Up-Regulation of *OsBIHD1*, a Rice Gene Encoding BELL Homeodomain Transcriptional Factor, in Disease Resistance Responses

H. Luo[1,2,5], F. Song[1,2,3], R. M. Goodman[4], and Z. Zheng[1,2]

[1] Institute of Biotechnology, Zhejiang University, Hangzhou, Zhejiang 310029, P.R. China
[2] Department of Plant Protection, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, Zhejiang 310029, P.R. China
[3] State Key Laboratory of Rice Biology, Zhejiang University, Hangzhou, Zhejiang 310029, P.R. China
[4] Department of Botany and Plant Pathology, Gaylord Nelson Institute for Environmental Studies, University of Wisconsin, Madison, WI 53706, USA
[5] Present address: Institute of Biotechnology, Hainan University, Haikou, Hainan 570000, P.R. China

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Abstract: **In the present study, we cloned and identified a full-length cDNA of a rice gene, *OsBIHD1,* encoding a homeodomain type transcriptional factor. *OsBIHD1* is predicted to encode a 642 amino acid protein and the deduced protein sequence of OsBIHD1 contains all conserved domains, a homeodomain, a BELL domain, a SKY box, and a VSLTLGL box, which are characteristics of the BELL type homedomain proteins. The recombinant OsBIHD1 protein expressed in *Escherichia coli* bound to the TGTCA motif that is the characteristic *cis*-element DNA sequence of the homeodomain transcriptional factors. Subcellular localization analysis revealed that the OsBIHD1 protein localized in the nucleus of the plant cells. The *OsBIHD1* gene was mapped to chromosome 3 of the rice genome and is a single-copy gene with four exons and three introns. Northern blot analysis showed that expression of *OsBIHD1* was activated upon treatment with benzothiadiazole (BTH), which is capable of inducing disease resistance. Expression of *OsBIHD1* was also up-regulated rapidly during the first 6 h after inoculation with *Magnaporthe grisea* in BTH-treated rice seedlings and during the incompatible interaction between *M. grisea* and a resistant genotype. These results suggest that OsBIHD1 is a BELL type of homeodomain transcription factor present in the nucleus, whose induction is associated with resistance response in rice.**

Key words: **Benzothiadiazole (BTH), rice (*Oryza sativa* L.), homeodomain; disease resistance response, *OsBIHD1, Magnaporthe grisea.***

Abbreviations:

ABA:	abscisic acid
BTH:	benzothiadiazole
HD:	homeodomain
ORF:	open reading frame
OsBIHD1:	*Oryza sativa* L. BTH-induced homeodomain protein 1
uORF:	upstream open reading frame

Footnote: **The nucleotide sequence reported in this paper has been deposited in GenBank database under the accession number of AY524972.**

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Introduction

Genes containing homeoboxes, a highly conserved DNA sequence motif, are present extensively in the genomes of animals, fungi, and plants (Chan et al., 1998). Homeobox genes were first characterized as transcriptional regulatory genes that control morphogenesis in *Drosophila* species (Gehring, 1987). The proteins encoded by the homeobox genes contain a unique domain known as the homeodomain (HD) (Desplan et al., 1988). The HD consists of a highly conserved 61-amino acid stretch containing three α-helices that form a helix-turn-helix-type DNA binding motif (Otting et al., 1990), which recognizes and binds to specific DNA sequences *in vivo* (Chang et al., 1997). Therefore, the homeodomain proteins are believed to regulate the expression of batteries of target genes by acting as transcription factors.

According to sequence conservation within the homeodomain and the presence of additional sequences, the plant homeodomain proteins can be subdivided into different families, including Knotted1, HD-Zip, Glabra2, PHD finger, and BELL1 (Chan et al., 1998). The functions of the homeodomain proteins in plants have been demonstrated to be involved in various developmental processes, e.g., maintenance of the shoot apical meristem, development of the epidermis, and integument specification (Kerstetter et al., 1997; Hung et al., 1998; Sentoku et al., 2000; Mussig et al., 2000; Ohashi et al., 2003). Most of the previous studies have been focused on the function of the homeodomain proteins belonging to the Knotted1, HD-Zip, Glabra2, and PHD finger families from various plant species. Recently, several genes encoding the BELL type of homeodomain proteins have been isolated from different plant species, including *Arabidopsis thaliana* (Reiser et al., 1995), barley (Muller et al., 2001), potato (Chen et al., 2003), and apple (Dong et al., 2000). The *Arabidopsis* BELL1 has been demonstrated to be involved in regulation of ovule development (Reiser et al., 1995) and might act, at least in part, to regulate ovule development by repressing the function of the organ identity gene AGAMOUS (Western and Haughn, 1999). The apple MDH1 and the potato StBEL5 have been shown to play an important role in fruit and tuber development (Chen et al., 2003; Dong et al., 2000).

On the other hand, plant homeodomain proteins may also be involved in responses to hormones as well as to environmental and biotic stress. Homeobox genes from *Arabidopsis thaliana,*

Craterostigma plantagineum, and sunflower have been shown to be inducible by abscisic acid (ABA) or water stress (Soderman et al., 1996; Lee and Chun, 1998; Frank et al., 1998; Soderman et al., 1999; Sakamoto et al., 2001; Deng et al., 2002; Gago et al., 2002) and some of them may regulate ABA responses (Masucci and Schiefelbein, 1996; Tamaoki et al., 1997; Kusaba et al., 1998; Himmelbach et al., 2002; Sawa et al., 2002; Johannesson et al., 2003). Moreover, some homeodomain proteins have been demonstrated to play important roles in transcriptional regulation of defence-related gene expression during disease resistance responses (Korfhage et al., 1994) and programmed cell death (Mayda et al., 1999). HD domain-containing nuclear proteins from *Arabidopsis* and parsley bound to an 11-bp motif (CTA ATT GTT TA) present in the parsley *PR2* gene promoter (Korfhage et al., 1994; Abe et al., 2001). A tomato gene, *H52,* encoding a HD-Zip transcription factor, has been shown to be involved in cellular protection by limiting spread of programmed cell death in plants (Mayda et al., 1999).

Several lines of evidence have showed that the plant homeodomain proteins play roles in regulation of expression of genes that are involved in development processes and responses to abiotic and biotic stress (Otting et al., 1990; Chang et al., 1997; Abe et al., 2001; Johannesson et al., 2001; Tang et al., 2001; Himmelbach et al., 2002). For example, while the *Arabidopsis* ATHB5 was shown to interact with a 9-bp pseudopalindromic DNA sequence, CAA TNA TTG (Johannesson et al., 2001), the ATHB1 was able to activate transcription from target sequences upstream of a reporter gene in tobacco cells (Aoyama et al., 1995). Recently, it has also been demonstrated that plant homeodomain proteins physically interact with other transcriptional factors to regulate gene expression via a direct or indirect effect on transcription of the target genes (Muller et al., 2001; Bellaoui et al., 2001; Smith et al., 2002; Chen et al., 2003).

We have identified over 200 differentially expressed cDNAs that were associated with disease resistance responses and demonstrated that genes encoding a mitogen-activated protein kinase and a phosphoinositide-specific phospholipase C play a role in rice defence responses (Song and Goodman, 2002 a, b). Here, we report the molecular cloning and characterization of a rice gene, *OsBIHD1,* which encodes a BELL type homeodomain protein. The expression of *OsBIHD1* was activated by treatment with BTH and by infection with the blast fungus in an incompatible interaction between a resistant rice line and *M. grisea.* The recombinant OsBIHD1 protein bound the TGTCA motif DNA sequence that is the characteristic *cis*-element DNA sequence of the homeodomain transcriptional factors. The OsBIHD1 protein localized in the nucleus of plant cells, as revealed by transient expression of OsBIHD1 in onion epidermal cells. Our results further suggest that *OsBIHD1* encodes a BELL type homeodomain transcriptional factor whose inducible expression might be associated with disease resistance response in rice.

Materials and Methods

Growth of rice seedlings and treatments

Rice cultivar Yuanfengzao (*Oryza sativa* L. indica type) and a pair of near-isogenic lines (H8S and H8R) were used in this study. Yuanfengzao is highly susceptible to the rice blast fungus *Magnaporthe grisea* strain 85-14B1, belonging to race ZB1. H8S is susceptible and H8R is resistant to *M. grisea* isolate 85-14B1.

Three-week-old seedlings grown in field soil in plastic pots (8 cm in diameter and 10 cm in height, 10 seedlings per pot) under greenhouse conditions at 22/27 °C (night/day) were used for all experiments. For analysis of benzothiadiazole (BTH)-induced gene expression, the seedlings were treated by spraying with 0.3 mmol/L BTH solution (Novartis Crop Protection Inc., Research Triangle Park, NC, USA) or with sterilized distilled water as a control. The third and the fourth leaves were collected at different time points after treatment and frozen at – 80 °C until use. For analysis of gene expression in rice seedlings after blast fungus infection, the seedlings were inoculated 3 days after BTH treatments with spore suspension (5×10^5 spores per ml in 0.05 % Tween-20) of *M. grisea* or with sterilized distilled water containing 0.05 % Tween-20. The inoculated and the uninoculated rice seedlings were kept at 100 % relative humidity in darkness for 36 h. Leaf samples were collected at different time points after inoculation and stored at – 80 °C.

To analyze the expression pattern of *OsBIHD1* in incompatible and compatible interaction between rice and the blast fungus, three-week-old seedlings of H8R and H8S were inoculated directly with spores of the fungus as described above. Leaf samples were collected at different time points after inoculation and stored at – 80 °C.

Cloning of the OsBIHD1 *cDNA*

In our previous study, differentially expressed cDNAs associated with induced disease resistance were isolated through suppression subtractive hybridization (SSH) (Song and Goodman, 2002 a). The differentially expressed clone, HIHN-w5, obtained form that study, contained a 733-bp insert. A sequence similarity search against the GenBank database revealed that the insert in the HIHN-w5 clone showed high level of similarity to *Arabidopsis thaliana* BELL1 (GenBank accession no. A57632) (Reiser et al., 1995) and potato BELL30 (GenBank accession no. AF406703) (Chen et al., 2003). To obtain the full-length cDNA of this putative BELL gene, the 5′-sequence flanking the sequence in the clone HIHN-w5 was amplified by PCR using phage DNA prepared from a rice cDNA library (Song and Goodman, 2002 a) as template. The gene-specific primer, HIHN-w5-1R (5′-CGT GTA CGA CGC GAC CAT GT-3′), which corresponded to sequences at positions 83 – 102 in the sequence of the clone HIHN-w5, and a vector primer T3-2 (5′-CCT GCA GGT CGA CAC TAG TG-3′) were used for amplification of the 5′-end. According to the result of 5′ sequence, a gene-specific primer in the 5′-UTR, HIHN-w5-1F (5′-TCT TGC TAA TTG AGT TGT CA-3′), and a vector primer T7-2 (5′-CCA CCC GGG TGG AAA ATC GA-3′) were used for amplification of the full-length cDNA using the phage DNA as template. All PCR products were purified using the DNA Gel Purification Kit (Sangon, Shanghai, China) and cloned into pGEM T-Easy vector (Promega, Madison, WI, USA) by T/A cloning. The plasmid containing the complete ORF and the UTR sequences of both ends was designated pUCm-HIHN-w5.

The entire open reading frame of the full-length cDNA was amplified using plasmid pUCm-HIHN-w5 as template, and a pair of specific primers HIHN-w5-2F (5′-**GGA TCC** ATG GCT ACT TAC TAC T-3′) and HIHN-w5-2R (5′-**AAG CTT** TCA GGC CAC AAA ATC ATG CA-3′), which contain a *Bam*HI and a *Hin*dIII site (bold), respectively. The entire ORF was cloned and confirmed by sequencing, yielding plasmid pUCm-OsHIHD1-1.

DNA sequencing and sequence analysis

DNA sequencing was performed on both strands on the MegaBACE 1000 DNA Analysis System (Amersham Biosciences, UK) at the Center of Analysis and Measurement in Zhejiang University. Similarity searches on nucleotide and amino acid sequences were carried out using BLAST at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). Sequences of the other plant homeodomain proteins were retrieved from GenBank. Sequence alignments were conducted using CLUSTAL (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994). A phylogenetic tree was constructed by the Clustal method using DNAStar software (LaserGene, Madison, WI, USA).

Purification of the recombinant OsBIHD1 protein and DNA binding assay

The coding region of *OsBIHD1* was released from plasmid pUCm-OsHIHD1-1 by digesting with *Bam*HI/*Hin*dIII and cloned into the pET-30a expression vector (NovaGen, Madison, WI, USA) in the sense orientation. The resulting plasmid, pET-OsBIHD1-1, was confirmed by sequencing and introduced into *Escherichia coli* BL21 (DE3) cells. Purification of the recombinant protein from *E. coli* was carried out with the His-Bind kit (NovaGen, Madison, WI, USA) following the manufacturer's instructions. The protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) following the recommended method.

DNA binding activity of the recombinant OsBIHD1 protein was analyzed by electrophoretic mobility shift assay. Sequences of four tandem repeats of the homeodomain binding motif (TGTCA) and its mutant version (TCTCA) were prepared by synthesizing both strands (Abe et al., 2001). Double stranded complementary fragments were annealed and labelled with [γ-^{32}P] ATP (Furui Biotech Co., Beijing, China) by T4 polynucleotide kinase (Promega, Madison, WI, USA). DNA binding reactions were performed at 25 °C for 30 min in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05 mg/l Poly[dI-dC]·poly [dI-dC]) and subjected to electrophoretic mobility shift assay using 10% polyacrylamide gels in 0.5× Tris-borate-EDTA buffer.

Subcellular localization analysis of OsBIHD1-GFP protein

To construct the OsBIHD1-GFP expression vector, the *OsBIHD1* coding region was amplified by PCR with a pair of primers containing a *Sal*I site upstream of the start codon and an *Nco*I site at the stop codon. This fragment was introduced into the *Sal*I/*Nco*I site of the pSGFP (S65T) vector (Chiu et al., 1996; Niwa et al., 1999) and translationally fused in-frame N-terminal to the Green Fluorescent Protein (GFP)-encoding sequence in pS6FP, yielding plasmid pSGFP-OsBIHD1.

This plasmid was introduced into onion epidermal cells by the particle bombardment method (Takeuchi et al., 1992). Onion cells were placed on filter paper in Petri dishes. Particle bombardment was performed with a PDS-1000 (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, using gold particles coated with plasmid DNA. Onion cells were bombarded once under a slight vacuum using a helium pressure of 1100 psi. After bombardment, onion peels were incubated with liquid Murashige-Skoog medium for 12 – 18 h and GFP was detected with an Olympus DP-50 fluorescent microscope.

Southern blot hybridization

Rice genomic DNA was isolated from three-week-old seedlings by the cetyltrimethylammonium bromide method (Murray and Thompson, 1980). Fifteen micrograms of the rice genomic DNA were digested completely with *Xba*I, *Bam*HI, or *Nde*I, separated by electrophoresis on a 0.8% agarose gel, and transferred by capillary action overnight onto Hybond-N$^+$ nylon membrane (Amersham Biosciences, Little Chalfont, UK) using 0.4 M NaOH/1.0 M NaCl. A 897-bp fragment was prepared by digestion of the *OsBIHD1* ORF sequence with *Pst*I and labelled with [α-^{32}P]-dCTP (3000 Ci mM-1) by the random priming method using a Random Primed DNA Labelling Kit (Takara, Dalian, China). Prehybridization was performed at 42 °C for 30 min in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) and hybridization was carried out overnight at 42 °C in the same hybridization buffer with the [α-^{32}P]-labelled probe. After hybridization, the blots were washed twice with 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS for 10 min each at 42 °C. After washing, the membrane was blotted between waxfilm (Whatman International, Maidstone, UK) and autoradiographed by exposure to X-ray film (Lucky Film Corporation, Baoding, China) for 2 d at – 80 °C.

Northern blot hybridization

Total RNA was extracted using acidic phenol-guanidine isothiocyanate-chloroform (Chomczynski et al., 1987). Twenty micrograms of total RNA were fractionated on 1.0% agarose-formaldehyde gel and transferred by capillary action overnight to a Hybond-N$^+$ nylon membrane (Amersham Biosciences, Little Chalfont, UK) using 20 × SSC. The RNA on the membrane was fixed by baking at 80 °C for 2 h. Probe labelling, hybridization, and detection were the same as in the procedure described for Southern blot hybridization.

Results

Cloning of OsBIHD1, *a gene encoding a rice homeodomain protein*

In our previous studies aimed at elucidating the molecular biology of disease resistance in rice, hundreds of differentially expressed cDNA clones associated with BTH-induced disease resistance response were isolated and identified by SSH (Song and Goodman, 2002a). Among these differentially expressed cDNAs, BLAST similarity searching against the GenBank database revealed that the 733 bp insert in clone HIHN-w5 showed a high level of similarity to genes encoding homeodomian proteins. ESTs associated with the sequence in clone HIHN-w5 were retrieved through database searches and a 2201-bp of

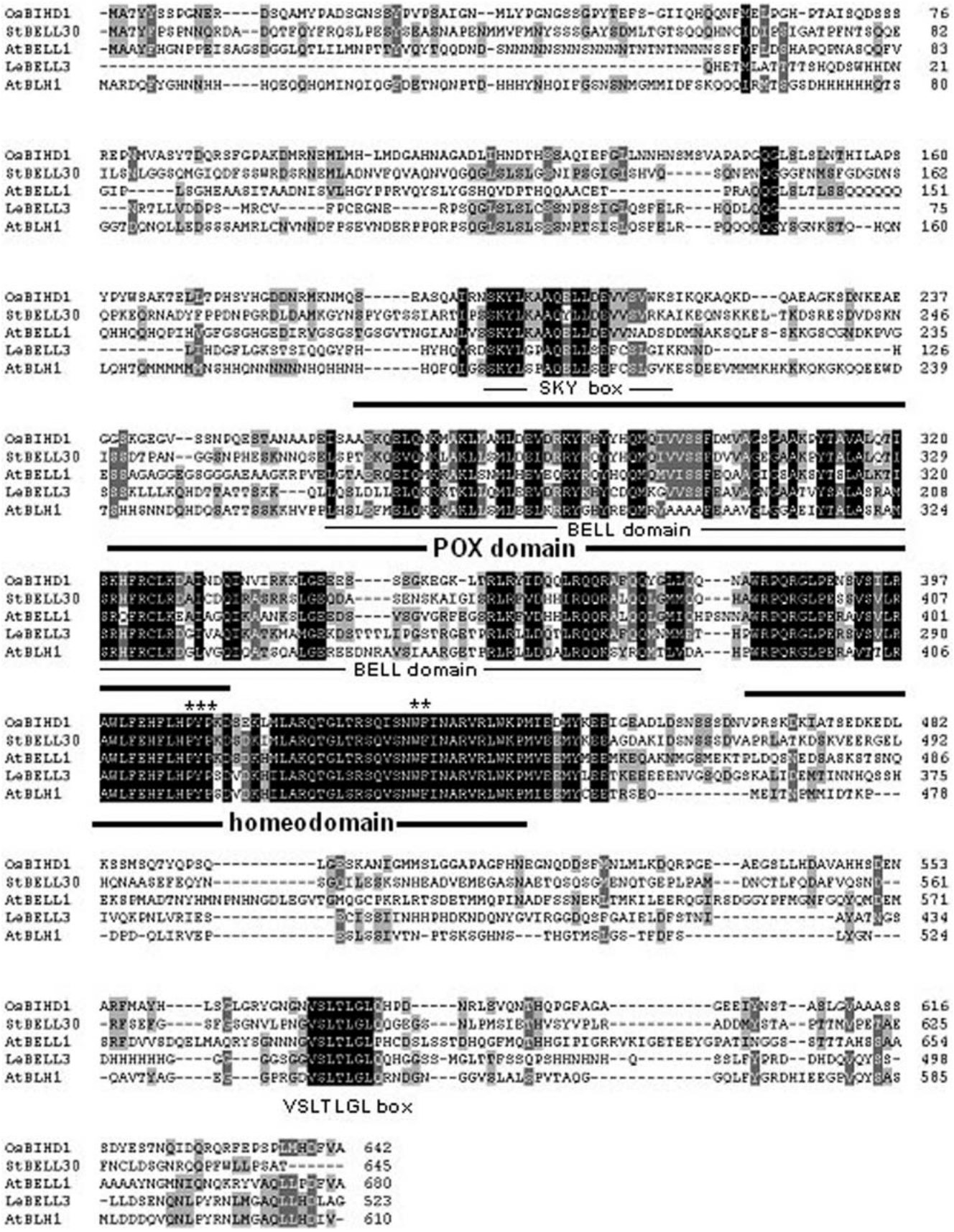

Fig. 1 Alignment of the OsBIHD1 amino acid sequence with those of other known plant homeodomain proteins. The plant homeodomain proteins used for alignment are: *Arabidopsis thaliana* AtBEL1 (A57632), AtBEL1-like homeodomain protein AtBLH1 (AAK43836), potato StBEL30 (AAN03627), and tomato LEBELL3 (AAP47032).

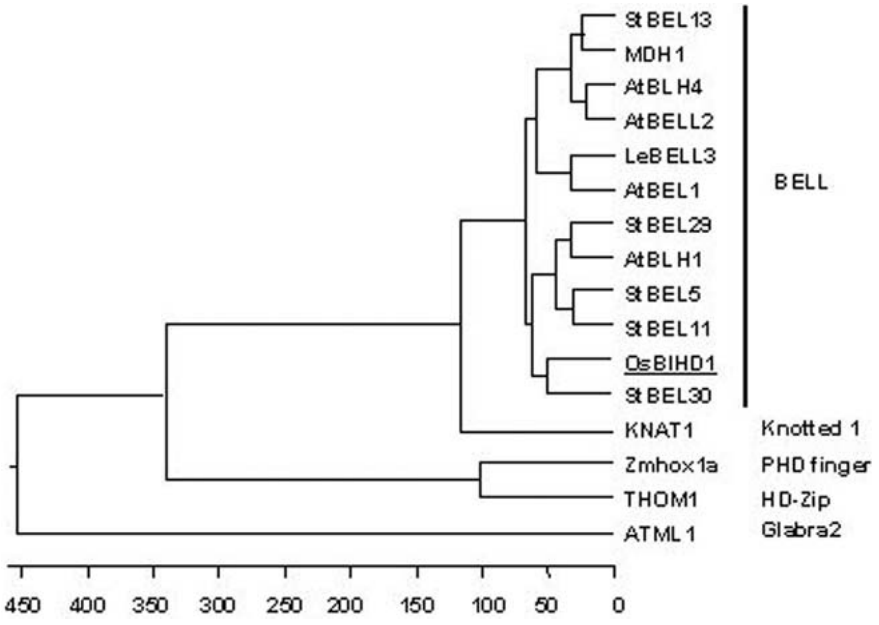

Fig. 2 Phylogenetic analysis of OsBIHD1 with other homeodomain proteins from various plant species. The plant BELL proteins used are: *Arabidopsis thaliana* AtBEL1 (A57632) (Reiser et al., 1995), AtBEL1-like homeodomain AtBLH1 (AAK43836), AtBLH2 (CAB80353), AtBLH4 (AAK43834); apple MDH1 (AAF43095) (Dong et al., 2000); *Solanum tuberosum* StBEL5 (AAN03621), StBEL11 (AAN03622), StBEL13 (AAN03623), StBEL29 (AAN03626), StBEL30 (AAN03627) (Chen et al., 2003), *Lycopersicon esculentum* LeBELL3 (AAP47023). Other homeodomain proteins belonging to Knotted1, glabra2, HD-Zip, and PHD finger families used were: *Arabidopsis thaliana* KNAT1 (AAA67881) (Lincoln et al., 1994), *Lycopersicon esculentum* THOM1 (CAA62608) (Meissner and Theres, 1995), *Zea mays* Zmhox1a (CAA47859) (Bellmann and Werr, 1992), *Arabidopsis thaliana* ATML1 (AAB49378) (Lu et al., 1996).

cDNA fragment was assembled. However, this cDNA fragment was still not the full-length cDNA of the gene, lacking approximately 300 bp sequence at the 5′ end. To obtain the full-length cDNA encoding this putative homeodomain protein, the 5′ end of the gene sequence was amplified with phage DNA prepared from a rice cDNA library as template, using HIHN-w5 sequence-specific and phage vector primers. One fragment of ~500 bp was amplified, cloned, and sequenced. The full-length cDNA of the gene was assembled, which was 2524 bp with a predicted 1929-bp open reading frame (ORF) and 257 bp and 339 bp of 5′- and 3′-UTR sequences, respectively. The ORF sequence was amplified using a pair of primers at the start and stop codons, designed based on the predicted ORF and verified by sequencing. This full-length cDNA was designated as *OsBIHD1* for ***O**ryza **s**ativa* L. **B**TH-**i**nduced **h**omeo**d**omain protein *1*. The 1929 bp ORF of the *OsBIHD1* predicted to encode a putative protein that has 642 amino acid residues with a calculated molecular weight of 71 kD and isoelectric point of 6.04.

We also found a putative upstream open reading frame (uORF) (from nucleotides 116 to 172) encoding 18 amino acids (MHATAINQLIQQGNSIGI) in the 257 bp 5′ UTR sequence of the OsBIHD1 cDNA. Such uORFs are often found in mRNAs encoding critical regulatory proteins such as transcription factors and involved in regulation of target genes (Damiani and Wessler, 1993; Quaedvlieg et al., 1995).

OsBIHD1 is a homeodomain protein belonging to the BELL family

Alignment of the deduced protein sequence of OsBIHD1 with other homeodomain proteins from various plant species indicated that the OsBIHD1 protein contained a highly conserved homeodomain. The homeodomain region (from Trp-382 to Met-443) in OsBIHD1 is almost identical to those of other known plant homeodomain proteins (Fig. **1**). Two conserved motifs, PYP (407 – 409) and WF (431 – 432), which are believed to interact directly with the target sequence (Nagasaki et al., 2001), were identified in the homeodomain region of the OsBIHD1 protein. In addition, three other conserved regions, a SKY box, a BELL domain, and a VSLTLGL box (Bellaoui et al., 2001), were also identified in the sequences outside the homeodomain. The SKY box consisted of 19 amino acids from Ser-197 to Lys-215 in OsBIHD1 and was nearly identical to those in other homeodomain proteins. The BELL domain consisted of the 120 amino acids starting at Ile-260 of the OsBIHD1 sequence, and showed from 36.7% to 55.8% identity to those in AtBEL1 (A57632), AtBLH1 (AAK43836), StBEL30 (AAN03627), and LeBELL3 (AAP47023) (Reiser et al., 1995; Chen et al., 2003). Moreover, the region from Ser-188 to Ile-334, which contained the SKY box and part of the BELL domain, also showed a high level of homology to the POX domain. However, the sequences outside these conserved regions showed high levels of sequence variation when compared to other plant BELL family members.

Phylogenetic tree analysis using the identified and putative BELL proteins, along with other homeodomain proteins belonging to Knotted1, HD-Zip, glabra2, and PHD finger families, revealed that the OsBIHD1 protein belonged to the BELL type of the homeodomain proteins and was closest to the potato homeodomain BELL protein StBel30 (AAN03627) (Chen et al., 2003) (Fig. **2**). Therefore, we concluded that the OsBIHD1 is a BELL type protein of the homeodomain family in rice.

Gene structure and genomic organization of OsBIHD1

Southern blotting analysis was performed to determine the number of the *OsBIHD1* genes in the rice genome. The rice genomic DNA isolated from 3-week-old leaves of rice seedlings was digested with *Xba*I, *Bam*HI, or *Nde*I, and hybridized with the 897-bp fragment of the 5′ end of the *OsBIHD1* cDNA as a probe. Only one band was detected in the rice genomic DNA digested with *Xba*I, *Bam*HI, or *Nde*I enzymes (data not shown). This result indicated that the *OsBIHD1* appeared to be a single

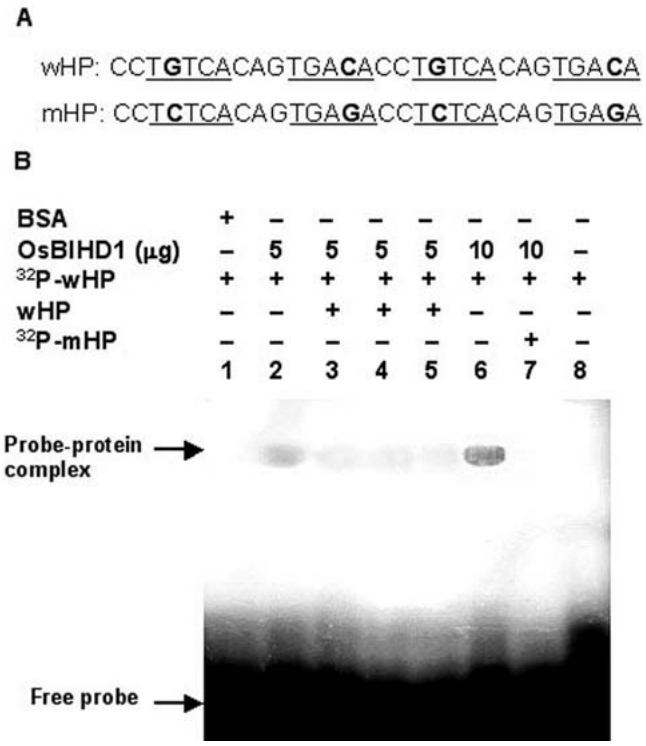

Fig. 3 DNA binding activity of the recombinant OsBIHD1 protein. Sequence of the wild type probe (wHP) and the mutant version (mHP) used for the DNA binding assays (**A**). DNA binding activity of the recombinant OsBIHD1 protein (**B**). Electrophoretic mobility shift assays were performed using the recombinant OsBIHD1 protein and 1 ng of [γ-^{32}P]-labelled either wild-type probe (lanes 2 and 6) or mutated probe (lane 7). The competition assay (lanes 3 to 5) was performed by adding unlabelled wild type probe in the binding reactions. The negative controls (lanes 1 and 8) contained the labelled wild type probe and BSA alone or no protein in the binding reactions. Specific DNA-protein complexes and free probes are indicated by the arrows.

copy gene in the rice genome. To elucidate the structure of the *OsBIHD1* gene, genomic sequence data was retrieved from GenBank using the ORF sequence of the *OsBIHD1* cDNA as query. A clone named OSJNBa0003G23 (GenBank accession no. AC079736), which located on chromosome 3 of the rice genome, was found to be identical to the ORF sequence. Alignment of the OSJNBa0003G23 sequence with the ORF sequence of the *OsBIHD1* cDNA revealed that the genomic sequence corresponding to the ORF of the *OsBIHD1* gene contains 3280 nucleotides and the *OsBIHD1* gene consists of four exons and three introns (data not shown).

Recombinant OsBIHD1 protein showed DNA binding activity

To determine whether the *OsBIHD1* gene encodes a homeodomain protein with DNA binding activity, we expressed the ORF sequence of the full-length cDNA in *E. coli* and purified the recombinant protein. In SDS-PAGE gel, the purified recombinant OsBIHD1 protein showed a single band with molecular weight of ~ 75 kD, which was in agreement with the calculated molecular weight from the predicted OsBIHD1 amino acid sequence (data not shown). Previous studies have shown that the homeodomain proteins bound to the *cis*-element sequence containing the TGTCA motif (Abe et al., 2001). Complementary four tandem repeated sequences of the TGTCA motif and a mutant version TCTCA were synthesized, annealed to form double stranded fragments and labelled with [γ-^{32}P] ATP. In our gel mobility shift assay, the negative controls, presence of BSA only or absence of the OsBIHD1 protein in the binding reactions containing the wild type probe, did not show any signal (Fig. **3**, lanes 1 and 8, respectively). The recombinant OsBIHD1 protein bound the wild type probe in a dosage-dependent manner, as the binding activity was much higher in 10 μg of OsBIHD1 protein than that in 5 μg of protein (Fig. **3**, lanes 2 and 6). However, the binding activity of the recombinant OsBIHD1 protein was dramatically reduced by competition with an unlabelled wild type probe (Fig. **3**, lanes 3 to 5). Moreover, the OsBIHD1 protein did not bind the mutant probe, in which the G residue within the TGTCA motif was replaced by C (Fig. **3**, Lane 7). These results suggest that the OsBIHD1 protein binds specifically to DNA sequence containing the TGTCA motif.

OsBIHD1 protein was targeted to the nucleus

Because the homeodomain proteins are believed to function as transcriptional factors (Chang et al., 1997), it is necessary to confirm that OsBIHD1 is localized in the nucleus. To study the *in vivo* subcellular localization of the OsBIHD1 protein, we cloned the *OsBIHD1* ORF sequence into pSGFP(S65 T) (Chiu et al., 1996; Niwa et al., 1999) to translationally fuse the OsBIHD1 sequence in-frame to GFP. We then used particle bombardment to transfer this construct encoding a translational fusion between GFP and OsBIHD1 in onion epidermal cells. As shown in Fig. **4**, GFP alone did not localize to a specific compartment and was detected in the whole cytoplasm and nucleus. As expected, the GFP-OsBIHD1 fusion protein was localized specifically in the nucleus. This result clearly demonstrated that the OsBIHD1 protein is targeted to the nucleus in plant cells.

OsBIHD1 *was differentially expressed in rice disease resistance responses*

To elucidate the possible involvement of *OsBIHD1* in rice disease resistance responses, we analyzed the expression patterns of *OsBIHD1* in response to BTH induction as well as to infection with the blast fungus, *M. grisea.* In Northern blot hybridization, the size of the hybridizing band was ~ 2.5 Kb, which was similar to the size of the full-length cDNA of *OsBIHD1.* Expression of the *OsBIHD1* gene was upregulated after BTH treatments, with a high level of induced expression within the first 12 h (Fig. **5A**), as compared to that in the water-treated seedlings, in which a relatively low level of *OsBIHD1* expression was maintained unchanged during the experimental period (Fig. **5B**). These results indicated that the expression of *OsBIHD1* gene was rapidly activated upon BTH induction. In the BTH-treated rice seedlings, expression of *OsBIHD1* gene was activated rapidly after infection by *M. grisea* (Fig. **6**). Induced expression of *OsBIHD1* was detected as early as 6 h after inoculation with the fungus and maintained a higher level of expression from 12 to 72 h (Fig. **6A**). Conversely, no significant induced expression of *OsBIHD1* was detected in water-treated rice seedlings within 24 h after inoculation with the fungus, but a relatively low level of induced expression was observed from 30 – 72 h after inoculation (Fig. **6B**).

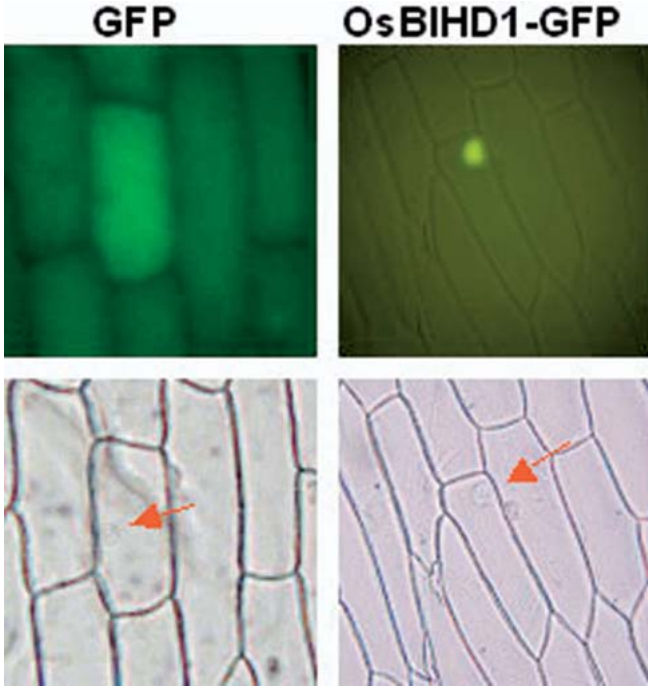

Fig. 4 Nuclear localization of the OsBIHD1 protein in onion epidermal cells. Onion epidermal cells were transiently transformed with constructs containing either control GFP (left) or OsBIHD1-GFP (right) by bombardment. The subcellular localization of the OsBIHD1-GFP fusion protein and GFP alone were viewed with a fluorescent microscope 18 h after bombardment. Bright-field (lower panel) and the corresponding epifluorescence images (upper panel) of representative cells expressing GFP or a OsBIHD1-1-GFP fusion protein are shown.

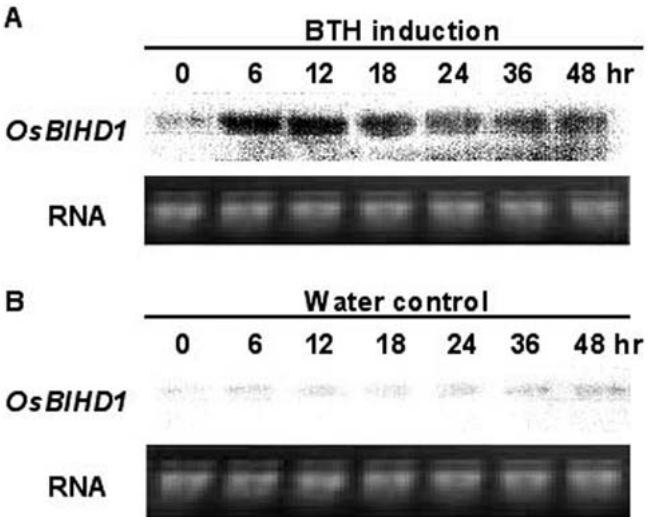

Fig. 5 Expression of *OsBIHD1* is activated by BTH induction. Rice seedlings were treated with BTH solution of 0.3 mmol/L (**A**) or water (**B**) and leaf samples were collected at time points as indicated. Twenty micrograms of total RNA were fractionated on a 1.2% agarose formaldehyde gel and hybridized with the ^{32}P-labelled 897-bp fragment of *OsBIHD1* cDNA as a probe. The corresponding ethidium bromide gel image shows the relative levels of RNA loaded for each sample.

The association of the induction of *OsBIHD1* gene expression and the disease resistance response in rice after inoculation with *M. grisea* was studied further using a pair of near-isogenic lines, H8R and H8S. The disease responses of H8R and H8S seedlings to infection by *M. grisea* represent incompatible and compatible interactions, respectively. The results showed that expression of *OsBIHD1* in H8R leaves was activated rapidly by infection with the blast fungus (Fig. **7**). As seen in the BTH-treated rice seedlings, expression of *OsBIHD1* in the incompatible interaction between rice and *M. grisea* was detected as early as 6 h after inoculation, and increased gradually at a higher level during 6 – 48 h (Fig. **7 A**). Compared with the expression in H8R, only a slight induced expression of *OsBIHD1* was observed in H8S leaves from 24 to 48 h (Fig. **7 B**). These results indicate that the expression of *OsBIHD1* was involved in a resistance response and/or an incompatible interaction.

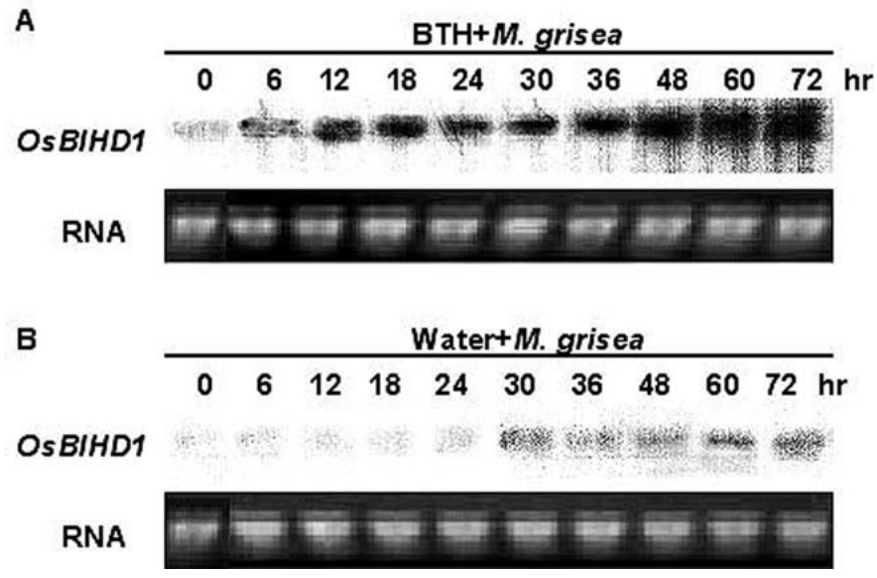

Fig. 6 Induced expression of the *OsBIHD1* gene is associated with disease resistance responses induced by BTH. Rice seedlings were treated with 0.3 mmol/L BTH solution (**A**) or water (**B**), and inoculated with *Magnaporthe grisea* 3 days after treatment. Leaf samples were collected at each time point (hr) as indicated. Twenty micrograms of total RNA were fractionated on a 1.2% agarose formaldehyde gel and hybridized with the ^{32}P-labelled 897-bp fragment of *OsBIHD1* cDNA as a probe. The corresponding ethidium bromide gel image shows the relative levels of RNA loaded for each sample.

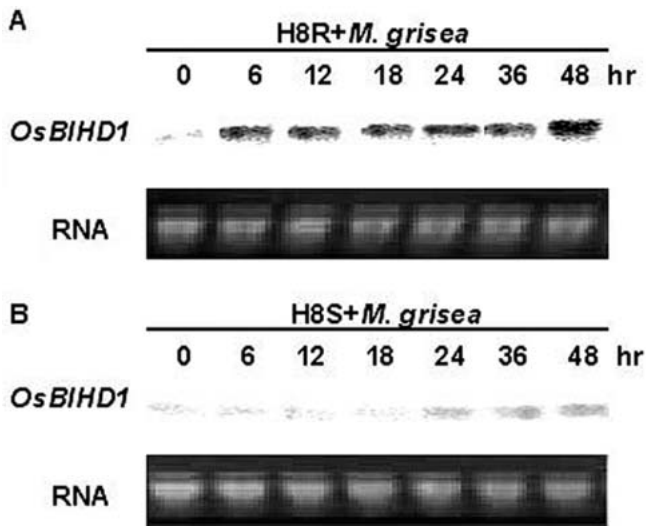

Fig. 7 Induced expression of the *OsBIHD1* gene is associated with incompatible interaction between rice and *Magnaporthe grisea.* Three-week-old rice seedlings of H8R (**A**) and H8S (**B**) were inoculated with *M. grisea.* Leaf samples were collected at each time point (hr) as indicated. Twenty micrograms of total RNA were fractionated on a 1.2% agarose formaldehyde gel and hybridized with the ^{32}P-labelled 897-bp fragment of *OsBIHD1* cDNA as a probe. The corresponding ethidium bromide gel image shows the relative levels of RNA loaded for each sample. Near-isogenic lines H8R and H8S represent incompatible and compatible interactions, respectively, with the tested strain of the blast fungus *M. grisea.*

Discussion

The homeodomain proteins have been extensively studied for their functions in plant development processes (Chan et al., 1998), as well as their involvement in adaptation to environment stress (Soderman et al., 1996; Frank et al., 1998; Lee and Chun, 1998; Soderman et al., 1999; Deng et al., 2002; Gago et al., 2002). In the present study, we cloned and identified a rice gene, *OsBIHD1,* encoding a BELL type homeodomain protein. The inducible expression of *OsBIHD1* by BTH induction and pathogen infection suggests a role for OsBIHD1 in rice disease resistance responses, providing more support for the function of the homeodomain proteins in the signalling pathways leading to activation of disease resistance. Our results further extend the knowledge for the functions of the homeodomain proteins.

The OsBIHD1 protein contains all conserved domains that are characteristics of the BELL type homedomain proteins (Fig. **1**). The homeodomain region has been demonstrated to contain a DNA binding motif and mediate an interaction with corresponding *cis*-element DNA sequences (Bellaoui et al., 2001; Himmelbach et al., 2002; Smith et al., 2002). The recombinant OsBIHD1 protein specifically bound the TGTCA motif in our EMSA assay (Fig. **3**). The BELL region has been suggested to form a coiled coil structure that could mediate interactions with other proteins and the first 80 amino acids of this domain are necessary to mediate the interactions (Reiser et al., 1995; Chen et al., 2003), leading to activation of the transcription of target genes (Bellaoui et al., 2001). A putative nuclear localization motif was also identified in the BELL domain (Reiser et al., 1995), indicating a role in the subcellular distribution of the BELL proteins in plant cells. In our study, we found that the OsBIHD1 protein was localized to the nucleus in onion epidermal cells (Fig. **4**). This result is in agreement with the finding that the *Arabidopsis* BELL1 protein targeted to the nucleus (Reiser et al., 1995). Previous studies showed that the SKY box plays a role in mediating interaction with other partner proteins through direct involvement in the interaction or by enhancing the binding affinity to partner proteins (Muller et al., 2001; Chen et al., 2003). Recently, the BELL proteins from *Arabidopsis* and potato were found to interact with KNOX type homeodomain proteins (Bellaoui et al., 2001; Chen et al., 2003). The function of the VSLTLGL box remains unknown, but it was not involved in protein-protein interactions (Chen et al., 2003). Moreover, an upstream ORF of 18 amino acids was also found in the *OsBIHD1* cDNA. Such upstream ORFs are believed to be involved in regulation of translation of regulatory protein such as transcription factors (Damiani and Wessler, 1993; Quaedvlieg et al., 1995; Dong et al., 2000). The DNA binding activity and nuclear localization of the OsBIHD1 protein, as well as the presence of a uORF, suggest that OsBIHD1 may function as a transcription factor.

Plant homeodomain proteins have been shown to be involved in responses to environmental and biotic stress. Expression of homeobox genes encoding HD-Zip proteins from different plant species was activated by water deficit stress or dehydration (Soderman et al., 1996; Frank et al., 1998; Lee and Chun, 1998; Soderman et al., 1999; Sakamoto et al., 2001; Deng et al., 2002; Gago et al., 2002). Binding activity of HD domain-containing nuclear proteins from *Arabidopsis* and parsley to a *cis*-element motif present in the parsley *PR2* gene promoter (Korfhage et al., 1994; Abe et al., 2001) and induction of the tomato gene, *H52,* encoding a HD-Zip protein, by infection with an incompatible pathogen (Mayda et al., 1999), suggest that homeodomain proteins may also play a role in plant disease resistance responses through regulating defence-related gene expression. Expression of the potato *BELL* genes and the apple *MDH* gene were detected in different organs, including flowers and leaves (Dong et al., 2000; Chen et al., 2003), and the expression of the potato *StBEL5* gene in leaves was increased by short light treatment (Chen et al., 2003). In our Northern analysis, the *OsBIHD1* gene showed a relatively high level of basal expression under normal growth conditions, implying *OsBIHD1* might have a role in development processes. However, expression of the *OsBIHD1* gene was induced rapidly by BTH induction and infection by the rice blast fungus. Most importantly, expression of *OsBIHD1* was specifically activated in the incompatible interaction between rice and *M. grisea.* These results demonstrate that *OsBIHD1* might be involved in a resistance response and/or an incompatible interaction between rice and the blast fungus.

Antisense inhibition of *H52* gene expression in transgenic tomato plants resulted in a mis-regulation of programmed cell death, activation of defence genes and enhanced disease resistance against virulent pathogens (Mayda et al., 1999). Thus, H52 might be a negative regulator of defence responses and protect from programmed cell death in plants. In another study, we introduced the *OsBIHD1* gene under the control of the CaMV 35S promoter into tobacco and our preliminary results show that overexpression of *OsBIHD1* in transgenic tobacco plants leads to constitutive expression of the defence-related gene, *PR-1,* and enhanced disease resistance against

viral and fungal pathogens (Luo, H., Song, F., and Zheng, Z., unpublished data). Together with the inducible expression in rice disease resistance responses, these results suggest that OsBIHD1 plays a role in a signalling pathway leading to activation of defence responses in rice.

The BELL type homeodomain proteins have been implicated in flower, fruit, and tuber development (Reiser et al., 1995; Dong et al., 2000; Chen et al., 2003). Transgenic potato plants that overexpressed *StBEL5* exhibited enhanced tuber formation, but did not exhibit significant morphological changes (Chen et al., 2003). However, transgenic *Arabidopsis* plants overexpressing the apple *MDH1* gene showed dwarfing, reduced fertility and changes in carpel and fruit (silique) shape (Dong et al., 2000). We also found that overexpression of *OsBIHD1* in transgenic tobacco plants resulted in some morphological abnormalities in the top buds and roots and some of the transgenic lines showed reduced fertility (Luo, H., Song, F., and Zheng, Z., unpublished data). Thus, OsBIHD1 proteins are capable of both determination of basic developmental processes and regulation of gene expression in defence responses. However, the precise functions of OsBIHD1 in rice development and disease resistance responses requires further study through evaluation of the phenotypes of transgenic rice plants with overexpression of *OsBIHD1* or suppression of the *OsBIHD1* expression by functional genomics approaches.

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F. Song

Department of Plant Protection
College of Agriculture and Biotechnology
Zhejiang University – Huajiachi Campus
Hangzhou
Zhejiang 310029
P.R. China

E-mail: fmsong@zju.edu.cn

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