Overexpressing the WRKY transcription factor OsWRKY97 improves drought tolerance in rice

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Abstract

Background: WRKY transcription factor (TF) is one of the largest TF families in plants and plays an important role in plant development and stress protection. However, the function of individual WRKY gene in plants are still under investigation. Here, we identified a new member of WRKY TF family, OsWRKY97, and analyze its role in stress resistance by using a series of transgenic plant lines.

Results: OsWRKY97, which positively regulates drought tolerance in rice. OsWRKY97 was expressed in all examined tissues and could be induced by various abiotic stresses. OsWRKY97 is located in the nucleus. Compared with the wild-type (WT), OsWRKY97-overexpressing (OE) plants enhanced the tolerance of rice to drought and osmotic stress. In addition, OE plants also showed higher sensitivity to exogenous abscisic acid (ABA). When exposed to drought stress, up-regulation OsWRKY97 increased the accumulation of ABA and reduced water loss. Furthermore, OE plants achieved higher proline content and reduced levels of malondialdehyde (MDA) and reactive oxygen species (ROS). The homeostasis of ROS was maintained via the activity of active oxygen scavenging enzyme, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), activated by OsWRKY97

Conclusions: These results indicate that OsWRKY97 plays a crucial role in the response to drought stress and may possess high potential value in improving drought tolerance in rice.

Background

Drought is one of the major stresses that seriously affects plant growth and reduces yield[1]. Plants have evolved various strategies to cope with drought stress for survival and development. The response process of plants to drought stress includes stress signal perception, signal transduction and amplification, and adaptation at morphological, physiological and molecular levels[2]. In these processes, many diverse stress-related proteins are expressed that enhance drought resistance via outputs such as osmotic adjustment, stomatal closure, and ROS scavenging[3].

ABA has been characterized extensively as an important plant hormone, which responds to drought stress by regulating stomatal closure and transpiration rate[4, 5]. It has been reported that most water stress inducing genes respond to treatment with exogenous ABA and relate to ABA signal transduction pathways[6]. ABA-mediated stomatal closure is usually accompanied by the production of H₂O₂[7]. It was reported that H₂O₂ participated in ABA signal transduction in plant guard cells and triggered stomatal closure by activating Ca²⁺ channels on cell membranes[8, 9]. It has also been reported that H₂O₂ can induce stomatal closure, which was independent of ABA pathway[10]. However, under stress conditions, the content of H₂O₂ increased sharply in plant cells, which posed a threat to plants, but it is also thought that ROS is a signal for activation of defense pathways[11].

The WRKY TF is one of the largest TF families and is a class of DNA-binding proteins in plants[12]. The WRKY protein has a highly conserved WRKY domain, containing an almost unchanged WRKYGQK sequence at the N-terminal, followed by a Cx4 – 5Cx22 – 23 HxH or Cx7Cx23 HxC zinc-finger motif[13].

Based on the number of WRKY domains and the types of zinc finger motifs, WRKY proteins can be divided into groups I-III. Group I WRKY contains two WRKY domains, Group II WRKY contains a WRKY domain, and the I-II groups all contain a Cx4 – 5Cx22 – 23 HxH zinc-finger motif. Group III WRKY contains a WRKY domain and a Cx7Cx23 HxC zinc-finger motif[14].

At present, studies have revealed the involvement of the WRKY family in plant responses to abiotic stresses. For example, Overexpression of TaWRKY2 reduced the water loss rate of transgenic wheat and enhanced its drought resistance[15]. Furthermore, many TFs have been reported to be involved in ABA-mediated signaling pathways of plant responses to drought stress[16]. CmWRKY10 is located in the nucleus, and overexpression of CmWRKY10 enhanced chrysanthemum tolerance to drought stress through the ABA-signaling pathway[17]. OsWRKY45 plays an important role in drought tolerance and ABA signal-regulation in rice[18]. In recent years, progress has been made in ABA sensing signal transduction pathway[19]. In the presence of ABA, the receptor Pyrabactin resistance (PYR) / PYR1-like (PYL) / Regulatory component of ABA receptors (RCARs) of ABA can disrupt the interaction between the SNF1-related protein kinase 2 (SnRK2s) and 2C-type protein phosphatases (PP2Cs), thus preventing the PP2C-mediated dephosphorylation of the SnRK2s and resulting in the activation of the SnRK2 kinases[20]. Further analyses revealed that the activation of SnRK2s plays a key role in the ABA-dependent phosphorylation of ABRE-binding protein (AREB)[21]. It has been reported that WRKY TFs regulate the expression of many genes related to ABA-induced physiology and development by binding the w-box sequence in the promoters of AREBs[22]. AtWRKY63 mutants impaired the sensitivity of ABA mediated stomatal closure and affects its drought tolerance[23]. It is reported that WRKY40 and WRKY60 transcription factors inhibit ABI4 gene expression. In addition, their mutual antagonism not only occurs between WRKY40 and WRKY60, but also between WRKY18 and WRKY40 to balance the repressive functions on the ABI4 gene. However, for ABI5 gene, WRKY60, WRKY18 and WRKY40 inhibited ABI5 independently, but WRKY60 also antagonized the inhibition of WRKY18 and WRKY18-WRKY40 heterodimer to balance the inhibition of ABI5 gene. These findings help to understand the complex mechanism of WRKY mediated ABA signal transduction[24].

The corresponding sequence of OsWRKY97 (LOC_Os12g02420.1) can be downloaded from Rice Genome Annotation Project (RGAP) (<http://rice.plantbiology.msu.edu/>) and OsWRKY97 belongs to the WRKY family according to the SMART database (<http://smart.embl-heidelberg.de/>). The OsWRKY97 gene was identified by analyzing rice drought stress microarray data generated by our lab (unpublished). The gene was strongly up-regulated under drought conditions in microarray data. So we think that OsWRKY97 may also play an important role in the response to abiotic stresses. In this study, we found that the expression level of OsWRKY97 in Nipponbare was significantly improved under abiotic stress, and overexpression of OsWRKY97 improved the tolerance of transgenic rice to drought and osmotic stress. OE plants also showed higher sensitivity to exogenous ABA and increased the accumulation of ABA under drought stress. In addition, we found that OE plants had higher activity of active oxygen scavenging enzymes, less membrane lipid peroxidation and higher accumulation of proline under drought conditions. These results suggest that OsWRKY97 positively regulates drought stress tolerance in rice.

Results

Expression patterns of *OsWRKY97*

We examined the expression of *OsWRKY97* in 15 different tissues by quantitative real-time PCR (qRT-PCR), so that the expression pattern of *OsWRKY97* in different tissues at different stages of rice was detected. *OsWRKY97* was expressed in various tissues of rice at different stages (Additional file 1:Figure S1). We investigated the expression patterns of *OsWRKY97* seedlings under various abiotic stresses by qRT-PCR. These results suggested that *OsWRKY97* may be involved in responses to various stresses (Figure 1). For drought treatment, the transcription level of *OsWRKY97* gradually increased until reaching a maximum at 8 h (Figure 1A). The transcription level of *OsWRKY97* reached its maximum after treatment with 50 μ M ABA for 12 hours, and similar results were obtained after treatment with 250 mM NaCl (Figure 1B,C). The expression of *OsWRKY97* increased significantly in 12 to 24 h under cold stress (Figure 1D). However, the transcription level of *OsWRKY97* under high temperature treatment was not significantly induced (Figure 1E).

Subcellular localization of *OsWRKY97*

To determine the subcellular localization of *OsWRKY97*, the full-length cDNA sequence of *OsWRKY97* was fused to GFP and driven by CaMV35S. The fusion protein and green fluorescent protein (GFP) control were transiently expressed in tobacco cells via *Agrobacterium* infiltration, and meanwhile, the carrier of the red fluorescent protein nuclear Localization Signal - red fluorescent protein (NLS-RFP) connected with the nuclear localization signal is used as the nuclear localization control. The fluorescence signal of the fusion protein was located in the nucleus (Figure 2A), while the fluorescence signal of the GFP control was located in the nucleus and cytoplasm (Figure 2B). *OsWRKY97*-GFP fusion protein and NLS-RFP were co-located in the nucleus, indicating that *OsWRKY97* is a nuclear protein.

Overexpression of *OsWRKY97* in rice improved osmotic stress tolerance at the germination and post-germination stages

In this study, we found that *OsWRKY97* was strongly induced by osmotic stress (Figure 1A). To further verify whether *OsWRKY97* was involved in regulating the sensitivity of rice to stress, we constructed an *OsWRKY97* overexpression vector and transferred it into Nipponbare. The expression level of *OsWRKY97* in overexpressed plants was analyzed by qRT-PCR. The results showed that the expression of *OsWRKY97* was significantly enhanced in transgenic lines (Figure 3A). Although we obtained 18 OE lines, only two independent lines, OE-1 and OE-23 were selected for future testing. Under normal conditions, the germination rates of WT and OE lines were not significantly different. However, the germination rate of WT seeds was less than 50%, however, after absorption for 5 days under the osmotic stress, the germination rate of overexpressed seed OE-1 was 76%, and that of OE-23 was 80%. (Figure 3B,C). To further understand the sensitivity of OE lines to osmotic stress at the post-germination stage, seedlings of WT and OE lines that were growing under normal conditions for 4 days were selected and transferred to the nutrient solution with or without 20% PEG6000. After 12 days, each material was photographed, and

its plant height was measured. Under normal conditions, the seedlings of WT and OE lines were similar in growth. Under osmotic stress, the plant height of WT seedlings was 8.6 cm, while the plant height of overexpression seedling OE-1 was 10.8 cm, and that of OE-23 was 10.6 cm (Figure 3D,E). These results showed that overexpression of *OsWRKY97* in rice did not affect seed germination and seedling growth under normal growth conditions, but significantly attenuated the inhibitory effects on seed germination and seedling under osmotic stress induced by 20% PEG6000.

Overexpression of *OsWRKY97* in rice enhanced drought stress tolerance

To further verify the biological function of *OsWRKY97*, we tested the drought stress tolerance of OE lines by withholding water. The seedlings of OE lines and WT in the same pots that were of similar vigor were not watered until the leaves curled and then recovered. Before the pressure was introduced, the growth conditions of all genotypes were similar. After drought treatment, the leaf rolling degree of WT plants was more serious than that of OE plants. After re-watering, The survival rate of overexpressed plant OE-1 was 53%, and that of OE-23 was 55%, however, the survival rate of WT was only 14%-16% (Figure 4A,B). These results indicated that overexpression of *OsWRKY97* enhances the drought tolerance of rice. The relative water loss rate of detached leaves is an important characteristic reflecting drought tolerance[25]. Leaves of 2-week-old transgenic plants and WT seedlings were removed and exposed to water-free air for dehydration. Compared with WT, the leaf water loss of OE lines was significantly slower (Figure 4C), which means that *OsWRKY97* played an active role in improving the water retention capacity of plants under dehydration conditions.

Effects of *OsWRKY97* overexpression on related physiological indexes under drought stress

To further clarify the physiological mechanism by which *OsWRKY97* confers tolerance to drought, we investigated the possible physiological basis related to the enhancement of drought resistance in OE plants. ROS were generated in plants when they were subjected to abiotic stresses such as drought, salinity, heat and cold[26]. H_2O_2 , which is an important second messenger, is one of the most significant of these ROS[27]. Therefore, we measured the accumulation of H_2O_2 in plants after drought treatment. We found that the accumulation of H_2O_2 in *OsWRKY97*-overexpressing plants after drought treatment was much lower than that of WT (Figure 5A). To explore the potential mechanism of the reduction of active oxygen level in transgenic plants, ROS scavenging enzyme activity was also measured[11]. As shown in Figure 6, there was no significant difference in SOD, CAT and POD activities between transgenic lines and WT under normal conditions, while the enzyme activities of transgenic lines were higher than those of WT lines under drought stress (Figure 5B-D). Abiotic stresses to plants lead to enhanced membrane peroxidation and accumulation of osmotic substances in plants to maintain osmotic potential tissues of leaves[28]. As shown in Figure 5E, under drought conditions, the MDA content of transgenic plants and WT plants increased, but the increase of MDA content in transgenic plants was significantly lower than that of WT plants, which indicated that overexpression of *OsWRKY97* in rice could reduce membrane lipid peroxidation under drought stress. Under drought stress, the proline content of transgenic plants was significantly higher than that of control plants (Figure 5F). This result clearly shows that

overexpression of *OsWRKY97* can increase proline synthesis and protect rice plants to better cope with drought stress.

***OsWRKY97* may be a positive regulator in ABA signaling under drought stress**

In this study, we found that *OsWRKY97* was also strongly induced by ABA (Figure 1B), to verify this expression pattern, the sensitivities of OE plants to exogenous ABA were examined. The results showed that the germination rate of WT plants was higher than OE plants under the treatment of exogenous ABA (Figure 6A,B). Therefore, we suspect that the drought tolerance of OE transgenic lines may be related to ABA. In order to verify this hypothesis, we measured the ABA content in OE lines and WT respectively under drought stress. The results showed that the endogenous ABA level was not significantly different between WT plants and OE plants under normal conditions. However, under drought stress, the endogenous ABA level in OE plants was significantly higher than WT plants (Figure 6C). In addition, we also analyzed the transcription level of response genes in ABA signaling pathway in WT plants and OE plants, including *OsRAB21*, *OsRD22*, *OsRAB16A*, *OsNCED3* [29, 30]. As shown in Figure 6D, the transcription level of these genes in OE plants was significantly higher than WT plants under drought stress. These results indicate that *OsWRKY97* may improve drought tolerance via ABA signaling pathway.

Discussion

Plants will suffer from various abiotic stresses in their natural environment, which will affect the normal development of plants and even affect their yield [31, 32]. At present, many studies have shown that WRKY TFs are negatively or positively involved in the integration of signaling pathways in abiotic stress responses [33]. However, the functions of many WRKY TFs in plants, especially stress responses, are still unclear. In this study, we examined whether *OsWRKY97* participates in the regulation of the response to drought stress and its effect on rice. In addition, we found that *OsWRKY97*-GFP subcellular localization was in the nucleus of tobacco epidermal cells (Fig. 2), which is consistent with previous studies on other WRKY TFs and may be related to its function [34].

Increasing evidence shows that WRKY TFs play an important role in the drought stress response, as ectopic expression of *OsWRKY11* enhanced tolerance to drought stress and induced constitutive expression of drought-responsive genes [35]. Overexpression of *GsWRKY20* from Glycine soja L.G07256 in *Arabidopsis* resulted in increased sensitivity to ABA when stomata were closed and stronger drought tolerance compared with the WT [36]. Similarly, our results showed that *OsWRKY97* expression was rapidly activated under drought stress, suggesting that *OsWRKY97* may play an important role in drought stress. Therefore, we constructed OE transgenic plants and tested their resistance to drought stress. The results showed that the germination rate of OE at the germination stage was significantly higher, and the growth inhibition of *OsWRKY97* at the seedling stage was also weakened compared with that of WT plants under osmotic stress (Fig. 3). These results suggest that *OsWRKY97* may positively regulate drought stress tolerance. In addition, this conclusion was supported by the result that the survival rate of OE plants was higher than that of WT plants under drought conditions (Fig. 4A,B).

Plants respond to water loss at physiological, cellular and molecular levels[37]. ABA is an important plant hormone involved in plant developmental process, which is widely considered as the main regulatory factor of plant response to drought. ABA reduces water loss by inducing stomatal closure and induces a number of stress response genes[38, 39]. It has been reported that ABA-independent and ABA-dependent regulatory systems both exist in response to drought stress[40]. In this study, First, we found that OsWRKY97 was strongly induced by exogenous ABA (Fig. 1B). Second, the sensitivities of OE plants to exogenous ABA was enhanced (Fig. 6C). Third, the expression level of OsWRKY97 induces ABA accumulation and the expression level of ABA responsive gene under drought stress (Fig. 6A,D), this result was consistent with the capability of OsWRKY97 to reduce the water loss rate of plants under drought conditions (Fig. 4C). These results intimate that OsWRKY97 improves drought tolerance by enhancing water retention of rice through ABA-dependent pathway. Meanwhile the up-regulation of OsWRKY97 expression under drought stress will lead to the up-regulation of ABA biosynthesis and response genes, resulting in ABA accumulation and increased sensitivity to exogenous ABA.

H₂O₂ is not only an important ROS but also the pivot for the mutual conversion of ROS, which is also an important signal molecule at normal levels[27]. ABA signal interacts with H₂O₂ in plant tissues. There is evidence that H₂O₂ acts upstream of ABA signaling pathway. Exogenous H₂O₂ increases ABA catabolism during seed germination by enhancing the expression of CYP707A genes [41, 42]. It has also been reported that H₂O₂ plays an important role as a second messenger in ABA-induced stomatal closure in guard cells[8, 9]. It is reported that H₂O₂ can be induced by ABA, which is mediated by inducing plant gene expression encoding NADPH oxidase to respond to ABA[43, 44]. However, the increase of H₂O₂ content induced by drought stress is more obvious than that caused by exogenous ABA, this shows that the water deficit signal enhances the production of ROS to a greater extent, which will pose a threat to plants[45]. As expected, the level of H₂O₂ in plants has increased under drought stress. However, the accumulation of H₂O₂ in WT plants was significantly higher than that in OE plants (Fig. 5A), and the activities of major oxygen scavenging enzymes, including SOD, POD, CAT, were also observably higher than those in OE plants (Fig. 5B-D). SOD, POD and CAT, which maintain the ROS homeostasis, were activated in OE plants.

As a product of ROS-stimulated lipid peroxidation, MDA contents can be used to evaluate the extent of ROS-mediated injuries in plants. In our research, we found that ROS in WT plants accumulated so much that MDA content increased correspondingly compared with OE plants under drought stress (Fig. 5E). In addition, we also found that drought stress led to the accumulation of osmotic compound, proline, in OE plants was more than WT plants (Fig. 5F). Under osmotic conditions, proline, as an important osmotic protective agent, can maintain low water potential of cells so as not to be damaged by active enzymes[46]. These results showed that WT was more seriously damaged than OE plants under osmotic conditions, thus increasing the probability of WT death.

Conclusions

In conclusion, we identified OsWRKY97, a transcription factor located in the nucleus. OsWRKY97 affects the sensitivity of rice to exogenous ABA and the accumulation of ABA content. In addition, OsWRKY97 affects the redox balance and drought resistance of rice. Redox related mechanisms might be involved in OsWRKY97 mediated drought tolerance, which might affect the content of proline and MDA in rice. All these results indicated that OsWRKY97 gene has a high potential in improving rice drought resistance.

Materials And Methods

Plant material and growth conditions

Japonica rice (*Oryza sativa* L. *japonica*. cv. Nipponbare) was selected as the WT in this study. Rice seeds were sterilized with 0.1% NaClO for 30 min before being soaked in distilled water for 2 days in the dark and were then transferred to a culture dish containing *Hoagland* nutrient solution and grown in a climate chamber (Southeast instrument, Ningbo, China) with the temperature of 28°C, the relative humidity of 70%, and the 14-hour light/10-hour dark photoperiod[47].

Abiotic treatments

To determine the expression pattern of *OsWRKY97* under different stress conditions, two-week-old seedlings of Nipponbare rice were subjected to various stress treatments. For drought treatment, seedlings were grown in culture solution containing 20% PEG6000. For salt treatment, NaCl solution was added to achieve a final concentration of 250 mM. ABA treatment was carried out by adding 50 μ M ABA to the culture solution. The seedlings were transferred to a 4°C climate chamber for cold treatment. For heat stress, the seedlings were subjected to 42°C heat shock treatment[48, 49]. Samples were collected at 0, 1, 2, 4, 8, 12, 16 and 24 h after treatment. Two-week-old seedlings of *OsWRKY97* overexpression lines were subjected to drought treatment, and samples were collected at 0h and 10h after treatment respectively.

RNA extraction and real-time PCR

The leaves of 14-day-old plants were sampled, and total RNA was extracted with TRIzol reagent (Invitrogen, Nanjing, China) according to the manufacturer's instructions. One microgram of DNase-treated RNA was reverse-transcribed using a RevertAid RT Reverse Transcription Kit (TaKaRa, Beijing, China) according to the manufacturer's protocol. Real-time PCR was performed on a Bio-Rad CFX96 real-time PCR system. Each reaction was performed in triplicate, and the reaction procedure was as follows: 95°C for 10 min and, then 39 cycles of 95°C for 10s and, 60°C for 30s. The data of relative expression level were analyzed by the $2^{-\Delta\Delta C_t}$ method[50]. The *OsActin* rice gene was used as an internal control gene, and relevant primer pairs are listed in Additional file 2:Table S1.

Vector construction and gene transformation

To construct the overexpression vector of *OsWRKY97*, cDNA from rice (*Oryza sativa* L. *japonica*. cv. Nipponbare) was used. Total RNA was used to amplify the open reading frame of *OsWRKY97*, and the relevant primer pairs are listed in Additional file 2:Table S1. The fragments confirmed by sequencing were digested and cloned into the pCambia1300 vector, and the digestion sites were *HindIII* and *Sall*. This gene was driven by the CaMV35S promoter (Additional file 3:Figure S2). All the vectors were transferred into Nipponbare plants via *Agrobacterium*-mediated transformation[51].

Determination of stress-associated physiological indicators

Seedlings growing under normal conditions for two weeks were transferred to a solution with or without 20% PEG6000. Samples were collected at 0 h and 10 h, and then, the corresponding physiological indexes were measured. MDA content was qualified by the reaction method as described by Gao[52]. H_2O_2 content was determined according to the method of Zhan[53]. For the water loss rate, two-week-old seedlings were selected, exposed to air under the condition of no water supply and weighed and recorded every half hour until the weight was constant. Water loss rate was calculated by comparing the measured weight from each indicated time with the measurement at time zero. It is expressed as percentage of initial fresh weight[54]. Proline was detected by following the reported methods[55]. The activity of active oxygen scavenging enzymes was determined according to previous methods[56].

Measurement of ABA content

We analyzed the content of ABA in rice by liquid chromatography-mass spectrometry (LC-MS)[57]. Briefly, 1 g of frozen leaf tissue was extracted in 10 ml of acetone/water/acetic acid (80:19:1, v/v). The complete homogenate was incubated overnight in darkness at 4°C. After that, they were vortexed and centrifuged at 15,000 rpm, 4°C for 10 minutes, and the crude extract supernatant was collected and dried in a rotavapor until an aqueous fractions were obtained. Dried samples were resuspended in 1 mL of acetonitrile/water/acetic acid (90:10:0.05, v/v), and filtered through a 0.45 µm PTFE filter (JET BIOFIL, Guangzhou, China). Quantification was done by the standard addition method by spiking control plant samples with ABA solutions.

Subcellular localization of *OsWRKY97*

The coding region of *OsWRKY97* was amplified and cloned into the pAcGFP1-N1 vector to generate the *OsWRKY97*-GFP (green fluorescent protein) fusion construct, which was inserted into the pCambia1300 vector, and the constructed vector was transferred into *Agrobacterium*. After sequencing confirmation, the fusion structure and the control (empty body) vector were co-transfected with another NLS-RFP vector respectively into tobacco cells by *Agrobacterium* mediated transient expression method, and the fluorescence signal was observed by confocal laser scanning microscope[58].

Analysis of stress tolerance

Two independent T2 over-expression lines and WT seeds were soaked in clear water for two days after being sterilized by 0.1% NaClO, and then, the seeds were transferred to culture solution for growth. When the plant height was approximately 1 cm, seedlings with similar growth potential were selected and transferred to nutrient solution containing 20% PEG, and their growth conditions were observed and recorded. To simulate an arid environment, the two-week-old seedlings were transferred to the sand, and watering was stopped after two days of normal irrigation. The mortality rates were determined. In order to test the sensitivity of ABA sensitivity at germination stage, seeds of OE plants and WT plants were germinated on culture solution containing 5 μ M ABA, and the germination rate was calculated on the 6th day after germination.

Statistical Analysis

Results are reported as the mean $\pm$ standard deviation (SD) values of the three independent biological experiments, all experiments were repeated at least three times, Statistical analysis was performed using the Student's t-test by SPSS 18.0 (Chicago, IL) software package, *p < 0.05, **p < 0.01.

Abbreviations

ABA

abscisic acid

WT

wild-type

MDA

malondialdehyde

ROS

reactive oxygen species

SOD

superoxide dismutase

CAT

catalase

POD

peroxidase

OE

OsWRKY97-overexpressing

GFP

green fluorescent protein

NLS-RFP

Nuclear Localization Signal - red fluorescent protein

PYR

Pyrabactin resistance

PYL

PYR1-like
RCARs
Regulatory component of ABA receptors
PP2C
2C-type protein phosphatase
SnRK2
SNF1-related protein kinase 2
TF
Transcription factor
AREB
ABRE-binding protein
ABRE
ABA-responsive element
qRT-PCR
quantitative real-time RT-PCR

Declarations

Contributions

ML, DH, TY, LZ, JF, JX, CL and LC carried out the experiments. SR, YD, WC, YS, ZH and JZ analyzed the data LL and ZX conceived and designed the research. LL, ML and XG wrote and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data analyzed during this study are included in this published article and its supplementary information files.

Competing interests

We declare that we have no conflict of interest.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

Figure 1

Expression analysis of the *OsWRKY97* gene in rice. (A-E) *OsWRKY97* expression analysis (qRT-PCR) in leaves of 2-week-old rice seedlings subjected to 20% PEG6000, 50 μ M ABA, 250 mM NaCl, cold (4°C) and heat (42°C) treatments, respectively. *OsActin* gene was used as internal controls. The data are represented as the mean $\pm$ SD (n=3), with three biological experiments. Asterisks indicates a significant difference from the value at 0h (T-test, *p < 0.05, **p < 0.01).

Figure 2

Subcellular localization of OsWRKY97-GFP. OsWRKY97 driven by the Cauliflower mosaic virus 35S promoter was transiently expressed in tobacco leaf epidermal cells and viewed with confocal microscopy. Nuclear and cytosolic localization of GFP protein was shown as a control. (A) 35S:OsWRKY97-GFP; (B) 35S: GFP. A vector harboring 35S::RFP::NLS (the RFP red fluorescent protein linked to a nuclear localization signal) was used as nucleus marker in transient co-expression assays. Bar = 50 μ m.

Figure 3

Phenotype of OsWRKY97-overexpressing plants under osmotic stress. (A) Two independent transgenic lines (OE-1, OE-23) of OsWRKY97 were verified by qRT-PCR. OsActin was analyzed as an internal control. Data were means $\pm$ SD with at least three biological replicates. Asterisks represent statistically significant differences between WT and OE lines (T-test, * $p < 0.05$, ** $p < 0.01$). The growth performance (B) and germination rate (C) of OE lines and wild-type at 5 days after germination in nutrient solution with or

without 20% PEG6000. The data are represented as the mean $\pm$ SD with at least three biological replicates and every replicate contains 20 individual plants. The growth phenotype (D) and plant height (E) of overexpression lines and WT, which were growing under normal conditions for 4 days, were transferred to nutrient solution with or without 20% PEG6000 for 12 days. Data are shown as the mean $\pm$ SD (n=4) with four biological replicates and every replicate contains 40 individual plants. The asterisk represents the statistically significant difference between the WT and OE lines under osmotic stress (T-test, *p < 0.05, **p < 0.01).

Figure 4

Drought stress analysis of wild-type plants and transgenic plants with OsWRKY97. (A) Performance of 2-week-old seedlings from OsWRKY97 transgenic and wild-type plants subjected to drought stress without water for 15 days and then recovered for 3 days. The experiment contained three biological replicates. (B) Survival rates of transgenic and WT plants under drought stress. Values represent the mean $\pm$ SD (n = 3) from three independent biological experiments and every replicate contains 12 individual plants. Statistically significant differences between WT and OE lines were indicated by asterisks (T-test, *p < 0.05, **p < 0.01). (C) Rate of water loss by detached leaves from control and transgenic plants. It is expressed as percentage of initial fresh weight. Values are the mean $\pm$ SD (n = 3) from three independent

biological experiments and every replicate contains 5 individual plants. The asterisk represents the statistically significant difference at the same time between the WT and OE lines (T-test, * $p < 0.05$, ** $p < 0.01$).

Figure 5

Physiological parameters of OE plants under Osmotic Stress. Two-week-old rice was transferred to nutrient solution with or without 20% PEG6000 (A) H₂O₂ content (B-D) CAT activity, POD activity, SOD activity. (E) MDA content (F) proline content. Values are shown as the mean ± SD (n=3) and the experiments were performed with at least three biological repetitions. The asterisk represents the statistically significant difference between the WT and OE lines under osmotic stress (T-test, * $p < 0.05$, ** $p < 0.01$).

Figure 6

ABA accumulation and sensitivity of OE plants. The growth performance (A) and The Germination rates (B) of OE and WT seeds under ABA treatment. Values are shown as the mean $\pm$ SD from three biological experiments, with 20 plants in each repeat. The asterisk represents the statistically significant difference between the WT and OE lines under exogenous ABA stress (T-test, * $p < 0.05$, ** $p < 0.01$). (C) ABA contents of OE and WT plants under normal and drought stress conditions. Results are means $\pm$ SD from three independent biological experiments. (D) Real-time PCR analysis of the expression of ABA biosynthesis and responsive genes under normal and drought stress conditions. The data are represented as the mean $\pm$ SD, with three biological experiments. The asterisk represents the statistically significant difference between the WT and OE lines under osmotic stress (T-test, * $p < 0.05$, ** $p < 0.01$).

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