Activated OsbZIP46 confers drought tolerance

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Journal research area:
Signal transduction, Environmental stress
Constitutive Activation of Transcription Factor OsbZIP46 Improves Drought Tolerance in Rice¹

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This work was supported by grants from the National Program for Basic Research of China (2012CB114305), the National Program on High Technology Development (2012AA100103), the National Natural Science Foundation of China (30725021 and 30921091), and the Project from the Ministry of Agriculture of China for Transgenic Research (2009ZX08001-021B, 2011ZX001-003).

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Abstract

OsbZIP46 is one member of the third subfamily bZIP transcription factors in rice. It has high sequence similarity to ABF/AREB transcription factors ABI5 and OsbZIP23, two transcriptional activators positively regulating stress tolerance in Arabidopsis and rice, respectively. Expression of OsbZIP46 was strongly induced by drought, heat, H$_2$O$_2$, and abscisic acid (ABA) treatment; however, it was not induced by salt and cold stresses. Overexpression of the native OsbZIP46 gene increased ABA sensitivity but had no positive effect on drought resistance. The activation domain of OsbZIP46 was defined by a series of deletions, and a region (domain D) was identified as having a negative effect on the activation. We produced a constitutive active form of OsbZIP46 (OsbZIP46CA1) with a deletion of domain D. Overexpression of OsbZIP46CA1 in rice significantly increased tolerance to drought and osmotic stresses. Gene chip analysis of the two overexpressors (native OsbZIP46 and constitutive active form OsbZIP46CA1) revealed that a large number of stress-related genes, many of them predicted to be downstream genes of ABF/AREBs, were activated in the OsbZIP46CA1 overexpressor but not (even down-regulated) in the OsbZIP46 overexpressor. OsbZIP46 can interact with homologs of SnRK2 protein kinases that phosphorylate ABFs in Arabidopsis. These results suggest that OsbZIP46 is a positive regulator of ABA signaling and drought stress tolerance of rice depending on its activation. The stress-related genes activated by OsbZIP46CA1 are largely different from those activated by the other rice ABF/AREB homologs (such as OsbZIP23), further implying the value of OsbZIP46CA1 in genetic engineering of drought tolerance.
An understanding of how plants respond to various adverse environmental stresses is a prerequisite for discovering promising genes and, therefore, such knowledge could provide useful insights for generating crop plants with improved stress tolerance. A complex network of stress signaling and regulation of gene expression exists in plants responding and adapting to the stresses. The stress signals are perceived through diverse known and unknown sensors and transduced by various signaling components including many second messengers, phytohormones, signal transducers (such as protein kinases and phosphatases), and transcriptional factors, resulting in activation of a large number of stress-related genes and synthesis of diverse functional proteins in plants that finally lead to various physiologic and metabolic responses to adapt to the stresses (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Hirayama and Shinozaki, 2010; Matsukura et al., 2010; Lata and Prasad, 2011).

The phytohormone abscisic acid (ABA) controls various processes of plant growth, including seed germination and development and abiotic stress tolerance (particularly drought tolerance). ABA has been the most extensively studied stress-related hormone in plants although the roles of other phytohormones, such as cytokinins, brassinosteroids, and auxins, in stress-related processes are emerging (Cutler et al., 2010; Hubbard et al., 2010; Peleg and Blumwald, 2011). Both ABA-dependent and ABA-independent processes are involved in stress responses (Shinozaki et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2006). The understanding of the molecular basis of ABA responses in plants has been improved dramatically mainly due to the recent exciting breakthroughs in unveiling the core signaling of ABA. The major events are the identification of the PYR/PYL/RCAR receptors of ABA and the establishment of the details of one of the ABA signaling pathways in Arabidopsis. According to the core signaling model, the binding of ABA to the receptors PYR/PYL/RCAR inhibits the type 2C protein phosphatases (PP2Cs), resulting in the activation of SNF1-related type 2 protein kinases (SnRK2s), which can target some ion channels and the ABA-dependent gene expression by phosphorylating the bZIP transcription factors (Geiger et al., 2009; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Cutler et al., 2010; Geiger et al., 2010; Hubbard et al., 2010; Nishimura et al., 2010; Raghavendra et al., 2010; Umezawa et al., 2010). Although the major components of ABA signaling remain unknown in other plants,
analyses of homologous ABA signaling genes and complementation tests suggest that a
similar pathway of ABA core signaling as in Arabidopsis also exists in economically
important crops such as rice (Kobayashi et al., 2004; Fujii and Zhu, 2009; Fujita et al.,
2009; Fujii et al., 2011).

In the ABA signaling-mediated stress responses, many transcription factors have
crucial regulatory roles in activating the ABA-dependent stress-responsive gene
expression. Among the transcription factors, members of the bZIP family, which contain
a basic region and a leucine zipper domain, have been identified for their function in the
ABA-dependent pathway by recognizing ABA-responsive elements (ABREs) containing
an ACGT core motif. As a big transcription factor family, quite a few bZIP transcription
factors have been well-characterized functionally with diverse roles in many aspects
including pathogen defense, light signaling, seed maturation, flower development, and
especially, abiotic stress responses and hormone signal transduction (Uno et al., 2000;
Jakoby et al., 2002; Nijhawan et al., 2008). Among the studies of bZIP family, a
noteworthy aspect is that the function of the third subfamily (also known as the
AREB/ABF/ABI5 subfamily) has been well elucidated because of the prominent roles of
this subfamily in the ABA signaling pathway. ABI5 is a genetically identified ABA
signaling component that plays an essential role in seed germination and ABA-triggered
developmental arrest processes after germination in Arabidopsis. In contrast, ABFs or
ABA-responsive element binding factors (AREBs) function mostly in the vegetative
stage (Choi et al., 2000; Uno et al., 2000; Kang et al., 2002; Kim et al., 2004; Fujita et al.,
2011). However, recent reports confirmed that the AREB/ABF/ABI5 members are major
targets of SnRK2 protein kinases in the ABA core signaling (Fujii and Zhu, 2009; Fujita
et al., 2009). Noticeably, the members AREB1, AREB2, and ABF3 are master
transcription factors that cooperatively regulate ABA signaling involved in multiple stress
responses and require ABA for full activation (Yoshida et al., 2010). AREB1/ABF2 has
been further documented for its posttranslational modification, which is related to its
activity regulation (Fujita et al., 2005; Furihata et al., 2006). Thus far,
SnRK2-AREB/ABF has been proven as a major module regulating ABA-mediated gene
expression in response to abiotic stresses in Arabidopsis (Kim et al., 2004; Fujita et al.,
2005; Furihata et al., 2006; Fujii et al., 2009; Yoshida et al., 2010).
Rice plants have 89 putative bZIP transcription factor genes (Nijhawan et al., 2008). Several members have been studied for their functions potentially related to stress response, such as *LIP19* (Shimizu et al., 2005), *OsBZ8* (Nakagawa et al., 1996; Mukherjee et al., 2006), and *RF2a* and *RF2b* (Dai et al., 2004; Dai et al., 2008). Noticeably, in contrast to the comprehensive studies of ABFs in Arabidopsis, mainly three members of the third subfamily in rice, *TRAB1*, *OsABI5*, and *OsbZIP23*, have been studied for their roles in ABA-mediated stress responses (Hobo et al., 1999; Xiang et al., 2008; Zou et al., 2008). *TRAB1*, which is identified by yeast two-hybrid screening for proteins that interact with the seed development-related transcription factor VP1, can be activated by ABA-dependent phosphorylation in its Ser102 residue (Hobo et al., 1999; Kagaya et al., 2002; Kobayashi et al., 2005), but its biologic function remains to be clarified. *OsABI5*, named according to its homology to *ABI5* in Arabidopsis, was suggested to be involved in ABA signal transduction and stress responses (Zou et al., 2007, 2008). *OsbZIP23* was characterized as a key player of the bZIP family for conferring ABA sensitivity, salinity, and drought tolerance of rice (Xiang et al., 2008). Several other members were also mentioned for their involvement in ABA and stress (Zou et al., 2007; Amir Hossain et al., 2009; Lu et al., 2009). Among the third (or *AREB/ABF/ABI5*) bZIP subfamily of rice, there are dozens of stress-responsive *ABF* homologs including the reported members (Xiang et al., 2008). The functional redundancy or specificity for a specific member of this family in the stress responses remain to be addressed.

In this paper, we report the functional analysis of *OsbZIP46*, another member of the third subfamily that has high sequence identity to *OsbZIP23*. This gene was also named as *ABI5-like1* (*ABL1*) based on the decreased ABA sensitivity of a knockout mutant of this gene (Yang et al., 2011). Overexpression of intact *OsbZIP46* showed no significant effect on drought resistance. We identified a constitutive active form of *OsbZIP46*, *OsbZIP46CA1*, by mutagenesis, and overexpression of *OsbZIP46CA1* could activate the expression of the downstream genes and increase drought resistance. We also found that the posttranslational modification of *OsbZIP46* may be required for its function.

**RESULTS**
Identification of OsbZIP46 as a Stress-Responsive bZIP Factor

We identified the full-length cDNA of OsbZIP46, designated according to Nijhawan et al. (2008), in a cDNA library of Minghui 63 (Oryza sativa L. ssp. indica) (Chu et al., 2003; Zhang et al., 2005) (Supplemental Fig. S1). To speculate on the function of OsbZIP46, we checked the expression profile of OsbZIP46 under different abiotic stresses and phytohormone treatments by real-time quantitative reverse transcription-PCR (RT-PCR). We found that the expression level of OsbZIP46 was induced by drought, ABA, and indole-3-acetic acid (IAA) (Fig. 1A and B), consistent with a previous report (Yang et al., 2011). In addition, we found that the expression of OsbZIP46 was affected by temperature stress (induced by heat but repressed by cold), oxidative stress (induced by H₂O₂), and cytokinin treatment (repressed by kinetin) (Fig. 1A and B), but only slightly by salt stress. The promoter sequence of OsbZIP46 contains many putative stress response-related cis-elements, such as ABRE element (9 hits), MYB recognition site (13 hits), MYC recognition site (10 hits), and one hit of DRE/CRT element (Fig. 1C). In addition, transient expression assays in rice protoplast suggested that the OsbZIP46-GFP fusion protein was located in the nucleus; the nuclear localization was confirmed by its colocalization with the CFP-fused nuclear protein GHD7 (Supplemental Fig. S2), indicating that OsbZIP46 is a nuclear protein, consistent with the result obtained in onion cells (Yang et al., 2011).

Increased ABA Sensitivity of the Transgenic Plants Overexpression OsbZIP46

According to the features of OsbZIP46 described above, we hypothesized that OsbZIP46 may have a positive role in ABA signaling in rice. To confirm this, we generated the transgenic rice overexpression line OsbZIP46. We first checked the ABA sensitivity of transgenic plants at the germination stage. Seeds of three independent overexpression lines, two negative lines, and the wild-type (WT) Zhonghua11 were germinated in 1/2MS medium containing ABA with a gradient of concentrations (0, 1, 3, 6 µM). The germination rate of the overexpression lines was identical to the negative lines and the WT Zhonghua11 at 0 and 1 µM ABA, but it was significantly lower than that of the negative lines and WT at 3 and 6 µM ABA (Fig. 2A and B), suggesting that the ABA sensitivity (in terms of seed germination) of OsbZIP46-overexpression plants was
increased. We also investigated the ABA sensitivity of transgenic plants at the 
post-germination stage. The lengths of shoot and root of overexpression seedlings grown 
for 2 weeks in 1/2MS medium containing 3 µM ABA were significantly shorter 
compared with those of the negative lines and WT, but no difference was observed for 
seedlings grown in the medium without ABA (Fig. 2C–E). These results suggested that 
overexpression of OsbZIP46 can increase ABA sensitivity at post-germination stages. 
Together with the decreased ABA sensitivity of the mutant of this gene (Yang et al., 2011), 
we propose that OsbZIP46 is a positive regulator the ABA signaling in rice.

**Performance of the Transgenic OsbZIP46-Overexpression Plants Under Abiotic Stress**

It has been generally accepted that a positive regulator of ABA signaling may also 
contribute to tolerance of plants to drought stress. Therefore, we examined the 
performance of OsbZIP46-overexpression plants under drought stress with four 
independent overexpression lines compared to the WT. To our surprise, the 
overexpression lines showed slightly decreased drought tolerance at the seedling and 
reproductive stages. At the seedling stage, the survival rate of overexpression lines was 
lower than that of WT after drought stress (Fig. 3A and B). At the reproductive stage, the 
overexpression lines, drought-stressed at the panicle development stage, showed lower 
relative spikelet fertility compared to WT (Fig. 3C and D). We repeated this testing 
several times and obtained the same results. We also tested the overexpression lines for 
salt stress tolerance but found no significant distance between the overexpression lines 
and WT (data not shown). These results suggest that overexpression of the native 
OsbZIP46 gene may have a negative effect on drought stress tolerance.

We checked the expression levels of RAB21, a commonly used marker gene activated 
by AREB in ABA and drought responses, in the OsbZIP46-overexpression plants. The 
expression level of RAB21 in the OsbZIP46-overexpression plants showed no difference 
compared to that in the WT under normal growth conditions; however, it was 
significantly increased after exogenous application of ABA (Fig. 3E). This result is in 
contrast to the result obtained in the OsbZIP23-overexpression rice plant in which the 
RAB21 showed constitutive elevated expression (Xiang et al., 2008). Actually, a large
number of drought-responsive genes were not up-regulated in the

\textit{OsbZIP46}-overexpression plant, as discussed below. Therefore, we suspected that the
native OsbZIP46 protein may not be able to activate the expression of downstream genes
and its activity may be activated through the ABA signaling pathways.

\textbf{Activation of the Transcriptional Activity of OsbZIP46}

The negative effect of \textit{OsbZIP46} overexpression on drought stress tolerance prompted us
to check the transcriptional activity of OsbZIP46 because it was predicted to be a typical
bZIP transcription factor. First, we tested transactivation activity of OsbZIP46 in yeast.
Unlike its close homolog OsbZIP23 (Xiang et al., 2008), the full length of OsbZIP46
protein fused with the GAL4-binding domain had no transactivation activity in yeast.
Partial fragments of OsbZIP46 with a series of deletions were then tested. Results showed
that the mutated forms with a deletion of 204 or 264 amino acids from the carboxyl
terminal could activate the expression of reporter gene, suggesting that the transactivation
domain of OsbZIP46 is located in the N-terminal of the protein. We noticed that the
activity was detected only when the domain D was absent, suggesting that the domain D
may have a prominent role in the regulation of the transactivation activity of OsbZIP46.
To confirm this, we generated a mutated form of OsbZIP46, which contains 120 amino
acids from the N-terminal (containing domains A, B, and C) as a transactivation domain
and 105 amino acids from C-terminal (containing the bZIP DNA-binding domain and the
domain E) while the middle part (99 amino acids containing the domain D) is absent. We
further checked the activity of the mutated form and found that it has constitutive
transactivation activity in yeast, and this mutated form was designated as \textit{OsbZIP46CA1}
(\textit{OsbZIP46} constitutive active form 1) (Fig. 4).

\textbf{Performance of the Transgenic Rice Overexpression \textit{OsbZIP46CA1} under ABA
Treatment or Abiotic Stress}

Because the domain D-missed mutated form OsbZIP46CA1 showed constitutive
transactivation activity in yeast, we wondered if it has functions different from the native
protein in rice. Therefore, transgenic rice overexpression \textit{OsbZIP46CA1} was generated
and tested for stress tolerance.
We first checked the sensitivity to ABA. As expected, the OsbZIP46CA1-overexpression (CA1-OE hereafter) plants showed increased ABA sensitivity just like the intact OsbZIP46 overexpressors (Fig. 5A). We were more interested in the performance of CA1-OE plants under drought stress. The CA1-OE lines showed significantly increased drought resistance at both the seedling and reproductive stages in contrast to the results of the overexpressor of full-length OsbZIP46 presented above. At the seedling stage, the survival rate of CA1-OE lines was significantly higher compared to the WT control after drought stress (Fig. 5B and C). At the reproductive stage in the field, the results indicated that overexpression of OsbZIP46 can also improve drought resistance (Fig. 5D and E). The water loss rate of the CA1-OE plants was significantly lower than that in the WT and the negative lines (Fig. 5F), which supported the improved drought resistance phenotype. In addition, the CA1-OE lines were tested for tolerance to osmotic stress in comparison with OsbZIP46-overexpression plants and the WT Zhonghua11. We evaluated the osmotic tolerance by using relative shoot length of plants as a criterion because the CA1-OE lines grew a little lower than the WT. OsbZIP46CA1 overexpression had positive effect on the osmotic stress tolerance, whereas OsbZIP46 overexpression had a slightly negative effect on osmotic tolerance (Supplemental Fig. S6).

Distinct Expression Profiles in the OsbZIP46CA1 and OsbZIP46 Transgenic Lines

The different effects of OsbZIP46 and OsbZIP46CA1 overexpression on drought resistance might be attributed to different downstream genes activated because OsbZIP46CA1 showed constitutive transactivation activity. To confirm this hypothesis and elucidate the molecular function of this gene, we compared expression profile changes in the OsbZIP46-OE and OsbZIP46CA1-OE plants (three independent lines were checked for each overexpressor) by using Affymetrix GeneChip. Compared to WT, transcriptomes of both overexpressors had significant changes. With a threshold of 2-fold change, a total of 391 and 469 genes were up- and down-regulated, respectively, in the OsbZIP46CA1-OE plants, whereas in the OsbZIP46-OE plants, 119 and 390 genes were up- and down-regulated, respectively (Fig. 6A and B). Among those genes, only 51 and 100 genes were up- and down-regulated, respectively, in both overexpressors.
Interestingly, 13 genes showed opposite expression change patterns between the two overexpressors (Fig. 6C). These results indicate that significantly more genes were affected by OsbZIP46CA1 overexpression than by the native OsbZIP46 overexpression, and most of the genes affected in the two overexpressors are different. Quantitative RT-PCR was performed to check the differently regulated genes in the two overexpressors. Among the nine genes checked, all showed the same expression patterns as in the gene chip results (Fig. 6E and Supplemental Fig. S7).

Gene ontology (GO) analysis of the differently regulated genes in the two overexpressors revealed that genes in several GO terms under the term “Biological processes” were significantly overrepresented. The GO with the highest proportion of the differently regulated genes is “Response to stimulus” (biotic, abiotic, and endogenous stimuli, etc.), followed by GO terms such as “Signal transduction,” “Protein modification,” “Transcription,” “Metabolism,” and “Biosynthesis’ (Supplemental Table S1). “Transcription factor activity” and “Nucleus” have the highest proportion of the differently regulated genes under the “Molecular function” and “Cellular component,” respectively (Supplemental Table S1).

Because the two overexpressors showed distinct transcriptome changes and most of the differently regulated genes were attributed to the “Response to stimulus” category, we further classified all these genes based on their change patterns in the overexpressors and responsiveness to drought stress. Based on the published microarray result (Jain et al., 2007), more than half of these genes were regulated by drought stress. Among the 1204 expression-affected genes, 455 and 202 were up- and down-regulated, respectively, by drought (Supplemental Tables S2–S5). Interestingly, most of the genes up-regulated and down-regulated in both overexpressors were down- and up-regulated, respectively, by drought stress (Fig. 6D). The genes differentially regulated in the two overexpressors can be classified into five groups. Most of the genes in group I (up-regulated in CA1-OE but not changed in OsbZIP46-OE) and III (up-regulated in CA1-OE but with opposite changes in OsbZIP46-OE) were induced by drought stress, whereas most of the genes in group II (down-regulated in CA1-OE but not changed in the OsbZIP46-OE) were suppressed by drought stress (Fig. 6D). Noticeably, many of the genes up-regulated only in CA1-OE have been annotated or confirmed for stress response or adaptation-related
functions, including dehydrin or late embryogenesis abundant (LEA) proteins (5 genes),
stress-related transcription factors (27 genes), kinases (7 genes), phosphatases (8 genes),
amino acid metabolism or transportation proteins (3 genes), aquaporin (2 genes), and
lipid transfer proteins (5 genes) (Supplemental Table S2). This implies that the increased
drought tolerance of CA1-OE may result from the up-regulation of these genes.
Interestingly, the differentially expressed genes only in OsbZIP46-OE (groups IV and V)
showed distinct trends of responsiveness to drought; most of the genes up-regulated in
OsbZIP46-OE showed drought-suppressed expression patterns, whereas most of the
down-regulated genes in OsbZIP46-OE showed drought-induced expression (Fig. 6D).
Especially, among these down-regulated genes in OsbZIP46-OE, there are many genes
annotated or confirmed for stress response or adaptation including many transcription
factors (40 genes), kinases (35 genes), and some other stress-related functional proteins
(LEA protein, lipid-transfer protein) (Supplemental Table S5). This result may partially
explain the reason overexpression of OsbZIP46 resulted in decreased drought tolerance.
Because so many genes were differentially regulated by OsbZIP46 and
OsbZIP46CA1 overexpression, we further analyzed the promoter sequences of these
genes. The result suggested that 54% of these genes have enriched cis-elements featuring
ABRE that are potential biding sites of ABF or AREB proteins homologous to OsbZIP46
(Supplemental Table S6). Interestingly, the genes up-regulated in CA1-OE but not
changed or with opposite change patterns in the OsbZIP46-overexpressor (groups I and
III) and genes down-regulated in OsbZIP46-OE (group IV) had higher ratios of the
enriched cis-elements (62% and 63%, respectively) than the other two groups (42% and
45%).
Three genes, Os11g26790 (also designated as RAB21 encoding a LEA protein),
Os03g19290 (a putative mitochondrial import inner membrane translocase subunit), and
Os05g38290 (a protein phosphatase 2C) that were up-regulated only in
OsbZIP46CA1-OE but not in OsbZIP46-OE, were selected to test if OsbZIP46 can
directly act on these differentially regulated genes. The pGAD-OsbZIP46 plasmid
(containing the putative DNA-binding domain of OsbZIP46 fused to the GAL4 activation
domain) and the reporter construct pHIS-cis (containing the putative ABRE-containing
promoters of the three genes) were co-transformed into yeast strain Y187. The reporter
gene was activated for all the three co-transformation (Supplemental Fig. S8), indicating that OsbZIP46 can bind the promoters of these genes.

OsbZIP46 May Be Activated by Phosphorylation

Significant differences in affecting gene expressions between the constitutive activated and native forms of OsbZIP46 indicated that the activity of native OsbZIP46 might be regulated by posttranslational modifications. In Arabidopsis, stress/ABA-activated protein kinases (SAPKs) or SnRK2 have been suggested for phosphorylating ABFs or AREBs (Furihata et al., 2006; Fujii and Zhu, 2009; Fujita et al., 2009; Yoshida et al., 2010). Therefore, we checked 9 putative SAPK family members from rice with OsbZIP46 for yeast two-hybrid analysis. The result suggested that OsbZIP46 could interact with OsSAPK2, 6, and 9 (Fig. 7A). The interaction between OsbZIP46 and OsSAPKs was further confirmed by Bimolecular Fluorescence Complementation (BiFC) (Fig. 7B). In vitro phosphorylation assay demonstrated that the OsbZIP46 protein could be phosphorylated by SAPK2 and SAPK6 (Fig. 7C).

Sequence analysis suggested that five putative phosphorylation target sites are present in the OsbZIP46 (Fig. 7D). We inferred that the transcriptional activity of OsbZIP46 might also be related to these putative phosphorylation target sites. To confirm this, we generated single or multiple amino acid substitutions (Ser/Thr to Asp) of OsbZIP46 (Asp provides a negative charge and can mimic the phosphorylated status) and checked their transactivation activity. The single-substitution mutant PA1 (phosphorylation active form 1, Thr 129-to-Asp substitution) showed slightly increased transactivation activity. But the multiple (3–5) amino acid substitutions could significantly enhance the transactivation activity of OsbZIP46 (Fig. 7D and E). Taken together, these results suggest that the native OsbZIP46 may be activated by posttranslational phosphorylation modification.

Discussion

Constitutive Activation Is a Useful Tool for Functional Analysis of Transcription Regulators
Many reports suggest that overexpression of some stress-inducible transcription factors can increase the abiotic stress tolerance of plants (Ciftci-Yilmaz and Mittler, 2008; Dubos et al., 2010; Matsukura et al., 2010; Lata and Prasad, 2011), but some special members need additional modifications to exhibit their full functions. For example, overexpression of \textit{DREB2A} in transgenic plants could not improve stress tolerance, but overexpression of \textit{DREB2ACA} carrying a deletion of the central negative regulatory domain (NRD) activated the expression of many stress-inducible genes and improved tolerance to drought and heat stress in transgenic Arabidopsis (Sakuma et al., 2006a, b). Similarly, the \textit{AREB1}, a homologous gene of \textit{OsbZIP46}, has been reported to fully function after posttranslational modification (Fujita et al., 2005). However, such modification of transcription factors for stress tolerance was seldom reported in crops.

Although \textit{OsbZIP46/ABL1} has been recently reported as a positive regulator of ABA signaling by mutant analysis of this gene (Yang et al., 2011), its actual role in regulating stress tolerance remains unknown. Normally, overexpression of positive regulators of ABA signaling from the bZIP family, such as \textit{OsbZIP23} (Xiang et al., 2008) and \textit{OsbZIP72} (Lu et al., 2009), can result in increased drought tolerance. However, \textit{OsbZIP46}-overexpression rice did not display an expected increase but a decrease of drought tolerance. We found that the intact \textit{OsbZIP46} was insufficient to induce the expression of the target gene unless the exogenous ABA was applied. In yeast, the full length of \textit{OsbZIP46} had no transactivation activity. Considering the fact that \textit{OsbZIP46} is a homolog of the previously reported ABA-dependent phosphorylation of the TRAB1 (Kagaya et al., 2002), these preliminary results suggest that the function of \textit{OsbZIP46} may also need posttranslational level modifications. Therefore, we managed to produce a constitutively active form of \textit{OsbZIP46} in yeast and tested its function for stress tolerance in rice.

As expected, \textit{OsbZIP46CA1} overexpression significantly increased the drought tolerance of rice at both seedling and reproductive stages, which is in contrast to the drought-sensitive phenotype of \textit{OsbZIP46}-overexpression plants. Microarray analysis of the two overexpressors provided strong evidence for the differences in drought tolerance. In short, many stress tolerance-related genes were activated only in the \textit{OsbZIP46CA1} overexpression lines but not in the \textit{OsbZIP46} overexpression lines, supporting the
hypothesis that OsbZIP46CA1 also possesses constitutive transcriptional activity in rice and thus activates the expression of stress-related target genes leading to increased drought tolerance. Therefore, generation of constitutive activated forms for posttranslational modification-required regulatory proteins (at least for some transcription factors) seems to be a promising way to probe their functions, which may be especially true when functional redundancy exists for the gene being studied or overexpression of the native form of the gene results in unexpected outcomes such as the case for OsbZIP46 discussed below.

**Negative Effect of OsbZIP46 Overexpression on Drought Tolerance**
We observed that overexpression of both OsbZIP46 and OsbZIP46CA1 increased ABA sensitivity. However, the OsbZIP46 overexpressor showed slightly reduced drought tolerance. Logically, increased expression of a positive regulator of ABA signaling will most likely have some positive effect, not negative at least, on stress tolerance, even if the regulator needs ABA- or stress-triggered posttranslational modification for its full activity. We propose that OsbZIP46 is an activation-required transactivator, but its activation varies depending on the stresses or ABA treatment. Because OsbZIP46 is one of the positive regulators of ABA signaling (Yang et al. 2011), we propose that overexpressed OsbZIP46 may just match up with the high dosage requirement for mediating the signaling triggered by exogenous ABA. While under the drought stress that triggers ABA signaling more slowly than does the exogenous application of ABA, the pre-accumulated OsbZIP46 in the overexpressor might have a negative effect on mediating the stress signaling through endogenous ABA. This is very likely true because the native OsbZIP46 contains a negative domain (domain D) for the activation activity in yeast. Microarray analysis revealed that arrays of drought stress-related genes were actually down-regulated in the OsbZIP46 overexpressor (Fig. 6D and Supplemental Table S5) and some of these genes were not induced or were significantly less induced by drought compared with those in the WT (data not shown). Nevertheless, the decreased drought resistance might also result from other unknown negative effects of the overexpression of OsbZIP46.
Possible Mechanisms for the Constitutive Activity of OsbZIP46CA1

Because deletion of domain D of OsbZIP46 resulted in constitutive transactivation activity in yeast (Supplemental Fig. S11), we proposed that domain D is a negative regulatory domain and has a pivotal role in the regulation of OsbZIP46 activity. It has been reported that the conserved Leu in the LxLxL motif is important for repression in some transcription repressors, such as the ethylene response factor (ERF)–associated amphiphilic repression (EAR) motif found in some ERFs and SUPERMAN (Fujimoto et al., 2000; Ohta et al., 2001; Hiratsu et al., 2002; Hiratsu et al., 2003; Kazan, 2006) and the repression domain found in auxin/indole-3-acetic acid (Aux/IAA) genes (Tiwari et al., 2004). We compared the sequence of domain D with the conserved EAR motif. Although the LxLxL motif was not found, the domain D contains a sequence signature of LxxxxLxxxL (Supplemental Fig. S1D). In addition, considering that DREB2A contains a PEST sequence in the NRD that plays a role in the stability of the protein (Sakuma et al., 2006a), and the PEST sequence, existing in some rapid-turnover proteins, serves as signals for proteolytic degradation and regulates the activity of proteins by controlling their accumulation (Rechsteiner and Rogers, 1996), we wondered whether domain D of OsbZIP46 has a similar role as that of NRD in DREB2A. However, no potential PEST sequence was identified in OsbZIP46 using the epestfind server (http://emboss.biocomputing.org/emboss/epestfind) (data not shown). It would be interesting to further test which part of domain D is essential for the negative effect on transactivation activity.

It cannot be excluded that deletion of domain D may cause a conformational change that mimics the changes resulting from posttranslational modification such as phosphorylation of the native OsbZIP46. Here, our results showed that the transactivation activity of OsbZIP46 in yeast was related to multiple putative phosphorylation sites and OsbZIP46 may be phosphorylated by three homologs of SnRK2 kinases, OsSAPK2, OsSAPK6, and OsSAPK9 (Fig. 7). In vitro phosphorylation assay confirmed that OsbZIP46 protein can be phosphorylated by OsSAPK2 and OsSAPK6. Because we failed to get soluble expressed proteins for OsbZIP46CA1, it remains to check if there is any change in phosphorylation of OsbZIP46CA1 by the OsSAPKs. It has been well documented that the phosphorylation of ABF/AREB by SnRK2 is a essential part of the
complete ABA signaling pathway in Arabidopsis (Fujii and Zhu, 2009; Fujita et al., 2009). Moreover, the genes of SnRK2 family (also designated SAPK for osmotic stress/ABA-activated protein kinase) in rice were identified for their ABA-inducible characteristics (Kobayashi et al., 2004). Especially, TRAB1, a homolog of OsbZIP46, has been identified as phosphorylated by SAPKs (Kagaya et al., 2002; Kobayashi et al., 2005). RAB21, the putative target gene of OsbZIP46, was activated in the OsbZIP46-overexpression rice plants only after treatment with exogenous ABA. These results together imply that the transcriptional activity of OsbZIP46 may be mainly regulated by ABA-dependent phosphorylation.

Specificity of OsbZIP46 Compared to Other ABF Members

OsbZIP46 is member of the third (or ABF/AREB/ABI5) subfamily of bZIP transcription factors. Although most of the ABF members are thought to be involved in ABA and abiotic stress responses according to the studies in Arabidopsis, the results of OsbZIP46 suggest it has some specificity in addition to its common features compared to other ABF members.

At the protein sequence level, most members in the third subfamily of bZIP family, including OsbZIP46, contain a typical basic region and leucine zipper domain and five other conserved motifs (named domain A, B, C, D and E, respectively) that are predicted to contain five phosphorylation sites, suggesting that most members of the ABF subfamily maybe involved in stress or ABA signaling through phosphorylation by the protein kinase. Moreover, the third subfamily bZIP members of rice and Arabidopsis could be further classified into four groups (I-IV) according to a phylogenetic analysis (Xiang et al., 2008). OsbZIP46 belonged to group II, which included the previously reported stress or ABA-related rice bZIP members (TRAB1, OsbZIP23, and OsABI5) and has the highest similarity to the ABF1–4 of Arabidopsis (Hobo et al., 1999; Xiang et al., 2008; Zou et al., 2008).

However, our results showed that the expression of OsbZIP46 is inducible by drought, heat, H2O2, and ABA treatment and repressed by cold, but apparently not affected by salt stress. These results suggest a different expression profile compared to OsbZIP23, in which expression is inducible by drought, salt, and ABA treatment but not
cold (Xiang et al., 2008). We compared the expression profile of OsbZIP46 across various tissues and stages with its homology members by searching the expression profiles database CREP (Wang et al., 2009) and found that their expression patterns were also different (Supplemental Fig. S3). The OsbZIP46-overexpression rice showed unexpectedly reduced drought tolerance, which is distinct from the OsbZIP23-overexpression rice that showed significantly increased drought and salt stress tolerance (Xiang et al., 2008). This may suggest that different members of the third bZIP subfamily may function in stress tolerance with a different spectrum of stresses or different degrees to the same stress. To support this, we compared the transcriptomes of the OsbZIP46 (OsbZIPCA1 as well) and OsbZIP23-overexpression plants and found that the genes affected by overexpression of the two ABF members are largely different (only less than a quarter of the regulated genes are overlapped) (Supplemental Fig. S9 and Table S7). Furthermore, unlike OsbZIP23 and most other members in this subfamily, the native form OsbZIP46 has no transactivation activity in yeast. This further suggests that the activity of OsbZIP46 in planta may strongly rely on the posttranslational modification. However, considering that the domain D sequence and putative phosphorylation target sites also exist in other ABF members, it remains intriguing to identify which part of the domain D or which phosphorylation target sites determines the specificity of stress responses.

CONCLUSION

We characterized a constitutive active form of transcription factor OsbZIP46. Comparison analysis revealed that OsbZIP46 is a positive regulator of ABA signaling and regulates drought stress resistance of rice by modulating many stress-related genes and its function on activating downstream genes depends on posttranslation modification. This constitutive active form of OsbZIP46 has promising usefulness in transgenic breeding of drought resistance.

MATERIALS AND METHODS

Generation of Transgenic Rice Plants

The full-length cDNA of OsbZIP46 was obtained from a cDNA library of Minghui 63
(Oryza sativa L. ssp. indica) and confirmed by sequencing with primer SP6
(5\'-ATTTAGGTGACACTATA-3\') and T7 (5\'-TAATACGACTCACTATAGGG-3\'). The sequence-confirmed clone containing the full-length cDNA of OsbZIP46 was
digested by KpnI and BamHI and cloned into the KpnI and BamHI sites of the binary
expression vector pCAMBIA1301U (driven by a maize ubiquitin promoter). The
construct was introduced into Zhonghua11 (Oryza sativa L. ssp. japonica) by
Agrobacterium-mediated transformation. To create the overexpression construct of
OsbZIP46CA1, the OsbZIP46CA1 coding region was PCR amplified with KpnI-BamHI
linker primers: 5\'-ATAggtaccATGGAGTTGCCGGCGGATG-3\' and
5\'-ATAggatccTCAGCATGGACCAGTCAGTG-3\' based on the template of a truncated
fragment of OsbZIP46 with an internal deletion. The sequence-confirmed PCR fragment
of OsbZIP46CA1 was also cloned into pCAMBIA1301U and then transformed into
Zhonghua11.

Stress Treatment of Plant Materials
To check the expression level of the OsbZIP46 gene under various abiotic stresses or
phytohormone treatment, the Zhonghua11 rice plants were grown in the soil (for drought, cold, and heat stress) or hydroponic culture medium (for others) for about 3 weeks under
normal conditions. The seedlings at the 4-leaf stage were treated with abiotic stress,
including drought stress (removing the water supply), salt stress (200 mM NaCl), heat
stress (exposing plants to 42°C), cold stress (seedlings were transferred to a growth
chamber at 4°C), and oxidative stress (treated with 1% H2O2 solution), followed by
sampling at the designated time. For phytohormone treatment, 0.1 mM abscisic acid
(ABA), brassinosteroid, indole-3-acetic acid, kinetin, gibberellic acid, and jasmonic acid
were sprayed on the leaves and added to the culture medium of the rice plants,
respectively.

To test the ABA sensitivity or osmotic stress tolerance of transgenic plants at the
seedling stage, positive transgenic lines were selected by germinating seeds on 1/2MS
medium containing 25 mg/L hygromycin; the negative transgenic lines and the wild type
(WT) were also germinated on normal 1/2MS medium. The seedlings (12 plants each, 3
repeats) were transplanted to normal1/2MS medium or 1/2MS medium containing 3 µM
ABA or 150 mM mannitol and grown for 14 days. For testing the ABA sensitivity of
transgenic plants at the germination stage, the positive T3 transgenic families, negative
transgenic lines, and the WT (30 seeds each, 3 repeats) were germinated on 1/2MS
medium containing gradient concentration of ABA (0, 1, 3, 6 µM) and the germination
rate of the treated seeds was calculated after 13 days.

To test the other abiotic stress tolerances of transgenic plants, the positive transgenic
lines were also germinated on hygromycin-containing medium. For testing at the seedling
stage, positive transgenic and WT plants (30 plants each, 3 repeats) were grown in a
half-and-half manner in the barrels filled with sandy soil. At the 4-leaf stage, stress
testing was conducted, including drought (irrigating was withheld for 1 week followed by
recovery for 1 week) and salt (irrigated with 200 mM NaCl solution for 1 week). Drought
testing at the reproductive stage was conducted in polyvinyl chloride (PVC) pipes; the
details of drought treatment and trait measurement for these tests were basically the same
as described previously (Yue et al., 2006; Xiao et al., 2007). One positive transgenic plant
and one WT plant each was planted per PVC tube (10 plants each). Drought stress was
initiated at the panicle development stage by discharging water plants and the plants were
recovered with irrigating at the flowering and seed maturation stages.

To detect the water loss rate under dehydration conditions, leaves of positive
transgenic plants, negative transgenic control, and the WT plants were cut and exposed to
air at room temperature and weighed at designated times.

Quantification of Gene Expression
Total RNAs of the rice leaves were extracted using the TRIzol reagent (Invitrogen)
according to the manufacturer’s instructions. The DNase-treated RNA was
reverse-transcribed using SuperScript™ reverse transcriptase (Invitrogen) according to
the manufacturer’s instructions. Real-time quantitative PCR was performed on an optical
96-well plate with an ABI PRISM 7500 real-time PCR system (Applied Biosystems)
using SYBR Premix Ex Taq™ (TaKaRa). The PCR thermal cycles were: 95°C for 10 s
and 45 cycles at 95°C, 5 s; 60°C for 34 s. Rice Actin1 gene was used as the endogenous
control and the relative expression levels were determined as described previously (Livak
and Schmittgen, 2001). For RNA gel blotting, 15 μg total RNA from each sample was
separated on a 1.2% agarose gel containing 1% formaldehyde and then transferred onto a nylon membrane. Hybridization and washing conditions were based on standard protocols (Sambrook et al., 1989).

**Subcellular Localization**

To determine the subcellular localization of OsbZIP46, OsbZIP46 was cloned into pM999-33 vector to produce a OsbZIP46-GFP fusion construct driven by a Cauliflower Mosaic virus 35S promoter (35S::OsbZIP46:GFP). Rice protoplasts prepared from etiolated shoots were co-transformed with 35S::OsbZIP46:GFP and 35S::Ghd7:CFP as a nuclear marker by polyethylene glycol treatment. The fluorescence signal was observed through a confocal microscope (Leica) at 24 hours after transformation.

**Transactivation and Two-Hybrid and One-Hybrid Assays in Yeast**

The single or multiple amino acid substitutions (Ser/Thr to Asp) of OsbZIP46 were produced with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). The procedure was performed according to the instruction manual. For the transactivation assay, the intact, point-mutated, or truncated fragments of OsbZIP46 were fused in frame with the yeast GAL4 DNA-binding domain in the vector pDEST32 (Invitrogen). Fusion proteins were expressed in yeast cells MaV203 (MATα; leu2–3,112; trpl–901; his3Δ200; ade2–101; gal4Δ; gal80Δ; SPAL10::URA3; GAL1::lacZ; HIS3UASGAL1::HIS3@LYS2; can1R; cyh2R) (Invitrogen). The colony-lift filter assay (X-gal assay) was performed according to the manufacturer’s manual (Invitrogen).

The yeast two-hybrid assay was performed using the ProQuest Two-Hybrid System (Invitrogen). The coding regions of OsbZIP46 and SAPKs were cloned into the vector pDEST32 and pDEST22 using the Gateway™ technology (Invitrogen) to generate bait and prey vector, respectively. The two vectors were co-transformed into the yeast strain MaV203 and the transformants were identified according to the manufacturer’s instructions.

For the one-hybrid assay, fusion protein of OsbZIP46 and the GAL4 activation domain were produced in the vector pGADT7-Rec2 (Clontech). Then the fusion protein was co-transformed with the reporter vector (pHIS2-cis) into yeast cells Y187 (MATα;
ura3-52; his3-200; ade2-101; trp1-901; leu2-3, 112; gal4Δ; gal80Δ; met–;
URA3::GAL1 UAS-GAL1 TATA-LacZ; MEL1 (Clontech). The DNA–protein interactions
were determined by the growth of the transformants on the nutrient-deficient medium.
The detailed procedure conducted was referred to the manufacturer’s manual (Clontech).

**Bimolecular Fluorescence Complementation**

Rice SAPK genes were cloned into the pVYCE vector and fused to the C-terminal
156–239 amino acids of YFP, and OsbZIP46 was cloned into pVYNE vector and fused to
the N-terminal 1–155 amino acids of YFP. Combinations of BiFC constructs were
expressed transiently in rice leaf protoplasts via PEG transformation. The fluorescence
was detected by confocal microscope (Leica).

**Protein Phosphorylation Assay**

GST-fused OsbZIP46 (or OsbZIP46CA1) and OsSAPK genes were constructed into
vector pGEX-6P (GE Healthcare) and were expressed in *E. coli* (BL21). GST-fusion
protein production was induced by isopropyl-β-D-thiogalactoside (IPTG, 0.1 mM) for
overnight at 18°C. Bacterial lysates were applied to glutathione sepharose™ (GE
Healthcare) and GST-fusion proteins were eluted with 10 mM glutathione by following
the manufacturer’s instruction. For the kinase assay, SAPK and OsbZIP46 proteins were
incubated in kinase assay buffer (50 mM Tris-HCl, 10 mM MgCl2, 10mM MnCl2, 1mM
DTT, 0.2mM ATP, 1 µCi γ-32P-ATP). The reaction was incubated at room temperature
for 30 min and terminated by the addition of 5 µL of sample buffer and heating at 100°C
for 5 min. After separation on a 12% SDS-PAGE gel, the gel was exposed to Kodak
X-ray film for detecting the protein phosphorylation.

**Microarray Analysis**

Three independent *OsbZIP46*-overexpression lines (30 positive transgenic plants each),
three independent OsbZIP46CA1-OE lines (30 positive transgenic plants each), and the
WT (two independent biological replicates, 30 plants each) were sampled for microarray
experiments. The process of microarray analysis was conducted according to the standard
protocol of Affymetrix GeneChip service (CapitalBio). The differentially expressed
genes (up- or down-regulated) between the overexpression transgenic plants and the control (the WT) were selected with a significance threshold of $P < 0.01$ and analyzed with Excel add-in and molecule annotation system (MAS) 3.0 (http://bioinfo.capitalbio.com/mas3/) (Supplemental Tables S1–S7). The results were then confirmed by real-time quantitative RT-PCR.

ACKNOWLEDGMENTS
We thank Jian Xu and Lei Wang (Huazhong Agricultural University) for providing plasmid pM999-33 and 35S::Ghd7:CFP, respectively.

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**Figure legends**

**Figure 1.** Expression analysis of OsbZIP46 under stress and hormone treatments. A, Expression level of OsbZIP46 under stresses including drought (time course 0 d, 3 d, 5, d, recover 12 h), salt (0 h, 3 h, 6 h, 12 h), cold (0 h, 3 h, 6 h, 12 h), heat (0 h, 2 h, 6 h, 12 h), and H2O2 (0 h, 3 h, 6 h, 12 h). B, Expression level of OsbZIP46 under phytohormone treatments including JA (0 h, 1 h, 3 h, 6 h) and ABA, IAA, GA, KT, and BR (0 h, 3 h, 6 h, 12 h). Error bars indicate SE based on 3 replicates. C, Distribution of major stress related cis-elements in the promoter region of OsbZIP46. JA, jasmonic acid; GA, gibberellic acid; KT, kinetin; BR, brassinosteroid.

**Figure 2.** Increased ABA sensitivity of OsbZIP46-overexpression plants at germination and seedling stages. A, Germination performance of OsbZIP46 overexpresser (OE15) and WT seeds on 1/2MS medium containing 0, 1, 3, 6 μmol/L ABA, respectively, at 6 d after initiation. B, Calculation of the germination rates of OsbZIP46 overexpressers (OE2, OE13, and OE15), negative transgenic lines (CK9 and CK15) and WT seeds. Error bars indicate SE based on 3 replicates. C, Performance of two independent OsbZIP46-overexpression transgenic lines (OE2 and OE15) and two negative transgenic lines (CK2 and CK15) in MS medium containing 3 μM ABA. D and E, Shoot and root lengths of the lines grown on normal (D) or ABA-containing medium (E). The shoot and root lengths were counted after growth for 14 d (n ≥ 9 each). Error bars indicate standard deviation.

**Figure 3.** Drought tolerance testing of OsbZIP46-overexpression transgenic rice. A, Phenotype of seedlings of OsbZIP46-overexpression plants under drought stress. Seedlings from four overexpression lines (OE2, OE9, OE13, and OE15) were grown in barrels each with wild-type (CK) as control. B, Survival rates of OsbZIP46-overexpression plants and wild type after drought stress. Error bars indicate SE based on 3 replicates. C, Phenotype of OsbZIP46-overexpression plants and WT plants in drought stress at flowering stage. Three overexpression lines (OE2, OE9, and OE15) were grown in PVC pipes each with WT (CK) plant as control. D, Relative spikelet fertility of the plants after drought stress at flowering stage. Error bars indicate
standard deviation (n=10). E, The expression level of $RAB21$ in the $OsbZIP46$-overexpression plants (OE) and WT (CK) under normal conditions or being treated with exogenous ABA.

Figure 4. Transactivation assay of OsbZIP46. A, The expression construct of $OsbZIP46$ in yeast. B, Transactivation assay of truncated OsbZIP46. Fusion proteins of the GAL4 DNA-binding domain and different portions of OsbZIP46 were checked for their transactivation activity in the yeast strain Mav203. FL indicates the full-length of OsbZIP46; dC1–dC6 and CA1 indicate the mutated forms of OsbZIP46. The results of colony-lift filter assay are shown at the right. + and − indicate positive and negative, respectively, for the transactivation activity.

Figure 5. ABA sensitivity and drought tolerance testing of $OsbZIP46CA1$-OE rice. A, Phenotype of native $OsbZIP46$ overexpression lines (FL-OE9 and FL-OE15) and $OsbZIP46CA1$-OE (CA1-OE5 and CA1-OE7) and WT under medium containing 3 μM ABA. B, Phenotype of seedlings of $OsbZIP46CA1$-OE plants (CA1-OE1 and CA1-OE7) and WT under drought stress. Seedlings before (upper) and after (lower) drought stress treatment are shown. C, Survival rate of $OsbZIP46CA1$-OE and WT plants after drought stress. Error bars indicate SE based on 3 replicates. D, Phenotype of $OsbZIP46CA1$-OE plants under drought stress at flowering stage. E, Relative yield of $OsbZIP46CA1$-OE (CA1-OE1, CA1-OE5, and CA1-OE7) and WT plants after drought stress at flowering stage. Error bars indicate standard deviation (n=10). F, Water loss rates in the leaves cut from the OsbZIP46CA1 overexpressor (CA1-OE1 and CA1-OE3), negative transgenic line control (NCK), and WT plants. Error bars indicate SE based on 3 replicates.

Figure 6. Gene chip analysis of $OsbZIP46$ overexpressors. A and B, Scatter plots of the expression profiles of whole-genome genes in FL-OE (A) and CA1-OE (B) compared against the WT. The X and Y axes indicate the chip hybridization signal in the overexpressor and WT, respectively. The red and green dots indicate the probe sets with signal ratios of OE/WT >2 and <0.5, respectively. C, Venn diagram for proportions of genes affected by overexpression of $OsbZIP46$ native form and $OsbZIP46CA1$. FL-up
and FL-down indicate genes up- and down-regulated, respectively, in OsbZIP46-OE. CA1-up and CA1-down indicate genes up- and down-regulated, respectively, in OsbZIP46CA1-OE. D, Drought-responsive patterns of all the differentially regulated genes in the two overexpressors. The symbol * indicates genes with the same regulated pattern between CA1-OE and FL-OE. Group I genes are up-regulated in CA1-OE; group II genes are down-regulated in CA1-OE; group III genes are up-regulated in CA1-OE but down-regulated in FL-OE; group IV genes are down-regulated in FL-OE; and group V genes are up-regulated in FL-OE. E, The differentially regulated patterns in the two overexpressors revealed by gene chip analysis are validated by real-time quantitative RT-PCR.

Figure 7. Interaction between OsbZIP46 and SAPKs and OsbZIP46 may be activated by phosphorylation. A, Yeast two-hybrid assays of OsbZIP46 and SAPK members. Control A and control C indicate the positive and negative control, respectively. SC-LTH, synthetic complete-Leu-Trp-His medium; 3-AT, 3-amino-1,2,4-triazole. B, Confirmation of the interaction of OsbZIP46 and SAPK2/SAPK6/SAPK9 by BiFC (Bimolecular fluorescence complementation). C, In vitro phosphorylation assay of OsbZIP46 by SAPK kinases. Molecular weight marker is indicated on the right. The arrow indicates the position of the GST-fused intact OsbZIP46 protein (60.7kD). D, Single or multiple amino acid substitutions (Ser/Thr to Asp) of OsbZIP46 by site-directed mutagenesis. WT, the native OsbZIP46; PA (phosphorylation active), the mutated forms of OsbZIP46. The hollow circles indicate the putative phosphorylation target sites, and the solid circles indicate the mutated sites in the mutated forms of OsbZIP46. E, Transactivation assay of OsbZIP46 mutants in yeast. X-gal assay and His test result are shown. CK− and CK+ are negative and positive controls, respectively.

Captions of supplemental data set

Supplemental Figure S1. Sequence analysis of OsbZIP46. A, Schematic exon-intron structure of the OsbZIP46 gene. B, Schematic bZIP domain and five other conserved motifs of the OsbZIP46 protein. C, Comparison of domain D sequence of OsbZIP46 with
several previously reported stress-related bZIP genes in rice and Arabidopsis. D, 
Sequence LOGO view of the consensus domain D sequences based on the 11 sequences 
presented in the panel C. The height of the letter (amino acid) at each position represents 
the degree of conservation.

Supplemental Figure S2. Subcellular localization analysis of OsbZIP46 in rice 
protoplast. A, 35S::GHD7:CFP and 35S::OsbZIP46::GFP were co-transformed into 
etiolated shoot protoplasts of rice. OsbZIP46-GFP fusion protein indicates nuclear 
subcellular localization in rice protoplast. B, 35S::GFP was transformed as control.

Supplemental Figure S3. Expression profile of OsbZIP46 and its homologous genes in 
various tissues and at different stages.

Supplemental Figure S4. Overexpression of OsbZIP46. A, Overexpression construct of 
OsbZIP46 for rice transformation. LB, left border; HPT, Hygromycin phosphotransferase; 
35S, cauliflower mosaic virus 35S promoter; Ubi, maize ubiquitin promoter; GUS, 
β-glucuronidase; RB, right border. B, Expression level analysis of OsbZIP46 in 
transgenic plants with northern blot. Ten overexpression lines were identified from 17 
transgenic plants produced. WT, wild type.

Supplemental Figure S5. Overexpression of OsbZIP46CA1. A, Overexpression 
construct of OsbZIP46CA1 for rice transformation. LB, left border; HPT, Hygromycin 
phosphotransferase; 35S, cauliflower mosaic virus 35S promoter; Ubi, maize ubiquitin 
promoter; GUS, β-glucuronidase; RB, right border. B, Expression level analysis of 
OsbZIP46CA1 in transgenic plants with real-time quantitative RT PCR. Eight 
overexpression lines were identified from 20 transgenic plants produced. WT, wild type.

Supplemental Figure S6. Osmotic stress tolerance testing of OsbZIP46 and 
OsbZIP46CA1 transgenic rice. A, Phenotype of native OsbZIP46 overexpressor 
(FL-OE15) and OsbZIP46CA1 overexpressor (CA1-OE1, CA1-OE9) and WT under 
normal condition (upper) or osmotic stress with 150 mM mannitol (lower). B, Relative
Supplemental Figure S7. Real-time quantitative RT-PCR for validating the result of gene chip. A, The expression level of OsbZIP46 in the overexpressors. CA1-OE5 and CA1-OE7 indicate the OsbZIP46CA1 overexpressors; FL-OE9 and FL-OE15 indicate the native OsbZIP46 overexpressors. B, Validation of the expression of the differential expressed genes in the overexpressors. The locus ID of genes checked are shown in the upper right of the figure. WT-1 and WT-2 are two biologic replications of the WT plants.

Supplemental Figure S8. Identification the putative target genes of OsbZIP46 by yeast one-hybrid assay. A, The schematic structure of the yeast expressed construct pGAD-OsbZIP46 and the reporter construct pHIS2-cis (cis indicates the fragment of candidate promoter containing ABRE cis-element). B, The two constructs were co-transformed into yeast strain Y187. The transformants were examined by growth performance on SD/-Leu/-Trp/-His plates in the absence or presence of 3-AT. OTG1, OTG2, and OTG3 (OsbZIP46 target gene 1, 2, 3) indicate the pGAD-OsbZIP46 plus pHIS2-cis (promoters of Os11g26790/RAB21, Os03g19290, Os05g38290 in pHIS2, respectively). CK+, positive control (p53HIS2 plus pGAD-53); CK−, negative control (p53HIS2 plus pGAD-OsbZIP46).

Supplemental Figure S9. Venn diagram shows proportion of genes regulated by OsbZIP46 overexpressors (native form FL-OE and constitutive active form CA1-OE) and OsbZIP23 overexpressor (OsbZIP23-OE) and the degree of overlapping. Up-regulated (left) and down-regulated (right) indicate the genes up- or down-regulated in the overexpressors, respectively.

Supplemental Figure S10. Germination performance of OsbZIP46 overexpressers (OE2 and OE13) and negative transgenic lines (CK9 and CK15) on 1/2MS medium containing 0, 1, 3, 6 μmol/L ABA, respectively.

Supplemental Figure S11. Transactivation assay of truncated OsbZIP46 without the D
domain (OsbZIP46-dD). A, The schematic fusion protein of the GAL4 DNA-binding
domain and OsbZIP46-dD. B, Transactivation activity of the fused protein in the yeast
strain Mav203 (colony-lift filter assay). CK+ and CK− indicate positive and negative
controls, respectively

Supplemental Table S1. Gene ontology analysis of the differential expressed genes

Supplemental Table S2. Up-regulated genes in transgenic rice plants overexpressing
OsbZIP46CA1

Supplemental Table S3. Down-regulated genes in transgenic rice plants overexpressing
OsbZIP46CA1

Supplemental Table S4. Up-regulated genes in transgenic rice plants overexpressing
native OsbZIP46

Supplemental Table S5. Down-regulated genes in transgenic rice plants overexpressing
native OsbZIP46

Supplemental Table S6. Genes that are differentially regulated by native OsbZIP46 and
OsbZIP46CA1 overexpression and contains enriched cis-element ABRE

Supplemental Table S7. Genes similarly regulated in both the OsbZIP23 and OsbZIP46
overexpression rice