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Functional analysis reveals pleiotropic effects of rice RING-H2 finger protein gene *OsBIRF1* on regulation of growth and defense responses against abiotic and biotic stresses

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Abstract RING finger proteins comprise a large family and play key roles in regulating growth/developmental processes, hormone signaling and responses to biotic and abiotic stresses in plants. A rice gene, *OsBIRF1*, encoding a putative RING-H2 finger protein, was cloned and identified. *OsBIRF1* encodes a 396 amino acid protein belonging to the ATL family characterized by a conserved RING-H2 finger domain (C-X2-C-X15-C-X1-H-X2-H-X2-C-X10-C-X2-C), a transmembrane domain at the N-terminal, a basic amino acid rich region and a characteristic GLD region. Expression of *OsBIRF1* was up-regulated in rice seedlings after treatment with benzothaidiazole, salicylic acid, l-aminocyclopropane-1-carboxylic acid and jasmonic acid, and was induced differentially in incompatible but not compatible interactions between rice and *Magnaporthe grisea*, the causal agent of blast disease. Transgenic tobacco plants that constitutively express *OsBIRF1* exhibit enhanced disease resistance against tobacco mosaic virus and *Pseudomonas syringae* pv. *tabaci* and elevated expression levels of defense-related genes, e.g. *PR-1*, *PR-2*, *PR-3* and *PR-5*. The *OsBIRF1*-overexpressing transgenic tobacco plants show increased oxidative stress tolerance to exogenous treatment with methyl viologen and H_2O_2, and up-regulate expression of oxidative stress-related genes. Reduced ABA sensitivity in root elongation and increased drought tolerance in seed germination were also observed in *OsBIRF1* transgenic tobacco plants. Furthermore, the transgenic tobacco plants show longer roots and higher plant heights as compared with the wild-type plants, suggesting that overexpression of *OsBIRF1* promote plant growth. These results demonstrate that OsBIRF1 has pleiotropic effects on growth and defense response against multiple abiotic and biotic stresses.

Keywords Rice · *OsBIRF1* · RING finger proteins · Disease resistance · Abiotic stress

Abbreviations

ABA	Abscisic acid
ACC	1-Amino cyclopropane-1-carboxylic acid
BTH	Benzothiadiazole
CaMV	Cauliflower mosaic virus
ET	Ethylene
H_2O_2	Hydrogen peroxide
JA	Jasmonic acid
MV	Methyl viologen
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PR	Pathogenesis-related
RT-PCR	Reverse transcription-PCR
SA	Salicylic acid
TMV	Tobacco mosaic virus

Introduction

Plants defend themselves against potential invading pathogens by activating a battery of defense mechanisms,

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in which thousands of defense-related genes are coordinately expressed. Extensive biochemical, genetic and genomic studies in Arabidopsis have revealed that different signaling pathways, e.g. salicylic acid (SA)-dependent and jasmonic acid (JA)/ethylene (ET)-dependent signaling pathways, are responsible to activate disease resistance responses by coordinately regulating expression of defense-related genes. Generally, the level and speed of de novo biosynthesis and accumulation of novel proteins (e.g. pathogenesis-related proteins, transcriptional factors, and other regulatory proteins) after pathogen infection are correlated to the degree and strength of the defense responses. In fact, most of the studies that have been made so far take advantage of an approach based on identification of novel proteins resulted from induction of gene expression during defense responses in order to elucidate the molecular basis of plant disease resistance. On the other hand, recent studies have also shed a new window that degradation of proteins is one of most important biochemical and physiological events that play critical roles in regulating almost all aspects of cellular processes.

The ubiquitin/26S proteasome system is a primary pathway for degradation of cellular proteins among eukaryotes (Glickman and Ciechanover 2002). Biochemical process of this system basically starts with ubiquitination of substrate proteins, which are then targeted for degradation by 26S proteasome (Hershko and Ciechanover 1998; Smalle and Vierstra 2004). Ubiquitination of proteins requires a cascade of enzymatic reactions involving three enzymes including ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin protein ligase E3 (Glickman and Ciechanover 2002). Among them, E3s interact with specific substrates and thereby determine specificity to the degradation process. Generally, E3s are grouped into four classes according to the characteristic domains: RING zinc finger, PHD finger, U-Box and HECT (Fang and Weissman 2004; Moon et al. 2004; Smalle and Vierstra 2004). The RING finger domain is defined by the consensus sequence CX2CX (9-39)CX(1-3)HX(2-3)C/HX2CX(4-48) CX2C, where X is any amino acid and the number of X residues varies in different fingers, and is further subcategorized into RING-HC and RING-H2 based on the presence of cysteine and histidine at the fifth coordination site, respectively (Saurin et al. 1996; Freemont 2000; Joazeiro and Weissman 2000).

RING finger proteins comprise a large family, which ubiquitously exist in all of eukaryotes including yeast, animals and plants. Database searches have identified 469 RING-finger proteins from the Arabidopsis genome and 218 cDNA clones in the rice full-length cDNA database (Stone et al. 2005; Katoh et al. 2005). Large number of the RING-finger proteins and their nature in determining specificity of the degradation process imply the diversity of their biological functions. The involvement and functions of RING finger proteins in plant growth/development and hormone responses have been well established recently. Genetic and biochemical studies have identified a number of RING finger proteins that play key roles in regulating growth and developmental processes. These include Arabidopsis COP1 and CIP8 (COP1-interacting protein 8) in photomorphogenesis, BIG BROTHER in organ size, RIE1 in seed development, SHA1 in shoot apical meristem maintenance, PEX10 in peroxisome biogenesis, and Arabidopsis XBAT32 and rice EL5 in root development (Hardtke et al. 2000, 2002; Osterlund et al. 2000; Ma et al. 2002; Lechner et al. 2002; Holm et al. 2002; Saijo et al. 2003; Xu and Li 2003; Seo et al. 2003, 2004; Duek et al. 2004; Nodzon et al. 2004; Jang et al. 2005; Yang et al. 2005; Disch et al. 2006; Koiwai et al. 2007; Sonoda et al. 2007; Schumann et al. 2007). There are many examples demonstrating that some RING finger proteins are directly involved in regulation of hormone signaling pathways by targeting of specific proteins for degradation. Arabidopsis SINAT5 attenuates the auxin signaling by targeting NAC1 for degradation (Xie et al. 2002). AIP2 negatively regulates ABA signaling by modulating its substrate ABI3 stability (Zhang et al. 2005), while SDIR1 positively regulates ABA signaling (Zhang et al. 2007). The Arabidopsis *BRH1* gene was rapidly down-regulated by brassinolide in wild-type plants but not in a BR-insensitive mutant plants, indicating a BR-dependent manner for its expression (Molnar et al. 2002). Furthermore, RING finger proteins also participate in regulation of symbiotic nodulation in Lotus (Nishimura et al. 2002; Shimomura et al. 2006).

In addition, recent studies have also showed that a number of RING finger proteins play important roles in regulating defense responses against abiotic and biotic stresses. The Arabidopsis HOS1, a negative regulator of cold signal transduction, physically interacts with ICE1, a transcription factor activating the expression of *CBF* genes, and mediates the ubiquitination of ICE1 (Lee et al. 2001; Dong et al. 2006). Cold stress induces the degradation of ICE1 in plants, and this degradation requires HOS1, indicating that HOS1 is a negative regulator of cold responses by mediating the degradation of the ICE1 protein (Dong et al. 2006). SDIR1 and XERICO have been shown to be positive regulators of drought tolerance through altering the homeostasis of ABA signaling pathway (Ko et al. 2006; Zhang et al. 2007). RING finger proteins are also involved in plant defense response. Arabidopsis *ATL2* is induced rapidly and transiently by chitin and cellulase treatments (Salinas-Mondragon et al. 1999) and the *eca* mutants with constitutive expression of *ATL2* gene exhibited up-regulated expression of defense-related genes and SA- and JA-responsive genes (Serrano and Guzman 2004). A T-DNA insertion mutant of Arabidopsis ATL9 results in

increased susceptibility to powdery mildew disease (Ramonell et al. 2005). Arabidopsis RIN2 and RIN3 and tobacco ACRE132 are involved in disease resistance gene-specific hypersensitive response (Kawasaki et al. 2005; Durrant et al. 2000). Furthermore, ectopic expression of genes encoding RING finger proteins in Arabidopsis alters disease resistance phenotype and expression of defense-related genes (Cheung et al. 2007; Hong et al. 2007).

Despite of large number of RING finger proteins in rice, only few of them has been identified so far for their possible functions (Katoh et al. 2005). The *EL5* gene, encoding a RING finger E3 ligase, is transiently induced by *N*-acetylchitoheptaose elicitor in rice cells, and functional analysis suggests that EL5 is involved in root development through the maintenance of cell viability (Takai et al. 2001, 2002; Katoh et al. 2003; Koiwai et al. 2007). *OsCOIN* is strongly induced by some abiotic stress factors including low temperature, salt and drought and overexpression in transgenic rice significantly enhanced their tolerance to cold, salt and drought stresses (Liu et al. 2007). *OsRING-1* and *OsRHC1* are induced by pathogen infection and defense-related signal molecules and overexpression of *OsRHC1* in Arabidopsis results in enhanced disease resistance (Meng et al. 2006; Cheung et al. 2007). Here we report the cloning and characterization of a novel rice RING-H2 finger protein gene, *OsBIRF1*. Expression of *OsBIRF1* was induced by defense-related signal molecules and during an incompatible interaction between rice and *Magnaporthe grisea*. Ectopic expression of *OsBIRF1* in transgenic tobacco plants results in enhanced disease resistance against viral and bacterial pathogens and constitutive expression of defense-related genes. Moreover, *OsBIRF1* transgenic tobacco plants also showed enhanced tolerance to drought and oxidative stress. Our results indicate that OsBIRF1 has pleiotropic effects on growth and defense response against multiple abiotic and biotic stresses.

Materials and methods

Plant growth and treatments

Rice cv. Yuanfengzao (*Oryza sativa* L. subsp. *indica*) and a pair of isogenic lines (H8R and H8S) were used in this study. Seedlings were grown in a growth room under a 14 h/10 h day/night regime at 22–27°C for 3 weeks. Seedlings of cv. Yuanfengzao were treated by foliar spraying with solutions of 0.3 mM benzothidiazole (BTH, Novartis Crop Protection Inc., USA), 1.5 mM salicylic acid (SA, pH 6.5) (Sigma-Aldrich, St. Lousi, USA), 100 μM jasmonic acid (JA) (Sigma-Aldrich, St. Lousi, USA) or 100 μM 1-amino cyclopropane-1-carboxylic acid (ACC) (Sigma-Aldrich, St. Lousi, USA), respectively. JA and ACC were dissolved in 0.1% ethanol. The controls were treated in the same way as spraying with 0.1% ethanol or distilled sterilized water. Inoculation of seedlings of H8R and H8S with *Magnaporthe grisea* (strain 85-14B1, race ZB1) was carried out as described previously (Luo et al. 2005a). Leaf samples were collected at different time points as indicated and stored at −80°C until use.

Tobacco plants (*Nicotiana tabacum*) were cultivated in a growth room under a 14 h/10 h day/night regime at 20–25°C.

Cloning of the OsBIRF1 cDNA

A differentially expressed cDNA clone, BIHN-w1, was obtained previously from a suppression subtractive hybridization library (Song and Goodman 2002). BIHN-w1 appears to be a part of a gene encoding a putative RING finger protein, but lacks most part of sequence at 5′-end. To obtain the full-length cDNA, BIHN-w1-specific primers BIHN-w1-1R (5′-ATCTCCAGTAGCATCTCTCT-3′) and BIHN-w1-2R (5′-CAG AGC TTA TGC CAG TAG CT-3′) and a vector primer T3-2 (5′-CCT GCA GGTCGA-CACTAGTG-3′) were used to amplify the 5′-end sequence using phage DNA prepared from a rice cDNA library as template. Amplified 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. A pair of gene-specific primers, BIHN-w1-orf-1F (5′-TCTCCTCCCAATCCGAGATTGC-3′) and BIHN-w1-orf-1R (5′-TAATCCTGAACAATCACAAGCA-3′), located in 5′- and 3′-UTRs, were designed and used to amplify the full-length cDNA. Plasmid containing the full-length cDNA was designated as pUCm-BIHN-w1-1.

DNA sequencing and sequence analysis

DNA sequencing was performed by Invitrogen sequencing company (Shanghai, China). Similarity searches were carried out using BLAST at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments and phylogenetic tree construction were performed using ClustalW program in DNAstar software (LaserGene, Madison, WI, USA).

Construction of binary vector and transformation of tobacco

The coding sequence was amplified using primers, BIHN-w1-orf-2F (5′-GGA TCC ATG GTT GCC GCA GTA GCA GCT-3′) (underlined is *Bam*HI site) and BIHN-w1-orf-2R (5′-GTC GAC TCA CAT ATT TGA CTT ATC TCC AGT-3′) (underlined is *Sal*I site), with plasmid

pUCm-BIHN-w1-1 as template. PCR product was cloned and confirmed by sequencing, yielding plasmid pUCm-BIHN-w1-2. The ORF sequence was released from pUCm-BIHN-w1-2 by digestion with *Bam*HI/*Sal*I and ligated into *Bam*HI/*Sal*I sites of the plant binary vector CHF3, obtaining plasmid CHF3-BIHN-w1, which was then introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation using a GENE PULSER II Electroporation System (Bio-Rad Laboratories, Hercules, CA, USA). Agrobacteria were grown at 28°C with shaking (200 rpm) in YEP broth containing 100 μg/ml streptomycin and 100 μg/ml spectinomycin and collected by centrifugation, followed by resuspending in MS medium to a final OD_{600} of approximately 0.8. Transformation of tobacco was performed using Agrobacterium-mediated leaf disc transformation as described previously (Luo et al. 2005b). Re-generated shoots were rooted on MS medium containing 200 μg/ml kanamycin and 250 μg/ml carbenicillin, and transferred to soil and grown in greenhouse. Seeds of the transgenic lines were germinated on 1/2 MS medium containing 200 μg/ml kanamycin and the survivals were transferred into natural soil and grown to set seeds. The transgenic lines were allowed to grow to three generations and homozygous and single-copy lines were screened and confirmed by segregation of antibiotic resistance marker.

Disease resistance assays

Eight-week-old wild-type and transgenic tobacco plants were used to assay disease resistance against tobacco mosaic virus (TMV). To prepare the inoculum, TMV-infected tobacco leaves were ground to a fine powder in a potassium phosphate buffer (50 mM, pH 7.0). Tobacco plants were inoculated with TMV using a standard mechanical rubbing method. Briefly, fully expanded leaves were dusted with dry carborundum and inoculated by gently rubbing the upper leaf surface with 100 μl of the viral suspension inoculum, followed by rinsing with distill sterilized water immediately. After inoculation, plants were maintained at 23–26°C under continuous illumination provided by fluorescent lamps. Lesion numbers on the inoculated leaves were counted 5 day after inoculation.

Pseudomonas syringae pv. *tabaci* was cultured in King's B broth at 28°C for 2 days, and a bacterial suspension was prepared in 10 mM $MgCl_2$ (5×10^4 colony-forming units ml^{-1}). Inoculation was performed by infiltration of the bacterial suspension using a 1-ml syringe without a needle into leaves of 8-week-old wild-type and transgenic tobacco plants. To determine bacterial titers, 10 infected leaves were collected after inoculation. Leaf discs of the same size made using a hole puncher were homogenized in 10 mM $MgCl_2$, then serial-diluted by 1:10, and plated onto King's B medium. Plates were incubated at 28°C for 2 days, and bacterial colonies appeared were counted.

Abiotic stress tolerance assay

Assays for oxidative stress tolerance were performed as follow. Fully expanded leaves from 8-week-old plants of wild-type and transgenic lines were briefly washed in sterile distill water, and leaf discs (13 mm in diameter) were punched out using a hole puncher. For methyl viologen (MV) treatment, leaf discs were floated on a solution containing different concentrations of MV and 0.1% Tween-20 in the dark for 1 h, followed by illumination at moderate light intensity (200 μmol m^{-2} s^{-1}) for 18 h at 25°C. For H_2O_2 treatment, leaf discs were incubated in MES buffer containing different concentrations of H_2O_2 for 1 day under the light of 200 μmol m^{-2} s^{-1}. Chlorophyll contents in leaf discs at 18 h after MV treatment or at 24 h after H_2O_2 treatment were measured as described previously (Veronese et al. 2003). Leaf disc samples were harvested 5 h after treatments with MV (2 μM), H_2O_2 (50 mM) or water as control and were subject to extraction of total RNA for analysis of gene expression by semi-quantitative RT-PCR.

ABA sensitivity and drought tolerance were assayed. Surface-sterilized seeds were sown on 1/2 MS medium containing different concentrations of ABA and seedlings were grown under16 h/8 h light/night regime at 22–25°C. Root lengths of the transgenic and wild-type seedlings were measured 2 weeks after germination and at least 40 individual seedlings were measured. To evaluate drought tolerance, transgenic and wild type seeds were placed on filter paper saturated with or without 200 mM mannitol or different concentrations of PEG6000 and incubated under16 h/8 h light/night regime at 22–25°C.

Gene expression analysis by RT-PCR

Total RNA was extracted from rice and tobacco leaf samples using TRIZOL reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Total RNA (500 ng) was reverse-transcribed using the Super-Script III Kit (Invitrogen, Shanghai, China). One microliter of the RT reaction and 10 pmol of each primer were used for semi-quantitative RT-PCR in a total volume of 25 μl. PCR conditions were set as 94°C 30 s, 48–65°C 30 s and 72°C 30 s for 25–30 cycles based on the abundance of transcript for each gene, followed by 7 min of final extension at 72°C. PCR products were electrophoresed on a 1.2% agarose gel. Information on gene-specific primers used was listed in Table 1.

Table 1 Primers used for RT-PCR analysis of gene expression

Name	Accession no.	Primer sequences (5′–3′)
OsBIRF1		GGATGGATTCCAATCTTCCAGAC
		GTCTGAACCAGCTGGCAGCTTGC
OsActin	X16280	ACTGCTCCCATCTATGAAGGA
		CTGCTGGAATGTGCTGAGAGA
NtPR1	X12737	GATGCCCATAACACAGCTCGTGC
		GCCTCTATAATTACCTGGAGGATC
NtPR2	M60460	GCAACATATTCAGGGATC
		ATTGAAATTGAGTTGATA
NtPR3	M29868	CCAGAGTGACAGATATTA
		GCCCTGGCCGAAGTTCCT
NtPR5	X03913	GTCAACCAATGCACCTAC
		GGTGGATCATCCTGTGGA
NtGST	D10524	GGCGATCAAAGTCCATGGTAG
		GCTTCTCCAATCCCTTAACCC
NtACT	U93244	GGATCCATACAAGTACCGTCC
		CAAGGACCCTCCAATTCTCCTG
NtAPX	AF443182	GCATGGCACTCTGCTGGTACC
		GGGGATTGGTAGTCCAAGGTC
NtActin	X69885	CTATTCTCCGCTTTGGACTTGGCA
		ACCTGCTGGAAGGTGCTGAGGGAA

Results

Cloning and characterization of rice *OsBIRF1*, encoding a RING finger protein

In our previous studies aimed at elucidating the molecular biology of rice defense response induced by BTH, a defense-related differentially expressed cDNA clones, BIHN-w1, was isolated and identified through approach of suppression subtractive hybridization (Song and Goodman 2002). Sequencing and BLAST similarity searching against the GenBank database revealed that BIHN-w1 is a part of a gene encoding a putative RING-H2 finger protein. The cDNA insert in BIHN-w1 seems to encode the C-terminal of this putative RING finger protein. The full-length cDNA of this putative RING finger gene was obtained by amplification of 5′-end sequences flanking the insert in clone BIHN-w1 and was named as *OsBIRF1* (*Oryza sativa* L. BTH-induced RING finger protein 1). The full-length cDNA of *OsBIRF1* is 1,873 bp with an open reading frame (ORF) of 1,191 bp. The *OsBIRF1* gene corresponds to locus Os02g50930 as annotated by the TIGR Rice Genome Annotation program, which is located on chromosome 2 of the rice genome and consists of a single exon without intron.

The *OsBIRF1* gene was predicted to encode a 396 amino acid protein with a calculated molecular weight of 43 kD and isoelectric point of 8.13. Database searches for conserved domains and alignment with ATL1D and ATL5I, two previously characterized Arabidopsis RING-H2 finger proteins, revealed that OsBIRF1 contains a highly conserved RING-H2 finger domain (C-X2-C-X15-C-X1-H-X2-H-X2-C-X10-C-X2-C) (Fig. 1a). In addition, a putative transmembrane domain from 58-A to 80-V, a basic amino acid region from 101-H to 124-S, and GLD domain from 125-G to 140-R, were identified (Fig. 1a). Protein similarity searches and phylogenetic tree analysis indicated that OsBIRF1 has high levels of similarity to Arabidopsis ATL1D and ATL5I, showing identity of 42–48% (Fig. 1b). The features of conserved domain organization in OsBIRF1 protein are similar to the characteristic regions present in the ATL family proteins (Salinas-Mondragon et al. 1999; Serrano et al. 2006), and therefore *OsBIRF1* encodes a rice RING-H2 finger protein belonging to the ATL family.

Expression patterns of *OsBIRF1* in rice disease resistance responses

The differentially expressed cDNA clone BIHN-w1 was originally obtained from a suppression subtractive hybridization library constructed by subtraction of cDNAs from BTH-induced and pathogen-infected rice leaf samples with those from water control (Song and Goodman 2002), suggesting that the gene corresponding to BIHN-w1 might be induced during rice defense response. We therefore analyzed by semi-quantitative RT-PCR whether expression of *OsBIRF1* in 3-week-old rice seedlings was induced by treatments with some of defense-related signal molecules including BTH, SA, ACC or JA. The results showed that expression of *OsBIRF1* was induced by all these defense-related signal molecules with similar kinetics. Up-regulation of *OsBIRF1* expression was detected at 12 h, peaked at 48 h, and maintained at a relatively high level during 12–72 h after BTH treatment (Fig. 2a). Likely, expression of *OsBIRF1* was induced in 6–72 h by ACC and JA, and in 24–72 h by SA. No significant induced expression of *OsBIRF1* was observed in water-treated seedlings during the experiment period, except for a low level of basal expression (Fig. 2a). These results indicate that *OsBIRF1* gene is responsible to multiple defense-related signal molecules, suggesting a role for *OsBIRF1* in induced defense response in rice.

To further elucidate the involvement of *OsBIRF1* in disease resistance response, the expression pattern of *OsBIRF1* in incompatible and compatible interactions between a pair of near-isogenic lines, H8R and H8S, and the blast fungus, *M. grisea*, was analyzed and compared. Once inoculated with spores of strain 85-14B1 of *M. grisea*, seedlings of H8R and H8S showed incompatible and

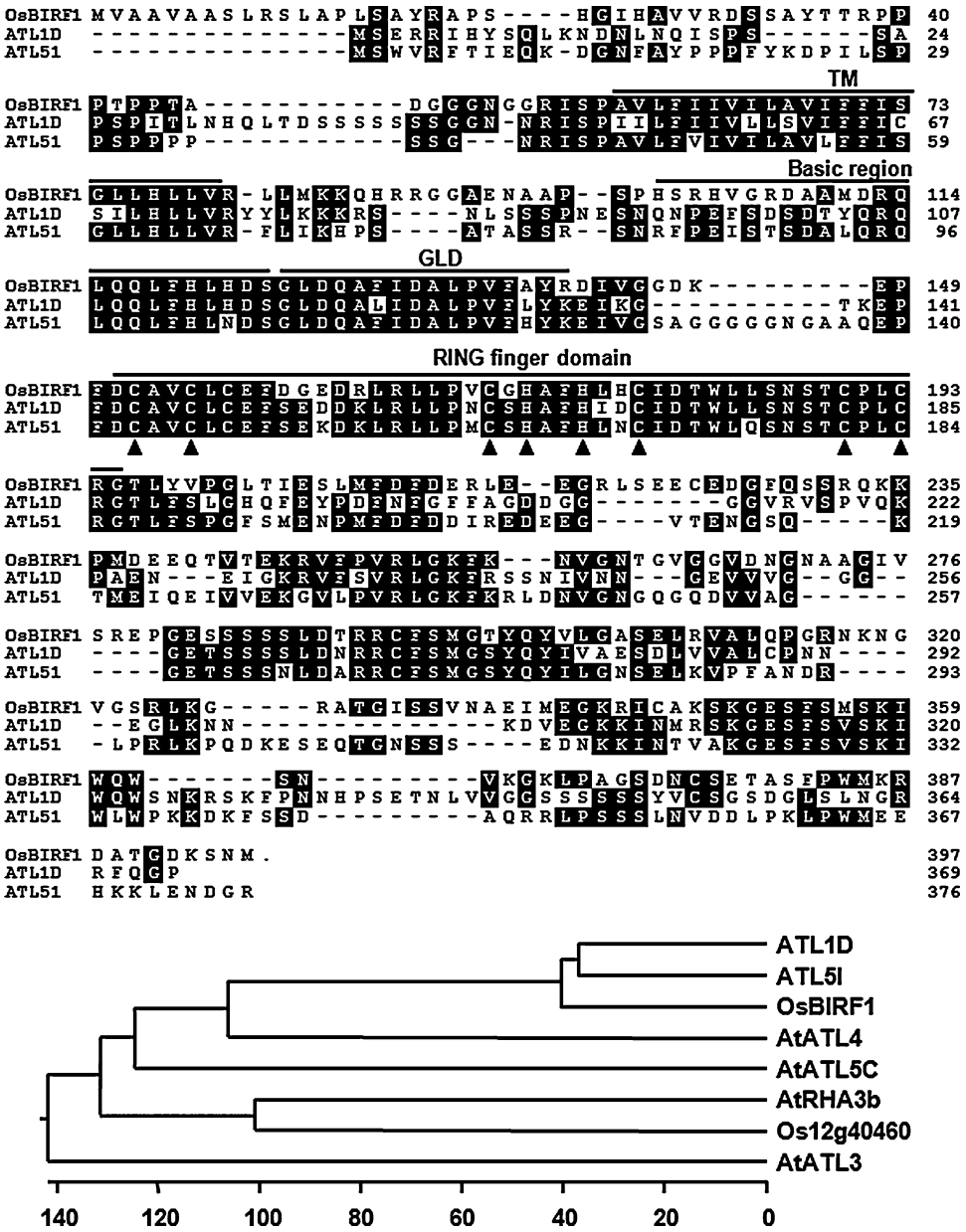

Fig. 1 OsBIRF1 is a RING finger protein. (**a**) Alignment of OsBIRF1 with *Arabidopsis thaliana* ATL1D (AAQ55274) and ATL5I (BAD44076). The putative transmembrane domain (TM), basic amino acid rich region, conserved ATL family GLD region and RING finger domain is indicated above the sequence and the conserved amino acid residues in the RING finger domain are indicated by filled triangles. (**b**) Phylogenetic tree analysis of OsBIRF1. The plant RING finger proteins used are *Arabidopsis thaliana* ATL1D (At1g23980, AAQ55274), ATL5I (At5g40250, BAD44076), AtATL3 (AT1G72310, AAM61130), AtRHA3b (At2g17450, AAM64643), AtATL4 (At3g60220, NP_191581) and AtATL5C (At5g05810, Q5EAE9); *Oryza sativa* Os12g40460 (ABA99194)

compatible interactions, resulting in disease resistance and susceptible responses, respectively. In leaves of H8R seedlings, expression of *OsBIRF1* was activated at 24 h, maintained a high level during 24–60 h after inoculation with *M. grisea* (Fig. 2b). However, expression of *OsBIRF1* was maintained unchanged in leaves of H8S seedlings after pathogen inoculation (Fig. 2b). These results indicate that expression of *OsBIRF1* is involved in the incompatible interaction between rice and *M. grisea*.

Generation of *OsBIRF1*-overexpressing transgenic plants

To study further the biological role of *OsBIRF1* in defense responses, a functional analysis of *OsBIRF1* in transgenic tobacco plants was performed. The coding sequence of *OsBIRF1* was cloned into a plant binary vector CHF3 under the control of CaMV 35S promoter and was introduced into tobacco using the *Agrobacterium*-mediated leaf disc transformation method. A total of 21 independent transgenic plants were obtained by screening of kanamycin and by PCR detection of the transgene with genomic DNA as template and five independent transgenic lines with single copy of *OsBIRF1* were obtained through screening based on segregation of antibiotic resistance marker in progenies. These single copy transgenic lines were allowed to grow for three generations and two homozygous lines were selected to for further studies. RT-PCR analysis indicated that *OsBIRF1* was expressed in these transgenic tobacco lines (Fig. 3a).

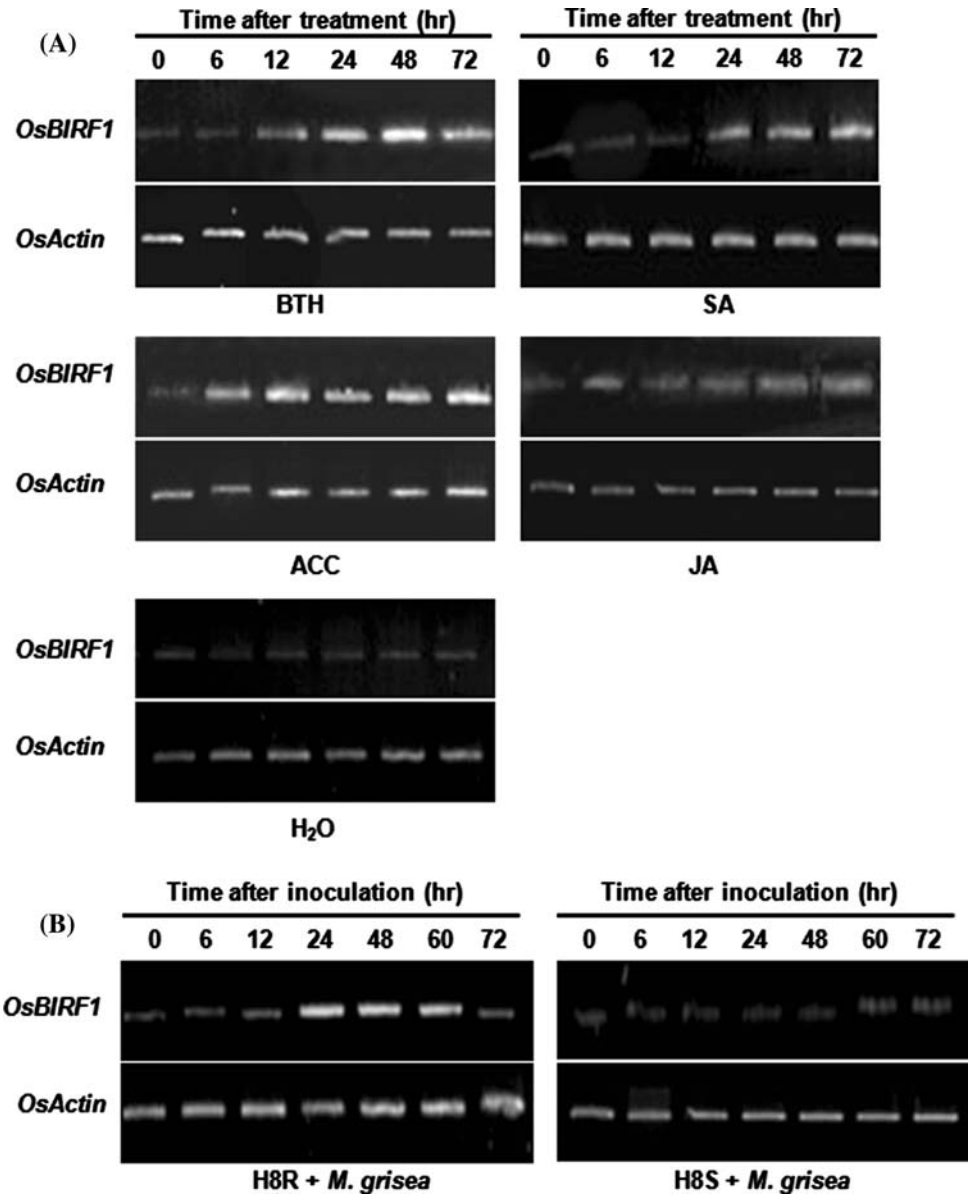

Fig. 2 Expression of *OsBIRF1* in rice disease resistance response. (**a**) Expression of *OsBIRF1* induced by BTH, ACC, SA and JA. Three-week-old rice seedlings were treated by foliar spraying with 0.3 mM BTH, 1.5 mM SA, 100 μM ACC, 100 μM JA or water. (**b**) Differential expression of *OsBIRF1* in interactions between rice and *Magnaporthe grisea*. Three-week-old rice seedlings of H8R and H8S were inoculated with *M. grisea* and leaf samples were collected at each time point (h) as indicated. Total RNA was extracted and used for semi-quantitative RT-PCR analysis

Overexpression of *OsBIRF1* promotes growth of transgenic tobacco plants

During screening the transgenic lines, we noted that the T0 transgenic plants grew faster and had more leaves than the wild-type plants. This implied that overexpression of *OsBIRF1* affected growth and development of the transgenic tobacco plants. We thus analyzed and compared growth and development phenotypes of the *OsBIRF1* transgenic plants with wild-type plants. The transgenic seedlings grown on 1/2 MS medium showed some morphological changes as compared with the wild-type seedlings (Fig. 3b, c). The transgenic seedlings had longer roots by 62–75% increase in root length and more leaves by 1–2 leaves than those of the wild-type seedlings when grown on 1/2 MS medium for 3 weeks (Fig. 3b, d). Heights of 3-week-old seedlings grown on 1/2 MS and of 10-week-old plants grown in soil also were significantly higher than those of wild-type plants (Fig. 3c, e). These results reveal that *OsBIRF1* might have a function in regulation of growth and development in transgenic tobacco plants as well as in rice.

Enhanced disease resistance in *OsBIRF1*-overexpressing transgenic plants

To clarify the possible role of *OsBIRF1* in defense responses, disease assays were performed to evaluate the disease resistance levels of the *OsBIRF1* transgenic tobacco plants against two different types of pathogens, TMV and *P. syringae* pv. *tabaci*. In TMV disease

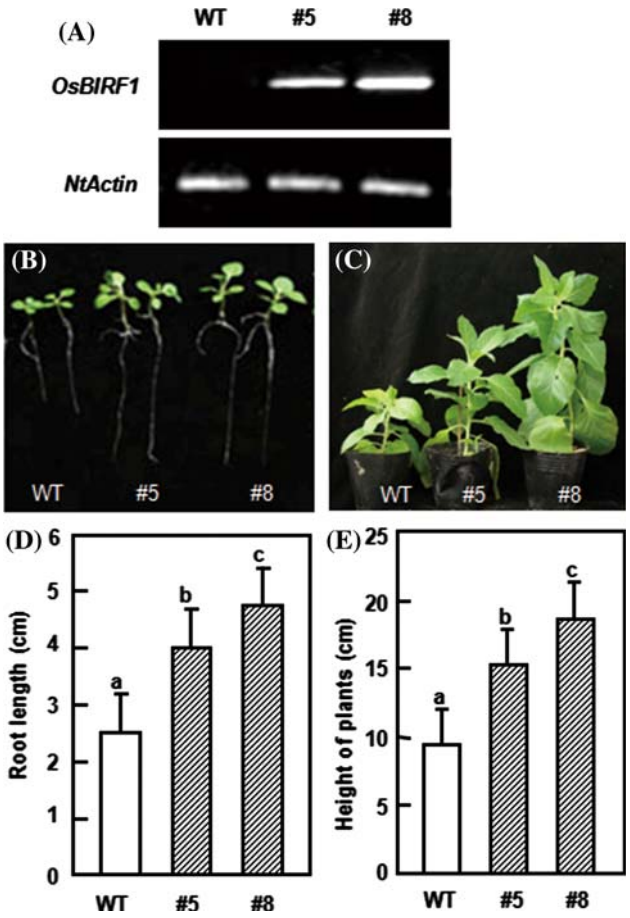

Fig. 3 Generation and growth of *OsBIRF1* transgenic tobacco plants. (**a**) Expression of *OsBIRF1* in transgenic tobacco plants. Total RNA was extracted from transgenic and wild-type plants grown under normal condition and used for semi-quantitative RT-PCR analysis. (**b**, **c**) Comparison of root elongation and growth of the transgenic and wild-type seedlings grown on 1/2 MS for 3 weeks (**b**) or in soil for 10 weeks (**c**). (**d**) Root length of the transgenic and wild-type seedlings grown on 1/2 MS for 3 weeks. (**e**) Height of the transgenic and wild-type plants grown in soil for 10 weeks. Values shown are the means ± SD from three independent experiments. Different letters above the columns indicate significant differences ($P < 0.05$). WT, wild-type plants; #5 and #8, independent transgenic lines

resistance assays, necrotic lesions were typically observed on the leaves of wild-type and the *OsBIRF1* transgenic plants 3 days after inoculation, and timing of symptom appearance was similar between the transgenic and wild-type plants (Fig. 4a). However, the lesion numbers in the leaves of the *OsBIRF1* transgenic plants was significantly reduced as compared with that in wild-type plants, resulting in reductions of 45 and 63% in transgenic lines #5 and #8, respectively (Fig. 4b). In addition, the disease resistance of the *OsBIRF1* transgenic plants against *P. syringae* pv. *tabaci* was also tested. Under our experimental conditions, disease symptom was observed around 3 days after inoculation on the leaves of wild type plants with yellowish chlorotic and necrotic areas, and the large necrotic lesions were formed at end of the experiments (Fig. 4c). On the transgenic plant leaves, the symptom was only visible 4–5 days after inoculation with relatively small necrotic areas (Fig. 4c). Measurement of bacterial titers in inoculated leaves of the *OsBIRF1* transgenic and wild-type plants further confirmed the observed phenotypes in *OsBIRF1* transgenic plants. The bacterial titers in inoculated leaves of the transgenic plants were markedly reduced as compared with those in wild-type plants at 4 day and 7 day after inoculation (Fig. 4d). These results suggest that overexpression of *OsBIRF1* in transgenic tobacco plants confers an enhanced disease resistance against TMV and *P. syringae* pv. *tabaci*.

Up-regulated expression of defense-related genes in *OsBIRF1* transgenic plants

To ascertain whether the observed enhanced disease resistance in *OsBIRF1* transgenic tobacco plants was resulted from activation of defense response by expression of *OsBIRF1*, we analyzed and compared the expression levels of some selected defense-related genes, e.g. *PR-1*, *PR-2*, *PR-3* and *PR-5*, in *OsBIRF1* transgenic and wild-type plants. As shown in Fig. 4e, under normal growth condition, no significant expression of *PR* genes was observed in the wild-type plants. However, up-regulated expression of *PR-1*, *PR-2* and *PR-3* were detected in the *OsBIRF1* transgenic plants (Fig. 4e), and relatively higher levels of expression for *PR-1* and *PR-3* in transgenic line #8 was observed. *PR-5* had slight increased expression in *OsBIRF1* transgenic plants as compared with that in wild-type plants (Fig. 4e). These results indicate that overexpression of *OsBIRF1* activates expression of *PR* genes in transgenic tobacco plants, resulting in constitutive activation of defense responses.

Enhanced oxidative stress tolerance in *OsBIRF1* transgenic plants

Possible roles of *OsBIRF1* in oxidative stress was further studied by testing the tolerance of leaves discs from 8-week-old transgenic and the wild-type plants to exogenous H_2O_2 or MV. As shown in Fig. 5a, c, no significant bleaching or chlorosis was observed in leaf discs from both transgenic and wild-type plants in normal medium without H_2O_2 or MV during the experimental period. After incubation in different concentrations of H_2O_2 or MV, symptoms of bleaching or chlorosis were observed in leaf discs both from transgenic and wild-type plants, but bleaching or chlorosis of leaf discs from wild-type plants was much more severe than those from transgenic plants (Fig. 5a, c). This was further confirmed by measuring chlorophyll contents in leaf discs from the transgenic and

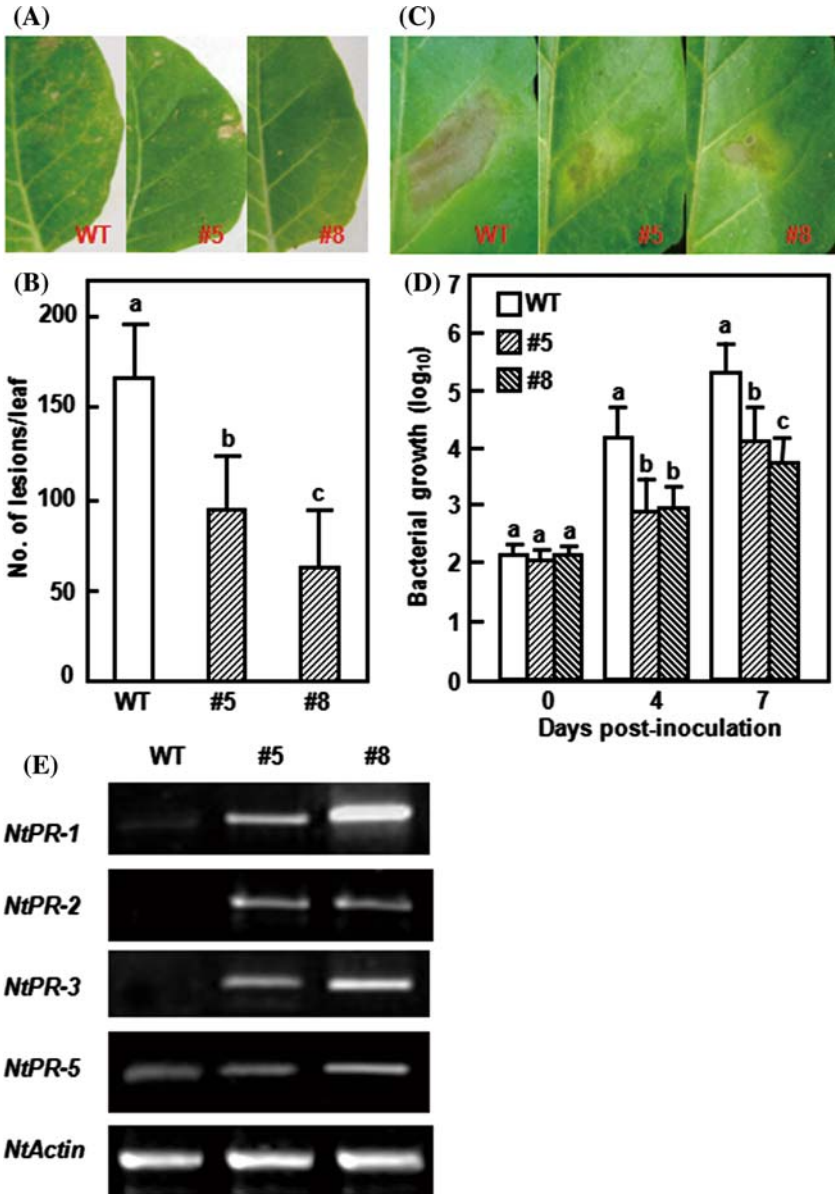

Fig. 4 Enhanced disease resistance and elevated expression levels of defense-related genes in *OsBIRF1* transgenic tobacco plants. (**a**, **b**) Symptom and severity of disease caused by tobacco mosaic virus in transgenic and wild-type plants. Photos were taken 5 days after inoculation. Disease severity as indicated by lesion numbers on the *OsBIRF1* transgenic and wild-type plants. (**c**, **d**) Symptom and severity of disease caused by *Pseudomonas syringae* pv. *tabaci* in transgenic and wild-type plants. Photos were taken 7 days after inoculation. Bacterial titers in inoculated leaves of the *OsBIRF1* transgenic and wild-type plants were measured at different time points after inoculation. Values shown are the means ± SD of three independent experiments. Different letters above the columns indicate significant differences ($P < 0.05$). (**e**) Expression of *PR* genes in transgenic and wild-type plants. Leaf samples were collected from transgenic and wild-type plants grown in soil under normal condition and expression of *PR* genes was analyzed by semi-quantitative RT-PCR. WT, wild-type plants; #5 and #8, independent transgenic lines

wild-type plants after H_2O_2 or MV treatments. Relative chlorophyll contents in leaf discs of the transgenic plants were markedly higher than those from the wild-type plants after treatments with different concentrations of H_2O_2 or MV (Fig. 5b, d).

Expression of oxidative stress-related genes, including *APX*, *CAT* and *GST*, was analyzed to gain insights into the possible mechanisms of the enhanced oxidative stress tolerance in *OsBIRF1* transgenic plants. Leaf discs from both transgenic and wild-type plants were treated by incubation in MV, H_2O_2 or water, and samples were collected to analyze expression of oxidative stress-related genes by RT-PCR. In water-treated controls, *CAT* and *APX* showed an increased expression in transgenic plants, and no significant expression of *GST* was detected in both wild-type and transgenic plants (Fig. 5e). After incubation with H_2O_2 or MV, expression of *APX*, *GST* and *CAT* was up-regulated in leaf discs of wild-type plants, indicating an oxidative stress was applied to leaf discs (Fig. 5e). However, the expression levels of *APX*, *GST* and *CAT* in leaf discs of the transgenic plants were higher than those in leaf discs of the wild-type plants (Fig. 5e). It was noted that expression of *CAT* showed no significant difference between the transgenic and wild-type plants after treatment with MV, but showed markedly increase in transgenic plants after treatment with H_2O_2 (Fig. 5e). It was thus concluded that overexpression of *OsBIRF1* in transgenic tobacco plants improves oxidative stress tolerance through up-regulating expression of oxidative stress-related genes.

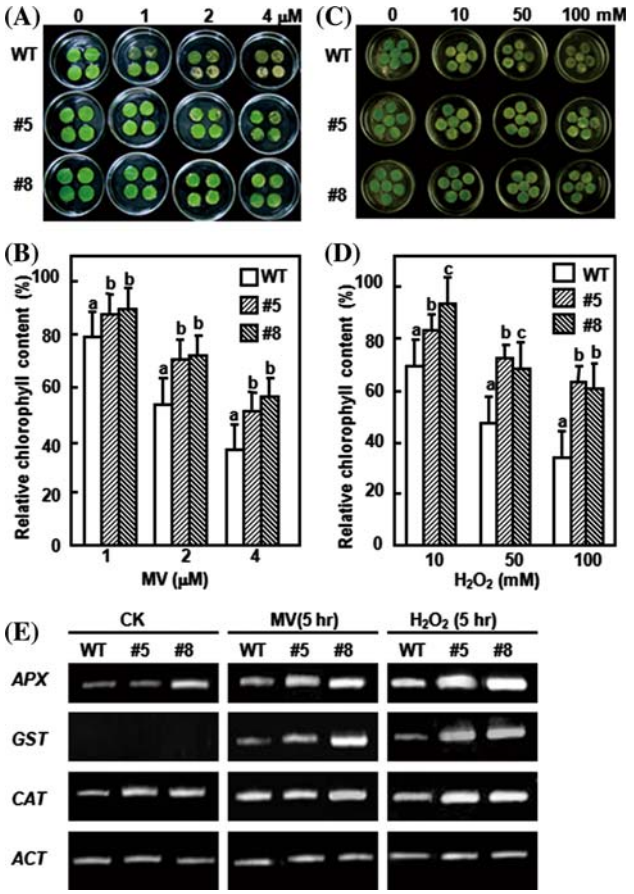

Fig. 5 Increased oxidative stress tolerance in *OsBIRF1* transgenic plants. (**a**) Comparison of sensitivity to methyl viologen (MV) between leaf discs from transgenic and wild-type plants. Leaf discs were collected from 8-week-old transgenic and wild-type plants and floated on solutions containing different concentrations of MV in dark for 1 h, and then under illumination at moderate light intensity (200 μmol m^{-2} s^{-1}) for 18 h at 25°C. Photos were taken 19 h after treatment. (**b**) Relative chlorophyll contents in leaf discs at 18 h after MV treatments. (**c**) Comparison of tolerance to H_2O_2 between leaf discs from transgenic and wild-type plants. Leaf discs were collected from 8-week-old transgenic and wild-type plants and floated on MES buffer containing different concentrations of H_2O_2 under illumination at moderate light intensity (200 μmol m^{-2} s^{-1}) for 24 h at 25°C. Photos were taken 24 h after treatment. (**d**) Relative chlorophyll contents in leaf discs at 24 h after H_2O_2 treatments. Values shown are the means ± SD from three independent experiments. Different letters above columns indicate significant differences ($P < 0.05$). (**e**) Expression of oxidative stress-related genes. Leaf discs were collected from 8-week-old transgenic and wild-type tobacco plants were treated with MV (2 μM) or H_2O_2 (50 mM) for 5 h. Leaf discs were harvested for extraction of total RNA and analysis of gene expression by semi-quantitative RT-PCR. WT, wild-type plants; #5 and #8, independent transgenic lines

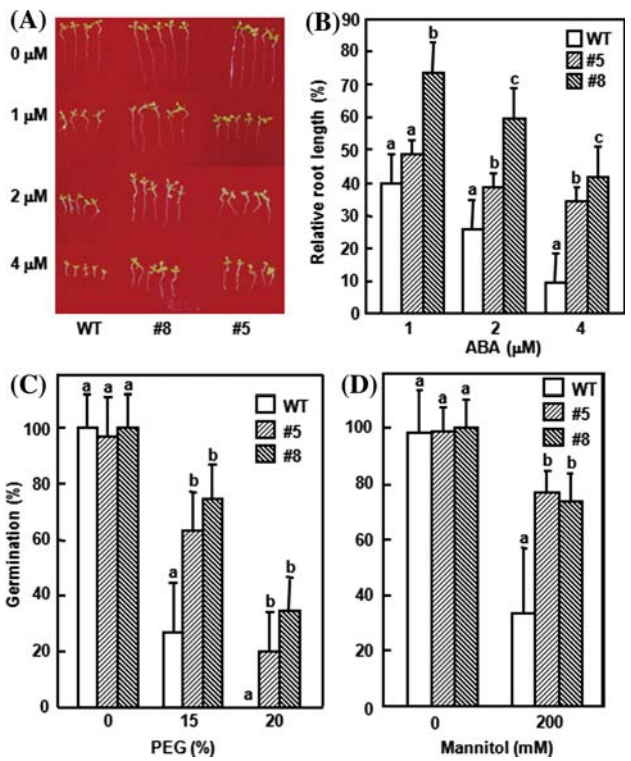

Fig. 6 Reduced ABA sensitivity and increased drought tolerance in *OsBIRF1* transgenic plants. (**a**, **b**) Inhibition of root elongation by ABA. (**a**) Growth of the transgenic and wild-type seedlings on 1/2 MS medium containing different concentrations of ABA. (**b**) Root lengths of the transgenic and wild-type seedlings 2 weeks after treatment with ABA. (**c**, **d**) Increased drought tolerance in *OsBIRF1* transgenic plants. Seed germination on 1/2 MS medium containing different concentration of PEG6000 (**c**) or mannitol (**d**). Values shown are means ± SD from three independent experiments. Different letters above the columns indicate significant differences ($P < 0.05$). WT, wild-type; #5 and #8, independent transgenic lines

Reduced ABA sensitivity and increased drought tolerance of the *OsBIRF1* transgenic plants

Previous studies have shown that RING finger proteins are involved in ABA signaling pathway and thus play an important role in drought tolerance (Ko et al. 2006; Zhang et al. 2005). To gain information on the function of *OsBIRF1* in drought tolerance, we further analyzed the sensitivity of *OsBIRF1* transgenic plants to exogenous ABA and drought tolerance. As mentioned above, roots of the *OsBIRF1* transgenic seedlings on 1/2 MS without ABA were longer than the wild-type seedlings (Fig. 6a). On 1/2 MS with exogenous ABA, root elongation of the wild-type seedlings was significantly inhibited; however, root elongation of the *OsBIRF1* transgenic seedlings was less inhibited (Fig. 6a). Compared with the wild-type seedlings, root growth of the transgenic seedlings was less inhibited by exogenous ABA, and this was much evident at high concentration of ABA supplemented in the medium (Fig. 6b). With the increase of ABA concentrations, inhibition of root length was much obvious in wild-type seedlings than in transgenic seedlings. Relative root length of wild-type seedlings grown on 1/2 MS containing 4 μM ABA was measured only ~10% of those grown on 1/2 MS

without ABA, while relative root length of the transgenic seedlings on ABA-containing MS was ~40% of those grown on ABA-free medium (Fig. 6b). These results suggest that overexpression of *OsBIRF1* in transgenic tobacco plants results in a reduced ABA sensitivity.

Drought tolerance of the *OsBIRF1* transgenic plants was studied by analyzing and comparing seed germination on PEG6000- or mannitol-containing medium with the wild-type. Germination rates of seeds from the transgenic and wild-type plants were comparable under normal condition without PEG6000 or mannitol (Fig. 6c, d). In the presence of PEG6000, germination rates of the wild-type seeds were significantly reduced, germination rates of the transgenic seeds were markedly higher than the wild-type on medium containing 15% PEG6000, giving 125–170% increase over the wild-type seeds. Under 20% PEG6000 condition, the wild-type seeds did not germinate, while there were 20–34% of the transgenic seeds germinated (Fig. 6c). Similarly, under 200 mM mannitol, only ~36% of the wild-type seeds germinated, while 74–78% of the transgenic seeds germinated, giving an increase of 105–117% over the wild-type seeds. These results indicate that overexpression of *OsBIRF1* in transgenic tobacco plants leads to an enhanced drought tolerance.

Discussion

RING finger proteins, comprising of a large protein family, ubiquitously exist in all of eukaryotes and, as a kind of ubiquitin E3 ligases, play key roles in ubiquitination of specific proteins for degradation by the ubiquitin/26S proteasome system (Freemont 2000; Joazeiro and Weissman 2000). Among the RING finger proteins, a specific family, called ATL family, was first identified in Arabidopsis (Salinas-Mondragon et al. 1999) and later found to distribute widely in plant species, for example, the Arabidopsis and rice ATL family contain 80 and 121 members, respectively (Serrano et al. 2006). In addition to a typical RING finger domain, the ATL family proteins contain common characteristic structural features, e.g. a predicted transmembrane domain, a basic amino acid rich region, a conserved GLD region and a highly diverse region in the C-terminal (Salinas-Mondragon et al. 1999). The fact that OsBIRF1 not only contains the above-mentioned characteristic structural features but also is phylogenetically related to Arabidopsis ATLs clearly demonstrates that it is a member of the rice ATL family. OsBIRF1 is an intronless gene, which is also similar to the previous observation that 90% of the Arabidopsis ATL genes are intronless (Serrano et al. 2006). Although more than 100 members were identified for the rice ATL family, only one member, EL5, has been studied in detail for its biochemical and biological function (Takai et al. 2001, 2002; Katoh et al. 2003; Koiwai et al. 2007). Our functional analysis in transgenic tobacco suggests important roles for *OsBIRF1* in growth and defense responses against biotic and abiotic stresses. These findings provide new insights into the biological functions of the rice ATL family.

It was previously found that expression of several ATL genes in Arabidopsis is induced by fungal elicitors (Serrano et al. 2006). Similarly, the *EL5* gene is transiently induced by *N*-acetylchitoheptaose elicitor in rice cells (Takai et al. 2002). However, there is no further evidence supporting a role for *EL5* in rice disease resistance response. *OsBIRF1* is up-regulated by some well-known defense-related signal molecules including SA and JA, which are believed to mediate the SA-dependent pathway and the JA/ET pathway in defense responses, respectively (Glazebrook 2005). Importantly, *OsBIRF1* is also differentially induced in incompatible but not in compatible interactions between rice and the blast fungus, which is in agreement with the notion that expression of *ACRE132*, a tobacco ATL gene, is induced in Avr9- and Cf9-mediated incompatible interaction (Durrant et al. 2000). The induced expression feature during defense responses provides preliminary evidence supporting a role for *OsBIRF1* in regulating disease resistance response in rice. Direct evidence supporting this conclusion came from our functional analysis of *OsBIRF1* in transgenic tobacco plants. In this study, the *OsBIRF1*-overexpressing transgenic tobacco plants showed enhanced disease resistance against at least two different types of pathogens, virus (TMV) and bacteria (*P. syringae* pv. *tabaci*), as revealed by reduced disease severity and bacterial population in the transgenic plants. Accompanied with the enhanced disease resistance phenotype in the *OsBIRF1* transgenic tobacco plants is the up-regulated expression of some defense-related genes in the absence of pathogen infection or elicitor induction. It was recently found that the Arabidopsis *eca* mutants, which showed constitutive expression of the *ATL2* gene, exhibits elevated expression levels of defense-related genes and SA- and JA-responsive genes and knockout mutation with T-DNA insertion in *ATL9* results in increased susceptibility to powdery mildew disease (Serrano and Guzman 2004; Ramonell et al. 2005). However, it is not clear yet whether the *eca* mutant plants have enhanced disease resistance phenotype and the *ATL9* T-DNA knockout plants impairs activation of defense-related genes in responding to pathogen infection (Serrano and Guzman 2004; Ramonell et al. 2005). The tomato *ATL6*-mediated ubiquitin/proteasome system contributes to fungal elicitor-activated defense response via the JA-dependent signaling pathway (Hondo et al. 2007). It was also reported that ectopic expression of the rice *OsRHC1*, encoding a RING finger protein other than the ATL family members, in Arabidopsis shows enhanced

disease resistance and elevated expression of defense-related genes (Cheung et al. 2007). On the other hand, overexpression of the *CaRFP1* gene in transgenic Arabidopsis plants leads to increased disease susceptibility and reduced PR gene expression (Hong et al. 2007). Therefore, the fact that enhanced disease resistance is associated with up-regulated PR gene expression in the transgenic tobacco plants suggests that the enhanced disease resistance in *OsBIRF1* transgenic plants is most likely to be the results of activation of defense-related gene expression.

It has been demonstrated that some of the RING finger proteins play important roles in regulation of abiotic stress tolerance (Dong et al. 2006; Ko et al. 2006; Zhang et al. 2007; Kam et al. 2007; Sahin-Cevik and Moore 2007). In this study, the *OsBIRF1* transgenic tobacco plants show enhanced tolerance to oxidative stress, as revealed by lowering in bleaching and chlorophyll loss of leaf discs after treatments with exogenous MV and H_2O_2. We also noted that in *OsBIRF1* transgenic tobacco plants, expression of oxidative stress-related genes, e.g. *APX*, *CAT* and *GST*, was up-regulated. It is well established 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). Interestingly, a further induction of *APX*, *CAT* and *GST* genes was observed in leaf discs of the transgenic plants after challenged with exogenous MV or H_2O_2, when compared to the expression levels in leaf discs without stress. This result suggests that (1) OsBIRF1 itself in the transgenic plants can elevate oxidative stress tolerance by activating expression of the oxidative stress-related genes in the absence of stress, and (2) OsBIRF1 can also potentitate or amplify the signal(s) required for activating expression of the oxidative stress-related genes when exposure to exogenous stress.

Some of RING finger proteins have been demonstrated to regulate ABA signaling and affect drought tolerance (Zhang et al. 2005, 2007). ABA treatment has been suggested to inhibit seed germination and seedling growth in plants (Finkelstein et al. 2002). The *OsBIRF1* transgenic plants show reduced ABA sensitivity, as revealed by reduced inhibition of primary root elongation with exogenous ABA treatments. This is similar to that of the Arabidopsis AIP2, whose constitutive expression in Arabidopsis resulted in insensitivity to ABA for primary root growth (Zhang et al. 2005), but is contrast to that of the *ATL43* gene, whose mutation led to ABA insensitivity (Serrano et al. 2006). Furthermore, results from seed germination on PEG6000- or mannitol-containing medium demonstrate that the *OsBIRF1* transgenic plants also have increased tolerance against drought stress. ABA is an essential mediator in triggering plant responses to most of the common abiotic stresses, including drought stress (Finkelstein et al. 2002). Reduced ABA sensitivity and increased drought tolerance were observed in the *OsBIRF1* transgenic tobacco plants, which is different from previous observation that constitutive expression of the Arabidopsis *SDIR1* resulted in increased ABA sensitivity and enhanced tolerance to multiple abiotic stresses including drought stress (Zhang et al. 2007). This may suggest that RING finger proteins have functions in ABA signaling and drought response through different mechanisms.

A number of RING finger proteins have been shown to regulate different aspects of growth and developmental processes (Moon et al. 2004). Transgenic alfalfa and Arabidopsis overexpressing the alfalfa *MsRH2-1*, an ortholog of Arabidopsis *ATL4*, exhibit shorter plant heights and abnormal lateral root development (Karlowski and Hirsch 2003). We also found that the *OsBIRF1* transgenic tobacco plants have longer primary roots and larger plant heights as compared with the wild-type plants. Interestingly, constitutive expression and suppressing by RNAi of *EL5* in rice did not cause any obvious phenotypic changes (Koiwai et al. 2007). Auxin signaling has been shown to be involved in RING finger proteins-mediated regulation of plant growth and development processes (Xie et al. 2002; Nodzon et al. 2004). The involvement of auxin or other phytohormones, if possible, in promotion of the *OsBIRF1* transgenic plants needs further investigation.

Taken together, our studies suggest that OsBIRF1 plays important roles in regulation of plant growth and defense response against biotic and abiotic stresses. Overexpression of *OsBIRF1* results in constitutive activation of defense-related genes in transgenic tobacco plants and thus OsBIRF1 appears to be a positive regulator of defense response to multiple environmental stresses. Considering that *OsBIRF1* encodes a putative RING-H2 finger ubiquitin E3 ligase, OsBIRF1 protein might target potential factors that are negative regulators of defense responses in plants. Further identification and functional analysis of the downstream targets will lead to a better understanding of the roles of OsBIRF1 in the cellular defense response to pathogen infection and environmental stresses. Generation of transgenic rice plants overexpressing or knocking down the *OsBIRF1* gene is underway. Results from these analyses will provide new insights into the biological functions of this gene in rice growth/development and defense responses against biotic and abiotic stresses.

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