Theor Appl Genet (2003) 107:1402–1409
DOI 10.1007/s00122-003-1378-x

ORIGINAL PAPER

Yu-Jun Wang · Zhi-Gang Zhang · Xin-Jian He · Hua-Lin Zhou · Yu-Xiang Wen · Ji-Xun Dai · Jin-Song Zhang · Shou-Yi Chen

A rice transcription factor OsbHLH1 is involved in cold stress response

Received: 25 April 2003 / Accepted: 16 June 2003 / Published online: 13 August 2003

Abstract Cold stress adversely affects plant growth and crop production. Some plants express a series of cold-responsive genes during cold acclimation to reduce the damage of cold stress. Among them, transcription factors play important roles in enhancing plant cold tolerance. A bHLH-type gene *OsbHLH1* was isolated from rice. The predicted OsbHLH1 protein has a putative nuclear-localization signal and a putative DNA binding-domain bHLH-ZIP. The genomic sequence of the *OsbHLH1* gene is unique in rice genome and has four introns. The transcription of the *OsbHLH1* gene was specifically induced in roots of rice seedlings by cold but not by NaCl, PEG and ABA treatments. The OsbHLH1 protein was located in the nucleus of plant cells and had the ability to activate the transcription of the reporter gene in yeast. In addition, OsbHLH1 had the ability to dimerize. These results indicate that the OsbHLH1 may function as a transcription factor in a cold signal-transduction pathway.

Keywords *Oryza sativa* · *OsbHLH1* · Transcription factor · Cold stress

Communicated by H. F. Linskens

Y.-J. Wang · Z.-G. Zhang · X.-J. He · H.-L. Zhou · Y.-X. Wen · J.-S. Zhang (✉) · S.-Y. Chen (✉)
Institute of Genetics and Developmental Biology,
Chinese Academy of Sciences,
100101 Beijing, PR China
e-mail: jszhang@genetics.ac.cn
Tel.: +86-10-64886859
Fax: +86-10-64873428
e-mail: sychen@genetics.ac.cn
Tel.: +86-10-64886859
Fax: +86-10-64873428

Y.-J. Wang · J.-X. Dai
College of Marine Life Sciences,
Ocean University of China,
266003 Qingdao, PR China

Introduction

Cold stress is one of the major environmental stresses that can delay plant growth and development, reduce productivity and in the extreme can cause the plant to die. To ensure the survival and prosperity of plants and their offspring, some plants have developed strategies to cope with the cold stress (Bohnert et al. 1995; Browse and Xin 2001). During cold acclimation, many genes are induced in plants (Hughes and Dunn 1996; Thomashow 1999) and the products of these genes are involved in the biosynthesis of compatible solutes (Chen and Murata 2002; Taji et al. 2002), the generation of antioxidants (Prasad et al. 1994) and the increase of membrane fluidity (Murata and Los 1997; Orvar et al. 2000). In addition, some induced proteins also function as regulatory molecules in the signal-transduction pathway of cold-stress responses (Karam et al. 2002).

Transcriptional control of the cold-regulated genes is a crucial part of the plant responses to cold stress (Liu et al. 1999; Karam et al. 2002). Much progress has been made in the past few years in the characterization of transcription factors. The CBF (CBF1, CBF2, CBF3)/DREB1 (DREB1B, DREB1C, DREB1A) proteins from *Arabidopsis* are transcriptional regulatory proteins involved in the expression of cold-regulated (*COR*) genes (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998). Similar proteins have also been found in *Arabidopsis* and other plants (Liu et al. 1998; Shen et al. 2003a, 2003b), and these proteins are involved in multiple-stress responses. The CBF/DREB1 proteins contain an AP2/EREBP DNA binding-domain, which can recognize the DNA regulatory element (TGCCGAC or TACCGACT) known as CRT (c-repeat)/DRE (dehydration-responsive element) (Stockinger et al. 1997; Medina et al. 1999). The CRT/DRE element is usually present in the promoters of cold and dehydration-inducible genes, including *COR15a, COR6.6/KIN2, COR47* and *COR78/RD29A* (Thomashow 1998). Under normal conditions, neither *CBF* nor *COR* genes were expressed. However, when treated with cold stress, the expression of the *CBF* genes were induced,

followed with the expression of CBF regulated-target genes. Furthermore, transgenic plants overexpressing *CBF1* showed increased tolerance to cold stress without a cold-acclimation period (Jaglo-Ottoson et al. 1998; Liu et al. 1998).

ABA-responsive element (ABRE), PyACGTGGC, and the transcription factors that bind to ABRE are also involved in cold-signal transduction. SGBF-1, a G-box binding ZIP transcription factor from soybean, can bind ABRE and regulates the expression of the *COR* genes. A zinc-finger protein SCOF-1 can interact with SGBF-1 and enhance its binding to ABRE. Transgenic *Arabidopsis* plants overexpressing *SCOF-1* were able to constitutively express *COR* genes (such as *COR15a, Rd29B* and *COR47*) in the absence of low temperature stimulus and improved the cold tolerance of non-acclimated transgenic plants (Kim et al. 2001).

In addition to the CBF/DREB1 and SCOF-1/SGBF-1 cold-response pathways, other regulatory pathway may also exist (Fowler and Thomashow 2002). The basic Helix-Loop-Helix (bHLH) proteins belong to a large family of transcription factors and play important roles in cell proliferation, determination and differentiation in animals, plants and yeast (Ledent and Vervoort 2001). In *Arabidopsis*, the bHLH protein rd22BP1 has been found to be involved in the dehydration and ABA response pathway (Abe et al. 1997). Here we report a novel cold-inducible and ABA-independent bHLH gene *OsbHLH1* from rice. Its genomic organization and expression under different stresses were investigated. Its localization and transcriptional activation activity were also characterized.

Materials and methods

Plant materials and treatments

Rice (*Oryza sativa* L. var. Lansheng, JX17, 9311) seeds were imbedded in water at 37°C for 2 days with water changed every day, and then grown on cheesecloth in a tray with the addition of water and mineral nutrient solution. The plants were grown under continuous illumination of approximately 2,500 lux at 25°C. The ten-day old rice seedlings were transferred to 4°C for various periods and both shoots and roots were harvested for RNA isolation. For ABA, NaCl and PEG treatments, the seedlings were transferred into solutions containing 100 μM of ABA, 250 mM of NaCl and 25% of PEG, respectively, for various periods and then harvested for RNA isolated.

Construction and screening of rice cDNA library

A cDNA library was constructed with the mRNA from the cold-treated rice seedlings (var. Lansheng). According to the manufacturer's protocol (Stratagene), the first-strand cDNAs were synthesized and followed by second-strand synthesis. The uneven termini of the double-stranded cDNA were filled in with *Pfu* DNA polymerase, and *Eco*RI adapters were ligated to the blunt ends. After *Xho*I digestion, the double-stranded cDNAs were ligated to the Uni-ZAP XR vector, and then packaged by Gigapack III Gold packaging extract. The library was amplified and screened by PCR with the following procedure (Takumi and Lodish 1994): (1) Lambda phages were excised in vivo into phagemid particles with the aid of helper phage. The phagemid particles were divided into 30 equal fractions. Each fraction was added with 200 μl of XLOLR cells (OD=5.0) and incubated in a micro-tube at 37°C for 20 min. The culture was transferred to 400 μl of LB containing Ampicillin and then cultured at 37°C overnight; (2) The culture from each fraction was condensed into 50 μl, and 2 μl was used as a template for PCR analysis. The plasmid harboring a *DREB1C* gene was used as a template for a positive control. The PCR condition was as follows: 94°C, 6 min, 1 cycle; 94°C, 1 min, 62°C, 1 min, 72°C, 2 min, 25 cycles with an extension of 10 min at 72°C. The 5′-primer was *5′-AATTAACCCTCACTAAAGGG-3′* (T3 primer), which was from the vector of pBluescript SK(+/−). The 3′-primer (annex primer) was *5′-(ATGC)GT(CT)TG(AG)AA(ATGC)GT (ATGC)CC(ATGC)AGCCA-3′*, which came from the conserved AP2/EREBP domain of CBF/DREB1. According to the presence and the length of the amplified DNA fragment, one fraction was chosen for further screening by repeating the procedures above, and one clone containing the putative target sequence was finally obtained. The inserted DNA in the plasmid was sequenced and further analyzed.

Southern analysis

DNA extraction was performed as described previously by Chen et al. (1991). Genomic DNA was fractionated in a 0.8% agarose gel after digestion with *Eco*RI, *Bam*HI, *Xho*I and *Xba*I, and blotted onto Hybond-N^+nylon membrane for Southern analysis. The hybridization procedures followed standard protocols. The membrane was washed once in 2×SSC plus 0.1% SDS at 65°C for 15 min, then in 1×SSC plus 0.1% SDS at 65°C for 8 min. The membrane was autoradiographed by using a phosphoimaging system (Amersham Pharmacia).

Northern analysis

RNA extraction was performed as described previously (Zhang et al. 1996). Total RNA (30 μg) was fractionated in a 1.2% agarose-containing formaldehyde and blotted onto Hybond-N^+ nylon membrane for Northern analysis. The full-length of *OsbHLH1* cDNA was labeled as a probe and used in hybridization. The membranes were washed in 2×SSC plus 0.1% SDS at 45°C for 15 min and in 1×SSC plus 0.1% SDS at 45°C for 5 min, The membranes were then autoradiographed by using a phosphoimaging system (Amersham Pharmacia).

OsbHLH1 expression in onion epidermal cells

The *OsbHLH1* coding sequence was amplified from the original plasmid with the 5′-primer *5′-TAGGGATCCGAGGTCGAGATG-GTGCCCAG-3′* (containing *Bam*HI site) and the 3′-primer *5′-GTGGTCGACAGATCTACCTATACGGTCTTC-3′* (containing the *Sal*I site). The product was fused to the green fluorescent protein (GFP) reporter gene in pUC18 and sequenced. The fusion construct was introduced into the onion epidermal-cells layer by particle bombardment. The transformed cells were cultured on MS medium at 28°C for 2 days and observed under a confocal microscope (Olympus FV500)(Xie et al. 2003). A *GFP* gene in pUC18 was used as a control, and both the fusion gene and the *GFP* gene were driven by the 35S promoter.

Analysis of OsbHLH1 transcriptional activation activity and the dimerization by a yeast two-hybrid assay

The yeast strains YRG-2 (*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538 LYS2::UAS$_{GAL1}$-TATA$_{GAL1}$-HIS3 URA3::UAS $_{GAL417mers(x3)}$-TATA$_{CYC1}$-lacZ*) containing the *HIS3* and the *lacZ* reporter genes was used as an assay system (Stratagene). The entire *OsbHLH1* coding sequence was obtained by PCR with the forward primer *5′-TAGGAATCCGAG-*

GTCGAGATGGTGCCCAG-3′ and the reverse primer *5′-GTAGTC-GACCTAAGATCTACCTATACGGTC-3′*. The product was in-frame fused to the GAL4 DNA binding-domain vector and the GAL4 activation-domain vector, respectively, to generate the plasmid pBD-OsbHLH1 and pAD-OsbHLH1. The GAL4 is a transcription factor involved in the expression of galactose-induced genes. According to the manufacturer's protocol (Stratagene), the plasmids of pBD (control), pGAL4 (control), pBD-OsbHLH1, and pAD-WT plus pBD-WT [interaction control, WT indicates wild-type fragment C of lambda cI repressor (aa 132–263)], respectively, were transformed into the yeast YRG-2. The transformed yeast cells were then streaked on the plates YAPD or SD-His medium (without His) to check the transcriptional activation activity according to their growth.

For dimerization analysis, the yeast cells (YRG-2) were transformed with plasmids of pBD vector plus pAD-OsbHLH1 (control), pAD vector plus pBD-OsbHLH1 (control), pAD-OsbHLH1 plus pBD-OsbHLH1, and pAD-WT plus pBD-WT (interaction control), respectively. The transformed yeast cells were then streaked on the YAPD medium or SD-His medium plus 10 mM of 3-AT. The plates were cultured at 30°C for 3 days and the status of yeast growth was examined (Xie et al. 2000). The transformed yeast cells were transferred onto the filter paper and incubated at 30°C for 3–8 h in the presence of X-gal to check the activity of β-galactosidase by observing the blue color produced.

Results

Isolation of the *OsbHLH1* gene from rice

We aimed to screen the rice cDNA library for the homologues of the *CBF/DREB*1 gene by the PCR method with the sense primer representing the sequence from the vector and the antisense primer representing the sequence from the conserved AP2/EREBP domain of CBF/DREB1. A clear band was stably amplified from one fraction of the *Eschericha coli* cells harboring the excised recombinant plasmids. The insert in the positive plasmid was sequenced and compared with those from the NCBI database. We found that the sequence was a full-length cDNA and its deduced amino-acid sequence exhibited a bHLH motif, characteristic of the bHLH-type transcription factors, but not an AP2/EREBP motif, characteristic of the CBF/DREB1-type transcription factors (Fig. 1A). The corresponding gene was thus designated as *OsbHLH1* (*O. sativa*, bHLH-like 1) and was further analyzed.

The *OsbHLH1* was 1,137 bp in length with an open reading frame (ORF) of 798 bp flanked by a 120-bp 5′-untranslated region (UTR) and a 219-bp 3′-UTR (Fig. 1A). The open reading frame encoded a putative protein of 266 amino acids with a predicted molecular mass of 42 kDa. By analysis via the SMART program (Letunic et al. 2002) and comparison with the amino-acid sequence from other bHLH proteins (Liu et al. 1999; Ledent and Vervoort 2001), several specific domains were identified. As shown in Fig. 1A, there was one short motif (KKCDKKAPKR) in the amino-terminal region, which was rich in basic residues such as lysine (K) and arginine (R). This basic motif may represent a putative nuclear localization signal. Following the putative nuclear localization signal was a bHLH motif containing 54 residues. The first 15 amino acids may represent a basic region overlapping with the putative nuclear localization signal, and the other residues formed two-helices separated by a variable loop region (Fig. 1B). A leucine-rich zipper-like (ZIP) motif followed the bHLH motif. The bHLH and ZIP motifs constituted a bHLH-ZIP domain, which may promote dimerization between the same or different members (Ledent and Vervoort 2001), and also may be the DNA binding-domain as reported in the rd22BP1 gene from *Arabidopsis* (Abe et al. 1997). This bHLH-ZIP domain shared 41%, 34% and 38% identity in amino acids with bHLH1 (AF251696), bHLH2 (AF488629) and bHLH3 (NP_188620) proteins from *Arabidopsis* respectively (Fig. 1B).

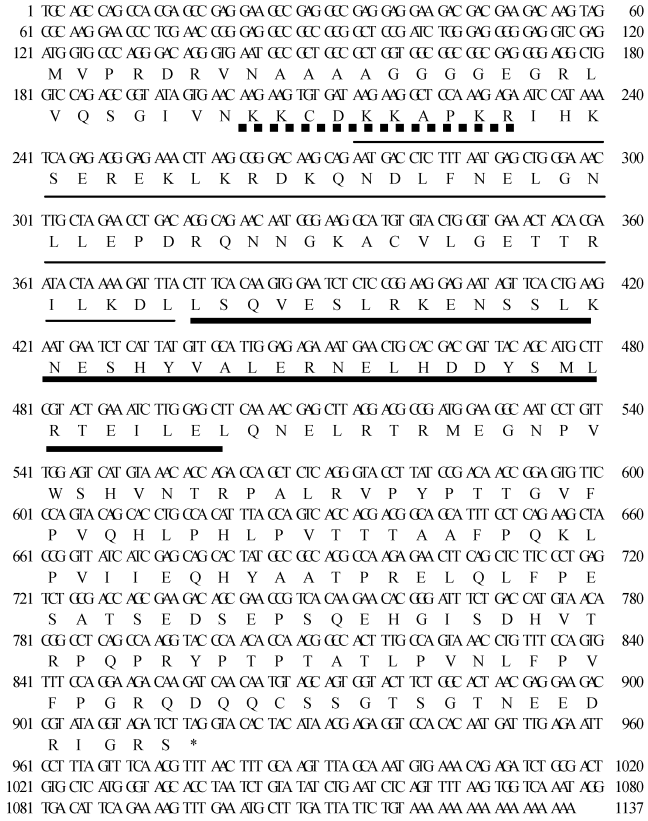
OsbHLH1 is a unique-copy gene

Rice genomic DNA was digested with various restriction endonucleases, fractionated on agarose gel, transferred onto filters and hybridized with the full-length *OsbHLH1* probe. It could be seen that one hybridization band was present in each digestion with *Bam*HI, *Xho*I and *Xba*I, and three bands were observed in *Eco*RI digestion (Fig. 2). The *Bam*HI, *Xho*I and *Xba*I hybridization patterns were consistent because there were no *Bam*HI, *Xho*I and *Xba*I cleavage sites in the *OsbHLH1* cDNA sequence. However, the *Eco*RI digested pattern was not consistent because there was only one site of *Eco*RI in *OsbHLH1* cDNA. We thus searched the rice genome DNA database (*Japonica* rice), and found that several parts of one sequence (AP004260.2) shared 99.9% identity with the nucleotide sequence of the *OsbHLH1* gene. This sequence was very likely to be the genomic sequence of the *OsbHLH1* gene. Compared with the cDNA sequence, the genomic sequence had 5 exons (108 bp, 81 bp, 119 bp, 130 bp and 699 bp) and four introns, and all the introns followed the GT/AG splicing rule. In the second intron, one more *Eco*RI site was found. Therefore, the *Eco*RI digestion pattern was still consistent with the two *Eco*RI sites found in the genomic sequence of the *OsbHLH1* gene. These results indicated that the *OsbHLH1* gene was unique in the rice genome.

Expression of the *OsbHLH1* gene in response to various treatments

The rice seedlings were subjected to various treatments and expressions of the *OsbHLH1* gene were examined. As shown in Fig. 3A, an increase of *OsbHLH1* mRNA was already detectable in rice seedlings (shoots plus roots) after being exposed to cold (4°C) for 30 min. The expression reached the maximal level at 30 min after the initiation of the cold stress, and this level lasted around 5 h; thereafter, the *OsbHLH1* expression was decreased. The seedlings were also treated with ABA, NaCl and PEG. However, after these treatments, the transcription level of the *OsbHLH1* gene did not change significantly,

A



B

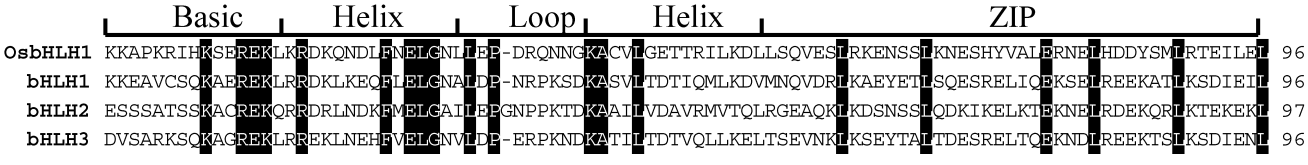


Fig. 1A,B Nucleotide sequence of*OsbHLH1* (AY222337) and comparison of its encoded protein with other bHLH-like proteins. **A** Nucleotide and amino-acid sequence of the *OsbHLH1* gene. The putative bHLH motif is marked by a thin line. The putative nuclear localization signal is indicated by a broken line. The putative ZIP motif is marked by a thick line. **B** Comparison of the amino-acid sequence of the bHLH-ZIP domain from OsbHLH1 and other bHLH-like proteins. bHLH1 (AF251696), bHLH2 (AF488629) and bHLH3 (NP_188620) are from *Arabidopsis*. The residues shaded in black indicate identity. Dashes were included for maximum alignment

indicating that OsbHLH1 may not mediate a plant response to these treatments.

The *OsbHLH1* expression was also compared in two rice subspecies, *Japonica* rice JX17 and *Indica* rice 9311. Under low temperature (4°C), the *OsbHLH1* gene was induced in the roots of both cultivars and at similar levels. In the leaves of both subspecies, the *OsbHLH1* expression was not detectable, implying that OsbHLH1 may not function in leaves but rather play roles in the cold responses of roots.

Nuclear localization of the OsbHLH1 protein

The deduced amino-acid sequence of the OsbHLH1 protein contains the KKCDKKAPKR motif, characterized by a core peptide enriched in lysine (K) and arginine (R), which was the potential nuclear-localization signal (NLS). To investigate the localization of the OsbHLH1 protein in plant cells, the *OsbHLH1* was fused to the *GFP* gene, and the fusion gene *OsbHLH1-GFP* in the pUC18 plasmid was generated. The fusion gene and the control *GFP* in pUC18, driven by the 35S promoter, were

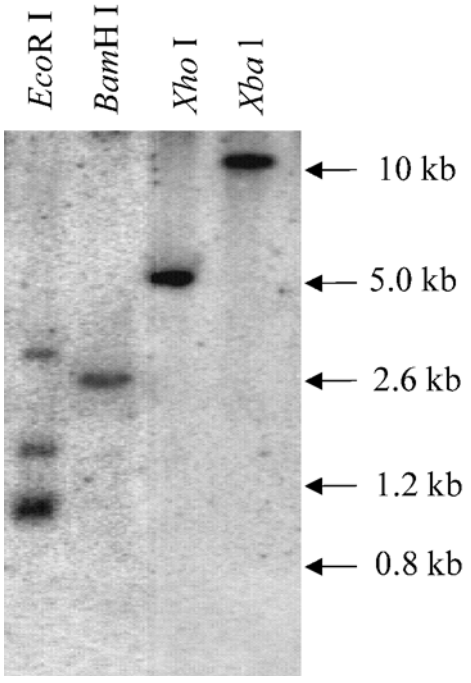


Fig. 2 Southern analysis of the *OsbHLH1* gene. Rice genomic DNA (about 8 μg) was digested with *Eco*RI, *Bam*HI, *Xho*I and *Xba*I. After electrophoresis, the DNA was transferred onto nylon membrane, and hybridized with the labeled full-length *OsbHLH1*. The position of molecular-size markers is illustrated on the left

transformed into the onion epidermal cells by particle bombardment. The protein expression was revealed by green fluorescence of GFP and observed under a confocal microscope. The result was presented in Fig. 4. It can be seen that the OsbHLH1-GFP fusion protein was targeted in the area of the nucleus of the cells (Fig. 4B). In contrast, all of the control GFP protein substantially remained in the cytoplasm (Fig. 4A). This result indicated that the OsbHLH1 was a nuclear protein, possibly functioning as a transcription factor.

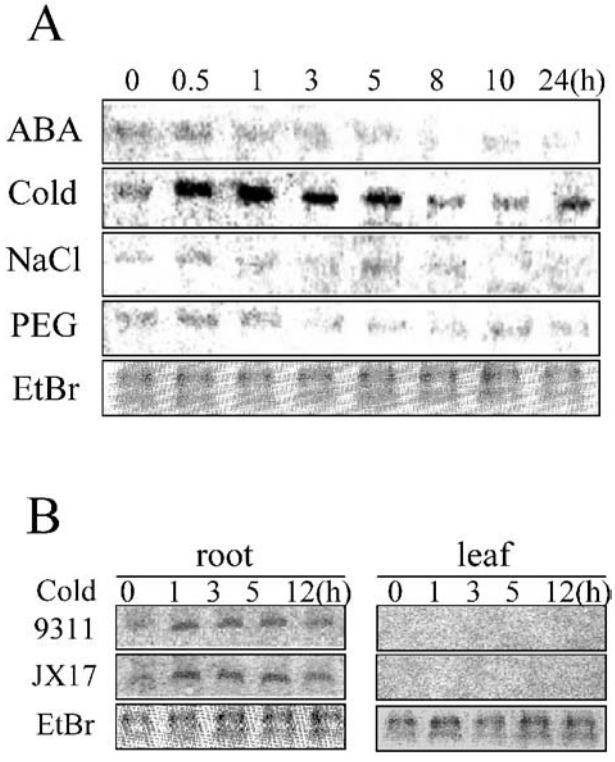


Fig. 3A,B *OsbHLH1* expression in rice seedlings. **A** *OsbHLH1* expression in response to ABA, cold (4°C), NaCl and PEG treatments. The rice seedlings were subjected to different treatments and total RNA was isolated. Each lane was loaded with 30 μg of total RNA. The RNA blot was hybridized with the labeled full-length *OsbHLH1* gene. The RNA was also stained with EtBr to check the equal loading of samples. **B** Expression of the *OsbHLH1* gene in the subspecies of rice under low temperature (4°C). The *Japonica* rice (JX17) and *Indica* rice (9311) were subjected to cold treatment. The total RNA was isolated from roots and leaves. Each lane was loaded with 30 μg of total RNA

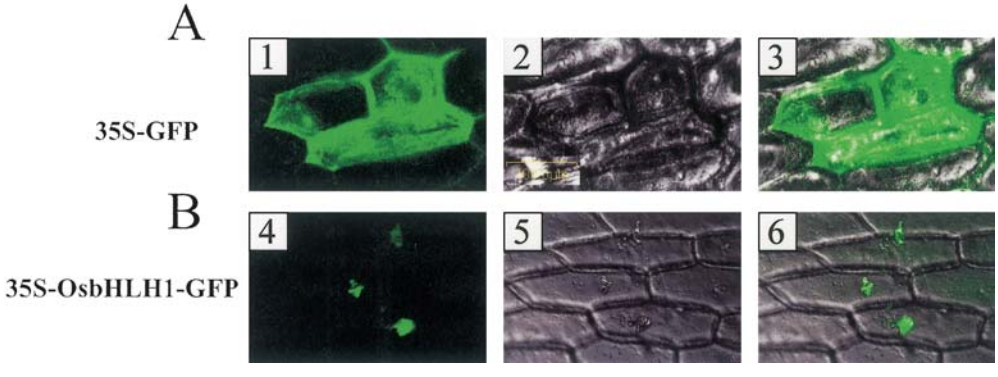


Fig. 4A,B Localization of the OsbHLH1 protein in onion epidermal cell. OsbHLH1-GFP fusion protein or GFP alone was expressed transiently under the control of the CaMV 35S promoter in onion epidermal cells and observed under a confocal microscope. The photographs were taken in the dark field for green fluorescence (part 1, 4), in the bright field for the morphology of the cell (part *2, 5*) and in combination (part *3, 6*). **A** Expression of the GFP control protein. **B** Expression of the OsbHLH1-GFP fusion protein

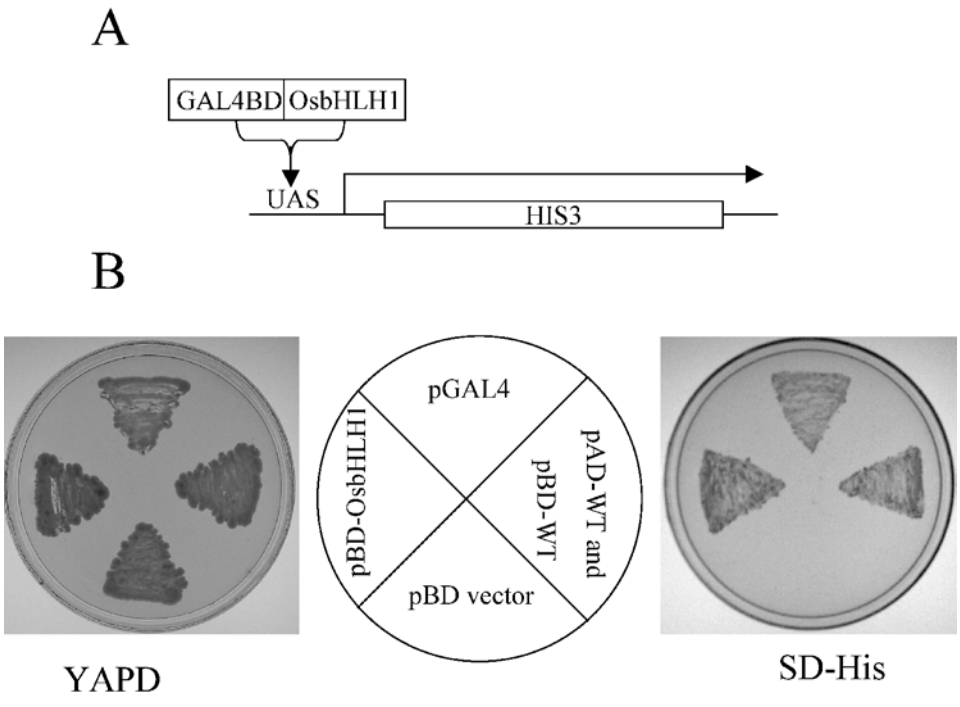


Fig. 5A,B Transactivation activity of OsbHLH1. **A** The *OsbHLH1* entire-coding sequence was fused to the GAL4 DNA binding domain (BD) vector to generate pBD-OsbHLH1. **B** The yeast YRG-2 was transformed with the plasmids of pBD, pGAL4, pBD-OsbHLH1 and pAD-WT plus pBD-WT. The transformants were streaked on the YAPD and SD-His medium respectively. The plates were incubated at 30°C for 3 days and the status of growth was examined

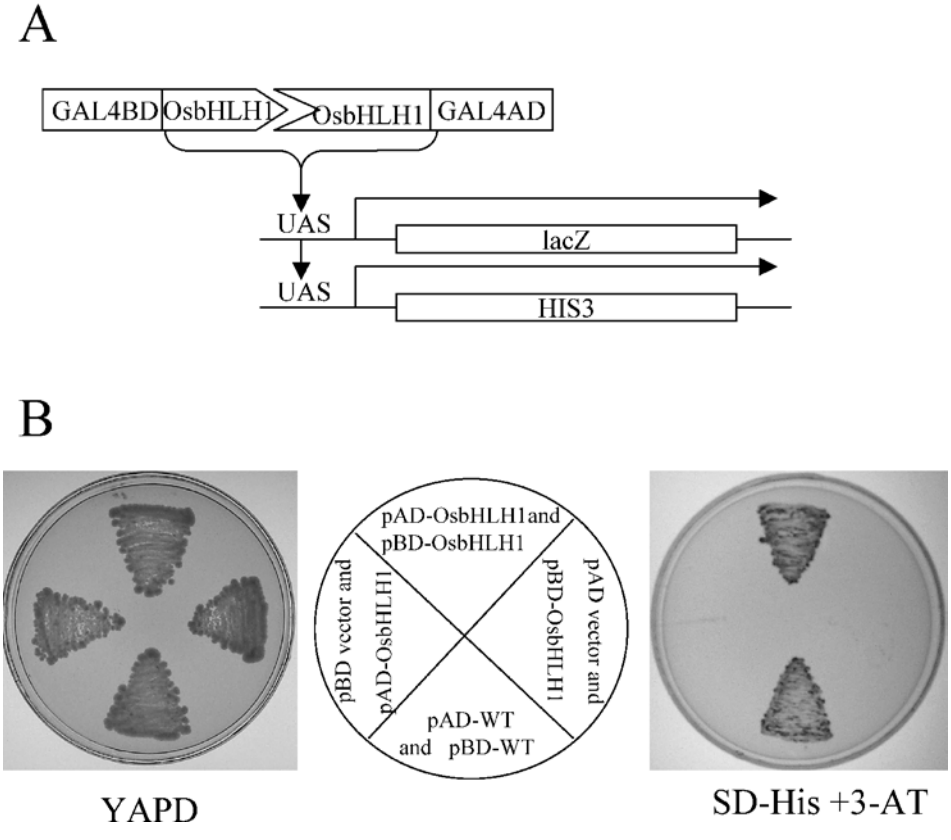


Fig. 6A,B Dimerization of OsbHLH1. **A** The entire *OsbHLH1* coding sequence was fused to the GAL4 binding-domain vector (pBD) and the GAL4 activation-domain vector (pAD) to generate pBD-OsbHLH1 and pAD-OsbHLH1 respectively. **B** The yeast cells (YRG-2) were transformed with plasmids pBD plus pAD-OsbHLH1, pAD plus pBD-OsbHLH1, pAD-OsbHLH1 plus pBD-OsbHLH1, and pAD-WT plus pBD-WT, respectively. The transformants were streaked on the YAPD medium or SD-His medium plus 10 mM 3-AT. The plates were cultured at 30°C for 3 days and the results were evaluated

Transcriptional activation activity of OsbHLH1

Since the OsbHLH1 protein has the structural feature similar to bHLH-type transcription factors, we used the yeast system to test whether an activation domain was present in OsbHLH1. The ORF of *OsbHLH1* was fused to the DNA sequence encoding the GAL4 DNA binding-domain, and the fusion plasmid pBD-OsbHLH1 was transformed into the yeast strain YRG-2 containing upstream activating sequences (UAS). The UAS can

regulate the expression of the reporter gene *HIS3* (Fig. 5A). If the yeast harboring the pBD-OsbHLH1 can grow on the medium without histidine (SD-His), then the reporter gene *HIS3* is activated, indicating that there is a transcriptional activation domain in the OsbHLH1. As seen in Fig. 5B (left panel), all the transformants containing the plasmid pGAL4 (the positive control), the pBD vector (negative control), the pAD-WT plus pBD-WT (interaction control), and the pBD-OsbHLH1 grew well on the YAPD medium. On the SD-His medium, the yeast cells containing pBD-OsbHLH1 also exhibited better growth, in addition to the growth of the yeast cells harboring the positive and the interaction-control plasmid. The yeast cells containing the pBD vector did not grow in the same medium (Fig. 5B, right panel). These results indicated that the fusion pBD-OsbHLH1 protein was bound to the UAS of the *HIS3* reporter gene and activated its expression. The expression of the *HIS3* gene helped the transformants grow well on the SD-His medium. Therefore, the OsbHLH1 protein had transcriptional activation activity.

Testing of the dimerization of OsbHLH1

The bHLH-ZIP domain may promote dimerization, allowing the formation of homodimeric complexes. To test the possibility of OsbHLH1 to dimerize, we made the construct pAD-OsbHLH1 by fusing the OsbHLH1 coding sequence to the coding sequence for the activation domain of GAL4, and examined its interaction with pBD-OsbHLH1 in a yeast two-hybrid assay (Fig. 6A). Figure 6B (left panel) showed that all the yeast cells transformed with different plasmids grew well on the YAPD medium. On SD-His medium plus 3-AT, the yeast cells containing pAD-OsbHLH1 plus pBD-OsbHLH1 grew well, indicating that the two proteins more likely formed a dimer and efficiently activated transcription of the *HIS3* gene. The yeast cells harboring the pBD-OsbHLH1 plus the pAD vector could also grow on the medium but in a very slow manner. The yeast cells containing the pAD-OsbHLH1 plus the pBD vector could not grow on the same medium. The yeast cells harboring the positive interaction control plasmids pAD-WT and pBD-WT grew normally. In the presence of X-gal, the yeast cells that could grow on SD-his plus 3-AT medium turned blue (data not show), indicating the activation of another reporter gene *lacZ*.

Discussion

Transcription factors of the bHLH family play important roles in the growth and development of organisms. Based on a phylogenetic analysis, these transcription factors were subdivided into four groups (Ledent and Vervoort 2001). In plants, a number of *bHLH* genes were annotated, and some of them were involved in cell development (Sorensen et al. 2003) and flower coloration (Spelt et al. 2000). The *bHLH* genes, including the *rd22BP1* and the present *OsbHLH1* gene, were also involved in abiotic stresses. The rd22BP1 was a binding protein of the *rd22* gene promoter (Abe et al. 1997). Both the rd22BP1 and the OsbHLH1 contained a bHLH-ZIP domain. However, there was low identity (25%) between them, and some of the identified amino acids in these regions were not conserved (data not show). The divergence suggested that the target element of OsbHLH1 was different from that of rd22BP1. The expression pattern was also different between the *OsbHLH1* and the *rd22BP1* gene. The *OsbHLH1* gene was cold-inducible and probably ABA-independent, and was expressed in a higher level in roots but undetectable in leaves. The *rd22BP1* gene was dehydration-inducible and ABA-dependent, and strongly expressed in siliques and stems but weak in leaves and roots. These results indicated that the OsbHLH1 and rd22BP1 were involved in different abiotic stresses, probably through ABA-independent and ABA-dependent signal-transduction pathways respectively.

Recently Chinnusamy et al. (2003) reported the identification of an *ICE1* gene from *Arabidopsis*. This gene encoded a bHLH transcription factor and regulated the transcription of *CBF* genes in the cold stress. *ICE1* can bind specifically to the MYC recognition sequences in the *CBF3* promoter. Over-expression of *ICE1* improved freezing tolerance of the transgenic plants (Chinnusamy et al. 2003). These data indicated that this bHLH transcription factor ICE1 was an upstream component of the CBF/DREB1 cold-signal transduction pathway, and the ICE1 and the CBF/DREB1 belong to the same pathway. Our present rice *OsbHLH1* gene also encoded a bHLH transcription factor. However, due to the limited identity to the bHLH-like proteins from *Arabidopsis*, it is not known whether the OsbHLH1 could also regulate the *CBF/DREB1* gene expression in rice. Possibilities exist that several cold-response pathways are present in plants (Fowler and Thomashow 2002).

By using a yeast assay system, we demonstrated the presence of an activation domain in OsbHLH1. The fusion of OsbHLH1 with the DNA binding-domain of GAL4 bound to UAS, activated the transcription of the reporter gene. However, where the activation domain was located in the OsbHLH1 protein remained unclear. Using the same system, we demonstrated that OsbHLH1 could form a dimer and more efficiently activated the transcription of the reporter gene. This phenomenon may suggest that in the in vivo situation, OsbHLH1 functioned as a dimeric complex. The bHLH or the ZIP motifs may be responsible for this dimerization (Ledent and Vervoort 2001). In the bHLH protein rd22BP1, the bHLH region has also been reported to be the DNA binding-domain, and could specifically bind to the MYC recognition site of the *rd22* promoter (Abe et al. 1997). Whether the bHLH region of the present OsbHLH1 also functions as a DNA binding-domain needs to be studied. Further research on these aspects will disclose more about the mechanism of the plant cold-stress response.

Acknowledgements This work was supported by the National Basic Research Program NKBRSF (G1999011703) and the National Hightech program (2001AA222131, 2001AA212121, 2002AA2Z1001). We are very grateful to the State Key Plant Cell and Chromosome Engineering Laboratory of the Institute of Genetics and Developmental Biology, CAS, for providing the confocal microscope (Olympus FV500).

References

Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K (1997) Role of *Arabidopsis* MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. Plant Cell 9:1859–1868

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptation to environmental stresses. Plant Cell 7:1099–1111

Browse J, Xin Z (2001) Temperature sensing and cold acclimation. Curr Opin Plant Biol 4:241–246

Chen SY, Zhu LH, Hong J, Chen SL (1991) Molecular biological identification of a salt-tolerant rice line. Acta Bot Sinica 33:569–573

Chen TH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol 5:250–257

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes Dev 17:1043–1054

Fowler S, Thomashow MF (2002) ***A****rabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14:1675–1690

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. Plant J 16:433–42

Hughes MA, Dunn MA (1996) The molecular biology of plant acclimation to low temperature. J Exp Bot 47:291–305

Jaglo-Ottoson KR, Klelf S, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidosis CBF1* expression induces COR genes and enhances freezing tolerance. Science 280:104–106

Karam BS, Rhonda CF, Luis OS (2002) Transcription factors in plant defense and stress responses. Curr Opin Plant Biol 5:430–436

Kim JC, Lee SH, Cheng YH, Yoo CM, Lee SI, Chun HJ, Yun DJ, Hong JC, Lee SY, Lim CO, Cho MJ (2001) A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. Plant J 25:247–259

Ledent V, Vervoort M (2001) The basic helix-loop-helix protein family: comparative genomics and phylogenetic analysis. Genome Res 11:754–770

Letunic I, Goodstadt L, Dickens NJ, Doerks T, Schultz J, Mott R, Ciccarelli F, Copley RR, Ponting CP, Bork P (2002) Recent improvements to the SMART domain-based sequence annotation resource. Nucleic Acids Res 30:242–244

Liu LS, Michael JW, Thomas HM (1999) Transcription factors and their genes in higher plants functional domains, evolution and regulation. Eur J Biochem 262:2472–2457

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10:1391–1406

Medina J, Bargues M, Terol J, Perez-Alonso M, Salinas J (1999) The *Arabidopsis* CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiol 119:463–470

Murata N, Los DA (1997) Membrane fluidity and temperature perception. Plant Physiol 115:875–879

Orvar BL, Sangwan V, Omann F, Dhindsa RS (2000) Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. Plant J 23:785–794

Prasad TK, Anderson MD, Martin BA, Steward CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell 6:65–74

Shen YG, Zhang WK, He SJ, Zhang JS, Liu, Q, Chen SY (2003a) An EREBP/AP2-type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. Theor Appl Genet 106:923–930

Shen YG, Zhang WK, Yan DQ, Du BX, Zhang JS, Liu Q, Chen SY (2003b) Characterization of a DRE-binding transcription factor from a halophyte *Atriplex hortensis*. Theor Appl Genet (DOI10.1007/soo122–003–1226-Z)

Sorensen AM, Krober S, Unte US, Huijser P, Dekker K, Saedler H (2003) The *Arabidopsis* ABORTED MICROSPORES (AMS) gene encodes a MYC class transcription factor. Plant J 33:413–423

Spelt C, Quattrocchio F, Mol JN, Koes R (2000) *anthocyanin1* of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. Plant Cell 12:1619–1632

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA 94:1035–1040

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought-and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana.* Plant J 29:417–426

Takumi T, Lodish HF (1994) cDNA cloning by PCR screening (RC-PCR). BioTechniques 17:443–444

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol 118:1–7

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Plant Mol Biol 50:571–599

Xie Q, Frugis G, Colgan D, Chua NH (2000) *Arabidopsis* NAC1 transduces the auxin signal downstream of TIR1 to promote lateral root development. Genes Dev 14:3024–3036

Xie C, Zhang JS, Zhou HL, Li J, Zhang ZG, Wang DW, Chen SY (2003) Serine/threonine kinase activity in the putative histidine kinase-like ethylene receptor NTHK1 from tobacco. Plant J 33:385–393

Zhang JS, Zhou JM, Zhang C, Chen SY (1996) Differential gene expression in a salt-tolerance rice mutant and its parental variety. Sci China (Series C) 39:310–319