

RESEARCH PAPER

# OsBIRH1, a DEAD-box RNA helicase with functions in modulating defence responses against pathogen infection and oxidative stress

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## Abstract

DEAD-box proteins comprise a large protein family with members from all kingdoms and play important roles in all types of processes in RNA metabolism. In this study, a rice gene *OsBIRH1*, which encodes a DEAD-box RNA helicase protein, was cloned and characterized. The predicted *OsBIRH1* protein contains a DEAD domain and all conserved motifs that are common characteristics of DEAD-box RNA helicases. Recombinant *OsBIRH1* protein purified from *Escherichia coli* was shown to have both RNA-dependent ATPase and ATP-dependent RNA helicase activities *in vitro*. Expression of *OsBIRH1* was activated in rice seedling leaves after treatment with defence-related signal chemicals, for example benzothiadiazole, salicylic acid, l-aminocyclopropane-1-carboxylic acid, and jasmonic acid, and was also up-regulated in an incompatible interaction between a resistant rice genotype and the blast fungus, *Magnaporthe grisea*. Transgenic *Arabidopsis* plants that overexpress the *OsBIRH1* gene were generated. Disease resistance phenotype assays revealed that the *OsBIRH1*-overexpressing transgenic plants showed an enhanced disease resistance against *Alternaria brassicicola* and *Pseudomonas syringae* pv. *tomato* DC3000. Meanwhile, defence-related genes, for example *PR-1*, *PR-2*, *PR-5*, and *PDF1.2*, showed an up-regulated expression in the transgenic plants. Moreover, the *OsBIRH1* transgenic *Arabidopsis* plants also showed increased tolerance to oxidative stress and elevated expression levels of oxidative defence genes, *AtApx1*, *AtApx2*, and *AtFSD1*. The results suggest that *OsBIRH1* encodes a functional DEAD-box RNA helicase and plays important roles in defence responses against biotic and abiotic stresses.

Key words: DEAD-box RNA helicase, disease resistance, *OsBIRH1*, oxidative stress, rice (*Oryza sativa* L.).

## Introduction

Plants, unlike animals, are sessile during their growth and development, and thus are unable to avoid unfavourable environmental conditions. To survive in stress environments, plants have developed a complex, precisely regulated defence network to cope with a variety of biotic and abiotic stresses. In particular, plant responses to invading pathogens involve a complex network of defence mechanisms, in which thousands of genes are coordinately activated and integrated upon recognition of pathogen-associated molecular patterns (Takken *et al.*, 2007). These coordinately activated genes encode quite different species of proteins involved in numerous biological processes that are favourable for the defence response against pathogen attack. Recent studies have revealed that expression of many genes with regulatory functions such as transcription factors, protein kinases and phosphatases, RNA-binding proteins, calcium-binding proteins, etc. is altered during the defence response (Xiong *et al.*, 2002; Zhu, 2002; Shinozaki *et al.*, 2003; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006).

RNA helicases, referred to as enzymes that use energy derived from the hydrolysis of a nucleotide triphosphate to unwind double-stranded RNAs, function as molecular motors that rearrange RNA secondary structure, potentially playing roles in many cellular process (de la Cruz *et al.*, 1999). DEAD-box and related DEAH, DExH, and DExD families, which are commonly referred to as the DExD/H helicase family, are members of the SF2 family,

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and share nine highly conserved motifs (Gorbalenya and Koonin, 1993; Tanner and Linder, 2001; Caruthers and McKay, 2002). These closely related families can be distinguished by variations within their conserved motifs. The DEAD-box family is by far the largest one, and is characterized by the presence of nine conserved motifs that are involved in ATPase and helicase activities and in their regulation (Tanner *et al.*, 2003). The conserved motifs of the DExD/H helicases are clustered in a central core region that spans 350–400 amino acids (Tanner and Linder, 2001; Caruthers and McKay, 2002). By contrast, the N- and C-terminal extensions are highly variable in size and composition (Caruthers and McKay, 2002).

The DEAD-box RNA helicases form a large family of proteins found in all eukaryotes, and most prokaryotes (Aubourg *et al.*, 1999; de la Cruz *et al.*, 1999; Rocak and Linder, 2004). For example, nearly 30 genes that encode DEAD-box RNA helicases were identified in genomes of *Caenorhabditis elegans* and *Drosophila melanogaster* (Boudet *et al.*, 2001). In *Arabidopsis*, >50 members of DEAD-box RNA helicases have been identified (Aubourg *et al.*, 1999; Boudet *et al.*, 2001). Despite the involvement of DEAD-box RNA helicases in many diverse biological processes, their precise functions and regulation largely remain to be elucidated.

Increasing evidence suggests that the DEAD-box RNA helicases play important roles in plant growth and development processes, probably through regulation of RNA metabolism and gene expression (Itadani *et al.*, 1994; Brander and Kuhlemeier, 1995; Okanami *et al.*, 1998; Jacobsen *et al.*, 1999; Li *et al.*, 2001; Dalmay *et al.*, 2001; Western *et al.*, 2002; Gendra *et al.*, 2004; Arciga-Reyes *et al.*, 2006; Yoine *et al.*, 2006a, b; Kobayashi *et al.*, 2007; Matthes *et al.*, 2007). ISE2, an *Arabidopsis* DEVH box RNA helicase, affects the structure of plasmodesmata in the embryo and genetic data, demonstrating that ISE2 is involved in determination of cell fate through a post-transcriptional gene silencing mechanism (Kobayashi *et al.*, 2007). The *Arabidopsis* recessive mutant *caf* converts the floral meristems to an indeterminate state and thus CAF, a DExH/DEAD-box RNA helicase, appears to suppress cell division in floral meristems (Jacobsen *et al.*, 1999). The tobacco VDL, a member of the DEAD-box RNA helicase family, controls early plastid differentiation and plant morphogenesis as its recessive mutant *vdl* showed variegated leaves and abnormal roots and flowers (Wang *et al.*, 2000).

Involvement of the DEAD-box RNA helicases in plant stress responses has been evident recently in several studies in *Arabidopsis*. A cold stress-regulated DEAD-box RNA helicase, LOS4, was found to be an early regulator of CBF transcription factor expression in response to chilling (Gong *et al.*, 2002, 2005). Genome-wide expression profiling has also identified additional

*Arabidopsis* RNA helicase genes whose expression is cold-stress regulated (Seki *et al.*, 2001, 2002; Kreps *et al.*, 2002). STRS1 and STRS2, encoding DEAD-box RNA helicases, were also identified in a functional genomics screening. Loss of function of the *STRS1* and *STRS2* genes led to an increased expression of stress-responsive transcriptional activators and abiotic stress tolerance, and thus both STRS1 and STRS2 appear to function as an upstream negative regulator of ABA-dependent and ABA-independent abiotic stress signalling networks (Kant *et al.*, 2007).

Recent studies have provided new insights into understanding the mechanism by which the DEAD-box RNA helicases exert their biological functions. The DEAD-box RNA helicases, CARPEL FACTORY/DICER-LIKE 1 (DCL1) and UPF1, are critical for the microRNA biogenesis and RNAi in *Arabidopsis* (Park *et al.*, 2002; Reinhart *et al.*, 2002). DCL1 and a dsRNA-binding protein, HYPOASTIC LEAVES 1 (HYL1), form a complex, which functions together in pri-miRNA processing in a distinct nuclear organelle (Kurihara *et al.*, 2006). DCL1 is also involved in processing of the natural antisense siRNAs from transcripts of protein-encoding genes (Borsani *et al.*, 2005). UPF1 is essential for the targeted destruction of mRNAs containing premature termination codons by nonsense-mediated mRNA decay, and *upf1* mutants contain high levels of aberrant mRNAs and exhibit a range of unexpected vegetative and floral abnormalities (Arciga-Reyes *et al.*, 2006; Yoine *et al.*, 2006a, b).

In this study, the characterization of a rice gene, *OsBIRH1*, which encodes a DEAD-box RNA helicase with a DEVH motif, is reported. The *OsBIRH1* protein has RNA-dependent ATPase and ATP-dependent RNA helicase activities *in vitro*. Expression of *OsBIRH1* was activated by pathogen infection as well as by treatment with disease resistance-related signal molecules. Ectopic expression of *OsBIRH1* in transgenic *Arabidopsis* led to increased expression of defence-related genes and enhanced disease resistance and oxidative stress tolerance. The results suggest that *OsBIRH1* encodes a functional DEAD-box RNA helicase, which functions as a positive regulator in defence responses against biotic and abiotic stresses.

## Materials and methods

### Plant growth and treatments

Rice cultivar Yuanfengzao (*Oryza sativa* L. subsp. *indica*) and a pair of isogenic lines, H8R and H8S, were used in this study. Cultivar Yuanfengzao is highly susceptible to rice blast fungus *Magnaporthe grisea* strain 85-14B1, belonging to race ZB1. H8S is susceptible, while H8R is resistant to *M. grisea* isolate 85-14B1. Seedlings were grown in soil in a growth chamber at 27 °C/22 °C with 14 h light/10 h dark (night/day). Three-week-old seedlings of cv. Yuanfengzao were used for most experiments unless indicated otherwise. Treatments with benzothiadiazole (BTH; Novartis, USA), salicylic acid (SA; Sigma-Aldrich, St Louis, MO, USA), jasmonic

acid (JA; Sigma-Aldrich), and 1-aminocyclopropane-1-carboxylate (ACC; Sigma-Aldrich) were performed by foliar spraying with solutions of 0.3 mM BTH, 1 mM SA (pH 6.5), 100  $\mu$ M JA, or 100  $\mu$ M ACC, and with sterilized distilled water as a control. Inoculation with *M. grisea* was done as described previously (Luo *et al.*, 2005a). Leaf samples were collected at different time points as indicated and stored at  $-80^{\circ}\text{C}$  until use.

*Arabidopsis* plants were grown in soil under fluorescent light ( $150 \mu\text{E m}^{-2} \text{s}^{-1}$ ) at  $22 \pm 3^{\circ}\text{C}$  with 60% RH and a 12 h light/12 h dark cycle. For axenic growth, seeds were sterilized and sown on a medium solidified with 1% agar that contained MS salts (Sigma-Aldrich) and 2% (w/v) sucrose. Conditions for axenic growth were 12 h of light of  $60 \mu\text{E m}^{-2} \text{s}^{-1}$ .

### Cloning of OsBIRH1

Differentially expressed clone BIHN-n5 was isolated from a suppression subtractive hybridization library and contained a 664 bp insert of cDNA, which showed a high level of similarity to plant DEAD-box RNA helicase proteins. The 5' and 3' ends flanking the sequence in the clone BIHN-n5 were amplified by PCR using phage DNA prepared from a rice cDNA library as template. Gene-specific primers used were BIHN-n5-1F (5'-ATG GAG GTG GCG GGT GCT CA-3') and BIHN-n5-1R (5'-CTA GAC GCA TGG CAC ATC GT-3'). Primer BIHN-n5-1R and a vector primer T3-2 (5'-CCT GCA GGT CGA CAC TAG TG-3') were used for amplification of the 5' end, and the primer BIHN-n5-1F and the vector-specific primer T7-2 (5'-CCA CCC GGG TGG AAA ATC GA-3') were used for amplification of the 3' end. All PCR products were purified using the DNA Gel Purification Kit (Sangon, Shanghai, China) and cloned into pUCm-T-vector (Sangon, Shanghai, China) by T/A cloning. The plasmid containing the complete open reading frame (ORF) and the UTR sequences of both ends was designated as pUCm-BIHN-n5. The coding sequence was amplified using plasmid pUCm-BIHN-n5 as template, and a pair of specific primers BIHN-n5-11F (5'-CGC GGA TCC ATG GAG GTG GCG GGT GCT C-3') and BIHN-n5-11R (5'-CCC AAG CTT CTA GAC GCA TGG CAC ATC G-3'), which contain a *Bam*HI and a *Hind*III site (underlined), respectively. The entire ORF was cloned and confirmed by sequencing, yielding plasmid pUCm-OsBIRH1-1.

### DNA sequencing and sequence analysis

DNA sequencing was performed on both strands on the MegaBACE 1000 DNA Analysis System (Amersham Biosciences, Piscataway, NJ, USA) 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/>). Sequence alignments were conducted using CLUSTAL (<http://www.ebi.ac.uk/clustalw/>). A phylogenetic tree was constructed by the Neighbor-Joining method using Mega3.1 software (<http://www.megasoftware.net/>; Kumar *et al.*, 2001).

### Purification of recombinant OsBIRH1 protein

The *OsBIRH1* coding region was released from pUCm-OsBIRH1-1 by digestion with *Bam*HI/*Hind*III and cloned into pRSET-A vector (Invitrogen, Carlsbad, CA, USA), which was then introduced into *Escherichia coli* strain BL21 plus (DE3) cells. Total proteins were induced by 1 mM isopropyl- $\beta$ -thiogalactoside at  $37^{\circ}\text{C}$  for 4 h. Purification of OsBIRH1 fusion protein was performed using a His-Bind Kit (Nova-Gen, Madison, WI, USA) following the manufacturer's instructions. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) following the recommended method.

### Preparation of short double-stranded RNA substrates

RNA substrates used for helicase assays were prepared as described (Iost *et al.*, 1999). Two double-stranded RNA molecules with different sizes of duplex were synthesized *in vitro*. Briefly, for L-dsRNA, two transcripts of 58 and 59 nucleotides (nt) were synthesized *in vitro* from pGEM-4Z (Promega, Madison, WI, USA) and the shorter one was labelled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP. The nucleotide sequences of the two strands are: 58 nt strand, 5'-UGC AUG CCU GCA GGU CGA CUC UAG AGG AUC CCC GGG UAC CGA GCU CGA AUU CGU AUU C-3'; 59 nt strand, 5'-CCC UCU GUU CGA ACG UAC GGA CGU CCA GCU GAG AUC UCC GGG UAC UAG GGG CCC AUG GCU CGA GC-3' (duplex regions underlined). For S-dsRNA, two transcripts of 45 nt and 34 nt were synthesized *in vitro* from pGEM-4Z and the shorter one was labelled. The sequence of the 45 nt strand is 5'-CCC UCU GUU CGA ACG UAC GGA CGU CCA GCU GAG AUC CUA GGG G-3' and that of the 34 nt strand 5'-AGG AUC CCC GGG UAC CGA GCU CGA AUU CGU AUU C-3' (with the duplex region underlined). The synthesized transcripts were annealed and the resulting partial duplex, which consists of a 47 bp and 9 bp region, respectively, was purified by native polyacrylamide gel electrophoresis.

### Biochemical assays for OsBIRH1 activity

ATPase activity was monitored continuously by a coupled spectrophotometric method as described previously (Panuska and Goldthwait, 1980). Reactions were performed at  $37^{\circ}\text{C}$  in a volume of 0.4 ml with buffer containing 20 mM TRIS-HCl, pH 8.0, 50 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 1 mM ATP, 300 mM NADH, 2 mM phosphoenolpyruvate, and 3 U  $\text{ml}^{-1}$  pyruvate kinase and lactate dehydrogenase. RNA and OsBIRH1 were added as indicated. The steady-state rate of ATP hydrolysis equals that of NADH oxidation, which was quantified using  $6300 \text{ M}^{-1} \text{ cm}^{-1}$  for the extinction coefficient of NADH. RNA helicase activity was measured according to a previously reported method (Iost *et al.*, 1999). Reaction mixtures of 15  $\mu\text{l}$  in total contained 20 mM TRIS-HCl, pH 8.0, 70 mM KCl, 2 mM  $\text{MgCl}_2$ , 2 mM dithiothreitol, 15 U of RNasin, 45 fmol (3 nM) of partial duplex, and 2 mM ATP, where indicated. Reactions were initiated by addition of OsBIRH1. After incubation for 10 min at  $37^{\circ}\text{C}$ , the reactions were stopped by addition of 4  $\mu\text{l}$  of a solution containing 1.2% SDS, 10 mM EDTA, 40% glycerol, bromphenol blue, xylene cyanol, and 250  $\text{mg ml}^{-1}$  proteinase K. For positive controls, duplex RNA was separated into monomers by heating at  $95^{\circ}\text{C}$  for 2 min, followed by rapid cooling on ice. Samples were electrophoresed on a 10% polyacrylamide gel. Labelled RNAs were visualized by autoradiography.

### Generation of OsBIRH1-overexpressing transgenic Arabidopsis

The *OsBIRH1* coding sequence was released from pUCm-OsBIRH1-1 by digestion with *Bam*HI/*Hind*III and was then cloned into *Bam*HI/*Hind*III sites of the binary vector pCAMBIA99-1 under control of the cauliflower mosaic virus (CaMV) 35S promoter in the sense orientation. The resulting plasmid, designated as pCAMBIA991-OsBIRH1, was introduced into *Agrobacterium tumefaciens* GV3101 by electroporation using a GENE PULSER II electroporation system (Bio-Rad Laboratories). Transformation of *Arabidopsis* was performed using the floral dip method as described previously (Clough and Bent, 1998). Transgenic plants were selected on MS media containing 50  $\text{mg l}^{-1}$  hygromycin and allowed to grow to third generations. The wild-type plants and homozygous transgenic T3 lines were used in all experiments.

Genomic DNA of transgenic *Arabidopsis* plants was extracted by the CTAB protocol (Luo *et al.*, 2005b). Approximately 100 ng of

genomic DNA was used for detection of the transgene in transgenic plants by PCR with primers BIHN-n5-11F (5'-CGC GGA TCC ATG GAG GTG GCG GGT GCT C-3') and BIHN-n5-11R (5'-CCC AAG CTT CTA GAC GCA TGG CAC ATC G-3'). Southern blotting analysis was performed in ULTRAhyb hybridization buffer and probed with a [ $\alpha$ - $^{32}$ P]dCTP-labelled 856 bp fragment of *OsBIRH1* cDNA. The membrane was blotted between waxfilm (Whatman International, Maidstone, UK) and autoradiographed by exposure to storage phosphor screens (Amersham BioScience) for 4 h.

#### Analysis of gene expression

Total RNA was extracted using an acid phenol-guanidine isothiocyanate-chloroform one-step method as described (Sambrook and Russell, 2001). Twenty micrograms of total RNA was used in northern blotting analysis as described above for Southern blot analysis.

Expression of defence-related genes in transgenic plants was analysed by reverse-transcriptase (RT)-PCR. Leaf samples were collected from 4-week-old plants and total RNA was extracted. Timers for the defence-related genes used in RT-PCR were as follows: AtPR1-F, 5'-TCGTCTTTGTAGCTCTTGTAGGTG-3'; AtPR1-R, 5'-TAGATTCTCGTAATCTCAGCTCT-3'; AtPR2-F, 5'-CGTTGTGGCTCTTTACAAACAACAAAAC-3'; AtPR2-R, 5'-GAAATTAACCTCATACTTAGACTGTGCGAT-3'; AtPR5-F, 5'-ATGGCAAATATCTCCAGTATTCACA-3'; AtPR5-R, 5'-ATGTCGGGGCAAGCCGCGTTGAGG-3'; AtPDF1.2-F, 5'-GCTAAGTTTGCTTCCATCATCACCCCTT-3'; AtPDF1.2-R, 5'-AAC-ATGGGACGTAACAGATACACTTGTG-3'; AtApx1-1F, 5'-CTGT-TGAGAAGTGCAGGAGGAAGC-3'; AtApx1-1R, 5'-CATGTGGGCCTCAGCGTAATCAGC-3'; AtApx2-1F, 5'-TGCTGTTGATGATCACTGGAGGAC-3'; AtApx2-1R, 5'-GATGAGCTTCCGTA-TAGTCTTCG-3'; AtFSD1-1F, 5'-AGTTCAATGCTGCTGCAGC-CACTC-3'; AtFSD1-1R, 5'-GCAGAACTCACTGTCCTGAAAGTC-3'; BIHN-n5-rt-1F, 5'-GATAATCGAGGAATCTCCTGTC-3'; BIHN-n5-rt-1R, 5'-TAGATGGTGCTATGTATGCTAG-3'; ATACTIN1-1F, 5'-GGCGATGAAGCTCAATCCAAACG-3'; ATACTIN1-1R, 5'-GGTCACGACCAGCAAGATCAAGACG-3'. AtACTIN1 was used as an internal control. One microgram of RNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and gene-specific primers. Each PCR was carried out with specific primers for 30 cycles of 94 °C 15 s, 60 °C 15 s, and 72 °C 1 min, using Taq DNA polymerase (PrimerStar, Takara, Dalian, China).

#### Disease assays

*Alternaria brassicicola* was grown on potato dextrose agar, and spores were collected and suspended in distilled water for inoculation. Disease assay for *A. brassicicola* was performed on detached leaves (Mengiste *et al.*, 2003). A single 5  $\mu$ l spore suspension ( $1 \times 10^6$  spores ml $^{-1}$ ) in water was dropped onto each detached leaf. Inoculated leaves were kept under a transparent cover to maintain high humidity and transferred to a growth chamber with 24 °C and a 12 h light/12 h dark cycle.

Leaves of 4-week-old plants were infiltrated with suspensions of *Pseudomonas syringae* pv. *tomato* DC3000 in 10 mM MgCl $_2$  (OD $_{600}$ =0.001). To determine the bacterial growth curve, 10 leaves each were collected at 0, 2, and 4 d after inoculation. Leaf discs of the same size were made using a hole puncher, and bacterial titre per leaf area was determined as described previously (Mengiste *et al.*, 2003).

#### Oxidative stress tolerance assays

Seeds were surface-sterilized and placed on 1/2 MS medium containing 1  $\mu$ M or 100  $\mu$ M methyl viologen (MV; Sigma-Aldrich) and were kept at 4 °C for 48 h to synchronize germination. Seed

germination (emergence of radicals) was scored daily. Detached leaf assay was used to determine the sensitivity of plants to oxidative stress induced by MV. Leaves from soil-grown plants were placed on 1/2 MS medium without sucrose, and a single drop of a 2  $\mu$ l solution with different concentrations of MV (0, 25, and 50  $\mu$ M) was placed on each leaf. Chlorophyll content was measured spectrophotometrically after treatments (Veronese *et al.*, 2003). Chlorophyll content was calculated according to the formula  $\text{Chl} (a+b) = 5.24A_{664} + 22.24A_{648}$ , where Chl is the chlorophyll concentration in micrograms per millilitre and A is the absorption (Lichtenthaler, 1987).

## Results

### Cloning of *OsBIRH1*, a rice gene encoding a DEAD-box RNA helicase

In previous studies aimed at elucidating the molecular biology of disease resistance in rice, differentially expressed cDNA clones associated with BTH-induced defence response were isolated and identified (Song and Goodman, 2002). Among these differentially expressed cDNAs, BLAST similarity searches revealed that the 664 bp insert in clone BIHN-n5 (GenBank accession no. BI118654) has a high level of similarity to genes encoding DEAD-box RNA helicases. Full-length cDNA of this putative DEAD-box RNA helicase gene was obtained by amplification of the 5' and 3' ends flanking the sequence in the clone BIHN-n5, and this gene was designated as *OsBIRH1* for *Oryza sativa* BTH-induced RNA helicase 1. The full-length cDNA of *OsBIRH1* was 2167 bp with a predicted 1926 bp ORF and has three nucleotide differences compared with the full-length cDNA AK065776 from *O. sativa* ssp. *japonica* but with no difference in predicted proteins. The *OsBIRH1* gene, the same as the predicted locus *Os03g01830* in rice genome sequence annotation, is localized on chromosome 3 of the rice genome and consists of eight exons and seven introns.

### *OsBIRH1* is a member of DEAD-box RNA helicases

*OsBIRH1* is predicted to encode a putative protein consisting of 641 amino acid residues with a calculated molecular weight of 71 kDa and isoelectric point of 9.1. Searching for conserved domains revealed that amino acids 242–455 and 473–613 in the *OsBIRH1* protein represent two typical conserved domains, DEADc (PF00270) and HELICs (helicase superfamily C-terminal domain, PF00271), which are characteristic of the DEAD-box protein family (Fig. 1A, B). *OsBIRH1* also possesses all nine highly conserved amino acid motifs characterizing the DEAD-box proteins (Fig. 1A). However, the sequences of these conserved motifs in *OsBIRH1* are not identical to those of the consensus sequences (Fig. 1C). Notably, the sequence of the motif II in *OsBIRH1* is DEVD, instead of the well-characterized motif sequences, for example, DEAD and DEAH, and thus *OsBIRH1* should belong to the DEXD

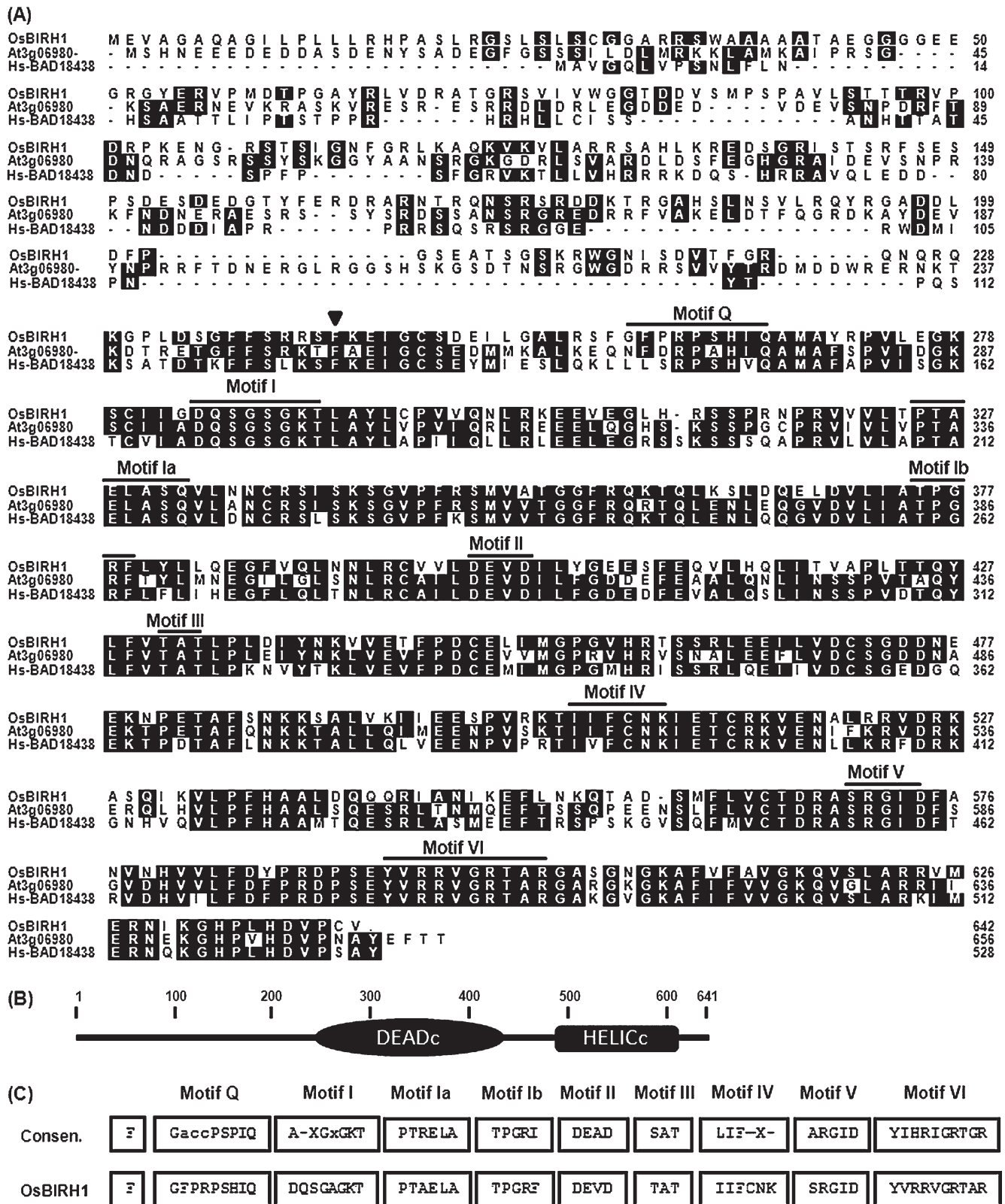


Fig. 1. Structure of OsBIRH1 protein. (A) Alignment of OsBIRH1 with *Arabidopsis* AtRH50 (At3g06980) and human BAD18438. Conserved motifs are indicated by short thick lines and the conserved phenylalanine residue is indicated by an inverted filled triangle. (B) Domain organization of the OsBIRH1 protein. The amino acid positions are indicated. (C) Comparison of the conserved motifs in OsBIRH1 with consensus from other DEAD-box RNA helicase proteins.

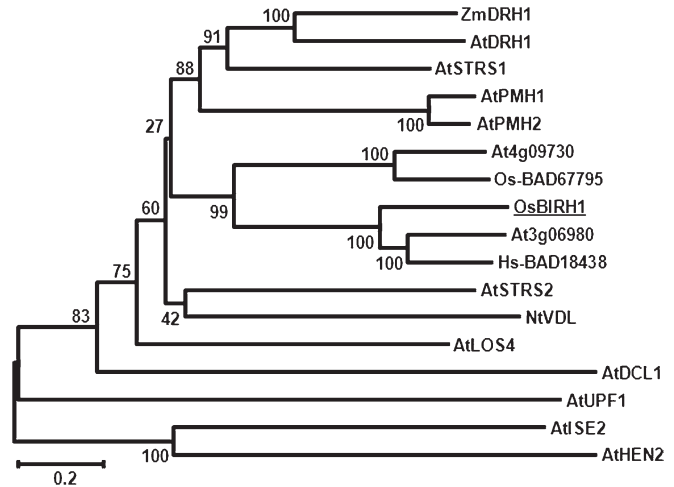
group (Cordin *et al.*, 2006). OsBIRH1 has a relatively long N-terminal extension of 241 amino acids, counting upstream from the conserved phenylalanine, but it has a short C-terminal extension of 39 amino acids, counting downstream from the conserved motif VI.

Alignment and phylogenetic tree analyses revealed that OsBIRH1 has a high level of identity to *Arabidopsis* AtRH50 (At3g06980) and a human protein BAD18438, showing identities of 53.6% and 46.7%, respectively (Fig. 1A). OsBIRH1, AtRH50, and human BAD18438 form a group of proteins, which all contain the conserved DEVD motif (Fig. 2). OsBIRH1 shows less identity to other known DEAD-box proteins identified so far. Sequence identity/similarity of OsBIRH1 to other DEAD-box proteins resides in the conserved motifs and the C-terminal extension, whereas there is no significant sequence similarity in the N-terminal extension.

#### *OsBIRH1* has RNA-dependent ATPase and ATP-dependent RNA helicase activity

Generally, as reported previously, the DEAD-box RNA helicases have both RNA-dependent RNA helicase activity and ATP-dependent RNA helicase activity (Cordin *et al.*, 2006). To determine whether *OsBIRH1* encodes a functional DEAD-box RNA helicase, ATPase and RNA helicase activity of recombinant OsBIRH1 protein were analysed. Combinant OsBIRH1 was purified from *E. coli* and the ATPase activity of OsBIRH1 was first measured using a continuous spectrophotometric method, in which ATP hydrolysis is coupled to NADH oxidation allowing monitoring of any change by measurement of the absorbance at 338 nm ( $A_{338}$ ). In the absence of RNA, no change of  $A_{338}$  was detected, indicating that OsBIRH1 has no ATPase activity by itself. By contrast, in the presence of total rice RNA, the  $A_{338}$  value decreased linearly over time during the experimental period (Fig. 3A). Addition of RNase A to the reaction abolished the activity, confirming that it was dependent on the added RNA (data not shown). These results confirm that OsBIRH1 has an RNA-dependent ATPase activity.

To examine RNA helicase activity of OsBIRH1, two different dsRNA substrates with partial duplexes of 47 bp and 9 bp, respectively, were synthesized. L-dsRNA was prepared by annealing a 59 nt ssRNA to a radiolabelled 58 nt ssRNA to form a 47 bp duplex; while S-dsRNA was made by annealing a 34 nt ssRNA to a longer radiolabelled 45 nt RNA to form a 9 bp duplex (Fig. 3B). As shown in Fig. 3C, the L-dsRNA and S-dsRNA duplexes migrated much slower than the corresponding ssRNAs (Fig. 3C, lanes 1 and 3). When the L-dsRNA was included as substrate, no duplex dissociation was detected in the presence or absence of ATP and even at higher concentrations of OsBIRH1 protein in the reactions (Fig. 3C). Similarly, when the S-dsRNA was included as

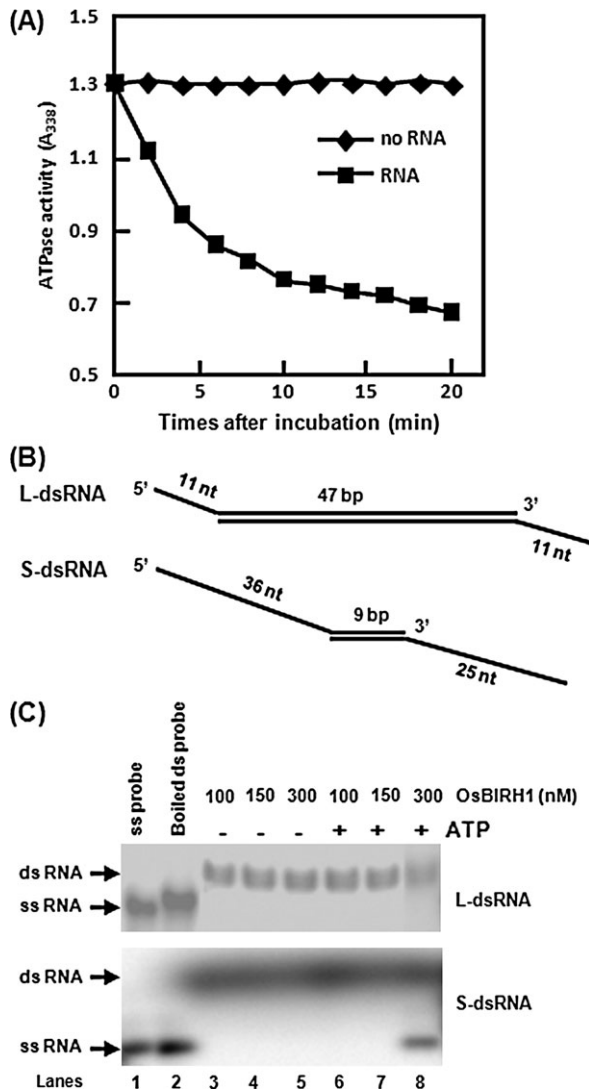


**Fig. 2.** Phylogenetic tree analysis of OsBIRH1 with DEAD-box RNA helicase proteins from other organisms. Phylogenetic trees were constructed using the Neighbor-Joining method and genetic distances were calculated using the Kimura two-parameter model. Bootstrap values from 1000 replicates were used to assess the robustness of the trees. The DEAD-box RNA helicase proteins used were: *Arabidopsis thaliana* At3g06980 (AAO00880, AY113064), At4g09730 (BAD43116, AK175435), AtLOS4 (At3g53110, BT002444), AtSTRS1 (At1g31970, AY080680), AtSTRS2 (At5g08620, AY035114), AtISE2 (At1g70070, AF387007), AtUPF1 (At5g47010, AF484122), AtDCL1 (At1g01040, AF292940), AtPMH1 (At3g22310, AY091091), AtPMH2 (At3g22330, AY062502), AtHEN2 (At2g06990, AY080791), and AtDRH1 (At3g01540, AY062591); *Oryza sativa* Os01g0184500 (BAD67795); *Homo sapiens* Hs-BAD18438 (BAD18438); *Zea mays* ZmDRH1 (AAR29370, AY466159); and *Nicotinana tabacum* NtVDL (AAG34873, AF261032). The OsBIRH1 protein is underlined.

substrate, no duplex dissociation was detected in reactions without ATP (Fig. 3C). In the presence of ATP, duplex dissociation was detected when a higher concentration of OsBIRH1 protein was included in the reactions (Fig. 3C, lower panel, lane 8). However, no duplex dissociation was observed when a low concentration of OsBIRH1 protein was used (Fig. 3C, lower panel, lanes 6 and 7). These results clearly indicate that OsBIRH1 has an ATP-dependent RNA helicase activity and that OsBIRH1 requires a high concentration for its RNA helicase activity.

#### *Expression of OsBIRH1 is induced in rice defence responses*

Originally, the differentially expressed cDNA clone BIHN-n5 was identified from a suppression subtractive hybridization library constructed by subtracting cDNAs from BTH-induced and *M. grisea*-inoculated rice leaf tissue with cDNAs from uninoculated control leaf tissue, implying that this gene was activated in rice defence responses (Song and Goodman, 2002). Therefore, an analysis was carried out to see if expression of *OsBIRH1* in rice seedlings was induced by well-known disease resistance-related signal molecules such as SA, BTH, JA, and ACC. Transcripts corresponding to *OsBIRH1* could be detected in water-treated healthy rice seedlings, but



**Fig. 3.** RNA-dependent ATPase and ATP-dependent RNA helicase activities of OsBIRH1. (A) ATPase activity was measured by a spectrophotometric assay in the presence or absence of total rice RNA. Oxidation of NADH, which is directly proportional to the rate of ATP hydrolysis, was continuously monitored by measuring the absorbance at 338 nm. Diamonds, OsBIRH1 (150 nM) without RNA; squares, OsBIRH1 (150 nM) with RNA (50 mg ml<sup>-1</sup>). (B) Scheme of the partial RNA duplexes used for unwinding activity assays. (C) RNA unwinding activity of OsBIRH1. Activity was measured by the ability of the protein to dissociate the partial RNA duplex. Fifty femtomol of labelled substrate was incubated with increasing concentrations of OsBIRH1 in the presence or absence of 2 mM ATP. The products of the reaction were separated by a 10% native PAGE and visualized by autoradiography. ss probe, Single strand probe as positive control; Boiled ds probe, the duplex was boiled before loading on the gel.

BTH treatment did induce expression of *OsBIRH1* (Fig. 4A). Induced expression of *OsBIRH1* was observed as early as 6 h after treatment, peaked at 12 h and maintained a relatively high level for 12–72 h. SA treatment also led to a rapidly induced expression of *OsBIRH1* within 6 h which was maintained at a relatively higher level for 12–72 h after treatment (Fig. 4A). It is likely that expression

of *OsBIRH1* was also induced by JA and ACC, with kinetics similar to those by BTH and SA (Fig. 4A).

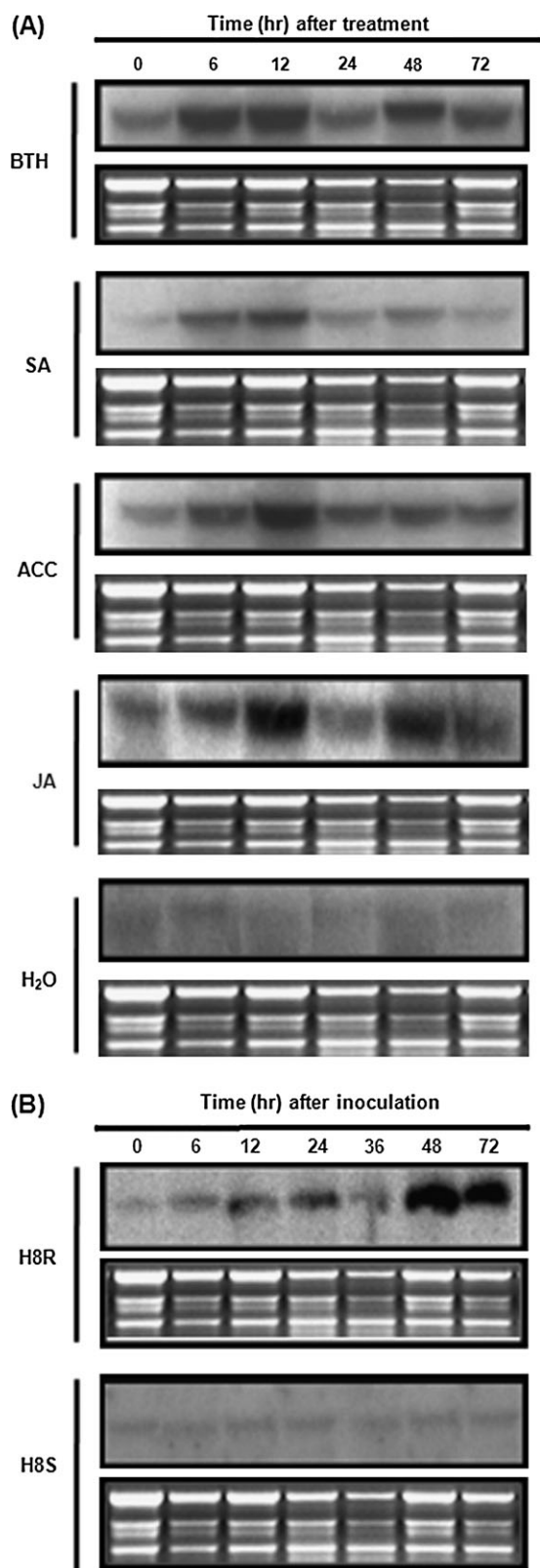
Expression of *OsBIRH1* in the disease resistance response was further examined using a pair of near-isogenic lines, H8R and H8S, which show incompatible and compatible interaction, respectively, with the tested strain of *M. grisea*. Similar to the kinetics after BTH treatment, expression of *OsBIRH1* in H8R leaves was induced as early as 6 h after inoculation with *M. grisea* and the expression levels increased progressively within 72 h after inoculation (Fig. 4B). By contrast, no significant induced expression was detected in H8S leaves after inoculation with *M. grisea* (Fig. 4B). These results suggest that expression of *OsBIRH1* is induced by multiple disease resistance-related signal molecules and pathogen infection, and indicate that induced expression of *OsBIRH1* only occurs in an incompatible interaction between rice and the blast fungus.

#### Generation of *OsBIRH1*-overexpressing transgenic *Arabidopsis* plants

To gain further information on the biological function of *OsBIRH1*, transgenic *Arabidopsis* lines that ectopically express *OsBIRH1* under the control of the CaMV 35S promoter were generated. A total of 15 independent transgenic lines were obtained through the floral dip transformation method and antibiotic screening. Southern blot analysis of genomic DNA from the transgenic plants confirmed that *OsBIRH1* was integrated into the genome of transgenic plants and that more than half of the transgenic lines contain two or more copies of the transgene (Fig. 5A). The transgenic lines with a single copy of the *OsBIRH1* gene were selected to grow for three generations to obtain homozygous lines for further studies. RT-PCR analysis was performed to examine expression levels of *OsBIRH1* in T3 plants of the transgenic lines (#2, #6, #7, and #9), and a high level of *OsBIRH1* expression was detected in these lines (Fig. 5B). During the experiments with these transgenic lines, no obvious differences in morphology, growth, and development under normal growth condition were observed (data not shown).

#### Overexpression of *OsBIRH1* in *Arabidopsis* confers enhanced disease resistance

First, the disease resistance phenotype of *OsBIRH1*-overexpressing plants was examined against *A. brassicicola*, a necrotrophic fungal pathogen causing early blight disease in cruciferous plants, using a detached leaf assay (Mengiste *et al.*, 2003). In wild-type plant leaves, typical disease symptoms were seen around 3 d after inoculation with spores of the fungus, and the lesion enlarged to form a yellow chlorotic area at 5 d after inoculation (Fig. 6A). By contrast, disease symptoms developed much more slowly and lesions were mainly restricted to a small area



**Fig. 4.** Expression patterns of *OsBIRH1* in rice seedlings after treatment with different resistance signal chemicals and in interactions between rice and *Magnaporthe grisea*. (A) Expression of *OsBIRH1* by disease resistance-related signal molecules. Rice seedlings were treated by spraying with solutions of 0.3 mM BTH, 1 mM SA, 100  $\mu$ M JA,

at the inoculation sites with less chlorosis at 5 d after inoculation (Fig. 6A). Further measurement of the disease lesions also revealed that lesion sizes in both of the transgenic lines tested were significantly reduced compared with the lesion size in the wild-type plants (Fig. 6A). These results indicate that overexpression of *OsBIRH1* in transgenic *Arabidopsis* enhances disease resistance against *A. brassicicola*.

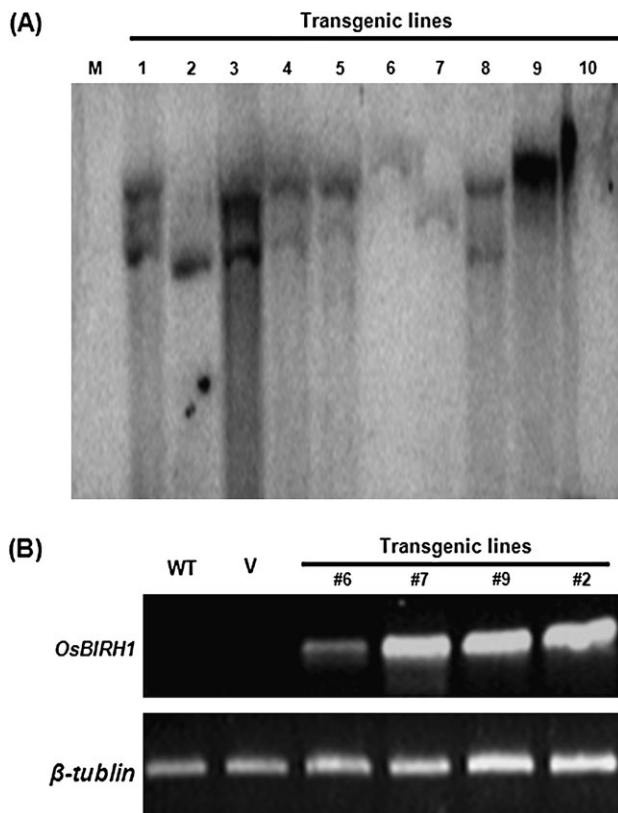
The disease resistance phenotype of *OsBIRH1*-overexpressing plants against *P. syringae* pv. *tomato* DC3000 was also examined. Leaves of wild-type and transgenic plants were infiltration-inoculated with *Pseudomonas syringae* pv. *tomato* suspension, and disease development was observed over 1 week after inoculation. Chlorosis symptoms were visible 3 d after inoculation under the experimental conditions and developed progressively on the infected leaves of the wild-type plants (Fig. 6B). Tissue collapse was also observed in some of the inoculated leaves of wild-type plants. By contrast, development of chlorosis symptoms in inoculated leaves of the transgenic plants was slower, typically seen 4–5 d after inoculation, and lesion size was also significantly reduced (Fig. 6B). Measurement of bacterial titres in inoculated leaves also indicated that the bacterial population in transgenic plant leaves was reduced by at least 10-fold compared with that in the wild-plant leaves (Fig. 6B). These results suggest that ectopic expression of *OsBIRH1* in transgenic *Arabidopsis* confers disease resistance against *P. syringae* pv. *tomato*.

#### *OsBIRH1*-overexpressing plants have up-regulated PR gene expression

To gain further information on the increased disease resistance phenotype observed in *OsBIRH1*-overexpressing *Arabidopsis* plants, the expression levels of some defence-related genes were analysed by RT-PCR. In the present experiments, induced expression of the selected defence-related genes in wild-type plants was not detected, suggesting that the growth regime did not cause significant stress. Results from RT-PCR experiments showed that the expression levels of *PR-1*, *PR-2*, *PR-5*, and *PDF1.2* were constitutively up-regulated in the *OsBIRH1*-overexpressing plants in the absence of the pathogen as compared with those in the wild-type plants (Fig. 7). *PR-2* and *AtPR-5* were up-regulated in all transgenic lines, while *PR-1* and *PDF1.2* were up-regulated in most of the transgenic lines tested.

100  $\mu$ M ACC, or water. (B) Induced expression of *OsBIRH1* in rice and the blast fungus interactions. Three-week-old rice seedlings of H8R and H8S were inoculated with *M. grisea*. Rice leaf samples were collected at each time point (hours) as indicated after treatment or inoculation. Twenty micrograms of total RNA were fractionated on a 1.2% agarose formaldehyde gel and hybridized with the  $\alpha$ - $^{32}$ P-labelled 856 bp fragment of *OsBIRH1* as a probe. The corresponding ethidium bromide gel images show equal loading of total samples.

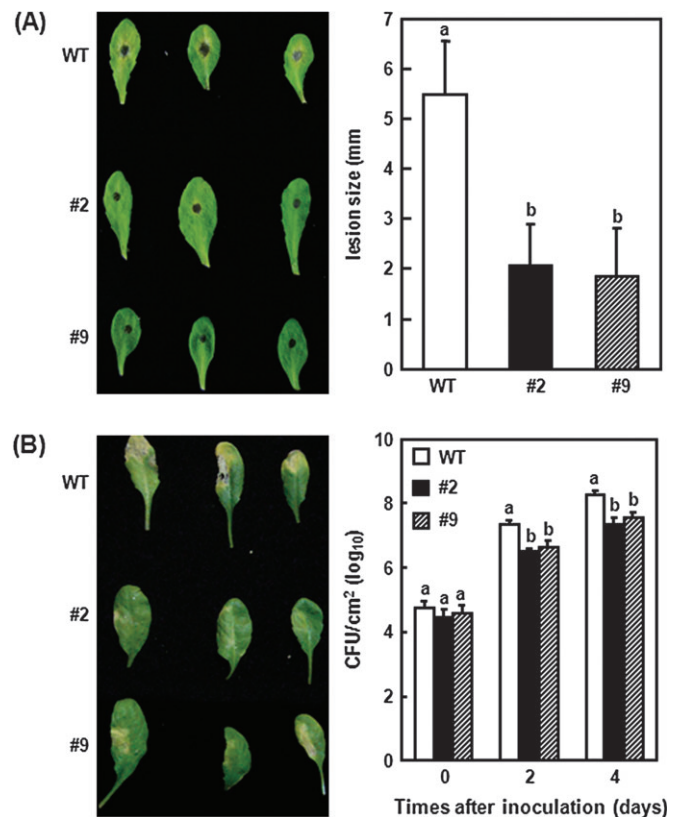




**Fig. 5.** Southern blot and RT-PCR analysis of the *OsBIRH1* transgenic plants. (A) Southern blot analysis of the transgenic lines. M, Non-transgenic plant as a negative control; 1–10, individual *OsBIRH1* transgenic plants. (B) RT-PCR analysis of *OsBIRH1* expression in homozygous transgenic lines. WT, Non-transgenic plant as a negative control (CK-); V, pCAMBIA 99-1 transgenic plants; #6, #7, #9, and #2, individual *OsBIRH1* transgenic plants carrying a single copy of the *OsBIRH1* gene.

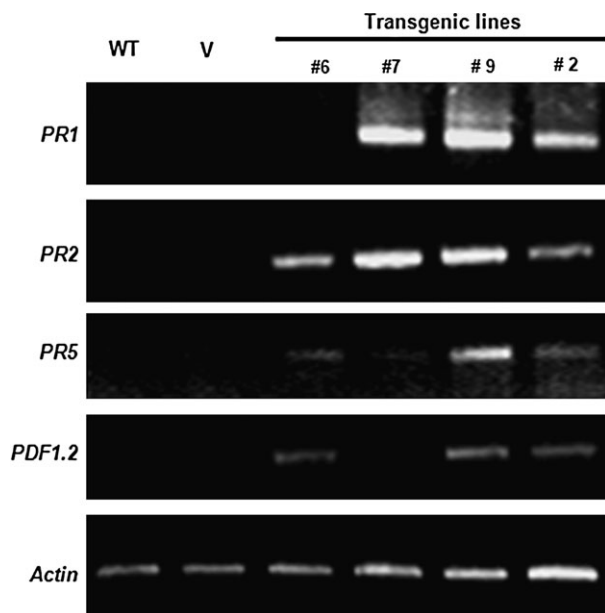
#### Increased tolerance of transgenic plants to oxidative stress

The possible function of *OsBIRH1* in the abiotic stress response was also examined by analysing tolerance of *OsBIRH1*-overexpressing plants to oxidative stress. Tolerance levels of the transgenic plants were tested at different stages, for example seed germination, seedling growth, and mature leaves of 4-week-old plants, using MV as the oxidative stress-generating chemical. In MS medium containing 100  $\mu$ M MV, seeds from the transgenic lines germinated much better than the wild-type seeds (Fig. 8A). At 7 d after treatment,  $\sim$ 70% and  $\sim$ 50% of seeds from transgenic lines #2 and #9, respectively, germinated, but the germination rate of the wild-type seeds was only about 20%. No difference in seed germination was observed between the transgenic line and wild-type plants on MS medium without MV (data not shown). Seedlings of the wild-type and the transgenic lines grown on MV-containing MS medium showed growth retardation, but oxidative stress-caused growth retardation in transgenic lines was less severe than in the wild-type as indicated by the seedling



**Fig. 6.** Enhanced disease resistance of *OsBIRH1*-overexpressing *Arabidopsis* plants against *Pseudomonas syringae* pv. *tomato* and *Alternaria brassicicola*. (A) Representative leaves showing disease symptoms (left panel) and lesion size (right panel) after inoculation with *Alternaria brassicicola*. Leaves from wild-type (Wt) and *OsBIRH1* transgenic plants were inoculated with a 4  $\mu$ l droplet of spores ( $10^6$  spores  $\text{ml}^{-1}$ ) and photographs were taken at 5 d after inoculation. Lesion size was measured 3 d after inoculation. Data presented are means  $\pm$ SD from a minimum of 40 lesions. (B) Representative leaves showing disease symptoms (left panel) and bacterial growth (right panel) after infiltration with *Pseudomonas syringae* pv. *tomato* DC3000. Bacterial growth [colony-forming units (CFU)  $\text{cm}^{-2}$  leaf area] in leaves at 0, 2, and 4 d after inoculation was measured. Photographs were taken 3 d after inoculation. Data presented are means  $\pm$ SD from three independent experiments. Different letters above the columns indicate significant differences at  $P=0.05$ . WT, Wild type; #2 and #9, *OsBIRH1*-overexpressing plants.

fresh weight (Fig. 8B, C). The fresh weight of the transgenic seedlings grown on MS medium containing 1  $\mu$ M MV was 2.5- to 4.0-fold higher than that of the wild-type seedlings (Fig. 8C). Tolerance of *OsBIRH1*-overexpressing *Arabidopsis* plants was further examined in mature leaves of plants. Detached leaves from wild-type plants showed significant necrosis and bleaching after treatment with 20  $\mu$ M or 50  $\mu$ M MV, while less necrosis and bleaching was observed in detached leaves from the transgenic plants (Fig. 8D). Total chlorophyll content in leaves of the wild-type plants decreased to 45% and 20% of the normal levels in untreated leaves after treatment with 20  $\mu$ M or 50  $\mu$ M MV, respectively, whereas the chlorophyll content in leaves of the transgenic plants was maintained at  $>60\%$  and  $40\%$  after MV treatment (Fig.



**Fig. 7.** Expression of defence-related genes in the *OsBIRH1*-overexpressing plants. Two-week-old seedlings grown on MS medium were collected for analysis of gene expression by RT-PCR. WT, Non-transgenic plant as a negative control (CK-); V, pCAMBIA 99-1 transgenic plants; #2, #6, #7 and #9, individual transgenic plants carrying single copy of *OsBIRH1* gene.

8E). These results suggest that overexpression of *OsBIRH1* in transgenic *Arabidopsis* plants confers enhanced tolerance to oxidative stress in all developmental stages.

To confirm further the oxidative stress tolerance phenotype in *OsBIRH1*-overexpressing transgenic plants, expression of some known oxidative stress defence genes was analysed (Mittler *et al.*, 2004). As shown in Fig. 9, the transgenic plants showed up-regulated expression of three different oxidative stress defence genes, *AtApx1* (ascorbate peroxidase 1), *AtApx2* (ascorbate peroxidase 1), and *AtFSD1* (Fe-superoxide dismutase 1), under normal growth conditions. Therefore, the enhanced tolerance observed in transgenic plants might be due to an increased capacity of anti-oxidative stress to be modulated by *OsBIRH1*.

## Discussion

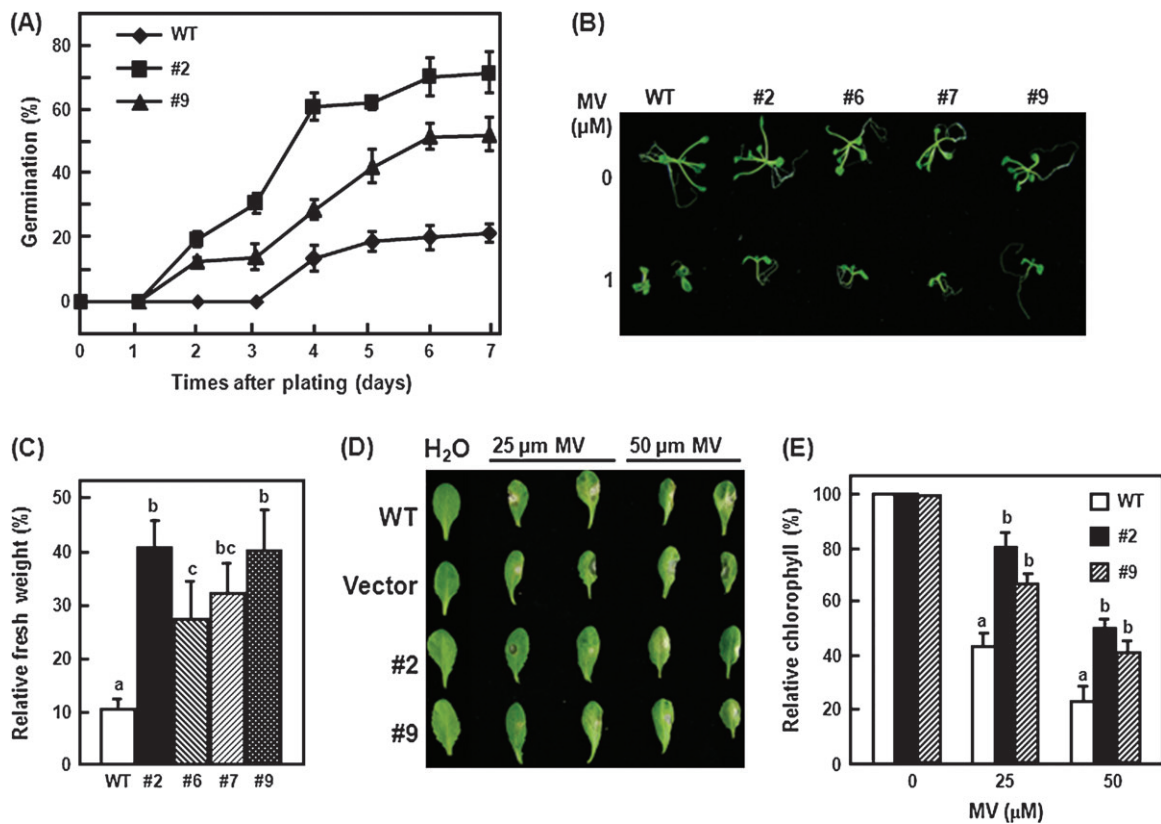
The involvement and significance of RNA helicases in response to biotic and abiotic stress have only recently begun to emerge (Owtrim, 2006; Vashisht and Tuteja, 2006). In the present study, the characterization of the rice *OsBIRH1* gene, which encodes a DEAD-box RNA helicase with RNA-dependent ATPase and ATP-dependent RNA helicase activities, is reported. Evidence that *OsBIRH1* plays important roles in disease resistance response and oxidative stress tolerance, as revealed by its inducible expression by pathogen infection and multiple defence signal molecules as well as by phenotypes of the *OsBIRH1*-overexpressing *Arabidopsis* plants, was also

presented. The findings not only extend knowledge of the biological function of the DEAD-box RNA helicases, but also provide new clues to explore further the significance of RNA helicase-mediated RNA metabolism in regulation of plant defence responses.

The *OsBIRH1* protein contains all conserved domains and motifs that are characteristic of the DEAD-box proteins. However, sequences of the conserved motifs in *OsBIRH1* are different from those of the consensus sequences; in particular, motif II of *OsBIRH1* is DEVD, instead of the well-characterized motif sequences, for example DEAD and DEAH, indicating that *OsBIRH1* is most likely to belong to the DExD group (Cordin *et al.*, 2006). In the phylogenetic tree analysis, *OsBIRH1*, *Arabidopsis* AtRH50 (At3g06980), and human BAD18438 form a small related subgroup. These three proteins not only contain the same conserved DEVD motif but also have almost identical sequences in the conserved motifs I–VI. However, neither AtRH50 or human BAD18438 has been characterized in terms of their function so far. The present results from the *OsBIRH1* studies might provide preliminary information on the biological function of this group.

Although a number of DEAD-box proteins or genes have been identified from various different plants, most of the proteins were not analysed for their biochemical activity *in vitro*. The *Arabidopsis* LOS4 was shown to have RNA-dependent ATPase activity *in vitro*, and AtDRH1 was found to possess both ATP-dependent RNA helicase and RNA-dependent ATPase activities (Okanami *et al.*, 1998; Gong *et al.*, 2005). In the present study, the recombinant *OsBIRH1* protein showed specific RNA-dependent ATPase and ATP-dependent RNA helicase activities *in vitro*, suggesting that *OsBIRH1* is a functional DEAD-box RNA helicase. It is considered that unwinding of RNA duplexes by DEAD-box RNA helicases occurs in a processive manner (Jankowsky *et al.*, 2000, 2005; Eoff and Raney, 2005; Cordin *et al.*, 2006). However, the DEAD-box proteins are feeble helicases in nature, and are thus supposed to act at key steps of RNA metabolism with short duplexes (<10 bp) preferred as their RNA substrates (Cordin *et al.*, 2006; Linder and Lasko, 2006). This better explains the observations that *OsBIRH1* was unable to unwind long RNA duplex substrates and its unwinding activity on the short RNA duplex required a high concentration of protein *in vitro*.

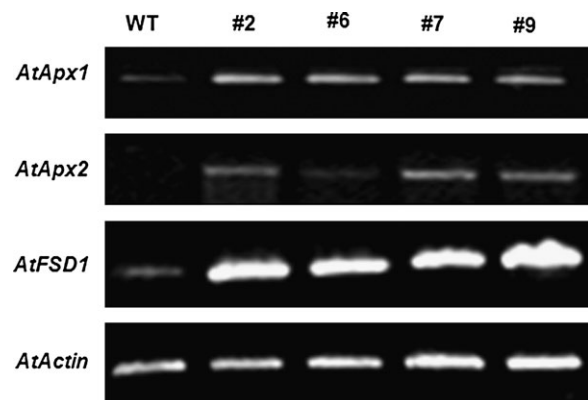
Previous studies have demonstrated that a number of DEAD-box RNA helicases are involved in regulating several key steps in development of plant embryos, plastids, and floral meristems (Jacobsen *et al.*, 1999; Wang *et al.*, 2000; Kobayashi *et al.*, 2007). A relatively high level of basal expression of the *OsBIRH1* gene under normal growth conditions may imply a function for *OsBIRH1* in growth and/or development processes, although ectopic expression in transgenic *Arabidopsis* does not cause any visible growth



**Fig. 8.** Enhanced tolerance to oxidative stress of *OsBIRH1*-overexpressing plants. (A) Percentage of seed germination of the wild-type (WT) and transgenic *Arabidopsis* lines on 1/2 MS agar medium containing 100 μM MV. Germination was scored when the radical tip had fully emerged from the seed coat. (B) Enhanced tolerance to MV of the transgenic plants at the seedling stage. Representative seedlings 14 d after MV treatment are shown. (C) Relative fresh weight of the WT or transgenic lines grown in the absence or presence of MV. Seedlings grown without MV are given a value of 100%. (D) Representative leaves from WT and transgenic plants 5 d after MV treatment are shown. (E) Chlorophyll concentration of the leaf tissues of the WT or transgenic lines was measured in the presence or absence of different MV concentrations. Data presented are means ± SD from three independent experiments. Different letters above the columns indicate significant differences at  $P=0.05$ .

and development changes. Thus, knockouts or knockdown mutants of rice *OsBIRH1* and *Arabidopsis AtRH50* (At3g06980) should be used to check whether these RNA helicases play a role in growth and development.

Evidence is accumulating that expression of RNA helicase genes or RNA helicase activity is regulated in response to changes in environmental conditions (Vashisht and Tuteja, 2006). Several DEAD-box RNA helicase genes were indeed identified from genome-wide expression profiling analysis, suggesting that these genes might be stress regulated (Seki *et al.*, 2001, 2002; Kreps *et al.*, 2002; Kant *et al.*, 2007). *STRS1* and *STRS2* were shown to be down-regulated by multiple abiotic stresses, for example salt, drought, and heat stress, but they both attenuated abiotic stress tolerance (Kant *et al.*, 2007). Although *OsBIRH1* has a relatively high level of basal expression, its expression was induced not only by pathogen infection, but also by several disease resistance-related signalling molecules, for example SA, JA, and ACC, suggesting a role for *OsBIRH1* in disease resistance signalling pathways. This is further supported by the observation that overexpression of *OsBIRH1* in transgenic



**Fig. 9.** Expression of oxidative stress defence genes in the *OsBIRH1*-overexpressing plants. Two-week-old seedlings grown on MS medium were collected for analysis of gene expression by RT-PCR. WT, Non-transgenic plant as a negative control (CK-); #2, #6, #7, and #9, individual transgenic plants carrying single copy of the *OsBIRH1* gene.

*Arabidopsis* plants enhanced disease resistance against infection by *A. brassicicola* and *P. syringae* pv. *tomato*. Studies with knockout or knockdown mutants of *OsBIRH1* will further provide direct genetic evidence to

demonstrate its function in disease resistance signalling pathways.

*Alternaria brassicicola* and *P. syringae* represent two different parasitic modes, necrotrophic and biotrophic, respectively. Correspondingly, plant defence responses against necrotrophic and biotrophic pathogens and the signalling pathways involved are also distinct in nature (Glazebrook, 2005). Generally, expression of *PR* genes and the SA-dependent signalling pathway are involved in the regulation of defence responses against biotrophic pathogens, while the *PDF1.2* gene and the JA/ET signalling pathway are involved in modulating defence responses against necrotrophic pathogens (Gupta *et al.*, 2000; Kunkel and Brooks, 2002; Spoel *et al.*, 2003). The results indicate that *OsBIRH1* might be involved in both of these two different signalling pathways. This is supported by several lines of evidence obtained from this study; for example, induction of *OsBIRH1* expression by SA and JA/ACC, representing the SA signalling pathway and the JA/ET signalling pathway, respectively, enhanced disease resistance against *A. brassicicola* and *P. syringae* pv. *tomato* in *OsBIRH1*-overexpressing transgenic *Arabidopsis*, and constitutively up-regulated expression of both *PR* genes and *PDF1.2* in transgenic plants. Such constitutively up-regulated expression of defence-related genes in transgenic plants indicates that ectopically expressed *OsBIRH1* activates endogenous signalling pathway(s) resulting in enhanced defence responses. However, the mechanisms linking the *OsBIRH1*-mediated signalling pathway and expression of defence-related genes remain unknown.

Some of the DEAD-box RNA helicases have been shown to integrate into multiple signalling pathways, leading to participation in regulation of different aspects of growth/development and stress responses. The cold stress-regulated *LOS4* was found to play an important role in responses to cold and heat stress (Gong *et al.*, 2002, 2005), while *STRS1* and *STRS2* were also shown to be negative regulator nodes in the multiple abiotic stress signalling subnetwork (Kant *et al.*, 2007). In the present studies, it was found that the ectopic expression of *OsBIRH1* in transgenic *Arabidopsis* plants led to enhanced tolerance of abiotic stresses, including oxidative stress and drought (D Li and F Song, unpublished data). It was also found that ectopic expression of *OsBIRH1* results in the enhanced expression of *AtApx1*, *AtApx2*, and *AtFSD1* in the transgenic plants under normal growth conditions. This is in agreement with previous observations that enhanced oxidative stress tolerance is associated with high levels of expression of genes that are involved in oxidative stress responses in plants (Mittler *et al.*, 2004, 2006). Furthermore, the preliminary results also revealed that the *OsBIRH1*-overexpressing transgenic *Arabidopsis* plants showed an increased sensitivity to ABA (D Li and F Song, unpublished data). Taken together, like *Arabidopsis* *STRS1* and *STRS2*, *OsBIRH1* might also partici-

pate in regulation of different signalling pathways leading to defence responses against biotic and abiotic stresses.

In summary, the present study has demonstrated that *OsBIRH1* is a functional DEVD-box RNA helicase belonging to the DEAD-box protein superfamily and that *OsBIRH1* plays important roles in regulating different defence signalling pathways against both biotic and abiotic stresses. However, many questions regarding the *OsBIRH1* function and its action mechanisms remain to be solved in the future; for example, how *OsBIRH1* protein regulates expression of defence-related genes in plants, what role *OsBIRH1* may play in plant growth and development, how *OsBIRH1* operates different stress signalling pathways, etc. On the other hand, DEAD-box RNA helicases belong to a large protein family, but only a few of them have been identified recently for their biological functions in plant growth/development and stress responses (Park *et al.*, 2002; Reinhart *et al.*, 2002; Gendra *et al.*, 2004; Gong *et al.*, 2005; Arciga-Reyes *et al.*, 2006; Yoine *et al.*, 2006a, b; Kant *et al.*, 2007; Kobayashi *et al.*, 2007; Matthes *et al.*, 2007). Definitely, additional extensive studies with knock-out or knockdown mutants in model species, for example *Arabidopsis* and rice, will be very helpful in elucidating both biochemical and biological functions of each member of this huge protein family in plants.

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