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ORIGINAL ARTICLE

OsbZIP42 is a positive regulator of ABA signaling and confers drought tolerance to rice

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Abstract

Main conclusion **OsbZIP42 is a positive regulator of ABA signaling and drought stress tolerance. The activation of OsbZIP42 depends on stress-/ABA-activated protein kinase 4 (SAPK4) and an additional ABA-dependent modification of OsbZIP42.**

Basic leucine zipper transcription factors (bZIP TFs) play important roles in the ABA signaling pathway in plants. Rice OsbZIP42 is a member of the group E bZIP, which is an ortholog of *Arabidopsis* group A bZIP. This latter group includes abscisic acid-responsive element (ABRE)-binding factors (ABFs) involved in abiotic stress tolerance. The expression of *OsbZIP42* was induced by ABA treatment, although it was not induced by drought and salt stresses. Unlike other bZIP TFs, OsbZIP42 contained two transcriptional activation domains. Although the full-length OsbZIP42 protein did not, the N-terminus of the protein interacted with SAPK4. Our results suggest that the activation of OsbZIP42 by SAPK4 requires another ABA-dependent modification of OsbZIP42. Transgenic rice overexpressing *OsbZIP42* (OsbZIP42-OX) exhibited a rapidly elevated expression of the ABA-responsive *LEA3* and *Rab16* genes and was hypersensitive to ABA. Analyses of the OsbZIP42-OX plants revealed enhanced tolerance to drought stress. These results suggest that OsbZIP42 is a positive regulator of ABA signaling and drought stress tolerance depending on its activation, which is followed by an additional ABA-dependent modification. We propose that OsbZIP42 is an important player in rice for conferring ABA-dependent drought tolerance.

Keywords Abiotic stress · Abscisic acid · Basic leucine zipper transcription factor · *Oryza sativa*

Abbreviations

SAPK	Stress-/ABA-activated protein kinase
SnRK2	SNF1-related type 2 protein kinase
TF	Transcription factor

Introduction

Abiotic and biotic stresses seriously affect crop yield. Abiotic stresses particularly cause an average yield loss of more than 50% in most major crop plants (Fujita et al. 2006). In particular, rice (*Oryza sativa* L.) production has been significantly affected by drought stress (Tuong and Bouman 2003). Rice is a notoriously drought-susceptible crop due in part to its small root system (Hirasawa 1999). Thus, understanding drought stress responses in rice is important for improving yield and quality. The ABA signal transduction pathway plays an important role in abiotic stress responses that modulate stress-responsive gene expression (Shinozaki and Yamaguchi-Shinozaki 1997; Verslues and Zhu 2005). In *Arabidopsis*, the ABA signaling model has been well established. The ABA and receptor (PYR/PYL/RCAR) complex inhibits type 2C protein phosphatases (PP2C), which dephosphorylate/inactivate SNF1-related protein kinase 2 (SnRK2) (Umezawa et al.

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2009; Vlad et al. 2009; Bhaskara et al. 2012). This deactivation leads to the activation of SnRK2, which phosphorylates/activates bZIP transcription factors (TFs; Schütze et al. 2008; Cutler et al. 2010). This type of posttranslational modification is one of the mechanisms that regulate the activities of bZIP TFs. bZIP TFs are defined by a conserved bZIP domain (Landschulz et al. 1988) and are involved in numerous response pathways in plants, such as light signaling, abiotic stress responses, biotic stress defense, seed maturation and flower development (Jakoby et al. 2002; Smeekens et al. 2010; Zg et al. 2014). bZIP TFs are present in all eukaryotes and are expressed by multiple genes. *Arabidopsis* encodes approximately 75 bZIP TFs (Jakoby et al. 2002), and rice encodes approximately 89 bZIP TFs (Nijhawan et al. 2008). The bZIP family is subdivided into ten groups (A–I plus S in *Arabidopsis* and A–J in rice) based on sequence similarity and conserved motifs.

bZIP TFs are involved in responses to ABA signaling and play important roles in ABA-dependent stress responses (Umezawa et al. 2010; Llorca et al. 2014). The *Arabidopsis* group A bZIP TFs include ABF1, ABF2/AREB1, ABF3, ABF4/AREB2, ABI5, GBF4, DPBF2, and DPBF4 (Jakoby et al. 2002). ABF1–ABF4 and ABI5 are expressed in vegetative tissues, and their expression is induced by various abiotic stresses (Choi et al. 2000; Finkelstein and Lynch 2000; Uno et al. 2000; Kang et al. 2002; Fujita et al. 2005). They play important roles in cooperatively regulating ABRE-dependent ABA signaling involved in abiotic stress tolerance (Uno et al. 2000; Kang et al. 2002; Yoshida et al. 2010). Furthermore, ABF2/AREB1, ABF4/AREB2, and ABF3 are the key TFs for ABA-dependent stress responses (Yoshida et al. 2010). These TFs are highly homologous to the members of rice group E bZIP TFs (Xiang et al. 2008; Lu et al. 2009). Members of rice group E, such as *OsbZIP10*/*OsABI5* (Zou et al. 2008), *OsbZIP12* (Amir Hossain et al. 2010; Joo et al. 2014), *OsbZIP23* (Xiang et al. 2008), *OsbZIP46* (Tang et al. 2012), *OsbZIP66*/*TRAB1* (Hobo et al. 1999), and *OsbZIP72* (Lu et al. 2009), are also involved in ABA signal transduction and abiotic stress responses. In addition, the members of rice group E bZIP TFs contain motifs 10–14, which contain potential phosphorylation sites (Nijhawan et al. 2008). These motifs have been identified in members of group A bZIP TFs in *Arabidopsis*. The activation of *Arabidopsis* group A bZIP TFs, which are key transcriptional regulators of ABA responses, is known to be regulated by ABA- and abiotic stress-induced protein kinases (Schütze et al. 2008). Rice group E bZIP TRAB1 is also phosphorylated in response to ABA stress. SnRK2 kinase directly phosphorylates TRAB1 in response to ABA (Kobayashi et al. 2005). The phosphorylation of bZIP proteins can modify homo-/heterodimerization and DNA-binding properties. Phosphorylation of bZIP proteins can also modify dimerization specificity (Lee et al. 2010), DNA-binding properties (Deppmann et al. 2003), and subcellular localization (Djamei et al. 2007; Ishida et al. 2008).

In this study, we characterized *OsbZIP42*, which belongs to subgroup II of group E. We determined the functional analysis of *OsbZIP42* by investigating the ABA and sugar sensitivity and the stress tolerance of transgenic plants overexpressing *OsbZIP42*. An analysis of the *cis*-acting elements in the promoter region and an expression profiling analysis were also performed. The results indicate that OsbZIP42 is a positive regulator of ABA signaling and confers ABA-dependent drought stress tolerance to rice.

Materials and methods

Plant materials and growth conditions

Both the transgenic and wild-type (WT) rice plants used in this study were of the *Oryza sativa* subsp. *japonica* cv. Nakdong background (seeds obtained from Professor Ju-kon Kim, Graduate School of International Agricultural Technology and Crop Biotechnology Institute/GreenBio Science and Technology, Seoul National University, Pyeongchang, Korea). Husked seeds were germinated in a half-strength Murashige–Skoog (MS) solid medium in a growth chamber, transplanted into soil in pots and grown in a greenhouse until further use (Joo et al. 2013b). The samples from each rice organ were prepared as described below. Seeds from the transgenic and WT rice were germinated in half-strength MS solid medium in a growth chamber at 28 °C for 3 days in the dark, followed by 3 days in the light. After germination, the seedlings were grown in a greenhouse for 3 weeks to obtain leaves, internodes, and roots for analysis. Panicles were obtained from field-grown rice plants during the pre- and postheading stages. The young panicles were harvested from the sheath, measured and categorized into three groups (P3 3–5 cm, P4 10–15 cm and P5 15–20 cm) based on the length of the panicle. The rice seeds were tagged on the day of pollination (0 DAP) and collected on each DAP from 0 to 29 DAP (S1 0–2 DAP, S2 3–4 DAP, S3 5–10 DAP, S4 11–20 DAP and S5 25–29 DAP).

Quantitative real-time PCR analysis

Total RNA was isolated from the rice tissue samples using TRI Reagent® (Molecular Research Center) according to the manufacturer's instructions. For quantitative real-time PCR (qRT-PCR), first-strand cDNA synthesis was performed using oligo$(dT)_{18}$ primers and 5 µg of total RNA as a template according to the manufacturer's instructions (RevertAid™ First-Strand cDNA Synthesis Kit, Fermentas). A one-third dilution of the cDNA synthesis reaction mixture

was used, and 1 µL of the diluted cDNA mixture was added as a template for the subsequent real-time PCR analyses using 2 × Real-time PCR Pre-Mix with Evagreen (SolGent). Thermocycling and fluorescence detection were performed using an Mx3000p real-time PCR machine (Stratagene). qRT-PCR was performed in triplicate, and each experiment was repeated three times. *OsUbi1* was used as a control to normalize the expression data. The primers for the qRT-PCR analyses are listed in Supplementary Table S1.

Promoter analysis

For the promoter analyses, 2-kb segments from the 5′ regulatory regions of the *OsbZIP42* gene were obtained from Oryzabase and scanned for the presence of putative *cis*-acting elements identical or highly similar to the motifs registered in PLACE (http://www.dna.affrc.go.jp/PLACE/). The accession number of OsbZIP42 in NCBI is Os05g0489700.

Transactivation and two-hybrid assays in yeast

A transactivation assay was performed using the vector pDEST32 (Invitrogen) and the yeast strain Mav203 (Invitrogen). The full-length *OsbZIP42* or fragments of *OsbZIP42* were fused in frame with the yeast GAL4 DNA-binding domain in the pDEST32 vector using Gateway LR ClonaseTM II enzyme mix (Invitrogen). A colony-lift filter assay (X-gal assay) was performed according to the manufacturer's manual (Invitrogen). A yeast two-hybrid assay was performed using a ProQuest two-hybrid system (Invitrogen). The coding region of *OsbZIP42* was cloned into the pDEST22 vector, and *SAPK*s were cloned into the pDEST32 vector using Gateway technology (Invitrogen) to generate the bait and prey vectors. The two constructed vectors were cotransformed into the Mav203 yeast strain. The transformed yeast strains were plated on minimal medium–Leu/–Trp and incubated for 4 days, and a colony-lift filter assay (X-gal assay) was performed as described by the manufacturer (Invitrogen). The primers for the plasmid construction are listed in Supplementary Table S1.

Plasmid construction and transformation of rice

The overexpression plasmids contained the *bar* gene under the control of the cauliflower mosaic virus *35S* promoter for herbicide-based selection. The rice *cytochrome c* promoter was used to drive constitutive expression. The coding region of the *OsbZIP42* gene used in this study was PCR-amplified using rice total RNA and a pair of primers containing the *attB* sequence for the Gateway® recombination site. The primers for construction are listed in Supplementary Table S1. The *att*B-PCR products were inserted into pMJ101 through BP and LR recombination reactions according to the manufacturer's instructions (Invitrogen). The plasmid for OsbZIP42-OX was confirmed by a sequencing analysis. The plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 through triparental mating, and embryogenic calli from mature rice seeds were transformed. Callus induction, cocultivation with *A. tumefaciens* and the selection of transformed calli were performed (Joo et al. 2013b).

Abiotic stress treatments and chlorophyll fluorescence measurement

For the abiotic stress treatments, 3-week-old WT plants (grown in a greenhouse) were washed to remove the soil from their roots and transferred to a growth chamber (16-h light/8-h dark cycle at 28 °C) for water adaptation for 3 days. After adaptation, the plants were treated with a solution containing 100 µM ABA, 250 mM NaCl, 100 µM gibberellin (GA), 100 mM glucose (Glc) or 100 mM sucrose (Suc) or were air-dried (Dry). The samples were obtained at the indicated times. To test the ABA sensitivity of the transgenic plants at the germination stage, the transgenic lines and the WT seeds were germinated in half-strength MS medium containing ABA (0, 2, and 6 µM), and the germination rate of the treated seeds was calculated after 5 days. To evaluate the responses of the OsbZIP42-OX plants to abiotic stress during germination, the seeds of WT and transgenic plants were germinated in water containing 400 mM mannitol or 250 mM NaCl, and then the germination rates were measured. To carry out the drought-resistance assays, the germinated transgenic OsbZIP42-OX and WT seedlings that were grown in half-strength MS solid medium in a growth chamber were transplanted into soil. Fifteen to twenty seedlings from each transgenic and WT plants were grown in pots (3 × 3 × 5 cm; one plant per pot) for 4 weeks before carrying out the drought stress experiments. To test for resistance to drought stress, 4-week-old WT and transgenic plants (grown in pots) were subjected to 2 days of drought followed by 10 days of watering in a greenhouse. Chlorophyll fluorescence was measured in 4-week-old WT and transgenic plants using a pulse modulation fluorometer (mini-PAM, Walz). For the leaf disc tests, the green portions of approximately ten seedlings were cut using scissors prior to in vitro stress treatments. Under continuous light at 150 µmol m^{-2} s^{-1}, the leaf discs were air dried for 2 h (for drought stress). For cold stress, the leaf discs were incubated in a 4 °C water bath for 5 h under the same light conditions as described above. After the stress treatments, the leaf discs were dark-adapted for 10 min, and the minimal fluorescence level (F_o) was measured. After the application of a saturating light pulse, the maximal fluorescence level (F_m) was measured, and the ratio of F_v to F_m ($F_v/F_m = F_m - F_0/F_m$), which represents the activity of photosystem II, was used to assess functional damage to the plants (Joo et al. 2013a).

Results

Transcriptional activity of OsbZIP42

We studied the molecular function of OsbZIP42 in subgroup II of the group E bZIP TFs. The amino acid sequence of OsbZIP42 was analyzed using an online motif scan tool (http://hits.isb-sib.ch/cgi-bin/PFSCAN), which revealed that it contained the bZIP domain, a nuclear localization signal (NLS), and five conserved phosphorylation sites (Fig. 1a). First, we checked the transactivation activity of OsbZIP42 in yeast. The full-length OsbZIP42 (42-FL) fused to the GAL4 DNA-binding domain had no transactivation activity in yeast (Fig. 1b). To map the transcriptional activation domains within OsbZIP42, we fused the GAL4 DNA-binding domain to the N-terminus or C-terminus with or without domain D. Domain D is a conserved 23-amino acid stretch that is only in the members of group E bZIP TFs (domain 13 in Nijhawan et al. 2008; Tang et al. 2012). A deletion assay of OsbZIP46 indicated that transactivation activity was detected only when domain D was absent. It was proposed that domain D is a negative regulatory domain and has a pivotal role in the regulation of OsbZIP46 activity (Tang et al. 2012). The N-terminus (42-N1, residues 1–128 without domain D) activated the expression of reporter genes, but the 42-N2 (residues 1–164 with domain D) did not. Interestingly, the C-terminus of OsbZIP42 (42-C1, residues 132–335 with domain D) activated the expression of reporter genes, but the 42-C2 (residues 159–335 without domain D) did not. These results show that OsbZIP42 contains two potential transcriptional activation domains.

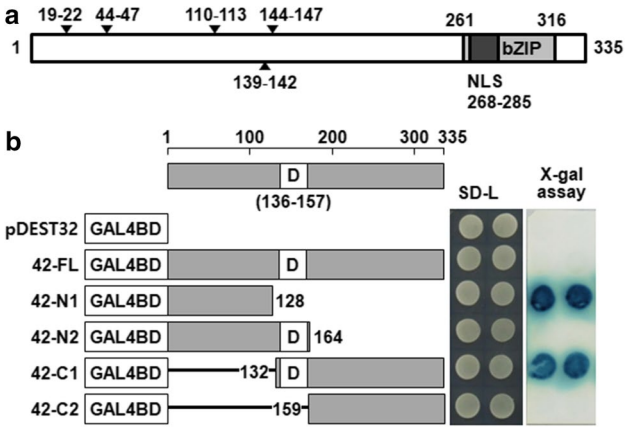

Fig. 1 Gene structures and transactivation activity assay of OsbZIP42. **a** The predicted domains and motifs of OsbZIP42 are shown. bZIP indicates the basic leucine zipper domain. NLS indicates a nuclear localization signal. The five arrowheads represent the conserved phosphorylation sites. The numbers indicate the amino acid position. **b** Fusion proteins of the GAL4 DNA-binding domain and five portions of OsbZIP42 were checked for their transactivation activity in yeast strain Mav203. The left panel displays schematic diagrams of the various constructs used for the transactivation activity assays. The results of a colony-lift filter assay are shown on the right. 42-FL shows the construct inserted with the full-length CDS of OsbZIP42. 42-N1 (1–128), 42-N2 (1–164), 42-C1 (132–335), and 42-C2 (159–335). *3-AT* 3-amino-1,2,4-triazole, *D* domain D (domain 13)

Analysis of the *cis*-acting elements in the 5′ regulatory regions of *OsbZIP42* and the expression profiles of *OsbZIP42*

The characterization of the *cis*-acting region bound by TFs that control gene expression often provides essential information regarding gene function. Thus, we checked the promoter sequence of the *OsbZIP42* gene (1500 bp upstream of the putative transcription start site) using the PLACE database (http://www.dna.affrc.go.jp/PLACE/) (Table 1). The expression of the *OsbZIP42* gene was analyzed following drought, high salinity and ABA treatments using qRT-PCR (Fig. 2). The *OsbZIP42* gene showed the highest expression in spikelets (Fig. 2a), and its promoter region contained two *cis*-acting elements associated with seed expression, the prolamin box and the RY repeat (Table 1). We performed qRT-PCR analysis at various stages of panicle and seed development (Fig. 2b). The transcript levels of *OsbZIP42* showed the highest levels during the early stage and decreased gradually during seed development.

Several functionally significant *cis*-acting elements associated with ABA and stress responses, such as the abscisic acid-responsive element (ABRE), the drought-responsive element (DRE), the GCC box, the MYB recognition site (MYBRS), the MYC recognition site (MYCRS), and the WRKY recognition site (WBOX), were identified in the *OsbZIP42* gene promoter. Under ABA treatment, the transcript level of *OsbZIP42* was increased in the leaves and roots (Fig. 2c, d). Unlike other members of rice subgroup II bZIP TFs (Lu et al. 2009), the expression of *OsbZIP42* was only slightly repressed by drought and high-salinity stress. The *cis*-acting elements associated with sugar responses, such as the sugar-repressive element (SRE), the sucrose-responsive element (SURE), and the TATCCAY motif, were identified in the *OsbZIP42* gene promoter. To verify the regulation of *OsbZIP42* gene expression by GA and sugar, we performed qRT-PCR under exogenous 100 μM GA, 100 mM glucose or 100 mM sucrose treatments (Fig. 3). The expression of *OsbZIP42* was slightly affected by the exogenous GA and sugar treatments.

Yeast two-hybrid assays of OsbZIP42 and SAPK members

The stress-/ABA-activated protein kinases (SAPKs) or SnRK2 has been suggested to phosphorylate ABFs/AREBs

Table 1 Potential *cis*-acting regulatory elements in the promoters of *OsbZIP42* gene

Class	Site name	Sequence	Copy number	Function
Stress	ABRE	MACGYGB	2	Abscisic acid-responsive element
	DRE	ACCGAGA	1	Drought-responsive element
	GCC box	GCCGCC	1	GCC box
	MYBRS	WAACCA	1	MYB recognition site
		CNGTTR	2	
		CCWACC	1	
	MYCRS	CANNTG	4	MYC recognition site
	WBOX	TTGAC	2	WRKY recognition site
		TGACY	5	
Auxin	ARF	TCATCAC	1	Auxin-response factor-binding site
Sugar response	SRE	TTATCC	3	Sugar-repressive element
	SURE	AATAGAAAA	1	Sucrose-responsive element
	TATCCAY motif	TATCCAY	1	Involved in sugar repression
Seed expression	PROLAMINBOX	TGCAAAG	1	Prolamine box
	RYREPEAT	CATGCAY	4	Quantitative seed expression
		CATGCATG	2	

in *Arabidopsis* (Furihata et al. 2006; Fujii and Zhu 2009) and OsbZIP46 in rice (Tang et al. 2012). We investigated the interaction between OsbZIP42 and ten putative rice SAPK family members (Kobayashi et al. 2004) using yeast two-hybrid assays. OsbZIP42 did not interact with any SAPKs (Supplementary Fig. S1). Unlike other bZIP TFs, the OsbZIP42 protein contained two transcriptional activation domains (Fig. 1b); therefore, we investigated the interaction activity of 42-N2 and 42-C2 separately (Fig. 4). Only 42-N2 could interact with SAPK4. This result suggests that OsbZIP42 may require another modification and/or conformational change mechanism to interact with SAPK4. To explain this result, the three-dimensional protein structure of OsbZIP42 was predicted using the I-TASSER online server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Supplementary Fig. S2). The predicted models of OsbZIP12, 42, 46, and 62 TFs exhibited very similar protein structures. In contrast, OsbZIP42 showed a different structure from those other models (Supplementary Fig. S2). OsbZIP12, 46, and 62 contained several well-developed long alpha helical structures, but OsbZIP42 contained very short alpha helical structures. This contrast in structure could lead to different interaction activities with SAPKs.

Overexpression of *OsbZIP42* leads to seedlings that are hypersensitive to ABA

To examine the roles of OsbZIP42 in rice plants, we constructed rice transformation plasmids (Fig. 5a), and transgenic rice plants overexpressing *OsbZIP42* (OsbZIP42-OX) were obtained via the *Agrobacterium*-mediated transformation method. T1–T4 generation seeds were collected from individual transgenic plants, and two independent homozygous T4 generation lines were subjected to further analysis. The ectopic expression of the transgenes in the OsbZIP42-OX plants was confirmed using qRT-PCR (Fig. 5b). The transcript levels of *OsbZIP42* were enhanced in the individual transgenic lines relative to the WT control.

The bZIP TFs are involved in various developmental and physiological processes in response to ABA signaling and play important roles in ABA-dependent stress responses (Llorca et al. 2014). To test whether ABA has an effect on the germination of OsbZIP42-OX plants, WT and transgenic seeds were germinated in half-strength MS medium with ABA (0, 2, and 6 μM). The germination rate of the OsbZIP42-OX transgenic lines was similar to that of WT control at 0 μM ABA (Fig. 5c). However, the germination rate of the OsbZIP42-OX transgenic lines was significantly decreased at 2 and 6 μM ABA. These results suggested that the sensitivity of seed germination of OsbZIP42-OX transgenic rice plants to ABA was increased. Next, we tested the effect of ABA on seedling development in OsbZIP42-OX plants. WT and transgenic seedlings were grown for 2 weeks in half-strength MS solid media with or without ABA (Fig. 5d), and the lengths of the shoots and roots were measured (Fig. 5e, f). There were no significant differences in shoot length between the WT and transgenic rice plants grown in the medium without ABA. The shoot and root lengths of the WT plants grown in the medium with 5 μM ABA were reduced by approximately 22% and 25% of those of the control plants grown in the medium without ABA, respectively. However, the shoot and root lengths of the OsbZIP42-OX transgenic lines were significantly reduced

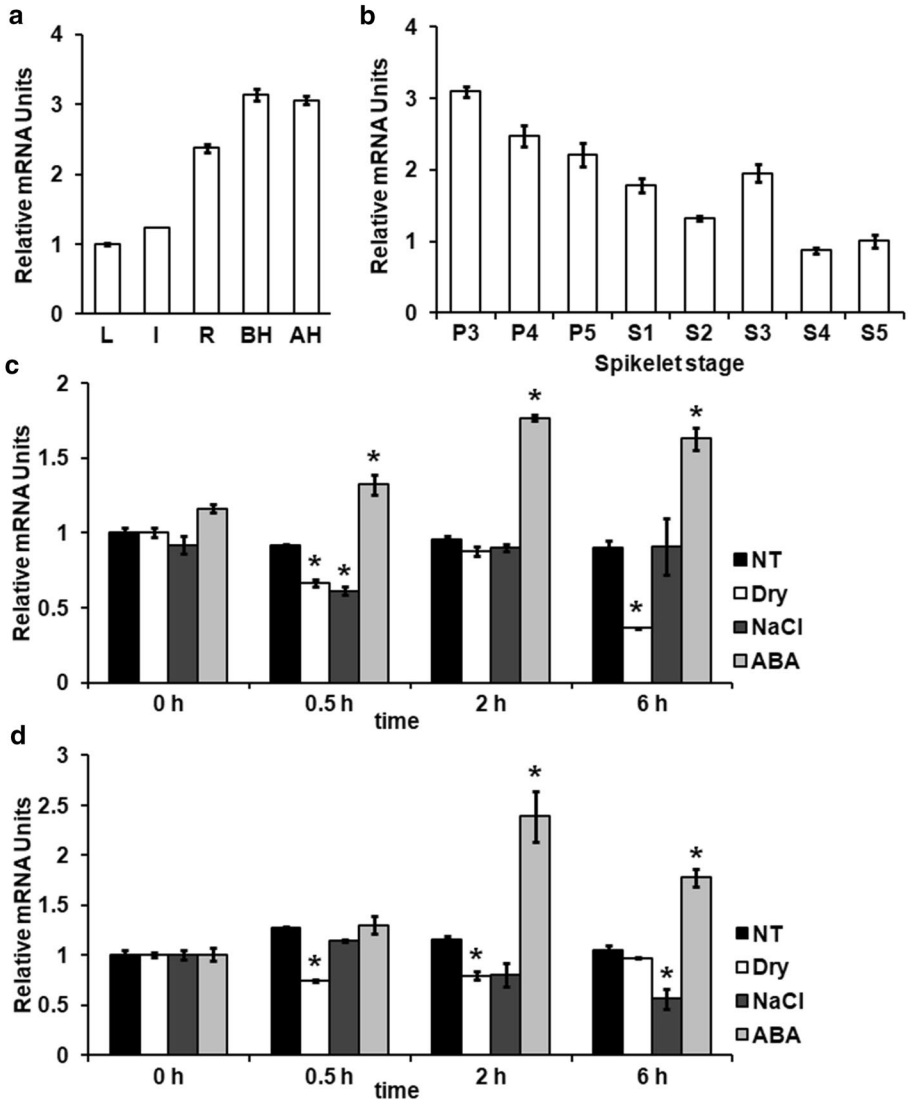

Fig. 2 Expression analyses of the *OsbZIP42* gene. **a** The transcript levels of *OsbZIP42* in different organs under normal conditions were analyzed using qRT-PCR. The relative transcript abundances of the tested genes were quantified relative to those in the leaves. The indicated organs, young leaves (L), internodes (I), young roots (R), prepollinated spikelets (BH), and postpollinated spikelets (AH), are shown. **b** The transcript levels of *OsbZIP42* at different reproductive stages were determined using qRT-PCR. The relative transcript abundances of the *OsbZIP42* gene were quantified relative to the S4 stage. The young panicles and developmental seeds were divided into three (P3 3–5 cm, P4 10–15 cm, P5 15–20 cm of panicle length) and five groups (S1 0–2 DAP, S2 3–4 DAP, S3 5–10 DAP, S4 11–20 DAP, S5 5–29 DAP), respectively. The transcript levels of *OsbZIP42* under stress conditions were analyzed in the leaves (**c**) and roots (**d**) using qRT-PCR. The relative transcript abundances of the tested genes were quantified relative to those of the NT (nontreated control) at 0 h. Three-week-old plants grown in soil were hydroponically adapted in water for 3 days and then transferred to fresh water containing either 250 mM NaCl (NaCl) or 100 μM ABA (ABA) in a greenhouse, or air-dried (Dry). NT indicates nontreated control plants. Total RNA was prepared during each condition at the indicated time points. The *OsUbi1* gene was used as a reference gene. The data are presented as the mean ± SE values ($n = 3$) from three independent experiments. Asterisks indicate statistically significant differences compared to the NT using Student's *t* test (*$P < 0.05$)

by 6–12% and 8–16%, respectively, compared to those of the control. These data indicate that the overexpression of *OsbZIP42* makes transgenic seedlings hypersensitive to ABA in comparison to WT plants, and thus OsbZIP42 is involved in ABA signaling in rice.

Expression analysis of ABA signal pathway marker genes in the transgenic plants

The ABA-dependent phosphorylation of bZIP TFs by SnRK2 or SAPK family protein kinases and dephosphorylation

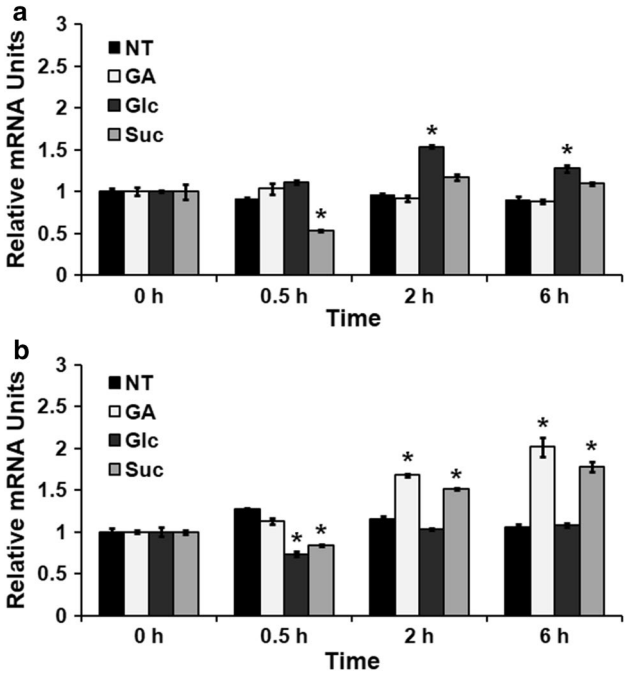

Fig. 3 The expression patterns of the *OsbZIP42* gene under gibberellin, glucose, and sucrose treatments were analyzed using qRT-PCR. The relative transcript abundances of the tested genes in the leaves (**a**) and roots (**b**) were quantified relative to those of the NT at 0 h. Three-week-old plants grown in soil were hydroponically adapted in water for 3 days and then transferred to fresh water containing either 100 µM gibberellin (GA), 100 mM glucose (Glc) or 100 mM sucrose (Suc) in a greenhouse. NT indicates nontreated control plants. Total RNA was prepared during each condition at the indicated time points. The *OsUbi1* gene was used as a reference gene. The data are presented as the mean ± SE values ($n = 3$) from three independent experiments. Asterisks indicate statistically significant differences compared to the NT using Student's *t* test (*$P < 0.05$)

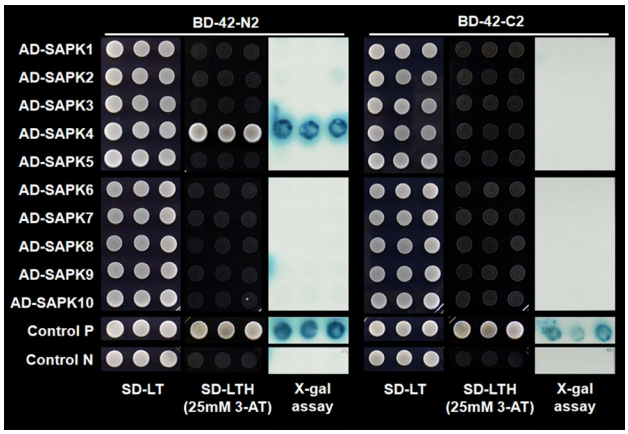

Fig. 4 Interaction between OsbZIP42 and rice SAPKs. Yeast two-hybrid assays of OsbZIP42-N2/OsbZIP42-C2 and SAPK members were performed. The results of a colony-lift filter assay are shown (X-gal assay). Control P and control N indicate the positive and negative controls, respectively. *3-AT* 3-amino-1,2,4-triazole, *SD-LTH* minimal medium–Leu–Trp–His

by PP2C-type protein phosphatases (PP2C) regulate their activation in plants. The expression of late-embryogenesis-abundant (*LEA*) genes, which participate in the acquisition of desiccation tolerance, could be induced by the activated ABF/ABRE system (Umezawa et al. 2010). To specify whether OsbZIP42 affects ABA signaling, three genes (*PP2C*, *LEA3* and *Rab16*) involved in ABA signal transduction were selected for expression level comparison between the WT and transgenic plants under ABA treatment; these included a PP2C family gene (*ABI2*, Os01g0583100) and two downstream genes (*LEA3*, Os05g0542500; *Rab16*, Os11g0454300). Under normal conditions, the three genes showed no differences in transcript levels between the WT and OsbZIP42-OX lines. Under 100 µM ABA, the transcript levels of *ABI2* (PP2C family gene) were decreased in the OsbZIP42-OX transgenic lines compared to those in the WT control (Fig. 6a). This result suggests that OsbZIP42 negatively regulates PP2C in the ABA signaling pathway. In the OsbZIP42-OX lines, the transcript levels of *LEA3* were 9.5- and 6.5-fold increased within 2 h compared to those of the WT controls. In contrast, these levels were 0.41- and 0.54-fold decreased at 6 h compared to those of the WT controls (Fig. 6b). The transcript levels of *Rab16* were 10.9- and 14.5-fold increased within 2 h compared to those of the WT controls, and they showed no significant difference at 6 h (Fig. 6c). These results support the involvement of OsbZIP42 in the ABA signal transduction pathway in rice. Furthermore, OsbZIP42 is involved in early ABA-responsive gene induction.

Abiotic stress tolerance of transgenic plants

To examine the responses of the OsbZIP42-OX plants to abiotic stress during the germination stage, the seeds of WT and transgenic plants were germinated in water containing 400 mM mannitol or 250 mM NaCl, and then the germination rates were measured (Fig. 7). There were no differences in the germination rates for WT and OsbZIP42-OX seeds under normal and 250 mM NaCl conditions. Under 400 mM mannitol, the OsbZIP42-OX seeds exhibit increased germination rates compared to the WT.

To evaluate the responses of the OsbZIP42-OX plants to water deficit, 4-week-old WT and OsbZIP42-OX seedlings were subjected to drought stress for 2 days, followed by rewatering. The WT and transgenic plants both exhibited leaf rolling and other stress-induced symptoms by 2 days of drought stress. During rewatering, the transgenic lines showed a faster recovery from drought stress and more stimulated growth than did the severely injured WT plants (Fig. 8a, R3 and R10). The survival rate of the OsbZIP42-OX transgenic lines was 83–94% (Fig. 8b). In contrast, the survival rate of the WT plants was only 40%. To further verify the stress tolerance of the OsbZIP42-OX plants, we

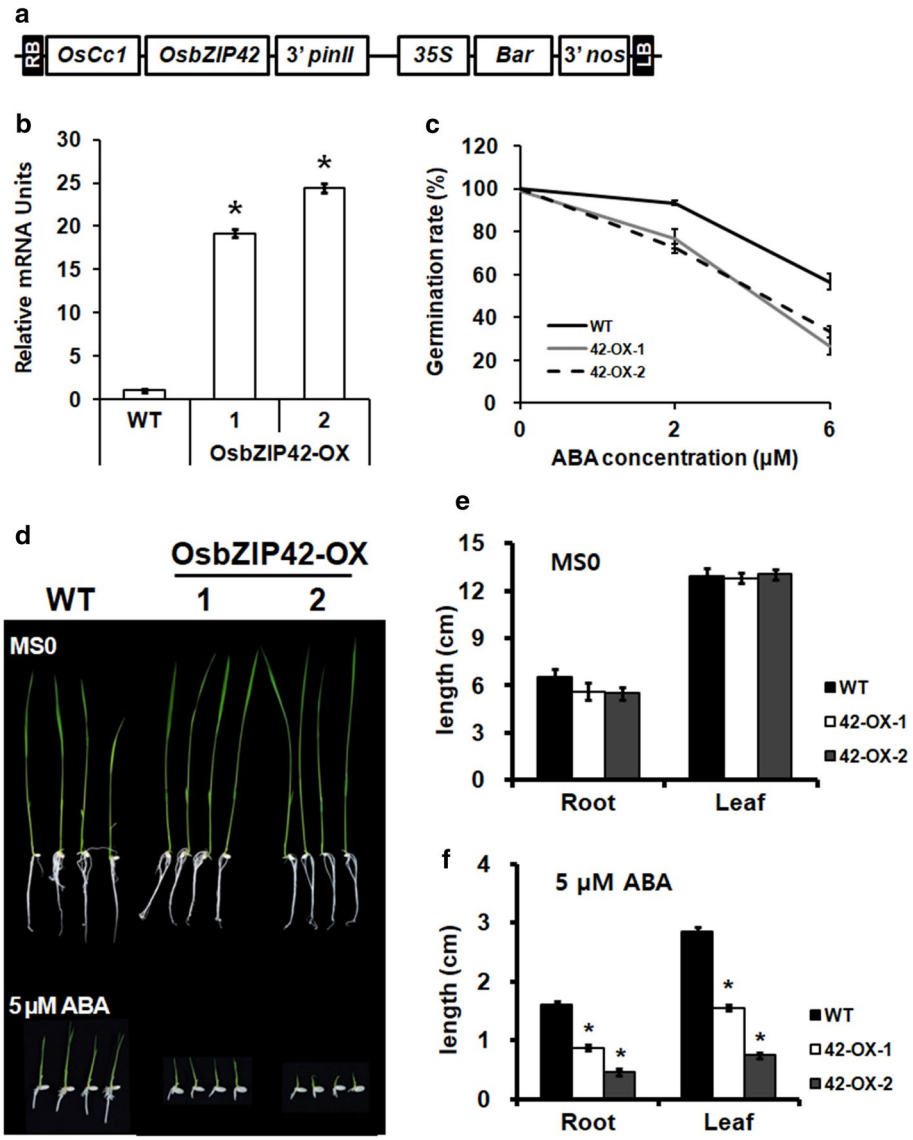

Fig. 5 Increased ABA sensitivity of *OsbZIP42* overexpression plants during germination and seedling stages. **a** The overexpression plasmid consisting of the constitutively expressed *OsCc1* promoter linked to the *OsbZIP42* coding region, which is transcribed with the 3′ UTR region of the potato proteinase inhibitor II gene (3′ *pinII*), also includes a Basta resistance (*Bar*) expression cassette containing the *35S* promoter, the bar-coding region and the 3′ UTR region of the nopaline synthase gene (3′ *nos*). **b** The transcript levels of the *OsbZIP42* gene in the leaves of T4 transgenic (OsbZIP42-OX) and control rice plants (WT) were analyzed using qRT-PCR. Those in the transgenic lines were quantified relative to those in the WT plants. The *OsUbi1* gene was used as a reference gene. The data are presented as the mean ± SE values ($n = 3$) from two independent experiments. **c** Germination assay of OsbZIP42-OX transgenic and WT seeds in half-strength MS medium containing 0, 2, and 6 μM ABA. The germination rate of the treated seeds was calculated after 5 days. **d** The OsbZIP42-OX transgenic and WT control plants were grown in MS0 and MS0 containing 5 μM ABA. Shoot (**e**) and root length (**f**) measurements of the rice plants are shown in panel **c**. The data are presented as the mean ± SE values ($n = 15$). Asterisks indicate statistically significant differences compared to the WT using Student's *t* test ($^*P < 0.05$)

measured the variations in the chlorophyll fluorescence ratio (F_v/F_m) after drought and cold stress treatments. Healthy plants typically achieve a maximum F_v/F_m value of approximately 0.8, and lower values are observed in plants that are exposed to abiotic stress factors. The values of F_v/F_m were 1.39- to 1.62-fold higher ($P < 0.001$) in the OsbZIP42-OX plants than in the WT plants under drought stress conditions (Fig. 8c). Under cold stress conditions, the OsbZIP42-OX plants showed 1.33- to 1.7-fold higher values for F_v/F_m than the WT plants (Fig. 8d). The results of the phenotypic and chlorophyll fluorescence analyses of the OsbZIP42-OX plants suggest that OsbZIP42 confers drought tolerance to rice.

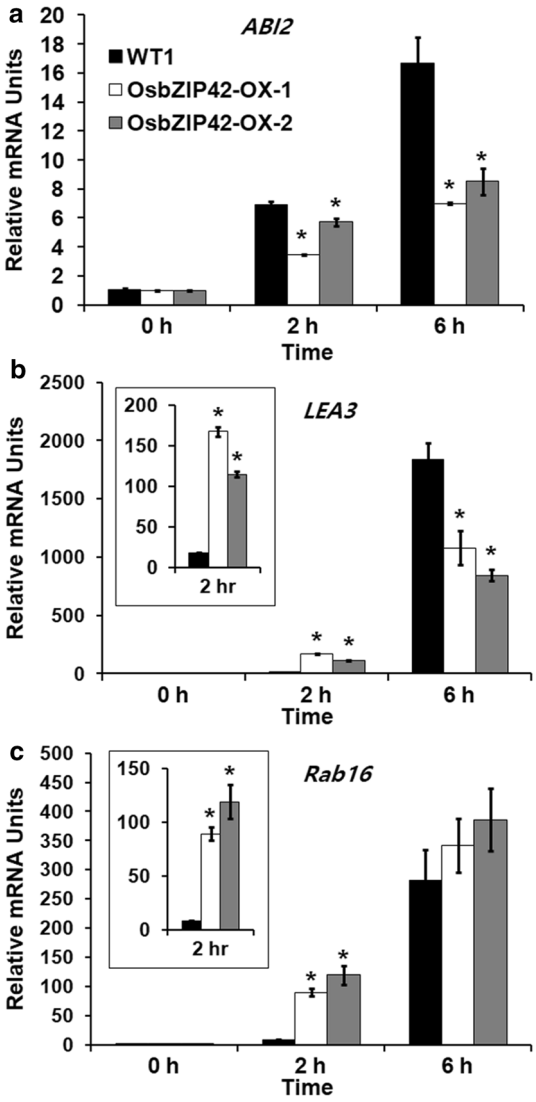

Fig. 6 Expression analysis of the ABA signaling pathway marker genes in OsbZIP42-OX transgenic plants. The relative transcript abundances of the *ABI2* (**a**), *LEA3* (**b**) and *Rab16* (**c**) genes in the leaves of the OsbZIP42-OX transgenic and WT plants under 100 μM ABA treatment were quantified relative to those in the WT plants at 0 h. Total RNA templates were prepared at the indicated time points. The *OsUbi1* gene was used as a reference gene. The data are presented as the mean ± SE values ($n = 3$) from three independent experiments. Asterisks indicate statistically significant differences compared to the WT using Student's *t* test (*$P < 0.05$)

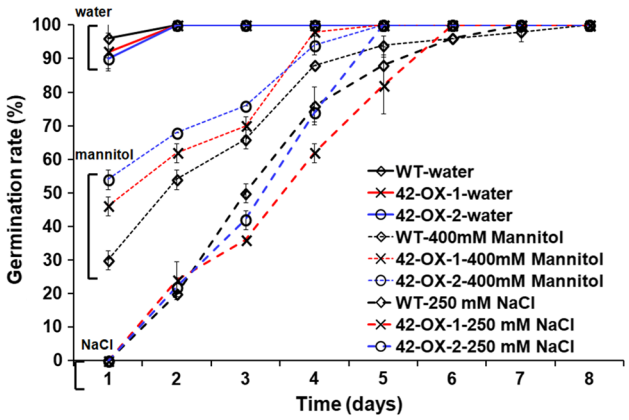

Fig. 7 Germination test of the OsbZIP42-OX seeds. The seeds of the WT and OsbZIP42-OX plants were germinated in water containing 400 mM mannitol or 250 mM NaCl, and then the germination rates were measured

Discussion

Plant bZIP TFs have been reported to be involved in various biological processes, including environmental stress responses. The *Arabidopsis* bZIP TFs, ABFs/AREBs, which play important roles in cooperatively regulating ABRE-dependent ABA signaling involved in abiotic stress tolerance, are classified as group A (Jakoby et al. 2002; Kang et al. 2002; Yoshida et al. 2010). The rice group E bZIP TFs are highly homologous to the *Arabidopsis* group A bZIP family members. Rice group E includes 11 members and is divided into three subgroups (Nijhawan et al. 2008; Lu et al. 2009). The members of subgroup I, OsABF1 (OsbZIP12), and subgroup III, OsABI5 (OsbZIP10), OsbZIP23, ABL1 (OsbZIP46), TRAB1 (OsbZIP66), and OsbZIP72, have been previously studied. *Arabidopsis* DPBF4 (AtbZIP12) and AREB3 (AtbZIP66) are classified as subgroup II members (Lu et al. 2009). However, the functions of subgroup II have not been well studied. In this study, we report on the functional characterization of OsbZIP42, which belongs to subgroup II. Our data suggest that OsbZIP42 is involved in ABA signaling and drought stress responses and is regulated through transcriptional control and posttranslational modification in rice.

Our results show that OsZIP42 contains two potential activation domains that are located in the N-terminus (residues 1–128) and C-terminus (residues 132–335). It has been reported that human LZIP or Luman, which is a basic leucine zipper protein that interacts with host cell factor-1, contains two transcriptional activation domains located in the N and C-termini (Luciano and Wilson 2000). However, the function of the C-terminal transcriptional activation domain has not been studied. In yeast assays, the full-length OsbZIP42 and N-terminal fragment 42-N1, which contains domain D, showed no transactivation activity in yeast (Fig. 1). Domain D is proposed as a negative regulatory domain in OsbZIP46, as the deletion of domain D from OsbZIP46 results in constitutive transactivation activity in yeast. It has been suggested that deletion of domain D may cause a conformational change that mimics the changes resulting from posttranslational modification (Tang et al. 2012). In contrast, the C-terminal fragment 42-C1, which contains domain D and the bZIP domain, exhibited transactivation activity in

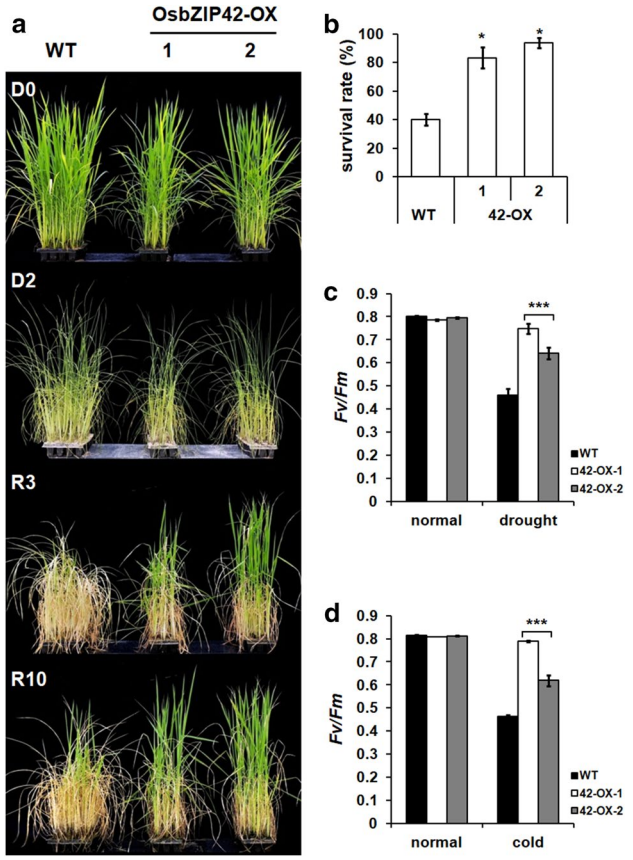

Fig. 8 Overexpression of *OsbZIP42* enhanced abiotic stress tolerance in rice. **a** The OsbZIP42-OX transgenic plants had an increased tolerance to abiotic stresses during the vegetative stage. The rice plants were subjected to drought stress by draining the water out of the pots (D0), and water was withheld for 2 days (D2) in a greenhouse. After 2 days of drought stress, the rice plants were rehydrated by resupplying water (R0). R3 and R10 indicate water supply for 3 and 10 days, respectively. Photographs were taken on the indicated days. **b** The survival rates of the OsbZIP42-OX transgenic and WT plants after drought stress. The data are presented as the mean ± SE values ($n = 20$) from two independent experiments. Changes in the chlorophyll fluorescence (F_v/F_m) of the leaf discs under drought (**c**) or cold stress (**d**). The data are presented as the mean ± SE values ($n = 9$) from two independent experiments. Asterisks indicate statistically significant differences compared to the WT using Student's *t* test (*$P < 0.05$, ***$P < 0.001$)

yeast. These results suggest that domain D may act only on the N-terminal transcriptional activation domain, which is commonly found in bZIP TFs, and/or has another function in addition to being a negative regulator.

Full-length OsbZIP42 showed no transactivation activity in the yeast assays (Fig. 1b). The *OsbZIP42* gene was induced only by ABA treatment in the leaves and roots (Fig. 2c, d). Furthermore, the target genes for OsbZIP42, *LEA3* and *Rab16*, were significantly more induced in the OsbZIP42-OX plants compared to in the WT plants under ABA treatment but showed no difference under normal conditions (Fig. 6). These results suggest that the transactivation activities of OsbZIP42 rely primarily on ABA-dependent modifications. Several *Arabidopsis* group A and rice group E bZIP TFs were reported to be activated by ABA-dependent phosphorylation (Uno et al. 2000; Kagaya et al. 2002; Schütze et al. 2008; Tang et al. 2012). These reports suggested that OsbZIP42 may also require ABA-dependent phosphorylation for transactivation activity. In the ABA signaling pathway, the phosphorylation of ABF/ABEB by SnRK2 in *Arabidopsis* and of TRAB1 by SAPK in rice have been well characterized (Kobayashi et al. 2005; Furihata et al. 2006; Fujii and Zhu 2009). TRAB1 can be activated via ABA-dependent phosphorylation. Its Ser-102 residue is critical for this function, and this residue is phosphorylated in response to ABA or hyperosmotic stress (Kagaya et al. 2002; Kobayashi et al. 2005). OsbZIP42 contains phosphorylation residues, and these conserved phosphorylation sites have been shown to be related to the ABA response and stress signaling.

Our results show that full-length OsbZIP42 did not interact with any SAPKs in yeast, but the N-terminal fragment 42-N2 (without domain D) interacted with SAPK4 (Supplementary Fig. S1, Fig. 4). This result suggests that OsbZIP42 likely requires additional modifications and/or conformational change mechanisms to interact with SAPK4. To investigate the effect of the structure of OsbZIP42 on its interactions with SAPKs, the predicted protein structure of OsbZIP42 was compared to the structures of other OsbZIP TFs using the I-TASSER online server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Supplementary Fig. S2). OsbZIP12, 46, and 62 contained several well-developed long alpha helical structures, but OsbZIP42 contained very short alpha helical structures. The results suggest that OsbZIP42 may have a different structure from those of other OsbZIP TFs. In addition, this difference in structure could require an additional modification and/or conformational change mechanism for OsbZIP42 to interact with SAPK4.

Several members of the rice group E bZIP TFs have been reported to be involved in the ABA signaling pathway. OsbZIP23, 46, and 72 are positive regulators of ABA signaling (Xiang et al. 2008; Lu et al. 2009; Tang et al. 2012). The overexpression of *OsbZIP42* induced the transgenic rice plants to become hypersensitive to ABA, indicating that *OsbZIP42* plays a role in the ABA signaling pathway. Under ABA conditions, the *LEA3* and *Rab16* genes, which are known to be downstream of *ABFs* in the ABA signaling pathway, were rapidly induced by ABA in the OsbZIP42-OX transgenic plants compared to in the WT plants (Fig. 6). These genes showed no significant difference or decrease at 6 h. These results suggest that OsbZIP42 may be involved early in the ABA signaling pathway. Our results show that the overexpression of *OsbZIP42* gene in transgenic rice plants confers increased

tolerance to drought stress during germination and in seedlings (Figs. 7, 8). Differences in the chlorophyll fluorescence analysis of the transgenic plants further support the increased drought tolerance phenotypes. The SnRK2 family is known to comprise the principal positive regulators of ABA and osmotic stress that act via the phosphorylation of AREB/ABF TFs (Fujita et al. 2013). The SnRK2 family consists of ten genes classified into three subclasses in *Arabidopsis* and rice (Kobayashi et al. 2004). SnRK2 subclass I member SAPK4, which interacts with OsbZIP42 (Fig. 4), has been reported to regulate stress-responsive gene expression in rice (Kobayashi et al. 2004; Diédhiou et al. 2008). In rice, only SnRK2 subclass III members are activated by ABA, and subclass I and II members are activated only by osmotic and salt stress (Kobayashi et al. 2004). Thus, OsbZIP42 could be phosphorylated in response to ABA by some other kinases. Calcium-dependent protein kinases (CPKs) have been found to phosphorylate ABF/AREBs (Choi et al. 2005; Zhu et al. 2007). CPKs are central components of ABA signal transduction (Choi et al. 2005). A SnRK3- type protein kinase CIPK11 is also found to phosphorylate ABI5 to regulate ABA response (Zhou et al. 2015). SAPK4 has been reported to interact with OsABF1/OsABI5/OREB1, which is highly phosphorylated in response to ABA and abiotic stress signals, in yeast and plants (Ding et al. 2009). The overexpression of *SAPK4* resulted in improved germination, growth and development under salt stress in both seedlings and mature plants. SAPK4-regulated genes are involved in ion homeostasis and oxidative stress response, vacuolar H^+-ATPase, the Na^+/H^+ antiporter *NHX1*, the Cl^- channel *OsCLC1* and a catalase (Diédhiou et al. 2008). The germination assay showed that overexpression of *OsbZIP42* resulted in improved germination under mannitol treatment but not under NaCl treatment (Fig. 8). These results suggest that OsbZIP42 is activated via ABA-dependent phosphorylation by SAPK4 and plays a role in ABA-dependent drought stress responses.

It has been determined that bZIP TFs bind to DNA predominantly as homo- or heterodimers (Landschulz et al. 1988; Nijhawan et al. 2008) and that homo- and heterodimers of bZIPs control gene expression during a variety of stress responses in plants (Schütze et al. 2008; Dietrich et al. 2011). Intact OsbZIP42 did not form homodimers (Supplementary Fig. S3). However, it may form heterodimers, and modified OsbZIP42 could form homodimers. This result could explain the enhancement in drought tolerance due to OsbZIP42 overexpression, even though the *OsbZIP42* gene is not induced by drought and high-salt stresses. Further studies are required to elucidate the heterodimerization and modification mechanisms of OsbZIP42 that are responsible for drought signal transduction.

Author contribution statement YHL and SIS conceived and designed the experiments; JJ and YHL performed the experiments; JJ and SIS wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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