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Molecular characterization of a defense-related AMP-binding protein gene, *OsBIABP1*, from rice*

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Abstract: We cloned and characterized a rice gene *OsBIABP1* encoding an AMP-binding protein. The full-length cDNA of *OsBIABP1* is 1912-bp long and is predicted to encode a 558-aa protein. OsBIABP1 contains a typical AMP-binding signature motif and shows high similarity to members of AMP-binding protein family. *OsBIABP1* is expressed in stems, leaves and flowers of rice plants, but is not expressed, or expressed at a very low level, in rice roots. The expression of *OsBIABP1* was induced by some defense-related signal molecules, e.g., salicylic acid (SA), benzothiadiazole, jasmonic acid (JA), and 1-amino cyclopropane-1-carboxylic acid, which mediate SA- and JA/ethylene (ET)-dependent defense signaling pathways, respectively. Furthermore, the expression of *OsBIABP1* is activated by the infection of *Magnaporthe oryzae*, and the induced expression is quicker and stronger during early stages of pathogenesis in incompatible interaction than that in compatible interaction between rice and *M. oryzae*. Our results suggest that OsBIABP1 may be a defense-related AMP-binding protein that is involved in the regulation of defense response through SA and/or JA/ET signaling pathways.

Key words: Rice, *Oryza sativa* L., *Magnaporthe oryzae*, Defense response, *OsBIABP1*, AMP-binding protein
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INTRODUCTION

Plants defend themselves against potential pathogens by activating a battery of defense mechanisms, in which thousands of defense genes are coordinately expressed. Plant responses in compatible and incompatible interactions between *Arabidopsis* and *Pseudomonas syringae* are qualitatively similar but quantitatively different soon after infection, and the amplitude of the responses in the compatible interaction increases later (Tao *et al*., 2003). Of all defense mechanisms, hypersensitive response (HR) often occurs at the infection site to restrict spreading of the invading pathogens from primary infection site to other parts of the plants. Occurrence of an HR usually is accompanied by systemic activation of defense responses throughout the whole plants, resulting in systemic acquired resistance (Durrant and Dong, 2004; Grant and Lamb, 2006). Recent biochemical, genetic and genomic studies in *Arabidopsis* have revealed that different signaling pathways are responsible to regulate the activation of disease resistance responses, among which salicylic acid (SA)- and jasmonic acid (JA)/ethylene (ET)-dependent signaling pathways are well documented (Beckers and Spoel, 2006; Wasternack, 2007; Kachroo and Kachroo, 2007). The SA- and JA/ET-dependent signaling pathways are believed to play pivotal roles in regulating active defense responses against biotrophic and necrotrophic pathogens, respectively (Glazebrook, 2005).

Disease resistance signaling pathways in rice share some common features with those in dicot plants including *Arabidopsis* (Song and Goodman,

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2001). *OsNPR1*, a homolog of *Arabidopsis NPR1*/*NIM1*, also defines a disease resistance pathway similar to the NPR1-mediated signaling pathway in *Arabidopsis*, because the overexpression of *Arabidopsis NPR1* or rice endogenous *OsNPR1* in transgenic rice resulted in enhanced disease resistance and activation of defense gene expression (Chern *et al*., 2001; 2005; Fitzgerald *et al*., 2004; Yuan *et al*., 2007). Meanwhile, JA has also been demonstrated to play important roles in rice defense response against pathogen infection (Mei *et al*., 2006; Yara *et al*., 2007; Qiu *et al*., 2007), probably coordinately operating with the SA-dependent signaling pathway (Qiu *et al*., 2007).

Previously, we demonstrated that benzothiadiazole (BTH), a structural analog of SA, can induce disease resistance response against rice blast disease (*Magnaporthe oryzae*), sheath blight disease (*Rhizoctonia solani*), and leaf bacterial blight disease (*Xanthomonas oryzae* pv. *oryzae*) (Ge *et al*., 1999; Song *et al*., 2001; Zhang *et al*., 2004). To study the molecular mechanism of the BTH-induced disease resistance in rice, we isolated and identified over 200 differentially expressed genes through suppression subtractive hybridization (Song and Goodman, 2002a). Recently, our further studies on some selected differentially expressed genes have revealed that genes encoding mitogen-activated protein kinases (*OsBIMK1* and *OsBIMK2*), protein phosphatase 2C (*OsBIPP2C1*), homeodomain and ERF transcriptional factors (*OsBIHD1* and *OsBIERF1~4*), phosphoinositide-specific phospholipase C (*OsPI-PLC1*), F-box protein (*OsDRF1*), diacylglycerol kinase (*OsBIDK1*), zinc finger protein (*OsBIRF1*), DEAD-box RNA helicase (*OsBIRH1*), and serine carboxypeptidase-like protein (*OsBISCPL1*) are involved in disease resistance responses and other abiotic stress (Song and Goodman, 2002a; 2002b; Luo *et al*., 2005a; 2005b; Hu *et al*., 2006; Cao *et al*., 2006a; 2006b; 2008; Song *et al*., 2006; Zhang *et al*., 2008; Liu *et al*., 2008a; 2008b; Li *et al*., 2008). In this study, we cloned and characterized a rice AMP-binding protein gene, *OsBIABP1*, and found that *OsBIABP1* gene can be induced by SA, BTH, JA and 1-amino cyclopropane-1-carboxylic acid (ACC), some well-known defense-related signaling molecules, and by infection with *M. oryzae*, causal agent of the blast disease. Our preliminary results suggest that OsBIABP1 may be involved in regulating rice disease resistance response through mediating different signaling pathways.

MATERIALS AND METHODS

Growth of rice plants, chemical treatments, and pathogen inoculation

Rice cultivar Yuanfengzao (*Oryza sativa* L. subsp. *indica*) and a pair of isogenic lines (H8R and H8S) were used in this study. Rice seedlings were grown in field soil packed in plastic pots (8 cm in diameter and 10 cm in height, 10 seedlings/pot) under 14 h light/10 h dark at 27/22 °C (day/night) in a growth room. Samples for tissue-specific expression were harvested from mature rice plants grown in a greenhouse with natural sunlight, frozen immediately in liquid nitrogen, and stored at −80 °C until use.

Three-week-old seedlings of cv. Yuanfengzao were treated by spraying with solutions of 0.3 mmol/L BTH (Novartis Crop Protection Inc., USA), 1.0 mmol/L SA (pH 6.5) (Sigma, St. Lousi, USA), 100 μmol/L JA (Sigma, St. Lousi, USA) or 100 μmol/L ACC (Sigma, St. Lousi, USA), respectively. JA and ACC were dissolved in 0.1% (v/v) ethanol. Control seedlings were treated in the same way as spraying with 0.1% (v/v) ethanol or distilled sterilized water. Three-week-old seedlings of H8R and H8S were inoculated by leaf spraying with spore suspension (5×10^5 spores/ml in 0.05% (v/v) Tween-20) of *M. oryzae* (race ZB1, strain 85-14B1). The inoculated seedlings were kept at 100% relative humidity in dark for 36 h and then returned to the growth chamber under 14 h light/10 h dark at 27/22 °C (day/night). Leaf samples were collected at different time points and stored at −80 °C until use.

Cloning of *OsBIABP1* cDNA and bioinformatics analysis

Differentially expressed cDNA clone BNHN-n7 contained a 284-bp insert, which showed a high level of similarity to putative AMP-binding protein (ABP) in plants. The 5′- and 3′-end fragments were amplified using phage DNA prepared from a rice cDNA library as template in order to obtain information of the full-length cDNA sequence of this putative ABP gene. The sequence-specific primers used were BNHN-n7-1F

(CATGGACAAGCTCGGCGCCA) and BNHN-n7-1R (CTGGCTAGATGCCGCTACA). The 5′-end fragment was amplified with BNHN-n7-1R and a vector primer T3-2 (CCTGCAGGTCGACACTAGTG), while the 3′-end fragment was amplified with BNHN-n7-1F, and a vector primer T7-2 (CCACCC GGGTGGAAAATCGA). A pair of gene-specific primers, BNHN-n7-2F (AAGAGCTCATGGACAA GCTCGGC, a *Sac*I site is underlined) and BNHN-n7-2R (CCCTCGAGCCGCTACATTTTACT, an *Xho*I site is underlined), was then used for the amplification of the entire open reading frame (ORF) sequence. All PCR products were purified and cloned into pUCm-T vector (Sangon, Shanghai, China). Plasmid containing the complete ORF sequence was designated pUCmT-BNHN-n7-1.

DNA sequencing was performed on both strands of the plasmids using a MegaBACE 1000 DNA Analysis System (Amersham Biosciences, UK). Similarity searches were carried out using BLAST programs at the National Center for Biotechnology Information (NCBI) GenBank database (http://www.nvbi.nlm.nih.gov/BLAST/). Sequence alignments and phylogenetic tree analysis were conducted using ClustalW method using DNAstar software (LaserGene, Madison, WI, USA).

RNA extraction and reverse transcription-polymerase chain reaction analysis of gene expression

Total RNA was extracted with Trizol reagent (Invitrogen, Shanghai, China) following the manufacturer's instruction. Samples of total RNA (0.5 μg) were reverse-transcribed using SuperScript III Kit (Invitrogen, Shanghai, China), followed by polymerase chain reaction (PCR) reaction using 1 μl of the RT product and 10 pmol of each primer in a total volume of 25 μl. The amplification conditions were as follows: 95 °C 3 min, 23~40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. PCR reactions were performed 23 cycles for *ACTIN1* gene, 40 cycles for tissue-specific expression, and 32 cycles for induction of *OsBIABP1* expression, and the PCR products were electrophoresed on a 1.2% (w/v) agarose gel. *OsBIABP1*-specific primers used are OsBIABP1-rt-1F (GAGGCGATCGGCTTCGAGGTC) and OsBIABP1-rt-1R (GTGGACGTCTTCGGTAA CTCGTC). Rice *ACTIN1* gene (accession No. X16280) was used as a control with primers of ACTIN1-1F (ACTGTCCCCATCTATGAAGGA) and ACTIN1-1R (CTGCTGGAATGTGCTGAGAGA).

RESULTS

Cloning of *OsBIAMP1* gene cDNA

In our previous studies, over 200 differentially expressed cDNA clones associated with BTH-induced defense response were identified (Song and Goodman, 2002a). BLAST searching revealed that a differentially expressed cDNA clone, BNHN-n7, containing a 284-bp insert, showed high similarity to genes encoding plant ABPs, indicating that the insert in BNHN-n7 may be a part of a rice ABP gene. To obtain this putative ABP gene, the 5′- and 3′-end sequences were amplified and a full-length cDNA was assembled. Because clone BNHN-n7 was identified originally from a suppression subtractive hybridization library constructed by subtracting cDNAs from BTH-induced rice leaf tissue with cDNAs from uninoculated control leaf tissue (Song and Goodman, 2002a), this gene was thus designated as *OsBIABP1* for *Oryza sativa* L. BTH-induced AMP-binding protein 1.

The full-length cDNA of *OsBIABP1* is 1912-bp long, which contains a predicted ORF of 1677 bp, and 5′- and 3′-UTR sequences of 87 and 148 bp, respectively. Database searches identified a full-length cDNA, AK106615, isolated from *Oryza sativa* L. subsp. *Japonica* by KOME project (http://cdna01.dna.affrc.go.jp/cDNA/), which has one nucleotide difference from *OsBIABP1* cDNA. However, this nucleotide difference does not cause amino acid difference in predicted proteins. The *OsBIABP1* gene corresponds to locus Os04g57850 as predicted by TIGR Rice Genome Annotation. *OsBIABP1* is localized at chromosome 3 of the rice genome and consists of 2 exons and 1 intron.

Characterization of OsBIAMP1 protein

The 1677-bp ORF of the *OsBIABP1* gene is predicted to encode a 558-aa protein with a calculated molecular weight of 60.4 kD and an isoelectric point of 7.87 (Fig.1). BLAST searches at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) indicate that

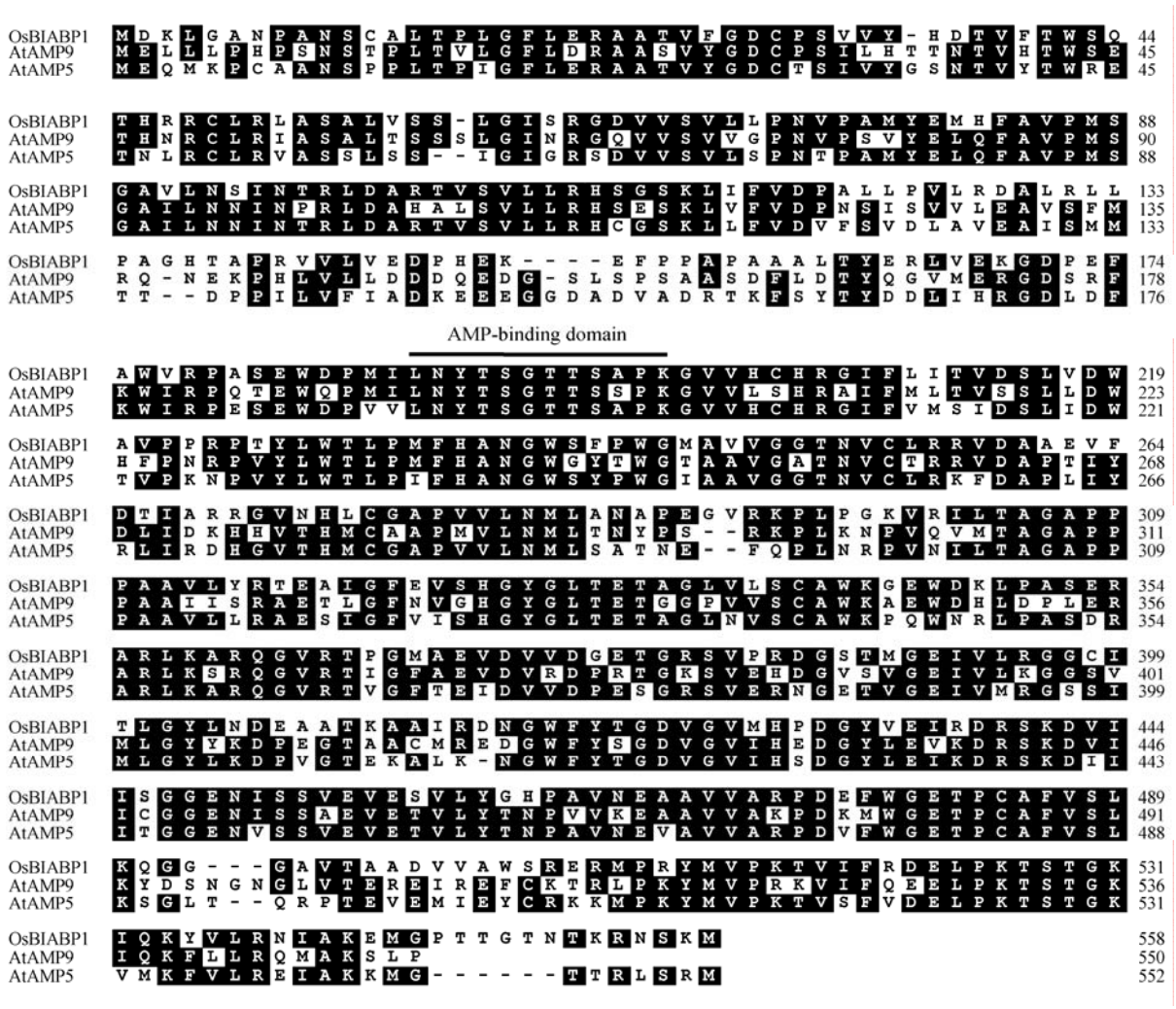

Fig.1 Comparison of OsBIABP1 with *Arabidopsis* AtAMP5 (At5g16370) and AtAMP9 (At1g21540)
Sequences were aligned using ClustalW program with DNAstar software. The conserved AMP-binding signature motif is indicated by a solid line on the sequences

OsBIABP1 contains a highly conserved region (from Leu-17 to Met-544), often seen in acyl-CoA synthetases (PRK08162, CaiC) that are involved in lipid/secondary metabolites biosynthesis, transport, and catabolism. Further domain search using PROSITE program at ExPASy server (http://au.expasy.org/) identified a conserved AMP-binding signature motif sequence LNYTSGTTSAPK (Fig.1). Similarity searches and phylogenetic tree analysis revealed that OsBIABP1 has high levels of similarity to *Atabidopsis* AtAMPs, specifically to AtAMP5, AtAMP6, and AtAMP8~10, giving 58.5%~64.5% identity at amino acid sequence level (Fig.2). OsBIABP1 and these *Arabidopsis* AMPs form a small group in the phylogenetic tree (Fig.2).

Tissue-specific expression of *OsBIABP1* in rice

Tissue-specific expression of *OsBIABP1* in rice plants grown under normal growth condition was examined by semi-quantitative RT-PCR. To avoid the risk to amplify homologous genes, downstream reverse primer was designed to be located in the UTR region at the 3′-end to improve specificity in PCR amplification. The specificity of the RT-PCR primers was further confirmed by sequencing of the PCR products. Under normal growth conditions, expression levels of *OsBIABP1* in different tissues of rice plants were very low, so we could not detect any product using 30~35 cycles of PCR. When RT-PCRs were increased to 40 cycles, a single band was observed. As shown in Fig.3, transcript of *OsBIABP1*

was detected in stems, leaves, and flowers, but not in root samples. *OsBIABP1* was expressed at relatively high levels in stems as compared with those in leaves and flowers (Fig.3). This result suggests that *OsBIABP1* is differentially expressed in a tissue-specific manner in rice.

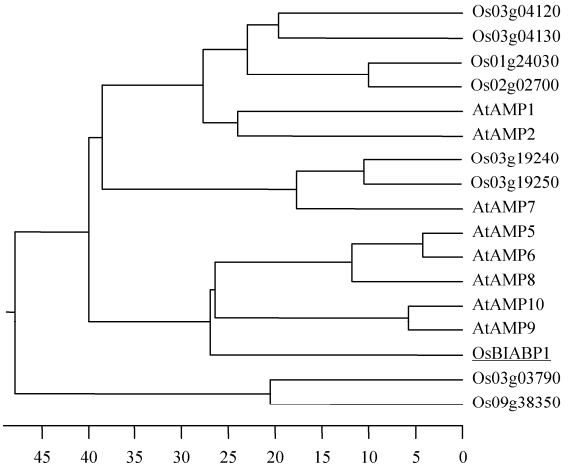

Fig.2 Phylogenetic tree analysis of OsBIABP1 with ABPs from rice and *Arabidopsis*

Sequence alignment and phylogenetic tree were constructed using ClustalW program in DNAstar software. The ABP proteins used for alignment are *Arabidopsis thaliana* AtAMP1 (At1g20560), AtAMP2 (At2g17650), AtAMP5 (At5g16370), AtAMP6 (At5g16340), AtAMP7 (At3g16910), AtAMP8 (At1g75960), AtAMP9 (At1g21540), and AtAMP10 (At1g21530); *Oryza sativa* L. Os03g04120, Os03g04130, Os01g24030, Os02g02700, Os03g19240, Os03g19250, Os03g03790, Os09g38350

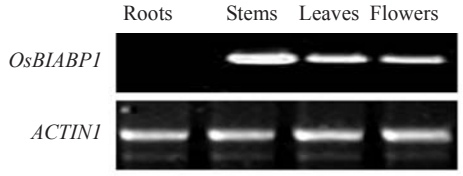

Fig.3 Expression of *OsBIABP1* in different tissues of rice plants grown under normal growth condition

Rice plants were grown in soil and samples from different tissues and organs were collected for analysis of gene expression. RT-PCR was performed 40 cycles for *OsBIABP1* using gene-specific primers and 23 cycles for *ACTIN1* used as a control

Induced expression of *OsBIABP1* by defense-related signaling molecules

The clone BNHN-n7 appeared to be differentially expressed in rice leaf tissue after treatment with BTH, indicating that *OsBIABP1* might be induced by BTH. We therefore first checked whether the expression of *OsBIABP1* in rice seedlings was induced by BTH. As expected, the induced expression of *OsBIABP1* was observed 24 h after treatment, peaked at 48 h, and maintained a relatively high level until 72 h after BTH treatment (Fig.4). We also analyzed whether the expression of *OsBIABP1* gene was induced by other different defense-related signal molecules including SA, JA, and ACC. Treatments with SA, JA or ACC all induced *OsBIRH1* expression in rice seedlings (Fig.4). SA treatment led to a rapid induced expression of *OsBIABP1* within 12 h and maintained to a relatively high level during 12~48 h after treatment (Fig.4). In JA-treated seedlings, the expression level of *OsBIABP1* increased significantly at 12 h, decreased slightly at 24 h, and increased again at 48 h after treatment (Fig.4). Similar to the kinetic in BTH-treated seedlings, ACC treatment induced markedly expression of *OsBIABP1* at 24 h, and a high level of expression was maintained during 24~72 h in rice seedlings (Fig.4). However, no significant expression of *OsBIABP1* was observed in water-treated

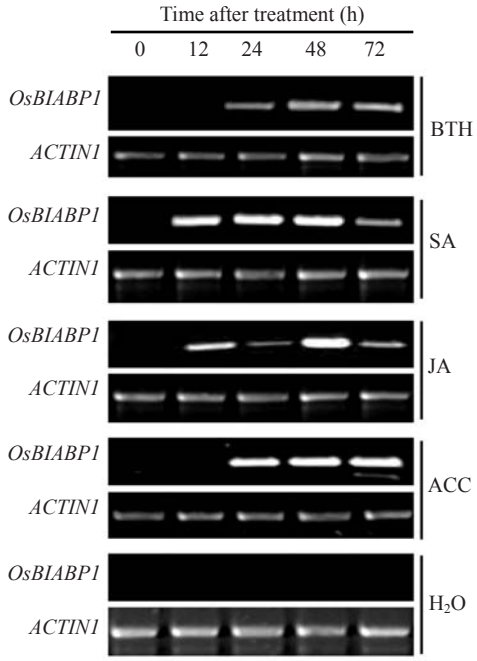

Fig.4 Induced expression of *OsBIABP1* in rice seedlings in response to treatments with different defense-related signal molecules

Three-week-old seedlings were treated by spraying with 0.3 mmol/L BTH, 1.0 mmol/L SA, 100 μmol/L JA, 100 μmol/L ACC or water, and leaf samples were collected at each time point after treatment. RT-PCR was performed 36 cycles for *OsBIABP1* using gene-specific primers and 23 cycles for *ACTIN1* used as a control

control seedlings during the experiment period (Fig.4). These results suggest that *OsBIABP1* is responsible to multiple defense signal molecules and therefore might be involved in different defense signaling pathways.

Expression patterns of *OsBIABP1* in rice-*M. oryzae* interactions

Expression patterns of *OsBIABP1* in incompatible and compatible interactions between rice and the blast fungus, *M. oryzae*, were also analyzed. In these experiments, a pair of isogenic lines, H8R and H8S, was used, in which disease responses of H8R and H8S seedlings to the tested strain of *M. oryzae* represent an incompatible interaction and a compatible interaction, respectively. Disease symptom usually was seen on three-week-old seedlings of H8R and H8S three days after inoculation with the fungus. As shown in Fig.5, the expression of *OsBIABP1* was induced in rice seedlings after inoculation with *M. oryzae*; however, the expression kinetics showed different patterns. In incompatible interaction of H8R seedlings with the blast fungus, the expression of *OsBIABP1* was detected at 12 h, peaked at 24 h, and maintained a relatively high level during 24~72 h after inoculation (Fig.5). By contrast, in compatible interaction of H8S seedlings with *M. oryzae*, significant expression of *OsBIABP1* was observed at 24 h, increased gradually, and peaked at 72 h after inoculation (Fig.5). These results indicate that the expression of *OsBIABP1* is activated by pathogen, and that the induced expression is much quicker and stronger during early stages of pathogenesis in incompatible interaction than in compatible interaction between rice and *M. oryzae*.

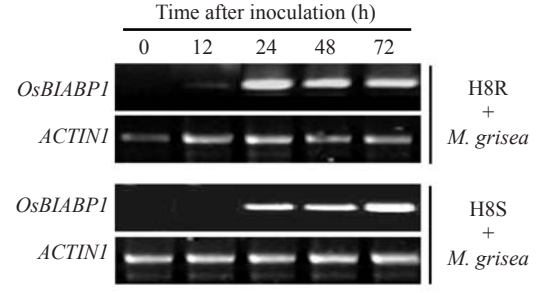

Fig.5 Induced expression of *OsBIABP1* in interactions between rice and *Magnaporthe oryzae*

Three-week-old rice seedlings of H8R and H8S were inoculated with *M. oryzae* and leaf samples were collected at each time point after inoculation. RT-PCR was performed 36 cycles for *OsBIABP1* using gene-specific primers and 23 cycles for *ACTIN1* used as a control

DISCUSSION

The AMP-binding proteins comprise a large family, whose members show diverse biochemical activities/functions, such as peptide antibiotic synthetases and siderophores, polyketide synthetases, 4-coumarate-CoA ligases, acetyl-CoA synthetases (ACSs), and acyl-CoA synthetases in plants (Shockey *et al*., 2000). In *Arabidopsis*, at least 44 genes encoding proteins containing the AMP-binding signature motif have been identified (Shockey *et al*., 2000; 2002). ACSs are the important members in this family because ACSs catalyze biochemically lipid metabolism, whose products have been shown to play crucial roles in a number of plant growth and development processes (Hayshi *et al*., 2002; Fulda *et al*., 2002; Schnurr *et al*., 2000; 2002). OsBIABP1 protein contains a conserved ACS domain and a characteristic AMP-binding signature motif sequence. However, results from phylogenetic tree analysis revealed that OsBIABP1 is much closer to *Arabidopsis* AMPBPs than to ACSs. It is therefore likely that OsBIABP1 is a member of AMPBPs rather than an ACS in rice. Biochemical activity for most members of the AMPBPs remains unclear. Thus, revealing if OsBIABP1 has ACS activity or not and exploring its novel biochemical activity will be the future challenges.

Analyses of expression patterns have revealed that *Arabidopsis* AMP binding protein (AMPBP) genes are expressed in a tissue-specific manner. The expression patterns of the AMPBP genes varied widely in different tissues with a high degree of overlap, indicating the complexity of function of this family in *Arabidopsis* (Shockey *et al*., 2000; 2002; Schnurr *et al*., 2000). Pepper *CaSIG4* encoding an ACS, rapeseed *ACS6* and another ACS gene, were also shown to be expressed in specific tissues, e.g., *CaSIG4* in flowers and rapeseed *ACS6* in lipogenic tissues, respectively (Fulda *et al*., 1997; Pongdontri and Hills, 2001; Lee *et al*., 2001). We found that *OsBIABP1* is expressed mainly in aboveground stems,

leaves, and flowers, indicating that *OsBIABP1* may function in rice growth and development of above-ground parts.

It was recently demonstrated that the AMP-binding proteins, including ACSs, play roles in plant adaption to environment stress. The *Arabidopsis ACN1*, encoding a peroxisome-localized ACS, is involved in acetate utilization by activating exogenous acetate to the coenzyme A form for entry into the glyoxylate cycle (Turner *et al*., 2005). The pepper *CaSIG4*, encoding a plasma membrane-localized ACS, was induced rapidly in leaves after SA treatment and infection with *Xanthomonas campestris* pv. *vesicatoria*, suggesting a role in defense response (Lee *et al*., 2001). Expression of *OsBIABP1* can be induced by SA, JA, and ACC, which mediate different plant defense signaling pathways, the SA-dependent pathway and the JA/ET pathway, respectively (Glazebrook, 2005). Importantly, pathogen-induced expression of *OsBIABP1* is much quicker and stronger during early stages of pathogenesis in incompatible interaction than in compatible interaction between rice and *M. oryzae*. These inducible features indicate that *OsBIABP1* has a function in defense response through mediating SA- and JA/ET-dependent defense signaling pathways in rice. This is partially supported by recent observations that some defense-related factors can coordinate both SA- and JA-dependent signaling pathways to regulate disease resistance responses (Mei *et al*., 2006; Qiu *et al*., 2007).

Lipid metabolism is a complex, highly regulated process that plays essential roles in normal plant growth and development as well as in response to environmental stress. Some classes of AMP-binding proteins, e.g., ACSs and long-chain ACS (LACSs), are directly or indirectly involved in fatty acid metabolism (Hayshi *et al*., 2002; Fulda *et al*., 2002; Schnurr *et al*., 2002; Weimar *et al*., 2002), which may yield important signaling molecules including the well-known JA. It has been recently shown that fatty acid metabolism-derived signaling plays a critical role in regulation of defense responses against pathogen infection in *Arabidopsis* (Kachroo *et al*., 2003; 2004; 2008; Chandra-Shekara *et al*., 2007). Characterization of *OsBIABP1* may provide an opportunity to explore the function of fatty acid metabolism in rice disease resistance responses. Further studies with knockout/knockdown mutants and/or overexpression in transgenic plants will be helpful in understanding of the biological function and the mechanism of *OsBIABP1* in rice growth/development and environmental stress responses.

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