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ABA-induced CCCH tandem zinc finger protein OsC3H47 decreases ABA sensitivity and promotes drought tolerance in *Oryza sativa*

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ABSTRACT

Water deficit causes multiple negative impacts on plants, such as reactive oxygen species (ROS) accumulation, abscisic acid (ABA) induction, stomatal closure, and decreased photosynthesis. Here, we characterized *OsC3H47*, which belongs to CCCH zinc-finger families, as a drought-stress response gene. It can be strongly induced by NaCl, PEG, ABA, and drought conditions. Overexpression of *OsC3H47* significantly enhanced tolerance to drought and salt stresses in rice seedlings, which indicates that *OsC3H47* plays important roles in post-stress recovery. However, overexpression of *OsC3H47* reduced the ABA sensitivity of rice seedlings. This suggests that *OsC3H47* is a newly discovered gene that can control rice drought-stress response, and it may play an important role in ABA feedback and post-transcription processes.

1. Introduction

Zinc-finger proteins are the most common DNA-binding protein in plants, animals, and bacteria [1]. Zinc-finger proteins consist of diversified types of Cys–Cys (CC) or Cys–His (CH) motifs, which give them nucleic-acid-binding abilities. Furthermore, the CCCH zinc-finger domain is able to induce protein DNA-specific recognition and single-strand RNA binding abilities [2–4]. Recent studies demonstrated that tandem CCCH proteins can traffic between the nucleus and cytoplasmic foci, and they mainly function as post-transcript regulators in mammalian cells [5]. However, plants, unlike in animals, have much larger tandem CCCH gene families. Proteins that contain multiple CCCH motifs have been reported as ubiquitously involved in multiple critical processes in plants, such as organ development, growth regulation, and stress responses [6–9].

Rice is an essential economic and food resource crop in East and South Asia. Water deficit has severe impacts on yield, and every year, droughts lead to massive yield losses worldwide [10]. Thus, identifying drought-resistance genes is urgent to overcome major yield losses. Genome-wide analysis revealed that rice has 67 CCCH family genes divided into 8 subfamilies. It has 13 pairs of duplicated genes, meaning that those genes are present before putative rice genome duplication events, and most genes have close homologs in *Arabidopsis* [11].

In rice, several CCCH genes have already been characterized. *OsGZF1* can directly bind to the promoter of Glub-1 and control glutelin accumulation during grain development [12]. Previously, *OsTZF1* was characterized as a senescence control gene whose overexpression can delay plant senescence and seed germination. However, *OsTZF1* can also be induced by drought, high salt stress, and ABA. Further investigation showed *OsTZF1* binds to 3′ untranslated region (UTR) of mRNA, which means the plant tandem CCCH protein may act similar to animal proteins and might play a crucial role in RNA metabolism of target genes [13]. In addition, OsDOS has also been reported as a leaf senescence-delaying factor associated with the jasmonic acid (JA) pathway, but the detailed mechanism remains unknown [14].

In order to better understand the role played by CCCH finger proteins in drought-stress and ABA responses, we investigated 10 rice CCCH genes. In this work, we applied NaCl and polyethylene glycol (PEG) as drought stresses to screen for the gene, which is responsible for the stress response. Our results demonstrated that *OsC3H47* enhanced drought resistance through its elevated sensitivity to ABA.

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2. Material and methods

2.1. Plant growth and stress treatments

Rice (*Oryza sativa* ssp. *japonica* 'Nipponbare') was used in the various experiments in this study. Rice seedlings were grown at 28 °C with 16/8 h light/dark cycles, 80% illumination, and 75% relative humidity in a greenhouse. For expression studies of 10 CCCH genes in response to various treatments, 2-week-old seedlings were transferred to Yoshida solution supplemented with 200 mM NaCl, 10 μM ABA, and 20% PEG4000. Seedlings grown in the same liquid medium without any supplementary component were used as controls.

Seeds of T2 *OsC3H47*-overexpressing plants and wild type (WT) were first germinated on double filter paper in Petri plates, followed by the transfer of uniformly germinated seeds to a 96-well plate from which the bottom was removed. For drought-resistance testing of transgenic rice at the seedling stage, 20-d-old seedlings were transferred to culture solution containing 200 mM NaCl. The survival rates were calculated after rewatering for 10 days. For PEG treatment, 20-d-old seedlings were transferred to culture solution containing 20% (w/v) PEG4000. The survival rates were calculated after rewatering for 15 days.

2.2. Vector construction and rice transformation

To generate *OsC3H47* overexpression plants, the full-length of *OsC3H47* cDNA using the primer sets 5′-GCTCTA-GAATGGCCGATCCCAACGGCC-3′ and 5′-CGGGGTACCCTA-TACGCCGCCATGGCA-3′was cloned into 1300-actin:NOS (nopaline synthase terminator) to produce the overexpression vector. The resultant vector was introduced into *Agrobacterium tumefaciens* strain EHA105, which was used to infect rice embryogenic calli from Nipponbare. Transgenic plants were screened by PCR amplification with the hygromycin B phosphotransferase gene (*Hpt*). All primers used in this study are listed in Table 1.

2.3. RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR (qRT-PCR)

Total RNAs were extracted from fresh shoots of the WT plant under normal and stress conditions using TRIzol reagent (Invitrogen). Reverse transcription (RT) was performed using Super-Script III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time RT-PCR analysis was conducted with the Lightcycler 480 machine using SYBR Green I (TAKARA). *UBIQUITIN* (*Os03g0234200*) mRNA was used as an internal control. The specific primers for quantitative real-time RT-PCR are listed in Table 1.

2.4. ABA treatment

To compare the difference in ABA sensitivity between WT and *OsC3H47*-overexpressing plants, rice seedlings were grown at 28 °C with 16/8 h light/dark cycles, 80% illumination, and 75% relative humidity in a greenhouse. One-week-old seedlings with a consistent shoot height (0.8 cm) were selected and then transferred to fresh culture solutions with different final concentrations of ABA. The shoot lengths of 20 rice seedlings were then measured.

3. Results and discussion

3.1. Expression of 10 CCCH genes under various drought-related conditions

Drought stress can lead to various physical and biochemical responses in plants, such as reactive oxygen species (ROS) accumulation, ABA induction, stomatal closure, and decreased photosynthesis [15]. However, the ABA biosynthetic and signaling pathway is the center of the integrating network of water-deficit signaling. After plants encounter drought stresses, ABA biosynthesis can be rapidly induced [16]. Exogenous ABA treatment can produce high CDPK activity that makes plants more tolerance to drought stresses [17]. In order to identify the drought stresses responsive to this gene, we performed a small-scale screen for the genes most closely related to drought-stress responses by following the protocol described earlier [18].

In this study, 10 CCCH genes were chosen for a drought-related induction assay. All of these 10 genes contain at least one CCCH zinc finger motif. Most of them are tandem CCCH zinc finger proteins, and they are distributed among chromosomes 1, 3, 4, 5, 7, and 9.

Real-time qPCR assay shows expression of most candidate genes was changed under different treatments. Among them, four genes (*OsC3H2*, *10*, *47*, and *61*) were induced by drought, five genes (*OsC3H2*, *10*, *32*, *35*, and *47*) were induced by salinity stress, four genes (*OsC3H2*, *3*, *47*, and *61*) were induced by ABA, and two genes (*OsC3H24 and 61*) were induced by PEG treatments. However, only one gene (*OsC3H47*) was detected as overexpressed under drought, salt, ABA, and PEG treatment. Other genes did not show the same pattern. As shown in Fig. 1, *OsC3H47* expression is 13-fold higher in water-deficit conditions than in well-watered conditions, 7-fold higher in salt conditions, 3-fold higher with extra ABA treatment, and 1.7-fold higher with PEG treatment. The overexpression of *OsC3H47* gradually decreased with intensity of stresses, and it showed overexpression in response to both in drought and exogenous ABA treatment. These results indicate that *OsC3H47* is a drought-stress responsive gene, and it is involved in the ABA-induced signaling pathway.

Table 1
The 10 CCCH genes and primers used for real-time qPCR assay.

Gene name	LOC no.	Exons	Chr.	Number of CCCH motifs	Primers for qRT-PCR	
					Forward primer (5′–3′)	Reverse primer (5′–3′)
OsC3H2	LOC_Os01g09620	1	1	2	CTCCAGCTTCATGCCCAACC	GCTCAGTCGCTCGAACACCTTC
OsC3H10	LOC_Os01g53650	1	1	2	TGCTTCTTCGCCCACAACG	TCGCCTTCCTCGCATCCA
OsC3H24	LOC_Os03g49170	1	3	2	GCTTGATGCCTGGTTCTC	TGCCTGGAAGGTGTAGTGT
OsC3H29	LOC_Os04g41060	2	4	1	GCAGACGGGAAGAAGATTGA	TCGCACCTTGAACTCGTTACAT
OsC3H32	LOC_Os05g57600	1	5	3	GACCCTCGCCGCTACTCCTACA	GGAGCCAGCACTCGAACACG
OsC3H35	LOC_Os05g10670	1	5	2	AGCTGGAAGAGGACGCTCCG	CGTGATCCGCTGTGGTGAAGA
OsC3H37	LOC_Os05g45020	1	5	2	AAGGAAGACTCGCCGCTGTCG	CCCGCATTGCGAACATCAACT
OsC3H47	LOC_Os07g04580	1	7	2	GTTGCCAATGCCCACCACA	GCACAATTCGTTGCTCCTCCTC
OsC3H50	LOC_Os07g38090	2	7	2	CTCCGAGGTTCCCTAATGAC	AATGGAGAATGTGCCAAAGAT
OsC3H61	LOC_Os09g36090	1	9	1	CAAGGCCACCGTGCTCAACC	GCTCATGCTCCGCAGCGTCT

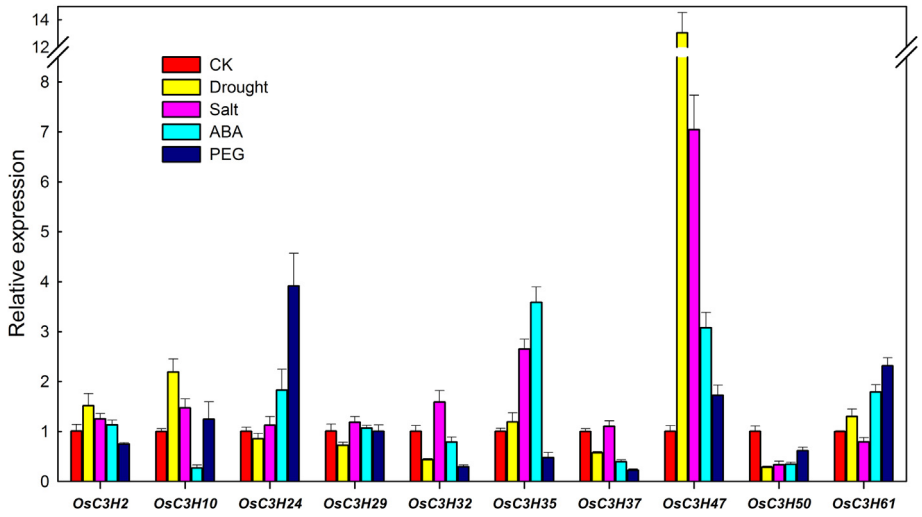

Fig. 1. Expression of 10 CCCH genes in seedlings under hormone and stress treatments, including ABA, PEG, salt, and drought. 5 of 2-weeks-old seedling was tested in each treatment, each treatment had 3 replicates, error bars means standard deviation.

3.2. OsC3H47 overexpression makes plants more tolerant to salt stresses

For further research on the role *OsC3H47* plays in the drought response of rice, *OsC3H47* overexpression transgenic lines were generated. In the overexpression construct p1300-ActinI:*OsC3H47*:NOS, *OsC3H47* was under the control of a constitutive promoter (rice actin 1) and NOS terminator (Fig. 2A). A total of 21 independent transgenic lines were identified carrying the p1300-ActinI:*OsC3H47*:NOS construct, and the *OsC3H47* expression of T6 and T17 lines was detected as 3.12- and 7.78-fold-higher than in WT plants, respectively (Data not shown). Thus, we chose the T6 and T17 lines for further investigation.

Transgenic seeds of two *OsC3H47*-overexpressed T1 generations were germinated on 1/2 MS medium containing hygromycin and then transferred into Yoshida solution with final concentrations of 200 mM NaCl for 12 days for salt tolerance testing. The transgenic and WT seedlings that showed the same growth vigor under

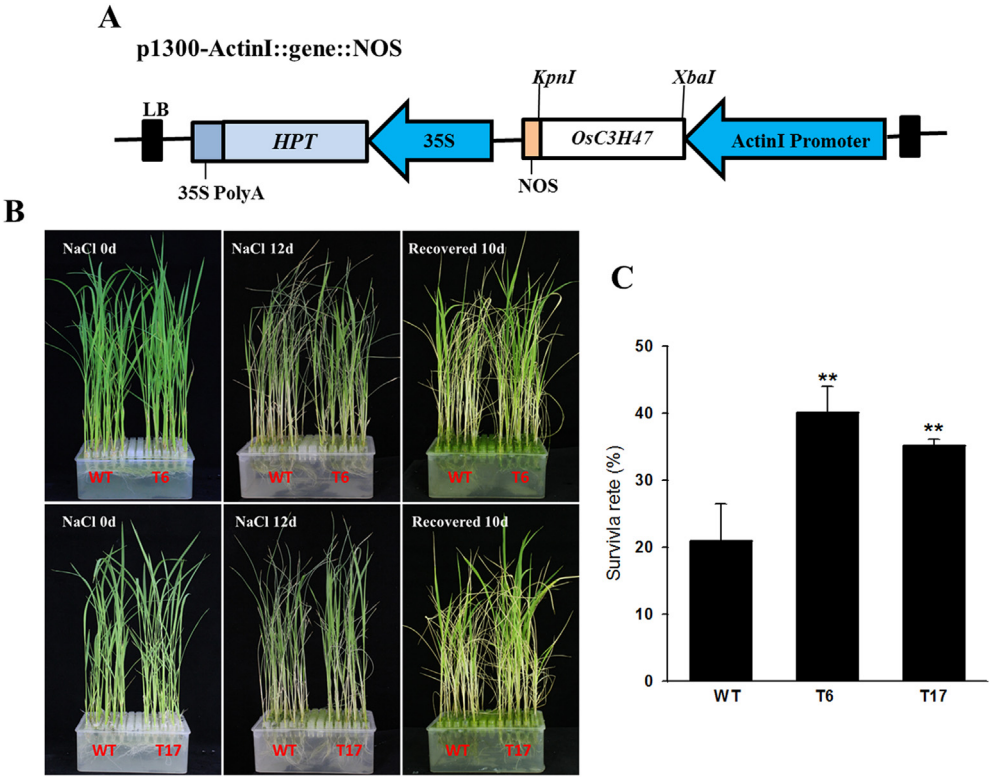

Fig. 2. The *OsC3H47*-overexpressing plants show tolerance to salt. (A) For production of overexpression vectors, the fragment was digested with *Xba*I and *Kpn*I and cloned into 1300-actin:NOS, (B) NaCl treatment of wild-type (WT) and transgenic lines. Twenty-day-old plants were treated with 200 mM for 12 days and then allowed to recover for 10 days., (C) Survival rate of transgenic lines and the wild type. Error bars are based on three replicates.

normal conditions were used in the salt tolerance assay. Twelve days of NaCl treatment apparently inhibited growth, rolled leaf, and accelerated leaf senescence in both plants. However, after 10 days of recovery, more seedlings of T6 and T17 lines than of WT survived after exposure to salt stresses for 12 days (Fig. 2B). Compared to the 20.97% survival of WT plants, 40.14% and 35.19% survival rates were noted for T6 and T17 plants, respectively (Fig. 2C), which means the overexpression of *OsC3H47* in rice plants renders more tolerance to salt stresses and plays an important role in post-stress recovery (*t* test, $P < 0.01$).

3.3. *OsC3H47 overexpression makes plants tolerant to PEG-induced drought stresses*

PEG had been widely used as a drought-stress stimulus in drought-stress studies [19]. We performed a PEG-induced drought assay on T6, T17, and WT seedlings. Transgenic seeds of two *OsC3H47*-overexpressed T1 generations were germinated on 1/2 MS medium containing hygromycin and then transferred into Yoshida solution with final concentrations of 20% (w/v) PEG4000 for 10 days for the PEG-induced drought assay.

The transgenic and WT seedlings that showed the same growth vigor under normal conditions were used in the PEG tolerance assay. Ten days of PEG treatment apparently inhibited growth, rolled leaf, and accelerated leaf senescence in both plants. However, after 15 days of recovery, more seedlings of T6 and T17 than of WT survived after exposure to drought stresses for 10 days (Fig. 3A). Compared with the 21.3% survival rate of WT plants, the survival rates in T6 and T17 plants were 59% and 63%, respectively (Fig. 3B), which means overexpression of *OsC3H47* makes rice plants more tolerant to PEG-induced stresses.

3.4. *OsC3H47 overexpression makes plants less sensitive to ABA*

As shown in Fig. 1, ABA treatment strongly induces expression of *OsC3H47*, and so, we hypothesize that this gene may be involved in an ABA-dependent pathway. In order to check the responses to ABA in plants with *OsC3H47* overexpression, transgenic and WT seedlings were treated with exogenous ABA. After treatment with different ABA concentrations, both the transgenic and WT plants showed growth inhibition, and the inhibition level increased with increasing ABA concentrations (Fig. 4A). However, the data indicated that the seedling length of the T17 transgenic lines is slightly longer than that of the WT. Under normal conditions, the T17 and WT seedlings showed no significant difference, but under 2, 5, and 8 μM ABA treatments, the T17 seedlings were, respectively, 16%, 12%, and 38% longer than the WT seedlings (Fig. 4B). These data indicate that overexpression of *OsC3H47* decreased the ABA sensitivity of rice seedlings. *OsC3H47* may participate in drought-stress response and after-stress recovery through an ABA-independent pathway.

In summary, we chose 10 CCCH genes for drought-stress screening. *OsC3H47* expression was found to be strongly induced by salt, PEG, drought, and ABA treatment (Fig. 1). We generated transgenic overexpression lines for further investigation, and T6 and T17 lines were chosen as the highest two overexpression lines for salt and drought tolerance assays. Plants with *OsC3H47* overexpression showed much more salt and drought tolerance than that by the WT seedlings (Figs. 2 and 3), but they also showed decreased ABA sensitivity compared to the WT seedlings (Fig. 4).

ABA has been widely reported as an important phytohormone integrating multiple abiotic stresses, such as salt, drought, and cold [20]. ABA biosynthesis was rapidly induced after stress, and it controls many plant adaptation responses to water deficit conditions [16]. The ABA signaling pathway begins with PYR1/ABA/PP2C complexes, which activate SnRK2 and CDPKs kinases [21,22]. Some of CDPKs were reported as drought tolerance enhancers, and most of their target genes were found as bZIP transcript factors [17,23]. *OsC3H47* has two CCCH zinc-finger motifs and belongs to the plant tandem zinc-finger (TZF) family. In rice, many TZF genes have already been characterized, and many of them were reported involved in seed development and abiotic stresses response. Many of them were reported to have RNA-binding ability, showing that plant TZFs, similar to their many animal homologous genes, are involved in post-transcript regulation and mRNA metabolism. Our results show that *OsC3H47* can be strongly induced by ABA

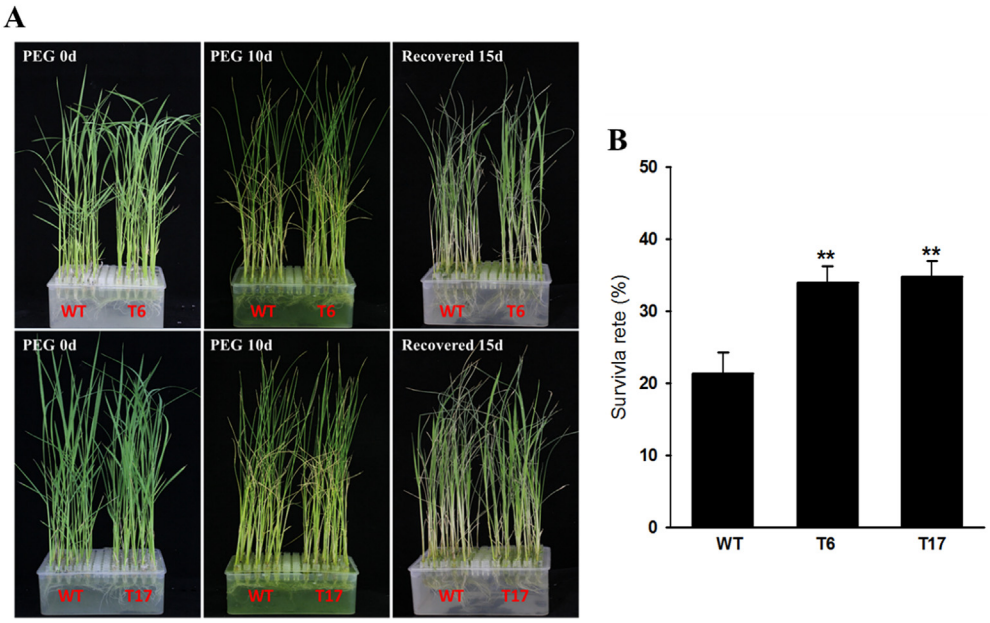

Fig. 3. Identification and drought-tolerance testing of *OsC3H47*-overexpressing plants. (A) PEG4000 treatment of wild-type (WT) and transgenic lines. Twenty-day-old plants were treated with 20% PEG4000 for 10 days and then allowed to recover for 15 days., (C) Survival rate of transgenic lines and the wild type. Error bars are based on three replicates.

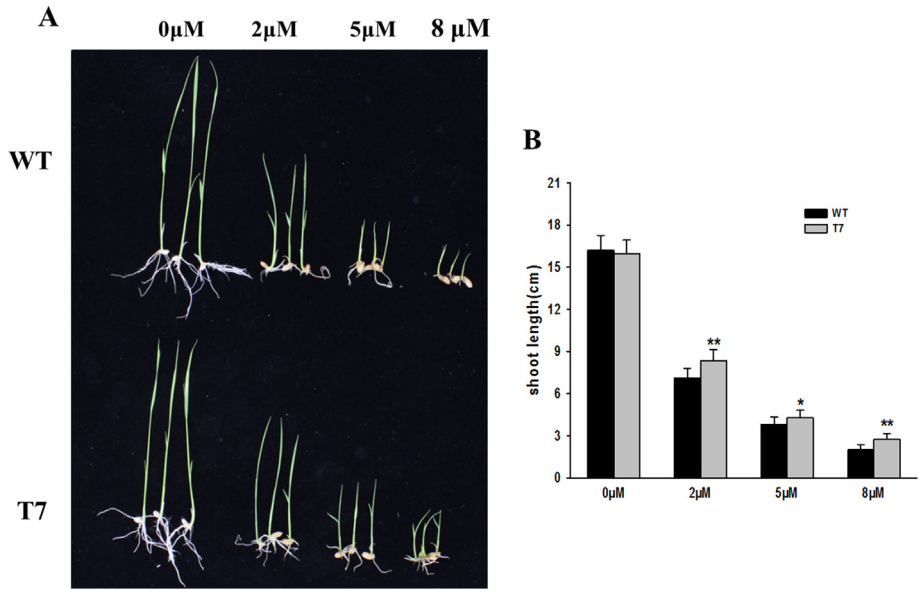

Fig. 4. Increased ABA sensitivity of *OsC3H47*-overexpressing plants at seedling stages., (A) The phenotype analysis of rice seedlings grown in culture solutions containing 0, 2, 5, and 8 μM ABA., (B) Response of *OsC3H47*-overexpressing plants and wild-type (WT) seedlings to different concentrations of ABA. Error bars indicate the SD ($n = 20$), $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ (Student's *t*-test).

treatment, but its overexpression decreases the ABA sensitivity of rice seedlings. This means it is located downstream of the ABA signaling pathway, and it may play an important role in ABA signaling feedback and post-transcript regulation.

Conflict of interest

The authors declare that there is no conflict of interest.

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