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Basic leucine zipper transcription factor OsbZIP16 positively regulates drought resistance in rice

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ABSTRACT

Abiotic stress has been shown to limit the growth, development, and productivity of crops. Here, we characterized the function of a rice bZIP transcription factor OsbZIP16 in drought stress. Expression of *OsbZIP16* was dramatically induced under drought conditions. Transient expression and transactivation assays demonstrated that OsbZIP16 was localized in the nucleus and had transactivation activity. At both the seedling and tillering stages, transgenic rice plants overexpressing *OsbZIP16* exhibited significantly improved drought resistance, which was positively correlated with the observed expression levels of *OsbZIP16*. Representative downstream drought-inducible genes were observed to have significantly higher expression levels in transgenic rice plants than in the wild type plants under drought conditions. *OsbZIP16* was shown to be induced by exogenous ABA treatment, while overexpression of *OsbZIP16* was observed to make transgenic plants more sensitive to ABA than wild type plants were. Transcriptome analysis identified a number of differentially expressed genes between wild type plants and plants overexpressing *OsbZIP16*, many of which are involved in stress response according to their gene ontologies. Overall, our findings suggest that OsbZIP16 positively regulates drought resistance in rice.

1. Introduction

Abiotic stresses, such as drought, high salinity and extreme temperature, seriously hamper the yield of crops all over the world, especially in developing countries that lack the technical and financial resources with which to combat these strains. Crops with higher abiotic stress resistance can adapt to substandard growth conditions, and thus contribute to increases in food production overall. Plants can initiate a variety of changes, including those at the molecular, cellular and physiological levels, to survive under stress. All of these adaptive processes, however, universally include changes in the expression of specific genes [1–3]. The products of these genes, in turn, have a variety of functions, ranging from regulating gene expression to acting as end effectors of stress resistance [4,5].

The concentration of phytohormone abscisic acid (ABA) has been observed to increase significantly under drought and high salinity stress conditions, resulting in major changes in both gene expression and the adaptive physiological response of plants [6,7]. Studies have shown, however, that exogenous application of ABA can only induce the expression of some of the genes known to be induced under drought stress [5,8–10]. This fact suggests the existence of both ABA-dependent and ABA-independent signal transduction pathways between the initial stress signals and the induction of specific stress-inducible genes in plants.

Transcription factors regulate almost every aspect of the plant life cycle by regulating the expression of specific genes, including the stress-responsive genes. Basic leucine zipper (bZIP) transcription factors make up one of the largest transcription factor families in plants. These transcription factors have been shown to play a variety of roles in many essential plant life processes [11–14]. In the dicotyledonous model plant *Arabidopsis* (*Arabidopsis thaliana*), 75 bZIP transcription factor genes have been designated (*AtbZIP1–AtbZIP75*) and classified into ten groups according to the sequence similarity of their basic region [15]. So far, however, only a few *Arabiodpsis* bZIP transcription factors have been functionally analyzed. For example, HY5, which mediates light response in *Arabidopsis*, is one of the few bZIP transcription factors that have been most thoroughly characterized to date [16,17]. Likewise, ATB2 has

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been shown to participate in both sucrose-specific sugar sensing and the signaling system associated with the transport and utilization of metabolites in *Arabidopsis* [18]. Furthermore, several members of the group A bZIP transcription factors in *Arabidopsis* have been shown to be involved in ABA-dependent stress signal transduction, and have thus been designated as ABA-responsive element binding proteins (AREBs), also known as ABRE binding factors (ABFs) [8,19–22]. Similarly, a recent analysis has identified 89 bZIP transcription factors in *indica* variety IR64, one of the monocotyledonous model plant rice (*Oryza sativa*) [23]. All of these bZIP transcription factors have been classified into 11 groups according to their DNA-binding specificity and the amino acid sequences in their basic and hinge regions [23]. To date, several rice bZIP transcription factors have been functionally characterized, including those shown to be responsive to abiotic stress. For example, *OSBZ8* has been shown to be strongly induced by ABA, and its expression pattern has been observed to positively correlate with salt resistance in rice [24,25]. Similarly, the expression of *LIP19* has been shown to be induced by low temperature, and *LIP19* functions as a molecular switch for low-temperature signal transduction in rice [26]. Previous research has also demonstrated that overexpression of *OsbZIP23* or *OsbZIP72* enhances the drought resistance of rice, and that these two transcription factors are involved in ABA-dependent drought signal transduction [13,27].

A global genome expression analysis of rice in response to drought stress has shown that *OsbZIP16* is strongly induced by drought stress [28]. Recent microarray data has also revealed that 26 rice bZIP transcription factor genes, including *OsbZIP16*, are up-regulated to be at least 2-fold under drought stress [23]. Furthermore, among these 26 genes, the expression level of *OsbZIP16* was observed to undergo the most significant change under drought conditions. These results have thus led us to further investigate the role(s) of OsbZIP16 in response to drought stress.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatments

The rice wild type plant utilized was Zhonghua 11 (ZH11). The rice seeds were first immersed in distilled water for 24 h, and then transferred to a growth chamber (30 °C, 50–60% relative humidity) with a 14 h light/10 h dark photoperiod. Six days later, the seedlings of transgenic rice and ZH11 were transferred to Yoshida's culture solution [29] with and without 1‰ hygromycin, respectively. The presence of 1‰ hygromycin was used to screen for positive transgenic plants. For dehydration treatment at the seedling stage, the water on the roots of the two-week-old seedlings was completely blotted with absorbent paper. The seedlings were then immediately transferred to dry flasks. After nine hours of dehydration, the dehydrated seedlings were recovered using a fresh culture solution. For the soil drought experiment, the rice seedlings were planted in PVC pots with holes at the bottom. Each pot contained the same weight of soil. After 25 days of growth in a greenhouse at a temperature of 24–30 °C and relative humidity of 50–60%, water was removed from the bottom of the PVC pots until the leaves curled completely (about 5 days). Water was then added for rehydration. For salt and ABA treatment, the two-week-old seedlings were transferred into culture solutions with final concentrations of 200 mM NaCl and 100 μM ABA, respectively.

2.2. Phylogenetic analysis

Multiple protein sequence alignments were performed using ClustalX 2.0 [30], while a phylogenetic tree was constructed by MEGA4.0.2 using a neighbor-joining method [31]. A bootstrap analysis was performed using 1000 replicates in MEGA to evaluate the reliability of the tree.

2.3. Generation of transgenic rice plants

To produce plants overexpressing *OsbZIP16*, the full-length CDS of *OsbZIP16* was amplified by RT-PCR using primers with *Xba* I and *Sal* I sites. The sequence-confirmed full-length CDS of *OsbZIP16* was then cloned into the *Xba* I-*Sal* I site of the binary expression vector pCactF to produce fusion proteins with 3× flag (Fig. 3A). The overexpression of *OsbZIP16* was driven by rice *Actin1* promoter. The final construct was introduced into *japonica* rice cultivar Zhonghua 11 via agrobacterium-mediated transformation [32]. Hygromycin was used to screen for positive transgenic plants. The primers are listed in Supplemental Table S1.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was isolated from the leaves of two-week-old rice seedlings with a TRIzol reagent (Invitrogen). The remaining potential genomic DNA contamination in the total RNA sample was eliminated by RNase-free DNase I (Takara). The total RNA was then used as a template for first-strand cDNA synthesis. ReverTra Ace -α- (Toyobo) was used to synthesize first-strand cDNA. Quantitative real-time PCR was performed on a Chromo 4™ Real-time PCR Detector (Bio-red) with SYBR Premix Ex Taq ™ (Takara). The cycling conditions were 30 s at 95 °C followed by 40 cycles of amplification (95 °C for 5 s, 60 °C for 30 s). Rice *Actin1* (TIGR ID: LOC_Os05g36290) was used as an endogenous control for normalization. The $\Delta\Delta$Ct method was used to analyze the data of real-time PCR [33]. The primers are listed in Supplemental Table S1. Three experiments using independently grown plant materials were performed in order to confirm the reproducibility of the results.

2.5. Subcellular localization and transactivation assay

The full-length CDS of *OsbZIP16* was cloned into the *Bgl* II-*Xba* I site of vector pRTL2-mGFP [34] in order to produce GFP-OsbZIP16 fusion proteins. In accordance with the particle bombardment procedure previously performed by Chen et al. [35], the plasmid pRTL2-mGFP-OsbZIP16 was introduced into onion cell layers. After bombardment, the onion cell layers were incubated at 22 °C for 24 h in the light, and then were stained with DAPI (4′,6-diamidino-2-phenylindole). Images were recorded using a fluorescence microscope.

The transactivation assay was performed using the vector pDEST32 (Invitrogen) and yeast strain AH109 (Clontech). The full-length CDS of *OsbZIP16* and its three DNA fragments (1–180 bp, 181–360 bp, 361–513 bp) were cloned into pDEST32 using the Gateway LR Clonase™ II Enzyme Mix (Invitrogen). All the constructs were transformed into yeast strain AH109. Yeast transformation was carried out as described in the Yeastmaker™ Yeast Transformation System 2 User Manual (Clontech). The transformants were serially diluted, and then dropped on -Leu/-His/-Ade SD (synthetic dropout) selection media. The plates were sealed with parafilm membranes and incubated upside down at 30 °C until colonies appeared (3–6 days). The primers are listed in Supplemental Table S1.

2.6. ABA sensitivity test

To compare the difference in ABA sensitivity between wild type and transgenic rice plants overexpressing *OsbZIP16*, rice seeds were sterilized with 75% ethanol, immersed in distilled water, and then transferred to a growth chamber. One-week-old seedlings with

a consistent shoot height (1 cm) were selected, and then transferred to fresh culture solutions with different final concentrations of ABA. After one week of treatment, the shoot heights of twenty rice seedlings were measured. This process was repeated two more times in order to confirm the reproducibility of the results.

2.7. cDNA library preparation and transcriptome sequencing

The two-week-old wild type and transgenic rice seedlings were grown under either normal conditions or three hours of dehydration. The seedlings were then collected and their total RNA was extracted for transcriptome sequencing. First-strand cDNA synthesis was performed by SuperScript™ II RT (Invitrogen) using random hexamer primers. Second-strand cDNA was synthesized using *E. coli* DNA polymerase I (Invitrogen). The paired-end cDNA library used for RNA sequencing was prepared according to Illumina's protocols. RNA sequencing was performed on a HiSeq 2000 platform. Transcriptome reads were mapped to the rice reference genome sequence (MSU 6.1) with tophat1.3.1 using the program's default parameters [36]. Mapped RNA-Seq reads were assembled into transcripts by Cufflinks [37]. Analysis of differential gene expression was performed using Cuffdiff. The genes with Benjamini-Hochberg corrected *P* values less than 0.05 were defined as differentially expressed. The differentially expressed genes were then categorized into gene ontologies using agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) [38]. Gene overrepresentations in the GO categories were calculated using a fisher test, using the criteria that the FDR *P*-values < 0.05. To visualize gene expression across 4 pairwise comparisons (ZH11-3 and ZH11-0, ox23-3 and ox23-0, ox23-0 and ZH11-0, and ox23-3 and ZH11-3), a heat map was generated using Treeview software (http://rana.lbl.gov/EisenSoftware.htm). The hierarchical clustering was created using the default complete linkage method included in the Cluster software package [39].

3. Results

3.1. Identification and sequence analysis of the bZIP transcription factor OsbZIP16

OsbZIP16 (TIGR ID: LOC_Os02g09830) is one of the bZIP transcription factors predicted by the MSU rice genome annotation project (http://rice.plantbiology.msu.edu). The 877 bp genomic sequence and the 513 bp coding sequence of *OsbZIP16* were cloned by PCR and RT-PCR, respectively. Both the DNA-binding specificity and amino acid sequence similarities observed in the basic and hinge regions of OsbZIP16 during prior analyses have led researchers to classify OsbZIP16 as a member of the group IV rice bZIP transcription factors [23]. Group IV, which has 22 members, is the largest group of bZIP transcription factors observed in rice, and can be divided into two subgroups (Fig. 1A). To date, the genes of seven members of this group have been shown to respond to abiotic stress [23,26]. Phylogenetic analysis has revealed that 14 *Arabidopsis* bZIPs have evolutionary homology with rice group IV bZIPs (Fig. 1A). A few of these AtbZIPs have previously been studied in their functions. AtbZIP63, for example, has been shown to be an important node in the glucose-ABA interaction network, and may also participate in the fine-tuning of ABA-mediated, sugar dependent, abiotic stress responses [40]. When we compared the OsbZIP16 protein sequence against the Pfam database [41], the bZIP domain and NLS (nuclear localization signal) were the only significant domains observed (Fig. 1B). An online motif scan tool (http://hits.isb-sib.ch/cgi-bin/PFSCAN) revealed three conserved phosphorylation sites, which have been shown to be related to the ABA response and stress signaling [8,42–47] (Fig. 1B). Both

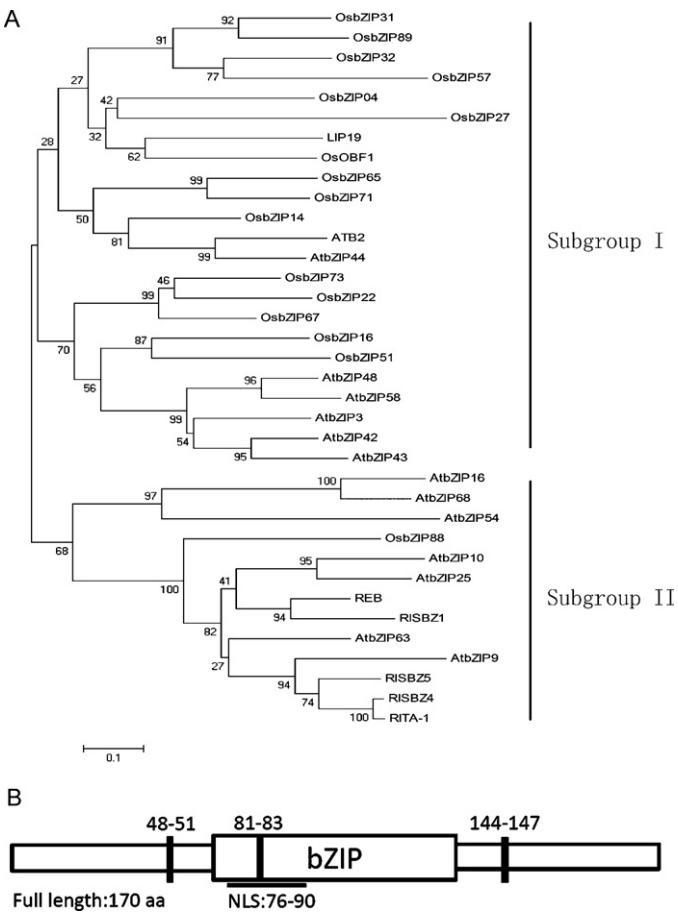

Fig. 1. Phylogenetic tree of bZIPs in rice and *Arabidopsis* and the protein sequence analysis of OsbZIP16. (A) The phylogenetic tree of rice group IV and orthologous *Arabidopsis* bZIPs. The alignment of protein sequences was performed using ClustalX 2.0, and the phylogenetic tree was constructed by MEGA4.0.2 using the neighbor-joining method. The numbers at each node indicate the percentage of bootstrap values from 1000 replicates. (B) The predicted domains and motifs of OsbZIP16. "aa" indicates amino acid. bZIP indicates basic leucine zipper domain. NLS indicates nuclear localization signal. Three vertical black lines represent conserved phosphorylation sites. The numbers indicate the amino acid positions of these domain and motifs.

OsbZIP16 and the other previously characterized members of rice group IV bZIPs were observed to be highly conserved within the basic leucine zipper region. Low homology was observed amongst these transcription factors elsewhere, however (Supplemental Fig. S1). This characterization of rice group IV bZIP transcription factors greatly contributes to our understanding of how the members in this group are able to fulfill such a wide variety of functions in rice [26,48–50].

3.2. Expression profiles of OsbZIP16 under several typical abiotic stresses

Real-time quantitative PCR (qPCR) was performed to investigate the expression of *OsbZIP16* in different rice tissues and organs under normal growth conditions. Transcripts of *OsbZIP16* were widely detected in the rice plants at both the seedling and heading stages (Fig. 2A). The mature leaves were observed to have the highest expression level of *OsbZIP16*. Both our previous genome analysis and other groups' recent microarray data have shown *OsbZIP16* to be up-regulated under drought and high salinity stress [23,28]. Given these results, *OsbZIP16* may be involved in the physiologic pathways that aid rice in adapting to less than habitable growth environments. In order to further study the expression

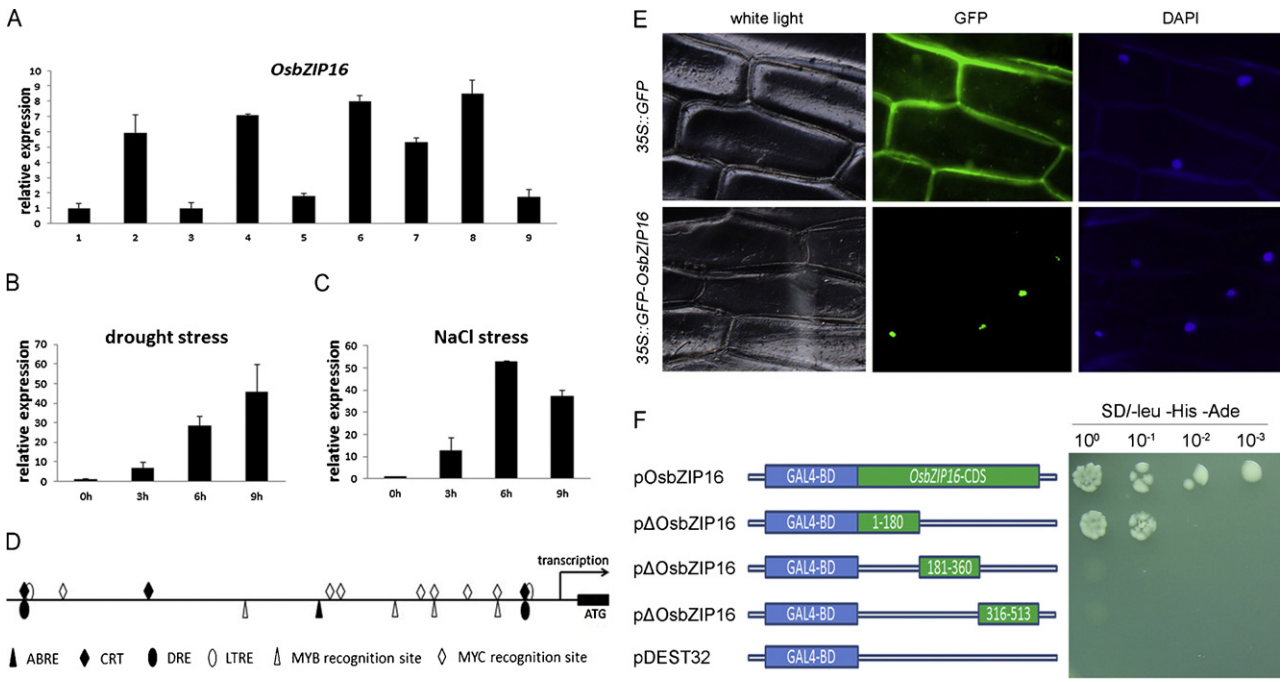

Fig. 2. The expression profiles, subcellular localization and transactivation activity assay of OsbZIP16. (A) The expression profiles of *OsbZIP16* in different tissues and organs under normal conditions. 1–4: root, culm, sheath and leaf at the seedling stage, respectively; 5–9: root, culm, sheath, leaf and panicle at the heading stage, respectively. (B) The induction profiles of *OsbZIP16* under drought stress. The error bars indicate standard deviations derived from three independent biological experiments. (C) The induction profiles of *OsbZIP16* under high salinity (200 mM NaCl) stress. The error bars indicate standard deviations derived from three independent biological experiments. (D) Distribution of several stress-related *cis*-elements in the promoter region (−1.5 kb) of *OsbZIP16*. (E) Subcellular localization of OsbZIP16. The bottom panels show the localization of GFP-OsbZIP16 in onion cells in a transient assay, while upper panels show the localization of GFP as a control. The same cells were stained by DAPI to visualize the nucleus positions. (F) Transactivation activity assay of OsbZIP16 in yeast strain AH109. The left panel displays schematic diagrams of various constructs used for the transactivation activity assay. pOsbZIP16 and pΔOsbZIP16 show the constructs inserted with the full-length CDS of *OsbZIP16* and its three fragments (nucleotide positions were labeled in the diagrams), respectively. Vector pDEST32 without exogenous fragment insertions was used as a negative control.

profiles of *OsbZIP16* under abiotic stress, we used qPCR to investigate the relative expression level of *OsbZIP16* induced by drought and high salinity stress in rice seedlings over time. The expression of *OsbZIP16* was rapidly induced within three hours under both drought and high salinity stress (Fig. 2B and C). The expression level of *OsbZIP16* under drought stress was observed to rapidly increase throughout the nine-hour testing period (Fig. 2B), while it was observed to plateau at the sixth hour under high salinity stress (Fig. 2C). Thus, these stress-induced expression profiles of *OsbZIP16* suggest that *OsbZIP16* is involved in rice's response to abiotic stress.

The increased expression levels of *OsbZIP16* observed under drought and high salinity stress led us to exam its promoter region (1500 bp upstream from transcription start site). As expected, a comparison of *OsbZIP16*'s promoter sequence with the online database PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) revealed that several putative stress-responsive elements, such as ABRE (ABA-responsive element, one hit) [19,51], DRE (dehydration responsive element, two hits) [52], LTRE (low temperature responsive element, two hits) [53], CRT (C-repeat element, three hits) [54], MYBRS (MYB recognition site, four hits) [55,56] and MYCRS (MYC recognition site, seven hits) [55,56], were indeed present (Fig. 2D). This analysis, combined with our experimental results, strongly suggests that OsbZIP16 has broad functions in abiotic stress signal transduction in general and may play a prominent role in rice's response to drought stress in particular.

3.3. *OsbZIP16 is localized in the nucleus and exhibits transactivation activity*

Although bioinformatics studies have predicted OsbZIP16 to be a putative transcription factor, there has been no experimental verification of its functionality to date. Consequently, we set out to determine whether OsbZIP16 possessed typical transcription factor characteristics. A GFP-OsbZIP16 fusion construct driven by a CaMV35S promoter was generated to determine the subcellular localization of OsbZIP16. A transient expression experiment showed that the fusion proteins only existed in the nucleus of onion epidermal cells (Fig. 2E).

To further analyze whether this nucleus-localized protein actually had transactivation activity, the full-length CDS of *OsbZIP16* and its three DNA fragments (1–180 bp, 181–360 bp, 361–513 bp) were introduced into the vector pDEST32. pDEST32 harbors a GAL4 DNA-binding domain (BD), and these generated constructs can express OsbZIP16- or ΔOsbZIP16-BD fusion proteins in yeast. These constructs were transformed into yeast strain AH109, serially diluted, and then dropped on -Leu/-His/-Ade SD selection media. The results confirmed that the full-length OsbZIP16 had transactivation activity, and showed that its N-terminal polypeptide was the primary contributor to this activity (Fig. 2F).

3.4. *Overexpression of OsbZIP16 enhances drought resistance in rice*

A number of transgenic rice plants overexpressing *OsbZIP16* under the control of a strong constitutive promoter were produced (Fig. 3A), and phenotype screens were performed to test the regulation of OsbZIP16 during the rice drought response. Seedlings of several transgenic rice lines exhibited a more complete recovery after quick dehydration than the wild types did (Fig. 3B). In the tillering stage, the plants of these transgenic lines also showed stronger resistance to drought stress than the wild type plants did (Fig. 3C). In order to determine whether this increase in drought resistance was actually the result of *OsbZIP16* overexpression, six

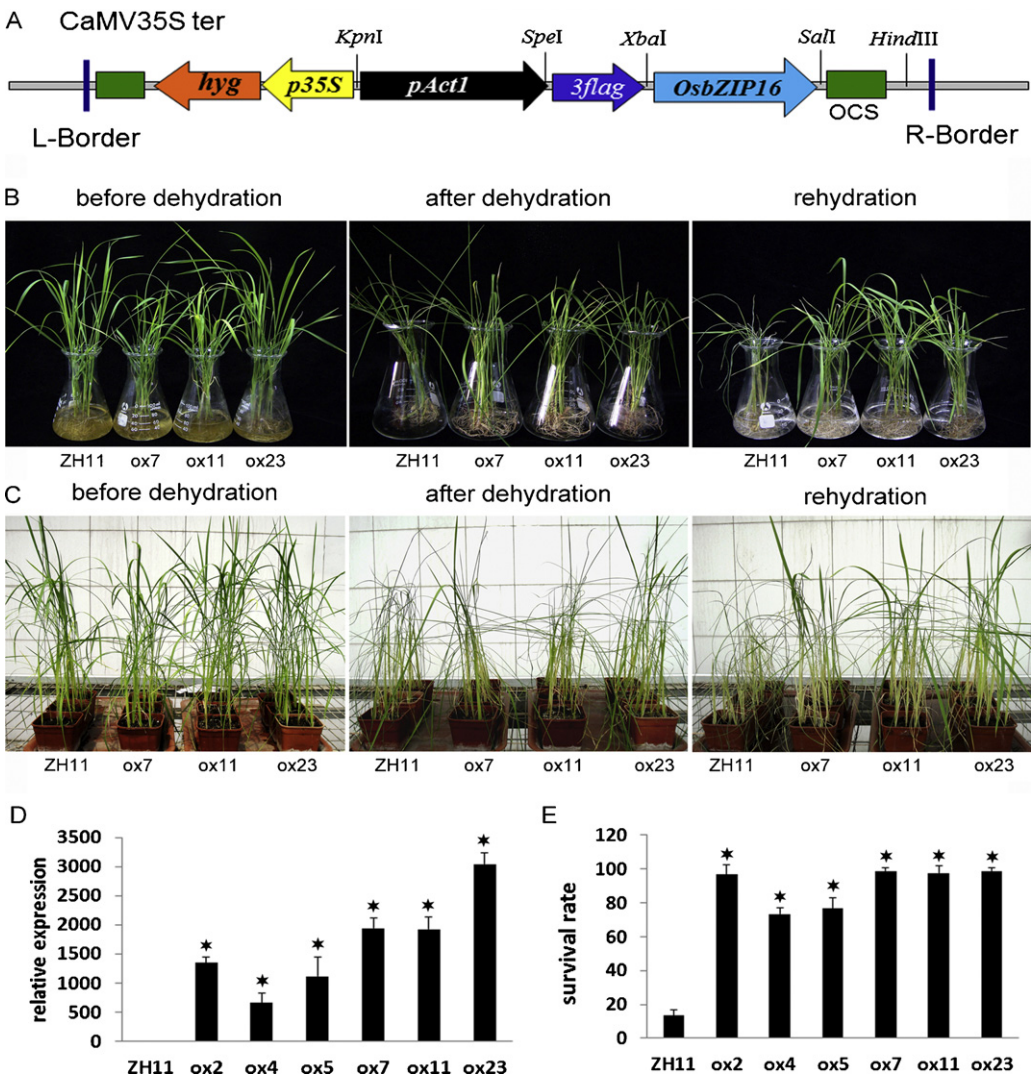

Fig. 3. Overexpression of *OsbZIP16* enhanced drought tolerance in rice. (A) The schematic diagram of the T-DNA region of construct pCactF-OsbZIP16. The full-length CDS of *OsbZIP16* was inserted into a *Xba* I-*Sal* I site. (B) Dehydration tolerance test among wild types and transgenic lines at the seedling stage. ZH11 is the wild type plant. The names of the transgenic lines were expressed as ox plus line numbers. Rice plants were grown in culture solution. (C) Drought tolerance test among wild type plants and transgenic lines at the tillering stage. Rice plants were planted in soil. (D) The expression levels of *OsbZIP16* in wild type and transgenic plants under normal conditions. The error bars indicate standard deviations derived from three independent biological experiments. (E) The survival rate statistics of wild types and transgenic lines after dehydration. After rehydration, rice plants with recovered leaves were considered to have survived. Standard deviations (triplicates; $n = 30$ each) are indicated. The stars in Fig. 3D and E indicate the highly significant differences ($P < 0.01$) between wild type and transgenic plants. The statistical analysis was performed using independent-samples *T* test.

transgenic lines with a better survival rate than wild type rice were used to further test the correlation between the expression level of *OsbZIP16* and drought resistance capability. As expected, all of these transgenic lines with high *OsbZIP16* expression levels showed dramatically increased survival rates compared with wild type plants (Fig. 3D and E). Furthermore, the transgenic lines (ox2, ox7, ox11, ox23) with higher expression levels of *OsbZIP16* exhibited higher survival rates than the transgenic lines (ox4, ox5) with lower expression levels (Fig. 3D and E). These results indicate that there is a positive correlation between the expression level of *OsbZIP16* and the plants' drought survival rate.

In order to gain further insight into the mechanisms driving the enhanced drought resistance observed in transgenic plants overexpressing *OsbZIP16*, the expression levels of three drought-inducible genes, *LEA7* (TIGR ID: LOC_Os03g62620) [57], *RAB21* (TIGR ID: LOC_Os11g26790) [58] and *RAB16D* (TIGR ID: LOC_Os11g26780) [57,59], were analyzed in wild type and transgenic plants under both normal and drought stress conditions. While the observed expression levels of these three genes in the transgenic and wild type plants were quite similar under normal conditions, all of these genes showed significantly higher expression levels in transgenic plants than in wild type plants under drought conditions (Fig. 4). Thus, it appears that *OsbZIP16* may positively mediate the transcript levels of some drought-resistant genes under drought conditions.

3.5. Overexpression of OsbZIP16 makes rice seedlings hypersensitive to ABA

Given the existence of both ABA-dependent and ABA-independent drought resistance pathways in higher plants [60] and the presence of an ABRE element in the promoter sequence of *OsbZIP16* (Fig. 2D), we next compared the responsiveness of wild type and transgenic rice plants overexpressing *OsbZIP16* to ABA treatment. Wild type and transgenic seedlings (ox7, ox11, ox23) with consistent shoot length (1 cm) were transferred into a series of culture solutions, each containing a different concentration of ABA. After one week of growth, the transgenic seedlings were observed to have significantly shorter shoot lengths than

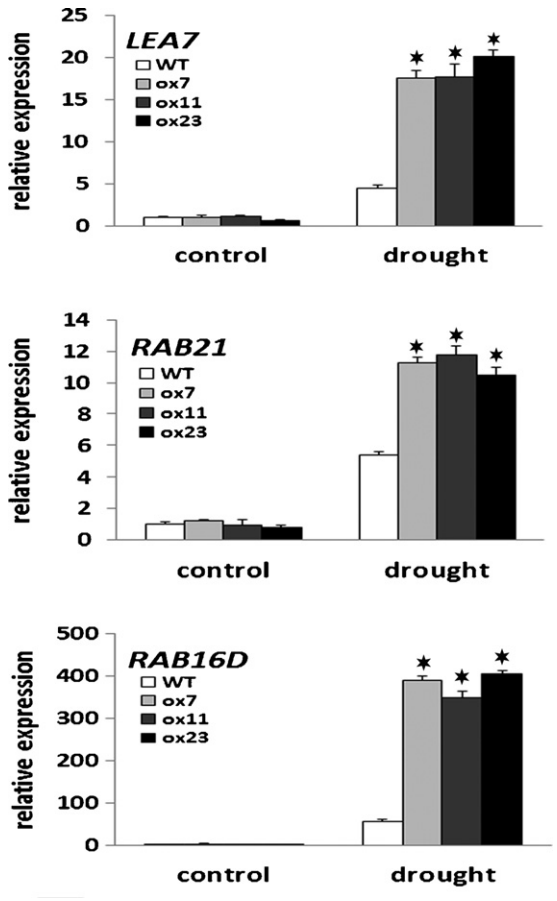

Fig. 4. The expression analyses of drought-inducible genes in wild type and transgenic rice plants under normal and drought conditions. The leaves were sampled from two-week-old seedlings under normal conditions (control) and six-hour dehydration conditions (drought), respectively. The error bars indicate standard deviations derived from three independent biological experiments. The stars indicate the significant differences ($P < 0.05$) between wild type and transgenic plants under drought conditions. The statistical analysis was performed using independent-samples *T* test.

the wild type plants had (Fig. 5A and B). This suggests that, in comparison to wild type plants, overexpression of *OsbZIP16* makes transgenic rice hypersensitive to ABA. In addition, when wild type rice seedlings were treated with exogenous ABA, the expression of *OsbZIP16* was observed to increase quickly and dramatically (Fig. 5C). These results thus suggest that *OsbZIP16* may be involved in the ABA signaling pathway in rice.

3.6. Expression analysis of ABA signaling marker genes in transgenic rice

In order to further specify whether OsbZIP16 affects ABA signaling, four genes (*SAPK10*, *PP2C*, *LEA3-1* and *RAB16C*) involved in ABA signal transduction were selected for an expression level comparison between wild type and transgenic plants. *SAPK10* (TIGR ID: LOC_Os03g41460) belongs to the SnRK2 (sucrose nonfermenting1-related protein kinase 2) protein kinase family, and *PP2C* (TIGR ID: LOC_Os09g15670) belongs to the protein phosphatase 2C protein family, both of which are upstream genes in the ABA signaling network, which responds to drought stress [61–65]. In contrast, *LEA3-1* (TIGR ID: LOC_Os05g46480) and *RAB16C* (TIGR ID: LOC_Os11g26760) both belong to the LEA (late embryogenesis abundant) protein family, which has been shown to be downstream genes in the ABA signaling pathway [57,58,66].

No difference in the expression levels of the two upstream genes, *SAPK10* and *PP2C*, was observed between the wild type and transgenic plants under either normal or drought conditions (Fig. 5D and E). In contrast, although the two downstream genes, *LEA3-1* and *RAB16C*, were observed to have no significant difference in expression levels under normal conditions, they showed much higher expression levels in transgenic plants than in wild type plants under drought conditions (Fig. 5F and G). These results suggest that *OsbZIP16* most likely participates in the ABA signaling pathway in rice.

3.7. Global expression changes in OsbZIP16 overexpression plants after drought treatment

RNA sequencing was performed in order to further elucidate the mechanisms of improved drought resistance mediated by the overexpression of *OsbZIP16*. Transgenic line ox23 was selected as sequencing material due to the fact that it was observed to have the highest *OsbZIP16* transcription level and the highest drought survival rate of all the transgenic lines (Fig. 3D and E). Wild type ZH11 was used as a control. Real-time PCR results confirmed that the accuracy of the RNA sequencing data was acceptable (Supplemental Figs. S2–S4, Table S2).

When the plants were dehydrated, 1629 and 2329 genes were found to be differentially expressed (P value < 0.05 and fold change > 2) in the wild type and transgenic plants, respectively, including many stress-related genes. The differentially expressed genes were all clustered (Fig. 6A) and functionally classified according to their Gene Ontologies (Supplemental Table S3). Two different groups of genes are thought to potentially play key roles in the OsbZIP16 regulated drought response. The first group of genes (Group 1) showed similar expression levels in both the wild type and transgenic plants under normal conditions, but were induced or reduced by drought in transgenic plants significantly more than in the wild types (Fig. 6A). The change in expression level of these genes after drought treatment was observed to be dependent on *OsbZIP16* overexpression. The second group of genes (Group 2) was observed to be differentially expressed between the wild type and transgenic plants under normal conditions, but showed similar expression levels under drought conditions (Fig. 6A). These group 2 genes may be influenced by *OsbZIP16* overexpression even under normal conditions in order to help the plant prepare to resist drought stress when it is encountered.

Although the transgenic plants shared 1114 differentially expressed genes in common with the wild type plants under drought stress, the transgenic specimens also had 1215 differentially expressed genes that were not detected at significant levels in the wild type samples (Fig. 6B). Also, we found 1065 differentially expressed genes between the wild type and transgenic plants under normal conditions, while there were 649 genes under drought conditions ($P < 0.05$) (Fig. 6B, Supplemental Fig. S5).

As shown in Fig. 6C, many of the differentially expressed genes known to function in redox reactions, catalytic activities, and transcription regulation were shown to be enriched in both the transgenic and wild type plants. In contrast, genes associated with antioxidant activity and peroxidase activity were enriched in transgenic plants, but not in the wild types (Fig. 6C). Several stress-related biological processes, including lipid metabolic process, lipid transport, lipid localization, response to water and response to oxidative stress, were significantly overrepresented in the transgenic plants when compared with biological processes in their wild type counterparts ($P > 0.05$ in wild types but $P < 0.05$ in transgenic plants) (Fig. 6D). These data thus suggest that the expression

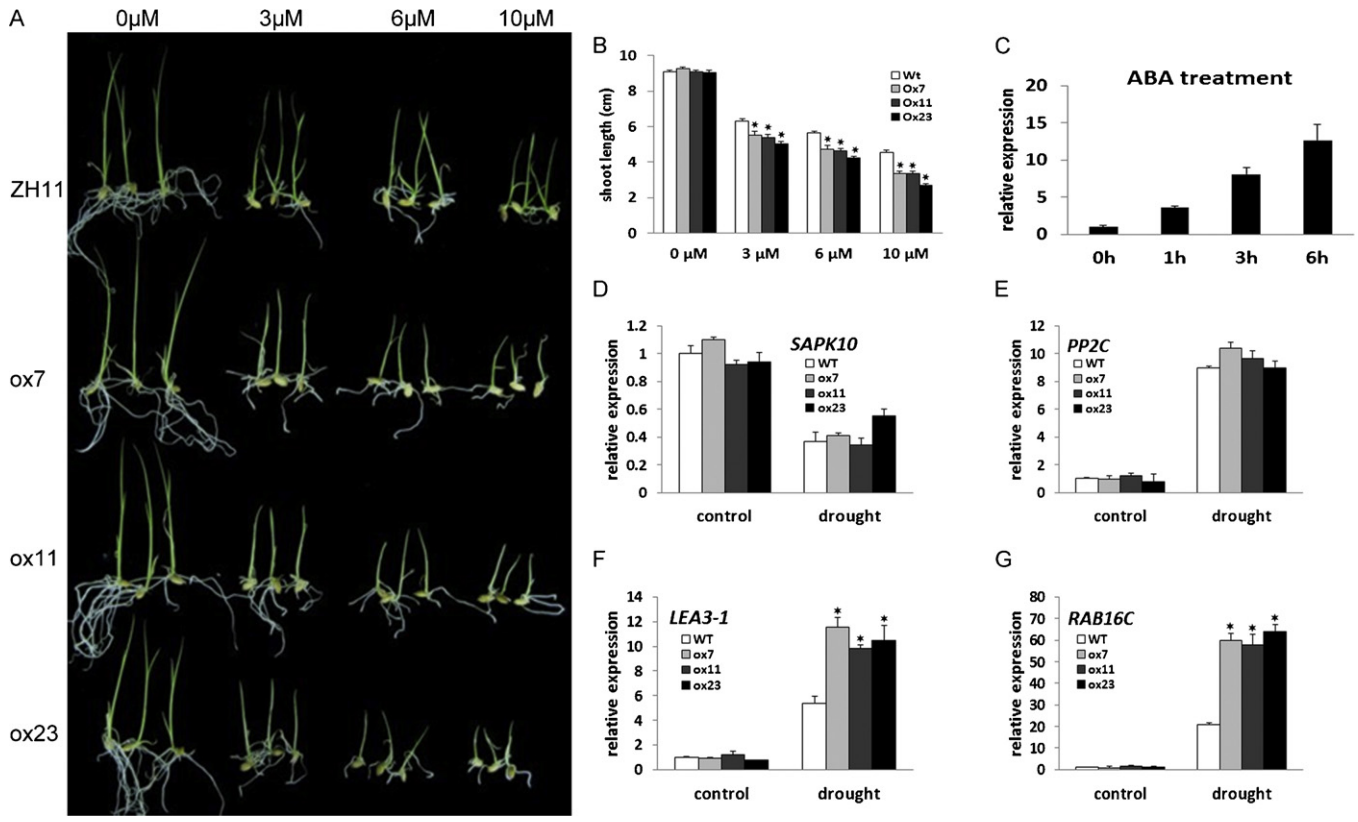

Fig. 5. OsbZIP16 involvement in ABA-dependent drought stress signal transduction. (A) The phenotype analysis of rice seedlings grown in culture solutions containing different concentrations of ABA. (B) Shoot length measurements of the rice plants shown in panel (A). Error bars indicate the standard deviations ($n = 20$). The stars indicate the highly significant differences ($P < 0.01$) of shoot lengths between wild type and transgenic plants. The statistical analysis was performed using independent-samples *T* test. (C) The induction profiles of *OsbZIP16* under 100 μM ABA treatment in wild type plants. The error bars indicate standard deviations derived from three independent biological experiments. (D–G) The expression analyses of four genes involved in ABA signaling. The error bars indicate standard deviations derived from three independent biological experiments. The stars indicate the significant differences ($P < 0.05$) of genes' relative expression levels between wild type and transgenic plants under drought conditions. The statistical analysis was performed using independent-samples *T* test.

of many stress-related genes was altered to enhance the drought resistance of the transgenic plants.

4. Discussion

OsbZIP16 has previously been classified as a member of the group IV bZIP transcription factors in rice [23]. Group IV, consisting of 22 members, is the largest bZIP transcription factor group found in rice. The group IV members studied to date show a variety of possible functions in the physiological processes of rice [26,48–50], while seven of them have been shown to respond specifically to abiotic stress [23,28]. Protein sequence alignments of these functionally studied group IV members have shown that their protein sequences have low homology outside of their bZIP regions (Supplemental Fig. S1). This might, in turn, explain the great diversity observed in the group IV bZIP transcription factors' documented functions.

After sequence analysis, several kinds of stress-responsive elements were found in the promoter of *OsbZIP16* (Fig. 2D). ABRE, DRE, MYBRS and MYCRS can, respectively, be recognized by the presence of AREB, DREB, MYB and MYC transcription factors. These transcription factors, in turn, have been shown to be involved in both ABA signaling and a variety of stress responses [56,67]. The enrichment of these stress-responsive elements in the *OsbZIP16* promoter sequence may thus explain why *OsbZIP16* expression was responsive to stress. A transactivation activity assay in yeast indicated that the N-terminal polypeptide of OsbZIP16 is necessary and sufficient for promoting transcription (Fig. 2F). This result is in agreement with a number of previously published studies [13,27,68,69].

Transgenic plants overexpressing *OsbZIP16* showed significantly improved drought resistance at both the seedling and tillering stages when compared with wild type plants (Fig. 3B and C). Furthermore, higher transcription levels of *OsbZIP16* were observed to lead to higher survival rates under drought stress conditions (Fig. 3D and E). Although dramatically increased levels of OsbZIP16 were observed in transgenic plants under both drought and normal conditions, transgenic plants overexpressing *OsbZIP16* were observed to possess higher transcription levels of some previously documented drought-inducible marker genes only under drought conditions (Fig. 4). Given that OsbZIP16 harbors three conserved phosphorylation sites, the higher expression of these genes may be dependent on the OsbZIP16's transcriptional activation activity that requires ABA-mediated phosphorylation under drought stress. If this is the case, it is also likely that OsbZIP16 positively mediates the expression of stress-responsive genes in transgenic plants under drought conditions, a role that has been shown to be common to many stress-responsive bZIP transcription factors. [8,42–47]. Overall, these results suggest that OsbZIP16 positively regulates drought resistance of rice.

Our data demonstrated that overexpression of *OsbZIP16* made transgenic plants more sensitive to exogenous ABA treatment than wild type plants were (Fig. 5A and B). This result, combined with the presence of an ABRE element in *OsbZIP16* promoter region and the observed induction of *OsbZIP16* following ABA application (Figs. 2D and 5C), led us to hypothesize that *OsbZIP16* may be involved in ABA signaling. Under drought conditions, the expression levels of two ABA signal pathway genes, *LEA3-1* and *RAB16C*, were shown to be much higher in transgenic plants than in wild

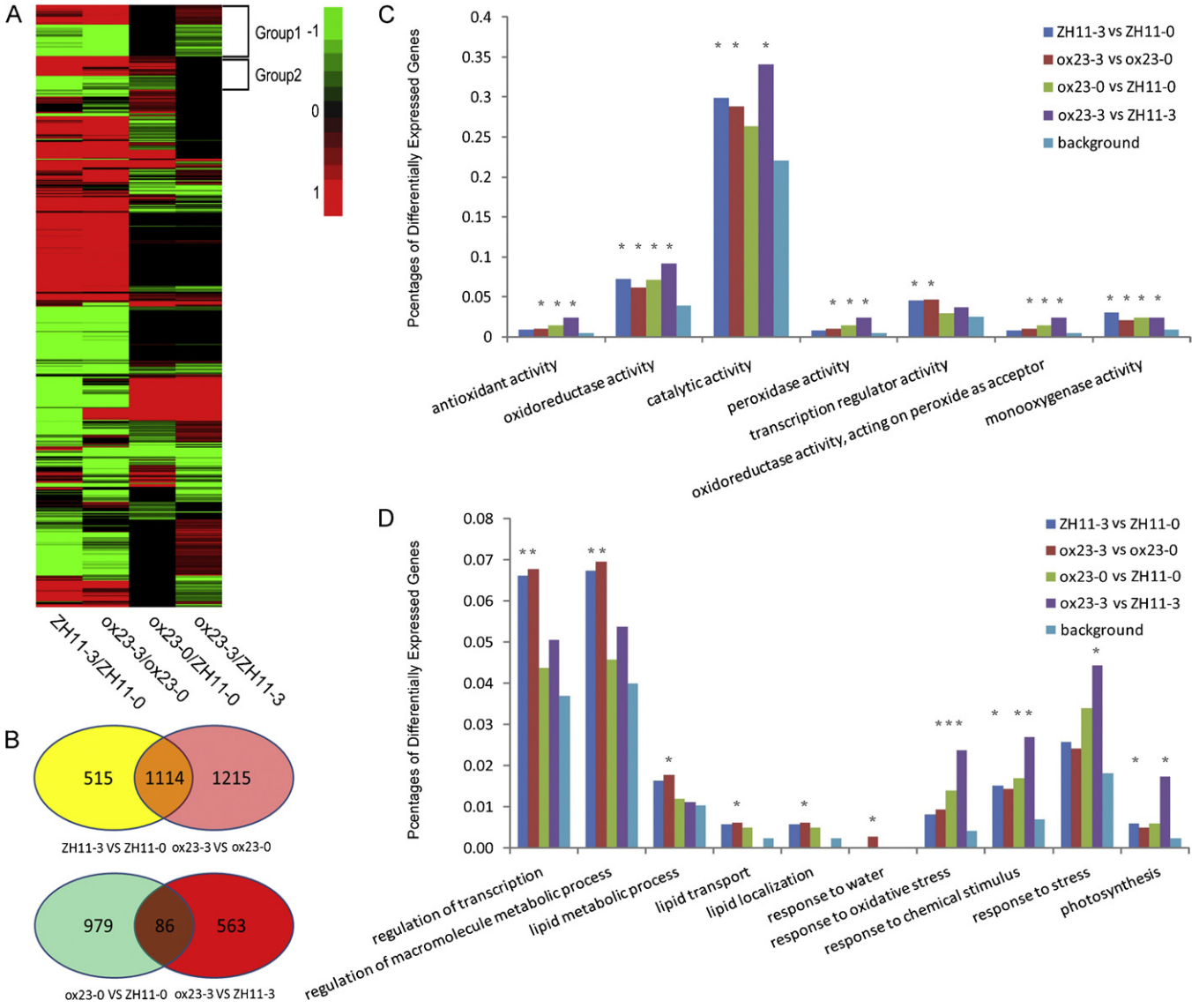

Fig. 6. The analyses of differentially expressed genes obtained from RNA sequencing data. (A) Heatmap of differential expression genes between 4 pairwise comparisons. The median ratio is $\log_2$ transformed and subject to complete linkage hierarchical clustering. ZH11-0 and ZH11-3 represent ZH11 grown under normal conditions or dehydrated 3 h, respectively. Ox23-0 and ox23-3 represent ox23 grown under normal conditions or dehydrated 3 h, respectively. Group 1 includes the genes that were not differentially expressed between wild type and transgenic plants under normal conditions, but were differentially expressed under drought conditions. Group 2 includes the genes that were differentially expressed between wild type and transgenic plants under normal conditions, but not under drought conditions. (B) Venn diagrams of differentially expressed genes. Yellow and pink represent the genes only differentially expressed in wild types and transgenic plants after drought treatment, respectively. Cyan and red represent the genes only differentially expressed between wild type and transgenic plants under normal and drought conditions, respectively. Orange represents the differentially expressed genes commonly observed in wild type and transgenic plants under drought conditions. Brown represents the differentially expressed genes commonly observed between wild type and transgenic plants under both normal and drought conditions. (C) Molecular function and (D) biological process of differentially expressed genes based on Gene Ontology (GO). For clarity, only the most enriched GO terms with fdr *P*-values <0.001 of each pair comparison are given. There were no significant enriched GO terms in cell components. Asterisks indicate significant difference (FDR $P < 0.05$).

type plants (Fig. 5F and G). As previously discussed, this increase in the expression levels of *LEA3-1* and *RAB16C* in the transgenic plants under drought conditions may rely on OsbZIP16's transcriptional activation activity, which, in turn, may be activated by ABA-mediated phosphorylation. Overall, the ABA sensitivity test and expression profiles of the ABA signaling-related genes together suggest that *OsbZIP16* may be involved in ABA signaling.

Although previous research has already identified some drought-inducible bZIP transcription factors within group IV, no functional analysis to date has determined whether these transcription factors play a role in ABA signaling [23,28]. Our data suggests that OsbZIP16 may also positively regulate the ABA response in rice. Previous research has indicated that heterodimers composed of bZIPs from either the same or two different subgroups control gene expression during a variety of stress responses in plants [70–72]. Given these findings, whether OsbZIP16 heterodimerizes with TRAB1-like bZIPs to accomplish drought signal transduction is a question worthy of further study.

Our RNA sequencing data demonstrated that overexpression of *OsbZIP16* can significantly change the expression level of numerous genes. The differentially expressed genes in transgenic plants were enriched in a number of stress-related biological processes, including lipid metabolic processes, lipid localization and lipid transport (Fig. 6D). Cell membranes are major targets of drought stress, and consequently drought stress can result in rapid degradation of the cell membrane and irreversible cell damage in sensitive plants [73]. Lipids are important membrane components. Maintenance of the membrane's appropriate lipid content may help to maintain

membrane integrity [74–76]. This, in turn, suggests that the genes induced or reduced by excessive OsbZIP16 expression may result in greater membrane stability, and thus, higher resistance to drought stress. According to our data, some of the differentially expressed genes showed greater enhancement in antioxidant activity, oxidoreductase activity and oxidative stress response in transgenic plants than in wild types (Fig. 6C and D). Under normal conditions, an overrepresentation of differentially expressed genes known to respond to oxidative stress was observed between wild type and transgenic plants (Fig. 6D). This result suggests that, under normal conditions, transgenic plants may accumulate stress resistance-related proteins early, so as to improve drought resistance for the moment when they eventually encounter drought. Drought stress produces an excessive amount of reactive oxygen species (ROS) that can result in cell damage or even death. This over accumulation of ROS, however, is generally kept under tight control by a versatile and cooperative antioxidant system [77,78]. The details of how these pathways are regulated by OsbZIP16, however, is a topic that will necessitate future research.

Overall, this study starts to investigate the functions of the drought-inducible group IV bZIP transcription factors in rice, and suggests that OsbZIP16 positively regulates drought resistance in rice. There is a possibility that OsbZIP16's contribution to drought resistance in rice is related to the ABA signaling pathway, but the exact mechanism of this connection needs to be flushed out over the course of future research. For example, a future test should include ABA signal pathway mutants in order to test whether OsbZIP16's drought resistance is dependent on ABA signaling. Overall, an analysis of both OsbZIP16's direct target genes and its relationship to other bZIP transcription factors involved in drought resistance may yield more information about how OsbZIP16 contributes to drought resistance in rice. Nevertheless, the fact that overexpression of OsbZIP16 dramatically increases drought resistance of rice suggests that further analyses of OsbZIP16 will have the potential to greatly improve stress resistance of crops [79,80].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2012.05.003.

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