Overexpression of OrbHLH001, a putative helix–loop–helix transcription factor, causes increased expression of AKT1 and maintains ionic balance under salt stress in rice

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The basic helix–loop–helix family of proteins, which function as transcription factors, have been intensively studied in plants and animals. However, the molecular mechanism of these factors contributing to stress tolerance is unknown. Here, we report on the overexpression of OrbHLH001 from Dongxiang wild rice (Oryza rufipogon) conferring salt tolerance in transgenic rice plants. The expression of OrbHLH001 was tissue specific, mainly in phloem tissues throughout the plant. Ion assay with the scanning ion-selective electrode technique showed that NaCl stress has a greater influence on Na+ influx and K+ influx in OrbHLH001-overexpressed plants than the wild type. OrbHLH001 protein can induce the expression of OsAKT1 to regulate the Na+/K+ ratio in OrbHLH001-overexpressed plants by specifically binding to an E-box motif in the promoter region of OsAKT1. The mechanism may have potential use in rice molecular breeding.

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I n t r o d u c t i o n

Salinity is the major environmental stress in rice and affects plant growth and agricultural production worldwide. Salt-stress signal transduction includesionic and osmotic homeostasis signaling pathways (Zhu, 2001, 2002; Xu et al., 2006). Transcription factors such as APETALA2, dehydration responsive element binding factor (DREB), and basic helix–loop–helix (bHLH) families are involved in this regulation network to confer stress tolerance (Chen et al., 2002; Pastori and Foyer, 2002; Seki et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). The factors can bind cis-acting elements in promoters and activate downstream stress-responsive genes under cold, drought and salt stress (Tran et al., 2004; Ma et al., 2009; Zhang and Gan, 2012).

The bHLH protein family of transcription factors have been intensively studied in plants and animals (Toledo-Ortiz et al., 2003; Sonnenfeld et al., 2005). With the genome-wide analysis of the bHLH transcription factor family in plants, 162 bHLH genes in Arabidopsis and 167 in rice were identified (Li et al., 2006). This family is defined by the bHLH signature domain, which is evolutionarily conserved (Murre et al., 1989; Ferre-D’Amare et al., 1994). Plant bHLH proteins bind to the E-box (CANNTG) motif in gene promoters; a consensus core element called G-box (CACGTG) is the most common form (Li et al., 2006).

On exposure to salt stress, the ionic balance (especially Na+/K+) ratio and distribution is the ultimate manifestation of several physiological processes in response to salt stress, including Na+ uptake and exclusion. A rice quantitative trait loci (QTLs), Shoot K+ Concentration 1 (SKC1) functions as a Na+-selective transporter and maintains K+ homeostasis under salt stress (Ren et al., 2005). OsAKT1, another quantitative trait loci (QTLs) controlling K+ uptake into the root and the Na+/K+ ratio in salt-stress has been reported as well (Koyama et al., 2001; Fuchs et al., 2005). Moreover, abundant evidence shows that changes in the expression of K+ transporters and channels can affect the response to reactive oxygen species and phytohormones, so K+ might have a specific regulatory role in plant stress responses (Ashley et al., 2006). A series of genes are induced to protect against stress or further control the expression of other target genes. In addition, some of the ion transporters need to be activated or have their activities enhanced, whereas others, such as some Na+ influx transporters, need to have their activities suppressed (Zhu, 2001). Thus, the overexpression of AKT1 from Puccinellia tenuiflora might enhance salt tolerance in Arabidopsis (Ardie et al., 2010). However, little is known about the genes that regulate ion transporters and affect iron uptake in response to salt stress.

Abbreviations: bHLH, helix–loop–helix; DREB, dehydration responsive element binding factor; GFP, green fluorescent protein; GUS, β-glucuronidase; ICE1, inducer of CBP expression 1; WT, wild type; OE, overexpression line.

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In Arabidopsis, a MYC-like bHLH transcriptional factor inducer of CBF expression 1 (ICE1) can be induced by NaCl, ABA, and cold treatments and its over expression enhances the freezing tolerance by positively regulating CBF3/DREB1A and CBF2/DREB1C transcription (Chinnusamy et al., 2003). Polyubiquitination and proteolysis of ICE1 is mediated by ubiquitin E3 ligase HOS1. During cold acclimation, SUMO E3 ligase SI2 mediates sumoylation of ICE1 and protects ICE1 from proteasomal degradation (Chinnusamy et al., 2007). One of the rice homologs of ICE1, OsICE2/OsHLH001, is induced by cold stress but its mRNA were not (Nakamura et al., 2011). In our previous study, it was found that OrbHLH001 shares the 40% amino acid identity with ICE1 and its transcription was not induced by cold stress in Dongxiang wild rice (O. rufipogon), but its over expression can confer freezing tolerance in Arabidopsis (Zhou et al., 2009; Li et al., 2010). OsICE1/OsHLH002 is another homolog of ICE1 and can be induced by cold stress (Nakamura et al., 2011). Overexpression of OrhHLH002 improves the tolerance to salt stress (Zhou et al., 2009). To better understand the mechanism of the bHLH response to salt tolerance in rice, we cloned OrbHLH001 from Dongxiang wild rice. We found that overexpression of OrbHLH001 enhances tolerance to salt stress by altering ion flux in roots under salt stress.

**Materials and methods**

**Plant materials**

Dongxiang wild rice (Oryza rufipogon Griff.) was used for gene cloning. Zhonghua 10 (Oryza sativa L. ssp. japonica) was used for gene transformation.

**Vector construction and rice transformation**

Total RNA from 14-day old Dongxiang wild rice was isolated with use of TRIzol reagent (Invitrogen, USA); cDNA was reverse transcribed by use of a reverse transcriptase kit (TaKaRa, Tokyo). The open reading frame of OrbHLH001 was amplified by RT-PCR with the primers forward, 5′-GCC TCT TTC TCA GAG CGC TCG GAG GTG-3′ (BamHI site italicized) and reverse, 5′-GCT GAC TAT CCC TCA TAG TGC-3′ (KpnI site italicized). The PCR products were digested with BamHI and KpnI and cloned into the pUN1301 expression vector, which was under the control of a maize ubiquitin promoter (Ge et al., 2004).

The promoter of OsHLH001 was amplified by PCR with the primers forward, 5′-GGG TCT TGG TGT ACC GAC CAC TCT CGG-3′ (BamHI site italicized), and reverse 5′-CCA TCC AAG GTC GCC GGT TCT TCA-3′ (NcoI site italicized). The product was inserted upstream of GUS at the BamHI and NcoI sites of the pCAMBIA1301 vector. Vectors were transformed into rice embryonic calli by transformation with Agrobacterium tumefaciens EHA105 (Ge et al., 2004).

**Quantitative real-time PCR**

Total RNA from wild-type or transgenic rice plants or various rice organs at different developmental stages was extracted by use of TRIzol. Total RNA, 2 μg, was reverse transcribed into cDNA by use of Superscript II (Invitrogen). The real-time PCR amplification involved 20 μL reaction solution containing 5 μL 50-fold-diluted cDNA, 0.2 μM each primer, and 10 μL SYBR Green PCR Master Mix (TaKaRa). Analysis involved the Mx3000P detection system (STRATAGENE). The relative quantification method (Delta–Delta CT) was used to evaluate variation in expression. The primers for gene-specific PCR were for OsACTIN, forward, 5′-ACC ACA GGT ATT GTG TTT GAC TAC-3′ and reverse, 5′-AGA GCA TAT CCT TCA TAG ATG G-3′; OsAKT1, forward, 5′-CAT CAC TGC TCG GGA GGT TCA-3′ and reverse, 5′-GGG TCT TGG TGT ACC GAC CAC TCT CGG-3′; and OrbHLH001, forward, 5′-AGA TCG ACA GAG GTC TTA CTA TCG-3′ and reverse, 5′-GAC TCA AGT TCA TGT TGA AGA TC-3′.

**Subcellular localization of OrbHLH001**

The coding sequence of OrbHLH001 was amplified with the primers forward, 5′-TCT AGA ATG GAC GAG GGC GAC G-3′ (XbaI site italicized) and reverse, 5′-GCT ACC CAT TGC GTG CTG AAG GCC-3′ (KpnI site italicized). The PCR products were digested with XbaI and KpnI and inserted into the pGFP221 vector pre-digested with Xbol and KpnI to create pGFP-OrbHLH001 under the control of a CaMV35S promoter. The construct was used for 2 different experiments. The transient transformation of onion epidermis involved particle bombardment (Varagona et al., 1992). Rice protoplasts were transformed as described (Asai et al., 2000; Bart et al., 2016). The onion cells were cultured on Murashige and Skoog medium in the dark for 24 h. The rice protoplasts cells were incubated at 28 °C in the dark for 14–16 h. All the transformed cells were observed by confocal microscopy (Zeiss LSM, Germany).

**Transcription activation of OrbHLH001**

The cDNA of OrbHLH001 was amplified with the primers forward, 5′-GAA TTC CAT CGA CGG GAG GCC GGC GTG-3′ (EcoRI site italicized), and reverse, 5′-GCT ACG ATC ACC AAA TCA CAC TCA AGC CCT CGG CCT CGT TCT TG-3′ (KpnI site italicized). The PCR products and vector pGBK7tk containing a GAL4 DNA binding domain were digested with EcoRI and BamHI and ligated to create pBD-OrbHLH001. The constructs pBD-OrbHLH001 and plasmid pBD (negative control) were transformed in yeast strain AH109 cells by the lithium–acetate (LiA)-mediated method (Gietz et al., 1992). Transformed yeast cells were selected on SD-Trp medium at 28 °C for 2 days. The X-gal transactivation assay was performed as described (Choi et al., 2004).

**Salt stress treatment in rice**

Seeds of transgenic lines and wild type Zhonghua 10 (WT) were grown on Kimura B solution for 14 days under 12-h light/12-h dark, then submersed in new Kimura B solution containing 150 mM NaCl for 6–7 days. Survival was measured after recovery for 10 days.

**Measurement of net Na⁺ and K⁺ flux**

Net Na⁺ and K⁺ flux was measured noninvasively by the scanning ion-selective electrode technique (SIET) (BIO-001A; Younger USA Sci & Tech. Corp.) (Zonia et al., 2002; Vincent et al., 2005). Wild-type and transgenic rice plants were grown on Kimura B solution for 10 days (Yoshida et al., 1976), then root tips were used for ion flux measurement.

For Na⁺ ion flux measurements, 10-d-old seedlings were transferred for 12 h to new Kimura B solution supplemented with 150-mM concentrations of NaCl or new Kimura B solution without 150 mM NaCl as a control, then roots were rinsed with redistilled water and incubated in measurement solution (0.1 mM KCl, 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.1 mM NaCl, 0.3 mM MES, 0.2 mM Na₂SO₄, 0.1% sucrose, and pH 6.0) for 30 min. The ionic flux was measured by moving the ion selective microelectrode between 2 positions close to the root tips (Sun et al., 2009). The ionic flux was calculated by use of MageFlux (http://xuyue.net/mageflux).

For K⁺ ion flux measurement, roots were incubated in the measuring solution (described above) to equilibrate for 30 min, the K⁺ ion flux measurements were recorded for 3 min. Then NaCl was added to a final concentration of 150 mM NaCl for salt shock treatment. After that, the K⁺ ion flux recording was restarted and
continued further for 20 min. The data measured during the first 3 min after the salt shock was discarded.

Yeast one-hybrid assay

The wild-type or mutated E-box of OsAKT1 promoter tandem repeat sequences (E-box, ATCCAAATGCCA; and mutated E-box, ATCTCGAAACCA) were placed upstream of the minimal promoter in pHiSi-1 vectors. The entire coding region of OrbHLH001 was fused to the pGAD424 vector pre-digested with EcoRI and BamHI (Clontech). pGAD424-OrbHLH001 was then transformed into the yeast strain YM4271, which carries the reporter gene His3 under the control of wild-type or mutated E-box, as described. The transformed yeast cells were selected on SD/-His/-Leu medium containing 0, 15, 30, 45, and 60 mM 3-AT (a competitive inhibitor of the HIS3 gene product) by standard protocols (Clontech).

Results

Molecular characteristics and phenotypes of transgenic rice

We performed a BLAST search of Arabidopsis ICE1 gene homologs in rice in the NCBI database (http://www.ncbi.nlm.nih.gov/blast). The protein AK102594, sharing 39.7% sequence identity with ICE1, was named OsbHLH001 (Li et al., 2006). The cDNA of OrbHLH001 was cloned from Dongxiang wild rice (O. rufipogon). By nucleic acid sequence analysis, OrbHLH001 and OsbHLH001 contain almost the same amino acid identity (Li et al., 2010). Protein secondary structure prediction indicates that the two proteins share the same structure (Supplemental Fig. S1). OrbHLH001 over expression lines (OE) were selected by use of hygromycin and confirmed by β-glucuronidase (GUS) staining. The expression was >30 times higher for OrbHLH001-OE lines than the wild type (WT) (Fig. 1C).

The mRNA levels of OrbHLH001 are specifically induced by salt stress and the protein level of OsbHLH001/OsICE2 is induced by cold (Li et al., 2010; Nakamura et al., 2011). Under normal growth conditions, the WT and OrbHLH001-OE lines do not differ in morphologic or developmental phenotypes (Fig. 1A and B). On exposure to NaCl stress, the transgenic lines displayed a salt-tolerance phenotype as compared with the WT: 14-d-old transgenic seedlings treated with 150 mM NaCl for 1 week showed etiolated leaf tips, whereas almost all wild type leaves were discolored. After recovery for 10 days, the survival was 46% and 33% for OE1 and WT, respectively (Fig. 1F). OrbHLH001 may have conferred salt tolerance in rice.

Na* and K* flux in OrbHLH001 transgenic lines

In general, plants can survive under high concentrations of salt depending on the ability to regulate ionic homeostasis under saline conditions. Among the altered ion relations, the maintenance of a low Na*/K* ratio in cells is a crucial trait for plant salt adaptation (Martinez-Atienza et al., 2007). Rice seedling absorbed Na* from roots and discharged K* under salt stress. We compare the Na*/K* ratio in shoots and roots in WT and OE when they were grown in 150 mM NaCl for 2 days. Under the normal condition, WT and the transgenic lines showed the similar Na*/K* ratio. Under 150 mM
NaCl stress, the Na+ content in root was lower in OE than in WT and K+ content was higher in OE than in WT (Fig. S3), which resulted in the lower Na+/K+ ratio by selective transportation K+ from stems and root to green leaves and absorbed less Na+ in stems and root (Fig. 2A).

To further determine whether OsbHLH001 can affect K+ and Na+ flux under high NaCl content, we used the SIET approach. With 150 mM NaCl for 12 h, the net Na+ efflux ranged from 0 to 250 pmol cm⁻² s⁻¹ in wild type rice roots but from 40 to 500 pmol cm⁻² s⁻¹ in OE1 roots (Fig. 2B and C). After salt shock treatment with 50 mM NaCl for 5 min, K+ flux in OE1 roots exhibited a two-phase response, with an instantaneous large increase in influx (1000–2200 pmol cm⁻² s⁻¹), then a continuous drift of efflux (Fig. 2D); however, in WT roots, K+ flux extended smoothly for a long time. K+ flux was greater in OE1 than WT roots with salt shock (Fig. 2E). SIET assay suggested that OsbHLH001 could maintain lower Na+/K+ ionic balance in saline environments by regulating Na+ efflux with long-term salt exposure and K+ influx with salt shock treatment.

Expression patterns of OsbHLH001 in rice

We used real-time PCR to investigate the expression of OsbHLH001 in various rice organs. OsbHLH001 expression was higher in seedlings than other tissues (Fig. 3A).

To understand the expression patterns of OsbHLH001 in vivo, we generated transgenic rice with a GUS gene expression construct driven by the native promoter of OsbHLH001. GUS signals were detected in all tissues including stem, leaf, root, leaf sheath, pistil, anther and especially in the phloem of stem, leaf, and root (Fig. 3B). The semi-quantitative RT-PCR showed that OsbHLH001 mRNA level was increased in 14-d-old seedlings (Fig. 3D) and the stronger GUS signals were found in roots (Fig. 3C) after 250 mM NaCl treatment. These results suggested that OsbHLH001 is a constitutively expressed gene and may have some potential effects on ions and assimilating transportation.

OsbHLH001 encodes a transcription activator and is localized in the nucleus

To examine the sub cellular localization of OsbHLH001, we fused it with green fluorescent protein (GFP) under the control of a CaMV35S promoter and transformed it into rice protoplasts and onion epidermal cells. Fluorescence image analysis revealed the OsbHLH001-GFP fusion protein was localized in the nucleus in onion cells (Fig. 4A) and rice protoplasts (Fig. 4B). In contrast, the GFP alone was localized at whole cell.

Bioinformatics analysis demonstrated that protein OsbHLH001 contains a conservative bHLH-ZIP transcription factor domain similar to Arabidopsis ICE protein (Fig. 5A), which suggested that the function of OsbHLH001 might be conserved like other bHLH-ZIP transcription factors. To confirm whether OsbHLH001 is a transcriptional factor, we investigated the activation of OsbHLH001 in a yeast GAL4 system. The coding region of OsbHLH001 was fused to the GAL4 DNA-binding domain of the pGKT7 vector, transformed into the yeast strain AH109 and screened on SD/-Trp medium. Compared with the empty vector control, only yeast containing pBD-OsbHLH001 and the positive control showed activity in the galactosidase assay (Fig. 4C).

OsbHLH001 binds to the E-box of the target gene promoter

E-box (CANNTG) is one of the cis-elements that bHLH proteins recognize (Toledo-Ortiz et al., 2003). Alignment analysis revealed that the cis-element (CAAAATG) is ~2296 in the promoter of the putative OsAKT1 gene (GenBank accession no. AK120308) (Fig. 5B). The transcription levels of K+ influx transporters OsHAK1 (Banuelos et al., 2002), OsAKT1 (Fuchs et al., 2005) and Na+ transporter OsHKT1 (Kader et al., 2006) were detected. The real-time PCR results showed that the transcription of OsAKT1 was increased to approximately 3 times the amount in OE1 when compared to that in WT and OsHAK1 and OsHKT1 transcriptional levels, but were not affected by OsbHLH001 overexpression (Fig. 5D).

Yeast one-hybrid assay was used to analyze the E-box binding function of OsbHLH001. Yeast strains transformed with
Fig. 3. Expression pattern of OsbHLH001 in rice organs. (A) Real-time PCR analysis of the expression of OsbHLH001 in root (R), seedlings (S), stem (ST), leaf sheath (LS), and spike (SP). Actin was an internal control. Data are mean ± SD of triplicate experiments. The whole plant was used as the control (100%). (B) GUS expression in OsbHLH001::GUS transgenic rice. (a) stem; (b) stem node; (c) pistil and anther; (d and f) leaf; (g) sheath; (e and h) young root. Bar = 1 mm in (a–f) and 0.1 mm in (g and h). (C) The histochemical staining of OsbHLH001::GUS transgenic roots treated with 250 mM NaCl for 0, 1 and 6 h. (D) The semi-quantitative RT-PCR of OsbHLH001 in wild type Zhonghua 10 after 250 mM NaCl treatment for various times.

pGAD424-OrbHLH001 and E-box-His3 could grow on SD media lacking His and containing 15 mM 3-AT, whereas transformants carrying pGAD424-OrbHLH001 and mE-box-His3 could not grow on the same media (Fig. 5C). Therefore, OrbHLH001 encodes a transcription factor that can specifically bind to E-box motifs in promoter regions of OsAKT1. We observed the phenotype of OE1 and OE4 under low K⁺ (10 μM). The tip of OrbHLH001-overexpression leaves turned dark brown as compared with WT leaves, which

Fig. 4. Subcellular localization and transcription activative of OrbHLH001. (A) Subcellular localization of OrbHLH001 protein in onion epidermal cells. (a–c) OrbHLH001-GFP protein showing signals in nucleus. (d–f) GFP protein alone showing fluorescent signals in nucleus, membrane and cytoplasm. Bar = 50 μm. (B) Subcellular localization of OrbHLH001 protein in rice protoplasts. (a–c) OrbHLH001-GFP protein showing signals in nucleus. (d–f) GFP protein alone showing fluorescent signals in nucleus, membrane and cytoplasm. Bar = 10 μm. (C) Transcription activative analysis of OrbHLH001 protein. (a) Clones were transformed to Whatman filter paper plus X-Gal. (b) Transformants with pBD and pBD-OrbHLH001 grew normally on SD-/Trp medium. (c) Corresponding positions of transformed yeast on the plates.
promoter line with porters

Fig. 5. OrbHLH001 binds to the E-box in OsAKT1 promoter. (A) Alignment of putative DNA binding domain of OrbHLH001 with that of OrbHLH002 and ICE1 proteins; the basic region of bHLH transcription factors determines the E-box (CANNTG) binding specificity. (B) Schematic representation of the E-box and mutated E-box in the OsAKT1 promoter region. E-box: CAXAXTC; mE-box: TGGAAA. (C) Yeast one-hybrid analysis of DNA-binding specificity of OrbHLH001. The yeast strain YM4271 was transformed with the constructs of pAD-OrbHLH001 and pHIS-E-box (upper plates); pAD-OrbHLH001 and pHIS-mE-box (middle plates); pAD-OrbHLH001 and pHISi (bottom plates) and grown with 0 or 15 mM 3-AT. (D) Real-time PCR analysis of the transcription levels of OsAKT1, OsHAK1 and OsHKT1 in wild type (WT) and OrbHLH001-overexpression line (OE). OsACTIN was used as an internal control. (E) Phenotype of WT and OrbHLH001-overexpression rice grown in Kimura B solution containing 1 mM K+ and 10 μM K+.

shows the low-K+-sensitive brown-spot phenotype (Fig. 5E). Overexpression of OsAKT1 may cause altered potassium uptake and transport.

Discussion

OrbHLH001 acts as a positive regulator in tolerance to salinity

The salt tolerance of rice (Oryza sativa) is associated with the ability to extrude Na+ from the shoot and maintain a low Na+/K+ ratio in the cell (Martinez-Atiencia et al., 2007). When plant roots are subjected to high NaCl environments, external Na+ and Cl− establish a large electrochemical gradient favoring the passive entry of salt ions through cation and anion channels and transporters in the plasma membrane. K+ is critical for Na+ tolerance because K+ and Na+ are similar chemically (Lin et al., 2004). When plants are under salt stress, some of the ion transporters need to be activated, whereas others, such as Na+ influx transporters, need to be suppressed (Zhu, 2001). We used SIEF to investigate K+ and Na+ flux in roots. The capacity to extrude Na+ after exposure to 150 mM NaCl for 12 h and intrude K+ after 150 mM NaCl salt shock was greater for OrbHLH001-overexpression line than wild-type plants. Therefore, OrbHLH001 overexpression can alternate ion flux in rice root under NaCl stress, which is consistent with OsbHLH001 predominant expression in phloem (Fig. 3B).

We further investigated the transcription of K+ influx transporters OsHAK1 (Banuelos et al., 2002), OsHKT1 (Kader et al., 2006), and OsAKT1 encoding a rice shaker K+ channel (Fuchs et al., 2005) in OrbHLH001-overexpression plants. The real-time PCR results indicated that only OsAKT1 mRNA levels was raised obviously in transgenic plants (Fig. 5D). Both OsAKT1 and OsbHLH001 have a similar transcription pattern in rice organs (Fig. 3A and Fig. 5E). Furthermore, OsAKT1 is localized in the epidermis and the endodermis in root and can also be detected in vasculature and the cortex (Goldack et al., 2003), which is similar to the expression of OrbHLH001 in root (Fig. 3). The OrbHLH001 protein specifically binds to E-box motifs in promoter regions of OsAKT1 in the yeast one-hybrid system (Fig. 5C).

In addition, OrbHLH001 overexpression in rice produced the low-K+-sensitive phenotype (Fig. 5E), which was not consistent with the increase of AKT1 in transgenic lines. One speculation is that low-K+ stress could result in a decrease of expression of one or more K+ channel genes in leaves, which cause disorder and turbulence of K+ transport. In fact, in Arabidopsis, AtAKT2 functions in phloem tissue for K+ long distance transport (Lacombe et al., 2000) and under K+ starvation conditions, AtRCK1 and AtAKT1 expression levels remained unchanged in roots and shoots. However, AKT2 mRNA was decreased in shoots (Pilot et al., 2003).

A novel role of OrbHLH001 gene in regulating rice salt tolerance

Genome-wide analysis of the bHLH transcription factor family in plants predicted or identified 167 bHLH genes in rice (Li et al., 2006). Previous reports have indicated that rice bHLH genes have a great influence on diverse cellular processes and stress responses. For example, OsbHLH122 is involved in shoot branching (Komatsu et al., 2003), OsbHLH164 in tapetum development (Jung et al., 2005), OsbHLH062 in cold response (Wang et al., 2003), and OsbHLH066 in wound and drought responses (Kiribuchi et al., 2004). Rice homologs of ICE1, OsbHLH001, and OsbHLH002 were obtained by BLAST search (Li et al., 2010). We cloned OrbHLH001 from Dongxiang wild rice. Overexpression of OrbHLH001 and OrbHLH002 enhances tolerance to salt stress in Arabidopsis (Zhou et al., 2009; Li et al., 2010). Antisense OrbHLH001 transgenic lines shows the same phenotypes with the wild type in morphologic and development, that may because of the gene redundancy (Supplemental Fig. S2). OsICE homologs OsbHLH001/OsICE2 and OsbHLH002/OsICE1 function in regulating OsDREB1B and OsHsfA3 involved in cold acclimation and trehalose synthesis (Nakamura et al., 2011).
OrbHLH001 shares a similar bHLH-ZIP transcription-factor domain with other ICE1 or ICE1-related proteins, and our data also demonstrated that OrbHLH001 proteins were localized in the nucleus and showed transcriptional activity (Fig. 4). OrbHLH001 may function in salt tolerance. When the plant suffers in a saline environment, the expression of both OrbHLH001 and OsbHLH001/OsICE2 can be induced quickly. The protein accumulates in 1 h (Li et al., 2010; Nakamura et al., 2011), and then its target gene OsAKT1 may be activated by the high expression of OrbHLH001 (Fig. 5D). The induced expression of OsAKT1 and, as a consequence, K+ intrudes via OsAKT1, which might result in less sensitivity to salt stress. To confirm this supposition, gel-shift assays and chromatim immuno-precipitation assays are needed for further testing.

Different homologs of ICE1 may mediate the regulation of salt tolerance in a different signal response process because OrbHLH2 modulates the salt-stress-related DREB/CBF pathway, while OrbHLH001 is involved in stress tolerance in a CBF/DREB1-independent pathway (Zhou et al., 2009; Li et al., 2010). As more members in the complex systems in stress response are reported, the function of ICE1 homologs and their specific interacting bHLH factors will be better understood.

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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.jplph.2012.08.019.

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