under mannitol stress (Fig. 3B, E), and presented a higher survival rate than WT plants under drought stress conditions (Fig. 4A, B). The ABA biosynthesis was activated with the *AtNCED3* accumulation (Fig. 5D), and the antioxidant system was also triggered (Fig. 4D-F) in the transgenic plants.

The functions of the *WRKY* genes in response to drought stress have been extensively established. For instance, Jiang et al. (2012) demonstrated that overexpression of *AtWRKY57* enhances *Arabidopsis*' ability to withstand drought. Also, by altering the root architecture's length, sorghum *SbWRKY30* has been reported to make rice more tolerant to drought stress (Yang et al. 2020). In this study, foxtail millet *SiWRKY89* over-expression plants showed longer roots under mannitol stress in comparison with wild type (Fig. 3B, E). *AtWRKY57* was highly expressed in rosette leaves (Jiang et al. 2012), but the expression of *SiWRKY89*

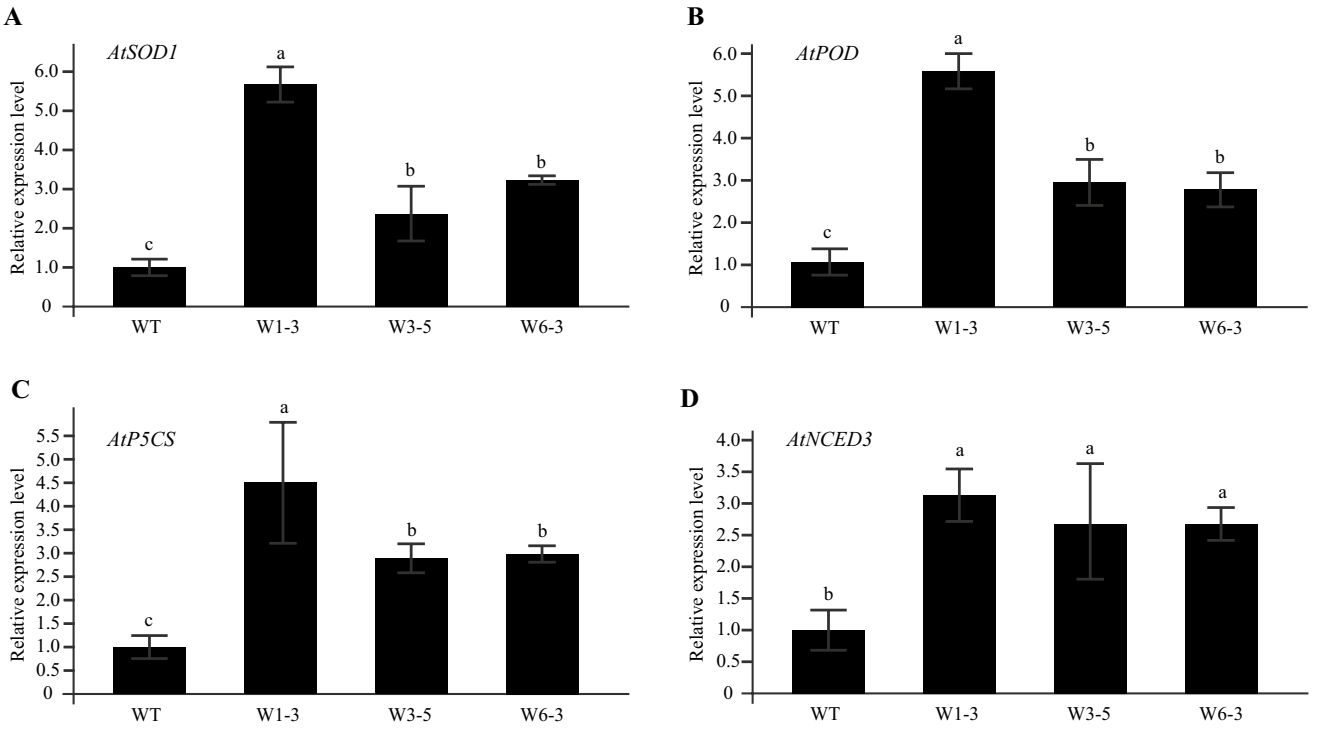

Fig. 5 qRT-PCR expression analysis of four stress-responsive genes. The expression level of four stress-responsive genes *AtNCED3, AtSOD1, AtPOD* and *AtP5CS* under drought stress was analysed in WT and three homozygous transgenic lines (*W1-3*, *W3-5*, and *W6-3*). Relative gene expression values were determined using the $2^{-\Delta\Delta Ct}$ method. The data are shown as mean ± SD of three replicates. *AtTUBULIN* was used as an internal control to normalise the data. Values are mean ± SD

was much higher in roots than in leaves and flowers (Zhang et al. 2017), and *SbWRKY30* is highly expressed in sorghum taproots (Yang et al. 2020). Notably, sorghum and foxtail millet are both natural stress tolerant crops. However, further studies are required to determine the mechanism by which *SiWRKY89* regulates root length.

A great deal of evidence has shown that abiotic stresses such as drought, cold, salinity, heat, and light could induce ROS generation in plant cells (Huang et al. 2019). As signalling molecules, ROS trigger signal transduction pathways in response to abiotic stresses and play key roles in the acclimation process of plants to abiotic stresses (Sarvajeet et al. 2010). However, more ROS accumulation could cause irreversible cellular damage through their strong oxidative properties. Antioxidant enzymes such as SOD, POD, CAT, and APX, et al. were regarded as the enzymatic system to scavenge excess ROS (Huang et al. 2019). The function of antioxidants in ROS scavenging were reviewed well in many reviews (Huang et al. 2019; Sarvajeet et al. 2010). In our study, the antioxidants SOD and POD, and the encoding genes *AtSOD1*, *AtPOD* were all up-regulated in transgenic plants (Fig. 4E, F; Fig. 5A, B), which was consistent with previous reports (Huang et al. 2019; Sarvajeet et al. 2010). Proline is an important osmolyte accumulated in plants under drought stress (Yoshiba et al. 1997; Kavi Kishor et al. 2014). It has been reported that an increased proline content could enhance drought tolerance in various plants overexpressing *WRKY* genes. For example, higher accumulation of proline has been shown to enhance the drought tolerance of *AtWRKY57* transgenic rice (Jiang et al. 2016). Overexpression of *MuWRKY3* from groundnut led to the accumulation of higher proline levels under drought stress (Kiranmai et al. 2018). In line with these earlier findings, we found that *SiWRKY89* overexpression led to a large increase of the proline-encoding gene *AtP5CS* (Fig. 5C) and proline content (Fig. 4D), and that these OX-plants had high drought tolerance. Thus, we came to the conclusion that *SiWRKR89* overexpressing plants' high drought tolerance was likely brought on by an abundance of antioxidant enzymes and proline, which lessened ROS damage. This is further supported by Nishizawa et al. (2008) who reported that proline performs the same role as antioxidant enzymes in scavenging reactive oxygen species.

By attaching to the W-box cis-element in the target gene promoter, WRKY TFs can control the expression of target genes (Rushton et al. 2010). The key enzyme gene in the ABA biosynthesis pathway is *AtNCED3* (Woo et al. 2011). According to a previous study, *AtWRKY57* could directly bind to the W-box of the *AtNCED3* promoter sequences, which led to high levels of *AtNCED3* expression and ABA

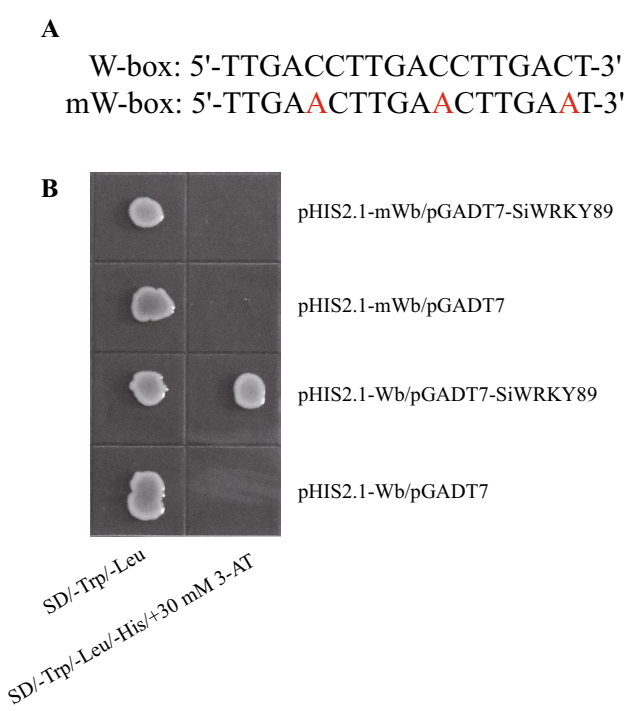

Fig. 6 DNA-binding assay of SiWRKY89. **A** The sequence of the triple tandem copies of the W-box and mW-box binding elements. **B** Yeast one-hybrid assay using the W-box or mW-box as bait. Yeast cells were co-transformed with the plasmid combination of pHIS2.1-Wb/pGADT7, pHIS2.1-Wb/pGADT7-SiWRKY89, pHIS2.1-mWb/pGADT7, and pHIS2.1-mWb/pGADT7-SiWRKY89 on SD/-Trp/-Leu or SD/-Trp/-Leu/-His + 30 mM 3-AT plates

content in transgenic plants with good drought tolerance (Jiang et al. 2012). *SiWRKY89* and *AtWRKY57* share the same WRKY conserved domain, although having an amino acid identity of only 22.30 percent (Fig. 1). Importantly, the yeast one-hybrid assay revealed that *SiWRKY89* could bind to the W-box elements of *AtNCED3* (Fig. 6), indicating that *SiWRKY89* could bind to the promoter of *AtNCED3* and regulate the plants' response to drought stress in a manner similar to that of *AtWRKY57* (Jiang et al. 2012).

Conclusions

We elucidated the role of *SiWRKY*89 in drought tolerance by exploring the consequences of overexpressing this gene in *Arabidopsis*. The functional analysis revealed that overexpressing *SiWRKY*89 enhanced drought tolerance in *Arabidopsis* by regulating the downstream gene of *AtNCED3* and activating the antioxidant system. Our study provides valuable information for analysing the basis of drought tolerance of *S. italica* and a candidate gene for breeding drought tolerant crops.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10725-022-00916-8.

Acknowledgements This work was financially supported by the National Key Research and Development Program (2020YFD1000803-2); State Key Laboratory of Sustainable Dryland Agriculture (202105D121008-2-5); the Natural Science Foundation of China (NSFC) (31860409; 32060509); Natural Science Foundation of Shanxi Province (201901D111221); Key Research and Development Program of Shanxi Province (201803D221019-1); Research Program Sponsored by Shanxi Key Laboratory of Minor Crops Germplasm Innovation and Molecular Breeding, Shanxi Agricultural University (202105D121010-06); Demonstration and Guidance Program for Technology People-Benefit in Qingdao (20-3-4-7-nsh), and the Agricultural Science and Technology Innovation Program (ASTIP No. CAAS-ZDRW202201).

Funding The State Key Laboratory of Sustainable Dryland Agriculture, 202105D121008-2-5, Li Zhang, Natural Science Foundation of Jilin Province,31860409, Li Zhang,32060509, Li Zhang, Key Technologies Research and Development Program,2020YFD1000803-2, AiYing Zhang, Natural Science Foundation of Shanxi Province, 201901D111221, Li Zhang

Data availability Not applicable.

Declarations

Conflicts of interest The authors declare no conflict of interest.

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